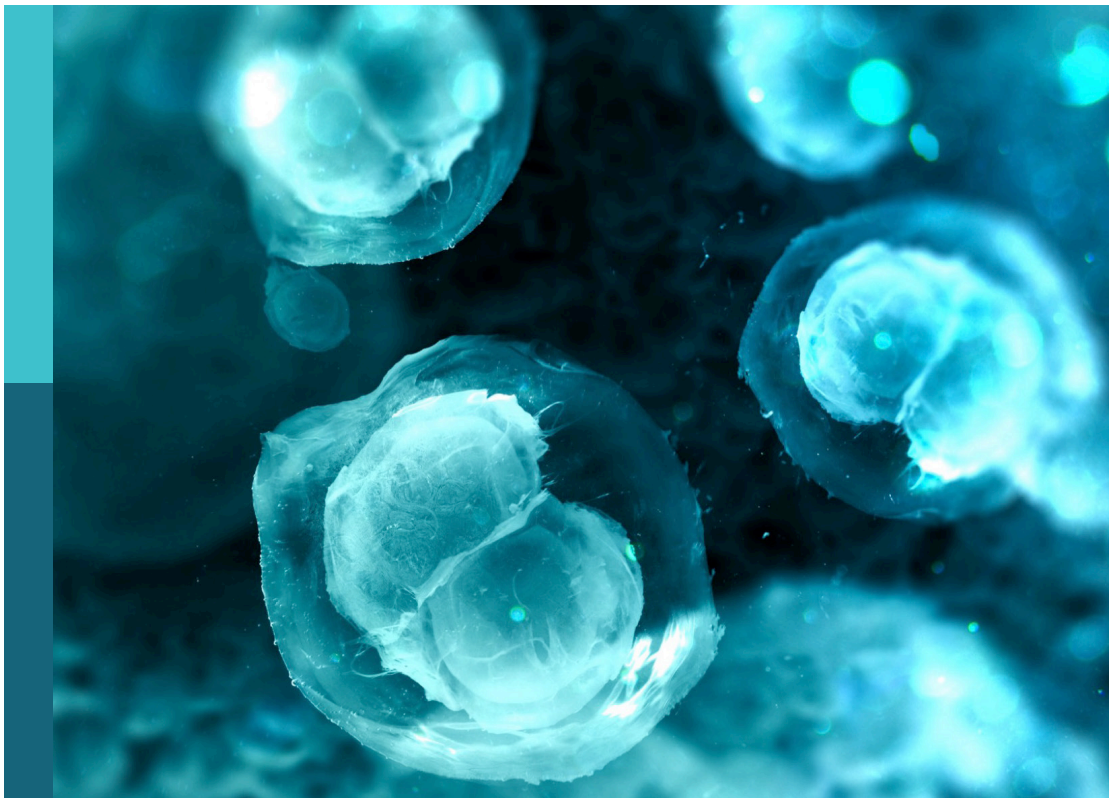
frontiers | Research Topics

Unconventional protein secretion: From basic mechanisms to dysregulation in disease

Edited by
Vivek Malhotra, Marioara Chiritoiu-Butnaru,
Sarah Elizabeth Stewart, Julien Villeneuve and Min Zhang

Published in
Frontiers in Cell and Developmental Biology



ISSN 1664-8714
ISBN 978-2-83251-047-6
DOI 10.3389/978-2-83251-047-6

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Unconventional protein secretion: From basic mechanisms to dysregulation in disease

Topic editors

Vivek Malhotra — Centre for Genomic Regulation (CRG), Spain
Marioara Chiritoiu-Butnaru — Institute of Biochemistry of the Romanian Academy, Romania
Sarah Elizabeth Stewart — La Trobe University, Australia
Julien Villeneuve — Institute for Functional Genomics (IGF), CNRS, France
Min Zhang — Tsinghua University, China

Citation

Malhotra, V., Chiritoiu-Butnaru, M., Stewart, S. E., Villeneuve, J., Zhang, M., eds. (2022). *Unconventional protein secretion: From basic mechanisms to dysregulation in disease*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-047-6

Table of contents

frontiers | Frontiers in Cell and Developmental Biology

TYPE Editorial
PUBLISHED 30 November 2022
DOI 10.3389/fcell.2022.1088002

OPEN ACCESS

EDITED BY
Vladimir Lupashin,
University of Arkansas for Medical Sciences, United States

REVIEWED BY
Catherine Rabouille,
Hubrecht Institute (KNAW), Netherlands

*CORRESPONDENCE
Julien Villeneuve,
julien.villeneuve@igf.cnrs.fr

SPECIALTY SECTION
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

RECEIVED 02 November 2022
ACCEPTED 21 November 2022
PUBLISHED 30 November 2022

CITATION
Chiritoiu-Butnaru M, Stewart SE, Zhang M, Malhotra V and Villeneuve J (2022), Editorial: Unconventional protein secretion: From basic mechanisms to dysregulation in disease. *Front. Cell Dev. Biol.* 10:1088002. doi: 10.3389/fcell.2022.1088002

Editorial: Unconventional protein secretion: From basic mechanisms to dysregulation in disease

Marioara Chiritoiu-Butnaru[1], Sarah E. Stewart[2], Min Zhang[3], Vivek Malhotra[4,5,6] and Julien Villeneuve[7]*

[1]Department of Molecular and Cellular Biology, Institute of Biochemistry of the Romanian Academy, Bucharest, Romania, [2]Department of Biochemistry and Chemistry, La Trobe Institute for Molecular Science, La Trobe University Bundoora, Melbourne, VIC, Australia, [3]Tsinghua University, School of Pharmaceutical Sciences, Beijing, China, [4]Centre for Genomic Regulation (CRG), The Barcelona Institute for Science and Technology, Barcelona, Spain, [5]Universitat Pompeu Fabra (UPF), Barcelona, Spain, [6]ICREA, Barcelona, Spain, [7]Institute of Functional Genomics, University of Montpellier, CNRS, INSERM, Montpellier, France

KEYWORDS

unconventional protein secretion (UPS), membrane trafficking, cell adaptation, disease, cell compartmentalization

Editorial on the Research Topic
Unconventional protein secretion: From basic mechanisms to dysregulation in disease

In eukaryotes, the classical view is that secreted proteins involved in intercellular communication are exported from cells through a highly conserved pathway generally termed the conventional secretory pathway, first postulated by Palade and co-workers (Palade, 1975). Over the past 5 decades, this pathway has been extensively studied, leading to an extensive characterization of key players involved in cargo recognition, packaging, sorting and transport. Briefly, proteins released by cells through the conventional secretory pathway, such as antibodies, collagens, mucins, cytokines, hormones, and neurotransmitters, contain a signal sequence that directs their translocation into the Endoplasmic Reticulum (ER) where they are packaged and sorted by the use of COPII coats and TANGO1 (transport and Golgi organization 1) to reach the Golgi apparatus (Raote et al., 2021). Then, cargo proteins are resorted at the Golgi apparatus and delivered by vesicular transport to their respective destinations within the cell and to the cell's exterior. Although challenging questions remain unanswered, understanding these processes has revealed their fundamental role in maintaining the specificity and communication of different organelles, in supporting cell-cell communication, and has highlighted how defects in this secretory route are connected to human diseases.

However, new findings have recently emphasized the critical role of alternative secretory pathways for the export of an increasing number of proteins lacking signal sequence (or leaderless proteins). These new routes, collectively designated as

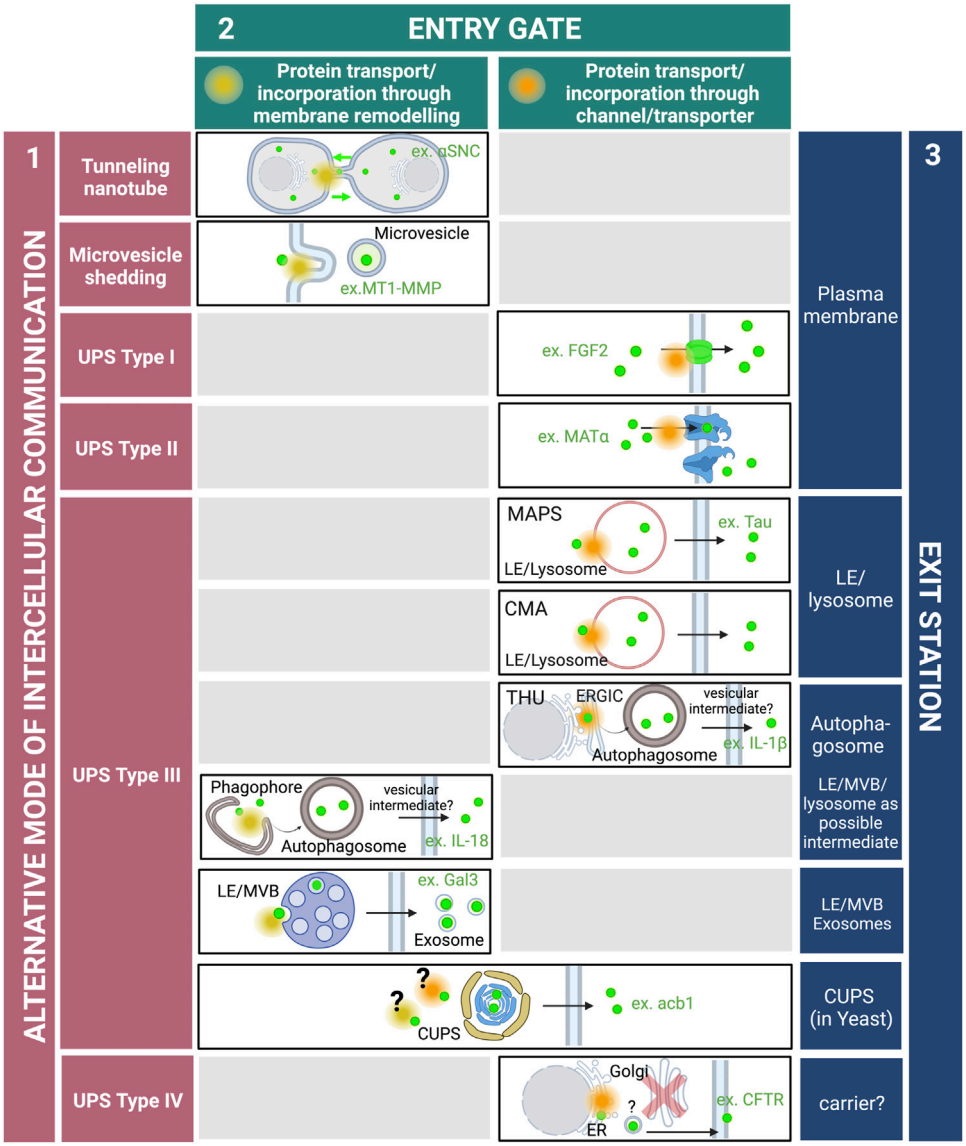


FIGURE 1
Roads and hubs of unconventional protein secretion. In addition to the conventional ER-Golgi secretory pathway, cells are endowed with additional routes for the export of cytoplasmic proteins, and multiple modes of intercellular communications have been characterized (purple). They include tunnelling nanotubes formed by membranous protrusions that emerge from the PM and connect adjacent cells. Tunnelling nanotubes allow the direct transfer of cytoplasmic proteins as well as whole organelles such as lysosomes. Outward budding of the PM produce microvesicles that are then released into the extracellular space. In types I and II UPS, cargo proteins are directly translocated across the PM by pore formation or ABC transporters, respectively. In type III UPS, cargo proteins are incorporated into intracellular compartments and transported through single or successive membrane intermediates that fuse with the PM. In type IV UPS or Golgi-bypass, integral membrane proteins are inserted into the ER and then reach the PM independently of the Golgi apparatus. Along these alternative secretory routes, cargo proteins are transported or incorporated within membrane compartments either by protein channels or by membrane remodeling defining distinct entry gates at the PM or along intracellular trafficking pathways (green). Cargo proteins can also be gathered into particular membrane intermediates prior to release into the extracellular space, defining distinct exit stations (blue). Abbreviations–αSNC: alpha-synuclein; acb1: acyl-CoA-binding protein; CFTR: cystic fibrosis transmembrane conductance regulator; CUPS: Compartment for unconventional protein secretion; CMA: Chaperone-mediated autophagy; ER: Endoplasmic reticulum; ERGIC: ER-Golgi intermediate compartment; FGF2: Fibroblast growth factor 2; Gal3: Galectin-3; IL: Interleukin; LE: Late endosome; MAPS: Misfolded-associated protein secretion; MT1-MMP: Membrane-type 1 matrix metalloproteinase; MVB: Multivesicular body; THU: TMED10-channeled UPS; UPS: Unconventional protein secretion. The figure was generating using BioRender.

unconventional protein secretion (UPS), have emerged as essential for maintaining cellular homeostasis and intercellular communication. Proposed pathways involve sequential events that take place at the plasma membrane (PM) where leaderless proteins can be directly translocated across the lipid bilayer through protein channels, or transferred to adjacent cells through microvesicles or tunnelling nanotubes. Other underlying mechanisms reflect the plasticity and dynamic properties of intracellular compartments that can be remodeled, rerouted or created *de novo* in response to intrinsic demands or external signals for secretion of signal sequence lacking proteins (Filaquier et al., 2022) (Figure 1).

This new paradigm led us to revisit the current framework of mechanisms that support protein trafficking and secretion. This Research Topic of 18 articles explores the general topic of UPS by covering basic mechanisms, their dysregulation in pathophysiological conditions, and the potential use of UPS for biomedical applications.

UPS mediated by vesicular intermediates (type III and IV UPS)

UPS has been identified in all eukaryotes including plants, yeast, flies and mammals to export a wide range of protein families such as cytokines, lipid chaperones, hydrolytic enzymes, or toxic aggregate-prone proteins, among others. This raises the central question of the evolutionary significance of UPS and the selective advantages gained by acquiring these alternative secretory routes. To address this issue, the comprehension of UPS mechanisms for multiple cargo proteins in various organisms is required. Maricchiolo et al. describe mammalian and plant UPS pathways, pointing similarities and differences, and propose revising UPS plant classification to converge on a single classification system based on features defined in mammalian UPS. While UPS relies on a striking diversity of mechanisms in both plants and animals, common processes are highlighted, as illustrated by intracellular compartments with equivalent functions, such as the vacuole in plants and lysosomes in animals that can be diverted into secretory organelles, thus representing important sorting stations for UPS. However, the involvement of additional vesicular intermediates that may derive from autophagosomes, multivesicular bodies (MVBs) or, as shown in yeast, from a transient and hybrid compartment formed by Golgi and endosome membranes called CUPS, have also been reported (Rabouille, 2017). Lee et al. describe recent advances in a specific UPS pathway called MAPS (Misfolded associated protein secretion) that requires the mobilization of consecutive vesicle carriers along the endo-lysosomal system. MAPS is used by cells as an additional protein quality control (PQC) mechanism in the context of proteotoxic stress to promote clearance of misfolded proteins. This UPS pathway is controlled by the coordinated action of the ER associated deubiquitylase USP19, the membrane-associated chaperone HSC70 and its co-chaperone DNAJC5/CSPα, the latter being able to couple MAPS with the endosomal microautophagy. Noh et al. focus on autophagy-related pathways and describe in detail how autophagy machineries can alter UPS. While autophagosomes could represent a major entry gate for many UPS cargo proteins, there is no evidence that autophagosomes directly fuse with the PM. Instead, autophagosomes might first fuse with other vesicular intermediates, such as late endosomes, MVBs or lysosomes, which then release their content into the extracellular space after exocytosis. Vats and Galli describe the role of the machinery involved in fusion events between distinct intracellular compartments and the PM. Specifically, they highlight the role of the vesicular SNARE protein VAMP7, which has been reported to be involved in the exosome-, lysosome- and autophagy-mediated secretion.

While UPS is mainly involved in the export of proteins lacking a signal sequence, mechanisms allowing the transport of proteins from the ER to the cell surface independently of the Golgi apparatus have also been indexed as UPS pathway. This is illustrated by the study of Dimou et al. which suggests the existence of distinct COPII carriers involved in the transport from the ER to the PM of the proton-pump ATPase PmaA and the PalI pH sensing component, in the filamentous fungus *Aspergillus nidulans*.

UPS mediated by direct translocation across the plasma membrane (type I UPS)

In addition to pathways mediated by vesicular intermediates, UPS is also achieved by pore-mediated translocation across the PM. This direct mode of secretion has been extensively studied in the case of Fibroblast growth factor 2 (FGF2), for which detailed mechanistic insights have been provided (Sparn et al., 2022). Briefly, FGF2 secretion is mediated by sequential interactions of FGF2 with Na,K-ATPase, tec kinase and phosphoinositide in the inner leaflet of the PM. These interactions trigger FGF2 oligomerization, leading to the formation of membrane-spanning FGF2 oligomers, recognized as a self-sustained translocation channel. Directional transport of FGF2 is then ensured by the interaction of FGF2 with heparan sulfate proteoglycans located on the outer leaflet of the PM. Here, Lolicato and Nickel discuss recent findings suggesting that this sequence of events occurs in specialized, liquid-ordered nanodomains of the PM enriched in cholesterol and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$). Other examples of UPS cargo proteins directly translocated across the PM include Tau, HIV-Tat and Engrailed 2 homeoprotein. Joliot and Prochiantz comprehensively summarize the mechanisms involved in the UPS of homeoproteins that also

require their physical interactions with $PI(4,5)P_2$ and proteoglycans, and focus on their physio-pathological functions once secreted and internalized by adjacent cells.

The mechanisms involved in the secretion of IL-1 family cytokines are also of major interest and perfectly illustrate how UPS cargo proteins can be directed to multiples UPS pathways (Semino et al., 2018). While several studies have demonstrated that IL-1 family members can be secreted after being incorporated into intracellular compartments, including autophagosomes or lysosomes (Pallotta and Nickel, 2020), compelling evidence have recently highlighted that, during acute inflammation, IL-1β is translocated across the PM through a channel formed after oligomerization of inflammasome-activated Gasdermin D (Evavold et al., 2018). In this context, Evavold and Kagan discuss appealing emerging ideas whereby host metabolic state dictates alternative or complementary pathways for IL-1β secretion.

Extracellular vesicles (EVs) and tunnelling nanotubes (TNTs)

UPS also includes secretory routes by which cargo proteins are released from cells enclosed in EVs. Meldolesi describes the general properties of the two main types of EVs, i.e. exosomes and microvesicles, and the mechanisms underlying their biogenesis. Briefly, exosomes derive from intraluminal vesicles (ILVs) formed within the endocytic pathway by inward budding of late endosomes. This generates MVBs that can then fuse with the PM, whereas microvesicles derive from outward budding of the PM. Although EVs differ in many features including size and molecular composition, the biogenesis of exosomes and microvesicles requires the endosomal sorting complex required for transport (ESCRT) machinery. Thuault et al. summarize the current knowledge of the molecular mechanisms involved in the loading of matrix metalloproteinases (MMPs) into both exosomes and microvesicles, focusing particularly on MT1-MMP, a membrane-associated MMP contributing to cell invasive behavior. They provide a detailed description of the underlying trafficking events that combine cycles of endocytosis, recycling and exocytosis. Bänfer et al. provide new insight in the loading of E-cadherin into ILVs, that depends on the ESCRT-I component Tsg101. Farley et al. summarize the state of knowledge on plant EVs, and present exciting perspectives on their potential use as drug delivery tools.

Finally, to complete the list of alternative modes of intercellular communication, Turos-Korgul et al. describe how membranous protrusions emerging from the PM to connect adjacent cells and called tunnelling nanotubes (TNTs) are established, and their role in the progression of a wide range of diseases.

UPS in disease

Several review in this Research Topic, as described above, have extended their discussion to the role of UPS in several disorders. In addition, Iglesia et al. provide a comprehensive overview of UPS involvement in brain tumor maintenance, and Pilliod et al. present new findings on the UPS of Tau, whose release from neurons may represent a critical step in Alzheimer's disease progression.

Thus, although further studies are needed to delineate the mechanisms and factors involved in the different UPS pathways, we now appreciate their fundamental role in cell biology and how their dysregulation is associated with diseases. Therefore, research on UPS will not only highlight processes conserved across many species, but also open new perspectives for the development of innovative therapeutic strategies. A promising direction will also be the development of new biotechnological applications, as illustrated by Philipp et al. who explore in a fungal model the use of UPS cargo proteins as a carrier for the production and export of heterologous proteins, including synthetic nanobodies directed against the SARS-CoV2 virus.

Concluding remarks

In conclusion, while initially perceived as an enigma by the scientific community studying protein trafficking and secretion processes, the pioneer studies that revealed the lack of a signal peptide in the IL-1β sequence, and the release of this secreted cytokine through an ER-Golgi-independent pathway (Auron et al., 1984; Rubartelli et al., 1990), have laid the foundation for an entirely new field of research. The field is now maturing, suggesting even more questions and challenges in the coming years. For example, how are UPS pathways integrated into the adaptive stress response to meet cellular needs, and what is the functional relationship between conventional and unconventional secretory pathways? The role of GRASP proteins could be of major importance in this context, given their function in Golgi organization and their relocation to distinct organelles involved in UPS during particular stress conditions (Cruz-Garcia et al., 2014; Zhang et al., 2019; Nüchel et al., 2021). The principles of selection and recognition of cargo proteins for UPS also warrant further exploration? Specific domains or amino acids have been recognized for a few UPS cargo proteins, but they remain elusive in most cases. Cargo selection for UPS likely relies on a combination of specific and complementary sequences, as illustrated by Biswal et al. Another challenge will be to obtain a comprehensive list of cargo proteins that are actively and selectively secreted by UPS. Poschmann et al. examine how recent advances in quantitative secretomics combined with pharmacological perturbation strategies (pharmocosecretomics) could achieve this critical objective.

Overall, these contributions are representative of the complexity and diversity of the mechanisms underlying UPS, which intersect with key processes within the cell, including protein sorting, membrane trafficking, and organelle dynamics. Undoubtedly, the knowledge gained about these fundamental principles of cell biology, combined with the prodigious technological advances made in recent years, guarantees exciting new discoveries about UPS in the near future.

Author contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Funding

MC acknowledges support from the Romanian Ministry of Research and Innovation, UEFISCDI grant TE156/2020 (PN-III-P1-1.1-TE-2019-1705). SES acknowledges support from the Australian Research Council, DE200100611. VM is an Institució Catalana de Recerca i Estudis Avançats professor at the Centre for Genomic Regulation. Work in the Malhotra lab is funded by grants from the Spanish Ministry of Economy and Competitiveness (Plan Nacional to VM: PID 2019-105518GB-I00) and the European Research Council Synergy Grant (ERC-2020-SyG - Proposal No. 951146). JV acknowledges support from the Fondation Vaincre Alzheimer, the association France Parkinson, CNRS, INSERM and the University of Montpellier.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Auron, P. E., Webb, A. C., Rosenwasser, L. J., Mucci, S. F., Rich, A., Wolff, S. M., et al. (1984). Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. *Proc. Natl. Acad. Sci. U. S. A.* 81, 7907–7911. doi:10.1073/pnas.81.24.7907

Cruz-Garcia, D., Curwin, A. J., Popoff, J. F., Bruns, C., Duran, J. M., and Malhotra, V. (2014). Remodeling of secretory compartments creates CUPS during nutrient starvation. *J. Cell Biol.* 207, 695–703. doi:10.1083/jcb.201407119

Evavold, C. L., Ruan, J., Tan, Y., Xia, S., Wu, H., and Kagan, J. C. (2018). The pore-forming protein gasdermin D regulates interleukin-1 secretion from living macrophages. *Immunity* 48, 35–44. doi:10.1016/j.immuni.2017.11.013

Filaquier, A., Marin, P., Parmentier, M. L., and Villeneuve, J. (2022). Roads and hubs of unconventional protein secretion. *Curr. Opin. Cell Biol.* 75, 102072. doi:10.1016/j.ceb.2022.02.006

Nüchel, J., Tauber, M., Nolte, J. L., Mörgelin, M., Türk, C., Eckes, B., et al. (2021). An mTORC1-GRASP55 signaling axis controls unconventional secretion to reshape the extracellular proteome upon stress. *Mol. Cell* 81, 3275–3293. doi:10.1016/j.molcel.2021.06.017

Palade, G. (1975). Intracellular aspects of the process of protein synthesis. *Science* 189, 347–358. doi:10.1126/science.1096303

Pallotta, M. T., and Nickel, W. (2020). FGF2 and IL-1β - explorers of unconventional secretory pathways at a glance. *J. Cell Sci.* 133, jcs250449. doi:10.1242/jcs.250449

Rabouille, C. (2017). Pathways of unconventional protein secretion. *Trends Cell Biol.* 27, 230–240. doi:10.1016/j.tcb.2016.11.007

Raote, I., Saxena, S., Campelo, F., and Malhotra, V. (2021). TANGO1 marshals the early secretory pathway for cargo export. *Biochim. Biophys. Acta. Biomembr.* 1863, 183700. doi:10.1016/j.bbamem.2021.183700

Rubartelli, A., Cozzolino, F., Talio, M., and Sitia, R. (1990). A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J.* 9, 1503–1510. doi:10.1002/j.1460-2075.1990.tb08268.x

Semino, C., Carta, S., Gattorno, M., Sitia, R., and Rubartelli, A. (2018). Progressive waves of IL-1β release by primary human monocytes via sequential activation of vesicular and gasdermin D-mediated secretory pathways. *Cell Death Dis.* 9, 1088. doi:10.1038/s41419-018-1121-9

Sparn, C., Meyer, A., Saleppico, R., and Nickel, W. (2022). Unconventional secretion mediated by direct protein self-translocation across the plasma membranes of mammalian cells. *Trends biochem. Sci.* 47, 699–709. doi:10.1016/j.tibs.2022.04.001

Zhang, X., Wang, L., Ireland, S. C., Ahat, E., Li, J., Bekier, M. E., et al. (2019). GORASP2/GRASP55 collaborates with the PtdIns3K UVRAG complex to facilitate autophagosome-lysosome fusion. *Autophagy* 15, 1787–1800. doi:10.1080/15548627.2019.1596480

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 10 January 2022
doi: 10.3389/fcell.2021.816335

A Novel Potent Carrier for Unconventional Protein Export in *Ustilago maydis*

*Magnus Philipp, Kai P. Hussnaetter, Michèle Reindl, Kira Müntjes, Michael Feldbrügge and Kerstin Schipper**

Institute for Microbiology, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

Recombinant proteins are ubiquitously applied in fields like research, pharma, diagnostics or the chemical industry. To provide the full range of useful proteins, novel expression hosts need to be established for proteins that are not sufficiently produced by the standard platform organisms. Unconventional secretion in the fungal model *Ustilago maydis* is an attractive novel option for export of heterologous proteins without *N*-glycosylation using chitinase Cts1 as a carrier. Recently, a novel factor essential for unconventional Cts1 secretion termed Jps1 was identified. Here, we show that Jps1 is unconventionally secreted using a fusion to bacterial β-glucuronidase as an established reporter. Interestingly, the experiment also demonstrates that the protein functions as an alternative carrier for heterologous proteins, showing about 2-fold higher reporter activity than the Cts1 fusion in the supernatant. In addition, Jps1-mediated secretion even allowed for efficient export of functional firefly luciferase as a novel secretion target which could not be achieved with Cts1. As an application for a relevant pharmaceutical target, export of functional bi-specific synthetic nanobodies directed against the SARS-CoV2 spike protein was demonstrated. The establishment of an alternative efficient carrier thus constitutes an excellent expansion of the existing secretion platform.

Keywords: luciferase, anti-Sars-CoV2 nanobody, unconventional secretion, *Ustilago maydis*, sybody

OPEN ACCESS

Edited by:
Julien Villeneuve, Institute for Functional Genomics (IGF), France

Reviewed by:
Carmen Faso, University of Bern, Switzerland
Kai Heimel, University of Göttingen, Germany
Rosana Puccia, Federal University of São Paulo, Brazil

****Correspondence:***
Kerstin Schipper
kerstin.schipper@uni-duesseldorf.de

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *16 November 2021*
Accepted: *17 December 2021*
Published: *10 January 2022*

Citation:
Philipp M, Hussnaetter KP, Reindl M, Müntjes K, Feldbrügge M and Schipper K (2022) A Novel Potent Carrier for Unconventional Protein Export in Ustilago maydis. *Front. Cell Dev. Biol. 9:816335. doi: 10.3389/fcell.2021.816335*

INTRODUCTION

The market for recombinant proteins like biopharmaceuticals is steadily increasing (Walsh 2018). As one example, the number of monoclonal antibody therapeutics entering phase 3 clinical trials has risen from 39 in 2014 to 88 in 2020 (Reichert 2015; Kaplon and Reichert 2021). Protein secretion into the culture broth is an excellent strategy for the production of recombinant proteins because it supports straight-forward and inexpensive downstream processing (Nicaud et al., 1986; Flaschel and Friehs 1993). In eukaryotes, proteins are mostly targeted via the endomembrane system by N-terminal signal peptides for secretion (Viotti 2016). By contrast, the term unconventional secretion describes protein export that does not occur via the classical endomembrane system including endoplasmic reticulum and Golgi apparatus (Nickel 2010). Various routes for such alternative secretion events exist, including direct transfer across the plasma membrane via transporters or self-sustained translocation or vesicular pathways where membrane vesicles are hitchhiked for export (Nickel 2010; Rabouille 2017).

Unconventional export of chitinase Cts1 in yeast cells of the fungal model *Ustilago maydis* is coupled to cytokinesis in a lock-type mechanism (Reindl et al., 2019). Upon formation of the

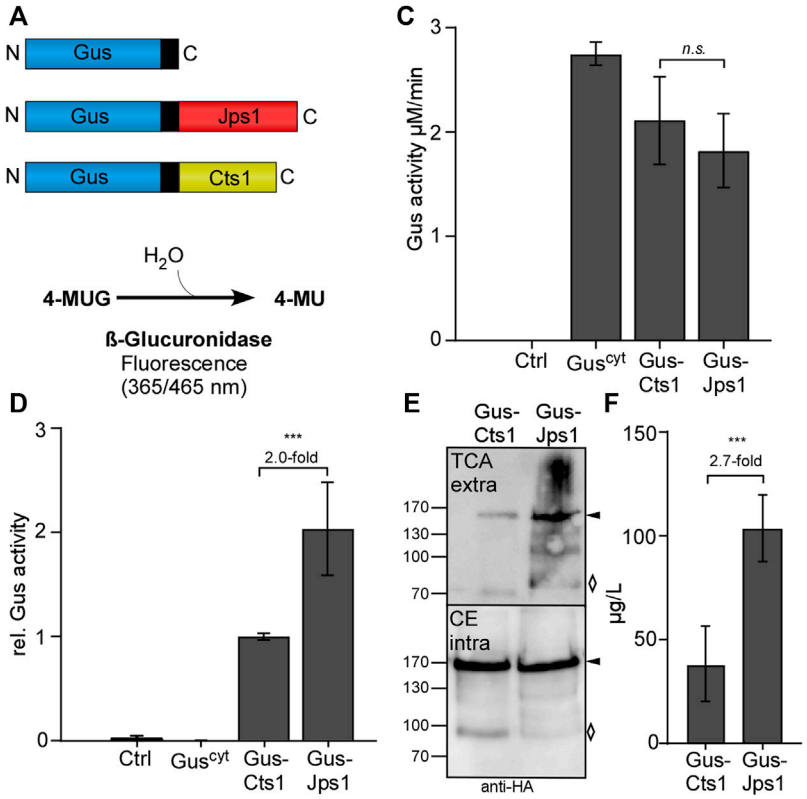


FIGURE 1 | Jps1 is unconventionally secreted and serves as an alternative carrier for Gus export. **(A)** Schematic display of the proteins expressed to study unconventional secretion. Cytoplasmic Gus (Gus^{cyt}) is used as a lysis control (top). Gus-Jps1 (middle) and Gus-Cts1 (bottom) harbor the respective carrier proteins at the C-terminus. All proteins carry an SHH (double Strep, ten times His, triple HA) tag indicated in black (Sarkari et al., 2014). All schemes are drawn to scale. **(B)** Enzymatic reaction mediated by β-glucuronidase. 4-methyl-umbeliferyl-β-D-glucuronide (4-MUG) and H_2O are converted to 4-methyl-umbelliferone which is a fluorescent molecule (365 nm excitation/465 nm emission). **(C)** Determination of intracellular Gus activity. Progenitor strain AB33P8Δ (Ctrl) and AB33 Gus^{cyt} expressing cytoplasmic Gus were included as controls. The experiment was conducted in three biological replicates. **(D)** Comparative extracellular Gus activity of strains using either Cts1 or Jps1 as a carrier. Enzyme activities were normalized to average values of the strain secreting Gus-Cts1. AB33P8Δ and AB33 Gus^{cyt} were used as a negative and lysis controls, respectively. The experiment was conducted in three biological replicates. **(E)** Representative Western blot analysis of Gus-Cts1 and Gus-Jps1 secretion. Extracellular protein was enriched from culture supernatants by TCA precipitation. Intracellular protein levels were visualized by cell extracts. Western blots show 1 ml of precipitated supernatants (TCA) and 10 µg cell extract (CE). Full length protein signal indicated by arrows, degradation bands with a rhombus. **(F)** Quantification of secreted protein using Western blot analysis. Supernatants of strains producing Gus-Jps1 or Gus-Cts1 were enriched by TCA precipitation and subjected to Western blot analysis. Signal intensities were compared to defined protein amounts of Multiple Tag protein (GenScript Piscataway, NJ, United States) included in the same gel. Bars show extrapolated protein amounts in µg/L. Western blots used for the analysis, see **Supplementary Figure S3**. Three biological replicates are shown; error bars in figures **(C)**, **(D)**, and **(F)** indicate standard deviation. Definition of statistical significance (***): p-value < 0.05. p-value derived from Student's unpaired t-test.

daughter cell at one growth pole of the cigar shaped mother cell, Cts1 is targeted to the so-called fragmentation zone delimited at the mother-daughter neck by consecutive formation of two septa (Langner et al., 2015). Here, the chitinase participates in separation of the two cells likely by degrading the remnant cell wall (Langner et al., 2015). Two septation factors, guanine nucleotide exchange factor (GEF) Don1 and kinase Don3, are essential for formation of the secondary septum and for Cts1 secretion (Weinzierl et al., 2002; Aschenbroich et al., 2019). Furthermore, a recently identified potential anchoring factor, Jps1, is crucial for chitinase localization and export (Reindl et al., 2020).

Importantly, unconventional Cts1 secretion can be exploited for co-export of heterologous proteins (Stock et al., 2012). Circumventing the classical secretion system is advantageous for the production of distinct proteins, because it avoids post-translational modifications like *N*-glycosylation occurring in the endomembrane system. In addition, there is no apparent size limitation (Stock et al., 2012). Successful examples are secretion of functional enzymes like β-glucuronidase or β-galactosidase, and antibody formats like single-chain variable fragments (scFv) or nanobodies (Stock et al., 2012; Sarkari et al., 2014; Terfrüchte et al., 2017; Reindl et al., 2020). While the secretion system is operational for several target proteins, low yields in the µg per liter range are currently limiting its applicability (Terfrüchte et al., 2017). Recently, major improvements were achieved by the generation of protease-deficient production strains, usage of strong constitutive promoters and medium optimization (Sarkari et al., 2014; Terfrüchte et al., 2018). However, novel strategies to further advance the system are needed.

In the present study we demonstrate that Jps1 is a novel potent carrier for co-export of heterologous proteins. We observed improved overall yields of secreted protein and export of firefly luciferase that was not functionally secreted via Cts1-fusions. As a proof-of-principle for pharmaceutical proteins we exported functional nanobodies directed against the receptor-binding domain (RBD) of the SARS-CoV2 spike protein. The novel carrier thus constitutes an important improvement of our expression system towards a competitive production platform.

RESULTS

Jps1 is a Potent New Carrier for Unconventional Protein Export

Previous experiments had shown that Jps1 co-localizes with Cts1 in the fragmentation zone (Reindl et al., 2020), suggesting that it might also be unconventionally secreted. To study this, we applied the well-established β-glucuronidase (Gus) reporter system (**Figure 1A,B**). This bacterial enzyme is largely inactivated upon secretion through the eukaryotic endomembrane system. By contrast, it is released in a functional state via unconventional secretion in yeast cells of *U. maydis* (Stock et al., 2012). To assay unconventional secretion of Jps1, a strain expressing a Gus-Jps1 fusion protein was generated in the background of the octuple protease-deletion laboratory strain AB33P8Δ (**Figure 1A**) (Terfrüchte et al., 2018). Microscopic analysis revealed that yeast cells expressing Gus-Jps1 did not show any morphological differences as compared to the progenitor (**Supplementary Figures S1, S2**). The Gus-Jps1 fusion did also not disturb Cts1 function as detected by determining extracellular chitinase activity of AB33P8Δ/Gus-Jps1 which was similar to the activity detected in a strain expressing Gus-Cts1 (**Supplementary Figure S1**). Subsequently, intra- and extracellular Gus activity was determined (**Figures 1C,D**). The progenitor strain AB33P8Δ was used as a negative control, while a strain expressing intracellular Gus served as a lysis control (AB33 Gus^{cyt}) (Stock et al., 2012). High Gus activity was present in cell extracts of all strains harboring the Gus enzyme but not in the progenitor AB33P8Δ lacking the enzyme (**Figure 1C**). Importantly, Gus activity was also detected in the supernatant of Gus-Jps1 expressing strains but not for the lysis control, confirming unconventional secretion of Jps1 (**Figure 1D**). At the same time, this experiment demonstrates, that Jps1—similar to Cts1—is able to act as a carrier for heterologous proteins. Notably, extracellular Gus activity levels were increased by about 2-fold in culture supernatants of Gus-Jps1 compared to Gus-Cts1 expressing strains (**Figure 1D**), suggesting that Jps1 might constitute a more effective carrier than Cts1. Both strains were also compared in terms of growth speed and strain fitness using online monitoring in a BioLector device (m2p-labs, Baesweiler, Germany) (Funke et al., 2010). The progenitor strain AB33P8Δ as well as AB33P8Δ/Gus-Cts1 and AB33P8Δ/Gus-Jps1 showed similar proliferation patterns and doubling times of about 3 h

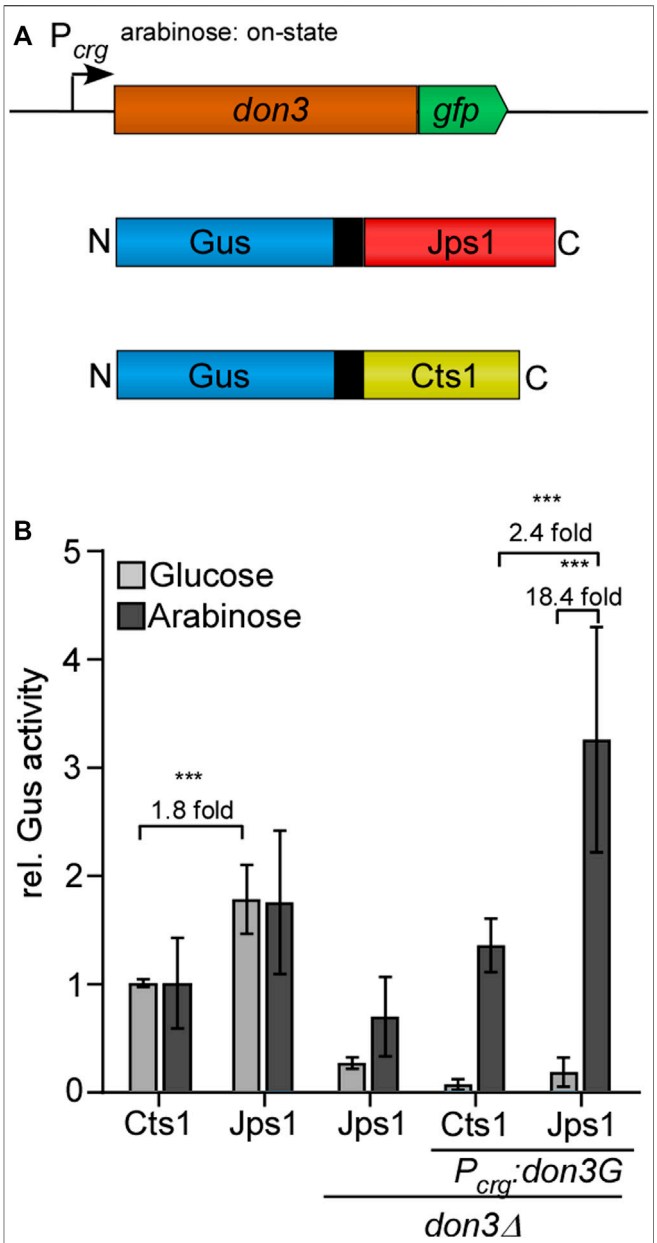


FIGURE 2 | Inducible secretion of Gus-Jps1 via transcriptional regulation of *don3*. **(A)** Schematic display of the inducible secretion system. *don3-gfp* is expressed under control of the arabinose-inducible promoter P_{crg}. Under glucose conditions the promoter is in its "off state", unconventionally secreted proteins under control of P_{oma} are thus expressed but not secreted. Under arabinose condition the promoter is in its "on state" and proteins are secreted. Gus is fused to either Cts1 or Jps1 including an internal SHH tag (double Strep, ten times His, triple HA). **(B)** Gus activity in culture supernatants of AB33 derivatives expressing Gus-Cts1 or Gus-Jps1 and their Δ*don3* variants. Enzymatic activity was normalized to average values of positive controls secreting Gus-Cts1 constitutively. The diagram represents the results of three biological replicates. Error bars depict standard deviation. Fold change of induced cultures depicted over brackets. Definition of statistical significance (***): *p*-value < 0.05. *p*-value derived from Student's unpaired *t*-test.

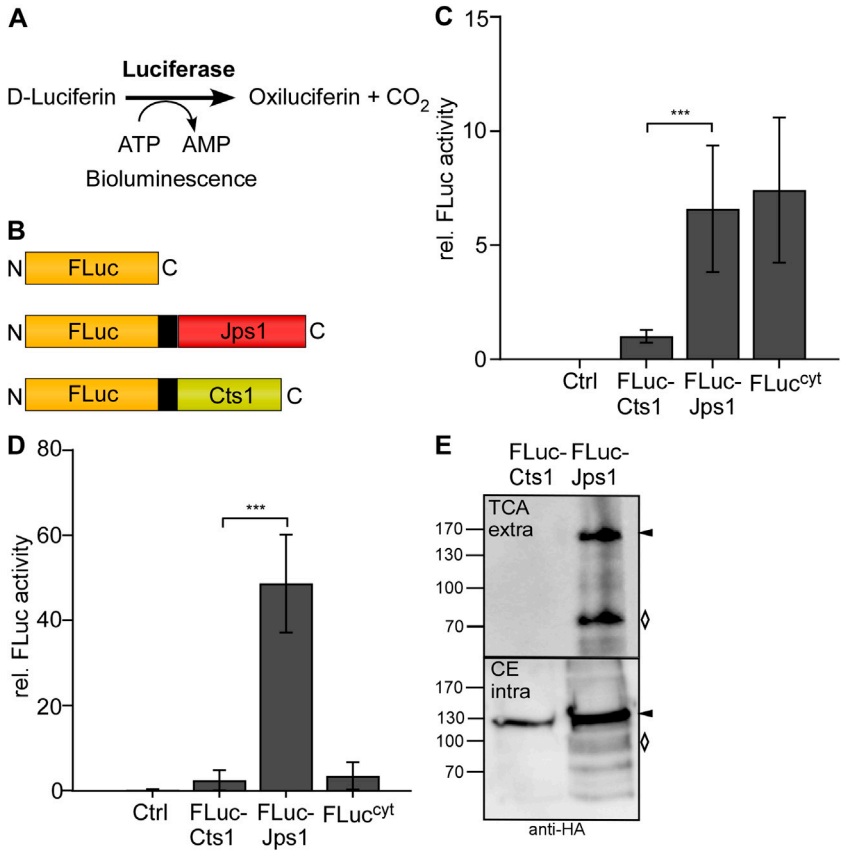


FIGURE 3 | Efficient Jps1-mediated export of firefly luciferase as a new reporter for unconventional secretion. **(A)** Schematic display of the proteins expressed to study unconventional secretion. Cytoplasmic FLuc ($FLuc^{cyt}$) was used as a lysis control (top). FLuc-Jps1 (middle) and FLuc-Cts1 (bottom) harbor the respective carrier proteins at the C-terminus. All proteins carry an SHH tag indicated in black (Sarkari et al., 2014). All schemes are drawn to scale. **(B)** Enzymatic reaction mediated by firefly luciferase: D-Luciferin and ATP are converted to oxiluciferin, AMP and CO_2. During this reaction excited intermediates emit energy in the form of light that can be detected as bioluminescence. **(C)** Comparison of intracellular FLuc activity of the strains AB33P8Δ/FLuc-Cts1 and AB33P8Δ/FLuc-Jps1. Enzymatic activity was normalized to average values of strain secreting FLuc-Cts1. The progenitor strain AB33P8Δ was used as a negative control. Strain AB33 $FLuc^{cyt}$ with intracellular FLuc expression dealt as positive control. Three biological replicates are shown. **(D)** Comparison of extracellular FLuc activity of strains harboring either Cts1 or Jps1 as a carrier. Enzymatic activity was normalized to average values of strain secreting FLuc-Cts1. Strain AB33 $FLuc^{cyt}$ with intracellular FLuc expression dealt as lysis control. Three biological replicates are shown. Error bars in figures **(C)** and **(D)** indicate standard deviation. Definition of statistical significance (***): p-value < 0.05. p-value was derived from Student's unpaired t-test. **(E)** Representative Western blot of FLuc-Cts1 and FLuc-Jps1. Secreted protein was enriched from the supernatant by TCA precipitation. Intracellular protein levels were visualized by cell extracts. Western blots show 1 ml of precipitated supernatants (TCA) and 10 µg cell extracts (CE). Full length protein signals indicated by arrows, degradation bands with a rhombus.

during the exponential growth phase when incubated in CM medium supplemented with 1% glucose (**Supplementary Figure S2**). Thus, Jps1 constitutes a promising candidate for a novel potent carrier for heterologous proteins.

To assay secretion on the protein level, Western blot analyses were conducted. These experiments showed that extracellular amounts of Gus-Jps1 were markedly increased as compared to Gus-Cts1, while intracellular levels were comparable. This confirms that Jps1 is secreted with enhanced efficiency in relation to Cts1 (**Figure 1E**, **Supplementary Figure S3**). To quantify this result distinct amounts of Multiple Tag protein (GenScript Biotech, Piscataway, NJ, United States) were included (**Supplementary Figure S4**). Quantification of the Western blot signals revealed that Gus-Cts1 levels in the supernatant reach concentrations of 38 µg/L while Gus-Jps1 is present at about 103 µg/L (about 2.7-fold increase; **Figure 1F**). In summary, these results demonstrate that Jps1 can deal as a powerful carrier for heterologous proteins with elevated levels in comparison to Cts1.

don3 Induced Secretion Further Enhances Gus-Jps1 Secretion

Recently, we have established a system that allows for the induction of unconventional secretion via regulation of kinase Don3, a gatekeeper of the fragmentation zone (Hussnaetter et al., 2021). To this end we used a arabinose-inducible promoter to control *don3* expression, which is prerequisite for secondary septum formation (Weinzierl et al., 2002). Unconventional secretion is only functional with a functional fragmentation zone consisting of two septa (Aschenbroich et al., 2019). Here

we reproduced these findings using Jps1 as a carrier as demonstrated by a strain which carried genetic modifications for transcriptional induction of *don3* and expressed the Gus-Jps1 reporter as read-out (**Figure 2A,B**) (Hussnaetter et al., 2021). Although we observed a slightly higher background activity in arabinose cultures, the induction was more than 18-fold and thus, significantly higher than for using Cts1 as a carrier protein, showing about 12-fold induction (**Figure 2B**). Furthermore, Gus-activity was elevated 2.4-fold compared to induced Gus-Cts1 secretion and more than 3-fold compared to regular Gus-Cts1 secretion. Hence, inducible Jps1 constitutes a powerful tool for unconventional secretion of heterologous proteins. Jps1 enables export of functional firefly luciferase.

Jps1 Enables Export of Functional Firefly Luciferase

Photinus pyralis luciferase FLuc was recently established for intracellular use in *U. maydis* (Müntjes et al., 2020). Bioluminescence would be a straight-forward alternative read-out for unconventional secretion because the signal can be detected directly from the culture broth while the established reporters Gus and β-galactosidase (LacZ) require more elaborate biochemical assays. Further advantages are low background signals and the use of the inexpensive substrate D-luciferin **Figure 3A** (Miska and Geiger 1987). To test bioluminescence as a read-out for unconventional secretion, an expression strain producing FLuc-Cts1 was generated in the background of the octuple protease deletion strain (AB33P8Δ/FLuc-Cts1). Similarly, a FLuc-Jps1 expressing strain was generated (AB33P8Δ/FLuc-Jps1) to evaluate the effect of the alternative carrier (**Figure 3B**). AB33 producing intracellular luciferase ($FLuc^{Cyt}$) was used as a positive control in all assays (Müntjes et al., 2020). Monitoring of proliferation revealed that growth speed was slightly reduced in AB33P8Δ/FLuc-Jps1 with a doubling time of 3.5 h, compared to the progenitor strain AB33P8Δ and AB33P8Δ/FLuc-Cts1 showing doubling times of 3 h in the exponential growth phase (**Supplementary Figure S2**). The slight reduction might eventually be caused by a minor increase in the number of abnormal cells growing in clusters in the FLuc-Jps1 expressing strain (**Supplementary Figure 2C**). Luciferase assays showed that intracellular activity was very low in the FLuc-Cts1 expressing strain compared to the strain producing cytoplasmic FLuc, while levels of Fluc-Jps1 expressing strains were comparable to the cytoplasmic control showing significant activity (**Figure 3C**). Importantly, in culture supernatants the observed effect was even more pronounced and extracellular FLuc activity for the strain producing FLuc-Jps1 was about 48-fold higher than activity of FLuc-Cts1 secreting cells for which no significant difference to the control strain could be observed (**Figure 3D**). These results were confirmed in Western blot analyses. While intracellular levels of FLuc-Cts1 were reduced in comparison to FLuc-Jps1 which showed an about 1.8-fold higher signal intensity, only FLuc-Jps1 was detectable in supernatants (**Figure 3E**; **Supplementary Figure S3**). This demonstrates that not only expression of FLuc-Cts1 was impaired but also detectable Cts1 based secretion was absent. The reason for the differential performance of the Cts1 and Jps1 fusions with FLuc remains unclear. The size of the FLuc-Cts1 fusion protein is likely not affecting its unconventional secretion, since larger fusions had been successfully exported in earlier studies (Stock et al., 2012). Eventually, structural interferences or other unknown features of this particular fusion lead to reduced protein production or its instability. These results further emphasize the advantage of having a second carrier for the unconventional secretion system at hands.

Unconventional Secretion of Functional Antibodies Against Sars-CoV2-Receptor Binding Domain

Next, we tested unconventional secretion of nanobodies directed against the SARS-CoV2 spike protein receptor binding domain (RBD) as a timely example of pharmaceutically relevant targets. Therefore, strains were generated in which two synergistic synthetic nanobodies (sybodies) directed against the Sars-CoV2 spike-RBD were combined (Walter et al., 2020). The bi-specific sybody was tagged with a 10× His-linker for purification and fused to either Cts1 or Jps1 for unconventional secretion (AB33P8Δ/$Sy^{68/15}$-Cts1 and AB33P8Δ/$Sy^{68/15}$-Jps1) (**Figure 4A**). Western blot analyses confirmed that both fusion proteins were synthesized. However, $Sy^{68/15}$-Cts1 was produced at a lower level compared to $Sy^{68/15}$-Jps1. The latter showed stronger degradation than observed for other Jps1 fusion proteins (see above). In supernatants only a very faint signal was present for $Sy^{68/15}$-Cts1 while for $Sy^{68/15}$-Jps1 a stronger signal and less degradation than in cell extracts was detected (**Figure 4B**). Quantification revealed an increase of about 18-fold in signal intensity for the Jps1 full-length fusion compared to the Cts1 full-length fusion (**Supplementary Figure S3**). Subsequently, the antigen-binding activity of the sybody was determined via direct confrontation with spike-RBD immobilized on ELISA plates and subsequent detection with an antibody sandwich **Figure 4C**. Immobilized bovine serum albumin (BSA) dealt as a negative control. ELISA experiments using cell extracts demonstrated that both sybody-fusion proteins were functional in detecting the cognate antigen. While the activity of $Sy^{68/15}$-Cts1 was only slightly above baseline, $Sy^{68/15}$-Jps1 showed strong volumetric activity (**Figure 4D**). Next, sybody-fusion proteins were IMAC purified from culture supernatants and applied to ELISA in up to 10-fold concentrated solutions **Figure 4E**. While no activity could be observed for $Sy^{68/15}$-Cts1, $Sy^{68/15}$-Jps1 showed volumetric binding activity on the antigen, confirming the secretion of the functional sybody fusion protein. Thus, pharmaceutically relevant nanobodies were exported in their functional form using Jps1 as a carrier for unconventional secretion.

DISCUSSION

Here we successfully evaluated the potential anchoring factor Jps1 as a novel carrier for the export of heterologous proteins by unconventional secretion in *U. maydis*. Carrier proteins are ubiquitously used in fungal protein expression systems based on conventional secretion (Fleissner and Dersch 2010). This is mainly due to the observation that homologous proteins like

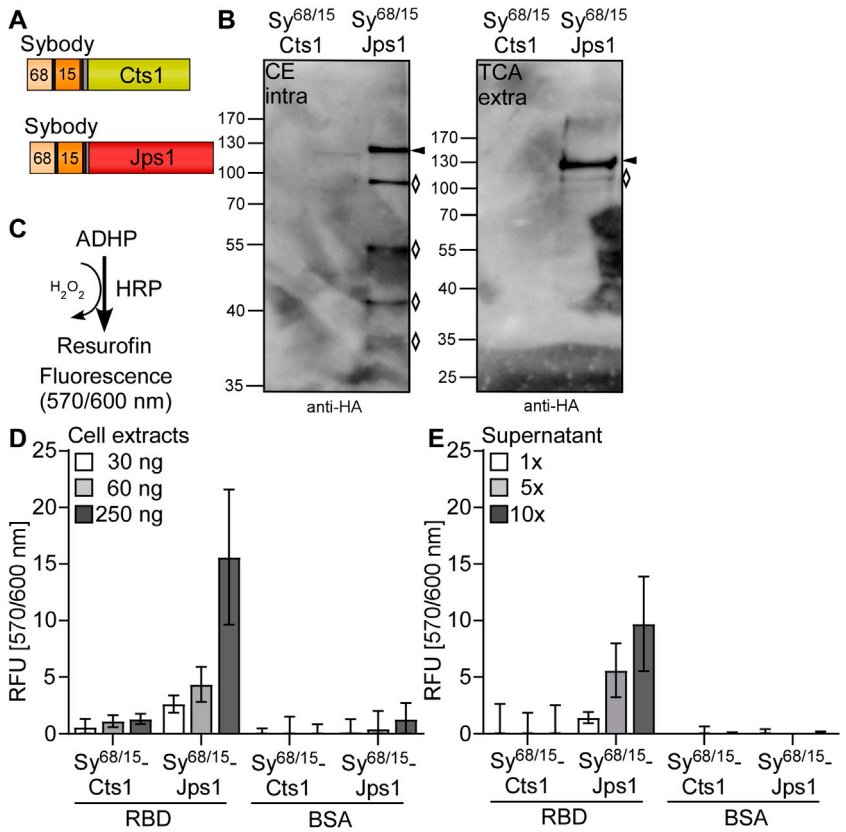


FIGURE 4 | Export of functional bi-specific Sars-CoV2 sybodies using Jps1 as a carrier for unconventional secretion. **(A)** Bi-specific anti SARS-CoV2 spike-RBD sybodies sy#15 and sy#68 (Walter et al., 2020) were tagged with a 10x His tag and fused to either Cts1 (top) or Jps1 (bottom) via a TEV protease cleavage site and an HA-tag. **(B)** Detection reaction for ELISA: Colorless 10-acetyl-3,7-dihydrophenoxazine (ADHP) is converted by horseradish peroxidase (HRP) using H_2O_2 to resurofin, a purple substance that emits strong fluorescence (excitation 570 nm, emission 600 nm). **(C)** Representative Western blot analyses of $Sy^{68/15}$-Cts1 and $Sy^{68/15}$-Jps1. Secreted protein was enriched from the supernatant by TCA precipitation. Intracellular protein levels were visualized by cell extracts. Western blots show 1 ml of precipitated supernatants (TCA) and 10 µg cell extracts (CE). Full length protein signals indicated by arrows, degradation bands with rhombi. **(D)** ELISA of cell extracts: 1 µg of RBD was immobilized per well. 1 µg BSA dealt as a negative control. Baseline was established by a well coated with RBD and only treated with anti-HA and anti-mouse-HRP. Serial dilutions of *U. maydis* cell extracts (30 ng, 60 ng, 250 ng per well) were applied in technical triplicates both to RBD and BSA coated wells. Detection was carried out with the before mentioned anti-HA-mouse and anti-mouse-HRP antibodies. Three biological replicates are shown. Error bars indicate standard deviation of biological replicates. **(E)** ELISA of protein purified from supernatants: ELISA wells were coated, and reactions detected as described in **(D)**. Culture supernatants containing sybody-fusion proteins were subjected to $Nickel^{2+}$-NTA IMAC and subsequently concentrated up to 10-fold. Serial dilutions of supernatants (1-fold, 5-fold, 10-fold concentrated supernatant) were mixed with blocking solution and added to ELISA wells in technical triplicates. Three biological replicates are shown. Error bars indicate standard deviation for biological replicates.

hydrolytic enzymes are secreted with very high titers compared to heterologous targets (Nevalainen and Peterson 2014). In our system, similar to the previously used carrier chitinase Cts1, Jps1 was fused to the C-terminus of heterologous target proteins to mediate their export via the fragmentation zone. Of note, one exception identified during this study was the reporter enzyme LacZ: Here, a LacZ-Cts1 fusion is functional and unconventionally secreted (Reindl et al., 2020) while strains producing the respective LacZ-Jps1 fusion showed growth retardation and were lacking detectable LacZ activity and LacZ-Jps1 protein in the culture supernatant (results not shown). We anticipate that this could be related to the formation of tetramers by LacZ which interfere with Jps1 but not with Cts1 secretion; however, this hypothesis needs to be verified. Nevertheless, the discovery of a second carrier for unconventional secretion in *U. maydis* is a very favorable addition to our expression system (Reindl et al., 2019; Wierckx et al., 2021): The choice between the two fusion proteins, Cts1 and Jps1, will greatly enhance the repertoire of our secretion targets. Jps1 proofed valuable for the export of proteins that were not secreted at significant levels as Cts1 fusions and showed promising secretion levels for these targets. This is for example true for the firefly luciferase FLuc or the bi-specific sybodies that were only secreted efficiently when fused to Jps1. As a positive side effect, the FLuc-Jps1 fusion protein is a valuable alternative that allows a quick and inexpensive quantification of unconventional secretion via Jps1 in future studies (Wider and Picard 2017; Branchini et al., 2018). On the contrary, the intrinsic feature of chitin binding of Cts1 is very attractive as a tool which can be developed for efficient *in situ* protein purification from culture broth (Terfrüchte et al., 2017). Hence, both carriers show

distinct advantages that can be exploited depending on the actual demands.

In line with our results, different carriers show varying efficiencies in other fungal systems. For example, glycoamylase or α-amylase have been described as a powerful tool for heterologous protein secretion in filamentous fungi like Aspergilli (Ward et al., 1990; Nakajima et al., 2006). Similarly, the choice of the conventional signal peptide for efficient entry into the endoplasmic reticulum has been described as a key factor for improving conventional secretion (Xu et al., 2018; Wang et al., 2020). While existence of a signal peptide remains elusive for lock-type unconventional secretion (Stock et al., 2012), it is conceivable that other unconventionally secreted proteins are still to be discovered that might constitute even more powerful carriers. Currently, we do not have a precise idea on why Jps1 mediates export of heterologous proteins more effectively than Cts1. Further studies on the molecular roles of Jps1 during Cts1 secretion might resolve this question in the future. Notably, unconventional secretion was also observed for septation factor Don3 (Aschenbroich et al., 2019) which may thus serve as such alternative carrier. However, Gus activity levels of unconventionally secreted Gus-Don3 are minute compared to Gus-Cts1, suggesting that it does not constitute a promising alternative (Aschenbroich et al., 2019). Hence, it is important to further study the mechanism of lock-type secretion and in particular, to identify further players that localize to the fragmentation zone for export during cytokinesis (Reindl et al., 2019; Wierckx et al., 2021).

The successful synthesis and functional export of nanobodies directed against the RBD of the surface spike protein of the SARS-CoV2 virus is a timely new addition to the repertoire of secreted targets. The current pandemics situation underpinned that it is important to develop novel methodology for quick, specific, and sensitive detection and treatment of viral infections in the future. On the one hand nanobodies are potent proteins for antigen detection (Muyldermans 2013) and thus very promising tools in the context of SARS-CoV2 detection. On the other hand, antibody-based pharmaceuticals like Casirivimab and Imdevimab are already used to treat COVID-19 infection (Sun and Ho 2020). Therefore, besides application in virus diagnostics, nanobodies directed against SARS-CoV2 could potentially even become novel pharmaceutical targets for therapeutic approaches (Dubey et al., 2020). The unique system of unconventional secretion in *U. maydis* now offers new possibilities for nanobody production without the risk of undesired modifications by *N*-glycosylation (Stock et al., 2012). This would eliminate the necessity to humanize llama derived nanobodies for safe use as pharmaceuticals to avoid allergic reaction in patients (Vincke et al., 2009; Dong et al., 2020). To achieve this, both the unconventional secretion system and specifically the production and application of nanobodies via this system have to be optimized, for example by further multimerization to increase valency and affinity (Wichgers Schreur et al., 2020; Koenig et al., 2021). By the establishment of a new carrier and export of functional SARS-CoV2 nanobodies we have thus laid a solid foundation for further exploitation and application of lock-type unconventional secretion.

MATERIAL AND METHODS

Molecular Biology Methods

All plasmids (pUMa/pUx vectors) generated in this study were obtained using standard molecular biology methods established for *U. maydis* including Golden Gate and Gibson cloning (Brachmann et al., 2004; Gibson 2011; Gibson et al., 2009; Terfrüchte et al., 2014). All plasmids were verified by restriction analysis and sequencing. Oligonucleotides applied for cloning are listed in **Table 1**. Genomic DNA of *U. maydis* strain UM521 was used as template for PCR reactions. The genomic sequence for this strain is stored at the EnsemblFungi database (EnsemblFungi). The generation of plasmids pUMa3329_Δupp1_P_{crg}-eGfp-T_{nos}-natR, pUMa2113_pRabX1-P_{oma}_gus-SHH-cts1, pUMa2240_Ip_Poma-his-αGfpllama-ha-Cts1-CbxR and pUMa3771_Δupp3_Potef_FLuc_NatR has been described previously (resulting strains, see **Table 2**). For the generation of pUMa3012_Ip_Poma_Gus-SHH-Jps1_CbxR the *jps1 gene* (*umag_03776*) was amplified from genomic DNA using primers oMB372 and oMB373 with AscI and ApaI hydrolyzation sites. Subsequently, the backbone of pUMa2113_Ip_Poma_Gus-SHH-Cts1_CbxR was used for restriction ligation cloning and *jps1* was inserted into the backbone instead of *cts1*. pUMa4131_Ip_Poma_FLuc-SHH-Cts1_CbxR was generated by amplification of the *U. maydis* dicodon-optimized *P. pyralis fluc* gene from pUMa3771_Δupp3_Potef_FLuc_NatR using oAB297 and oAB298 with BamHI and SfiI hydrolyzation sites. pUMa2113_Ip_Poma_Gus-SHH-Cts1_CbxR was then hydrolyzed with BamHI and SfiI and *fluc* was inserted into the backbone instead of *gus* via restriction/ligation cloning. A restriction/ligation cloning approach was applied for pUMa4566_Ip_Poma_FLuc-SHH-Jps1_CbxR. *jps1* was excised from pUMa3012_Ip_Poma_Gus-SHH-Jps1_CbxR using AscI and ApaI and inserted into pUMa4131_Ip_Poma_FLuc-SHH-Cts1_CbxR, also hydrolyzed with AscI and ApaI. pUx1_Ip_Poma-Sy#68-his-Sy#15-ha-Cts1-CbxR was generated by amplification of genes $sy^{\#68}$ and $sy^{\#15}$ (Walter et al., 2020) from a synthetic gBlock (Integrated DNA Technology, Coralville, Iowa, United States) using primers oAB908 and oAB909 for $sy^{\#15}$ adding BamHI and SpeI hydrolyzation sites and oCD234 and oCD235 for $sy^{\#68}$ with complementary overhangs for Gibson cloning. Subsequently, pUMa2240_Ip_Poma-his-αGfpllama-ha-Cts1-CbxR (Terfrüchte et al., 2017) was hydrolyzed with BamHI and SpeI and gene $sy^{\#15}$ was inserted via restriction ligation cloning, replacing *αgfpllama* and thereby generating pUMa4678. pUMa4678 was then hydrolyzed with BamHI and the sequence encoding for $sy^{\#68}$ was inserted via Gibson cloning (Gibson et al., 2009), generating pUx1. For the generation of pUx8 *jps1* was excised from pUMa3012 using AscI and ApaI and inserted into the AscI and ApaI hydrolyzed backbone of pUx1.

TABLE 1 | DNA oligonucleotides used in this study.

Designation	Nucleotide sequence (5′- 3′)
oMB372_jps1_fw	TTAGGCGCGCCATGCCAGGCATCTCC
oMB373_jps1_rev	TTAGGGCCCTTAGGATTCCGCATCGATTGGGG
oMF502_ip_fw	ACGACGTTGTAAAACGACGGCCAG
oMF503_ip_rev	TTCACACAGGAAACAGCTATGACC
oAB297_fluc_fw	AAATTGGATCCATGGAGGACGCCAAGAACATCAAG
oAB298_fluc_rev	AATAGGCCGCGTTGGCCACGGCGATCTTGCCACCCTT
oAB908_sy$^{\#15}$_fw	ATATAGGATCCATGGCGGCCCATCACCACCATCACC ACCATCACCACCATCATATGCAGGTGCAGCTCG
oAB909_sy$^{\#15}$_rev	ATATAACTAGTCGAGACGGTGACCTGGGTGC
oCD234_sy$^{\#68}$_fw	CTACCTTACTCTATCAGGATCATGCAGGTGCAGCTC GTCG
oCD235_sy$^{\#68}$_rev	GGTGATGGGCCGCCATGGATCCCGAGACGGTGACCT GGGTGC

Strain Generation

U. maydis strains used in this study were obtained by homologous recombination yielding genetically stable strains (Bösch et al., 2016) (**Table 2**). For genomic integrations at the *ip* locus, integrative plasmids were used (Stock et al., 2012). These plasmids contained the ip^r allele, promoting carboxin resistance. For integration, plasmids were linearized within the ip^r allele to allow for homologous recombination with the ip^s locus. For transformation, integrative plasmids were hydrolyzed within the ip^r locus using the restriction endonuclease SspI, resulting in a linear DNA fragment. For genetic modifications in other loci, plasmids with about 1 kb flanking regions and a resistance cassette were generated (Brachmann et al., 2004; Terfrüchte et al., 2014). For transformation, the insertion cassette was excised from the plasmid backbone using SspI or SwaI (Terfrüchte et al., 2014). For all genetic manipulations, *U. maydis* protoplasts were transformed with linear DNA fragments for homologous recombination. All strains were verified by Southern blot analysis (Southern 1974). For *in locus* modifications the flanking regions were amplified as probes. For *ip* insertions, the probe was obtained by PCR using the primer combination oMF502/oMF503 and the template pUMa260 (Keon et al., 1991; Brachmann et al., 2004). Primer sequences are listed in **Table 1**.

Cultivation

U. maydis strains were grown at 28°C in complete medium supplemented with 1% (w/v) glucose (CM-glc) or with 1% (w/v) arabinose (CM-ara) if not described differently (Holliday 1974; Tsukuda et al., 1988). Solid media were supplemented with 2% (w/v) agar agar. Growth phenotypes were evaluated using the BioLector microbioreactor (m2p-labs, Baesweiler, Germany) (Funke et al., 2010). MTP-R48-B(OH) round plates were inoculated with 1.5 ml culture per well and incubated at 1,000 rpm at 28°C. Backscatter light with a gain of 25 or 20 was used to determine biomass.

Quantification of Unconventional Secretion Using the Gus Reporter

Extracellular Gus activity was determined to quantify unconventional Cts1 secretion using the specific substrate 4-methylumbelliferyl β-D galactopyranoside (MUG, bioWORLD, Dublin, OH, United States)) (Koepke et al., 2011; Stock et al., 2012; Stock et al., 2016). Cell-free culture supernatants were mixed 1:1 with 2× Gus assay buffer (10 mM sodium phosphate buffer pH 7.0, 28 μM β-mercaptoethanol, 0.8 mM EDTA, 0.0042% (v/v) lauroyl-sarcosin, 0.004% (v/v) Triton X-100, 2 mM MUG, 0.2 mg/ml (w/v) BSA) in black 96-well plates. Relative fluorescence units (RFUs) were determined using a plate reader (Tecan, Männedorf, Switzerland) for 100 min at 28°C with measurements every 5 min (excitation/emission wavelengths: 365/465 nm, gain 60). For quantification of conversion of MUG to the fluorescent product 4-methylumbelliferone (MU), a calibration curve was determined using 0, 1, 5, 10, 25, 50, 100, 200 μM MU.

Determination of Extracellular Cts1 Activity

Extracellular Cts1 activity was analyzed using 4-methylumbelliferyl β-D cellobioside (MUC, Sigma-Aldrich, Billerica, MA, United States) as a substrate (Koepke et al., 2011). Whole cell cultures were mixed 3:7 with KHM Buffer (110 mM CH_3CO_2K, 20 mM HEPES, 2 mM $MgCl_2$, 2 mM MUC) in black 96 well plates. Relative fluorescence units were determined using a plate reader (Tecan, Männedorf, Switzerland) by fluorescence measurement at 28°C for 100 min every 2 min (360 nm excitation and 450 nm emission, gain 100).

Quantification of Unconventional Secretion Using Luciferase Reporter

Extra- and intracellular luciferase activity was determined using D-luciferin (Biosynth Carbosynth, Compton, United Kingdom). Cell-free supernatants or whole cell cultures in CM medium were mixed 8:2 with luciferin substrate mix (20 mM tricine, 2.67 mM $MgSO_4{\times}7H_2O$, 0.1 mM EDTA×2 H_2O, 33.3 mM DTT, 0.524 mM ATP, 0.269 mM acetyl-CoA, 0.467 mM D-luciferin, 5 mM NaOH, 0.264 mM $MgCO_3{\times}5H_2O$) in white 96-well plates. Relative photon count (RPC) was determined using a Mithras LB 940 plate reader (Berthold technologies, Bad Wildbad, Germany) for 20 min with measurements every 20 s.

Quantification of Unconventional Secretion by Western blot analysis

Gus-Cts1 and Gus-Jps1 secretion was analyzed by trichloroacetic acid (TCA) precipitation of culture broths. 1 ml of cell-free supernatants of cultures grown in Verduyn medium (55.5 mM Glucose, 74.7 mM NH_4Cl, 0.81 mM $MgSO_4{\times}7H_2O$, 0.036 mM $FeSO_4{\times}7H_2O$, 36.7 mM KH_2PO_4, 100 mM MES pH 6.5, 0.051 mM EDTA, 0.025 mM $ZnSO_4{\times}7H_2O$, 0.041 mM $CaCl_2$, 0.016 mM H_3bBO_3, 6.7 μM $MnCl_2{\times}2H_2O$, 2.3 μM $CoCl_2{\times}6H_2O$, 1.9 μM $CuSO_4{\times}5H_2O$, 1.9 μM $Na_2MoO_4{\times}2H_2O$, 0.6 μM KI) to an OD_{600} of 3 were chilled on ice and mixed with 400 μl 50% (v/v) TCA solution and incubated on ice at 4°C overnight. Subsequently, precipitated protein pellets were harvested by centrifugation at 11,000 x g at 4°C for 30 min. Supernatants were discarded and pellets were washed with 300 μl of-20°C acetone followed by centrifugation at 11,000 × g at 4°C for

TABLE 2 | *U. maydis* strains used in this study.

Strains	Relevant genotype/Resistance	Strain collection no. (UMa[a])	Plasmids transformed/Resistance[b]	Manipulated locus (*umag* gene identifier)	Progenitor (UMa[a])	References
AB33	*a2 P_{nar}bW2bE1 PhleoR*	133	pAB33	*b*	FB2 (55)	Brachmann et al. (2004)
AB33 Gus-Cts1	*a2 P_{nar}bW2bE1 PhleoR ip^S(P_{oma}gus:shh:cts1)ip^{R}CbxR*	1289	pUMa2113/CbxR	*ip*	133	Sarkari et al. (2014)
AB33don3Δ/Gus-Cts1	*a2 P_{nar}bW2bE1 PhleoR ip^S(P_{oma}gus:shh:cts1)ip^R CbxR umag_don3Δ_HygR*	1742	pUMa2717/HygR	*umag_05543 (don3)*	1289	Aschenbroich et al. (2019)
AB33don3Δ	*a2 P_{nar}bW2bE1 PhleoR umag_don3Δ_HygR*	2028	pUMa2717/HygR	*umag_05543 (don3)*	133	Aschenbroich et al. (2019)
AB33don3Δ/P_{crg}don3-gfp/Gus-Cts1	*a2 P_{nar}bW2bE1 PhleoR ip^S(P_{oma}gus:shh:cts1)ip^R CbxR umag_don3Δ_HygR upp1:(P_{crg}don3:gfp) NatR*	2302	pUMa3330/NatR	*umag_02178* (*upp1*)	1742	Aschenbroich et al. (2019)
AB33P8ΔGus-Cts1	*a2 P_{nar}bW2bE1 PhleoR FRT10(um04641Δ:hyg) FRT11(um03947Δ) FRT6(um03975Δ) FRT5(um04400Δ) FRT3(um11908Δ) FRT2(um00064Δ) FRTwt[um02178Δ) FRT1(um04926Δ) HygR ip^S(P_{oma}gus:shh:cts1)ip^R CbxR*	2418	pUMa2113	*lp*	2413	Terfrüchte et al. (2018)
AB33don3Δ/Gus-Jps1	*a2 P_{nar}bW2bE1 PhleoR ip^S(P_{oma}gus:shh:cts1)ip^R CbxR umag_don3Δ_HygR*	2734	pUMa3012	*lp*	2028	This study
AB33don3Δ/P_{crg}don3-gfp/Gus-Jps1	*a2 P_{nar}bW2bE1 PhleoR ip^S(P_{oma}gus:shh:cts1)ip^R CbxR umag_don3Δ_HygR upp1:(P_{crg}don3:gfp) NatR*	2776	pUMa3330/NatR	*umag_02178* (*upp1*)	2734	This study
AB33P8ΔGus-Jps1	*a2 P_{nar}bW2bE1 PhleoR FRT10(um04641Δ:hyg) FRT11(um03947Δ) FRT6(um03975Δ) FRT5(um04400Δ) FRT3(um11908Δ) FRT2(um00064Δ) FRTwt[um02178Δ) FRT1(um04926Δ) HygR ip^S(P_{oma}gus:shh:jps1)ip^R CbxR*	2900	pUMa3012	*lp*	2413	this study
AB33P8Δ FLuc-Cts1	*a2 P_{nar}bW2bE1 PhleoR FRT10(um04641Δ:hyg) FRT11(um03947Δ) FRT6(um03975Δ) FRT5(um04400Δ) FRT3(um11908Δ) FRT2(um00064Δ) FRTwt[um02178Δ) FRT1(um04926Δ) HygR ip^S(P_{oma}fluc:shh:cts1)ip^R CbxR*	3151	pUMa4131	*lp*	2413	this study
AB33P8Δ FLuc-Jps1	*a2 P_{nar}bW2bE1 PhleoR FRT10(um04641Δ:hyg) FRT11(um03947Δ) FRT6(um03975Δ) FRT5(um04400Δ) FRT3(um11908Δ) FRT2(um00064Δ) FRTwt[um02178Δ) FRT1(um04926Δ) HygR*	3214	pUMa4566	*ip*		this study

(Continued on following page)

TABLE 2 | (*Continued*) *U. maydis* strains used in this study.

Strains	Relevant genotype/Resistance	Strain collection no. (UMa[a])	Plasmids transformed/Resistance[b]	Manipulated locus (*umag* gene identifier)	Progenitor (UMa[a])	References
AB33P8ΔSy#68/#15-Cts1	*ip^{S}(P$_{oma}$fluc:shh:jps1)ip^{R} CbxR a2 P$_{nar}$bW2bE1 PhleoR FRT10(um04641Δ:hyg) FRT11(um03947Δ) FRT6(um03975Δ) FRT5(um04400Δ) FRT3(um11908Δ) FRT2(um00064Δ) FRTwt[um02178Δ) FRT1(um04926Δ) HygR ip^{S}(P$_{oma}$antirbdsybody#68:his:antirbdsybody#15:ha:cts1)ip^{R} CbxR*	Ux1	pUx1	*ip*	2413	this study
AB33P8ΔSy#68/#15-Jps1	*a2 P$_{nar}$bW2bE1 PhleoR FRT10(um04641Δ:hyg) FRT11(um03947Δ) FRT6(um03975Δ) FRT5(um04400Δ) FRT3(um11908Δ) FRT2(um00064Δ) FRTwt[um02178Δ) FRT1(um04926Δ) HygR ip^{S}(P$_{oma}$antirbdsybody#68:his:antirbdsybody#15:ha:jps1)ip^{R} CbxR*	Ux8	pUx8	*ip*	2413	this study

[a]*Internal strain collection numbers (UMa/Ux codes).*

[b]*Plasmids generated in our working group are integrated in a plasmid collection and termed pUMa, or pUx plus a number as 4-digit number as identifier.*

20 min twice. Pellets were dried at room temperature and resuspended in Laemmli buffer containing 0.12 M NaOH. Resuspended pellets were denatured at 95°C for 10 min and then subjected to SDS-PAGE and Western blot analysis. To determine protein concentration obtained by TCA precipitation a standard ladder of 50, 100, 200 and 500 ng of Multiple Tag protein (GenScript Biotech, Piscataway, NJ, United States) was loaded onto the SDS-PAGE next to obtained samples. Western blot signals were quantified using image studio lite version 5.2 (Li-Cor Biosciences, Lincoln, NE, United States) and the standard curve obtained by quantification of Multiple Tag protein signals was used to determine protein concentrations in culture supernatants.

SDS PAGE and Western Blot Analysis

To verify protein production and secretion in cell extracts and supernatants, respectively, Western Blot analysis was used. 20 ml cultures were grown to an OD_{600} of 1.0 and harvested at 1,500 × g for 5 min in centrifugation tubes. Until further preparation, pellets were stored at −20°C. For preparation of cell extracts, cell pellets were resuspended in 1 ml cell extract lysis buffer (100 mM sodium phosphate buffer pH 8.0, 10 mM Tris/HCl pH 8.0, 8 M urea, 1 mM DTT, 1 mM PMSF, 2.5 mM benzamidine, 1 mM pepstatin A, 2× complete protease inhibitor cocktail (Roche, Sigma/Aldrich, Billerica, MA, United States) and cells were crushed by agitation with glass beads at 2,500 rpm for 12 min at 4°C. After centrifugation (11,000 × g for 30 min at 4°C), the supernatant was separated from cell debris and was transferred to a fresh reaction tube. Protein concentration was determined by Bradford assay (BioRad, Hercules, CA, United States) (Bradford 1976) and 10 µg total protein was used for SDS-PAGE. SDS-PAGE was conducted using 10% (w/v) acrylamide gels. Subsequently, proteins were transferred to methanol activated PVDF membranes using semi-dry Western blotting. SHH-tagged Gus-Cts1 was detected using a primary anti-HA (1:3,000, Millipore/Sigma, Billerica, United States). An anti-mouse IgG-horseradish peroxidase (HRP) conjugate (1:3,000 Promega, Fitchburg, United States) was used as secondary antibody. HRP activity was detected using the Amersham ™ ECL ™ Prime Western Blotting Detection Reagent (GE Healthcare, Chalfont St Giles, United Kingdom) and a LAS4000 chemiluminescence imager (GE Healthcare Life Sciences, Freiburg, Germany).

IMAC Purification of Supernatants

For the purification of recombinant unconventionally secreted protein from *U. maydis*, cells were grown in CM-glucose (1% w/v) medium buffered with 0.05 M MES pH 6.5.200 ml of culture supernatants were harvested at and OD_{600} of 0.8 by centrifugation at 5,000 × g for 3 min. Harvested supernatants were chilled to 4°C and treated with a protease inhibitor tablet of cOmplete protease inhibitor (Roche, Sigma/Aldrich, Billerica, MA, United States). 2 ml of $Nickel^{2+}$-NTA matrix was equilibrated with 50 ml lysis buffer (10 mM imidazole 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). 22 ml of 10 times concentrated lysis buffer were added to the supernatants and subsequently $Nickel^{2+}$-NTA matrix was added

to the supernatant. The mixture was batched by gentle stirring on a magnetic stirrer at 4°C for 1 h. Following batching supernatant flow-through was discarded via a PD-10 column. Matrix was collected in the PD-10 column during the process. Collected matrix was washed with 50 ml of wash buffer (lysis buffer, 20 mM Imidazole) and protein was eluted with 2 ml elution buffer (lysis buffer, 250 mM imidazole). In the last step supernatants were concentrated via Amicon Ultra 50 k 0.5 ml centrifugal filter devices (Merck Millipore, Burlington, Massachusetts, United States) and the buffer exchanged to PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) and applied for ELISA.

Enzyme-Linked Immunosorbent Assay

For detection of nanobody binding activity protein adsorbing 384-well microtiter plates (Nunc® Maxisorp™, ThermoFisher Scientific, Waltham, MA, United States) were used. Wells were coated with 1 µg commercially available Sars-CoV2 spike-RBD-domain protein (GenScript Biotech, Piscataway, NJ, United States). 1 µg BSA per well dealt as negative control (NEB, Ipswich, MA, United States). Samples were applied in a final volume of 100 µl coating buffer (100 mM Tris-HCL pH 8, 150 mM NaCl, 1 mM EDTA) per well at 4°C for at least 16 h. Blocking was conducted for at least 4 h at 4°C with 5% (w/v) skimmed milk in coating buffer. Subsequently, 5% (w/v) skimmed milk in PBS was added to defined protein amounts of nanobody samples from cell extracts or purified from culture supernatants and respective controls. 100 µl of sample was added to wells coated with Sars-CoV2 spike-RBD and BSA. The plate was incubated with samples and controls over night at 4°C. After 3x PBS-T (PBS supplemented with 0.05% (v/v) Tween-20, 100 µl per well) washing, a mouse anti-HA antibody (Millipore/Sigma, Billerica, United States) 1: 5,000 diluted in PBS supplemented with skimmed milk (5% w/v) was added (100 µl per well) and incubated for 2 h at room temperature. Then wells were washed again three times with PBS-T (100 µl per well) and incubated with an anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Promega, Fitchburg, United States) (50 µl per well) for 1 h at room temperature [1:5,000 in PBS supplemented with skimmed milk (5% w/v)]. Subsequently wells were washed three times with PBS-T and three times with PBS and incubated with Quanta Red™ enhanced chemifluorescent HRP substrate (50: 50:1, 50 µl per well) (ThermoFisher Scientific, Waltham, MA, United States) at room temperature for 15 min. The reaction was stopped with 10 µl per well Quanta RedTM stop solution and fluorescence readout was performed at 570 nm excitation and 600 nm emission using an Infinite M200 plate reader (Tecan, Männedorf, Switzerland).

Microscopic Analyses

Microscopic analyses were performed with immobilized early-log phase budding cells on agarose patches (3% w/v f. c.) using a wide-field microscope setup from Zeiss (Oberkochen, Germany) Axio Imager M1 equipped with a Spot Pursuit CCD camera (Diagnostic Instruments, Sterling Heights, United States) and the objective lenses Plan Neofluar (×40, NA 1.3), Plan Neofluar (63×, NA 1.25) and Plan Neofluar (100×, NA 1.4). The microscopic system was controlled by the software MetaMorph (Molecular Devices, version 7, Sunnyvale, United States). Image processing including rotating and cropping of images, scaling of brightness, contrast and fluorescence intensities as well as insertion of scale bars was performed with MetaMorph. Arrangement and visualization were performed with Canvas 12 (ACD Systems).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MP, KPH and MR designed the experiments. MP and KPH conducted the experiments. KS, KM and MF supervised the project. KS, MP and KM prepared the manuscript with input from all co-authors. MP and KH prepared figures and tables.

FUNDING

KH was supported by the CLIB-Competence Center Biotechnology (CKB) funded by the European Regional Development Fund ERDF (34. EFRE-0300096). KS and MR received funding from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)—Projektnummer 267205415—SFB 1208.

ACKNOWLEDGMENTS

We are thankful to B. Axler for excellent technical support of the project. We gratefully acknowledge support in microscopic imaging and analysis by N. Heßler.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.816335/full#supplementary-material

REFERENCES

Aschenbroich, J., Hussnaetter, K. P., Stoffels, P., Langner, T., Zander, S., Sandrock, B., et al. (2019). The Germinal centre Kinase Don3 Is Crucial for Unconventional Secretion of Chitinase Cts1 in Ustilago Maydis. *Biochim. Biophys. Acta (Bba)—Proteins Proteomics* 1867, 140154, 2019 . Epub 2018/10/15. doi:10.1016/j.bbapap.2018.10.007

Bösch, K., Frantzeskakis, L., Vraneš, M., Kämper, J., Schipper, K., and Göhre, V. (2016). Genetic Manipulation of the Plant Pathogen Ustilago Maydis to Study Fungal Biology and Plant Microbe Interactions. *JoVE* 30, 20160930. doi:10.3791/54522

Brachmann, A., König, J., Julius, C., and Feldbrügge, M. (2004). A Reverse Genetic Approach for Generating Gene Replacement Mutants in Ustilago Maydis. *Mol. Genet. Genomics* 272, 216–226. Epub 2004/08/19. doi:10.1007/s00438-004-1047-z

Bradford, M. M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* 72 (72), 248–254. Epub 1976/05/07. doi:10.1006/abio.1976.9999

Branchini, B. R., Southworth, T. L., Fontaine, D. M., Kohrt, D., Florentine, C. M., and Grossel, M. J. (2018). A Firefly Luciferase Dual Color Bioluminescence Reporter Assay Using Two Substrates to Simultaneously Monitor Two Gene Expression Events. *Sci. Rep.* 8 (8), 5990, 2018 . Epub 2018/04/18. doi:10.1038/s41598-018-24278-2

Dong, J., Huang, B., Jia, Z., Wang, B., Gallolu Kankanamalage, S., Titong, A., et al. (2020). Development of Multi-specific Humanized Llama Antibodies Blocking SARS-CoV-2/ace2 Interaction with High Affinity and Avidity. *Emerging Microbes & Infections* 9, 1034–1036. Epub 2020/05/15. doi:10.1080/22221751.2020.1768806

Dubey, A., Dahiya, S., Rouse, B. T., and Sehrawat, S. (2020). Perspective: Reducing SARS-CoV2 Infectivity and its Associated Immunopathology. *Front. Immunol.* 11, 11. doi:10.3389/fimmu.2020.581076

Flaschel, E., and Friehs, K. (1993). Improvement of Downstream Processing of Recombinant Proteins by Means of Genetic Engineering Methods. *Biotechnol. Adv.* 11, 31–77. doi:10.1016/0734-9750(93)90409-G

Fleissner, A., and Dersch, P. (2010). Expression and export: Recombinant Protein Production Systems for Aspergillus. *Appl. Microbiol. Biotechnol.* 87, 1255–1270. Epub 2010/06/10. doi:10.1007/s00253-010-2672-6

Funke, M., Buchenauer, A., Schnakenberg, U., Mokwa, W., Diederichs, S., Mertens, A., et al. (2010). Microfluidic Biolector-Microfluidic Bioprocess Control in Microtiter Plates. *Biotechnol. Bioeng.* 107, 497–505. Epub 2010/06/03. doi:10.1002/bit.22825

Gibson, D. G. (2011). Enzymatic Assembly of Overlapping DNA Fragments. *Methods Enzymol.* 498, 349–361. Epub 2011/05/24. doi:10.1016/B978-0-12-385120-8.00015-2

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., 3rd, and Smith, H. O. (2009). Enzymatic Assembly of DNA Molecules up to Several Hundred Kilobases. *Nat. Methods* 6, 343–345. Epub 2009/04/14. doi:10.1038/nmeth.1318

Holliday, R. (1974). "Ustilago Maydis," in *Handbook of Genetics* (New York, NY: Plenum Press), 575–595. doi:10.1007/978-1-4899-1710-2_31

Hussnaetter, K. P., Philipp, M., Müntjes, K., Feldbrügge, M., and Schipper, K. (2021). Controlling Unconventional Secretion for Production of Heterologous Proteins in Ustilago Maydis through Transcriptional Regulation and Chemical Inhibition of the Kinase Don3. *JoF* 7, 179, 2021 . Epub 2021/04/04. doi:10.3390/jof7030179

Kaplon, H., and Reichert, J. M. (2021). Antibodies to Watch in 2021. *mAbs* 13, 1860476. doi:10.1080/19420862.2020.1860476

Keon, J. P. R., White, G. A., and Hargreaves, J. A. (1991). Isolation, Characterization and Sequence of a Gene Conferring Resistance to the Systemic Fungicide Carboxin from the maize Smut Pathogen, Ustilago Maydis. *Curr. Genet.* 19, 475–481. Epub 1991/06/01. doi:10.1007/bf00312739

Koenig, P.-A., Das, H., Liu, H., Kümmerer, B. M., Gohr, F. N., Jenster, L.-M., et al. (2021). Structure-guided Multivalent Nanobodies Block SARS-CoV-2 Infection and Suppress Mutational Escape. *Science* 371, 371, 2021 . Epub 2021/01/14. doi:10.1126/science.abe6230

Koepke, J., Kaffarnik, F., Haag, C., Zarnack, K., Luscombe, N. M., König, J., et al. (2011). The RNA-Binding Protein Rrm4 Is Essential for Efficient Secretion of Endochitinase Cts1. *Mol. Cell Proteomics* 10, M111 011213, 2011 . Epub 2011/08/03. doi:10.1074/mcp.M111.011213

Langner, T., Öztürk, M., Hartmann, S., Cord-Landwehr, S., Moerschbacher, B., Walton, J. D., et al. (2015). Chitinases Are Essential for Cell Separation in Ustilago Maydis. *Eukaryot. Cel* 14, 846–857. Epub 2015/05/03. doi:10.1128/EC.00022-15

Miska, W., and Geiger, R. (1987). Synthesis and Characterization of Luciferin Derivatives for Use in Bioluminescence Enhanced Enzyme Immunoassays. *New Ultrasensitive Detect. Syst. Enzyme Immunoassays,* 25:23–30. doi:10.1515/cclm.1987.25.1.23

Müntjes, K., Philipp, M., Hüsemann, L., Heucken, N., Weidtkamp-Peters, S., Schipper, K., et al. (2020). Establishing Polycistronic Expression in the Model Microorganism Ustilago Maydis. *Front. Microbiol.* 11, 1384, 2020 . Epub 2020/07/17. doi:10.3389/fmicb.2020.01384

Muyldermans, S. (2013). Nanobodies: Natural Single-Domain Antibodies. *Annu. Rev. Biochem.* 82, 775–797. Epub 2013/03/19. doi:10.1146/annurev-biochem-063011-092449

Nakajima, K.-i., Asakura, T., Maruyama, J.-i., Morita, Y., Oike, H., Shimizu-Ibuka, A., et al. (2006). Extracellular Production of Neoculin, a Sweet-Tasting Heterodimeric Protein with Taste-Modifying Activity, by Aspergillus oryzae. *Appl. Environ. Microbiol.* 72, 3716–3723. doi:10.1128/aem.72.5.3716-3723.2006

Nevalainen, H., and Peterson, R. (2014). Making Recombinant Proteins in Filamentous Fungi- Are We Expecting Too Much? *Front. Microbiol.* 5, 75, 2014 . Epub 2014/03/01. doi:10.3389/fmicb.2014.00075

Nicaud, J.-M., Mackman, N., and Holland, I. B. (1986). Current Status of Secretion of Foreign Proteins by Microorganisms. *J. Biotechnol.* 3, 255–270. doi:10.1016/0168-1656(86)90008-8

Nickel, W. (2010). Pathways of Unconventional Protein Secretion. *Curr. Opin. Biotechnol.* 21, 621–626. Epub 2010/07/20. doi:10.1016/j.copbio.2010.06.004

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cel Biol.* 27, 230–240. Epub 2016/12/19. doi:10.1016/j.tcb.2016.11.007

Reichert, J. M. (2015). Antibodies to Watch in 2015. *mAbs* 7, 1–8. doi:10.4161/19420862.2015.988944

Reindl, M., Hänsch, S., Weidtkamp-Peters, S., and Schipper, K. (2019). A Potential Lock-type Mechanism for Unconventional Secretion in Fungi. *Ijms* 20, 460. doi:10.3390/ijms20030460

Reindl, M., Stock, J., Hussnaetter, K. P., Genc, A., Brachmann, A., and Schipper, K. (2020). A Novel Factor Essential for Unconventional Secretion of Chitinase Cts1. *Front. Microbiol.* 11, 1529, 2020 . Epub 2020.02.07. doi:10.1101/2020.02.07.93861310.3389/fmicb.2020.01529

Sarkari, P., Reindl, M., Stock, J., Müller, O., Kahmann, R., Feldbrügge, M., et al. (2014). Improved Expression of Single-Chain Antibodies in Ustilago Maydis. *J. Biotechnol.* 191, 165–175. doi:10.1016/j.jbiotec.2014.06.028

Southern, E. M. (1974). Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis. *J. Mol. Biol.* 98, 503–517. doi:10.1016/s0022-2836(75)80083-0

Stock, J., Sarkari, P., Kreibich, S., Brefort, T., Feldbrügge, M., and Schipper, K. (2012). Applying Unconventional Secretion of the Endochitinase Cts1 to export Heterologous Proteins in Ustilago Maydis. *J. Biotechnol.* 161, 16180–16191. Epub 2012/03/27. doi:10.1016/j.jbiotec.2012.03.004

Stock, J., Terfrüchte, M., and Schipper, K. (2016). A Reporter System to Study Unconventional Secretion of Proteins Avoiding N-Glycosylation in Ustilago Maydis. *Unconventional Protein Secretion: Methods Protoc. Springer Protoc.* 1459, 149–160. doi:10.1007/978-1-4939-3804-9_10

Sun, Y., and Ho, M. (2020). Emerging Antibody-Based Therapeutics against SARS-CoV-2 during the Global Pandemic. *Antibody Ther.* 3, 246–256. doi:10.1093/abt/tbaa025

Terfrüchte, M., Joehnk, B., Fajardo-Somera, R., Braus, G. H., Riquelme, M., Schipper, K., et al. (2014). Establishing a Versatile Golden Gate Cloning System for Genetic Engineering in Fungi. *Fungal Genet. Biol.* 62, 1–10. doi:10.1016/j.fgb.2013.10.012

Terfrüchte, M., Reindl, M., Jankowski, S., Sarkari, P., Feldbrügge, M., and Schipper, K. (2017). Applying Unconventional Secretion in Ustilago Maydis for the export of Functional Nanobodies. *Ijms* 18, 937. doi:10.3390/ijms18050937

Terfrüchte, M., Wewetzer, S., Sarkari, P., Stollewerk, D., Franz-Wachtel, M., Macek, B., et al. (2018). Tackling Destructive Proteolysis of Unconventionally Secreted Heterologous Proteins in Ustilago Maydis. *J. Biotechnol.* 284, 28437–28451. Epub 2018/08/01. doi:10.1016/j.jbiotec.2018.07.035

Tsukuda, T., Carleton, S., Fotheringham, S., and Holloman, W. K. (1988). Isolation and Characterization of an Autonomously Replicating Sequence from Ustilago Maydis. *Mol. Cel. Biol.* 8, 3703–3709. Epub 1988/09/01. doi:10.1128/mcb.8.9.3703-3709.1988

Vincke, C., Loris, R., Saerens, D., Martinez-Rodriguez, S., Muyldermans, S., and Conrath, K. (2009). General Strategy to Humanize a Camelid Single-Domain Antibody and Identification of a Universal Humanized Nanobody Scaffold. *J. Biol. Chem.* 284, 2843273–2843284. Epub 2008/11/18. doi:10.1074/jbc.M806889200

Viotti, C. (2016). ER to Golgi-dependent Protein Secretion: The Conventional Pathway. *Methods Mol. Biol.* 1459, 3–29. Epub 2016/09/26. doi:10.1007/978-1-4939-3804-9_1

Walsh, G. (2018). Biopharmaceutical Benchmarks 2018. *Nat. Biotechnol.* 36, 1136–1145. doi:10.1038/nbt.4305

Walter, J. D., Hutter, C. A. J., Zimmermann, I., EgloffWyss, P., Sorgenfrei, M., Hürlimann, L. M., et al. (2020). *Sybodies Targeting the SARS-CoV-2 Receptor-Binding Domain*. bioRxiv. doi:10.1101/2020.04.16.045419

Wang, Q., Zhong, C., and Xiao, H. (2020). Genetic Engineering of Filamentous Fungi for Efficient Protein Expression and Secretion. *Front. Bioeng. Biotechnol.* 8, 293, 2020 . Epub 2020/04/24. doi:10.3389/fbioe.2020.00293

Ward, M., Wilson, L. J., Kodama, K. H., Rey, M. W., and Berka, R. M. (1990). Improved Production of Chymosin in Aspergillus by Expression as a Glucoamylase-Chymosin Fusion. *Nat. Biotechnol.* 8, 435–440. Epub 1990/05/01. doi:10.1038/nbt0590-435

Weinzierl, G., Leveleki, L., Hassel, A., Kost, G., Wanner, G., and Bolker, M. (2002). Regulation of Cell Separation in the Dimorphic Fungus Ustilago Maydis. *Mol. Microbiol.* 45, 219–231. Epub 2002/07/09. doi:10.1046/j.1365-2958.2002.03010.x

Wichgers Schreur, P. J., van de Water, S., Harmsen, M., Bermúdez-Méndez, E., Drabek, D., Grosveld, F., et al. (2020). Multimeric Single-Domain Antibody Complexes Protect against Bunyavirus Infections. *eLife* 9, e52716. doi:10.7554/eLife.52716

Wider, D., and Picard, D. (2017). Secreted Dual Reporter Assay with Gaussia Luciferase and the Red Fluorescent Protein mCherry. *PLoS One* 12, e0189403, 2017 . Epub 2017/12/09. doi:10.1371/journal.pone.0189403

Wierckx, N., Miebach, K., Ihling, N., Hussnaetter, K. P., Büchs, J., and Schipper, K. (2021). Perspectives for the Application of Ustilaginaceae as Biotech Cell Factories. *Essays Biochem. Jul* 65 (65), 365–379. Epub 2021/04/17. doi:10.1042/EBC20200141

Xu, Y., Wang, Y.-h., Liu, T.-q., Zhang, H., Zhang, H., and Li, J. (2018). The GlaA Signal Peptide Substantially Increases the Expression and Secretion of α-galactosidase in Aspergillus niger. *Biotechnol. Lett.* 40, 949–955. doi:10.1007/s10529-018-2540-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Cell and Developmental Biology

PERSPECTIVE
published: 22 March 2022
doi: 10.3389/fcell.2022.878027

Secretomics—A Key to a Comprehensive Picture of Unconventional Protein Secretion

*Gereon Poschmann[1], Jasmin Bahr[2], Jürgen Schrader[2], Ioana Stejerean-Todoran[3], Ivan Bogeski[3] and Kai Stühler[1,4]**

[1]Institute for Molecular Medicine, Proteome Research, University Hospital and Medical Faculty, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany, [2]Department of Molecular Cardiology, University Hospital and Medical Faculty, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany, [3]Molecular Physiology, Institute for Cardiovascular Physiology, University Medical Center, Georg August University Göttingen, Göttingen, Germany, [4]Molecular Proteomics Laboratory, Biological Medical Research Center, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany

For a long time, leaderless secreted proteins (LLSP) were neglected as artifacts derived from dying cells. It is now generally accepted that secretion of LLSP–as a part of the collective term unconventional protein secretion (UPS) - is an evolutionarily conserved process and that these LLSP are actively and selectively secreted from living cells bypassing the classical endoplasmic reticulum-Golgi pathway. However, the mechanism of UPS pathways, as well as the number of LLSP and which part of a protein is involved in the selection of LLSPs for secretion, are still enigmatic and await clarification. Secretomics-a proteomics-based approach to identify and quantify all proteins secreted by a cell-is inherently unbiased toward a particular secretion pathway and offers the opportunity to shed light on the UPS. Here, we will evaluate and present recent results of proteomic workflows allowing to obtain high-confident secretome data. Additionally, we address that cell culture conditions largely affect the composition of the secretome. This has to be kept in mind to control cell culture induced artifacts and adaptation stress in serum free conditions. Evaluation of click chemistry for secretome analysis of cells under serum-containing conditions showed a significant change in the cellular proteome with longer incubation time upon treatment with non-canonical amino acid azidohomoalanine. Finally, we showed that the number of LLSP far exceeds the number of secreted proteins annotated in Uniprot and ProteinAtlas. Thus, secretomics in combination with sophisticated microbioanalytical and sample preparation methods is well suited to provide a comprehensive picture of UPS.

Keywords: Secretome, mass spectrometry, unconventional protein secretion, comparative secretomics, pharmacosecretomics, high-confident secretome (Min.5-Max. 8), proteomics

OPEN ACCESS

Edited by:
Min Zhang,
Tsinghua University, China

Reviewed by:
Liang Ge,
Tsinghua University, China

****Correspondence:***
Kai Stühler
kai.stuehler@hhu.de

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *17 February 2022*
Accepted: *07 March 2022*
Published: *22 March 2022*

Citation:
Poschmann G, Bahr J, Schrader J, Stejerean-Todoran I, Bogeski I and Stühler K (2022) Secretomics—A Key to a Comprehensive Picture of Unconventional Protein Secretion. Front. Cell Dev. Biol. 10:878027. doi: 10.3389/fcell.2022.878027

INTRODUCTION

Secretomics - a proteomics-based approach to identify and quantify all proteins secreted by a cell - is inherently unbiased towards a specific secretion pathway and has been successfully applied in several research areas (for a detailed review, see (Mukherjee and Mani, 2013; Schaaij-Visser et al., 2013; Wei et al., 2021). However, sophisticated data analysis and experimental design allow meaningful conclusions about the underlying secretion pathways. To date, secreted proteins have been

broadly classified into two classes. One class comprises secreted proteins that carry an N-terminal signal peptide that directs them to the ER-Golgi pathway. This secretion pathway, also known as "classical protein secretion," is well proven and it had been shown that the signaling peptide hypothesis was both correct and universal because this process occurs in the same way in yeast, plant, and animal cells.

In contrast, the other classes of protein secretion, collectively termed "unconventional protein secretion" (UPS), are characterized by bypassing the ER-Golgi pathway and represents a group of proteins of unknown size. Currently, four types of pathways for UPS have been proposed (Rabouille, 2017; Dimou and Nickel, 2018). Three of them refer to soluble leaderless secreted proteins (LLSP) in the cytoplasm that are secreted either by 1) direct protein translocation through lipid pores in the plasma membrane (type I UPS), 2) plasma membrane-resident ABC transporters, with cargo proteins modified by acylation (type II UPS), or by uptake into endocytic compartments that mature and subsequently fuse with the plasma membrane (type III UPS). Type IV refers to plasma membrane proteins (with signal peptide) that are taken up into the ER but bypass the Golgi on their way to the cell surface. Detailed information is available only for a small group of LLSP that includes medically relevant proteins such as the cytokines FGF-1 and 2, IL-1a, IL-1b, IL-6, IL-18, and IL-33. To date, fibroblast growth factor-2 (FGF-2) is the best characterized candidate protein among these candidate proteins, exhibiting non-vesicular translocation through lipid-induced oligomerization and membrane insertion (Steringer et al., 2017).

The main reasons for the limited knowledge of UPS in contrast to classical protein secretion are that 1) UPS cannot be accurately predicted due to the still missing signaling patterns, 2) some UPS proteins also have an intracellular function (moonlighting), and therefore 3) the assignment of proteins to unconventional secretory pathways ultimately requires extensive experimental confirmation of extracellular function.

However, we believe that secretomics, in combination with rigorous experimental design and focused data analysis, is the key to a comprehensive picture of UPS. In this perspective, we will focus on recent advances and experimental settings in mass spectrometry (MS)-based secretomics that allow to shed light on the UPS. Here, we follow the definition that the secretome (as part of the conditioned medium) comprises bona fide secreted proteins whose abundance can be explained by experimental data (enrichment value), established knowledge of secretion, or predictions.

High-Confident Secretome Data by Quantitative Secretomics

Quantitative protein analysis by mass spectrometry is the method of choice in proteomics to characterize cellular compartments (Itzhak et al., 2017), interaction with proteins (Bensimon et al., 2012), nucleotides (Brillen et al., 2017), or drugs (Savitski et al., 2014), as this, in combination with specific fractionation steps, allows the determination of a significantly enriched protein population. We and others have shown that bona fide secreted proteins can be identified regardless of the secretion pathway when secretome and proteome data are compared by so-called "comparative secretomics" approach (Poschmann et al., 2021). Thus, we demonstrated that, depending on the cell line analyzed, comparative secretomics results in a high proportion of bona fide secreted proteins, with more than 30–70% being classically secreted proteins and 4–29% being candidate proteins released *via* unconventional secretion pathways (**Figure 1A**). Using this approach, more than 180 UPS candidate proteins have been identified, allowing the development of a novel prediction tool "OutCyte" [see below (Zhao et al., 2019)].

Quantitative secretomics has also been used to characterize leaderless secreted proteins through the so-called stable isotope dynamic labeling of secretomes (SIDLS) approach (Hammond et al., 2018). Dynamic isotope labeling of secretion kinetics can distinguish between secretory proteins and intracellular proteins released by cancer and stromal cells in culture. Interestingly, this study revealed a large number of LLSP with secretion kinetics comparable to classical secretory proteins, suggesting that the SIDLS approach is suitable for identifying continuously LLSP such as HDGF, PRDX2, AKR1B10, and C1QBP (Hammond et al., 2018).

Currently, we are working on combining quantitative secretomics and target identification by thermal proteome profiling in a so-called pharmacosecretomics approach. This approach aims to characterize the unconventional secretory pathways through a small molecule perturbation strategy. To date, there are only a limited number of small molecules with known targets that can be used for pharmacological modulation of UPS (Rodriguez-Furlan et al., 2017). Small molecule modulators that disrupt leaderless protein secretion allow functional dissection of components, their connectivity, and their regulators, especially within protein families (Rodriguez-Furlan et al., 2017). As suggested by Hick and Raikhe, this approach can be specific, robust, conditional, efficient, reversible, tunable, rapid, and simple (Hicks and Raikhel, 2012). In the first step, the change in secretory phenotype is determined by quantitative secretomics after treatment with small molecules with a previously unknown target spectrum. In the second step, the protein target involved in the secretory pathway is identified by thermal proteomic profiling (Savitski et al., 2014). Following this concept, new components and cargo associations of unconventional secretory pathways will become accessible.

In summary, quantitative secretomics provides the ability to identify LLSP either by their enrichment in the secretome or based on secretion kinetics. Thus, quantitative secretomics adds another level of quality control in addition to the simple bioinformatics filtering steps. However, these methods tend to be conservative, failing to detect LLSP with higher intracellular concentrations or slow secretion kinetics. In addition, quantitative secretomics combined with a small molecule perturbation strategy (pharmacosecretomics) has the potential to characterize secretory pathways and define subsets of LLSP that are preferentially secreted through specific pathways.

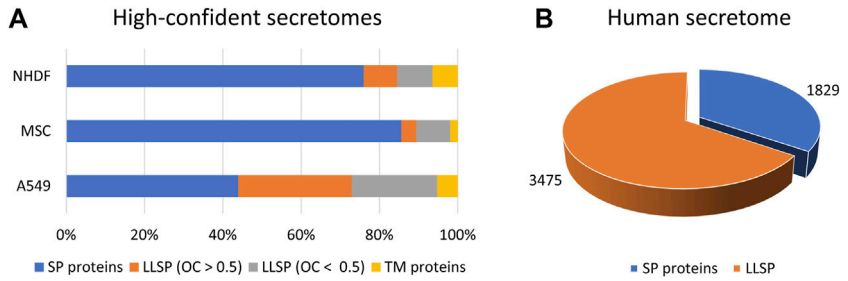


FIGURE 1 | High-confident secretomes and the human secretome. **(A)** By means of comparative secretomics approach we were able to generate lists of high-confident secreted proteins of NHDF, MSC and A549 cells including 72–88% of proteins which were predicted to be secreted (Poschmann et al., 2021). **(B)** Our prediction tool OutCyte was used to estimate the number of candidates LLSP in the human secretome to 3,475 (Zhao et al., 2019). LLSP: leaderless secreted proteins. SP proteins: signal peptide containing proteins. OC: OutCyte score. TM proteins: transmembrane proteins.

Prediction of UPS and Current State of Secretome Data Bases

The development of prediction tools for UPS and thus the prediction of LLSP is more challenging than for classical protein secretion, because there are only a limited number of LLSP known and little is known about the different pathways involved. Currently, there are a number of prediction tools such as Outcyte, SecretomeP, SecretP, SPRED, or SRTpred, some of which differ in terms of sample set, taxa, algorithm, and prediction performance (for more details, see (Nielsen et al., 2019). For example, SecretomeP and SPRED use classical secretory proteins by removing their signal peptides based on the hypothesis that all secretory proteins share common features regardless of the specific secretory pathways. However, a recent benchmark has shown that SecretomeP performs much worse than originally thought, casting doubt on the underlying hypothesis. We therefore developed OutCyte, an integrated tool with two modules for predicting unconventional secretory proteins in eukaryotes (Zhao et al., 2019). In contrast to existing tools, the module for predicting potential UPS (OutCyte-UPS) was created with our in-house experimental secretome datasets using features generated directly from protein sequences. This allowed us to demonstrate that Outcyte outperforms SecretomeP and its successors, and we obtained information on important individual feature contributions for predictions using OutCyte. Among 61 tested physicochemical features, eight features were finally selected for tree boosting based machine learning. Among them, most important for the prediction of UPS were a higher frequency of arginine and other positively charged amino acids within the complete protein sequence as well as a relatively low molecular weight (~21 kDa average MW of UPS candidates). Further features include the frequency of the aromatic amino acids tryptophan and phenylalanine as well as the frequency in the C-terminal 50 amino acids of: small amino acids, hydrophobic amino acids and polar amino acids. (Zhao et al., 2019).

Using Outcyte, we were able to roughly estimate the number of secreted proteins from the human proteome. Of 20,170 proteins, 1,829 were predicted to contain a signal for classical secretion (**Figure 1B**). This is consistent with other prediction tools/repositories: 1,836 proteins (SignalP 4.1), 1,693 proteins (DeepSig), and 1,999 proteins (UniProt). Surprisingly, we predicted 3,475 candidate LLSP proteins using Outcyte (**Figure 1B**). This far exceeds the number of the secretome annotated proteins (classically secreted and LLSP) in Uniprot (2,044 proteins) and ProteinAtlas (2,641 proteins) (Uhlen et al., 2019)).

The underrepresentation of LLSP in public available repositories interferes with comprehensive characterization extracellular space. For example, in an approach that aims to discover endocrine interactions by the integration of global multi-tissue expression and publicly available resources, LLSP might be underrepresented due to the incomplete annotation in UniProt KB (338 LLSP out of 2,248 proteins; 11%; Seldin et al., 2018). Nevertheless, this method, termed Quantitative Endocrine Network Interaction Estimation (QENIE), revealed an endocrine relationship between different tissues for seven LLSP (Xdh, Csn2, Nampt, Otop1, C1qbp, Ctf1, Fgf1), resulting in an overrepresentation of LLSP among the total number of candidate proteins (7 LLSP out of 47 proteins; 15%, $p = 0.0499$).

This example highlights both the biological role of LLSP and the need for dissemination and access to high-confidence secretome data through public repositories. In addition, the increasing amount of highly reliable secretome data and recent developments in machine learning are opening up new avenues that can not only allow to improve the prediction tools of unconventional protein secretion but also provide access to information on sequence motifs and cellular interactions.

In Vivo and *in Vitro* Methods Allowing to Manage Secretion Artefacts

The main drawback in identifying of bona fide secreted proteins using secretomics from cultured cells are proteinaceous artefacts originating form cell culture medium, dying cells and artificial culture conditions that do not perfectly mimic the physiological environment. Although serum deprivation might impact cells' viability, conditioned media obtained from serum-free cell cultivation is still the gold standard for secretomics. Nevertheless, in a number of studies, only limited information is available about detailed culture conditions and viability and

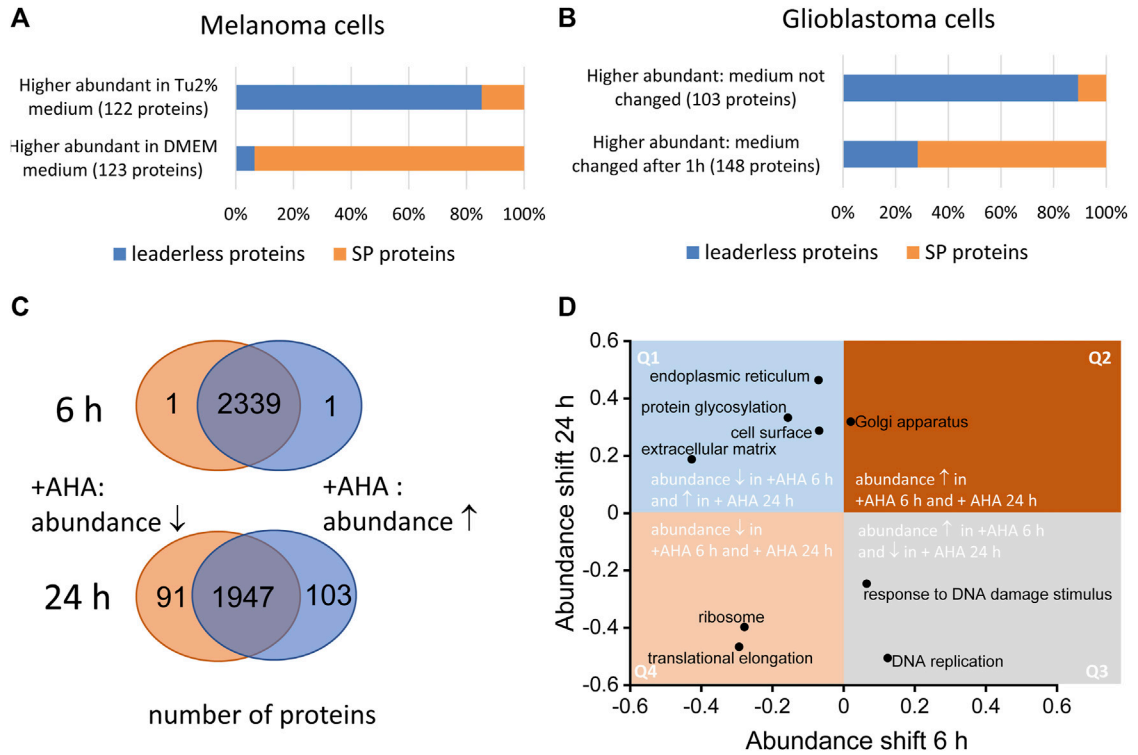


FIGURE 2 | Effect of cell culture conditions on the secretome. Both serum-free and serum containing approached might influence the composition of secretomes. **(A)** WM3918 melanoma cells (n = 3 dishes per group) were cultivated for 24 in serum free medium. Cells expanded in DMEM showed a significantly higher proportion (*p*-value 2.2E-16, Fisher's exact test) of signal-peptide containing proteins (SP proteins) at higher abundances in secretomes, whereas in Tu2% medium based secreteomes, putative LLSP showed higher abundances in comparison to expansion in DMEM. **(B)** LN18 glioblastoma cells (n = 3 per group) were incubated for 24 h in serum-free medium. After 1 h, the medium was replaced in one set of samples. In this samples, signal peptide containing proteins showed higher intensities in resulting secretome samples whereas in samples in which the medium was not changed after 1 h, a significant higher proportion of putative LLSP could be found at higher intensities (*p*-value 2.2E-16, Fisher's exact test). **(C)** Normal human dermal foreskin fibroblasts were cultured with azidohomoalanine (AHA) or methionine as control for 6 and 24 h (n = 5 dishes per group). After MS analysis, different abundant proteins were determined by the Student's *t*-test based significance analysis of microarrays approach (Tusher et al., 2001). Whereas after 6 h only 2 proteins (of 2441 cellular proteins) showed a significant AHA induced abundance change, 194 proteins (of 2141 cellular proteins) showed an abundance change after 24 h **(D)**. This dataset was also used for the analysis of global shifts in abundance on the level of proteins grouped by gene ontology annotations (Cox and Mann, 2012). Selected GOCC and GOBP categories are shown indicating the size of the abundance shift of associated proteins. Whereas proteins for some categories show an abundance change in the same direction after 6 and 24 h AHA incubation (found in quadrant Q2 and Q4), other protein groups show an AHA induced abundance shift in the opposite direction at the two timepoints (found in quadrant Q1 and Q3).

changes of cells after serum deprivation. In this context, cell culture conditions should be optimized allowing a high viability of the cells without extensive amount of protein supplements (for more details we refer to (Schira-Heinen et al., 2019). It is important to note that the choice of medium can also have a major impact on the secretome of the cells. We found that, for example, WM3918 melanoma cells released a much higher proportion of signal peptide containing proteins and a lower proportion of LLSP in Dulbecco's Modified Eagle Medium (DMEM) compared to expansion in Tu2% medium (**Figure 2A**). In secretomes from WM3918 cells expanded in the latter medium, we found the opposite: a large proportion of predicted LLSP and a comparable low number of signal peptide containing proteins. Tu2% medium is a mixture of 80% MCDB153 basal medium and 20% Leibovitz's L-15 medium and contains 2% fetal calf serum and insulin whereas the used DMEM contained 10% fetal calf serum.

Furthermore, we revealed that renewal of serum-free medium after a short period (1 h) helps to avoid or measure potential stress artifacts originating from the adaptation to the serum-free medium conditions. In secretomes from LN18 cells cultured for 24 h under serum-free condition, the proportion of signal peptide containing proteins increased when the serum-free medium was exchanged after 1 h. When cells were incubated in serum-free medium for 24 h without exchanging the medium after 1 h, the proportion of putative LLSP in the secretome was significantly higher (**Figure 2B**).

However, in secretome approaches based on serum-free medium, contaminating proteins from serum that were not completely removed during the washing steps could remain in the secretome. Some groups use labeling of cells with heavy isotope labeled amino acids to deal with this issue (Polacek et al., 2010). Proteins which were only found with light amino acids included can be identified as serum contaminants in this approach. We found that -at least for cultures from human cells–it might also be possible to control contaminants from bovine serum just by tagging the respective proteins by

contaminant lists as they were already included for example, in MaxQuant (Tyanova et al., 2016). In a study with normal human dermal foreskin fibroblasts which previously were labeled with heavy amino acids, we found 72 proteins with no intensity values in non-light channels which were all included in the contaminant list of MaxQuant and could therefore be removed without the need of additional isotope labeling.

As not every cell type can be cultured under serum deprivation without significant changes in cell viability, protein abundance or posttranslational modification (Hasan et al., 1999; Cooper, 2003), methods were developed to enable secretome preparation in serum-containing medium (Eichelbaum et al., 2012; Lai et al., 2013). For example, Eichelbaum and others used a two-dimensional metabolic labelling strategy based on pulsed stable isotope labelling of amino acids in cell culture (pSILAC) (Eichelbaum and Krijgsveld, 2014) and labelling with the biorthogonal, non-canonical amino acid azidohomoalanine (AHA) (Eichelbaum et al., 2012). The labeling strategy enables an enrichment of newly synthesized low abundant proteins by click chemistry on the azide-group of the AHA label as well as direct comparison of two different conditions by SILAC labeling. Although successfully applied caution have to be paid, when applying AHA labelling. We and others have shown that AHA-labelling changes the proteome of cells especially at longer incubation times (**Figure 2C**; (Eichelbaum et al., 2012)). Therefore, it is important to find a good compromise between longer incubation times, which might be necessary to collect a sufficient amount of secreted proteins for analysis and a potentially undesirable AHA-induced changes in cellular pathways. Those changes could already be detected after 6 h as for examples, ribosomal and translation associated proteins show a lower abundance in normal human dermal foreskin fibroblasts after incubation with AHA which is even more pronounced after 24 h (**Figure 2D**). Moreover, proteins associated with extracellular matrix, glycosylation and endoplasmic reticulum exhibit higher abundances in the cell upon 24 h AHA labeling and lower abundances after 6 h AHA labeling. This suggests that at least the classical secretion of proteins may be impaired during longer AHA incubation times, as we found related proteins predominately inside the cell at this time and in lesser amounts in the secretome.

Glycocapture, which is an additional tool to enrich secreted proteins under serum containing conditions (Lai et al., 2013) will probably more suited for secreted proteins of type IV UPS as glycosylation is a hallmark of secreted proteins facilitating the ER-Golgi route.

To make secretome analysis less susceptible to cell culture artifacts, both *ex vivo* and *in vivo* methods have been developed. For example, Roelofsen and others developed a method based on comparison of incorporation rates of isotope-labeled amino acids (CILAIR) to determine the secretome of human adipose tissue (Roelofsen et al., 2009). After incubation of human visceral adipose tissue from a patient in medium containing [^{13}C]-lysine, 156 potentially secreted proteins were identified based on significant incorporation rates. Although this method allows the determination of secreted proteins from *ex vivo* tissue samples, it is biased toward proteins with rapid rates of protein synthesis and secretion and is therefore less suitable for proteins that are not continuously secreted. Although most LLSP are secreted under certain conditions such as stress, Hammond and others have shown that a large number of LLSP proteins also have a high secretion constant (Hammond et al., 2018). This suggests that the CILAIR approach is not only suitable for classical secreted proteins and allows LLSP to be monitored under *ex vivo* conditions from human tissue.

Recently, two publications used proximity biotinylation to characterize tissue-specific *in vivo* secretion in mice. Liu and others introduce the "secretome mouse," a genetic platform that allows rapid identification of the cell- or tissue-specific *in vivo* secretome under basal conditions or after physiological or pathophysiological stress (Liu et al., 2021). Although they used an ER-BioID^{HA} construct containing the promiscuous biotinylation enzyme BioID2 in conjunction with the C-terminal ER retention sequence KDEL, they were confident in detecting LLSP of type III UPS in addition to classically secreted proteins because this pathway involves late endosomes, multivesicular bodies, or autophagosomes whose membrane is thought to be of ER origin. A similar approach that additionally targets UPS uses the Cyto-TurboID construct in combination with the Mem-TurboID and ER-TurboID constructs (Wei et al., 2021). After infection with the lentiviral constructs and feeding the mice with biotin, cell type-specific expression of the constructs was applied in mice to characterize nutrient-dependent reprogramming of the hepatocyte *in vivo* secretome. Thus, increased abundance of LLSP betaine homocysteine S-methyltransferase (BHMT) with a high cell type specific secretion was observed and functionally validated.

Although these approaches are unbiased toward a specific secretory pathway, they address a major pitfall in the field of UPS research, namely the avoidance of artifacts from *in vitro* cultivation.

CONCLUDING REMARKS

As an omics technique, secretomics aims for the generation of a comprehensive picture of all proteins secreted by different cell types. Thus, secretomics will provide access to a vast array of LLSP which have been neglected so far. This will on the one hand enable to characterize the composition of the extracellular microenvironment leading to a deeper understanding of tissue homeostasis and cell-cell-communication and on the other hand give access to the elucidation of unconventional secretory pathways in respect of cargo selection and transport to the extracellular space. Regarding the latter aspect it will be interesting to follow how secretomics combined with machine learning will accelerate the elucidation of novel associations between cargo motifs and secretory pathway, as known for the IL-1b and motif-1 in TMED10-channeled UPS (Zhang et al., 2020) or the basic clusters of FGF2 in the lipid-induced oligomerization and membrane insertion associated UPS (Steringer et al., 2017).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/pride/archive/, PXD018895.

AUTHOR CONTRIBUTIONS

KS and GP developed the concept, performed biological experiments, analysed data and wrote the manuscript. JS, JB, and IS-T performed biological experiments. IB supervised melanoma cell-related experiments.

ACKNOWLEDGMENTS

We thank Sabrina Cappello and Andrea Paluschkiwitz for helping with the WM3918 melanoma cell sample preparation. IB acknowledges the financial support by DFG funded SFB1027 and SFB1190 grants.

REFERENCES

Bensimon, A., Heck, A. J. R., and Aebersold, R. (2012). Mass Spectrometry-Based Proteomics and Network Biology. *Annu. Rev. Biochem.* 81, 379–405. doi:10.1146/annurev-biochem-072909-100424

Brillen, A. L., Schöneweis, K., Walotka, L., Hartmann, L., Müller, L., Ptok, J., et al. (2017). Succession of Splicing Regulatory Elements Determines Cryptic 5′ss Functionality. *Nucleic Acids Res.* 45, 4202–4216. doi:10.1093/nar/gkw1317

Cooper, S. (2003). Reappraisal of Serum Starvation, the Restriction point, G0, and G1 Phase Arrest Points. *FASEB j.* 17, 333–340. doi:10.1096/fj.02-0352rev

Cox, J., and Mann, M. (2012). 1D and 2D Annotation Enrichment: a Statistical Method Integrating Quantitative Proteomics with Complementary High-Throughput Data. *Bmc Bioinformatics* 13 (Suppl. 16), S12. doi:10.1186/1471-2105-13-S16-S12

Dimou, E., and Nickel, W. (2018). Unconventional Mechanisms of Eukaryotic Protein Secretion. *Curr. Biol.* 28, R406–R410. doi:10.1016/j.cub.2017.11.074

Eichelbaum, K., and Krijgsveld, J. (2014). Combining Pulsed SILAC Labeling and Click-Chemistry for Quantitative Secretome Analysis. *Methods Mol. Biol.* 1174, 101–114. doi:10.1007/978-1-4939-0944-5_7

Eichelbaum, K., Winter, M., Diaz, M. B., Herzig, S., and Krijgsveld, J. (2012). Selective Enrichment of Newly Synthesized Proteins for Quantitative Secretome Analysis. *Nat. Biotechnol.* 30, 984–990. doi:10.1038/nbt.2356

Hammond, D. E., Kumar, J. D., Raymond, L., Simpson, D. M., Beynon, R. J., Dockray, G. J., et al. (2018). Stable Isotope Dynamic Labeling of Secretomes (SIDLS) Identifies Authentic Secretory Proteins Released by Cancer and Stromal Cells. *Mol. Cell Proteomics* 17, 1837–1849. doi:10.1074/mcp.tir117.000516

Hasan, N. M., Adams, G. E., and Joiner, M. C. (1999). Effect of Serum Starvation on Expression and Phosphorylation of PKC-? and P53 in V79 Cells: Implications for Cell Death. *Int. J. Cancer* 80, 400–405. doi:10.1002/(sici)1097-0215(19990129)80:3<400::aid-ijc11>3.0.co;2-u

Hicks, G. R., and Raikhel, N. V. (2012). Small Molecules Present Large Opportunities in Plant Biology. *Annu. Rev. Plant Biol.* 63, 261–282. doi:10.1146/annurev-arplant-042811-105456

Itzhak, D. N., Davies, C., Tyanova, S., Mishra, A., Williamson, J., Antrobus, R., et al. (2017). A Mass Spectrometry-Based Approach for Mapping Protein Subcellular Localization Reveals the Spatial Proteome of Mouse Primary Neurons. *Cel Rep.* 20, 2706–2718. doi:10.1016/j.celrep.2017.08.063

Lai, Z. W., Nice, E. C., and Schilling, O. (2013). Glycocapture-based Proteomics for Secretome Analysis. *Proteomics* 13, 512–525. doi:10.1002/pmic.201200414

Liu, J., Jang, J. Y., Pirooznia, M., Liu, S., and Finkel, T. (2021). The Secretome Mouse Provides a Genetic Platform to Delineate Tissue-specific *In Vivo* Secretion. *Proc. Natl. Acad. Sci. U S A.* 118, e2005134118. doi:10.1073/pnas.2005134118

Mukherjee, P., and Mani, S. (2013). Methodologies to Decipher the Cell Secretome. *Biochim. Biophys. Acta (Bba) - Proteins Proteomics* 1834, 2226–2232. doi:10.1016/j.bbapap.2013.01.022

Nielsen, H., Petsalaki, E. I., Zhao, L., and Stühler, K. (2019). Predicting Eukaryotic Protein Secretion without Signals. *Biochim. Biophys. Acta (Bba) - Proteins Proteomics* 1867, 140174. doi:10.1016/j.bbapap.2018.11.011

Polacek, M., Bruun, J.-A., Johansen, O., and Martinez, I. (2010). Differences in the Secretome of Cartilage Explants and Cultured Chondrocytes Unveiled by SILAC Technology. *J. Orthop. Res.* 28, 1040–1049. doi:10.1002/jor.21067

Poschmann, G., Brenig, K., Lenz, T., and Stühler, K. (2021). Comparative Secretomics Gives Access to High Confident Secretome Data: Evaluation of Different Methods for the Determination of Bona Fide Secreted Proteins. *Proteomics* 21, e2000178. doi:10.1002/pmic.202000178

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cel Biol.* 27, 230–240. doi:10.1016/j.tcb.2016.11.007

Rodriguez-Furlan, C., Raikhel, N. V., and Hicks, G. R. (2017). Merging Roads: Chemical Tools and Cell Biology to Study Unconventional Protein Secretion. *J. Exp. Bot.* 69, 39–46. doi:10.1093/jxb/erx261

Roelofsen, H., Dijkstra, M., Weening, D., de Vries, M. P., Hoek, A., and Vonk, R. J. (2009). Comparison of Isotope-Labeled Amino Acid Incorporation Rates (CILAIR) Provides a Quantitative Method to Study Tissue Secretomes. *Mol. Cell Proteomics* 8, 316–324. doi:10.1074/mcp.m800254-mcp200

Savitski, M. M., Reinhard, F. B. M., Franken, H., Werner, T., Savitski, M. F., Eberhard, D., et al. (2014). Tracking Cancer Drugs in Living Cells by thermal Profiling of the Proteome. *Science* 346, 1255784. doi:10.1126/science.1255784

Schaaij-Visser, T. B. M., de Wit, M., Lam, S. W., and Jiménez, C. R. (2013). The Cancer Secretome, Current Status and Opportunities in the Lung, Breast and Colorectal Cancer Context. *Biochim. Biophys. Acta (Bba) - Proteins Proteomics* 1834, 2242–2258. doi:10.1016/j.bbapap.2013.01.029

Schira-Heinen, J., Grube, L., Waldera-Lupa, D. M., Baberg, F., Langini, M., Etemad-Parishanzadeh, O., et al. (2019). Pitfalls and Opportunities in the Characterization of Unconventionally Secreted Proteins by Secretome Analysis. *Biochim. Biophys. Acta (Bba) - Proteins Proteomics* 1867, 140237. doi:10.1016/j.bbapap.2019.06.004

Seldin, M. M., Koplev, S., Rajbhandari, P., Vergnes, L., Rosenberg, G. M., Meng, Y., et al. (2018). A Strategy for Discovery of Endocrine Interactions with Application to Whole-Body Metabolism. *Cell Metab.* 27, 1138–1155.e1136. doi:10.1016/j.cmet.2018.03.015

Steringer, J. P., Lange, S., Čujová, S., Šachl, R., Poojari, C., Lolicato, F., et al. (2017). Key Steps in Unconventional Secretion of Fibroblast Growth Factor 2 Reconstituted with Purified Components. *Elife* 6, e28985. doi:10.7554/eLife.28985

Tusher, V. G., Tibshirani, R., and Chu, G. (2001). Significance Analysis of Microarrays Applied to the Ionizing Radiation Response. *Proc. Natl. Acad. Sci. U.S.A.* 98, 5116–5121. doi:10.1073/pnas.091062498

Tyanova, S., Temu, T., and Cox, J. (2016). The MaxQuant Computational Platform for Mass Spectrometry-Based Shotgun Proteomics. *Nat. Protoc.* 11, 2301–2319. doi:10.1038/nprot.2016.136

Uhlén, M., Karlsson, M. J., Hober, A., Svensson, A. S., Scheffel, J., Kotol, D., et al. (2019). The Human Secretome. *Sci. Signal.* 12, eaaz0274. doi:10.1126/scisignal.aaz0274

Wei, W., Riley, N. M., Yang, A. C., Kim, J. T., Terrell, S. M., Li, V. L., et al. (2021). Cell Type-Selective Secretome Profiling *In Vivo*. *Nat. Chem. Biol.* 17, 326–334. doi:10.1038/s41589-020-00698-y

Zhang, M., Liu, L., Lin, X., Wang, Y., Li, Y., Guo, Q., et al. (2020). A Translocation Pathway for Vesicle-Mediated Unconventional Protein Secretion. *Cell* 181, 637–652. doi:10.1016/j.cell.2020.03.031

Zhao, L., Poschmann, G., Waldera-Lupa, D., Rafiee, N., Kollmann, M., and Stühler, K. (2019). OutCyte: a Novel Tool for Predicting Unconventional Protein Secretion. *Sci. Rep.* 9, 19448. doi:10.1038/s41598-019-55351-z

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

MINI REVIEW
published: 01 April 2022
doi: 10.3389/fcell.2022.864257

A Role for Liquid-Ordered Plasma Membrane Nanodomains Coordinating the Unconventional Secretory Pathway of Fibroblast Growth Factor 2?

Fabio Lolicato and Walter Nickel**

Heidelberg University Biochemistry Center, Heidelberg, Germany

Fibroblast growth factor 2 (FGF2) is a tumor cell survival factor that belongs to a subgroup of extracellular proteins lacking N-terminal signal peptides. Whereas this phenomenon was already recognized in the early 1990s, detailed insights into the molecular mechanisms underlying alternative pathways of protein secretion from eukaryotic cells were obtained only recently. Today, we know about a number of alternative secretory mechanisms, collectively termed unconventional protein secretion (UPS). FGF2 belongs to a subgroup of cargo proteins secreted by direct translocation across the plasma membrane. This feature has been classified as type I UPS and is shared with other unconventionally secreted proteins, such as HIV-Tat and Tau. FGF2 translocation across the membrane is initiated through sequential interactions with the Na,K-ATPase, Tec kinase, and phosphoinositide PI(4,5)P_2 at the inner plasma membrane leaflet. Whereas the first two are auxiliary factors of this pathway, the interaction of FGF2 with PI(4,5)P_2 triggers the core mechanism of FGF2 membrane translocation. It is based on a lipidic membrane pore that is formed by PI(4,5)P_2-induced oligomerization of FGF2. Membrane-inserted FGF2 oligomers are recognized as translocation intermediates that are resolved at the outer plasma membrane leaflet by glypican-1, a heparan sulfate proteoglycan that captures and disassembles FGF2 oligomers on cell surfaces. Here, we discuss recent findings suggesting the molecular machinery mediating FGF2 membrane translocation to be highly organized in liquid-ordered plasma membrane nanodomains, the core process underlying this unusual pathway of protein secretion.

Keywords: fibroblast growth factor 2, unconventional protein secretion, protein self-translocation across membranes, lipidic membrane pores, phosphoinositides, heparan sulfates, glypican

OPEN ACCESS

Edited by:
Julien Villeneuve, Institute for Functional Genomics (CNRS), France

Reviewed by:
Bruno Beaumelle, UMR9004 Institut de Recherche en Infectiologie de Montpellier (IRIM), France

****Correspondence:***
Fabio Lolicato
fabio.lolicato@bzh.uni-heidelberg.de
Walter Nickel
walter.nickel@bzh.uni-heidelberg.de

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *28 January 2022*
Accepted: *18 February 2022*
Published: *01 April 2022*

Citation:
Lolicato F and Nickel W (2022) A Role for Liquid-Ordered Plasma Membrane Nanodomains Coordinating the Unconventional Secretory Pathway of Fibroblast Growth Factor 2? Front. Cell Dev. Biol. 10:864257. doi: 10.3389/fcell.2022.864257

INTRODUCTION

The Unconventional Secretory Pathway of FGF2

As with many cargo proteins transported into the extracellular space by various types of unconventional protein secretion (UPS) pathways (Malhotra, 2013; Rabouille, 2017; Dimou and Nickel, 2018; Pallotta and Nickel, 2020), fibroblast growth factor 2 (FGF2) is a growth factor involved in fundamental biological processes, such as angiogenesis and wound healing (Beenken and Mohammadi, 2009). These functions of FGF2 are linked to its ability to form ternary signaling complexes with heparan sulfates and FGF high affinity receptors on cell surfaces (Plotnikov et al.,

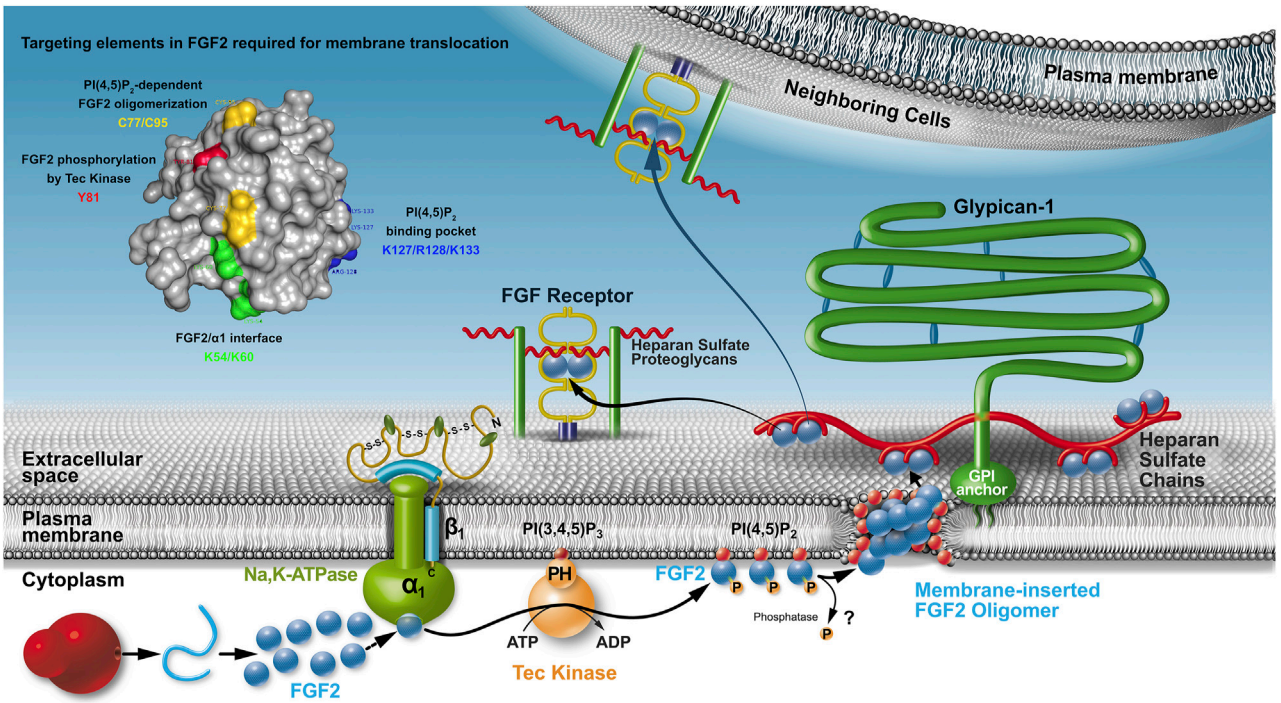


FIGURE 1 | The unconventional secretory pathway of FGF2. Localization, molecular machinery, and mechanism as well as *cis* elements in FGF2 that are required for FGF2 translocation across the plasma membrane of eukaryotic cells. Whereas it is clear that the Na,K-ATPase are upstream of $PI(4,5)P_2$, the order of sequential FGF2 interactions with the Na,K-ATPase and Tec kinase remains to be determined. It is also unclear whether FGF2 is secreted as a phosphorylated protein or whether a phosphatase removes this modification before FGF2 membrane translocation occurs.

1999; Schlessinger et al., 2000). In addition to its role in development, FGF2 also plays key roles under pathophysiological conditions with both cancer cells and cells from their microenvironment producing vast amounts of FGF2 to trigger tumor-induced angiogenesis (Akl et al., 2016). Under certain circumstances, signaling cascades initiated by FGF2 can trigger immune escape mechanisms that lead to a block of apoptotic programs (Noh et al., 2014). For example, FGF2 causes chemoresistance in patients suffering from acute myeloid leukemia (Traer et al., 2016; Javidi-Sharifi et al., 2019). Despite the requirement of FGF2 to have access to the extracellular space to activate FGF receptors on cell surfaces, the analysis of its primary structure revealed the absence of a signal peptide for ER/Golgi-dependent protein secretion. However, even though major efforts were made, the proposed existence of alternative pathways of protein secretion (Muesch et al., 1990; Nickel, 2003) remained a hypothesis for decades as detailed insights into the molecular mechanism by which FGF2 and other UPS cargoes are transported into the extracellular space could be obtained only recently (Malhotra, 2013; Rabouille, 2017; Dimou and Nickel, 2018; Steringer and Nickel, 2018; Pallotta and Nickel, 2020).

All components of the molecular machinery mediating unconventional secretion of FGF2 have been found to be localized to the plasma membrane (**Figure 1**). These factors include the Na, K-ATPase (Zacherl et al., 2015; Legrand et al., 2020), Tec kinase containing a PH domain that binds to the phosphoinositide $PI(3,4,5)P_3$ (Ebert et al., 2010; La Venuta et al., 2016; Steringer et al., 2012) as well as $PI(4,5)P_2$, another phosphoinositide enriched in the inner leaflet of the plasma membrane (Nickel, 2011; Temmerman et al., 2008; Temmerman and Nickel, 2009). A cluster of amino acids with basic side chains (K127, R128, and K133; **Figure 1**) mediates $PI(4,5)P_2$-dependent membrane recruitment of FGF2 (Temmerman et al., 2008; Steringer et al., 2017; Müller et al., 2015). This interaction initiates the core mechanism of FGF2 membrane translocation, a process that involves membrane insertion of FGF2 oligomers (Steringer and Nickel, 2018; Steringer et al., 2012; Steringer et al., 2017). Their biogenesis depends on the formation of intermolecular disulfide bridges (Dimou and Nickel, 2018; Steringer et al., 2017; Müller et al., 2015). As illustrated in **Figure 1**, the lipidic membrane pore that is induced by FGF2 oligomers is characterized by a toroidal architecture (Steringer et al., 2012; Müller et al., 2015; Steringer and Nickel, 2018). Several experimental observations support this view, such as the simultaneous membrane passage of fluorescent tracers and the transbilayer diffusion of membrane lipids that can be observed concomitant with $PI(4,5)P_2$-dependent membrane insertion of FGF2 oligomers (Steringer et al., 2012; Steringer and Nickel, 2018). Furthermore, diacylglycerol, a cone-shaped lipid that interferes with $PI(4,5)P_2$-induced positive membrane curvature inhibits membrane pore formation by FGF2 oligomers (Steringer et al., 2012; Steringer and Nickel, 2018). Finally, fusion proteins, such as

FGF2-GFP form lipidic membrane pores with an increased pore size cutoff, a phenomenon that is reported previously for toroidal membrane pores (Gilbert et al., 2014). Therefore, $PI(4,5)P_2$ plays multiple roles in FGF2 secretion with 1) mediating FGF2 membrane recruitment, 2) initiating FGF2 oligomerization, and 3) stabilizing positive membrane curvature to trigger the conversion of the lipid bilayer into a toroidal membrane pore with membrane-inserted FGF2 oligomers accommodated in its hydrophilic center (Dimou and Nickel, 2018; Steringer and Nickel, 2018). In this context, because FGF2 can attract multiple $PI(4,5)P_2$ molecules, a strong local accumulation of this bilayer perturbing membrane lipid is proposed to compromise the integrity of the plasma membrane facilitating a membrane remodeling process converting the lipid bilayer into a toroidal membrane pore (Steringer et al., 2017; Dimou and Nickel, 2018; Pallotta and Nickel, 2020).

Based on the findings described above, membrane-inserted FGF2 oligomers are believed to be membrane translocation intermediates as part of an assembly/disassembly pathway that drives directional transport of FGF2 into the extracellular space (Dimou and Nickel, 2018; Steringer and Nickel, 2018). The final step of this process is mediated by cell surface heparan sulfate proteoglycans that capture FGF2 at the outer leaflet of the plasma membrane (Nickel, 2007; Nickel and Rabouille, 2009; Nickel and Seedorf, 2008; Zehe et al., 2006). Of note, compared with $PI(4,5)P_2$, heparan sulfates exhibit an approximately hundredfold higher affinity toward FGF2 (Temmerman et al., 2008; Temmerman and Nickel, 2009; Steringer et al., 2017; Raman et al., 2003). Furthermore, the binding site in FGF2 for heparan sulfates and $PI(4,5)P_2$ overlaps with some key residues, such as K133 being essential for both types of interactions (Temmerman et al., 2008; Temmerman and Nickel, 2009; Steringer et al., 2017). Consistently, interactions of FGF2 with heparan sulfate chains and $PI(4,5)P_2$ are shown to be mutually exclusive (Steringer et al., 2017). These findings reveal a key aspect of the molecular mechanism of FGF2 membrane translocation providing a compelling explanation of how FGF2 assembles at the inner leaflet in a $PI(4,5)P_2$-dependent manner into membrane-inserted oligomers that are captured and disassembled at the outer leaflet by cell surface heparan sulfate chains (Dimou and Nickel, 2018; Pallotta and Nickel, 2020; Rabouille, 2017). Thus, heparan sulfates mediate the last step of FGF2 membrane translocation with FGF2 being retained on cell surfaces (**Figure 1**). Following translocation into the extracellular space, FGF2 is capable of spreading to neighboring cells, probably mediated by direct exchange between heparan sulfate chains that are linked to proteoglycans on cell surfaces that are in close proximity (Zehe et al., 2006). Thus, from the biosynthesis of FGF2 on free ribosomes all the way to the cell surface, heparan sulfate proteoglycans exert multiple functions with 1) mediating the final step of FGF2 secretion (Nickel, 2007; Zehe et al., 2006), 2) protecting FGF2 on cell surfaces against degradation and denaturation (Nugent and Iozzo, 2000) and 3) mediating FGF2 signaling through ternary complexes in which FGF2, heparan sulfate chains, and FGF high-affinity receptors are engaged (Presta et al., 2005; Belov and Mohammadi, 2013; Ribatti et al., 2007). In conclusion, directional transport of FGF2 into the extracellular space depends on sequential interactions of FGF2 with $PI(4,5)P_2$ at the inner leaflet and, bridged my membrane translocation intermediates, interactions with heparan sulfates on the cell surface (**Figure 1**). The proposed mechanism is further supported by previous studies demonstrating that FGF2 remains in a fully folded state during all steps of its unconventional secretory route (Backhaus et al., 2004; Torrado et al., 2009; Nickel, 2011), a phenomenon that reflects the requirement for the formation of defined oligomers during membrane insertion. These findings imply a quality control step that ensures secretion to be limited to FGF2 species that are biologically active (Torrado et al., 2009; Nickel, 2011).

Another aspect of quality control as part of the unconventional secretory pathway of FGF2 might be related to the role of the Na, K-ATPase in this process. Whereas its function may be restricted to building a landing platform as the first contact point of FGF2 at the inner plasma membrane leaflet (Legrand et al., 2020), it is also speculated that unconventional secretion of FGF2 could be linked to the regulation of the ATPase activity of the Na,K-ATPase (Pallotta and Nickel, 2020). Because FGF2 secretion involves the formation of a transient lipidic pore in the plasma membrane and FGF2 binds to a region in the cytoplasmic domain of the α-subunit of the Na,K-ATPase that contains its enzymatic activity (Legrand et al., 2020), it appears to be an intriguing hypothesis that FGF2 might upregulate the ATPase activity of this Na,K exchanger. This, in turn, might help to maintain the membrane potential under circumstances that trigger the formation of lipidic membrane pores during unconventional secretion of FGF2, a process that does not appear to compromise cell viability (Dimou and Nickel, 2018; Steringer and Nickel, 2018; Pallotta and Nickel, 2020).

The molecular mechanism illustrated in **Figure 1** is also relevant for other unconventionally secreted proteins. For example, HIV-Tat and Tau are shown to directly translocate across plasma membranes to get access to the extracellular space. Like FGF2, these processes require physical interactions with $PI(4,5)P_2$ at the inner leaflet and heparan sulfates at the outer leaflet of the plasma membrane (Rayne et al., 2010; Debaisieux et al., 2012; Zeitler et al., 2015; Agostini et al., 2017; Katsinelos et al., 2018; Merezhko et al., 2018). In addition, certain aspects of this process may also be relevant to the unconventional secretory mechanism of interleukin 1β, a process that, under certain physiological conditions, is based upon the formation of membrane pores that are triggered by $PI(4,5)P_2$-dependent oligomerization of inflammasome-activated Gasdermin D (He et al., 2015; Martín-Sánchez et al., 2016; Brough et al., 2017; Evavold et al., 2017; Monteleone et al., 2018).

Recent Evidence Suggesting Liquid-Ordered Nanodomains to Play a Role in Organizing the Molecular Machinery Mediating FGF2 Membrane Translocation

The molecular principles of the unconventional secretory pathway of FGF2 could be recapitulated recently by two complementary experimental approaches, the biochemical reconstitution of FGF2 membrane translocation using purified

components and giant unilamellar vesicles (GUVs) (Steringer et al., 2017) as well as the real-time imaging of FGF2 membrane translocation in living cells using single molecule TIRF microscopy (Dimou et al., 2019). In these studies, the molecular mechanism of this process has been validated and is now established in the field as the best-characterized example for a UPS Type I pathway (**Figure 1**) (Dimou and Nickel, 2018; Pallotta and Nickel, 2020). However, a striking difference was observed when comparing these experimental systems concerning the kinetics by which FGF2 can physically traverse the membrane. Using purified components to reconstitute FGF2 membrane translocation, incubation times in the range of several tens of minutes were required to observe a substantial amount of GUVs into which FGF2 had translocated (Steringer et al., 2017). Similar observations are made in experimental systems reconstituting $PI(4,5)P_2$-dependent FGF2 oligomerization and membrane insertion (Steringer et al., 2012). By contrast, the time interval required for FGF2 translocation from the inner to the outer leaflet of the plasma membrane in living cells was found to be in the range of 200 ms (Dimou et al., 2019). Thus, whereas the molecular requirements were found to be identical with $PI(4,5)P_2$-dependent oligomerization and heparan sulfate-mediated capturing of FGF2 being essential for FGF2 membrane translocation in both experimental systems, a vast difference was observed with regard to kinetics. Most likely, several factors contribute to this phenomenon. For example, in the biochemical reconstitution system, $PI(4,5)P_2$ is not present in an asymmetric distribution between the two leaflets that characterizes native plasma membranes. Further, whereas heparan sulfate chains on cell surfaces are contained in proteoglycans positioning them in a membrane-proximal manner, soluble heparin was added to the lumen of GUVs in reconstitution experiments (Steringer et al., 2017). Finally, auxiliary factors, such as the Na, K-ATPase, and Tec kinase, were absent in the *in vitro* reconstitution experiments. Whereas these factors probably affect the kinetics of FGF2 membrane translocation, they are unlikely to fully explain the vast difference of minutes versus milliseconds observed for this process when *in vitro* conditions (Steringer et al., 2012; Steringer et al., 2017) were compared with the authentic action observed in living cells (Dimou et al., 2019). What could be a compelling and testable explanation for the observed differences? An intriguing hypothesis would be the existence of nanodomains in native plasma membranes in which all components of the FGF2 secretion machinery are brought into proximity. As detailed below, recent studies indeed provide initial evidence for the structural organization of the FGF2 secretion machinery in specialized plasma membrane nanodomains.

A Role for Liquid-Ordered Nanodomains as a Structural Platform of the FGF2 Secretion Machinery?

Several lines of evidence support the idea of a subpopulation of liquid-ordered membrane domains enriched in cholesterol and $PI(4,5)P_2$ as platforms that host the machinery mediating FGF2 membrane translocation. First, in a recent study, cholesterol is demonstrated to be a critical factor affecting the ability of FGF2 to get recruited to membranes in a $PI(4,5)P_2$-dependent manner with high binding strength and fast kinetics (Lolicato et al., 2021). The physiological relevance of this phenomenon could be confirmed in intact cells with increased levels of cholesterol resulting in higher efficiencies of FGF2 transport into the extracellular space. This study further provides insights into the molecular mechanism by which cholesterol affects both $PI(4,5)P_2$-dependent recruitment and membrane translocation of FGF2 using molecular dynamics simulations. An increase of cholesterol at the expense of phosphatidylcholine, a scenario that mimics the changes in membrane lipid compositions when plasma membranes are compared with the endoplasmic reticulum, caused two phenomena. First, the visibility of the head group of $PI(4,5)P_2$ was found to be increased, facilitating FGF2 binding to lipid bilayers. Second, in the presence of increased levels of cholesterol, $PI(4,5)P_2$ was found to cluster forming trimers and tetramers. This, in turn, causes an increase in avidity, explaining faster binding kinetics and an enhanced binding strength of FGF2 toward $PI(4,5)P_2$ (Lolicato et al., 2021). The observed effects of cholesterol could also be directly relevant for the subsequent oligomerization of FGF2. Using molecular dynamics simulations, it was found that cholesterol-containing membranes stabilize an orientation of $PI(4,5)P_2$ that promotes the formation of disulfide-linked dimers of FGF2 (Steringer et al., 2017). Under these conditions, in addition to the defined high-affinity $PI(4,5)P_2$ binding site in FGF2, additional $PI(4,5)P_2$ molecules were found to bind to FGF2 at other sites. The role of $PI(4,5)P_2$ in this process is a highly specific one as artificial membrane anchors, such as a Ni-NTA lipid along with a His-tagged version of FGF2, were found incapable of forming functional FGF2 oligomers that form lipidic membrane pores as transient intermediates in unconventional secretion of FGF2 (Steringer et al., 2012; Steringer et al., 2017). Beyond the abovementioned studies on FGF2, an enrichment of $PI(4,5)P_2$ in liquid-ordered domains organized by cholesterol has indeed been recognized in other studies as well (Myeong et al., 2021; Wen et al., 2021).

Another recent study added further support to the idea that FGF2 membrane translocation occurs in cholesterol-enriched plasma membrane nanodomains characterized by a liquid-ordered state. In a BioID screen probing for proteins that are in proximity of FGF2 at any time point of its lifetime in cells, a specific type of heparan sulfate proteoglycan has been identified as the key driver of FGF2 secretion, Glypican-1 (GPC1) (Sparn et al., 2021). Whereas a knockout of GPC-1 was found to cause a substantial decrease in FGF2 secretion efficiencies, overexpression of GPC1 did not only rescue to wild-type levels but rather significantly increased FGF2 secretion rates. Furthermore, biochemical analyses revealed that the heparan sulfate chains of GPC1 contain high-affinity sites for FGF2 that are less present in other heparan sulfate proteoglycans, including members of the glypican and syndecan families. Of note, like all glypicans, GPC1 is associated with the outer leaflet of the plasma membrane *via* a GPI anchor (Filmus et al., 2008). Like other membrane proteins with GPI anchors, GPC1 is known to partition into liquid-ordered plasma membrane domains on cell

surfaces. In addition, GPC1 contains a large N-terminal domain that builds a lid-like structure with a length of about 10 nm on top of the membrane. The N-terminal lid domain is connected to the GPI anchor *via* a linker to which three heparan sulfate chains are attached. They are oriented in a highly membrane-proximal manner with the distance between them and the membrane surface being just about 3 nm. These observations imply that the prominent role of GPC1 in unconventional secretion of FGF2 is promoted by its unique structure that appears to form a microenvironment between the GPC1 lid and the membrane surface in which high-affinity binding sites for FGF2 are arranged in a membrane-proximal and highly concentrated manner.

DISCUSSION

Whereas the principal molecular components and mechanisms of the molecular machinery mediating unconventional secretion of FGF2 have been identified, its spatiotemporal organization at the plasma membrane is unknown. With recent findings demonstrating a role for cholesterol promoting efficient binding of FGF2 to $PI(4,5)P_2$ concomitant with increased FGF2 secretion rates, the identification of $PI(4,5)P_2$ clusters in liquid-ordered domains and the identification of GPC1, a GPI-anchored heparan sulfate proteoglycan that partitions into liquid-ordered domains, being the key driver of the unconventional secretory pathway of FGF2, we propose all components of this pathway to reside in plasma membrane nanodomains in a highly organized manner. It will be of great interest to challenge this hypothesis further, in particular with regard to the Na, K-ATPase that is the initial contact point for FGF2 at the inner plasma membrane leaflet. It will be an important future goal to reconstitute this type of nanodomain with purified components to uncover the mechanisms underlying the vast kinetic differences that have been found between *in vitro* reconstitution experiments and the authentic action observed in living cells. A comprehensive understanding of this pathway will not only solve a long-standing problem in molecular cell biology, but will also pave the way for the development of new inhibitors that have great potential for cancer therapy, for example, in fighting chemoresistances that are caused by FGF2 in acute myeloid leukemia.

AUTHOR CONTRIBUTIONS

FL and WN: Conceptual design, development of hypotheses and writing the manuscript.

FUNDING

Deutsche Forschungsgemeinschaft (SFB/TRR 83, SFB/TRR 186, DFG Ni 423 / 9-1, DFG Ni 423 / 10-1, DFG Ni 423 / 12-1).

REFERENCES

Agostini, S., Ali, H., Vardabasso, C., Fittipaldi, A., Tasciotti, E., Cereseto, A., et al. (2017). Inhibition of Non Canonical HIV-1 Tat Secretion through the Cellular Na + ,K + -ATPase Blocks HIV-1 Infection. *EBioMedicine* 21, 170–181. doi:10.1016/j.ebiom.2017.06.011

Akl, M. R., Nagpal, P., Ayoub, N. M., Tai, B., Prabhu, S. A., Capac, C. M., et al. (2016). Molecular and Clinical Significance of Fibroblast Growth Factor 2 (FGF2/bFGF) in Malignancies of Solid and Hematological Cancers for Personalized Therapies. *Oncotarget* 7, 44735–44762. doi:10.18632/oncotarget.8203

Backhaus, R., Zehe, C., Wegehingel, S., Kehlenbach, A., Schwappach, B., and Nickel, W. (2004). Unconventional Protein Secretion: Membrane Translocation of FGF-2 Does Not Require Protein Unfolding. *J. Cel Sci* 117, 1727–1736. doi:10.1242/jcs.01027

Beenken, A., and Mohammadi, M. (2009). The FGF Family: Biology, Pathophysiology and Therapy. *Nat. Rev. Drug Discov.* 8, 235–253. doi:10.1038/nrd2792

Belov, A. A., and Mohammadi, M. (2013). Molecular Mechanisms of Fibroblast Growth Factor Signaling in Physiology and Pathology. *Cold Spring Harb Perspect. Biol.* 5, a015958. doi:10.1101/cshperspect.a015958

Brough, D., Pelegrin, P., and Nickel, W. (2017). An Emerging Case for Membrane Pore Formation as a Common Mechanism for the Unconventional Secretion of FGF2 and IL-1β. *J. Cel Sci* 130, 3197–3202. doi:10.1242/jcs.204206

Debaisieux, S., Rayne, F., Yezid, H., and Beaumelle, B. (2012). The Ins and Outs of HIV-1 Tat. *Traffic* 13, 355–363. doi:10.1111/j.1600-0854.2011.01286.x

Dimou, E., Cosentino, K., Platonova, E., Ros, U., Sadeghi, M., Kashyap, P., et al. (2019). Single Event Visualization of Unconventional Secretion of FGF2. *J. Cel Biol* 218 (2), 683–699. doi:10.1083/jcb.201802008

Dimou, E., and Nickel, W. (2018). Unconventional Mechanisms of Eukaryotic Protein Secretion. *Curr. Biol.* 28, R406–R410. doi:10.1016/j.cub.2017.11.074

Ebert, A. D., Laussmann, M., Wegehingel, S., Kaderali, L., Erfle, H., Reichert, J., et al. (2010). Tec-kinase-mediated Phosphorylation of Fibroblast Growth Factor 2 Is Essential for Unconventional Secretion. *Traffic* 11, 813–826. doi:10.1111/j.1600-0854.2010.01059.x

Evavold, C. L., Ruan, J., Tan, Y., Xia, S., Wu, H., and Kagan, J. C. (2017). The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages. *Immunity* 48 (1), 35–44.e6. doi:10.1016/j.immuni.2017.11.013

Filmus, J., Capurro, M., and Rast, J. (2008). Glypicans. *Genome Biol.* 9, 224. doi:10.1186/gb-2008-9-5-224

Gilbert, R. J. C., Serra, M. D., Froelich, C. J., Wallace, M. I., and Anderluh, G. (2014). Membrane Pore Formation at Protein-Lipid Interfaces. *Trends Biochem. Sci.* 39, 510–516. doi:10.1016/j.tibs.2014.09.002

He, W.-t., Wan, H., Hu, L., Chen, P., Wang, X., Huang, Z., et al. (2015). Gasdermin D Is an Executor of Pyroptosis and Required for Interleukin-1β Secretion. *Cell Res* 25, 1285–1298. doi:10.1038/cr.2015.139

Javidi-Sharifi, N., Martinez, J., English, I., Joshi, S. K., Scopim-Ribeiro, R., Viola, S. K., et al. (2019). FGF2-FGFR1 Signaling Regulates Release of Leukemia-Protective Exosomes from Bone Marrow Stromal Cells. *Elife* 8, e40033. doi:10.7554/eLife.40033

Katsinelos, T., Zeitler, M., Dimou, E., Karakatsani, A., Müller, H.-M., Nachman, E., et al. (2018). Unconventional Secretion Mediates the Trans-cellular Spreading of Tau. *Cel Rep.* 23, 2039–2055. doi:10.1016/j.celrep.2018.04.056

La Venuta, G., Wegehingel, S., Sehr, P., Müller, H.-M., Dimou, E., Steringer, J. P., et al. (2016). Small Molecule Inhibitors Targeting Tec Kinase Block Unconventional Secretion of Fibroblast Growth Factor 2. *J. Biol. Chem.* 291, 17787–17803. doi:10.1074/jbc.m116.729384

Legrand, C., Saleppico, R., Sticht, J., Lolicato, F., Müller, H.-M., Wegehingel, S., et al. (2020). The Na,K-ATPase Acts Upstream of Phosphoinositide PI(4,5)P2 Facilitating Unconventional Secretion of Fibroblast Growth Factor 2. *Commun. Biol.* 3, 141. doi:10.1038/s42003-020-0871-y

Lolicato, F., Saleppico, R., Griffo, A., Pokrandt, B., Müller, H. M., Ewers, H., et al. (2021). Cholesterol Promotes Both Head Group Visibility and Clustering of

PI(4,5)P2 Driving Unconventional Secretion of Fibroblast Growth Factor 2. *bioRxiv*. doi:10.1101/2021.04.16.440132

Malhotra, V. (2013). Unconventional Protein Secretion: an Evolving Mechanism. *EMBO J.* 32, 1660–1664. doi:10.1038/emboj.2013.104

Martín-Sánchez, F., Diamond, C., Zeitler, M., Gomez, A. I., Baroja-Mazo, A., Bagnall, J., et al. (2016). Inflammasome-dependent IL-1β Release Depends upon Membrane Permeabilisation. *Cell Death Differ* 23, 1219–1231. doi:10.1038/cdd.2015.176

Merezhko, M., Brunello, C. A., Yan, X., Vihinen, H., Jokitalo, E., Uronen, R.-L., et al. (2018). Secretion of Tau via an Unconventional Non-vesicular Mechanism. *Cel Rep.* 25, 2027–2035. doi:10.1016/j.celrep.2018.10.078

Monteleone, M., Stanley, A. C., Chen, K. W., Brown, D. L., Bezbradica, J. S., von Pein, J. B., et al. (2018). Interleukin-1β Maturation Triggers its Relocation to the Plasma Membrane for Gasdermin-D-dependent and -Independent Secretion. *Cel Rep.* 24, 1425–1433. doi:10.1016/j.celrep.2018.07.027

Muesch, A., Hartmann, E., Rohde, K., Rubartelli, A., Sitia, R., and Rapoport, T. A. (1990). A Novel Pathway for Secretory Proteins? *Trends Biochem. Sci.* 15, 86–88. doi:10.1016/0968-0004(90)90186-f

Müller, H.-M., Steringer, J. P., Wegehingel, S., Bleicken, S., Münster, M., Dimou, E., et al. (2015). Formation of Disulfide Bridges Drives Oligomerization, Membrane Pore Formation, and Translocation of Fibroblast Growth Factor 2 to Cell Surfaces. *J. Biol. Chem.* 290, 8925–8937. doi:10.1074/jbc.m114.622456

Myeong, J., Park, C. G., Suh, B. C., and Hille, B. (2021). Compartmentalization of Phosphatidylinositol 4,5-bisphosphate Metabolism into Plasma Membrane Liquid-Ordered/raft Domains. *Proc. Natl. Acad. Sci. U S A.* 118. doi:10.1073/pnas.2025343118

Nickel, W., and Rabouille, C. (2009). Mechanisms of Regulated Unconventional Protein Secretion. *Nat. Rev. Mol. Cel Biol* 10, 148–155. doi:10.1038/nrm2617

Nickel, W., and Seedorf, M. (2008). Unconventional Mechanisms of Protein Transport to the Cell Surface of Eukaryotic Cells. *Annu. Rev. Cel Dev. Biol.* 24, 287–308. doi:10.1146/annurev.cellbio.24.110707.175320

Nickel, W. (2003). The Mystery of Nonclassical Protein Secretion. *Eur. J. Biochem.* 270, 2109–2119. doi:10.1046/j.1432-1033.2003.03577.x

Nickel, W. (2011). The Unconventional Secretory Machinery of Fibroblast Growth Factor 2. *Traffic* 12, 799–805. doi:10.1111/j.1600-0854.2011.01187.x

Nickel, W. (2007). Unconventional Secretion: an Extracellular Trap for export of Fibroblast Growth Factor 2. *J. Cel Sci* 120, 2295–2299. doi:10.1242/jcs.011080

Noh, K. H., Kim, S.-H., Kim, J. H., Song, K.-H., Lee, Y.-H., Kang, T. H., et al. (2014). API5 Confers Tumoral Immune Escape through FGF2-dependent Cell Survival Pathway. *Cancer Res.* 74, 3556–3566. doi:10.1158/0008-5472.can-13-3225

Nugent, M. A., and Iozzo, R. V. (2000). Fibroblast Growth Factor-2. *Int. J. Biochem. Cel Biol.* 32, 115–120. doi:10.1016/s1357-2725(99)00123-5

Pallotta, M. T., and Nickel, W. (2020). FGF2 and IL-1β - Explorers of Unconventional Secretory Pathways at a Glance. *J. Cel Sci* 133. doi:10.1242/jcs.250449

Plotnikov, A. N., Schlessinger, J., Hubbard, S. R., and Mohammadi, M. (1999). Structural Basis for FGF Receptor Dimerization and Activation. *Cell* 98, 641–650. doi:10.1016/s0092-8674(00)80051-3

Presta, M., Dell'Era, P., Mitola, S., Moroni, E., Ronca, R., and Rusnati, M. (2005). Fibroblast Growth Factor/fibroblast Growth Factor Receptor System in Angiogenesis. *Cytokine Growth Factor. Rev.* 16, 159–178. doi:10.1016/j.cytogfr.2005.01.004

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cel Biol.* 27, 230–240. doi:10.1016/j.tcb.2016.11.007

Raman, R., Venkataraman, G., Ernst, S., Sasisekharan, V., and Sasisekharan, R. (2003). Structural Specificity of Heparin Binding in the Fibroblast Growth Factor Family of Proteins. *Proc. Natl. Acad. Sci.* 100, 2357–2362. doi:10.1073/pnas.0437842100

Rayne, F., Debaisieux, S., Yezid, H., Lin, Y.-L., Mettling, C., Konate, K., et al. (2010). Phosphatidylinositol-(4,5)-bisphosphate Enables Efficient Secretion of HIV-1 Tat by Infected T-Cells. *EMBO J.* 29, 1348–1362. doi:10.1038/emboj.2010.32

Ribatti, D., Vacca, A., Rusnati, M., and Presta, M. (2007). The Discovery of Basic Fibroblast Growth Factor/fibroblast Growth Factor-2 and its Role in Haematological Malignancies. *Cytokine Growth Factor. Rev.* 18, 327–334. doi:10.1016/j.cytogfr.2007.04.011

Schlessinger, J., Plotnikov, A. N., Ibrahimi, O. A., Eliseenkova, A. V., Yeh, B. K., Yayon, A., et al. (2000). Crystal Structure of a Ternary FGF-FGFR-Heparin Complex Reveals a Dual Role for Heparin in FGFR Binding and Dimerization. *Mol. Cel* 6, 743–750. doi:10.1016/s1097-2765(00)00073-3

Sparn, C., Dimou, E., Meyer, A., Saleppico, R., Wegehingel, S., Gerstner, M., et al. (2021). Glypican-1 Drives Unconventional Secretion of Fibroblast Growth Factor 2. *bioRxiv*. doi:10.1101/2021.11.11.468179

Steringer, J. P., Lange, S., Čujová, S., Šachl, R., Poojari, C., Lolicato, F., et al. (2017). Key Steps in Unconventional Secretion of Fibroblast Growth Factor 2 Reconstituted with Purified Components. *Elife* 6, e28985. doi:10.7554/eLife.28985

Steringer, J. P., Bleicken, S., Andreas, H., Zacherl, S., Laussmann, M., Temmerman, K., et al. (2012). Phosphatidylinositol 4,5-Bisphosphate (PI(4,5)P2)-dependent Oligomerization of Fibroblast Growth Factor 2 (FGF2) Triggers the Formation of a Lipidic Membrane Pore Implicated in Unconventional Secretion. *J. Biol. Chem.* 287, 27659–27669. doi:10.1074/jbc.m112.381939

Steringer, J. P., and Nickel, W. (2018). A Direct Gateway into the Extracellular Space: Unconventional Secretion of FGF2 through Self-Sustained Plasma Membrane Pores. *Semin. Cel. Dev. Biol.* 83, 3–7. doi:10.1016/j.semcdb.2018.02.010

Temmerman, K., Ebert, A. D., Müller, H.-M., Sinning, I., Tews, I., and Nickel, W. (2008). A Direct Role for Phosphatidylinositol-4,5-Bisphosphate in Unconventional Secretion of Fibroblast Growth Factor 2. *Traffic* 9, 1204–1217. doi:10.1111/j.1600-0854.2008.00749.x

Temmerman, K., and Nickel, W. (2009). A Novel Flow Cytometric Assay to Quantify Interactions between Proteins and Membrane Lipids. *J. Lipid Res.* 50, 1245–1254. doi:10.1194/jlr.d800043-jlr200

Torrado, L. C., Temmerman, K., Müller, H.-M., Mayer, M. P., Seelenmeyer, C., Backhaus, R., et al. (2009). An Intrinsic Quality-Control Mechanism Ensures Unconventional Secretion of Fibroblast Growth Factor 2 in a Folded Conformation. *J. Cel Sci* 122, 3322–3329. doi:10.1242/jcs.049791

Traer, E., Martinez, J., Javidi-Sharifi, N., Agarwal, A., Dunlap, J., English, I., et al. (2016). FGF2 from Marrow Microenvironment Promotes Resistance to FLT3 Inhibitors in Acute Myeloid Leukemia. *Cancer Res.* 76, 6471–6482. doi:10.1158/0008-5472.can-15-3569

Wen, Y., Vogt, V. M., and Feigenson, G. W. (2021). PI(4,5)P2 Clustering and its Impact on Biological Functions. *Annu. Rev. Biochem.* 90, 681–707. doi:10.1146/annurev-biochem-070920-094827

Zacherl, S., La Venuta, G., Müller, H.-M., Wegehingel, S., Dimou, E., Sehr, P., et al. (2015). A Direct Role for ATP1A1 in Unconventional Secretion of Fibroblast Growth Factor 2. *J. Biol. Chem.* 290, 3654–3665. doi:10.1074/jbc.m114.590067

Zehe, C., Engling, A., Wegehingel, S., Schafer, T., and Nickel, W. (2006). Cell-surface Heparan Sulfate Proteoglycans Are Essential Components of the Unconventional export Machinery of FGF-2. *Proc. Natl. Acad. Sci.* 103, 15479–15484. doi:10.1073/pnas.0605997103

Zeitler, M., Steringer, J. P., Müller, H.-M., Mayer, M. P., and Nickel, W. (2015). HIV-tat Protein Forms Phosphoinositide-dependent Membrane Pores Implicated in Unconventional Protein Secretion. *J. Biol. Chem.* 290, 21976–21984. doi:10.1074/jbc.m115.667097

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 07 April 2022
doi: 10.3389/fcell.2022.852028

Golgi-Bypass Is a Major Unconventional Route for Translocation to the Plasma Membrane of Non-Apical Membrane Cargoes in *Aspergillus nidulans*

Sofia Dimou[1†], *Mariangela Dionysopoulou*[1†], *Georgia Maria Sagia*[1] *and George Diallinas*[1,2*]

[1]Department of Biology, National and Kapodistrian University of Athens, Panepistimioupolis, Athens, Greece, [2]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion, Greece

Nutrient transporters have been shown to translocate to the plasma membrane (PM) of the filamentous fungus *Aspergillus nidulans via* an unconventional trafficking route that bypasses the Golgi. This finding strongly suggests the existence of distinct COPII vesicle subpopulations, one following Golgi-dependent conventional secretion and the other directed towards the PM. Here, we address whether Golgi-bypass concerns cargoes other than nutrient transporters and whether Golgi-bypass is related to cargo structure, size, abundance, physiological function, or polar vs. non-polar distribution in the PM. To address these questions, we followed the dynamic subcellular localization of two selected membrane cargoes differing in several of the aforementioned aspects. These are the proton-pump ATPase PmaA and the PalI pH signaling component. Our results show that neosynthesized PmaA and PalI are translocated to the PM *via* Golgi-bypass, similar to nutrient transporters. In addition, we showed that the COPII-dependent exit of PmaA from the ER requires the alternative COPII coat subunit LstA, rather than Sec24, whereas PalI requires the ER cargo adaptor Erv14. These findings strengthen the evidence of distinct cargo-specific COPII subpopulations and extend the concept of Golgi-independent biogenesis to essential transmembrane proteins, other than nutrient transporters. Overall, our findings point to the idea that Golgi-bypass might not constitute a fungal-specific peculiarity, but rather a novel major and cargo-specific sorting route in eukaryotic cells that has been largely ignored.

Keywords: traffic, secretion, polarity, fungi, COPII, endoplasmic reticulum, Pma1, pH sensing

OPEN ACCESS

Edited by:
Julien Villeneuve, Institute for Functional Genomics (IGF) (CNRS), France

Reviewed by:
Andrea Pompa, University of Urbino Carlo Bo, Italy
Christos Gournas, National Centre of Scientific Research Demokritos, Greece

***Correspondence:**
George Diallinas
diallina@biol.uoa.gr

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *10 January 2022*
Accepted: *03 March 2022*
Published: *07 April 2022*

Citation:
Dimou S, Dionysopoulou M, Sagia GM and Diallinas G (2022) Golgi-Bypass Is a Major Unconventional Route for Translocation to the Plasma Membrane of Non-Apical Membrane Cargoes in Aspergillus nidulans. Front. Cell Dev. Biol. 10:852028. doi: 10.3389/fcell.2022.852028

INTRODUCTION

In eukaryotes, newly made plasma membrane (PM) proteins are thought to be directly sorted from ribosomes to the membrane of the endoplasmic reticulum (ER) *via* a co-translational translocation process (Voorhees and Hegde, 2016). After ER translocation, PM proteins are sorted into nascent ER-exit sites (ERes) and enter into COPII secretory vesicles, which fuse to the *early*- or *cis*-Golgi and then reach the *late*- or *trans*-Golgi network (TGN) *via* Golgi maturation (Zanetti et al., 2012; D'Arcangelo et al., 2013; Feyder et al., 2015; Gomez-Navarro and Miller, 2016; Casler et al., 2019). From the TGN, membrane proteins destined for the PM are thought to be secreted *via* AP-1/

clathrin-coated vesicles, either directly or indirectly through the endosomal compartment, *via* a mechanism controlled by multiple Rab GTPases, a process also requiring microtubule and actin polymerization (Robinson, 2015; Zeng et al., 2017). However, this conventional secretory route of PM proteins has been characterized by studies on a limited number of specific transmembrane or extracellularly secreted cargoes. Noticeably, very little is known on how transporters and receptors, the two most abundant types of PM proteins, are translocated to the PM. In fact, several findings challenge the long-standing mechanism of export from the ER in small COPII-coated vesicles, reporting alternative mechanisms of unconventional protein secretion (UPS) that either do not use COPII components, bypass the Golgi, or exit the TGN in carriers other than the standard AP-1/clathrin-coated vesicles (Rabouille, 2017; Gee et al., 2018). In addition, recently proposed models also challenge the role of COPII in coating ER-budding vesicles, proposing that COPII helps to select secretory cargo, but does not coat the membrane carriers leaving the ER. Notably, these models propose that budding from the ER takes place *via tubular* structures or *tunnels* fusing with distal compartments (Phuyal and Farhan, 2021; Raote and Malhotra, 2021; Shomron et al., 2021; Weigel et al., 2021).

The discovery of alternative membrane cargo trafficking mechanisms opens new questions. The most obvious of these is how cargo specificity for these pathways is determined. Does size, oligomerization state, abundance, or targeting to specific PM micro-domains of cargoes drive distinct ER-exit mechanisms and trafficking to the PM? A cargo-centric view of ER-exit, which constitutes the first step in cargo trafficking, also opens the issue of whether distinct cargoes interact with specific ER-associated *trans*-effectors (e.g., *adaptors, chaperones,* and *v-SNARES*) to form structurally and functionally distinct COPII subpopulations (vesicles, tubules, or tunnels) and how this might be achieved. Experimental evidence supporting the existence of distinct subpopulations of COPII vesicles has been reported, but studies on the issue are limited. Several reports concern observations that GPI-anchored proteins are selectively accumulated in ERes distinct from those of other cargo proteins (Muñiz et al., 2001; Morsomme et al., 2003; Castillon et al., 2009; Bonnon et al., 2010). Other reports have shown that Sec24 isoforms (paralogs) are selective towards distinct cargoes (Roberg et al., 1999; Miller et al., 2002) or are non-uniformly distributed to ERes in *S. cerevisiae* (Iwasaki et al., 2015). In addition, in the moss *Physcomitrella patens*, COPII Sec23 isoforms have been shown to form specific ER-exit sites with differential effects on polarized growth (Chang et al., 2021). A hypothesis that cargo identity may define the formation of distinct COPII complexes and vesicle populations during plant development and in response to stress has also been proposed (Tanaka et al., 2013; Chung et al., 2016). A particular case concerns COPII vesicles involved in the ER-exit of bulky cargoes, which are too big to fit into 'canonical' COPII carriers. In the case of collagen, for example, co-packaging of specific cargo receptors, such as TANGO1 or cTAGE5 and also the COPII initiating factor Sec12 (normally excluded from small COPII vesicles), has been shown to drive the formation of large COPII-coated vesicles (Saito et al., 2009; Yuan et al., 2018; Raote and Malhotra, 2021).

In recent years, we have developed a controllable genetic system for studying membrane cargo trafficking in *Aspergillus nidulans*, a filamentous fungus that emerges as a powerful organism for studying cell biology *in vivo* (Scazzocchio, 2009; Diallinas, 2016; Steinberg et al., 2017; Dimou and Diallinas, 2020; Etxebeste and Espeso, 2020; Pinar and Peñalva, 2021). Through this system we established, in line with results from other fungal groups, that membrane proteins necessary for growth (e.g., cell wall or PM biosynthesis enzymes) follow the conventional secretory route to be *polarly* positioned at the growing tip of elongating cells (*germlings* and *hyphae*). These studies also showed that continuous local endocytic recycling of *apical* cargoes is essential to conserve their localization and function at the tip. Surprisingly, however, our most recent study, using as model membrane cargoes nutrient transporters, which are evenly distributed in the hyphal PM, challenged the concept of Golgi-dependent secretion as the sole major route for membrane cargo subcellular trafficking (Dimou et al., 2020). More specifically, we have obtained experimental evidence that the trafficking of neosynthesized transporters, after COPII-depended exit from the ER, occurs *via* Golgi-bypass and is independent of conventional post-Golgi secretion [e.g., AP-1 clathrin adaptor, Rab11 GTPase, or microtubule polymerization; (Robinson, 2015; Zeng et al., 2017; Pinar and Peñalva, 2021)]. These findings strongly support the existence of distinct, cargo-specific, ER-exit mechanisms and predict the existence of alternative COPII subpopulations.

Here, we examine whether the trafficking of neosynthesized membrane cargoes, other than nutrient transporters, also bypasses the Golgi. The rationale of cargoes selected to be studied is given in the *Results* section. Our study identifies two novel cargoes bypassing the Golgi, namely, the proton-pump ATPase PmaA and the PalI pH sensing component, reinforcing the concept that Golgi-bypass reflects a major mechanism of membrane trafficking in eukaryotic cells. Furthermore, we provide evidence for the existence of distinct cargo-specific COPII carriers based on the observation that the ER-exit of PmaA and PalI necessitates different cargo receptors (Sec24 vs. LstA), while PalI also requires the ER adaptor Erv14.

RESULTS

Rationale of Cargoes Selected to be Studied and Experimental Design

Our primary question addressed in this study was whether Golgi-bypass concerns the sorting of membrane cargoes other than nutrient transporters and whether this mechanism is related to cargo structure, size, abundance, physiological function, or polar (apical) *vs.* non-polar (non-apical) distribution in the PM. To answer these questions, we selected two major *A. nidulans* PM cargoes differing in several of the aforementioned aspects. These are the main H^+ pump ATPase $PmaA^{Pma1}$, which is essential for the PM electrogenic potential needed for the functioning of transporters, and enzymes, regulation of pH, and cell

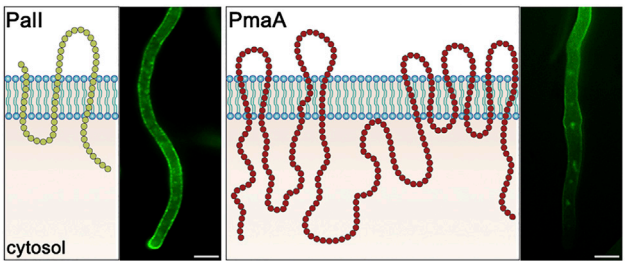


FIGURE 1 | PM topology and subcellular localization of PalI and PmaI. PalI is a 3-TMS protein, with an N-terminal signal peptide and a C-basic terminus facing the cytosol (Calcagno-Pizarelli et al., 2007). PalI-GFP appears evenly distributed to the PM with a prominent apical polarization. PmaA is a 10-TMS protein, with both termini located in the cytosol (Ferreira et al., 2001). PmaA-GFP localizes homogenously to the PM of subapical hyphal regions and is almost absent from the growing apical tip. Scale bars: 5 μm.

homeostasis (Reoyo et al., 1998; Ambesi et al., 2000), and $PalI^{Rim9}$, a component of the tripartite complex involved in signaling a response to an ambient pH value (Peñalva et al., 2014). These cargoes differ in size, number of transmembrane segments, oligomerization status, and essentiality for the cell. Previous studies have shown that PalI is not only non-polarly distributed along the entire PM but also forms cortical puncta of undefined nature (see also **Figure 1**). PmaA localization has not been studied in *A. nidulans*, but indirect evidence from studies of PmaA homologs in *Neurospora crassa* and *Saccharomyces cerevisiae* also point to a non-polar distribution along the PM (Fajardo-Somera et al., 2013; Henderson et al., 2014), as will also be confirmed herein (**Figure 1**).

We followed the dynamic subcellular localization of functional fluorescent-tagged versions of these cargoes, expressed from controllable promoters, in wild-type and mutant genetic backgrounds conditionally blocked in steps of the conventional secretory pathway. More specifically, we used mutant backgrounds where the transcriptional expression of key proteins involved in COPII formation (Sec24 or Sec13), early ($SedV^{Sed5}$, $GeaA^{Gea1}$) or late Golgi ($HypB^{Sec7}$) functioning, post-Golgi vesicle formation ($RabE^{Rab11}$, AP-1^{σ}, $ClaH^{Clh1}$), and sorting or recycling endosome functioning ($RabA/B^{Rab5}$) can be tightly repressed (in superscript names of well-studied true orthologs in *S. cerevisiae*). Transcriptional repression of these factors was achieved using alleles where native promoters of the endogenous genes were replaced by the thiamine-repressible $thiA_p$ promoter, as described in detail by Dimou et al. (2020) and in brief in *Materials and methods*. In all cases, the trafficking of neosynthesized cargoes was examined after the establishment of repression and depletion of factors essential for conventional Golgi-dependent trafficking, as shown by Dimou et al. (2020). In addition, we studied the trafficking of neosynthesized PmaA and PalI in the presence of drugs leading to depolymerization of tubulin (benomyl) or actin (latrunculin), also as described by Dimou et al. (2020). Finally, we performed co-localization studies of these two cargoes with key fluorescent molecular markers of the conventional secretory pathway, such as SedV, PH^{OSBP}, and RabE, as established by Dimou et al. (2020). Results obtained were compared with those from a nutrient transporter (e.g., UapA) and a standard non-polar cargo, SynA (synaptobrevin secretory v-SNARE). The results obtained are described and discussed in the next sections.

PmaA Translocation to the PM Bypasses the Conventional Golgi-Dependent Route

PmaA-GFP was expressed initially *via* its native promoter in a strain where the endogenous gene had been replaced *via* targeted homologous recombination with a GFP-tagged version of PmaA. The wild-type-like phenotype of this strain strongly suggests that the PmaA-GFP is functional in all growth media and pH tested (**Figure 2A**). Under epifluorescence microscopy, PmaA-GFP showed the expected PM-associated localization at all developmental stages tested (germinating conidiospores, germlings, and hyphae; **Figure 2B**; **Supplementary Figure S1**). As the main aspect of our study is the trafficking of newly synthesized membrane cargoes, we asked whether different physiological conditions might regulate the abundance of PmaA to the PM at a level that could be visibly monitored by epifluorescence microscopy. In *S. cerevisiae*, Pma1 is highly regulated by the presence of glucose, both transcriptionally and post-translationally, and by the decrease to the intracellular pH (Serrano, 1983; Cyert and Philpott, 2013). In our case, cellular expression and high steady-state levels of PmaA proved to be similar in minimal media differing in the carbon source or/and pH values (**Figure 2B**). The absence of glucose induction of PmaA expression was mentioned previously not only for *A. nidulans* (Abdallah et al., 2000) but also for its ortholog in *Penicillium simplicissimum* (Burgstaller et al., 1997), unlike what has been reported in *S. cerevisiae*. Overall, these results confirmed that GFP tagging has not affected PmaA cellular expression and PM localization and function and that PmaA expression is constitutive in *A. nidulans*.

In order to follow the localization of *de novo* made PmaA and given the constitutive expression of native PmaA, we replaced its endogenous promoter with the regulatable $alcA_p$ promoter. $alcA_p$ has been used previously for regulating the expression of other cargoes (Martzoukou et al., 2015; Dimou et al., 2020). In brief, transcription *via* $alcA_p$ is tightly repressed in the presence of glucose, but derepressed upon a shift of cells to fructose media. Levels of proteins expressed upon $alcA_p$ derepression are considered moderate. The strain expressing $alcA_p$-PmaA-GFP was used to examine the dynamic localization of *de novo*-made PmaA-GFP in single growing cells at an early stage of development (e.g., germlings) and mature hyphae. **Figure 2C** shows that, after derepression of transcription, PmaA first localizes in a membranous mesh and few static cytosolic puncta (best seen at 110–140 min) and progressively labels more abundant cortical puncta (>140–230 min), to eventually label the entire PM in a rather homogenous manner (230 min). This picture, obtained in all cells examined (>100), resembles the one observed with nutrient transporters, rather than those obtained with apical cargoes involved in polar growth (for a comparison with the dynamics of localization of transporters and apical markers, see Dimou et al., 2020). Thus, PmaA seems to

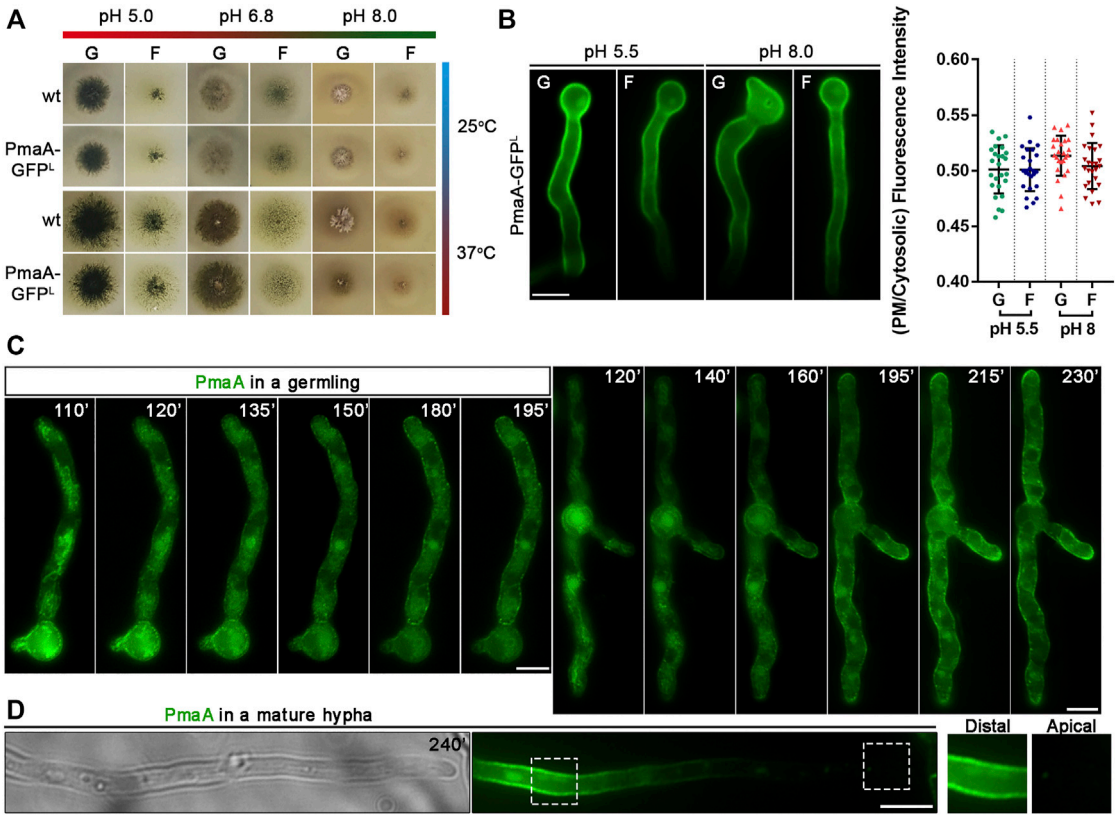


FIGURE 2 | Growth phenotypes of strains expressing PmaA-GFP and subcellular localization at different development stages or pH. **(A)** Comparative growth test analysis of a strain expressing the GFP-tagged version of PmaA with an isogenic wild-type control strain on selected C sources [G: 1% (w/v) glucose; F: 0.1% (w/v) fructose] at 25°C or 37°C, at pH 5, 6.8, or 8. It is noted that the growth rate and morphology of the strain expressing the in-locus GFP-tagged version of PmaA are identical to those of the control strain, in all conditions tested. **(B)** Epifluorescence microscopy of a strain expressing the in-locus GFP-tagged version of PmaA at acidic (pH 5.5) or alkaline pH (pH 8) with glucose [G: 1% (w/v)] or fructose [F: 0.1% (w/v)] as the sole C source. It is noted that PmaA-GFP fluorescence is identical in all conditions. Results shown are confirmed by quantification (right panel) of PmaA-GFP PM/cytosolic intensity ratios for all conditions tested (for details, see *Materials and Methods*). Mean intensity ratios of PmaA-GFP are 0.501 ± 0.022 in glucose pH 5.5, 0.501 ± 0.019 in fructose pH 5.5, 0.513 ± 0.018 in glucose pH 8, and 0.504 ± 0.021 in fructose pH 8. For the statistical analysis, Tukey's multiple comparison test was performed (one-way ANOVA). No statistically significant differences were found between different conditions. Biological/technical replicates for each condition: 2/25. **(C)** Epifluorescence microscopy of newly synthesized PmaA-GFP in two germlings after derepression of transcription (110–230 min). PmaA appears firstly in a membranous mesh and various cytosolic puncta (110–140 min) and steadily labels cortical puncta and finally the entire PM. **(D)** In mature hyphae, PmaA is practically no longer found in the PM of the apical region. Scale bars: 5 μm.

label an ER-like membrane mesh, rather than small Golgi-like foci. We also notice that, while in germlings PmaA labeled the growing tip (see 195–230 min), in more mature hyphae it was absent from the growing apical region (**Figure 2D**). The almost absent fluorescence from the tip area comes in agreement with observations of PMA-1-GFP localization in *N. crassa* (Fajardo-Somera et al., 2013). This localization is similar to nutrient transporters, and as reasoned by Dimou et al. (2020), strongly suggests that in mature hyphae neosynthesized PmaA and transporters are directly localized in the PM *via* lateral translocation from internal membranes, rather than being sorted to the apical tip and then diffusing laterally to the posterior PM.

The strain expressing PmaA-GFP from $alcA_p$ was genetically crossed with strains carrying $thiA_p$-repressible alleles of key proteins involved in Golgi functioning and conventional cargo secretion. Appropriate isogenic progeny carrying $alcA_p$-PmaA-GFP and repressible trafficking alleles were selected and were used to further study the sorting mechanism of PmaA. The corresponding strains do not form colonies under repressing conditions (presence of thiamine in the growth medium), except for $thiA_p$-hypB which forms a slow-growing colony, although HypB is not detected in western blot analysis under repressing conditions (**Figure 3A**; Dimou et al., 2020). **Figures 3B–E** show representative results obtained with several cells in each experiment (n > 100). In all cases, we followed the final localization of PmaA-GFP upon 300 min of *de novo* expression, initiated after depletion of key proteins involved in conventional trafficking [i.e., overnight 14–16 h of growth in the presence of thiamine; Dimou et al. (2020)]. Thus, PmaA localization was performed for 5 h in cells where the conventional trafficking pathway was severally repressed. Notice that, in our conditions, upon repression of the conventional secretory pathway, *A. nidulans* cells stop growing and their apical regions swell, but remain alive for at least 10–12 h (Dimou et al., 2020). Collectively, our results show that PmaA is

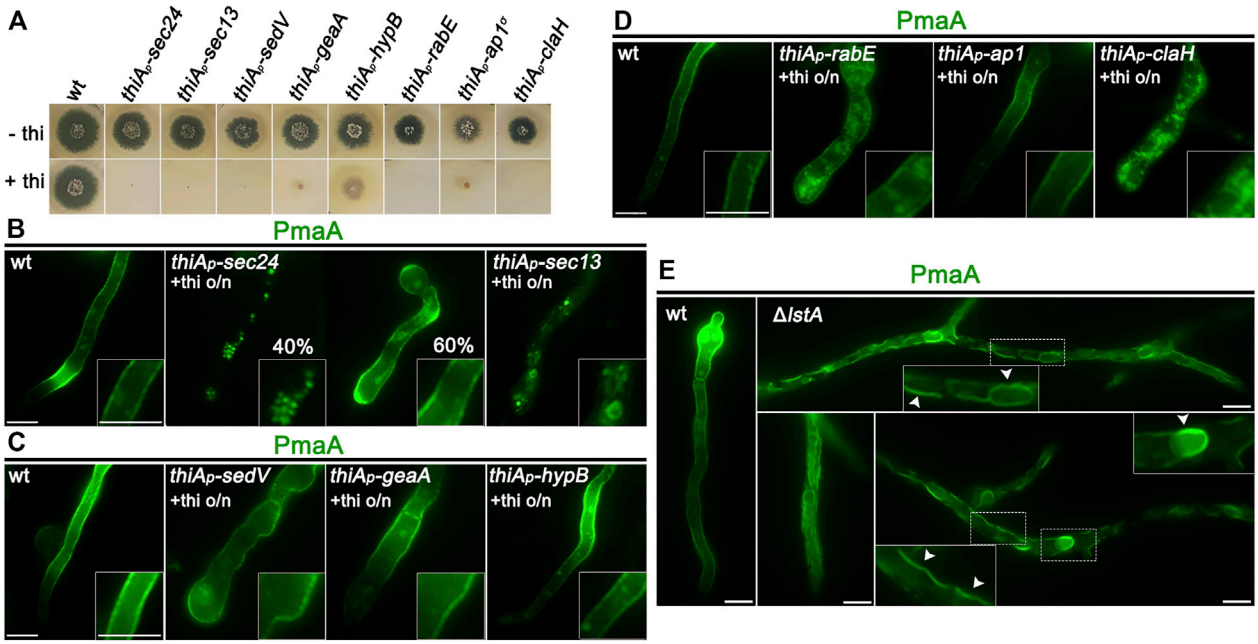


FIGURE 3 | Subcellular localization of neosynthesized PmaA in trafficking mutant backgrounds. **(A)** Growth tests showing that, under repressing conditions (presence of thiamine in the growth medium), strains selected to follow the *de novo* expression of PmaA-GFP do not form proper colonies, confirming that all genes expressed under the $thiA_p$ promoter are tightly repressed. Under derepressing conditions (absence of thiamine from the growth medium), all strains grow almost as the isogenic wild-type control strain (upper row). **(B)** Epifluorescence microscopic analysis of *de novo*-made PmaA-GFP in strains where *sec24* and *sec13* transcription is *ab initio* repressed by thiamine. o/n (overnight) means addition of thiamine from the onset of germination. The total lack of PM-associated signal of PmaA under conditions of Sec13 repression is noted, while Sec24 repression affects the ER-exit of PmaA in only ~40% of the cell population (n = 92). **(C)** Epifluorescence microscopic analysis of *de novo*-made PmaA-GFP in strains where *sedV*, *geaA*, or *hypB* transcription is *ab initio* repressed by thiamine. It is noted that repression of these key Golgi proteins does not affect at all the proper localization of PmaA-GFP to the PM. **(D)** Epifluorescence microscopic analysis of *de novo*-made PmaA-GFP in strains where *rabE*, *ap-1*$^{\sigma}$, or *claH* transcription is *ab initio* repressed by thiamine. Notice that when *rabE* or *ap-1*$^{\sigma}$ is repressed, PmaA-GFP still reaches the PM, but in the latter, there is a clear accumulation of membranous cytoplasmic structures. Repression of *claH* totally abolishes the labeling of the PM and leads to PmaA retention in the cytosolic membrane or aggregates. **(E)** Epifluorescence microscopic analysis of *de novo*-made PmaA-GFP in strains where *lstA* is knocked out. In this case, PmaA fails to reach the PM and remains instead in the ER. The accumulation of PmaA inside the cortical ER and perinuclear ER rings is noted, as highlighted by white arrows. Scale bars: 5 μm.

localized to the PM in all repressible trafficking mutants used, except $thiA_p$-Sec13 and $thiA_p$-ClaH (**Figures 3B–D**). Noticeably, however, repression of RabE (i.e., in $thiA_p$-RabE) led to significant accumulation of cytoplasmic structures resembling membrane aggregates, suggesting the possible indirect involvement of RabE in the efficiency of the localization of PmaA to the PM (**Figure 3D**). Overall, the picture obtained with PmaA expressed in repressible trafficking mutant backgrounds was very similar to that of nutrient transporters, except in the case of $thiA_p$-Sec24, where PmaA translocation to the PM was defective in only 40% of cells (see **Figure 3B**).

The partial independence of PmaA localization from Sec24 expression, not seen with the trafficking of nutrient transporters, which are fully dependent on Sec24 for PM localization (Dimou et al., 2020), suggested that PmaA might also be recognized by an alternative cargo receptor in specific COPII complexes. In *S. cerevisiae*, the ortholog of PmaA, Pma1, is indeed recognized by the Sec24 paralog Lst1, although both receptors co-operate in its ER-exit (Shimoni et al., 2000; Geva et al., 2017). Thus, we considered whether there is a true ortholog of Lst1 in *A. nidulans* and whether this operates in the PmaA ER-exit and further traffic to the PM. An *in silico* search and phylogenetic analysis showed that the product of AN3080 (https://fungidb.org) is a putative Lst1 ortholog and was, thus, named LstA (**Supplementary Figure S2A**, upper left panel). We knocked out (KO) the *lstA* gene by standard reverse genetics and showed that the mutant was viable, albeit showing a severe growth defect, more prominent at acidic conditions (**Supplementary Figure S2A**, upper right panel). $alcA_p$-PmaA-GFP was introduced by genetic crossing in the background of Δ*lstA* and PmaA subcellular localization was followed, as described before. **Figure 3E** shows that the lack of LstA led to retention of PmaA-GFP in perinuclear and cortical ER membranes. The reduced flux of PmaA out of the ER is also compatible with the sensitivity of Δ*lstA* to low pH (**Supplementary Figure S2A**, upper right panel), as also reported in *S. cerevisiae* (Roberg et al., 1999). Thus, COPII vesicles carrying PmaA include mostly LstA, rather than Sec24, similar to *S. cerevisiae*, which in turn suggests that they are distinct in composition from COPII vesicles carrying nutrient transporters or polar markers studied up to date in *A. nidulans*.

The localization of *de novo*-made PmaA to the PM was abolished when actin polymerization was blocked by latrunculin B but was not affected when microtubule

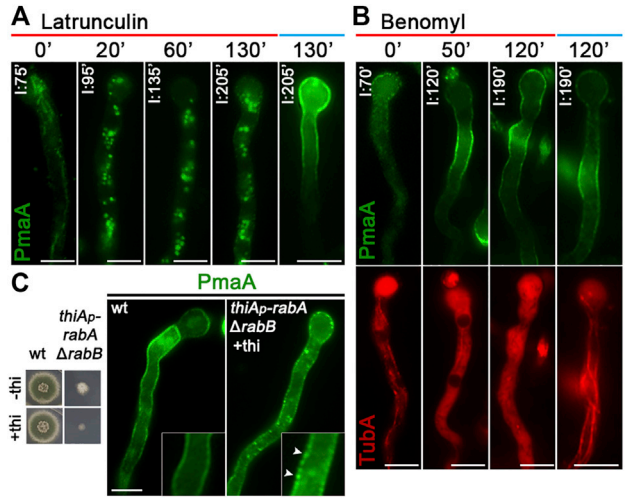


FIGURE 4 | Role of actin, tubulin, or Rab5 endosome function in PmaA subcellular localization. **(A)** Time course of treatment with the actin polymerization drug latrunculin B for 0–130 min of a strain expressing neosynthesized PmaA-GFP under conditions of derepression compared to an untreated strain included as control. In all cases, latrunculin B was added at 75 min of PmaA derepression so that the total time of PmaA-GFP expression was 75–230 min in the different samples. It is noted that PmaA fails to reach the PM when latrunculin is present in the medium, in contrast to the control untreated strain. **(B)** Time course of treatment of strains expressing neosynthesized PmaA-GFP and mCherry-TubA with benomyl for 0–120 min. The anti-microtubule drug was added at 70 min of PmaA derepression so that the total time of PmaA-GFP expression was 70–190 min. Depolymerization of microtubules upon treatment with benomyl was evident by the diffused cytoplasmic signal of mCherry-TubA. It is noted that, in this case, PmaA normally reaches the PM. **(C)** Localization of newly synthesized PmaA-GFP in the absence of both RabA and RabB. *rabA* transcription is *ab initio* repressed (o/n) *via* thiamine, while *rabB* is genetically deleted. The double knockout/knockdown strain fails to form a proper colony in the presence of thiamine, as seen in the growth test on the left. It is noted that PmaA localization to the PM is not affected by the absence of proper endosomal functioning. Scale bars: 5 μm.

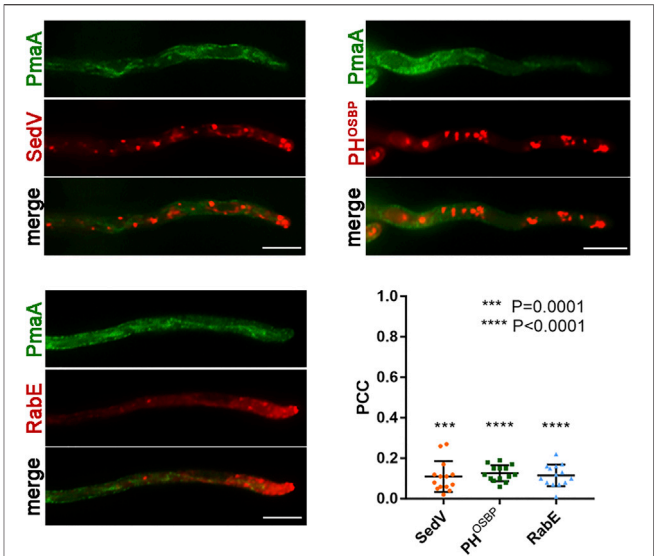


FIGURE 5 | Co-localization of PmaA with Golgi markers. Co-localization analysis and relevant quantification of strains co-expressing *de novo*-made PmaA-GFP with mCherry-SedV, mRFP-PH^{OSBP}, and mRFP-RabE. Quantification by calculating Pearson's correlation coefficient (PCC) shows a clear non-co-localization of PmaA with all the Golgi markers tested, as confirmed by the one-sample *t*-test (PCC = 0.11 ± 0.08 with $^{***}p = 0.0001$ for SedV, PCC = 0.13 ± 0.04 with $^{****}p < 0.0001$ for PH^{OSBP}, and PCC = 0.12 ± 0.05 with $^{****}p < 0.0001$ for RabE). Biological/technical replicates: 3/14 for each strain.

polymerization was blocked by benomyl (**Figures 4A,B**). PmaA localization to the PM was also examined when Rab5-dependent endosomes were repressed or knocked out (i.e., in *thiA$_p$-rabA*/Δ*rabB* double mutant). **Figure 4C** shows that when the functioning Rab5 endosomes were blocked, the great majority of PmaA still reached the PM normally. Although the appearance of a few cytoplasmic PmaA-GFP in *thiA$_p$-rabA*/Δ*rabB* foci might suggest a minor role of Rab5-like endosomes in PmaA exocytosis or recycling, this is in sharp contrast with the absolute PM delocalization effect that the depletion of Rab5-containing endosomes has on some polar secreted cargoes (Hernández-González et al., 2018b).

To obtain further evidence supporting the Golgi-bypass of *de novo*-made PmaA translocation to the PM, we performed a series of key co-localization studies with established red fluorescent protein markers of the early (mCherry-SedV) and late (mRFP-PH^{OSBP}) Golgi, as well as of post-Golgi vesicles (mRFP-RabE). The necessary isogenic strains were constructed by standard crossing, as described in *Materials and methods*. Notice that, in these experiments, we follow the *ab initio* dynamic co-localization of PmaA with the other protein markers, rather than recording its terminal localization to the PM. **Figure 5** shows that PmaA-GFP did not co-localize with any of the three molecular markers used, unlike what had been reported for conventional Golgi-dependent cargoes (i.e., SynA; see Dimou et al., 2020). Overall, our results strongly supported that PmaA, similar to nutrient transporters, does not follow the conventional post-Golgi trafficking route to localize in the PM.

PalI Translocation to the PM Bypasses the Conventional Golgi-Dependent Route

As in the case of PmaA, in order to regulate the expression of PalI and follow the translocation of neosynthesized protein to the PM, we replaced the constitutive and a very weak endogenous promoter with *alcA$_p$* (i.e., *alcA$_p$*-PalI-GFP). Previous studies by the Peñalva group have also used a similar construct to study the role of PalI pH sensing (Calcagno-Pizarelli et al., 2007). We first followed the *de novo* appearance of PalI-GFP upon derepression in single cells. **Figure 6** shows representative results (n = 100), which revealed that PalI initially labels a clear membranous network, coincident with a few cortical or cytoplasmic puncta (60–80 min), and progressively accumulates more and more in cortical foci all along hyphal PM, with a rather homogenous distribution (100–180 min). In some samples, PalI also appears early in septa (80 min), indicating a very fast PM localization. In more grown cells (250 min), PalI labels the entire PM with still

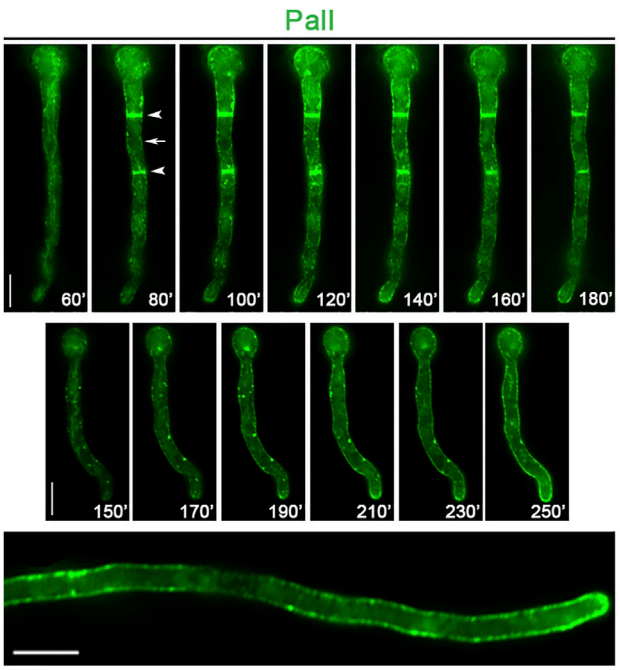


FIGURE 6 | Subcellular localization of neosynthesized PalI. *In vivo* epifluorescence microscopy of *de novo*-made PalI-GFP observed in two single germlings at various times (60–250 min, upper and middle panels) after transcriptional derepression. PalI initially labels a membranous network (best seen at 60 min in the upper panel) and later ER rings and septa (see arrows in the upper panel). Notice also the frequent appearance of cytosolic or cortical immotile foci (better detected at 150–230 min in the middle panel). In a more mature hypha, PalI (360 min) is homogenously localized at the plasma membrane, with still some prominent cortical foci (lower panel). Scale bars: 5 μm.

some distinct cortical foci, as also shown in the work of Calcagno-Pizarelli et al. (2007).

The strain expressing *alcA*$_p$-PalI-GFP was genetically crossed with strains carrying selected *thiA*$_p$-repressible alleles of key proteins involved in Golgi functioning and conventional cargo secretion, as described for PmaA. Appropriate isogenic progeny carrying *alcA*$_p$-PalI-GFP and repressible alleles were used to study the trafficking route of PalI. Most of the selected strains did not form proper colonies in the presence of thiamine in the growth medium, confirming the efficient repression of genes involved in Golgi-dependent trafficking in all cases (**Figure 7A**). **Figures 7B–D** show representative results obtained from several cells in each experiment (n > 100). In all cases, we followed the final localization of PalI-GFP upon 300 min of *de novo* expression, initiated after the full repression of trafficking protein expression (14–16 h of growth in the presence of thiamine). PalI localization to the PM was found to be absolutely dependent on Sec24, Sec13, or clathrin heavy chain (ClaH), but fully independent of SedV, GeaA, and HypB, and partially affected by RabE or AP-1. PalI translocation to the PM was also independent of LstA, in line with its full dependence on Sec24. Through the use of cytoskeleton polymerization drugs, PalI localization proved to be actin dependent, but microtubule and Rab5-like endosome independent, as shown previously for PmaA and nutrient transporters (Dimou et al., 2020) (**Figures 8A,B**).

Further evidence supporting the Golgi-bypass of *de novo*-made PalI translocation to the PM was obtained through co-localization studies with key fluorescent markers of the early (mCherry-SedV) and late (mRFP-PH^{OSBP}) Golgi, or of conventional post-Golgi vesicles (mRFP-RabE). The necessary isogenic strains were constructed as described in

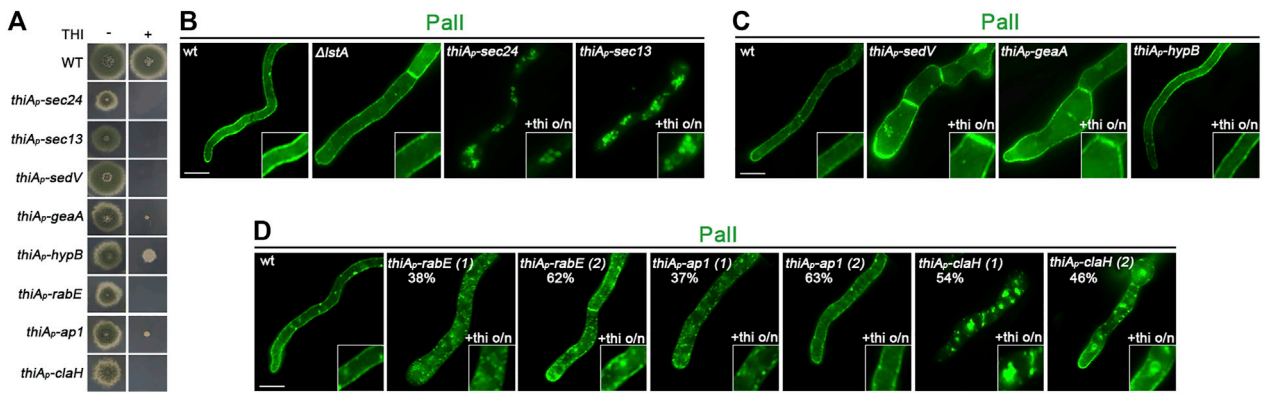


FIGURE 7 | Subcellular localization of neosynthesized PalI in trafficking mutant backgrounds. **(A)** Growth tests showing that, in the presence of thiamine in the growth medium, strains selected to study the *do novo* localization of PalI-GFP do not form proper colonies, confirming that all genes expressed under the *thiA*$_p$ promoter are tightly repressed. In the absence of thiamine from the growth medium (derepressed conditions), the corresponding strains grow nearly as an isogenic wild-type control. **(B)** Epifluorescence analysis of *de novo*-made PalI-GFP when the transcription of *sec24* or *sec13* is repressed or *lstA* is genetically deleted. Transcription of *sec24* and *sec13* was *ab initio* blocked by the addition of thiamine in the media. It is noted that PalI is properly localized at the PM in the Δ*lstA* background, whereas it is dramatically abolished when *sec24* and *sec13* are repressed. **(C)** Epifluorescence analysis of *de novo*-made PalI-GFP when *sedV*, *geaA*, or *hypB* transcription is repressed by *ab initio* addition of thiamine. PalI-GFP translocates to the PM in all cases. **(D)** Epifluorescence analysis of *de novo*-made PalI-GFP when *rabE, ap-1*$^{\sigma}$, or *claH* transcription is repressed by *ab initio* addition thiamine. PalI translocation to the PM was detected when *rabE* or *ap-1*$^{\sigma}$ was repressed in the majority of germlings or hyphae (n = 100). In a minority of hyphae, PalI-GFP labeled static cytoplasmic structures, possibly membranous aggregates. Repression of *claH* transcription significantly blocked PalI-GFP translocation to the PM in the majority of cells. Scale bars: 5 μm.

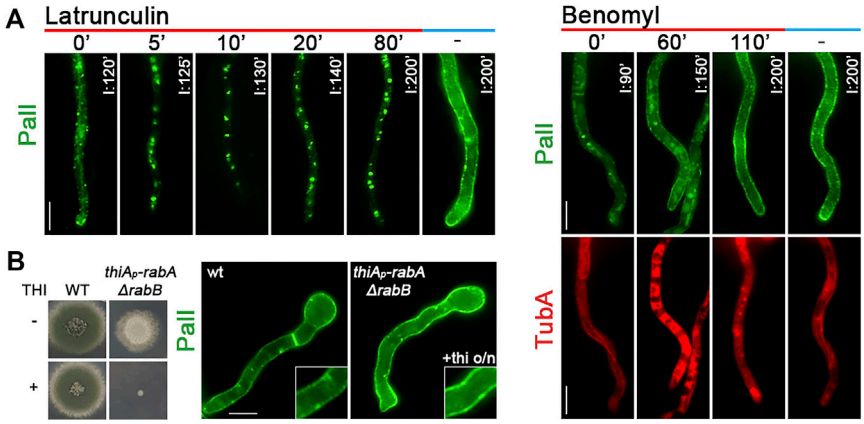


FIGURE 8 | Effect of actin or tubulin depolymerization in PalI subcellular localization. **(A)** ***Left panel***: time course treatment with the actin depolymerization drug latrunculin B for 0, 5, 10, 20, or 80 min of a strain expressing neosynthesized PalI-GFP under conditions of derepression compared to an untreated strain included as control (200 min). Latrunculin B was added at 120 min of derepression so that the total time of PalI-GFP expression was 120, 125, 130, 140, and 200 min in the different samples. The abolishment of sorting of PalI to the PM after 5 min of latrunculin B addition is noted in the growth medium. ***Right panel***: time course treatment of strains co-expressing neosynthesized PalI-GFP and mCherry-TubA with the anti-microtubule drug benomyl, for 0, 60, or 110 min. In all cases, benomyl was added at 90 of PalI derepression so that the total time of PalI-GFP expression was 90, 150, or 200 min. Benomyl abolished the thread-like appearance of microtubules in all samples added, evident by the diffuse cytoplasmic signal of mCherry-TubA. It is noted that PalI normally reaches the PM (best detected at 200 min of derepression). **(B)** When Rab5-dependent endosomes were knocked down/knocked out (i.e., in *thiA*$_p$*-rabA*/Δ*rabB*), fungal growth was arrested (as depicted in the growth test shown at the left panel); however, PalI-GFP was properly localized at the PM (right panel). Scale bars: 5 μm.

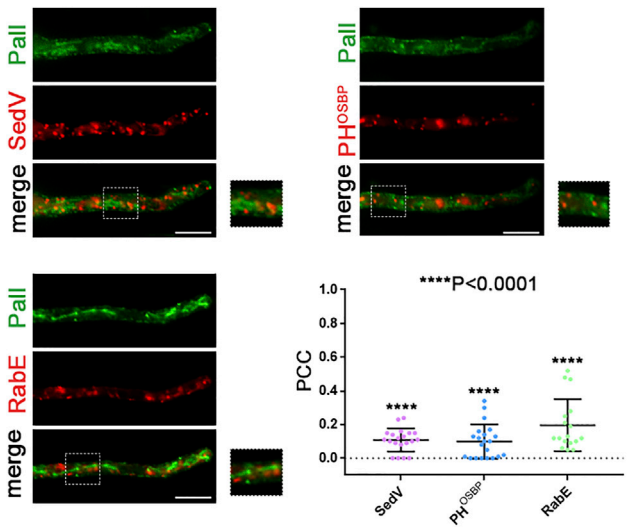


FIGURE 9 | Co-localization study of PalI with early and late Golgi markers. Localization of *de novo*-made PalI-GFP in three different strains co-expressing mCherry-SedV, mRFP-PH^{OSBP}, or mRFP-RabE in single hyphae. Quantification (lower right panel) by calculating Pearson's correlation coefficient shows no significant co-localization of PalI with the Golgi markers tested, as confirmed by the one-sample *t*-test (PCC = 0.11 ± 0.07, 0.1 ± 0.10, or 0.2 ± 0,15, with ****$p < 0.0001$ for mCherry-sedV, PH^{OSBP}, or RabE respectively). Biological/technical replicates: 2/20, 2/21, and 2/17 for each strain respectively.

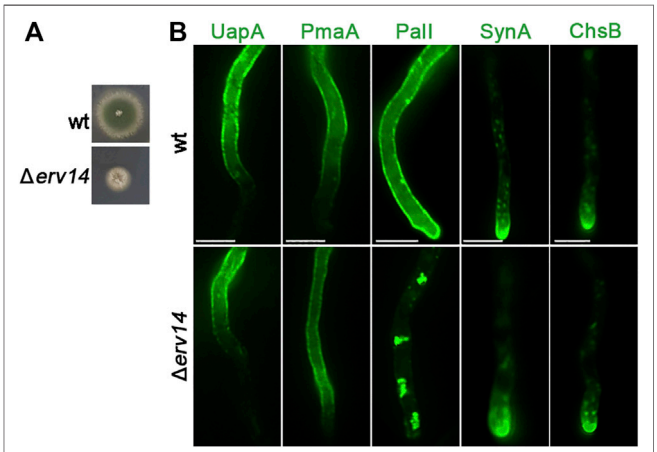


FIGURE 10 | Growth phenotype of Δerv14 and subcellular localization of UapA, PmaA, PalI, SynA, and ChsB in Δerv14. **(A)** Colony growth of Δ*erv14* compared to an isogenic wild-type control strain (wt). **(B)** Epifluorescence microscopic analysis of non-polar and polar cargoes in conditions of Erv14 depletion (lower panel). It is noted that the lack of Erv14 does not affect the localization of UapA, PmaA, SynA, and ChsB, but leads to the retention of PalI in cytosolic aggregates. Scale bars: 5 μm.

Materials and methods. **Figure 9** shows that PalI-GFP does not co-localize significantly with the Golgi or post-Golgi markers used.

Collectively, our findings confirm that neosynthesized PalI traffics to the PM *via* a Golgi-independent route that shares similar features with that employed by neosynthesized nutrient transporters and PmaA. Thus, the sole evident differences related to Golgi-bypass cargoes concerned the COPII cargo adaptor involved (Sec24 *vs.* LstA) and the level of dependence on the post-Golgi effectors RabE or AP-1.

The Erv14 Cargo Adaptor Is Essential for PalI ER-Exit

Erv14 is a COPII accessory protein involved in specific cargo packaging and vesicle formation in yeast (Powers and Barlowe, 2002). This cargo receptor belongs to the well-conserved Erv14/cornichon protein family (Erv14 in yeast, cornichon in *Drosophila*, and CNIH in mammals) which mediates the ER export of many transmembrane proteins, such as plasma membrane permeases, anti-porters, and multi-drug transporters (Herzig et al., 2012; Pagant et al., 2015; Rosas-Santiago et al., 2017). We examined whether the homologs of Erv14 have a crucial role in the ER-exit of Golgi bypassers and/or conventional apical cargoes. We, thus, identified *via in silico* searches the single homolog of Erv14 of *A. nidulans* as the product of the gene-annotated AN5195 (**Supplementary Figure S2B**). We constructed the KO *erv14* mutant by standard reverse genetics (Δ*erv14*). **Figure 10A** depicts its growth phenotype, showing a significantly reduced rate of growth and altered colony morphology. Δ*erv14* could germinate to germlings and hyphae in liquid cultures, in line with the viability of the analogous null mutant in yeast. Δ*erv14* was crossed with strains expressing not only GFP-tagged PmaA or PalI but also the UapA transporter, as another Golgi-bypasser, and SynA or ChsB, which are standard Golgi-dependent cargoes. Appropriate progeny from these crosses was used for studying the effect Erv14 on the trafficking of these cargoes. Erv14 expression was crucial only for PalI trafficking, as in its absence PalI showed extremely reduced translocation to the PM, concomitant with the appearance of fluorescent cytosolic aggregates, most probably the result of ER-associated aberrant accumulation (**Figure 10B**). No other cargo tested showed dependence on Erv14 (**Figure 10B**). This is in line with reports in yeast or mammals showing that cargo receptor complexes involving Erv-like proteins are required for export from the ERes of only some specific cargoes (Herzig et al., 2012). Overall, our results showed that Erv14 acts as a crucial and specific cargo receptor of PalI during COPII formation, but is dispensable for all other cargoes tested.

DISCUSSION

A major question that has arisen from the recent discovery showing that *de novo* made transporters, after COPII-dependent exit from the ER, find their way to the PM without passing from the Golgi and without employing a conventional post-Golgi mechanism, is whether other membrane proteins can also use the same unconventional route to be targeted to the PM. The present study has given a definite answer to this question. By selecting two well-characterized major membrane cargoes, the H^+ pump ATPase PmaA and the pH-sensing PalI component, we showed that both proteins bypass the Golgi and translocate to the PM without the need of microtubule polymerization or endosomal functioning.

Overall, our previous (Dimou et al., 2020) and present results show that several nutrient transporters, the major H^+ pump ATPase PmaA and a component of a pH sensor, all use a similar Golgi-independent route to translocate from the ER to the PM. This route is clearly distinct from the well-established Golgi- and microtubule-dependent trafficking of cargoes needed for *A. nidulans* polar growth, including chitin synthase ChsB (Fukuda et al., 2009; Hernández-González et al., 2018a), the synaptobrevin-like secretory v-SNARE SynA (Taheri-Talesh et al., 2008; Pantazopoulou and Peñalva, 2011; Martzoukou et al., 2018), the lipid flippases DnfA and DnfB (Schultzhaus et al., 2015, 2017; Martzoukou et al., 2018), the glycosylphosphatidylinositol-anchored protein (GPI-AP) EglC (Peñalva et al., 2020), or the soluble extracellular inulinase InuA (Hernández-González et al., 2018b). The membrane cargoes SynA, ChsB, DnfA, and DnfB all show strict polar localization at the Spitzenkörper/SPK [a vesicle supply apical center; (Zhou et al., 2018)] and the apical plasma membrane, co-localize dynamically with Golgi markers, and their biogenesis is aberrant in conditional mutants of key Golgi proteins (e.g., SedV, HypA, HypB, Tlg2, or RabO). Polarization of these cargoes occurs by direct vesicular sorting (i.e., Rab5 endosome independent), coupled with endocytic recycling at a subapical endocytic region and subsequent trafficking to TGN. The periplasmically secreted EglC enzyme, which also requires functional key Golgi proteins for its trafficking (e.g., TRAPPII complex and RabE-dependent), is translocated in a polarized fashion but then rapidly redistributes towards apico-distal regions. The extracellularly secreted InuA also follows the conventional Golgi-dependent secretory pathway, its secretion being blocked in temperature-sensitive mutants of *sedV, rabO, hypA,* or *hypB*. The biogenesis of all aforementioned cargoes necessitates functional COPIIs and seems microtubule dependent. Thus, the overall picture emerging from previous studies and results presented herein and by Dimou et al. (2020) is that the mechanism of trafficking and the steady-state localization of polarly secreted cargoes and Golgi-bypassers are markedly different. This difference seems to be related to physiological functions, as polar cargoes are related to biosynthesis or modification of cell wall and plasma membrane and are thus restricted in apical tips (Steinberg et al., 2017; Martzoukou et al., 2018), whereas Golgi bypassers serve cell nutrition and pH homeostasis in all compartments of hyphae and are thus sorted all over the PM. In other words, conventional, Golgi-dependent, trafficking seems to serve polar growth, while Golgi-bypass concerns nutrient supply and cell homeostasis.

Interestingly, the steady state, Golgi-dependent, anti-polar localization of EglC in hyphae somehow resembles that of nutrient transporters and PmaA, which are Golgi bypassers. It has been suggested that EglC is delivered from apical regions to basolateral parts of hyphae *via* an unknown mechanism. This seems quite feasible as this cargo accumulates in the outer space of the PM and, thus, might freely diffuse in the periplasm. This mechanism of anti-polar diffusion of a soluble cargo is not compatible with Golgi bypassers for several reasons. First, all Golgi bypassers studied are large oligomerizing transmembrane proteins. For example, PmaA is a ~100 kDa transmembrane protein forming 6-12mers in the PM (Zhao et al., 2021), UapA is a 123 kDa tight dimer which might oligomerize

further (Martzoukou et al., 2015; Alguel et al., 2016), and PalI is part of the heterocomplex of ~180 kDa (Calcagno-Pizarelli et al., 2007). It is strongly unlikely that such protein oligomers will diffuse rapidly long distances (>100 μm) within a lipid bilayer (Valdez-Taubas and Pelham, 2003). Second, transporters, PmaA and PalI appear in the PM in a non-continuous manner at several apical-distant cortical foci, rather than forming a gradient from the apex. In fact, transporters and PmaA are absent from apical regions of mature hyphae, dismissing the idea of lateral diffusion from the apex of growing cells. Finally, it is rather hard to consider that very long hyphal cells would translocate transporters, PmaA and PalI exclusively at their growing tips, while posterior distant parts of the cell are 'striving' to adapt to environmental fluctuations in nutrient availability and pH.

In an article from 2011, it has been speculated that, in *S. cerevisiae,* Pma1 is sorted to the Golgi before translocation to the PM (Huang and Chang, 2011). However, there is no formal evidence in this report for Golgi-dependent biogenesis of *de novo*-made Pma1. On the contrary, Huang and Chang (2011) have shown that Pma1 translocates properly to the PM in null *aps1*Δ mutants (i.e., absence of the AP-1 function), which are defective in the formation of post-Golgi vesicles, which also suggests the existence of a Golgi-independent sorting route for Pma1 in yeast. Notably, several reports stating that a cargo is sorted *via* the Golgi do not distinguish neosynthesized from recycling fractions, which might explain why Golgi-bypass has been overlooked in membrane trafficking studies. Notice also that yeast Pma1 and Rim9 (the yeast ortholog of PalI) are non-glycosylated proteins (Holcomb et al., 1988; Chang and Slayman, 1991; Obara et al., 2012). It has also been reported that, in *S. cerevisiae,* lipid rafts help to carry Pma1 through the Golgi to the plasma membrane (Bagnat et al., 2001). However, the entry of the proton ATPase into rafts and oligomerization seem to occur earlier, as raft-associated Pma1 oligomers can be isolated from COPII vesicles (Lee et al., 2002). PmaA, PalI, and nutrient transporters of *A. nidulans* are also not glycosylated. Absence of glycosylation is a strong indication that a protein does not pass from the Golgi. It is noticed, however, that the opposite is not true, as core N-glycosylation of membrane proteins takes place in the ER (Aebi, 2013).

Interestingly, an anti-polar localization (i.e., absence from growing apical regions) has also been observed for the orthologous H^+ ATPase PMA-1 in *Neurospora crassa* (Fajardo-Somera et al., 2013). In this report, the authors state that PMA-1 traffics to the PM *via* the Golgi. However, their work does not distinguish neosynthesized from recycling PMA-1, the latter being sorted to the PM *via* the endosomal compartment and TGN. In contrast to the anti-polar localization of PmaA and nutrient transporters, PalI appears rather homogenously in apical and subapical regions (i.e., non-polar distribution). This suggests that PalI might be needed for proper apical growth. In fact, previous reports have provided evidence for the need of alkaline pH gradient for proper polarized growth of fungal hyphae (Robson et al., 1996). The authors of this report proposed that pH sensing is critical in regulating the local assembly of cytoskeletal components (e.g., actin) and specific vesicle tethering at the apex, required for hyphal extension. In Dimou et al. (2020), a possible mechanistic explanation has been proposed for the absence of transporters from the apical tips of hyphae, based on actin filament re-distribution during transition from germlings to hyphae. How PalI remains in the apical tip during growth, while PmaA and transporters are absent from tip areas, remains elusive.

The experimental evidence presented in our previous (Martzoukou et al., 2018; Dimou et al., 2020) and present reports show that multiple trafficking mechanisms co-exist to serve the differential physiological roles of cargoes and the mode of cell growth. As most animal and plant cells are polar, the identification of distinct Golgi-dependent and Golgi-independent trafficking mechanisms seemingly associated with polar and non-polar targeting might not be an *A. nidulans* or fungal particularity, but rather reflect two major cargo trafficking mechanisms present also in other eukaryotes. This idea is supported by reports showing that specific mammalian transporters might also bypass the Golgi under specific physiological or stress conditions. These include the insulin-regulated human glucose transporter GLUT4 (Camus et al., 2020), a mutant version of the CFTR transporter associated with cystic fibrosis (Gee et al., 2018), and a handful of specifically localized mammalian cargoes in neurons, such as glutamate receptor GluA1, neuroligin, or the potassium channel Kv2.1 (Arnold and Gallo, 2014; Bowen et al., 2017; Stampe Jensen et al., 2017). Recently, the ER chaperone BiP/HSPA5/GRP78, a major regulator of the unfolded protein response (UPR), has been found to accumulate in the PM, where it assumes novel functions associated with signal transduction and cancer metastasis, also *via* Golgi-bypass. In this case, PM translocation is mediated by Rab4/Rab11/Rab15 GTPases and necessitates the ER v-SNARE Bet1 and endosomal t-SNARE syntaxin 13, suggesting vesicular transfer from the ER to the PM *via* specialized endosomes (Van Krieken et al., 2021). Interestingly, a recent report provided evidence that the assembly and cellular secretion of coronaviruses and other budding viruses employs a direct connection of ERGIC with endosomes, bypassing the passage from Golgi stacks (Saraste and Prydz, 2021). Cargo Golgi-bypass has also been recently speculated in *Physcomitrella patens*, based on the observation that Sec23 isoforms form distinct and functionally specific COPII/ERes, with some of them affecting ER to Golgi trafficking and polarized growth (i.e., conventional secretion), while others proved unrelated to polarized growth, while affecting specific cargo secretion (Chang et al., 2021). In fact, the multiple isoforms of COPII components present in plants (Chung et al., 2016) and mammals (Jensen and Schekman, 2011) may point to functional diversity, rather than redundancy, related not only to the cargoes selected but also the trafficking route followed after ER-exit. Thus, Golgi-independent cargo trafficking is emerging as a major trafficking route of membrane proteins in eukaryotes, serving bulk and/or non-directional cargo sorting.

MATERIALS AND METHODS

Media, Strains, Growth Conditions, and Transformation

Standard complete and minimal media for *A. nidulans* were used (FGSC, http://www.fgsc.net). Media and chemical reagents were obtained from Sigma-Aldrich (Life Science Chemilab SA, Hellas) or AppliChem (Bioline Scientific SA, Hellas). Glucose 1% (w/v)

or fructose 0.1% (w/v) was used as the carbon source. NH_4^+(di-ammonium tartrate) and $NaNO_3$ were used as nitrogen sources at 10 mM. Thiamine hydrochloride was used at a final concentration of 10–20 μM as a repressor of the $thiA_p$ promoter (Apostolaki et al., 2012) in microscopy or western blot analysis. *A. nidulans* transformation was performed by generating protoplasts from germinating conidiospores using TNO2A7 as a recipient strain that allows the selection of transformants *via* complementation of a pyrimidine autotrophy (Nayak et al., 2006). Integrations of gene fusions with fluorescent tags, promoter replacement fusions, or deletion cassettes were selected using the *A. fumigatus* markers orotidine-5-phosphate-decarboxylase (AF*pyrG*, Afu2g0836), GTP-cyclohydrolase II (AF*riboB*, Afu1g13300), or a pyridoxine biosynthesis gene (AF*pyroA*, Afu5g08090), resulting in the complementation of the relevant auxotrophies. Transformants were verified by PCR and Southern analyses. Combinations of mutations and fluorescent epitope-tagged strains were generated by standard genetic crossing and progeny analysis. The *E. coli* strains used were DH5a. *A. nidulans* strains used are listed in **Supplementary Table S1**.

Nucleic Acid Manipulations and Plasmid Constructions

Genomic DNA extraction was performed as described in FGSC (http://www.fgsc.net). All DNA fragments used in the various constructs were amplified from a TNO2A7 strain. Plasmid preparation and DNA gel extraction were performed using the Nucleospin Plasmid and the Nucleospin Extract-II kits (Macherey-Nagel, Lab Supplies Scientific SA, Hellas), restriction enzymes were from Takara Bio (Lab Supplies Scientific SA, Hellas), DNA sequences were determined by Eurofins-Genomics (Vienna, Austria), conventional PCRs and high-fidelity amplifications were performed using KAPA Taq DNA and Kapa HiFi polymerases (Kapa Biosystems, Roche Diagnostics, Hellas), and gene cassettes were generated by sequential cloning of the relevant fragments in the pGEM-T plasmid, which served as a template to PCR-amplify the relevant linear cassettes.

Conditions Used to Repress–Derepress Cargo Expression

For following the subcellular trafficking and localization of *de novo* made PmaA-GFP or PalI-GFP, we used the regulatable $alcA_p$ promoter (Waring et al., 1989) combined with a repression–derepression setup analogous to the one described by Dimou et al. (202) or by Calcagno-Pizarelli et al. (2007), respectively. In brief, cargo expression was repressed by overnight growth (for 12–14 h, at 25°C) in the presence of glucose as the sole carbon source and derepressed by a change to fructose (Dimou et al., 2020) or ethanol media (Calcagno-Pizarelli et al., 2007), for the following 1–8 h of growth. For following the trafficking of other control cargoes (UapA, ChsB, or SynA), we used an analogous setup. In experiments aiming at repressing key trafficking proteins expressed from the $thiA_p$ promoter, 10 mM thiamine was used throughout growth. For the microscopic analysis of trafficking markers tagged with fluorescent epitopes, we used their native promoter (RabE), $alcA_p$ (TubA), or the strong constitutive promoter $gpdA_p$ (SedV, PH^{OSBP}), as described by Dimou et al. (2020). All relevant strains carrying mRFP/mCherry-tagged versions of the trafficking markers were the product of in-locus gene replacements.

Protein Extraction and Western Blots

Total protein extraction was performed as previously described by Dimou et al. (2020), using dry mycelia from cultures grown in minimal media supplemented with $NaNO_3$ at 25°C. Total proteins (50 μg, estimated by Bradford assays) were separated in a 6% (w/v) polyacrylamide gel and were transferred on PVDF membranes (GE Healthcare Life Sciences, Amersham). Immunodetection was performed with an anti-GFP monoclonal antibody (11814460001, Roche Diagnostics), an anti-actin monoclonal (C4) antibody (SKU0869100-CF, MP Biomedicals, Europe), and an HRP-linked antibody (7076, Cell Signaling Technology Inc.). Blots were developed using the Lumi Sensor Chemiluminescent HRP Substrate kit (Genscript, United States) and SuperRX Fuji medical X-Ray films (Fuji FILM, Europe).

Fluorescence Microscopy and Statistical Analysis

Conidiospores were incubated overnight in glass-bottom 35 mm l-dishes (ibidi, Lab Supplies Scientific SA, Hellas) in liquid minimal media, for 16–22 h at 25°C, under conditions of transcriptional repression of cargoes expressed from the $alcA_p$ promoter [1% (w/v) glucose or 10 mM NH_4^+] and repression of the selected trafficking proteins expressed under the $thiA_p$ promoter (10 mM thiamine). Transcriptional derepression of cargoes was performed through a shift in media containing fructose or ethanol as sole carbon source for PmaA or PalI respectively. Derepression periods ranged from 60 min to 12 h, according to experiments. Benomyl (Sigma-Aldrich) and latrunculin B (Sigma-Aldrich) were used at 2.5 and 100 μg/ml final concentrations, respectively. To determine the effect of extracellular pH in PmaA-GFP fluorescence, conidiospores were cultured in an acidic (pH 5.5 with 25 mM KH_2PO_4) or alkaline medium (pH 8 with 23.5 mM K_2HPO_4, 0.15 mM KH_2PO_4). The images were obtained using an inverted Zeiss Axio Observer Z1 equipped with an Axio Cam HR R3 camera. Contrast adjustment, area selection, and colour combining were made using the Zenlite 2012 software. Scale bars were added using the FigureJ plugin of the ImageJ software. The images were further processed and annotated in Adobe Photoshop CS4 Extended version 11.0.2. Technical replicates correspond to different hyphal cells observed within each sample, while biological replicates correspond to different samples (Martzoukou et al., 2017). For quantifying co-localization (Dunn et al., 2011), Pearson's correlation coefficient (PCC) above thresholds, for a selected region of interest (ROI), was calculated using the ICY co-localization studio plugin (pixel-based method) (http://icy.bioimageanalysis.org/). A one-sample *t*-test was performed to see whether the mean PCC value was significantly greater than 0, using the GraphPad Prism software (Mcdonald and

Dunn, 2013). Confidence interval was set to 95%. For quantifying the fluorescence of PmaA in **Figure 2**, two ROIs in the same region were drawn manually, using the area selection tool in ICY, one including both the PM and the cytoplasm and another identical one, excluding the PM (Dimou et al., 2020). PM/cytoplasmic mean fluorescence intensity ratios for each condition are shown in box scatter plots, using the GraphPad Prism software. To test the significance of differences in PM/cytoplasmic fluorescence of measurements, Tukey's multiple comparison test was performed (one-way ANOVA).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SD and MD performed experiments, wrote parts of the draft manuscript, prepared figures, and contributed to discussion. GS performed experiments and prepared figures. GD designed experiments, wrote the draft and final manuscript, and obtained funding.

FUNDING

This work was supported by research grants of the *Stavros Niarchos Foundation* (KE 14315) and *Fondation Santé* (KE 17237). SD was supported by the "Stavros Tsakyrakis" fellowship.

ACKNOWLEDGMENTS

The authors thank Sotiris Amillis for help in the construction of the null *erv14* and *lstA* mutants.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.852028/full#supplementary-material

REFERENCES

Abdallah, B. M., Simões, T., Fernandes, A. R., Strauss, J., Seiboth, B., Sá-Correia, I., et al. (2000). Glucose Does Not Activate the Plasma-Membrane-Bound H + -ATPase but Affects pmaA Transcript Abundance in Aspergillus nidulans. *Arch. Microbiol.* 174, 340–345. doi:10.1007/s002030000212

Aebi, M. (2013). N-linked Protein Glycosylation in the ER. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1833, 2430–2437. doi:10.1016/j.bbamcr.2013.04.001

Alguel, Y., Amillis, S., Leung, J., Lambrinidis, G., Capaldi, S., Scull, N. J., et al. (2016). Structure of Eukaryotic purine/H+ Symporter UapA Suggests a Role for Homodimerization in Transport Activity. *Nat. Commun.* 7, 11336. doi:10.1038/ncomms11336

Ambesi, A., Miranda, M., Petrov, V. V., and Slayman, C. W. (2000). Biogenesis and Function of the Yeast Plasma-Membrane H(+)-ATPase. *J. Exp. Biol.* 203, 155–160. doi:10.1242/jeb.203.1.155

Apostolaki, A., Harispe, L., Calcagno-Pizarelli, A. M., Vangelatos, I., Sophianopoulou, V., Arst Jr, H. N., et al. (2012). Aspergillus nidulansCkiA Is an Essential Casein Kinase I Required for Delivery of Amino Acid Transporters to the Plasma Membrane. *Mol. Microbiol.* 84, 530–549. doi:10.1111/j.1365-2958.2012.08042.x

Arnold, D. B., and Gallo, G. (2014). Structure Meets Function: Actin Filaments and Myosin Motors in the Axon. *J. Neurochem.* 129, 213–220. doi:10.1111/jnc.12503

Bagnat, M., Chang, A., and Simons, K. (2001). Plasma Membrane Proton ATPase Pma1p Requires Raft Association for Surface Delivery in Yeast. *MBoC* 12, 4129–4138. doi:10.1091/mbc.12.12.4129

Bonnon, C., Wendeler, M. W., Paccaud, J.-P., and Hauri, H.-P. (2010). Selective export of Human GPI-Anchored Proteins from the Endoplasmic Reticulum. *J. Cel Sci.* 123, 1705–1715. doi:10.1242/jcs.062950

Bowen, A. B., Bourke, A. M., Hiester, B. G., Hanus, C., and Kennedy, M. J. (2017). Golgi-Independent Secretory Trafficking through Recycling Endosomes in Neuronal Dendrites and Spines. *Elife* 6, e27362. doi:10.7554/eLife.27362

Burgstaller, W., Müller, B., and Schinner, F. (1997). The *In Vivo* Effect of Glucose and Extracellular pH on the Plasma Membrane H+-ATPase of Penicillium simplicissimum. *FEMS Microbiol. Lett.* 147, 109–114. doi:10.1111/j.1574-6968.1997.tb10228.x

Calcagno-Pizarelli, A. M., Negrete-Urtasun, S., Denison, S. H., Rudnicka, J. D., Bussink, H.-J., Mu´nera-Huertas, T., et al. (2007). Establishment of the Ambient pH Signaling Complex in Aspergillus nidulans : PalI Assists Plasma Membrane Localization of PalH. *Eukaryot. Cel* 6, 2365–2375. doi:10.1128/EC.00275-07

Camus, S. M., Camus, M. D., Figueras-Novoa, C., Boncompain, G., Sadacca, L. A., Esk, C., et al. (2020). CHC22 Clathrin Mediates Traffic from Early Secretory Compartments for Human GLUT4 Pathway Biogenesis. *J. Cel Biol.* 219, e201812135. doi:10.1083/jcb.201812135

Casler, J. C., Papanikou, E., Barrero, J. J., and Glick, B. S. (2019). Maturation-driven Transport and AP-1-dependent Recycling of a Secretory Cargo in the Golgi. *J. Cel Biol.* 218, 1582–1601. doi:10.1083/jcb.201807195

Castillon, G. A., Watanabe, R., Taylor, M., Schwabe, T. M. E., and Riezman, H. (2009). Concentration of GPI-Anchored Proteins upon ER Exit in Yeast. *Traffic* 10, 186–200. doi:10.1111/j.1600-0854.2008.00857.x

Chang, A., and Slayman, C. W. (1991). Maturation of the Yeast Plasma Membrane [H+]ATPase Involves Phosphorylation during Intracellular Transport. *J. Cel Biol.* 115, 289–295. doi:10.1083/jcb.115.2.289

Chang, M., Wu, S.-Z., Ryken, S. E., O'Sullivan, J. E., and Bezanilla, M. (2021). COPII Sec23 Proteins Form Isoform-specific Endoplasmic Reticulum Exit Sites with Differential Effects on Polarized Growth. *Plant Cell* 34, 333–350. doi:10.1093/plcell/koab229

Chung, K. P., Zeng, Y., and Jiang, L. (2016). COPII Paralogs in Plants: Functional Redundancy or Diversity? *Trends Plant Sci.* 21, 758–769. doi:10.1016/j.tplants.2016.05.010

Cyert, M. S., and Philpott, C. C. (2013). Regulation of Cation Balance in *Saccharomyces cerevisiae*. *Genetics* 193, 677–713. doi:10.1534/genetics.112.147207

D'Arcangelo, J. G., Stahmer, K. R., and Miller, E. A. (2013). Vesicle-mediated export from the ER: COPII Coat Function and Regulation. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1833, 2464–2472. doi:10.1016/j.bbamcr.2013.02.003

Diallinas, G. (2016). Dissection of Transporter Function: From Genetics to Structure. *Trends Genet.* 32, 576–590. doi:10.1016/j.tig.2016.06.003

Dimou, S., and Diallinas, G. (2020). Life and Death of Fungal Transporters under the challenge of Polarity. *Ijms* 21, 5376. doi:10.3390/ijms21155376

Dimou, S., Martzoukou, O., Dionysopoulou, M., Bouris, V., Amillis, S., and Diallinas, G. (2020). Translocation of Nutrient Transporters to Cell Membrane via Golgi Bypass in Aspergillus nidulans. *EMBO Rep.* 21, e49929. doi:10.15252/embr.201949929

Dunn, K. W., Kamocka, M. M., and McDonald, J. H. (2011). A Practical Guide to Evaluating Colocalization in Biological Microscopy. *Am. J. Physiology-Cell Physiol.* 300, C723–C742. doi:10.1152/ajpcell.00462.2010

Etxebeste, O., and Espeso, E. A. (2020). Aspergillus nidulans in the post-genomic Era: a Top-Model Filamentous Fungus for the Study of Signaling and Homeostasis Mechanisms. *Int. Microbiol.* 23, 5–22. doi:10.1007/s10123-019-00064-6

Fajardo-Somera, R. A., Bowman, B., and Riquelme, M. (2013). The Plasma Membrane Proton Pump PMA-1 Is Incorporated into Distal Parts of the Hyphae Independently of the Spitzenkörper in Neurospora Crassa. *Eukaryot. Cel* 12, 1097–1105. doi:10.1128/EC.00328-12

Ferreira, T., Mason, A. B., and Slayman, C. W. (2001). The Yeast Pma1 Proton Pump: A Model for Understanding the Biogenesis of Plasma Membrane Proteins. *J. Biol. Chem.* 276, 29613–29616. doi:10.1074/jbc.R100022200

Feyder, S., De Craene, J.-O., Bär, S., Bertazzi, D., and Friant, S. (2015). Membrane Trafficking in the Yeast *Saccharomyces cerevisiae* Model. *Ijms* 16, 1509–1525. doi:10.3390/ijms16011509

Fukuda, K., Yamada, K., Deoka, K., Yamashita, S., Ohta, A., and Horiuchi, H. (2009). Class III Chitin Synthase ChsB of Aspergillus Nidulans Localizes at the Sites of Polarized Cell wall Synthesis and Is Required for Conidial Development. *Eukaryot. Cel.* 8, 945–956. doi:10.1128/EC.00326-08

Gee, H. Y., Kim, J., and Lee, M. G. (2018). Unconventional Secretion of Transmembrane Proteins. *Semin. Cel Developmental Biol.* 83, 59–66. doi:10.1016/j.semcdb.2018.03.016

Geva, Y., Crissman, J., Arakel, E. C., Gómez-Navarro, N., Chuartzman, S. G., Stahmer, K. R., et al. (2017). Two Novel Effectors of Trafficking and Maturation of the Yeast Plasma Membrane H+ -ATPase. *Traffic* 18, 672–682. doi:10.1111/tra.12503

Gomez-Navarro, N., and Miller, E. (2016). Protein Sorting at the ER-Golgi Interface. *J. Cel Biol.* 215, 769–778. doi:10.1083/jcb.201610031

Henderson, K. A., Hughes, A. L., and Gottschling, D. E. (2014). Mother-daughter Asymmetry of pH Underlies Aging and Rejuvenation in Yeast. *Elife* 3, e03504. doi:10.7554/eLife.03504

Hernández-González, M., Pantazopoulou, A., Spanoudakis, D., Seegers, C. L. C., and Peñalva, M. A. (2018b). Genetic Dissection of the Secretory Route Followed by a Fungal Extracellular Glycosyl Hydrolase. *Mol. Microbiol.* 109, 781–800. doi:10.1111/mmi.14073

Hernández-González, M., Bravo-Plaza, I., Pinar, M., de los Ríos, V., Arst, H. N., and Peñalva, M. A. (2018a). Endocytic Recycling via the TGN Underlies the Polarized Hyphal Mode of Life. *Plos Genet.* 14, e1007291. doi:10.1371/journal.pgen.1007291

Herzig, Y., Sharpe, H. J., Elbaz, Y., Munro, S., and Schuldiner, M. (2012). A Systematic Approach to Pair Secretory Cargo Receptors with Their Cargo Suggests a Mechanism for Cargo Selection by Erv14. *Plos Biol.* 10, e1001329. doi:10.1371/journal.pbio.1001329

Holcomb, C. L., Hansen, W. J., Etcheverry, T., and Schekman, R. (1988). Secretory Vesicles Externalize the Major Plasma Membrane ATPase in Yeast. *J. Cel Biol.* 106, 641–648. doi:10.1083/jcb.106.3.641

Huang, C., and Chang, A. (2011). pH-Dependent Cargo Sorting from the Golgi. *J. Biol. Chem.* 286, 10058–10065. doi:10.1074/jbc.M110.197889

Iwasaki, H., Yorimitsu, T., and Sato, K. (2015). Distribution of Sec24 Isoforms to Each ER Exit Site Is Dynamically Regulated inSaccharomyces Cerevisiae. *FEBS Lett.* 589, 1234–1239. doi:10.1016/j.febslet.2015.04.006

Jensen, C. S., Watanabe, S., Stas, J. I., Klaphaak, J., Yamane, A., Schmitt, N., et al. (2017). Trafficking of Kv2.1 Channels to the Axon Initial Segment by a Novel Nonconventional Secretory Pathway. *J. Neurosci.* 37, 11523–11536. doi:10.1523/JNEUROSCI.3510-16.2017

Jensen, D., and Schekman, R. (2011). COPII-mediated Vesicle Formation at a Glance. *J. Cel Sci.* 124, 1–4. doi:10.1242/jcs.069773

Lee, M. C. S., Hamamoto, S., and Schekman, R. (2002). Ceramide Biosynthesis Is Required for the Formation of the Oligomeric H+-ATPase Pma1p in the Yeast Endoplasmic Reticulum. *J. Biol. Chem.* 277, 22395–22401. doi:10.1074/jbc.M200450200

Martzoukou, O., Amillis, S., Zervakou, A., Christoforidis, S., and Diallinas, G. (2017). The AP-2 Complex Has a Specialized Clathrin-independent Role in Apical Endocytosis and Polar Growth in Fungi. *Elife* 6, e20083. doi:10.7554/elife.20083

Martzoukou, O., Diallinas, G., and Amillis, S. (2018). Secretory Vesicle Polar Sorting, Endosome Recycling and Cytoskeleton Organization Require the AP-1 Complex in Aspergillus Nidulans. *Genetics* 209, 1121–1138. doi:10.1534/genetics.118.301240

Martzoukou, O., Karachaliou, M., Yalelis, V., Leung, J., Byrne, B., Amillis, S., et al. (2015). Oligomerization of the UapA Purine Transporter Is Critical for ER-Exit, Plasma Membrane Localization and Turnover. *J. Mol. Biol.* 427, 2679–2696. doi:10.1016/j.jmb.2015.05.021

Mcdonald, J. H., and Dunn, K. W. (2013). Statistical Tests for Measures of Colocalization in Biological Microscopy. *J. Microsc.* 252, 295–302. doi:10.1111/jmi.12093

Miller, E., Antonny, B., Hamamoto, S., and Schekman, R. (2002). Cargo Selection into COPII Vesicles Is Driven by the Sec24p Subunit. *EMBO J.* 21, 6105–6113. doi:10.1093/emboj/cdf605

Morsomme, P., Prescianotto-Baschong, C., and Riezman, H. (2003). The ER V-SNAREs Are Required for GPI-Anchored Protein Sorting from Other Secretory Proteins upon Exit from the ER. *J. Cel Biol.* 162, 403–412. doi:10.1083/jcb.200212101

Muñiz, M., Morsomme, P., and Riezman, H. (2001). Protein Sorting upon Exit from the Endoplasmic Reticulum. *Cell* 104 (2), 313–320. doi:10.1016/S0092-8674(01)00215-X

Nayak, T., Szewczyk, E., Oakley, C. E., Osmani, A., Ukil, L., Murray, S. L., et al. (2006). A Versatile and Efficient Gene-Targeting System for Aspergillus nidulans. *Genetics* 172, 1557–1566. doi:10.1534/genetics.105.052563

Obara, K., Yamamoto, H., and Kihara, A. (2012). Membrane Protein Rim21 Plays a Central Role in Sensing Ambient pH in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 287, 38473–38481. doi:10.1074/jbc.M112.394205

Pagant, S., Wu, A., Edwards, S., Diehl, F., and Miller, E. A. (2015). Sec24 Is a Coincidence Detector that Simultaneously Binds Two Signals to Drive ER export. *Curr. Biol.* 25, 403–412. doi:10.1016/j.cub.2014.11.070

Pantazopoulou, A., and Peñalva, M. A. (2011). Characterization of Aspergillus nidulans RabC/Rab6. *Traffic* 12, 386–406. doi:10.1111/j.1600-0854.2011.01164.x

Peñalva, M. A., Lucena-Agell, D., and Arst, H. N. (2014). Liaison Alcaline: Pals Entice Non-endosomal ESCRTs to the Plasma Membrane for pH Signaling. *Curr. Opin. Microbiol.* 22, 49–59. doi:10.1016/j.mib.2014.09.005

Peñalva, M. A., Moscoso-Romero, E., and Hernández-González, M. (2020). Tracking Exocytosis of a GPI-Anchored Protein in Aspergillus nidulans. *Traffic* 21 (11), 675–688. doi:10.1111/tra.12761

Phuyal, S., and Farhan, H. (2021). Want to Leave the Er? We Offer Vesicles, Tubules, and Tunnels. *J. Cel Biol.* 220, e202104062. doi:10.1083/jcb.202104062

Pinar, M., and Peñalva, M. A. (2021). The Fungal RABOME: RAB GTPases Acting in the Endocytic and Exocytic Pathways of Aspergillus nidulans (With Excursions to Other Filamentous Fungi). *Mol. Microbiol.* 116, 53–70. doi:10.1111/mmi.14716

Powers, J., and Barlowe, C. (2002). Erv14p Directs a Transmembrane Secretory Protein into COPII-Coated Transport Vesicles. *MBoC* 13, 880–891. doi:10.1091/mbc.01-10-0499

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cel Biol.* 27, 230–240. doi:10.1016/j.tcb.2016.11.007

Raote, I., and Malhotra, V. (2021). Tunnels for Protein Export from the Endoplasmic Reticulum. *Annu. Rev. Biochem.* 90, 605–630. doi:10.1146/annurev-biochem-080120-022017

Reoyo, E., Espeso, E. A., Peñalva, M. A., and Suárez, T. (1998). The EssentialAspergillus nidulansGenepmaAEncodes an Homologue of Fungal Plasma Membrane H+-ATPases. *Fungal Genet. Biol.* 23, 288–299. doi:10.1006/fgbi.1998.1039

Roberg, K. J., Crotwell, M., Espenshade, P., Gimeno, R., and Kaiser, C. A. (1999). LST1 Is a SEC24 Homologue Used for Selective export of the Plasma Membrane ATPase from the Endoplasmic Reticulum. *J. Cel Biol.* 145, 659–672. doi:10.1083/jcb.145.4.659

Robinson, M. S. (2015). Forty Years of Clathrin-Coated Vesicles. *Traffic* 16, 1210–1238. doi:10.1111/tra.12335

Robson, G. D., Prebble, E., Rickers, A., Hosking, S., Denning, D. W., Trinci, A. P. J., et al. (1996). Polarized Growth of Fungal Hyphae Is Defined by an Alkaline pH Gradient. *Fungal Genet. Biol.* 20, 289–298. doi:10.1006/fgbi.1996.0043

Rosas-Santiago, P., Lagunas-Gomez, D., Yáñez-Domínguez, C., Vera-Estrella, R., Zimmermannová, O., Sychrová, H., et al. (2017). Plant and Yeast Cornichon

Possess a Conserved Acidic Motif Required for Correct Targeting of Plasma Membrane Cargos. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1864, 1809–1818. doi:10.1016/j.bbamcr.2017.07.004

Saito, K., Chen, M., Bard, F., Chen, S., Zhou, H., Woodley, D., et al. (2009). TANGO1 Facilitates Cargo Loading at Endoplasmic Reticulum Exit Sites. *Cell* 136, 891–902. doi:10.1016/j.cell.2008.12.025

Saraste, J., and Prydz, K. (2021). Assembly and Cellular Exit of Coronaviruses: Hijacking an Unconventional Secretory Pathway from the Pre-golgi Intermediate Compartment via the Golgi Ribbon to the Extracellular Space. *Cells* 10, 503. doi:10.3390/cells10030503

Scazzocchio, C. (2009). "Aspergillus: A Multifaceted Genus," in *Encyclopedia of Microbiology*. 3rd Edn. Editor M. Schaechter (Academic Press), 401–421. doi:10.1016/B978-012373944-5.00337-0

Schultzhaus, Z., Yan, H., and Shaw, B. D. (2015). Aspergillus Nidulansflippase DnfA Is Cargo of the Endocytic Collar and Plays Complementary Roles in Growth and Phosphatidylserine Asymmetry with Another Flippase, DnfB. *Mol. Microbiol.* 97, 18–32. doi:10.1111/mmi.13019

Schultzhaus, Z., Zheng, W., Wang, Z., Mouriño-Pérez, R., and Shaw, B. (2017). Phospholipid Flippases DnfA and DnfB Exhibit Differential Dynamics within the A. nidulans Spitzenkörper. *Fungal Genet. Biol.* 99, 26–28. doi:10.1016/j.fgb.2016.12.007

Serrano, R. (1983). *In Vivo* glucose Activation of the Yeast Plasma Membrane ATPase. *FEBS Lett.* 156 (1), 11–14. doi:10.1016/0014-5793(83)80237-3

Shimoni, Y., Kurihara, T., Ravazzola, M., Amherdt, M., Orci, L., and Schekman, R. (2000). Lst1p and Sec24p Cooperate in Sorting of the Plasma Membrane ATPase into COPII Vesicles in *Saccharomyces cerevisiae*. *J. Cel Biol.* 151, 973–984. doi:10.1083/jcb.151.5.973

Shomron, O., Nevo-Yassaf, I., Aviad, T., Yaffe, Y., Zahavi, E. E., Dukhovny, A., et al. (2021). Copii Collar Defines the Boundary between Er and Er Exit Site and Does Not Coat Cargo Containers. *J. Cel Biol.* 220, e201907224. doi:10.1083/jcb.201907224

Steinberg, G., Peñalva, M. A., Riquelme, M., Wösten, H. A., and Harris, S. D. (2017). Cell Biology of Hyphal Growth. *Microbiol Spectr.* 5 (2). doi:10.1128/microbiolspec.FUNK-0034-2016

Taheri-Talesh, N., Horio, T., Araujo-Bazán, L., Dou, X., Espeso, E. A., Peñalva, M. A., et al. (2008). The Tip Growth Apparatus ofAspergillus Nidulans. *MBoC* 19, 1439–1449. doi:10.1091/mbc.E07-05-0464

Tanaka, Y., Nishimura, K., Kawamukai, M., Oshima, A., and Nakagawa, T. (2013). Redundant Function of Two Arabidopsis COPII Components, AtSec24B and AtSec24C, Is Essential for Male and Female Gametogenesis. *Planta* 238, 561–575. doi:10.1007/s00425-013-1913-1

Valdez-Taubas, J., and Pelham, H. R. B. (2003). Slow Diffusion of Proteins in the Yeast Plasma Membrane Allows Polarity to Be Maintained by Endocytic Cycling. *Curr. Biol.* 13, 1636–1640. doi:10.1016/j.cub.2003.09.001

Van Krieken, R., Tsai, Y.-L., Carlos, A. J., Ha, D. P., and Lee, A. S. (2021). ER Residential Chaperone GRP78 Unconventionally Relocalizes to the Cell Surface via Endosomal Transport. *Cell. Mol. Life Sci.* 78 (12), 5179–5195. doi:10.1007/s00018-021-03849-z

Voorhees, R. M., and Hegde, R. S. (2016). Toward a Structural Understanding of Co-translational Protein Translocation. *Curr. Opin. Cel Biol.* 41, 91–99. doi:10.1016/j.ceb.2016.04.009

Waring, R. B., May, G. S., and Morris, N. R. (1989). Characterization of an Inducible Expression System in Aspergillus nidulans Using alcA and Tubulincoding Genes. *Gene* 79 (1), 119–130. doi:10.1016/0378-1119(89)90097-8

Weigel, A. V., Chang, C.-L., Shtengel, G., Xu, C. S., Hoffman, D. P., Freeman, M., et al. (2021). ER-to-Golgi Protein Delivery through an Interwoven, Tubular Network Extending from ER. *Cell* 184, 2412–2429. doi:10.1016/j.cell.2021.03.035

Yuan, L., Kenny, S. J., Hemmati, J., Xu, K., and Schekman, R. (2018). TANGO1 and SEC12 Are Copackaged with Procollagen I to Facilitate the Generation of Large COPII Carriers. *Proc. Natl. Acad. Sci. USA* 115, E12255–E12264. doi:10.1073/pnas.1814810115

Zanetti, G., Pahuja, K. B., Studer, S., Shim, S., and Schekman, R. (2012). COPII and the Regulation of Protein Sorting in Mammals. *Nat. Cel Biol.* 14, 20–28. doi:10.1038/ncb2390

Zeng, J., Feng, S., Wu, B., and Guo, W. (2017). Polarized Exocytosis. *Cold Spring Harb. Perspect. Biol.* 9, a027870. doi:10.1101/cshperspect.a027870

Zhao, P., Zhao, C., Chen, D., Yun, C., Li, H., and Bai, L. (2021). Structure and Activation Mechanism of the Hexameric Plasma Membrane H+-ATPase. *Nat. Commun.* 12. doi:10.1038/s41467-021-26782-y

Zhou, L., Evangelinos, M., Wernet, V., Eckert, A. F., Ishitsuka, Y., Fischer, R., et al. (2018). Superresolution and Pulse-chase Imaging Reveal the Role of Vesicle Transport in Polar Growth of Fungal Cells. *Sci. Adv.* 4, e1701798. doi:10.1126/sciadv.1701798

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Cell and Developmental Biology

MINI REVIEW
published: 28 April 2022
doi: 10.3389/fcell.2022.895853

Unconventional Pathways of Protein Secretion: Mammals *vs*. Plants

Elisa Maricchiolo[1†], Eleonora Panfili[2†], Andrea Pompa[1], Francesca De Marchis[3], Michele Bellucci[3] and Maria Teresa Pallotta[2]**

[1]Section of Biological and Biotechnological Sciences, Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy, [2]Section of Pharmacology, Department of Medicine and Surgery, University of Perugia, Perugia, Italy, [3]Institute of Biosciences and Bioresources, National Research Council of Italy, Perugia, Italy

In eukaryotes, many proteins contain an N-terminal signal peptide that allows their translocation into the endoplasmic reticulum followed by secretion outside the cell according to the classical secretory system. However, an increasing number of secreted proteins lacking the signal peptide sequence are emerging. These proteins, secreted in several alternative ways collectively known as unconventional protein secretion (UPS) pathways, exert extracellular functions including cell signaling, immune modulation, as well as moonlighting activities different from their well-described intracellular functions. Pathways for UPS include direct transfer across the plasma membrane, secretion from endosomal/multivesicular body-related components, release within plasma membrane-derived microvesicles, or use of elements of autophagy. In this review we describe the mammals and plants UPS pathways identified so far highlighting commonalities and differences.

Keywords: extracellular vesicles, vacuole, extracellular space, cell signaling, unconventional protein secretion

OPEN ACCESS

Edited by:
Julien Villeneuve, Institute for Functional Genomics (IGF) (CNRS), France

Reviewed by:
Yonglun Zeng, The Chinese University of Hong Kong, China
Cecilia Rodriguez-Furlán, University of California, Riverside, United States

****Correspondence:***
Michele Bellucci
michele.bellucci@ibbr.cnr.it
Maria Teresa Pallotta
maria.pallotta@unipg.it

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *14 March 2022*
Accepted: *13 April 2022*
Published: *28 April 2022*

Citation:
Maricchiolo E, Panfili E, Pompa A, De Marchis F, Bellucci M and Pallotta MT (2022) Unconventional Pathways of Protein Secretion: Mammals vs. Plants. Front. Cell Dev. Biol. 10:895853. doi: 10.3389/fcell.2022.895853

1 INTRODUCTION

Eukaryotic cells secrete soluble and membrane proteins during organism development or after induction by different types of stress. The discovery of protein trafficking describes the classical secretion pathway (Vitale and Denecke, 1999), in which proteins are translocated into the endoplasmic reticulum (ER) by a co-translational mechanism that involves the interaction in the cytosol of an N-terminal signal peptide (SP), or a transmembrane domain, with a signal recognition particle (SRP). SRP directs the protein to an ER-localized SRP receptor which, together with an ER-localized translocon complex (Sec61 complex), initiates the ER translocation (Osborne et al., 2005; Shan and Walter, 2005). Secretory proteins are then transported through the Golgi apparatus, to be sorted and targeted to the extracellular space or to the subsequent endomembrane compartments (plasma membrane, vacuoles in yeast and plants, lysosomes in animals, etc.). In recent years, an increasing number of proteins, either with or without an N-terminal SP (leaderless proteins) have been found to reach their final destinations by alternative pathways that bypass the Golgi, leading to the conclusion that this kind of transport is a very important type of protein traffic inside the cell (Goring and Di Sansebastiano, 2017; Pompa et al., 2017). Such proteins, very well represented in the eukaryotic secretome, reach their destination by an "unconventional" mechanism, of which determinants have not yet been clearly defined. In fact, neither the amino acid structural motifs that direct a protein along an unconventional protein secretion (UPS) pathway nor all the biological mechanisms that determine the UPS pathways and the molecular events involved, have been fully characterized. Another aspect related to the UPS definition is that some mechanisms involved in this process are superimposable with other cellular processes such as autophagy or programmed cell

death (PCD). Even if the crosstalk between organelles of different pathways may occur in specific situations, UPS is clearly determined by literature as a distinct pathway from the conventional exo–endocytic trafficking which regulates the turnover of plasma membrane proteins (Zhang et al., 2019) and the autophagic route as well (Hu et al., 2020). Currently investigated in many organisms, UPS seems to be related to several physiological processes like immune responses, abiotic stress responses and cell proliferation in normal growth conditions (Pallotta and Nickel, 2020; Balmer and Faso, 2021). In this review, along with a presentation of differences and commonalities between the UPS mechanisms in mammals and plants, we try to summarize the latest research on UPS to combine the molecular mechanisms and the physiological issues of this type of protein transport.

1.1 Mechanisms and Physiological Role of Unconventional Protein Secretion in Mammalian Cells

In mammals, many leaderless proteins can be secreted outside the cells through different UPS mechanisms. Such secretion is biologically controlled because these proteins can exploit distinct extracellular functions, like immune modulation or cell signaling, activities different from their intracellular ones (Cohen et al., 2020). Some proteins are directly translocated across the plasma membrane forming pore structures. Generally, these proteins bind lipids, undergo a conformational change facilitated by other proteins, and then pass through the plasma membrane (Stewart et al., 2018). Only in a few cases protein secretion turns out to be mediated by ABC transporters, while a wide range of proteins is taken up into intracellular vesicle intermediates and released upon fusion with the plasma membrane in a free form or into vesicles (Cocozza et al., 2020). Moreover, integral membrane proteins lacking SP are translocated from the ER to the plasma membrane without the passage through the Golgi apparatus (Pallotta and Nickel, 2020).

1.1.1 Type I Pathway: Golgi-Bypass Pathway for Leaderless Proteins

In mammals, UPS type I is a secretory pathway wherein soluble leaderless proteins directly translocate across the plasma membrane. One of the first and most studied proteins that undergo this pathway is Fibroblast Growth Factor 2 (FGF2), which is recruited at the inner plasma membrane leaflet through interaction with the α1-subunit of the Na/K-ATPase (Legrand et al., 2020). This event promotes FGF2 binding to the phosphoinositide $PI(4,5)P_2$ and the recruitment of the kinase Tec. FGF2 first oligomerizes, to be then phosphorylated by kinase Tec forming lipidic membrane pores. Lastly, membrane inserted FGF2 oligomers are disassembled at the outer plasma membrane leaflet by membrane proximal heparan sulfate proteoglycans, and FGF2 appears on the cell surface (Legrand et al., 2020; Pallotta and Nickel, 2020).

The UPS mechanism of FGF2 has proved to be relevant also for other functionally different proteins such as Tau and human immunodeficiency virus type 1 transactivator of transcription (HIV-Tat). Like FGF2, the secretory process of these proteins occurs by direct translocation across the plasma membrane and requires both $PI(4,5)P_2$ for the binding to the inner leaflet and heparan sulfates for the release from the outside leaflet (Mele et al., 2018; Merezhko et al., 2020). Furthermore, the secretion of HIV-Tat involves the binding to the α1-subunit of the Na/K-ATPase as well (Agostini et al., 2017). The recent case of the protein engrailed-2 homeoprotein (EN2) translocated across the plasma membrane due to its interaction with $PI(4,5)P_2$ also suggests that the EN2 secretion may rely on a UPS type I pathway (Amblard et al., 2020).

Interestingly, several aspects of the FGF2 secretion pathway also seem to be relevant for interleukin-1β (IL-1β). IL-1β, an essential cytokine necessary for acute inflammatory responses, is produced in the cytosol as a precursor (pro-IL1β). After the cleavage by caspase-1 into a mature form (mIL-1β), mIL-1β, like FGF2, is targeted to the plasma membrane in a $PI(4,5)P_2$-dependent manner and then exits the cell through membrane pores. However, unlike FGF2, mIL-1β does not appear to interact directly with $PI(4,5)P_2$. The membrane pores formation that allows mIL-1β passage is triggered by phosphoinositide-dependent oligomerization of the N-terminal domain of the cytosolic protein Gasdermin D, which is generated through proteolytic cleavage by inflammasome-activated caspases (Chan and Schroder, 2020). Moreover, by forming pores in the plasma membrane, the cleaved Gasdermin D ultimately causes cell lysis in a cell death process named pyroptosis (Evavold et al., 2018).

1.1.2 Type II Pathway: ABC Transporter-Based Secretion

In mammalian cells, a few proteins are known to be secreted through the Type II UPS pathway, which allows protein translocation through the plasma membrane *via* ATP-binding cassette (ABC) transporters (Dimou and Nickel, 2018). The first member of the ABCA subfamily, named ABCA1, promotes the secretion of several proteins, such as acetylated apurinic (apyrimidinic) endonuclease-1/redox factor-1 (AcAPE1/Ref-1) (Chen et al., 2021) and macrophage migration–inhibitory factor (MIF) (Sitia and Rubartelli, 2020). Heat shock 70-kDa protein (HSP70), which can be secreted through Type II UPS, appears to enter into endolysosomal vesicles with the aid of ABC transporters spanning the lysosomal membrane and to exit from mammalian cells *via* these vesicles (Cohen et al., 2020). Moreover, HSP70 seems to be capable of mediating a mechanism of type I UPS by itself. In fact, HSP70 associates with lipid membranes and, upon membrane insertion, oligomerizes and forms ion conductance channels. As a result, it mediates the extracellular secretion of different proteins (De Maio and Hightower, 2021).

1.1.3 Type III Pathway: Organelle-Based Translocation and Extracellular Vesicles

Type III UPS pathway involves different types of organelles that are in some cases intracellular vesicle intermediates, especially secretory lysosomes, multivesicular bodies (MVBs) and secretory autophagosomes. Endosomes, autophagosomes, and lysosomes

are membrane-bound organelles with their normal cellular functions, but turn out to be secretory organelles after induction by stress signaling pathways (Pallotta and Nickel, 2020).

In mammals, MVBs are crucial components of the endolysosomal system, which leads to endocytosis, recycling, and degradation of different kinds of macromolecules, including proteins. The membrane of MVBs invaginates, captures membrane and cytosolic proteins into vesicles and forms intraluminal vesicles (ILVs). Upon fusion of these compartments with the plasma membrane, proteins are secreted outside the cell into extracellular vesicles (EVs) named exosomes (Cocozza et al., 2020). Exosomes represent one of the major types of EVs (Mathieu et al., 2019) with contents and markers defined by previous studies (Jeppesen et al., 2019). The membrane of classical exosomes contains CD63, CD81, CD9, flotillin -1 and -2, EGFR, integrin beta1 and alpha2, and Na/K-ATPase. Four different complexes named endosomal sorting complexes required for transport 0-III (ESCRT 0-III) control the generation of ILVs, in particular monomers of the ESCRT-III protein Snf7, which polymerize, deform the membrane and allow the vesicles fission (Cohen et al., 2020).

Different types of vesicles can be released directly from the plasma membrane, such as ectosomes, microvesicles, microparticles, large oncosomes and apoptotic bodies (Cocozza et al., 2020).

Being derived from the pinching outwards of the plasma membrane, microvesicles can recruit cytosolic leaderless proteins. Like the blebbing mechanism, firstly the cytoskeleton adjacent to the site of shedding on the plasma membrane is disassembled, then the phosphatidylserine is translocated to the outer leaflet causing the plasma membrane to bulge (Cohen et al., 2020). Functional microvesicles are involved in several physiopathologic conditions, such as inflammation, oxidative stress, and senescence (Hijmans et al., 19852019). These vesicles differ from exosomes not only in formation mechanism, but also in size and molecular markers. Being heterogeneous, bigger than exosomes (50–1,000 nm in diameter whereas exosomes are between 30 and 150 nm (van Niel et al., 2018)), they contain glucose-regulated protein 94 (GRP94, also known as GP96), tumor susceptibility gene 101 (TSG101), annexin A1 and ADP-ribosylation factor 6 (ARF6) as markers (Jeppesen et al., 2019).

An interesting secretion route involves secretory lysosomes. Lysosomes can generate not only protein degradation, but also protein secretion. For this purpose, these vesicles release proteins by fusing with the plasma membrane and liberating their contents in the extracellular space. Studies on fatty acid-binding protein FABP4 show how a protein is secreted by this mechanism (Villeneuve et al., 2018).

Another way of leaderless protein release outside the cell is mediated by the so-called misfolding-associated protein secretion (MAPS) pathway. Cytotoxic polypeptides, such as alpha-synuclein, Tau and other cytosolic misfolded proteins, are delivered to late endosomes, which then fuse to the plasma membrane, releasing their contents (Sitia and Rubartelli, 2020).

Structures involved in autophagy are also critical for UPS of leaderless proteins, such as autophagosomes and amphisomes. Autophagosomes are double-membrane organelles formed under starvation and exogenous stresses to break down cellular components, but they are also constitutively formed to maintain the turnover of self-components. Moreover, autophagy can selectively degrade harmful substances that cannot be digested by other pathways such as the proteasomal degradation pathway (Kawabata and Yoshimori, 2020). Being capable of capturing other organelles and large areas of cytoplasm, autophagosomes deliver the materials to lysosomes or MVBs or the extracellular space for recycling, degradation or secretion of the cargo (Pallotta and Nickel, 2020). In particular, when autophagosomes fuse with MVBs, structures called amphisomes are formed, which can later fuse with the plasma membrane and deliver cargo to the external environment as a UPS mechanism (Cohen et al., 2020). An example of protein secreted by this mechanism is histone H3 (Jeppesen et al., 2019), while IL-1β can be released outside the cells through autophagosomes. Cytokine IL-1β can be released by either pyroptosis and pore formation (Type I UPS) or autophagy-mediated UPS mechanism (Type III UPS). Recent studies have revealed the mechanism by which IL-1β and other leaderless cargoes enter into the lumen of intracellular vesicle intermediates, in order to be secreted by the type III UPS pathway (Zhang et al., 2020). The transmembrane p24 trafficking protein 10 (TMED10) plays a crucial role in vesicle entry, as well as the secretion of many leaderless cargoes, like IL-1β. The unfolded form of this cytokine is bound to the cytoplasmic chaperone heat shock 90-kDa protein (HSP90A), which directs the protein to TMED10 localized in the ER-Golgi intermediate compartment (ERGIC). TMED serves as a protein channel and directs the entry of cargoes into this structure (Zhang et al., 2020). Besides, components of the early secretory pathway named Golgi reassembly and stacking proteins (GRASP, in mammals GRASP55 and GRASP65), are involved in the biogenesis of the vesicle intermediates, turning out to be important for IL-1β secretion (Chiritoiu et al., 2019).

1.1.4 Type IV Pathway: Bypassing the Golgi With SP/Transmembrane Domain-Containing Proteins

In mammals, type IV UPS is a pathway where integral membrane proteins translocated into the ER reach the plasma membrane bypassing the Golgi apparatus (therefore defined as Golgi-bypass). It is mostly associated with cellular stress signals generated during nutrient starvation, mechanical stress and ER stress. Indeed, proteins specialized in recognizing misfolded proteins and implicated in ER stress response, like IRE1, GRASPs, heat shock proteins as well as their cofactors and molecular chaperones, take part in the Golgi-bypass of different cargo proteins (Gee et al., 2018).

Well-known examples of transmembrane proteins that undergo the type IV UPS pathway are pendrin and cystic fibrosis transmembrane conductance regulator (CFTR). Disease-causing mutations of both CFTR and pendrin lead to, proteins misfolding and retention in the ER. Studies have demonstrated that under blocked ER-to-Golgi transport or ER stress conditions, immature core-glycosylated CFTR and pendrin

can reach the plasma membrane *via* the Golgi-bypass UPS pathway and retain their anion transporting activity. The basic mechanisms by which these two proteins reach the cell membrane *via* UPS appear to be similar, both enhanced by IRE1α kinase pathway activation (Park et al., 2020). However, some key molecules controlling the UPS of these two membrane proteins are not identical. For example, GRASP55 is required for the UPS of CFTR, whereas the HSP70 co-chaperone DNAJC14 is involved in the UPS of pendrin (Gee et al., 2018; Zhang and Wang, 2020). Furthermore, vesicular components related to autophagosome formation are involved in UPS. For instance, only knockdown of components in the autophagosome formation (ATG1, ATG5, ATG7, and ATG8), but not that of vacuole fusion (Vamp7), inhibits unconventional surface trafficking of the mutated form of CTFR (ΔF508-CFTR) (Gee et al., 2018). Moreover, secretory autophagy machinery and vesicular trafficking components have been demonstrated to take part in the secretory pathway of high mobility group box 1 (HMGB1), a leaderless protein whose unconventional secretion mechanism has recently been clarified (Kim et al., 2021). In particular, the machinery of HMGB1 secretion is mediated by Golgi reassembly stacking protein 2 (GORASP2), secretion associated Ras-related GTPase 1A ($SAR1A^{T39N}$), ADP ribosylation factor 1 ($ARF1^{Q71L}$) and MVBs formation (Kim et al., 2021). However, important questions regarding the mechanism of autophagy-mediated UPS of transmembrane proteins remain to be elucidated (Noh et al., 2018).

It should be noted that certain cargoes can enter different types of UPS pathways based on the physiological context. Indeed, some proteins that undergo the type I pathway can reach the extracellular space through other UPS mechanisms as well. Typical examples are Tau undergoing both type I UPS and UPS by EVs, and IL-1β going through both type I and type III UPS (Pallotta and Nickel, 2020).

2.2 Mechanisms and Physiological Role of Unconventional Protein Secretion in Plants

Numerous review articles have already described UPS in plants (De Marchis et al., 2013a; Davis et al., 2016; Robinson et al., 2016) or compared conventional protein secretion with UPS in plants (Goring and Di Sansebastiano, 2017; Wang et al., 2017). In one of these papers, (Ding et al., 2014) the authors have suggested a classification for the different types of UPS existing in plants: type I, a Golgi-bypass pathway for SP-lacking polypeptides, type II, a secretion route mediated by the vacuole, or (type III) mediated by MVBs, or (type IV) mediated by an exocyst-positive organelle (EXPO). Unfortunately, other authors have denominated UPS types in animals and yeast as type I-IV, and at least in two cases, very different UPS mechanisms share the same name. For example, the type IV pathway in mammalian cells involves SP- and/or transmembrane domain-containing proteins which are translated in the ER and then targeted to the plasma membrane without passing through the Golgi (Rabouille, 2017). Conversely, the type IV pathway in plant cells corresponds to a secretory pathway mediated by EXPO, a double-membrane-bound organelle that fuses with the plasma membrane and releases leaderless cytosolic proteins (Ding et al., 2014; Wang et al., 2020). Therefore, we describe in this paper how the UPS plant classification system should be revised in comparison to the UPS general categories identified by Pallotta and Nickel (Pallotta and Nickel, 2020).

2.2.1 Type I Pathway: Golgi-Bypass Pathway for Leaderless Proteins

Both mammalian and plant cells use this UPS route, and many leaderless secretory proteins have been described in the plant secretome (Agrawal et al., 2010; Krause et al., 2013), but in plants, there are only a few published examples of leaderless proteins secreted in the apoplast bypassing the Golgi apparatus. One involves a leaderless heterologous protein of bacterial origin, hygromycin phosphotransferase (HYGR), which is secreted, when expressed in transgenic Arabidopsis plants, from the cytosol to the apoplast, i.e the plant extracellular space, bypassing the Golgi (Zhang et al., 2011). With this aim, brefeldin A (BFA), an inhibitor of protein traffic through the Golgi apparatus caused by deregulated fusion of the ER with the Golgi cisternae, has been used. BFA treatment does not inhibit HYGR secretion (Zhang et al., 2011), nor does it impede the secretion of another protein, mannitol dehydrogenase (MTD) (Cheng et al., 2009). MTD, the only other example, converts mannitol to mannose and it is localized in the cell (cytoplasm, nucleus, etc.) but secreted into the apoplast after treatment with salicylic acid, an endogenous inducer of plant defense responses (Cheng et al., 2009). MTD secretion may represent part of a plant defense mechanism against mannitol-secreting fungal pathogens, and a very preliminary effort has recently been made to identify the cytoplasmic components of the MTD secretory machinery following salicylic acid treatment (Ho et al., 2022). Indeed, no information about the translocation mechanisms of HYGR and MTD is available yet.

2.2.2 Type II Pathway: ABC Transporter-Based Secretion

No plant-secreted protein seems to follow this route involving lipidated cargoes and being mediated by ABC transporters (Dimou and Nickel, 2018).

2.2.3 Type III Pathway: Organelle-Based Translocation and Extracellular Vesicles

Through similar mechanisms used by mammalian cells, the endomembrane trafficking system in plants is tightly linked to cellular stresses in order to rapidly adapt the cellular processes to the new physiological conditions (Wang et al., 2020). The vacuole is the largest membrane-bounded compartment in plant cells with multiple functions essential for plant growth and development, and some of these functions, like cellular waste degradation, are similar to those of lysosomes. In case of a pathogen attack, vacuoles can turn into secretory organelles and fuse with the plasma membrane at pathogen entry sites releasing antibacterial proteins like aleurain, aspartyl protease, and carboxypeptidase Y (Hatsugai et al., 2009). These hydrolytic enzymes enter the ER due to their N-terminal SP and then traffic along the conventional protein secretion pathway to reach the

vacuole where they normally degrade cellular proteins. Their induced release into the apoplast carries out both antibacterial activity and cell death-inducing activity, leading to PCD as a defense strategy developed by plants for lack of immune cells (Ruano and Scheuring, 2020).

In plants, another UPS route should be comprised of the type III pathway because it is organelle-mediated and the secreted proteins are released as part of vesicles: UPS mediated by EVs. Plant EVs can be secreted from either exocyst-positive organelles (EXPOs) or MVBs.

EXPOs, double-membrane organelles from 500 to 800 nm in diameter (similar to autophagosomes), are Exo70E2-positive structures because immunolabelling studies have shown the exocyst subunit Exo70E2 co-localized with them (Wang et al., 2010). EXPOs deliver cargo-containing vesicles into the apoplast by fusion of the outer membrane with the PM, while the inner boundary membrane is subjected to degradation (Ding et al., 2012). EXPO characteristics have recently been revised (Cui et al., 2020), so here we only underline that EXPOs seem to be involved in releasing exosomes containing leaderless proteins with a role in growth regulation and plant cell wall remodeling. De Caroli and colleagues (De Caroli et al., 2021) have shown that two out of three xyloglucan endotransglucosylase/hydrolases (XTHs) involved in cell wall assembly are targeted to the cell wall and plasma membrane through a conventional protein secretion pathway. Conversely, the other leaderless protein (XTH29), released in the apoplast by a UPS route mediated by EXPOs, appears to be upregulated in response to abiotic stresses.

Plant MVBs (alternatively named prevacuolar compartments or late endosomes) are organelles of conventional secretion pathway mediating the transport from the Golgi to vacuoles (Hu et al., 2020), but MVBs can also participate in UPS pathways by fusing with the plasma membrane to release their ILVs to the apoplast. These ILVs, referred to as exosomes, take part in intercellular communication and carry small RNAs and proteins (Cai et al., 2018). Exosomes belong to an EVs subpopulation of 30–150 nm in diameter, isolated by differential ultracentrifugation (Rutter and Innes, 2020; Kim, 2021) and enriched in membrane proteins used as biomarkers like the syntaxin AtSYP121/PENETRATION1 (PEN1) and the tetraspanin (TET) 8 and TET9 (Rutter and Innes, 2017; Cai et al., 2018). Transmission electron microscopy images of MVBs and exosomes have been shown in plant leaves (An et al., 2006; Liu et al., 2020) and stigmatic papillae (Goring, 2017), moreover, exosomes have been isolated from external fluids of leaves (Rutter and Innes, 2017), pollen grains (Prado et al., 2014) and seeds (Pinedo et al., 2012). However, there is limited experimental evidence of MVB-plasma membrane fusion resulted in the release of exosomes into the apoplast (Movahed et al., 2019). Since the secretion of plant exosomes/EVs is enhanced in response to pathogen infection (Hansen and Nielsen, 2017; Rybak and Robatzek, 2019), and proteomic analyses of exosomes/EVs isolated from plant tissues have identified enrichment of proteins involved in cell wall remodeling enzymes and defense/stress-related proteins (Regente et al., 2017; Rutter and Innes, 2017), it is widely accepted that plant exosomes/EVs perform a function in plant growth and development (de la Canal and Pinedo, 2018), including regulation of plant-microbe interactions (Ivanov et al., 2019; Roth et al., 2019; Cui et al., 2020). Nevertheless, many questions remain to be answered, like which cargo proteins are involved and how they are loaded in plant exosomes.

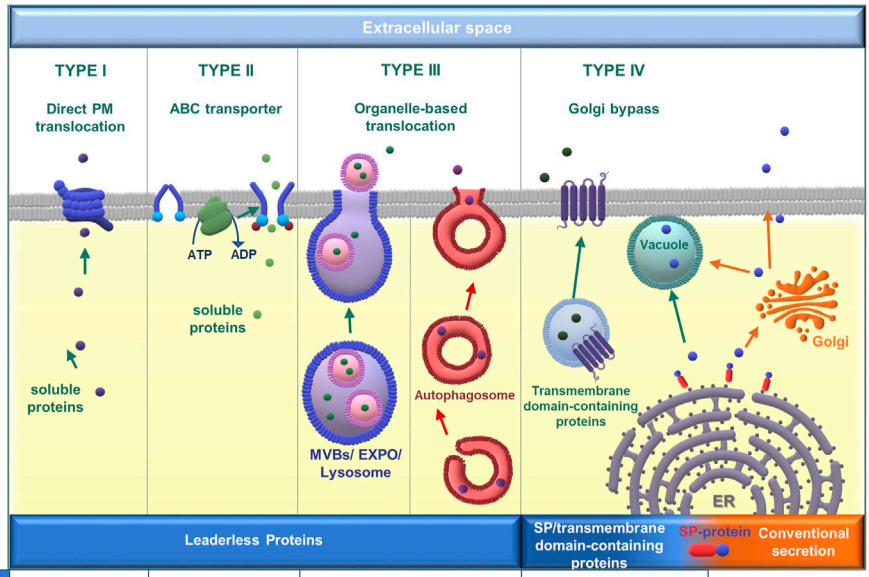
2.2.4 Type IV Pathway: Bypassing the Golgi With SP/Transmembrane Domain-Containing Proteins

Integral membrane proteins, synthesized in the ER and bypassing the Golgi during their journey to the plasma membrane, are comprised in this route. In plants, there is no solid result able to demonstrate protein traveling from the ER to the plasma membrane or the apoplast bypassing the Golgi apparatus. Alternatively, there are many examples of proteins directly delivered from the ER to the vacuole, which have extensively been reviewed (Pedrazzini et al., 2016; Bellucci et al., 2017). Both soluble and membrane proteins with ER-targeting signals can traffic to the vacuole bypassing the Golgi, including proteins aggregated in large polymers (ER bodies), which are stored either in seed tissues to allow seed germination or in vegetative tissues to join the plant defense against abiotic stresses. Examples of such ER bodies are the precursor-accumulating vesicles (PACs) and the ER bodies described in plants of the *Brassicales* order. PACs are ER-derived spherical bodies that accumulate storage proteins and, after being released in the cytoplasm, fuse with the protein storage vacuoles (Hara-Nishimura et al., 1998). In *Arabidopsis thaliana* (Brassicaceae) seedlings, ER bodies accumulate mainly proteases and fuse to the vacuoles in presence of salt stress, thus assisting the cell death under stress conditions (Hayashi et al., 2001).

Heterologous expression of soluble glycoproteins, like human lysosomal alpha-mannosidase (MAN2B1) and mouse IgG1 14D9, demonstrates that these proteins directly reach the vacuole after translocation into the ER (De Marchis et al., 2013b; Ocampo et al., 2016). It is not clear how they are delivered to the vacuole and if glycosylation is relevant for their trafficking. Interestingly, in the case of cardosins, plant vacuolar aspartic proteinases, when the C-terminal vacuolar sorting domain (VSD) is artificially removed, a second domain named plant-specific insert (PSI) acts as a VSD in specific conditions or developmental stages. In the species artichoke (*Cynara Cardunculus*) and soybean (*Glycine max*), the glycosylation status of the PSI domain seems to play an important role in determining if the cardosin should go through or bypass the Golgi in their route to the vacuole (Vieira et al., 2019). As regards membrane proteins, several proteins located in the vacuole membrane like calcineurin B-like (CBL) 6, soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) VAM3 and α-TIP, traffic through this UPS pathway (Di Sansebastiano et al., 2017).

2 DISCUSSION

We are firmly convinced that a unique classification system in mammals and plants for the UPS pathways based on their different molecular mechanisms should be an important prerequisite for biological research to avoid confusion. After all, similar motivations have driven the community to conceive biological classification systems (Marakeby et al., 2014). A re-classification



UPS in Mammals	FGF2 Tau HIV-Tat EN2 IL-1β Hsp70	AcAPE1/Ref-1 MIF Hsp70	Through different types of intracellular vescicles intermediates Examples: FABP4 IL-1β Histone H3 α-synuclein	Pendrin CFTR HMGB1
UPS in Plants	MTD HYGR	To date not known plant-secreted proteins following this route	Through different types of intracellular vescicle intermediates Examples: aleurain aspartyl protease carboxypeptidase Y XTH29	From ER to plasma membrane: to date not known From the ER to the vacuole: many examples

FIGURE 1 | Overview of unconventional protein secretion (UPS) pathways in mammals and plants, with a description of proteins following different UPS routes. From left to right: UPS type I, UPS type II, and UPS type III are used by leaderless proteins which employ different methods to go through the plasma membrane (PM). In type III autophagosomes are inserted to represent the crosstalk between UPS and autophagy (see Paragraph 1.1.3). The scheme of UPS type IV represents the destiny of SP/transmembrane domain-containing proteins translocated in the ER, the next journey to the plasma membrane in mammals, and the direct delivery to the vacuole in plants. However, such proteins normally traffic along the conventional secretory pathway and transit through the Golgi apparatus.

of the UPS pathways can especially benefit the plant scientific community because in plants the understanding of UPS mechanisms is still very restricted and the assignment of a particular protein transport route to a type of UPS pathway can arise debate (Pompa et al., 2017). This is the case of EXPO organelles, and in fact for some authors the question if these organelles should be considered part of an autophagic transport to the vacuole rather than part of a UPS pathway that releases leaderless proteins in the apoplast, is still in discussion (Kulich et al., 2013; Lin et al., 2015). However, with the scientific data available about the UPS pathways, abundant in mammals and few in plants, we've succeeded in finding some common categories/types (**Figure 1**). For example, there are many similarities between animals and plants in UPS type III, where organelles normally involved in the endomembrane trafficking system of the classical secretion pathway (vacuoles in plants and lysosomes in animals) become UPS organelles, but there are also differences due to specific membrane-bound organelles like the EXPOs in plants. Moreover, specialized UPS organelles, such as the yeast cup-shaped membranes (CUPS), are present neither in animals nor in plants (Bruns et al., 2011). It has been difficult for us to distinguish proteins secreted by UPS routes from those secreted by autophagy or PCD mechanisms because interaction takes place between UPS and autophagy/PCD. Conversely, we have unanimously decided to exclude from this review plasmodesmata and tunnelling nanotubes which are types of cell-to-cell transport based on intercellular channels (Knox and Benitez-Alfonso, 2014). By writing this review, we have realized that knowledge is still very limited of the molecular machineries involved in the secretion of proteins by unconventional pathways both in animals and in plants. The UPS topic will become more and more important in the coming years and an increasing understanding of these secretion mechanisms will provide unique opportunities for applied biology.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work has been supported by the PRIN program of Italian Ministry of Education, University and Research (MIUR, grant number PRIN 2017WJZ9W9) to MTP.

REFERENCES

Agostini, S., Ali, H., Vardabasso, C., Fittipaldi, A., Tasciotti, E., Cereseto, A., et al. (2017). Inhibition of Non Canonical HIV-1 Tat Secretion through the Cellular Na + ,K + -ATPase Blocks HIV-1 Infection. *EBioMedicine* 21, 170–181. doi:10.1016/j.ebiom.2017.06.011

Agrawal, G. K., Jwa, N.-S., Lebrun, M.-H., Job, D., and Rakwal, R. (2010). Plant Secretome: Unlocking Secrets of the Secreted Proteins. *Proteomics* 10 (4), 799–827. doi:10.1002/pmic.200900514

Amblard, I., Dupont, E., Alves, I., Miralvès, J., Queguiner, I., and Joliot, A. (2020). Bidirectional Transfer of Homeoprotein EN2 across the Plasma Membrane Requires PIP2. *J. Cel Sci* 133 (13), jcs244327. doi:10.1242/jcs.244327

An, Q., Huckelhoven, R., Kogel, K.-H., and van Bel, A. J. E. (2006). Multivesicular Bodies Participate in a Cell wall-associated Defence Response in Barley Leaves Attacked by the Pathogenic Powdery Mildew Fungus. *Cell Microbiol* 8 (6), 1009–1019. doi:10.1111/j.1462-5822.2006.00683.x

Balmer, E. A., and Faso, C. (2021). The Road Less Traveled? Unconventional Protein Secretion at Parasite-Host Interfaces. *Front. Cel Dev. Biol.* 9, 662711. doi:10.3389/fcell.2021.662711

Bellucci, M., De Marchis, F., and Pompa, A. (2017). The Endoplasmic Reticulum Is a Hub to Sort Proteins toward Unconventional Traffic Pathways and Endosymbiotic Organelles. *J. Exp. Bot.* 69 (1), 7–20. doi:10.1093/jxb/erx286

Bruns, C., McCaffery, J. M., Curwin, A. J., Duran, J. M., and Malhotra, V. (2011). Biogenesis of a Novel Compartment for Autophagosome-Mediated Unconventional Protein Secretion. *J. Cel Biol* 195 (6), 979–992. doi:10.1083/jcb.201106098

Cai, Q., Qiao, L., Wang, M., He, B., Lin, F.-M., Palmquist, J., et al. (2018). Plants Send Small RNAs in Extracellular Vesicles to Fungal Pathogen to Silence Virulence Genes. *Science* 360 (6393), 1126–1129. doi:10.1126/science.aar4142

Chan, A. H., and Schroder, K. (2020). Inflammasome Signaling and Regulation of Interleukin-1 Family Cytokines. *J. Exp. Med.* 217 (1), e20190314. doi:10.1084/jem.20190314

Chen, W., Wang, S., and Xing, D. (2021). New Horizons for the Roles and Association of APE1/Ref-1 and ABCA1 in Atherosclerosis. *Jir* 14, 5251–5271. doi:10.2147/jir.s330147

Cheng, F.-y., Zamski, E., Guo, W.-w., Pharr, D. M., and Williamson, J. D. (2009). Salicylic Acid Stimulates Secretion of the Normally Symplastic Enzyme Mannitol Dehydrogenase: a Possible Defense against Mannitol-Secreting Fungal Pathogens. *Planta* 230 (6), 1093–1103. doi:10.1007/s00425-009-1006-3

Chiritoiu, M., Brouwers, N., Turacchio, G., Pirozzi, M., and Malhotra, V. (2019). GRASP55 and UPR Control Interleukin-1β Aggregation and Secretion. *Dev. Cel* 49 (1), 145–155. doi:10.1016/j.devcel.2019.02.011

Cocozza, F., Grisard, E., Martin-Jaular, L., Mathieu, M., and Théry, C. (2020). SnapShot: Extracellular Vesicles. *Cell* 182 (1), 262. doi:10.1016/j.cell.2020.04.054

Cohen, M. J., Chirico, W. J., and Lipke, P. N. (2020). Through the Back Door: Unconventional Protein Secretion. *Cel Surf.* 6, 100045. doi:10.1016/j.tcsw.2020.100045

Cui, Y., Gao, J., He, Y., and Jiang, L. (2020). Plant Extracellular Vesicles. *Protoplasma* 257 (1), 3–12. doi:10.1007/s00709-019-01435-6

Davis, D. J., Kang, B.-H., Heringer, A. S., Wilkop, T. E., and Drakakaki, G. (2016). Unconventional Protein Secretion in Plants. *Methods Mol. Biol.* 1459, 47–63. doi:10.1007/978-1-4939-3804-9_3

De Caroli, M., Manno, E., Piro, G., and Lenucci, M. S. (2021). Ride to Cell wall: Arabidopsis XTH11, XTH29 and XTH33 Exhibit Different Secretion Pathways and Responses to Heat and Drought Stress. *Plant J.* 107 (2), 448–466. doi:10.1111/tpj.15301

de la Canal, L., and Pinedo, M. (2018). Extracellular Vesicles: a Missing Component in Plant Cell wall Remodeling. *J. Exp. Bot.* 69 (20), 4655–4658. doi:10.1093/jxb/ery255

De Maio, A., and Hightower, L. (2021). The Interaction of Heat Shock Proteins with Cellular Membranes: a Historical Perspective. *Cell Stress and Chaperones* 26 (5), 769–783. doi:10.1007/s12192-021-01228-y

De Marchis, F., Bellucci, M., and Pompa, A. (2013). Unconventional Pathways of Secretory Plant Proteins from the Endoplasmic Reticulum to the Vacuole Bypassing the Golgi Complex. *Plant Signal. Behav.* 8 (8), e25219. doi:10.4161/psb.25129

De Marchis, F., Bellucci, M., and Pompa, A. (2013). Traffic of Human α-Mannosidase in Plant Cells Suggests the Presence of a New Endoplasmic Reticulum-To-Vacuole Pathway without Involving the Golgi Complex. *Plant Physiol.* 161 (4), 1769–1782. doi:10.1104/pp.113.214536

Di Sansebastiano, G. P., Barozzi, F., Piro, G., Denecke, J., and de Marcos Lousa, C. (2017). Trafficking Routes to the Plant Vacuole: Connecting Alternative and Classical Pathways. *J. Exp. Bot.* 69 (1), 79–90. doi:10.1093/jxb/erx376

Dimou, E., and Nickel, W. (2018). Unconventional Mechanisms of Eukaryotic Protein Secretion. *Curr. Biol.* 28 (8), R406–r410. doi:10.1016/j.cub.2017.11.074

Ding, Y., Robinson, D. G., and Jiang, L. (2014). Unconventional Protein Secretion (UPS) Pathways in Plants. *Curr. Opin. Cel Biol.* 29, 107–115. doi:10.1016/j.ceb.2014.05.008

Ding, Y., Wang, J., Wang, J., Stierhof, Y.-D., Robinson, D. G., and Jiang, L. (2012). Unconventional Protein Secretion. *Trends Plant Sci.* 17 (10), 606–615. doi:10.1016/j.tplants.2012.06.004

Evavold, C. L., Ruan, J., Tan, Y., Xia, S., Wu, H., and Kagan, J. C. (2018). The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages. *Immunity* 48 (1), 35–44. doi:10.1016/j.immuni.2017.11.013

Gee, H. Y., Kim, J., and Lee, M. G. (2018). Unconventional Secretion of Transmembrane Proteins. *Semin. Cel Dev. Biol.* 83, 59–66. doi:10.1016/j.semcdb.2018.03.016

Goring, D. R., and Di Sansebastiano, G. P. (2017). Protein and Membrane Trafficking Routes in Plants: Conventional or Unconventional? *J. Exp. Bot.* 69 (1), 1–5. doi:10.1093/jxb/erx435

Goring, D. R. (2017). Exocyst, Exosomes, and Autophagy in the Regulation of Brassicaceae Pollen-Stigma Interactions. *J. Exp. Bot.* 69 (1), 69–78. doi:10.1093/jxb/erx340

Hansen, L. L., and Nielsen, M. E. (2017). Plant Exosomes: Using an Unconventional Exit to Prevent Pathogen Entry? *J. Exp. Bot.* 69 (1), 59–68. doi:10.1093/jxb/erx319

Hara-Nishimura, I., Shimada, T., Hatano, K., Takeuchi, Y., and Nishimura, M. (1998). Transport of Storage Proteins to Protein Storage Vacuoles Is Mediated by Large Precursor-Accumulating Vesicles. *The Plant Cell* 10 (5), 825–836. doi:10.2307/3870668

Hatsugai, N., Iwasaki, S., Tamura, K., Kondo, M., Fuji, K., Ogasawara, K., et al. (2009). A Novel Membrane Fusion-Mediated Plant Immunity against Bacterial Pathogens. *Genes Dev.* 23 (21), 2496–2506. doi:10.1101/gad.1825209

Hayashi, Y., Yamada, K., Shimada, T., Matsushima, R., Nishizawa, N., Nishimura, M., et al. (2001). A Proteinase-Storing Body that Prepares for Cell Death or Stresses in the Epidermal Cells of Arabidopsis. *Plant Cel Physiol* 42 (9), 894–899. doi:10.1093/pcp/pce144

Hijmans, J. G., Stockelman, K., Levy, M., Brewster, L. M., Bammert, T. D., Greiner, J. J., et al. (19852019). Effects of HIV-1 Gp120 and TAT-Derived Microvesicles on Endothelial Cell Function. *J. Appl. Physiol. (1985)* 126 (5), 1242–1249. doi:10.1152/japplphysiol.01048.2018

Ho, T. C., Blackburn, R. K., Goshe, M. B., and Williamson, J. D. (2022). Identification of Multiple Proteins Whose Interaction with Mannitol Dehydrogenase Is Induced by Salicylic Acid: Implications for Unconventional Secretion. *Proteomics* 22 (1-2), e2100091. doi:10.1002/pmic.202100091

Hu, S., Li, Y., and Shen, J. (2020). A Diverse Membrane Interaction Network for Plant Multivesicular Bodies: Roles in Proteins Vacuolar Delivery and Unconventional Secretion. *Front. Plant Sci.* 11, 425. doi:10.3389/fpls.2020.00425

Ivanov, S., Austin, J., Berg, R. H., and Harrison, M. J. (2019). Extensive Membrane Systems at the Host-Arbuscular Mycorrhizal Fungus Interface. *Nat. Plants* 5 (2), 194–203. doi:10.1038/s41477-019-0364-5

Jeppesen, D. K., Fenix, A. M., Franklin, J. L., Higginbotham, J. N., Zhang, Q., Zimmerman, L. J., et al. (2019). Reassessment of Exosome Composition. *Cell* 177 (2), 428–445. doi:10.1016/j.cell.2019.02.029

Kawabata, T., and Yoshimori, T. (2020). Autophagosome Biogenesis and Human Health. *Cell Discov* 6 (1), 33. doi:10.1038/s41421-020-0166-y

Kim, K. W. (2021). *In Situ* and *Ex Situ* Imaging of Plant Extracellular Vesicles as Nanovectors for Cross-domain Communication. *J. Phytopathol* 169 (9), 515–524. doi:10.1111/jph.13022

Kim, Y. H., Kwak, M. S., Lee, B., Shin, J. M., Aum, S., Park, I. H., et al. (2021). Secretory Autophagy Machinery and Vesicular Trafficking Are Involved in HMGB1 Secretion. *Autophagy* 17 (9), 2345–2362. doi:10.1080/15548627.2020.1826690

Knox, J. P., and Benitez-Alfonso, Y. (2014). Roles and Regulation of Plant Cell walls Surrounding Plasmodesmata. *Curr. Opin. Plant Biol.* 22, 93–100. doi:10.1016/j.pbi.2014.09.009

Krause, C., Richter, S., Knöll, C., and Jürgens, G. (2013). Plant Secretome - from Cellular Process to Biological Activity. *Biochim. Biophys. Acta (Bba) - Proteins Proteomics* 1834 (11), 2429–2441. doi:10.1016/j.bbapap.2013.03.024

Kulich, I., Pečenková, T., Sekereš, J., Smetana, O., Fendrych, M., Foissner, I., et al. (2013). Arabidopsis Exocyst Subcomplex Containing Subunit EXO70B1 Is Involved in Autophagy-Related Transport to the Vacuole. *Traffic* 14 (11), 1155–1165. doi:10.1111/tra.12101

Legrand, C., Saleppico, R., Sticht, J., Lolicato, F., Müller, H.-M., Wegehingel, S., et al. (2020). The Na,K-ATPase Acts Upstream of Phosphoinositide PI(4,5)P2 Facilitating Unconventional Secretion of Fibroblast Growth Factor 2. *Commun. Biol.* 3 (1), 141. doi:10.1038/s42003-020-0871-y

Lin, Y., Ding, Y., Wang, J., Shen, J., Kung, C. H., Zhuang, X., et al. (2015). Exocyst-Positive Organelles and Autophagosomes Are Distinct Organelles in Plants. *Plant Physiol.* 169 (3), 1917–1932. doi:10.1104/pp.15.00953

Liu, N.-J., Wang, N., Bao, J.-J., Zhu, H.-X., Wang, L.-J., and Chen, X.-Y. (2020). Lipidomic Analysis Reveals the Importance of GIPCs in Arabidopsis Leaf Extracellular Vesicles. *Mol. Plant* 13 (10), 1523–1532. doi:10.1016/j.molp.2020.07.016

Marakeby, H., Badr, E., Torkey, H., Song, Y., Leman, S., Monteil, C. L., et al. (2014). A System to Automatically Classify and Name Any Individual Genome-Sequenced Organism Independently of Current Biological Classification and Nomenclature. *PLoS One* 9 (2), e89142. doi:10.1371/journal.pone.0089142

Mathieu, M., Martin-Jaular, L., Lavieu, G., and Théry, C. (2019). Specificities of Secretion and Uptake of Exosomes and Other Extracellular Vesicles for Cell-To-Cell Communication. *Nat. Cel Biol* 21 (1), 9–17. doi:10.1038/s41556-018-0250-9

Mele, A. R., Marino, J., Chen, K., Pirrone, V., Janetopoulos, C., Wigdahl, B., et al. (2018), Defining the Molecular Mechanisms of HIV-1 Tat Secretion: PtdIns(4,5)P2 at the Epicenter. *Traffic*, 4, doi:10.1111/tra.12578

Merezhko, M., Uronen, R.-L., and Huttunen, H. J. (2020). The Cell Biology of Tau Secretion. *Front. Mol. Neurosci.* 13, 569818. doi:10.3389/fnmol.2020.569818

Movahed, N., Cabanillas, D. G., Wan, J., Vali, H., Laliberté, J.-F., and Zheng, H. (2019). Turnip Mosaic Virus Components Are Released into the Extracellular Space by Vesicles in Infected Leaves. *Plant Physiol.* 180 (3), 1375–1388. doi:10.1104/pp.19.00381

Noh, S. H., Gee, H. Y., Kim, Y., Piao, H., Kim, J., Kang, C. M., et al. (2018). Specific Autophagy and ESCRT Components Participate in the Unconventional Secretion of CFTR. *Autophagy* 14 (10), 1761–1778. doi:10.1080/15548627.2018.1489479

Ocampo, C. G., Lareu, J. F., Marin Viegas, V. S., Mangano, S., Loos, A., Steinkellner, H., et al. (2016). Vacuolar Targeting of Recombinant Antibodies in Nicotiana Benthamiana. *Plant Biotechnol. J.* 14 (12), 2265–2275. doi:10.1111/pbi.12580

Osborne, A. R., Rapoport, T. A., and van den Berg, B. (2005). Protein Translocation by the Sec61/SecY Channel. *Annu. Rev. Cel Dev. Biol.* 21, 529–550. doi:10.1146/annurev.cellbio.21.012704.133214

Pallotta, M. T., and Nickel, W. (2020). FGF2 and IL-1β - Explorers of Unconventional Secretory Pathways at a Glance. *J. Cel Sci* 133 (21), jcs250449. doi:10.1242/jcs.250449

Park, H., Shin, D. H., Sim, J. R., Aum, S., and Lee, M. G. (2020). IRE1α Kinase-Mediated Unconventional Protein Secretion Rescues Misfolded CFTR and Pendrin. *Sci. Adv.* 6 (8), eaax9914. doi:10.1126/sciadv.aax9914

Pedrazzini, E., Mainieri, D., Marrano, C. A., and Vitale, A. (2016). Where Do Protein Bodies of Cereal Seeds Come from? *Front. Plant Sci.* 7, 1139. doi:10.3389/fpls.2016.01139

Pinedo, M., Regente, M., Elizalde, M., Y. Quiroga, I., A. Pagnussat, L., Jorrin-Novo, J., et al. (2012). Extracellular sunflower Proteins: Evidence on Non-classical Secretion of a Jacalin-Related Lectin. *Ppl* 19 (3), 270–276. doi:10.2174/092986612799363163

Pompa, A., De Marchis, F., Pallotta, M. T., Benitez-Alfonso, Y., Jones, A., Schipper, K., et al. (2017). Unconventional Transport Routes of Soluble and Membrane Proteins and Their Role in Developmental Biology. *Int. J. Mol. Sci.* 18 (4), 703. doi:10.3390/ijms18040703

Prado, N., de Dios Alché, J., Casado-Vela, J., Mas, S., Villalba, M., Rodríguez, R., et al. (2014). Nanovesicles Are Secreted during Pollen Germination and Pollen Tube Growth: a Possible Role in Fertilization. *Mol. Plant* 7 (3), 573–577. doi:10.1093/mp/sst153

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cel Biol.* 27 (3), 230–240. doi:10.1016/j.tcb.2016.11.007

Regente, M., Pinedo, M., San Clemente, H., Balliau, T., Jamet, E., and de la Canal, L. (2017). Plant Extracellular Vesicles Are Incorporated by a Fungal Pathogen and Inhibit its Growth. *J. Exp. Bot.* 68 (20), 5485–5495. doi:10.1093/jxb/erx355

Robinson, D. G., Ding, Y., and Jiang, L. (2016). Unconventional Protein Secretion in Plants: a Critical Assessment. *Protoplasma* 253 (1), 31–43. doi:10.1007/s00709-015-0887-1

Roth, R., Hillmer, S., Funaya, C., Chiapello, M., Schumacher, K., Lo Presti, L., et al. (2019). Arbuscular Cell Invasion Coincides with Extracellular Vesicles and Membrane Tubules. *Nat. Plants* 5 (2), 204–211. doi:10.1038/s41477-019-0365-4

Ruano, G., and Scheuring, D. (2020). Plant Cells under Attack: Unconventional Endomembrane Trafficking during Plant Defense. *Plants (Basel)* 9 (3), 389. doi:10.3390/plants9030389

Rutter, B. D., and Innes, R. W. (2017). Extracellular Vesicles Isolated from the Leaf Apoplast Carry Stress-Response Proteins. *Plant Physiol.* 173 (1), 728–741. doi:10.1104/pp.16.01253

Rutter, B. D., and Innes, R. W. (2020). Growing Pains: Addressing the Pitfalls of Plant Extracellular Vesicle Research. *New Phytol.* 228 (5), 1505–1510. doi:10.1111/nph.16725

Rybak, K., and Robatzek, S. (2019). Functions of Extracellular Vesicles in Immunity and Virulence. *Plant Physiol.* 179 (4), 1236–1247. doi:10.1104/pp.18.01557

Shan, S.-o., and Walter, P. (2005). Co-translational Protein Targeting by the Signal Recognition Particle. *FEBS Lett.* 579 (4), 921–926. doi:10.1016/j.febslet.2004.11.049

Sitia, R., and Rubartelli, A. (2020). Evolution, Role in Inflammation, and Redox Control of Leaderless Secretory Proteins. *J. Biol. Chem.* 295 (22), 7799–7811. doi:10.1074/jbc.rev119.008907

Stewart, S. E., Ashkenazi, A., Williamson, A., Rubinsztein, D. C., and Moreau, K. (2018). Transbilayer Phospholipid Movement Facilitates the Translocation of Annexin across Membranes. *J. Cel Sci* 131 (14), jcs217034. doi:10.1242/jcs.217034

van Niel, G., D'Angelo, G., and Raposo, G. (2018). Shedding Light on the Cell Biology of Extracellular Vesicles. *Nat. Rev. Mol. Cel Biol* 19 (4), 213–228. doi:10.1038/nrm.2017.125

Vieira, V., Peixoto, B., Costa, M., Pereira, S., Pissarra, J., and Pereira, C. (2019). N-linked Glycosylation Modulates Golgi-independent Vacuolar Sorting Mediated by the Plant Specific Insert. *Plants (Basel)* 8 (9), 312. doi:10.3390/plants8090312

Villeneuve, J., Bassaganyas, L., Lepreux, S., Chiritoiu, M., Costet, P., Ripoche, J., et al. (2018). Unconventional Secretion of FABP4 by Endosomes and Secretory Lysosomes. *J. Cel Biol* 217 (2), 649–665. doi:10.1083/jcb.201705047

Vitale, A., and Denecke, J. (1999). The Endoplasmic Reticulum: Gateway of the Secretory Pathway. *The Plant Cell* 11 (4), 615–628. doi:10.2307/3870888

Wang, J., Ding, Y., Wang, J., Hillmer, S., Miao, Y., Lo, S. W., et al. (2010). EXPO, an Exocyst-Positive Organelle Distinct from Multivesicular Endosomes and Autophagosomes, Mediates Cytosol to Cell Wall Exocytosis inArabidopsisand Tobacco Cells. *Plant Cell* 22 (12), 4009–4030. doi:10.1105/tpc.110.080697

Wang, X., Chung, K. P., Lin, W., and Jiang, L. (2017). Protein Secretion in Plants: Conventional and Unconventional Pathways and New Techniques. *J. Exp. Bot.* 69 (1), 21–37. doi:10.1093/jxb/erx262

Wang, X., Xu, M., Gao, C., Zeng, Y., Cui, Y., Shen, W., et al. (2020). The Roles of Endomembrane Trafficking in Plant Abiotic Stress Responses. *J. Integr. Plant Biol.* 62 (1), 55–69. doi:10.1111/jipb.12895

Zhang, H., Zhang, L., Gao, B., Fan, H., Jin, J., Botella, M. A., et al. (2011). Golgi Apparatus-Localized Synaptotagmin 2 Is Required for Unconventional Secretion in Arabidopsis. *PLoS One* 6 (11), e26477. doi:10.1371/journal.pone.0026477

Zhang, L., Xing, J., and Lin, J. (2019). At the Intersection of Exocytosis and Endocytosis in Plants. *New Phytol.* 224 (4), 1479–1489. doi:10.1111/nph.16018

Zhang, M., Liu, L., Lin, X., Wang, Y., Li, Y., Guo, Q., et al. (2020). A Translocation Pathway for Vesicle-Mediated Unconventional Protein Secretion. *Cell* 181 (3), 637–652. doi:10.1016/j.cell.2020.03.031

Zhang, X., and Wang, Y. (2020). Nonredundant Roles of GRASP55 and GRASP65 in the Golgi Apparatus and beyond. *Trends Biochem. Sci.* 45 (12), 1065–1079. doi:10.1016/j.tibs.2020.08.001

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

REVIEW
published: 10 May 2022
doi: 10.3389/fcell.2022.906453

Safeguarding Lysosomal Homeostasis by DNAJC5/CSPα-Mediated Unconventional Protein Secretion and Endosomal Microautophagy

*Juhyung Lee, Yue Xu and Yihong Ye**

Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, United States

OPEN ACCESS

Edited by:
Julien Villeneuve, Institute for Functional Genomics (IGF), France

Reviewed by:
Robert J. Huber, Trent University, Canada
Alexandre Mezghrani, INSERM U1051 Institut des Neurosciences de Montpellier (INM), France
Mathieu Bourdenx, University College London, United Kingdom

****Correspondence:***
Yihong Ye
yihongy@mail.nih.gov

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *28 March 2022*
Accepted: *25 April 2022*
Published: *10 May 2022*

Citation:
Lee J, Xu Y and Ye Y (2022) Safeguarding Lysosomal Homeostasis by DNAJC5/CSPα-Mediated Unconventional Protein Secretion and Endosomal Microautophagy. Front. Cell Dev. Biol. 10:906453. doi: 10.3389/fcell.2022.906453

Neuronal ceroid lipofuscinosis (NCL) is a collection of genetically inherited neurological disorders characterized by vision loss, seizure, brain death, and premature lethality. At the cellular level, a key pathologic hallmark of NCL is the build-up of autofluorescent storage materials (AFSM) in lysosomes of both neurons and non-neuronal cells. Molecular dissection of the genetic lesions underlying NCLs has shed significant insights into how disruption of lysosomal homeostasis may lead to lipofuscin accumulation and NCLs. Intriguingly, recent studies on DNAJC5/CSPα, a membrane associated HSC70 co-chaperone, have unexpectedly linked lipofuscin accumulation to two intimately coupled protein quality control processes at endolysosomes. This review discusses how deregulation of unconventional protein secretion and endosomal microautophagy (eMI) contributes to lipofuscin accumulation and neurodegeneration.

Keywords: DNAJC5/CSPα, cysteine string protein, ceroid lipofuscinosis neuronal, lysosome, endosomal microautophagy, misfolding-associated protein secretion (MAPS), protein quality control, unconventional protein secretion

INTRODUCTION

Neuronal Ceroid Lipofuscinosis (NCL, also named Batten diseases) refers to a group of genetically inherited lysosomal storage diseases that impact primarily neuronal functions in the central nervous system (Mole and Cotman, 2015). The diseases are rare with incidence rates varying from 1:14,000 to 1:1,000,000 depending on the geographic region (Williams, 2011). The diseases mostly affect infants and juveniles, although adult onset NCLs (ANCL) were recently reported. As expected, the infantile and juvenile forms (INCL and JNCL) are more severe, often associated with vision loss, seizure, dementia, and premature death at young ages (Cotman et al., 2013). By contrast, ANCL has relatively milder symptoms. Nevertheless, ANCL patients usually die within 10 years after diagnosis (Naseri et al., 2021).

At the cellular level, NCL is associated with progressive accumulation of autofluorescent lipopigments (lipofuscin) in both neurons and non-neuronal tissues (Haltia, 2006; Anderson et al., 2013; Naseri et al., 2021). These lipopigments appear to originate from endolysosomes as they often bear proteins of either endosomes or lysosomes. Lipid analysis has identified free fatty acids such as palmitic acid and arachidonic acid as the major lipid component in lipofuscin, which

TABLE 1 | A list of genes associated with various forms of CLN. Please add a reference column.

Human Disease	Gene	Protein	Protein Localization	Protein Function
CLN1	*PPT1* Vesa et al. (1995)	Palmitoyl-protein thioesterase 1	Lysosome	Protein localization regulation Gorenberg et al. (2021)
CLN2	*TPP1* Sleat et al. (1997)	Tripeptidyl-peptidase 1	Lysosome	Lysosomal protease Lin et al. (2001)
CLN3	*CLN3* Mitchison et al. (1997)	Battenin	Endolysosome	Lysosomal acidification Pearce et al. (1999)
CLN4	*DNAJC5* Nosková et al. (2011)	CSPα/DNAJC5	Endolysosome	Co-chaperone Braun et al. (1996)
CLN5	*CLN5* Savukoski et al. (1998)	CLN5	Lysosome	Lysosome to TGN trafficking Mamo et al. (2012)
CLN6	*CLN6* Gao et al. (2002)	CLN6	ER	Cargo trafficking Bajaj et al. (2020)
CLN7	*MFSD8* Siintola et al. (2007)	MFSD8	Lysosome	Transporter Sharifi et al. (2010)
CLN8	*CLN8* Ranta et al. (1999)	CLN8	ER	Cargo trafficking di Ronza et al. (2018)
CLN10	*CTSD* Siintola et al. (2006)	Cathepsin D	Lysosome	Lysosomal protease Cullen et al. (2009)
CLN11	*GRN* Smith et al. (2012)	Granulin	Lysosome	Lysosomal regulation Kao et al. (2017)
CLN12	*ATP13A2* Bras et al., (2012)	ATP13A2	Endolysosome	Polyamine transporter van Veen et al. (2020)
CLN13	*CTSF* Smith et al., (2013)	Cathepsin F	Lysosome	Lysosomal protease Shi et al. (2000)
CLN14	*KCTD7* Staropoli et al., (2012)	KCTD7	Cytosol	Unknown

may result from increased phospholipase activities and/or abnormal membrane trafficking (Bazan et al., 1990).

To date, 13 types of NCLs have been clinically characterized (**Table 1**). While most NCL cases (those known as Batten diseases) are autosomal recessive, an autosomal dominant form of NCL referred to as Kufs disease was recently reported (Naseri et al., 2021). Genetic studies have identified many NCL-associated genetic mutations (Cotman et al., 2013; Specchio et al., 2020; Mole and Cotman, 2015) (**Table 1**). While most of the identified genes are linked to either INCL or JNCL, several ANCL-associated mutations have been found in *DNAJC5*, *CLN5*, *GRN*, and *CTSF* genes (**Table 1**). These genes, designated as CLNs (for ceroid lipofuscinosis neuronal), mostly encode proteins that regulate either lysosome dependent protein processing (e.g. PPT1 and CTSD) (Cotman et al., 2013) or the trafficking of lysosomal resident proteins (e.g. CLN6 and CLN8) (Bajaj et al., 2020; di Ronza et al., 2018). These findings further strengthen the tie between lipofuscin accumulation and endolysosomes, suggesting that neurodegeneration in NCLs may result from a deregulation in endolysosome homeostasis.

Lysosome Homeostasis Regulation

Lysosomes have long been recognized as critical metabolic compartments that break down not only proteins but also lipids, which make them a central hub of cellular homeostasis regulation (Pillay et al., 2002). Lysosomes receive proteins and lipids via both vesicular and non-vesicular trafficking routes. For example, lysosomes can fuse with vesicles originated from either the trans-Golgi network or the plasma membrane. While Golgi-derived vesicles deliver most lysosomal resident proteins, plasma membrane-originated vesicles are responsible for targeting cell surface molecules for lysosomal degradation. Under stress conditions (e.g., amino acid starvation), autophagy, a collection of "self-eating" mechanisms including macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) are activated, which recycle unwanted proteins to re-sculp the cellular proteome. Macroautophagy uses autophagosomes, a double membrane-encircled structure, to degrade cytosolic proteins as well as damaged or unwanted organelles such as endoplasmic reticulum (ER) and mitochondria (Dikic and Elazar, 2018). By contrast, microautophagy and CMA do not involve any vesicle intermediates. Instead, microautophagy moves cytosolic proteins or endosomal membranes into the lumen of late endosomes via inward membrane invagination, while CMA is believed to translocate cargos directly across the lysosomal membrane with the assistance of an oligomerized type I membrane protein named LAMP2A (Tekirdag and Cuervo, 2018; Fleming et al., 2022)

Lysosome Biogenesis and Lysosomal Secretion

Given the essential role of lysosomes in protein homeostasis regulation, eukaryotic cells have adopted a conserved strategy to fine-tune the lysosomal degradation capacity in response to "lysosomal stress" conditions. A central regulator in this process is the transcription factor EB (TFEB), which under normal conditions, is phosphorylated by lysosome-associated kinase mTORC1 (Martina et al., 2012). Phosphorylated TFEB is sequestered in the cytosol in an inactive form due to association with scaffolding proteins of the YWHA (14-3-3) family. Under stress conditions such as amino acid starvation, ER stress etc., mTORC1 is released from lysosomes, causing dephosphorylation of TFEB. Dephosphorylated TFEB is then dissociated from YWHA and translocated into the nucleus to activate genes involved in lysosome biogenesis (Settembre et al., 2011).

Besides lysosome biogenesis, stressed cells can also activate another process termed lysosomal secretion or lysosomal exocytosis. In this process, lysosomes fuse with the plasma membrane to release luminal contents. This mechanism is thought to "purge" lysosomes of undegradable contents, and therefore "rejuvenate" stressed lysosomes. In a multicellular organism like humans, proteins released by lysosomal exocytosis may be internalized and degraded by cells specialized in "garbage-processing" such as macrophages.

Lysosomal secretion was first reported by Gilbert Vaes in 1968. While studying bone resorption, he observed that several acid hydrolases of lysosomes were released into the medium to catalyze bone absorption (Vaes, 1968). This phenomenon was

later confirmed by other studies (Lee and Ye, 2018). In 1972, Miklos Muller showed that the release of hydrolases from *T. pyriformis* was caused by active secretion from what appears to be a special population of "lysosomes", thus for the first time linking lysosomes to a secretory process (Müller, 1972). Subsequent studies showed that upon activation by calcium, cytotoxic T cells and natural killer cells could release cytolytic proteins that had been stored in secretory granules, which shared features of lysosomes as they contained hydrolytic enzymes and lysosomal membrane proteins (Blott and Griffiths, 2002). Subsequent work by Andrews and colleagues showed that lysosomal secretion was tightly regulated in many cell types including fibroblast, myoblast and epithelial cells (Rodríguez et al., 1997; Jaiswal et al., 2002). The precise mechanism underlying lysosomal secretion is unclear. Several studies have implicated a GTP-dependent step involving the ADP-ribosylation factor 1 (ARF1), phospholipase D, and a phosphatidylinositol transfer protein (PITP) in lysosomal secretion (Stutchfield and Cockcroft, 1993; Fensome et al., 1996; Jones et al., 1999), but how these factors act in concert to facilitate lysosomal exocytosis is unknown. Importantly, it remains to be demonstrated whether lysosomal secretion occurs at mature degradation-competent lysosomes or at a pre-lysosomal compartment originated from the Golgi system, which still retains secretory capacity (Borland and Vilhardt, 2017).

Lysosome Repair and Lysophagy

When the integrity of the endolysosomal membrane is damaged, a membrane repairing pathway is activated, which was revealed recently with the application of a lysosomotropic dipeptide, L-leucyl-L-leucine methyl ester (LLOMe) (Thiele and Lipsky, 1990). When cells are treated with LLOMe, it is rapidly internalized into endolysosomes. In this acidic environment, LLOMe is condensed into small crystals that can permeabilize the endolysosomal membrane. This results in the rapid recruitment of endosomal sorting complex required for transport (ESCRT) proteins to endolysosomes (Radulovic et al., 2018; Skowyra et al., 2018). ESCRT complexes (0, I, II, and III) were initially identified as key regulators that control the sorting of endosomal membrane and cytosolic cargos via the so called multivesicular body pathway in *S. cerevisiae*. These complexes act in sequential order to recruit ubiquitinated cargos to the vacuole or lysosome surface, driving the inward budding of membranes to form intralumenal vesicles (Katzmann et al., 2001; Shields et al., 2009). Additional studies have attributed several topologically related functions to ESCRTs including cytokinesis, viral budding, plasma membrane repair (Vietri et al., 2020). In the lysosome repairing pathway, the recruitment of ESCRTs to endolysosomes is triggered by calcium efflux from damaged lysosomes, which activates ALIX, a lipid binding component of the ESCRTs (Skowyra et al., 2018). Recruited ESCRTs may serve as patches to temporarily seal damaged membranes, but permanent removal of the damaged membrane may require the budding of membranes into the lumen of endolysosomes, which is driven by the assembly of the filamentous ESCRT III complex.

When damages to lysosomes are too severe to be repaired, cells use a specialized macroautophagy mechanism termed lysophagy to remove damaged lysosomes (Papadopoulos et al., 2020). Unlike lysosome repair, lysophagy was triggered by the exposure of glycans in certain glycoproteins that normally reside only in the lumen of lysosomes (Jia et al., 2020). Given the size of these proteins and the bulky glycans attached, it is generally assumed that the exposure of these glycans on the surface of lysosomes would require either a full rupture or damages that are big enough to allow the movement of these proteins across the lysosomal membrane. The exposed glycans can be sensed by a group of cytosolic lectins named Galectin, which in turn recruits ubiquitination machinery such as the E3 ubiquitin ligase TRIM16 (Chauhan et al., 2016). Alternatively, exposed glycans may directly recruit certain ubiquitin ligases that have a glycan-binding activity (e.g., FBXO27) (Yoshida et al., 2017). Additionally, a recent study identified UBE2QL1, a ubiquitin conjugating enzyme (E2) as a critical regulator of lysophagy (Koerver et al., 2019). The recruitment of these ubiquitination factors led to massive ubiquitination of proteins on damaged endolysosomes, which then further engage downstream effectors such as the AAA (ATPase associated with diverse cellular activities) ATPase VCP to clear damaged lysosomes.

DNAJC5/CSPα is Membrane-Associated Protein That has a Neuroprotective Function

How can deregulation in lysosome homeostasis cause NCL? The answer to this question is poorly understood, but recent genetic and biochemical studies on a HSC70/HSP70 co-chaperone named DNAJC5/CSPα have provided some important clues.

CSPα (also named as DNAJC5 or CLN4) is a member of the HSP40 co-chaperone family that serves as a cofactor for the major heat shock protein HSC70/HSP70. Like other HSP40 family members, CSPα can stimulate the ATPase activity of HSC70/HSP70 (Braun et al., 1996; Russell et al., 1999). In addition to *DNAJC5/CSPα*, the human genome also contains two other CSPα-related genes, *CSPβ* and *CSPγ*. The encoded proteins share ~80% similarity with CSPα. Because the expression and function of CSPβ and CSPγ appear to be restricted to the testis (Fernández-Chacón et al., 2004; Gorleku and Chamberlain, 2010), we focus our discussions on CSPα in this review.

Human CSPα encodes a 198 amino-acid long polypeptide that contains three conserved domains: an amino-terminal (N) HSC70-binding J-domain, a central cysteine string (CS) domain, and a linker (LN) domain between the J- and the CS domains (Chamberlain and Burgoyne, 2000) (**Figure 1A**). Additionally, CSPα also contains a relatively long C-terminal segment that is predicted to be largely unstructured, and a small N-terminal segment preceding the J-domain (**Figure 1B**). The latter contains several putative phosphorylation sites that may regulate CSPα activities (**Figure 1C**) (see below). The cysteine residues in the CS domain are known to undergo palmitoylation (Greaves and Chamberlain, 2006; Greaves et al., 2008). Several

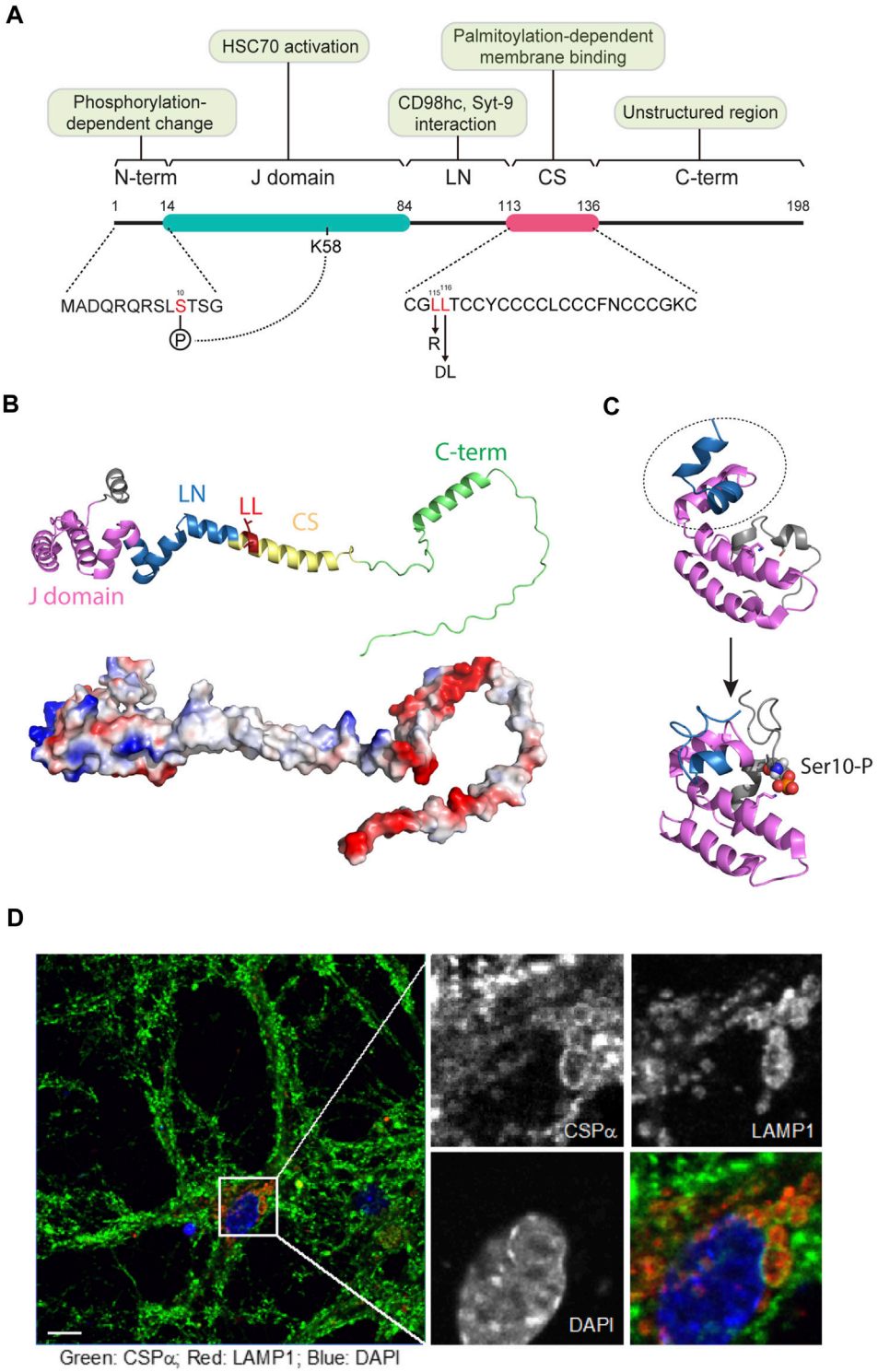


FIGURE 1 | The structure and subcellular localization of CSPα. **(A)** The domain structure of CSPα. CSPα consists of 5 domains, a small N-terminal (N-term.) segment, a DnaJ (J) domain, a hydrophobic linker (LN), a cysteine string (CS) domain, and a disordered C-term domain. Phosphorylation at Ser10 in the N-domain by PKA or PKB may allow CSPα activation by forming an intramolecular interaction between p-Ser10 and Lys58 in the J domain. The conserved J domain is essential for HSC70 interaction and activation. The LN domain can interact with other proteins such as Synaptotagmin-9 and CD98hc, which regulate SNARE complex assembly and MAPS, respectively. The CS domain possesses 14 cysteine residues for palmitoylation, engaging CSPα to membrane compartments. Mutations in two leucine residues (L115R and ΔL116) within the CS domain are linked to ANCL disease. **(B)** Upper panel, A ribbon model of full length human CSPα predicted by Alphafold (Identifier, AF-Q9H3Z4-F1). Each domain is labeled in colors. N-terminal domain, grey; J domain, pink; LN domain, blue; cysteine-string, yellow; C-terminal domain, light

(*Continued*)

FIGURE 1 | green. The ANCL-linked mutations in the CS domain are highlighted in red. Lower panel, a surface electrostatic potential view of the CSPα Alphafold model. **(C)** A phosphorylation dependent conformational change in the CSPα J domain as revealed by NMR. PDB: 2N04 and 2N05. Notice that the subdomain labeled in dashed oval rotates down to pack on the other subdomain labeled in magenta when Ser10 is phosphorylated. **(D)** The subcellular localization of CSPα in primary neurons. Murine primary hippocampal neurons at DIV10 were stained by antibodies for CSPα (green) and the lysosomal marker LAMP1 (red). Note that CSPα in Soma is localized to vesicular structures that overlap with LAMP1. Nuclei were labeled by DAPI in blue. Scale bars, 10 μm.

palmitoyl transferases are capable of palmitoylating CSPα when overexpressed, but DHHC5/HIP14 appears to be the major one responsible for proper membrane localization of endogenous CSPα (Ohyama et al., 2007; Stowers and Isacoff, 2007). CSPα palmitoylation can be reversed by the action of PPT1 (Henderson et al., 2016), a depalmitoylating enzyme encoded by the *CLN1* gene. The N terminal J-domain consists of four α-helices, which are packed into a tightly folded domain. It contains a highly conserved histidine-, proline-, and aspartic acid-containing motif (HPD), which is crucial for the HSC70/HSP70 binding and ATPase-stimulating activities (Jiang et al., 2007).

Two mutations in *DNAJC5* are associated with ANCL (Noskova et al., 2011; Benitez et al., 2011; Cadieux-Dion et al., 2013). These mutations result in either a substitution of Leu115 to Arg (L115R) or the deletion of Leu116 (ΔL116), both of which are located within the CS domain (**Figures 1A,B**). Recent studies suggest that these mutations reduce CSPα palmitoylation while increasing its aggregation propensity (Benitez and Sands, 2017; Diez-Ardanuy et al., 2017; Imler et al., 2019; Naseri et al., 2020). Additionally, these mutations cause the mis-localization of the mutant proteins in cells (Imler et al., 2019). Accordingly, ANCL-associated *DNAJC5* mutations are thought to reduce the CSPα chaperoning function (Naseri et al., 2020).

DNAJC5 is widely expressed in a variety of human tissues (Coppola and Gundersen, 1996). In neurons, CSPα is mainly detected on synaptic vesicles at the presynaptic terminal (Zinsmaier et al., 1990; Ohyama et al., 2007; Tobaben et al., 2001), but a fraction was also seen on lysosomes (**Figure 1D**) (Benitez and Sands, 2017). In non-neuronal cells, CSPα is more prominently localized to late endosomes/lysosomes with a fraction detected in a peri-nuclear compartment and some on the cell surface (Xu et al., 2018; Lee et al., 2022).

Genetic studies in mice and model organisms such as fruit flies have suggested a neuroprotective role for CSPα. *D. Melanogaster* has only one *CSP* gene and its inactivation results in embryonic lethality with a small percent of flies (<5%) surviving to adult stage. These escapers bear a variety of neurological phenotypes including sluggishness, uncoordinated movement, and premature death (Zinsmaier et al., 1990; Burgoyne and Morgan, 2015). Surprisingly, CSPα knockout mice are viable at birth, but these mice usually suffer age-dependent synapse loss and massive neurodegeneration, particularly in the retina. These mice usually die at 8 weeks of age (Fernandez-Chacon et al., 2004; Schmitz et al., 2006; Garcia-Junco-Clemente et al., 2010). Primary neurons isolated from CSPα knockout mice also undergo neurodegeneration *in vitro* (Garcia-Junco-Clemente et al., 2010). These observations have unambiguously established an essential role for CSPα in neuronal development.

MOLECULAR FUNCTIONS OF DNAJC5/CSPA

A Chaperoning Function in Membrane Trafficking

How does inactivation of *DNAJC5/CSP* cause the above-mentioned phenotypes? Early studies in flies suggested that neurodegeneration might be caused by a defect in calcium-elicited neurotransmitter release (Umbach et al., 1994; Zinsmaier, 2010). This finding, together with the reported interaction of CSPα with membrane fusion regulators such as synaptobrevin and synaptotagmin, prompted the idea that CSPα may regulate exocytosis by modulating the stability/activity of these SNARE proteins (Evans and Morgan, 2002; Boal et al., 2004).

Given the well-established role of CSPα as a HSC70/HSP70 co-chaperone, significant efforts were made in search of CSPα substrates. Presumably, substrates should associate with CSPα either directly or indirectly and they should either accumulate in an unfolded state or undergo rapid degradation by a protein quality control mechanism in CSPα deficient cells. Protein binding analyses suggested several candidate substrates including VAMP-1, G-protein subunits, SNAP25, and N-type calcium channels (Chamberlain et al., 2001). Among them, SNAP25 is a synaptic SNARE protein that has been extensively characterized. SNAP25 interacts with CSPα via HSC70 and is subject to ubiquitination and proteasomal degradation in CSPα deficient cells (Chandra et al., 2005; Sharma et al., 2011). Lentivirus-mediated overexpression of SNAP25 rescued neurodegeneration in CSPα deficient animals, confirming it as a mediator of cell death in CSPα null neurons (Sharma et al., 2012). Since SNAP25 is a component of a t-SNARE complex that mediates membrane fusion in exocytosis, its downregulation in CSPα knockout neurons offers a seemingly straightforward explanation for the neurotransmission defect in CSPα deficient animals. However, an alternative explanation was proposed when subsequent studies identified a vesicle recycling defect in CSPα deficient cells, which was attributed to deregulation of another CSPα substrate, the endocytic GTPase Dynamin-1 (Rozas et al., 2012; Zhang et al., 2012). CSPα not only maintains the stability of Dynamin-1 but also promotes its oligomerization during endocytosis. These findings raise the possibility that CSPα may couple exocytosis to endocytosis to ensure efficient synaptic vesicle recycling (Gross and von Gersdorff, 2016). Thus, defects in exocytosis may be secondary due to lack of endocytosis, which leads to a depletion of synaptic vesicles.

Intriguingly, neurodegeneration associated with CSPα depletion can be at least in part rescued by overexpression of

α-synuclein (α-syn) (Chandra et al., 2005), another synaptic vesicle-associated protein well known for its presence in Lewy bodies in Parkinson disease (Spillantini et al., 1997). Moreover, genetic mutations or gene duplication in the α-syn-encoding gene *SCNA* are linked to a familial form of Parkinson disease (Stefanis, 2012). Although α-syn has been subject to extensive study, its physiological function remains poorly understood. The genetic interaction of *SCNA* with *DNAJC5* suggests α-syn as a potential regulator of synaptic exocytosis or vesicle recycling. Since overexpression of α-syn does not rescue the SNAP25 downregulation phenotype in CSPα knockout animals, it may act downstream or in parallel to SNAP25 in membrane trafficking.

Eliminating Misfolded Proteins via MAPS

Protein misfolding imposes a major threat to cell homeostasis because misfolded proteins are not only defective in functions but also prone to aggregation. To cope with protein misfolding-associated proteotoxic stress, eukaryotic cells have evolved a variety of protein quality control (PQC) mechanisms, which include the ubiquitin-proteasome system, macroautophagy, microautophagy and CMA. Many chaperones such as HSC70/HSP70 and members of the HSP40 family play pivotal roles in these processes. Intriguingly, recent studies have underscored an unexpected PQC mechanism that exports misfolded proteins to the cell exterior by CSPα-assisted unconventional protein secretion (Fontaine et al., 2016; Xu et al., 2018; Lee et al., 2022; Wu et al., 2022).

Unconventional protein secretion refers to a collection of protein trafficking mechanisms that either export proteins lacking an endoplasmic reticulum (ER)-targeting signal sequence or transport proteins from the ER to the cell surface independent of the Golgi system (Nickel and Rabouille, 2009; Malhotra, 2013; Zhang and Schekman, 2013). To date, only a handful of unconventional secretion substrates have been characterized, which include FGF2, IL1β, α-syn, and Tau etc. but the list of unconventional secretion substrates is rapidly expanding. Unconventional secretion cargos can use a vesicle intermediate to reach the cell exterior (Rabouille, 2017), or in the case of FGF2 and Tau, direct translocation across the plasma membrane has been reported (Steringer et al., 2017; Katsinelos et al., 2018; Merezhko et al., 2018). Although many unconventional secretion cargos characterized to date are released in a native form to exert their functions in the extracellular environment, our recent work suggested that higher eukaryotic cells can also release misfolded cytosolic proteins via a secretion mechanism termed as misfolding-associated protein secretion (MAPS) (Lee et al., 2016).

MAPS was discovered serendipitously while we characterized an ER-associated deubiquitinase named USP19, which also harbors a chaperone activity and a C-terminal transmembrane domain (Lee et al., 2016). Biochemical study showed that USP19 binds to two major heat shock proteins HSC70 and HSP90 in cells, suggesting a possible role in PQC (Lee et al., 2014). Although the localization of USP19 to the ER suggested a possible function in ER-associated protein degradation (ERAD), this model has not been conclusively established. Instead, we found that USP19 overexpression promoted the release of certain cytosolic proteins while its inactivation inhibited unconventional protein secretion in mammalian cells. In this regard, USP19 preferentially promotes the secretion of misfolded proteins such as engineered mutant proteins, unassembled protein subunits, and some wild-type proteins that are prone to misfolding such as Tau and α-syn, which are known contributors to Alzheimer and Parkinson diseases, respectively. Many MAPS substrates are also subject to degradation by the ubiquitin-proteasome system. Thus, it appears that MAPS may act as a supplementary protein quality control mechanism to enhance the clearance of misfolded proteins. Consistent with this model, USP19 deficient cells are more sensitive to proteasome inhibitor-induced cytotoxicity (Lee et al., 2016).

Many neurodegenerative disease-associated MAPS substrates are also known to bind to HSC70 and/or CSPα. Consistent with this finding, Fontaine et al. showed that CSPα could act together with HSC70 to promote the release of Tau, TDP-13, and α-syn from both non-neuronal cells and neurons (Fontaine et al., 2016). These disease-associated misfolded proteins were released largely in a free form, not associated with any extracellular vesicles (Lee et al., 2016). A subsequent study showed that both CSPα and HSC70 functioned downstream of USP19 to promote MAPS as knockdown of CSPα or HSC70 inhibited USP19-induced protein secretion (Xu et al., 2018).

How do cells secrete misfolded cytosolic proteins lacking a signal sequence? Several lines of evidence suggest that MAPS substrates probably use one or more vesicle carriers as a secretory intermediate compartment, and it is possible that for a given substrate like Tau, multiple secretion routes are involved. Several types of vesicles, endolysosomes in particular, have been suggested to function in unconventional secretion given the previously documented lysosomal exocytosis (see above) (Lee and Ye, 2018). In *S. cerevisiae*, a Golgi-derived membrane compartment termed CUPS (Compartment for Unconventional Protein Secretion) was reported as a major mediator for nitrogen starvation-induced unconventional protein secretion (Malhotra, 2013). Our recent work suggested a peri-nuclear membrane compartment in proximity to the Golgi system as a CUPS equivalent compartment in mammalian cells (**Figure 2A**). A fraction of CSPα is localized to this compartment, which is regulated by SLC3A2/CD58hc, a common adaptor for several amino acid transporters (Lee et al., 2022). The peri-nuclear CSPα appears to retrieve misfolded cargos from ER-localized USP19 and accompany them to the CUPS for secretion (Xu et al., 2018). As expected, the CS domain essential for palmitoylation is crucial for localizing CSPα to the peri-nuclear compartment and for MAPS (Xu et al., 2018; Lee et al., 2022). Importantly, CS-mediated palmitoylation appears to drive CSPα into a large oligomeric assembly, which stimulates protein secretion (Wu et al., 2022). It is noteworthy that neither USP19 nor CSPα is absolutely essential for MAPS because knockout of either of these genes only led to a partial defect in MAPS (Xu et al., 2018), suggesting functional redundancy with other membrane-associated chaperones or the existence of parallel secretion mechanisms.

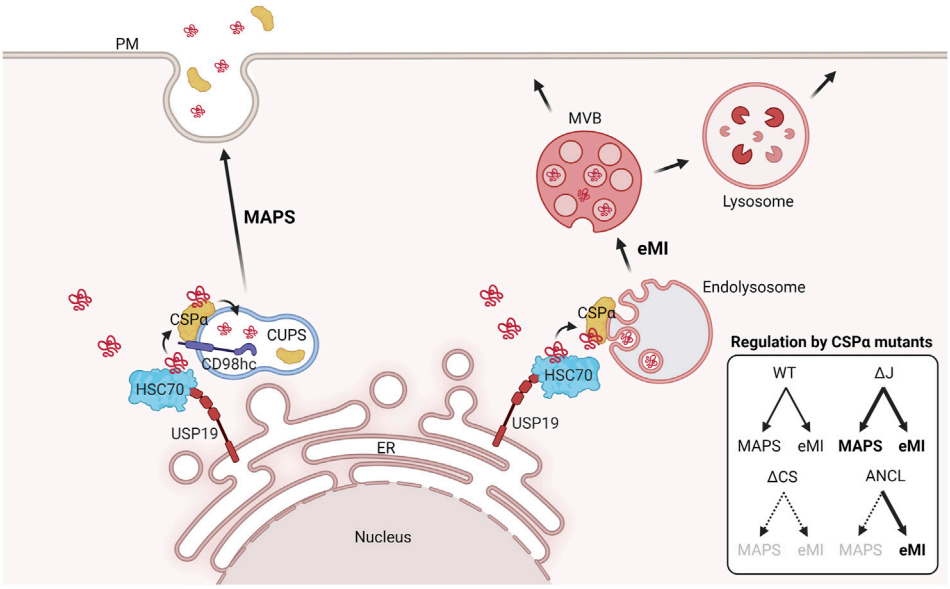


FIGURE 2 | CSPα couples MAPS to eMI to promote lysosome homeostasis. In mammalian cells, CSPα triages cytosolic misfolded proteins by two different mechanisms: misfolding-associated protein secretion and eMI-mediated degradation. In both cases, an ER-associated deubiquitinase USP19 recruits and deubiquitinates misfolded substrates at the ER. For MAPS, CSPα and HSC70 guide the cargos to a peri-nuclear secretory compartment for unconventional protein secretion (CUPS). CD98hc is required for targeting of CSPα/substrate complexes to this compartment. After CSPα chaperones substrates to the lumen of CUPS, the encapsulated MAPS cargos and CSPα are secreted possibly by vesicular trafficking between the CUPS and plasma membrane (PM). For eMI, CSPα also escorts misfolded proteins into endolysosomes by an ESCRT-dependent mechanism. The resulting multivesicular bodies containing misfolded cargos can be degraded or secreted after the fusion of MVBs with lysosomes or plasma membrane. The box indicates how these two processes can be differentially regulated by different CSPα mutant proteins. The figure was created by BioRender.

How to translocate cargoes into the lumen of the CUPS is currently a major open question. A recent study using the unconventional secretion cargo IL1β as a bait identified a membrane protein named TMED10, which appeared to mediate protein translocation across the membrane in unconventional protein secretion (Zhang et al., 2020). TMED10 is a single-spanning membrane protein localized to the ERGIC (ER and Golgi intermediate compartment). It belongs to the EMP24/GP25L/p24 cargo receptor family, which is generally involved in ER to Golgi trafficking (Strating and Martens, 2009). Knockout of TMED10 reduced not only IL1β secretion but also the release of many other unconventional secretion cargos. *In vitro* reconstitution experiments suggested that TMED10 might facilitate cargo translocation into the lumen of a secretory compartment by binding to a consensus motif in cargos (Zhang et al., 2020). However, whether TMED10 forms a protein-conducting channel or uses other means to promote unconventional protein secretion remains to be elucidated.

Protein Quality Control by Endosomal Microautophagy

Endosomal microautophagy (eMI) refers to a special form of autophagy in which late endosomes or lysosomes take up cytoplasmic materials by membrane invagination and pinching off, forming multivesicular bodies (MVBs) (also called intralumenal vesicles) (Marzella et al., 1981; Oku and Sakai, 2018). This process is conserved from *S. cerevisiae* to humans, involving several ESCRT machinery proteins (Zhang et al., 2021). Because MVB formation is coupled to the engulfment of a portion of the cytosol into late endosomes, which is then degraded together with the invaginated membranes by lysosomes, eMI-mediated protein and membrane turnover appears to be largely non-selective. However, recent studies have revealed several types of selective eMI in yeast, fruit flies and mammalian cells (Sahu et al., 2011; Mukherjee et al., 2016; Mejlvang et al., 2018; Lee et al., 2020; Lee et al., 2022).

Selective eMI was initially suggested when eMI cargos were found to undergo ubiquitination in yeast (Katzmann et al., 2001). Subsequent studies identified several ubiquitin binding motifs in ESCRT complexes (Shields et al., 2009), which function in cargo selection and recruitment (MacDonald et al., 2012). Selective eMI was later confirmed in mammalian cells (Sahu et al., 2011). Using an *in vitro* reconstitution system, Sahu and colleagues demonstrated that cytosolic proteins bearing a KFERQ-containing motif could be directly translocated into late endosomes in a LAMP2A independent but HSC70-, KFERQ-, and ESCRT-dependent manner. Further analyses suggested that HSC70 binds to eMI substrates and then uses a cationic domain to associate with endosomal membrane phosphatidylserines, linking substrates to late endosomes (Morozova et al., 2016; Uytterhoeven et al., 2015). Interestingly, the KFERQ-motif has also been known to direct proteins to the CMA pathway. A recent study on Tau suggests that this misfolding-prone protein is constitutively degraded by CMA because of multiple KFERQ-like motifs. However, upon acetylation, Tau is rerouted to eMI for

degradation or release by exosomal secretion (Caballero et al., 2021). In addition to KFERQ-dependent eMI, our recent study showed that CSPα also participated in selective eMI (**Figure 2B**) (Lee et al., 2022). In both neuron and non-neuronal cells, a fraction of CSPα is tightly associated with endolysosomal membranes. Intriguingly, despite the lack of the KFERQ motif, endolysosome-associated CSPα can efficiently enter into multivesicular bodies together with bound cargos (Lee et al., 2018). As expected, this process involves the ESCRT machinery, but surprisingly, is independent of the J domain of CSPα (Lee et al., 2022). How CSPα recruits substrates to endolysosomes and how it cooperates with HSC70 in eMI remain to be determined. Additionally, the role of CSPα palmitoylation in eMI also needs to be better defined.

Regulation of MAPS and eMI

In general, the ubiquitin-proteasome system and macroautophagy degrade substrates quite efficiently. By contrast, MAPS appears to operate only at low capacity under normal conditions because both USP19 and CSPα contain an autoinhibitory domain that restricts their activities in this process. The autoinhibitory domain of USP19 is a UBL (ubiquitin like)-containing domain inserted in the middle of the USP (ubiquitin specific protease) domain (Xu et al., 2018). For CSPα, the autoinhibitory domain is the HSC70-binding J-domain (Lee et al., 2022). When these domains are removed, the resulting truncated proteins are significantly more activated than the wild-type counterpart in MAPS. These autoinhibitory mechanisms appear to be applicable to eMI as the J-domain deleted CSPα mutant is more efficiently translocated into endolysosomes than wild-type CSPα (Lee et al., 2022). These observations raise the possibility that these proteins may be activated under stress conditions to promote substrate flow to eMI. Consistent with this notion, eMI is indeed upregulated under the conditions of nutrient starvation, DNA damage, and oxidative stress, although whether this is achieved via activating USP19 or CSPα remains to be established (Mukherjee et al., 2016; Lee et al., 2020; Mesquita et al., 2020).

Thus, understanding the regulatory mechanism of USP19 and CSPα may provide some clues on when and how MAPS and CSPα-dependent eMI are activated. Due to limited structural information, the regulation of USP19 is poorly understood. However, our proteomic study identified HSC70 and HSP90 as two major binding partners of USP19 (Lee et al., 2014). We further showed that HSC70 but not HSP90 was required for USP19-mediated MAPS (Xu et al., 2018). These findings corroborate the idea that MAPS might be regulated by proteotoxic stress, a notion further supported by the finding that the secretion of misfolded proteins is generally upregulated in cells treated with proteasome inhibitors (Lee et al., 2016; Lee et al., 2005). For CSPα, NMR studies suggested that the J-domain, when phosphorylated at Ser10, was packed into a globular domain, but dephosphorylation disrupted the interdomain interaction (**Figure 1C**), resulting in a conformational change that may be essential for the function of CSPα (Patel et al., 2016).

Intriguingly, the MAPS and eMI pathways appear to be tightly coupled as conditions that increase eMI often stimulate MAPS as well. Therefore, for a long time, it was assumed that misfolded proteins might use endolysosomes as a secretory intermediate compartment in MAPS. However, several lines of evidence now suggests that these two processes are parallel mechanisms coupled by CSPα. First, while the J domain-deleted CSPα mutant has a much-increased activity in promoting α-syn secretion, it only modestly promotes the translocation of α-syn into endolysosomes. More importantly, a dominant negative VPS4 mutant that disrupts the function of the ESCRT III complex in eMI can increase the secretion of several MAPS substrates although it completely blocks the endosomal translocation of these proteins (Lee et al., 2022).

DNAJC5/CSPα Dysfunction in ANCL

Although loss of CSPα function in animals causes neurodegeneration, the ANCL-associated CSPα mutations do not seem to act as a loss-of-function allele because lipofuscin accumulation, albeit being readily observed in cells overexpressing CSPα L115R or ΔL116 mutants (Naseri et al., 2020; Lee et al., 2022), has not been reported in CSPα deficient cells (Schmitz et al., 2006).

How do mutations in CSPα cause lipofuscin accumulation and neurodegeneration? Our recent study suggests that lipofuscin accumulation may be caused by abnormal membrane flow due to an imbalance between unconventional protein secretion and eMI (Lee et al., 2022). The fact that CSPα activation stimulates both MAPS and eMI suggests a necessity to couple these two quality control pathways, which conceivably may prevent the overflow of misfolded proteins and membranes into endolysosomes and thus inhibit lipofuscin biogenesis. Several lines of evidence indicate that inhibiting MAPS while maintaining eMI is sufficient to induce lipofuscin accumulation (**Figure 2**). First, both L115R and ΔL116 CSPα mutants are defective in MAPS (Lee et al., 2022; Wu et al., 2022). However, these mutants are capable of translocating into endolysosomes via eMI. Likewise, a CSP mutant lacking the linker domain is also defective in MAPS, but active in eMI, and overexpression of this mutant induces lipofuscin accumulation similarly as the disease-associated mutants (Lee et al., 2022). Finally, knockout of SLC3A2/CD98hc inhibits MAPS but does not significantly affect eMI. Intriguingly, a significant fraction of CD98hc deficient cells contain a single giant 'lysobody', which is a sphere-shaped autofluorescent organelle. This organelle is wrapped around by CSPα and late endosomal proteins such as LAMP1 and Rab9, indicating endolysosomes as its precursor. Given that many CLN-associated mutations known to date are recessive loss-of-function alleles that cause a deficiency in lysosomal degradation, our study underscores a special class of CLN mutations that cause abnormal flow of membranes and misfolded proteins into endolysosomes, which dominantly disrupts lysosomal function. Our model is also

consistent with recent studies implicating several other CLN proteins in unconventional protein secretion (Huber, 2021).

CONCLUSION

The implication of CSPα in eMI and unconventional protein secretion has significantly expanded the functional repertoire of CSPα, which establishes it as a key protein quality control regulator. These new findings, while providing new insights on the pathogenic mechanisms underlying NCL, also raise many questions pertaining to the role of endolysosomal trafficking in lipofuscin biogenesis. Most importantly, it would be important to gather more evidence to support the hypothesis that abnormal MAPS and eMI are a key contributor to neuronal lipofuscinosis and neuronal cell death. Given the specific lipid composition of the lipofuscin, it would be important to determine whether MVB formation in eMI has specific lipid requirement or involves specific lipases, which may lead to increased deposit of certain lipids in endolysosomes when this pathway is deregulated. Additionally, a thorough understanding of the physiological relevance of eMI requires a better characterization of the cellular mechanisms that activate eMI, particularly regarding how CSPα is regulated and what physiological eMI substrates are.

AUTHOR CONTRIBUTIONS

JL, YX, and YY designed and conceptualized the research; JL and YY wrote the original draft; JL and YX prepared the figures; JL, YX, and YY reviewed, edited and proofed the manuscript.

FUNDING

The work in the YY laboratory is supported by the intramural research program of the National Institute of Diabetes, Digestive, and Kidney Diseases in the National Institutes of Health.

REFERENCES

Anderson, G. W., Goebel, H. H., and Simonati, A. (2013). Human Pathology in NCL. *Biochimica Biophysica Acta (BBA) - Mol. Basis Dis.* 1832, 1807–1826. doi:10.1016/j.bbadis.2012.11.014

Bajaj, L., Sharma, J., Di Ronza, A., Zhang, P., Eblimit, A., Pal, R., et al. (2020). A CLN6-CLN8 Complex Recruits Lysosomal Enzymes at the ER for Golgi Transfer. *J. Clin. Invest.* 130, 4118–4132. doi:10.1172/JCI130955

Bazan, H. E., Bazan, N. G., Feeney-Burns, L., and Berman, E. R. (1990). Lipids in Human Lipofuscin-Enriched Subcellular Fractions of Two Age Populations. Comparison with Rod Outer Segments and Neural Retina. *Invest. Ophthalmol. Vis. Sci.* 31, 1433

Benitez, B. A., Alvarado, D., Cai, Y., Mayo, K., Chakraverty, S., Norton, J., et al. (2011). Exome-sequencing Confirms DNAJC5 Mutations as Cause of Adult Neuronal Ceroid-Lipofuscinosis. *PLoS One* 6, e26741. doi:10.1371/journal.pone.0026741

Benitez, B. A., and Sands, M. S. (2017). Primary Fibroblasts from CSPα Mutation Carriers Recapitulate Hallmarks of the Adult Onset Neuronal Ceroid Lipofuscinosis. *Sci. Rep.* 7, 6332. doi:10.1038/s41598-017-06710-1

Blott, E. J., and Griffiths, G. M. (2002). Secretory Lysosomes. *Nat. Rev. Mol. Cell Biol.* 3, 122–131. doi:10.1038/nrm732

Boal, F., Zhang, H., Tessier, C., Scotti, P., and Lang, J. (2004). The Variable C-Terminus of Cysteine String Proteins Modulates Exocytosis and Protein–Protein Interactions. *Biochemistry* 43, 16212–16223. doi:10.1021/bi048612+

Borland, H., and Vilhardt, F. (2017). Prelysosomal Compartments in the Unconventional Secretion of Amyloidogenic Seeds. *Ijms* 18, 227. doi:10.3390/ijms18010227

Bras, J., Verloes, A., Schneider, S. A., Mole, S. E., and Guerreiro, R. J. (2012). Mutation of the Parkinsonism Gene ATP13A2 Causes Neuronal Ceroid-Lipofuscinosis. *Hum. Mol. Genet.* 21, 2646–2650. doi:10.1093/hmg/dds089

Braun, J. E. A., Wilbanks, S. M., and Scheller, R. H. (1996). The Cysteine String Secretory Vesicle Protein Activates Hsc70 ATPase. *J. Biol. Chem.* 271, 25989–25993. doi:10.1074/jbc.271.42.25989

Burgoyne, R. D., and Morgan, A. (2015). Cysteine String Protein (CSP) and its Role in Preventing Neurodegeneration. *Seminars Cell & Dev. Biol.* 40, 153–159. doi:10.1016/j.semcdb.2015.03.008

Caballero, B., Bourdenx, M., Luengo, E., Diaz, A., Sohn, P. D., Chen, X., et al. (2021). Acetylated Tau Inhibits Chaperone-Mediated Autophagy and Promotes Tau Pathology Propagation in Mice. *Nat. Commun.* 12, 2238. doi:10.1038/s41467-021-22501-9

Cadieux-Dion, M., Andermann, E., Lachance-Touchette, P., Ansorge, O., Meloche, C., Barnabé, A., et al. (2013). Recurrent Mutations inDNAJC5cause Autosomal Dominant Kufs Disease. *Clin. Genet.* 83, 571–575. doi:10.1111/cge.12020

Chamberlain, L. H., and Burgoyne, R. D. (2000). Cysteine-String Protein: The Chaperone at the Synapse. *J. Neurochem.* 74, 1781–1789. doi:10.1046/j.1471-4159.2000.0741781.x

Chamberlain, L. H., Graham, M. E., Kane, S., Jackson, J. L., Maier, V. H., Burgoyne, R. D., et al. (2001). The Synaptic Vesicle Protein, Cysteine-String Protein, Is Associated with the Plasma Membrane in 3T3-L1 Adipocytes and Interacts with Syntaxin 4. *J. Cell Sci.* 114, 445–455. doi:10.1242/jcs.114.2.445

Chandra, S., Gallardo, G., Fernández-Chacón, R., Schlüter, O. M., and Südhof, T. C. (2005). α-Synuclein Cooperates with CSPα in Preventing Neurodegeneration. *Cell* 123, 383–396. doi:10.1016/j.cell.2005.09.028

Chauhan, S., Kumar, S., Jain, A., Ponpuak, M., Mudd, M. H., Kimura, T., et al. (2016). TRIMs and Galectins Globally Cooperate and TRIM16 and Galectin-3 Co-direct Autophagy in Endomembrane Damage Homeostasis. *Dev. Cell* 39, 13–27. doi:10.1016/j.devcel.2016.08.003

Coppola, T., and Gundersen, C. (1996). Widespread Expression of Human Cysteine String Proteins. *FEBS Lett.* 391, 269–272. doi:10.1016/0014-5793(96)00750-8

Cotman, S. L., Karaa, A., Staropoli, J. F., and Sims, K. B. (2013). Neuronal Ceroid Lipofuscinosis: Impact of Recent Genetic Advances and Expansion of the Clinicopathologic Spectrum. *Curr. Neurol. Neurosci. Rep.* 13, 366. doi:10.1007/s11910-013-0366-z

Cullen, V., Lindfors, M., Ng, J., Paetau, A., Swinton, E., Kolodziej, P., et al. (2009). Cathepsin D Expression Level Affects Alpha-Synuclein Processing, Aggregation, and Toxicity *In Vivo*. *Mol. Brain* 2, 5. doi:10.1186/1756-6606-2-5

Di Ronza, A., Bajaj, L., Sharma, J., Sanagasetti, D., Lotfi, P., Adamski, C. J., et al. (2018). CLN8 Is an Endoplasmic Reticulum Cargo Receptor that Regulates Lysosome Biogenesis. *Nat. Cell Biol.* 20, 1370–1377. doi:10.1038/s41556-018-0228-7

Diez-Ardanuy, C., Greaves, J., Munro, K. R., Tomkinson, N. C. O., and Chamberlain, L. H. (2017). A Cluster of Palmitoylated Cysteines Are Essential for Aggregation of Cysteine-String Protein Mutants that Cause Neuronal Ceroid Lipofuscinosis. *Sci. Rep.* 7, 10. doi:10.1038/s41598-017-00036-8

Dikic, I., and Elazar, Z. (2018). Mechanism and Medical Implications of Mammalian Autophagy. *Nat. Rev. Mol. Cell Biol.* 19, 349–364. doi:10.1038/s41580-018-0003-4

Evans, G. J. O., and Morgan, A. (2002). Phosphorylation-dependent Interaction of the Synaptic Vesicle Proteins Cysteine String Protein and Synaptotagmin I. *Biochem. J.* 364, 343–347. doi:10.1042/BJ20020123

Fensome, A., Cunningham, E., Prosser, S., Khoon Tan, S., Swigart, P., Thomas, G., et al. (1996). ARF and PITP Restore GTPγS-Stimulated Protein Secretion from Cytosol-Depleted HL60 Cells by Promoting PIP2 Synthesis. *Curr. Biol.* 6, 730–738. doi:10.1016/s0960-9822(09)00454-0

Fernández-Chacón, R., Wölfel, M., Nishimune, H., Tabares, L., Schmitz, F., Castellano-Muñoz, M., et al. (2004). The Synaptic Vesicle Protein CSPα Prevents Presynaptic Degeneration. *Neuron* 42, 237–251. doi:10.1016/s0896-6273(04)00190-4

Fleming, A., Bourdenx, M., Fujimaki, M., Karabiyik, C., Krause, G. J., Lopez, A., et al. (2022). The Different Autophagy Degradation Pathways and Neurodegeneration. *Neuron* 110, 935–966. doi:10.1016/j.neuron.2022.01.017

Fontaine, S. N., Zheng, D., Sabbagh, J. J., Martin, M. D., Chaput, D., Darling, A., et al. (2016). DnaJ/Hsc70 Chaperone Complexes Control the Extracellular Release of Neurodegenerative-associated Proteins. *EMBO J.* 35, 1537–1549. doi:10.15252/embj.201593489

Gao, H., Boustany, R.-M. N., Espinola, J. A., Cotman, S. L., Srinidhi, L., Antonellis, K. A., et al. (2002). Mutations in a Novel CLN6-Encoded Transmembrane Protein Cause Variant Neuronal Ceroid Lipofuscinosis in Man and Mouse. *Am. J. Hum. Genet.* 70, 324–335. doi:10.1086/338190

Garcia-Junco-Clemente, P., Cantero, G., Gomez-Sanchez, L., Linares-Clemente, P., Martinez-Lopez, J. A., Lujan, R., et al. (2010). Cysteine String Protein- Prevents Activity-dependent Degeneration in GABAergic Synapses. *J. Neurosci.* 30, 7377–7391. doi:10.1523/JNEUROSCI.0924-10.2010

Gorenberg, E. L., Tieze, S. M., Yücel, B., Zhao, H. R., Chou, V., Wirak, G. S., et al. (2021). Identification of Synaptic PPT1 Substrates Highlight Roles of Depalmitoylation in Disulfide Bond Formation and Synaptic Function. *bioRxiv* 2020, 074302. doi:10.1101/2020.05.02.074302

Gorleku, O. A., and Chamberlain, L. H. (2010). Palmitoylation and Testis-Enriched Expression of the Cysteine-String Protein β Isoform. *Biochemistry* 49, 5308–5313. doi:10.1021/bi100550h

Greaves, J., and Chamberlain, L. H. (2006). Dual Role of the Cysteine-String Domain in Membrane Binding and Palmitoylation-dependent Sorting of the Molecular Chaperone Cysteine-String Protein. *MBoC* 17, 4748–4759. doi:10.1091/mbc.e06-03-0183

Greaves, J., Salaun, C., Fukata, Y., Fukata, M., and Chamberlain, L. H. (2008). Palmitoylation and Membrane Interactions of the Neuroprotective Chaperone Cysteine-String Protein. *J. Biol. Chem.* 283, 25014–25026. doi:10.1074/jbc.M802140200

Gross, O. P., and Von Gersdorff, H. (2016). Recycling at Synapses. *Elife* 5, 17692. doi:10.7554/eLife.17692

Haltia, M. (2006). The Neuronal Ceroid-Lipofuscinoses: from Past to Present. *Biochimica Biophysica Acta (BBA) - Mol. Basis Dis.* 1762, 850–856. doi:10.1016/j.bbadis.2006.06.010

Henderson, M. X., Wirak, G. S., Zhang, Y.-q., Dai, F., Ginsberg, S. D., Dolzhanskaya, N., et al. (2016). Neuronal Ceroid Lipofuscinosis with DNAJC5/CSPα Mutation Has PPT1 Pathology and Exhibit Aberrant Protein Palmitoylation. *Acta Neuropathol.* 131, 621–637. doi:10.1007/s00401-015-1512-2

Huber, R. J. (2021). Altered Protein Secretion in Batten Disease. *Dis. Model Mech.* 14, 49152. doi:10.1242/dmm.049152

Imler, E., Pyon, J. S., Kindelay, S., Torvund, M., Zhang, Y.-q., Chandra, S. S., et al. (2019). A Drosophila Model of Neuronal Ceroid Lipofuscinosis CLN4 Reveals a Hypermorphic Gain of Function Mechanism. *Elife* 8, 46607. doi:10.7554/eLife.46607

Jaiswal, J. K., Andrews, N. W., and Simon, S. M. (2002). Membrane Proximal Lysosomes Are the Major Vesicles Responsible for Calcium-dependent Exocytosis in Nonsecretory Cells. *J. Cell Biol.* 159, 625–635. doi:10.1083/jcb.200208154

Jia, J., Claude-Taupin, A., Gu, Y., Choi, S. W., Peters, R., Bissa, B., et al. (2020). Galectin-3 Coordinates a Cellular System for Lysosomal Repair and Removal. *Dev. Cell* 52, 69–87. doi:10.1016/j.devcel.2019.10.025

Jiang, J., Maes, E. G., Taylor, A. B., Wang, L., Hinck, A. P., Lafer, E. M., et al. (2007). Structural Basis of J Cochaperone Binding and Regulation of Hsp70. *Mol. Cell* 28, 422–433. doi:10.1016/j.molcel.2007.08.022

Jones, D. H., Bax, B., Fensome, A., and Cockcroft, S. (1999). ADP Ribosylation Factor 1 Mutants Identify a Phospholipase D Effector Region and Reveal that Phospholipase D Participates in Lysosomal Secretion but Is Not Sufficient for Recruitment of Coatomer I. *Biochem. J.* 341 (Pt 1) (Pt 1), 185–192. doi:10.1042/bj3410185

Kao, A. W., Mckay, A., Singh, P. P., Brunet, A., and Huang, E. J. (2017). Progranulin, Lysosomal Regulation and Neurodegenerative Disease. *Nat. Rev. Neurosci.* 18, 325–333. doi:10.1038/nrn.2017.36

Katsinelos, T., Zeitler, M., Dimou, E., Karakatsani, A., Müller, H.-M., Nachman, E., et al. (2018). Unconventional Secretion Mediates the Trans-cellular Spreading of Tau. *Cell Rep.* 23, 2039–2055. doi:10.1016/j.celrep.2018.04.056

Katzmann, D. J., Babst, M., and Emr, S. D. (2001). Ubiquitin-dependent Sorting into the Multivesicular Body Pathway Requires the Function of a Conserved Endosomal Protein Sorting Complex, ESCRT-I. *Cell* 106, 145–155. doi:10.1016/s0092-8674(01)00434-2

Koerver, L., Papadopoulos, C., Liu, B., Kravic, B., Rota, G., Brecht, L., et al. (2019). The Ubiquitin-conjugating Enzyme UBE 2 QL 1 Coordinates Lysophagy in Response to Endolysosomal Damage. *EMBO Rep.* 20, e48014. doi:10.15252/embr.201948014

Lee, C., Lamech, L., Johns, E., and Overholtzer, M. (2020). Selective Lysosome Membrane Turnover Is Induced by Nutrient Starvation. *Dev. Cell* 55, 289–297. doi:10.1016/j.devcel.2020.08.008

Lee, H.-J., Patel, S., and Lee, S. J. (2005). Intravesicular Localization and Exocytosis of -Synuclein and its Aggregates. *J. Neurosci.* 25, 6016–6024. doi:10.1523/JNEUROSCI.0692-05.2005

Lee, J.-G., Kim, W., Gygi, S., and Ye, Y. (2014). Characterization of the Deubiquitinating Activity of USP19 and its Role in Endoplasmic Reticulum-Associated Degradation. *J. Biol. Chem.* 289, 3510–3517. doi:10.1074/jbc.M113.538934

Lee, J.-G., Takahama, S., Zhang, G., Tomarev, S. I., and Ye, Y. (2016). Unconventional Secretion of Misfolded Proteins Promotes Adaptation to Proteasome Dysfunction in Mammalian Cells. *Nat. Cell Biol.* 18, 765–776. doi:10.1038/ncb3372

Lee, J., Xu, Y., Saidi, L., Xu, M., Zinsmaier, K., and Ye, Y. (2022). Abnormal Triaging of Misfolded Proteins by Adult Neuronal Ceroid Lipofuscinosis-Associated DNAJC5/CSPα Mutants Causes Lipofuscin Accumulation. *Autophagy*. doi:10.1080/15548627.2022.2065618

Lee, J., Xu, Y., Zhang, T., Cui, L., Saidi, L., and Ye, Y. (2018). Secretion of Misfolded Cytosolic Proteins from Mammalian Cells Is Independent of Chaperone-Mediated Autophagy. *J. Biol. Chem.* 293, 14359–14370. doi:10.1074/jbc.RA118.003660

Lee, J., and Ye, Y. (2018). The Roles of Endo-Lysosomes in Unconventional Protein Secretion. *Cells* 7, 198. doi:10.3390/cells7110198

Lin, L., Sohar, I., Lackland, H., and Lobel, P. (2001). The Human CLN2 Protein/tripeptidyl-Peptidase I Is a Serine Protease that Autoactivates at Acidic pH. *J. Biol. Chem.* 276, 2249–2255. doi:10.1074/jbc.M008562200

Macdonald, C., Buchkovich, N. J., Stringer, D. K., Emr, S. D., and Piper, R. C. (2012). Cargo Ubiquitination Is Essential for Multivesicular Body Intralumenal Vesicle Formation. *EMBO Rep.* 13, 331–338. doi:10.1038/embor.2012.18

Malhotra, V. (2013). Unconventional Protein Secretion: an Evolving Mechanism. *EMBO J.* 32, 1660–1664. doi:10.1038/emboj.2013.104

Mamo, A., Jules, F., Dumaresq-Doiron, K., Costantino, S., and Lefrancois, S. (2012). The Role of Ceroid Lipofuscinosis Neuronal Protein 5 (CLN5) in Endosomal Sorting. *Mol. Cell Biol.* 32, 1855–1866. doi:10.1128/mcb.06726-11

Martina, J. A., Chen, Y., Gucek, M., and Puertollano, R. (2012). MTORC1 Functions as a Transcriptional Regulator of Autophagy by Preventing Nuclear Transport of TFEB. *Autophagy* 8, 903–914. doi:10.4161/auto.19653

Marzella, L., Ahlberg, J., and Glaumann, H. (1981). Autophagy, Heterophagy, Microautophagy and Crinophagy as the Means for Intracellular Degradation. *Virchows Arch. B Cell Pathol.* 36, 219–234. doi:10.1007/BF02912068

Mejlvang, J., Olsvik, H., Svenning, S., Bruun, J.-A., Abudu, Y. P., Larsen, K. B., et al. (2018). Starvation Induces Rapid Degradation of Selective Autophagy Receptors by Endosomal Microautophagy. *J. Cell Biol.* 217, 3640–3655. doi:10.1083/jcb.201711002

Merezhko, M., Brunello, C. A., Yan, X., Vihinen, H., Jokitalo, E., Uronen, R.-L., et al. (2018). Secretion of Tau via an Unconventional Non-vesicular Mechanism. *Cell Rep.* 25, 2027–2035. e4. doi:10.1016/j.celrep.2018.10.078

Mesquita, A., Glenn, J., and Jenny, A. (2020). Differential Activation of eMI by Distinct Forms of Cellular Stress. *Autophagy* 17, 1828–1840. doi:10.1080/15548627.2020.1783833

Mitchison, H. M., Munroe, P. B., O'Rawe, A. M., Taschner, P. E. M., De Vos, N., Kremmidiotis, G., et al. (1997). Genomic Structure and Complete Nucleotide Sequence of the Batten Disease Gene,CLN3. *Genomics* 40, 346–350. doi:10.1006/geno.1996.4576

Mole, S. E., and Cotman, S. L. (2015). Genetics of the Neuronal Ceroid Lipofuscinoses (Batten Disease). *Biochimica Biophysica Acta (BBA) - Mol. Basis Dis.* 1852, 2237–2241. doi:10.1016/j.bbadis.2015.05.011

Morozova, K., Clement, C. C., Kaushik, S., Stiller, B., Arias, E., Ahmad, A., et al. (2016). Structural and Biological Interaction of Hsc-70 Protein with Phosphatidylserine in Endosomal Microautophagy. *J. Biol. Chem.* 291, 18096–18106. doi:10.1074/jbc.M116.736744

Mukherjee, A., Patel, B., Koga, H., Cuervo, A. M., and Jenny, A. (2016). Selective Endosomal Microautophagy Is Starvation-Inducible in Drosophila. *Autophagy* 12, 1984–1999. doi:10.1080/15548627.2016.1208887

Müller, M. (1972). Secretion of Acid Hydrolases and its Intracellular Source in Tetrahymena Pyriformis. *J. Cell Biol.* 52, 478–487. doi:10.1083/jcb.52.2.478

Naseri, N. N., Ergel, B., Kharel, P., Na, Y., Huang, Q., Huang, R., et al. (2020). Aggregation of Mutant Cysteine String Protein-α via Fe-S Cluster Binding Is Mitigated by Iron Chelators. *Nat. Struct. Mol. Biol.* 27, 192–201. doi:10.1038/s41594-020-0375-y

Naseri, N., Sharma, M., and Velinov, M. (2021). Autosomal Dominant Neuronal Ceroid Lipofuscinosis: Clinical Features and Molecular Basis. *Clin. Genet.* 99, 111–118. doi:10.1111/cge.13829

Nickel, W., and Rabouille, C. (2009). Mechanisms of Regulated Unconventional Protein Secretion. *Nat. Rev. Mol. Cell Biol.* 10, 148–155. doi:10.1038/nrm2617

Nosková, L., Stránecký, V., Hartmannová, H., Přistoupilová, A., Barešová, V., Ivánek, R., et al. (2011). Mutations in DNAJC5, Encoding Cysteine-String Protein Alpha, Cause Autosomal-Dominant Adult-Onset Neuronal Ceroid Lipofuscinosis. *Am. J. Hum. Genet.* 89, 241–252. doi:10.1016/j.ajhg.2011.07.003

Ohyama, T., Verstreken, P., Ly, C. V., Rosenmund, T., Rajan, A., Tien, A.-C., et al. (2007). Huntingtin-interacting Protein 14, a Palmitoyl Transferase Required for Exocytosis and Targeting of CSP to Synaptic Vesicles. *J. Cell Biol.* 179, 1481–1496. doi:10.1083/jcb.200710061

Oku, M., and Sakai, Y. (2018). Three Distinct Types of Microautophagy Based on Membrane Dynamics and Molecular Machineries. *Bioessays* 40, 1800008. doi:10.1002/bies.201800008

Papadopoulos, C., Kravic, B., and Meyer, H. (2020). Repair or Lysophagy: Dealing with Damaged Lysosomes. *J. Mol. Biol.* 432, 231–239. doi:10.1016/j.jmb.2019.08.010

Patel, P., Prescott, G. R., Burgoyne, R. D., Lian, L.-Y., and Morgan, A. (2016). Phosphorylation of Cysteine String Protein Triggers a Major Conformational Switch. *Structure* 24, 1380–1386. doi:10.1016/j.str.2016.06.009

Pearce, D. A., Ferea, T., Nosel, S. A., Das, B., and Sherman, F. (1999). Action of BTN1, the Yeast Orthologue of the Gene Mutated in Batten Disease. *Nat. Genet.* 22, 55–58. doi:10.1038/8861

Pillay, C. S., Elliott, E., and Dennison, C. (2002). Endolysosomal Proteolysis and its Regulation. *Biochem. J.* 363, 417–429. doi:10.1042/0264-6021:363041710.1042/bj3630417

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cell Biol.* 27, 230–240. doi:10.1016/j.tcb.2016.11.007

Radulovic, M., Schink, K. O., Wenzel, E. M., Nähse, V., Bongiovanni, A., Lafont, F., et al. (2018). ESCRT -mediated Lysosome Repair Precedes Lysophagy and Promotes Cell Survival. *EMBO J.* 37, 9753. doi:10.15252/embj.201899753

Ranta, S., Zhang, Y., Ross, B., Lonka, L., Takkunen, E., Messer, A., et al. (1999). The Neuronal Ceroid Lipofuscinoses in Human EPMR and Mnd Mutant Mice Are Associated with Mutations in CLN8. *Nat. Genet.* 23, 233–236. doi:10.1038/13868

Rodríguez, A., Webster, P., Ortego, J., and Andrews, N. W. (1997). Lysosomes Behave as Ca2+-Regulated Exocytic Vesicles in Fibroblasts and Epithelial Cells. *J. Cell Biol.* 137, 93–104. doi:10.1083/jcb.137.1.93

Rozas, J. L., Gómez-Sánchez, L., Mircheski, J., Linares-Clemente, P., Nieto-González, J. L., Vázquez, M. E., et al. (2012). Motorneurons Require Cysteine String Protein-α to Maintain the Readily Releasable Vesicular Pool and Synaptic Vesicle Recycling. *Neuron* 74, 151–165. doi:10.1016/j.neuron.2012.02.019

Russell, R., Karzai, A. W., Mehl, A. F., and Mcmacken, R. (1999). DnaJ Dramatically Stimulates ATP Hydrolysis by DnaK: Insight into Targeting of Hsp70 Proteins to Polypeptide Substrates. *Biochemistry* 38, 4165–4176. doi:10.1021/bi9824036

Sahu, R., Kaushik, S., Clement, C. C., Cannizzo, E. S., Scharf, B., Follenzi, A., et al. (2011). Microautophagy of Cytosolic Proteins by Late Endosomes. *Dev. Cell* 20, 131–139. doi:10.1016/j.devcel.2010.12.003

Savukoski, M., Klockars, T., Holmberg, V., Santavuori, P., Lander, E. S., and Peltonen, L. (1998). CLN5, a Novel Gene Encoding a Putative Transmembrane Protein Mutated in Finnish Variant Late Infantile Neuronal Ceroid Lipofuscinosis. *Nat. Genet.* 19, 286–288. doi:10.1038/975

Schmitz, F., Tabares, L., Khimich, D., Strenzke, N., De La Villa-Polo, P., Castellano-Muñoz, M., et al. (2006). CSPα-deficiency Causes Massive and Rapid Photoreceptor Degeneration. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2926–2931. doi:10.1073/pnas.0510060103

Settembre, C., Di Malta, C., Polito, V. A., Arencibia, M. G., Vetrini, F., Erdin, S., et al. (2011). TFEB Links Autophagy to Lysosomal Biogenesis. *Science* 332, 1429–1433. doi:10.1126/science.1204592

Sharifi, A., Kousi, M., Sagné, C., Bellenchi, G. C., Morel, L., Darmon, M., et al. (2010). Expression and Lysosomal Targeting of CLN7, a Major Facilitator Superfamily Transporter Associated with Variant Late-Infantile Neuronal Ceroid Lipofuscinosis. *Hum. Mol. Genet.* 19, 4497–4514. doi:10.1093/hmg/ddq381

Sharma, M., Burré, J., Bronk, P., Zhang, Y., Xu, W., and Südhof, T. C. (2012). CSPα Knockout Causes Neurodegeneration by Impairing SNAP-25 Function. *EMBO J.* 31, 829–841. doi:10.1038/emboj.2011.467

Sharma, M., Burré, J., and Südhof, T. C. (2011). CSPα Promotes SNARE-Complex Assembly by Chaperoning SNAP-25 during Synaptic Activity. *Nat. Cell Biol.* 13, 30–39. doi:10.1038/ncb2131

Shi, G.-P., Bryant, R. A. R., Riese, R., Verhelst, S., Driessen, C., Li, Z., et al. (2000). Role for Cathepsin F in Invariant Chain Processing and Major Histocompatibility Complex Class II Peptide Loading by Macrophages. *J. Exp. Med.* 191, 1177–1186. doi:10.1084/jem.191.7.1177

Shields, S. B., Oestreich, A. J., Winistorfer, S., Nguyen, D., Payne, J. A., Katzmann, D. J., et al. (2009). ESCRT Ubiquitin-Binding Domains Function Cooperatively during MVB Cargo Sorting. *J. Cell Biol.* 185, 213–224. doi:10.1083/jcb.200811130

Siintola, E., Partanen, S., Strömme, P., Haapanen, A., Haltia, M., Maehlen, J., et al. (2006). Cathepsin D Deficiency Underlies Congenital Human Neuronal Ceroid-Lipofuscinosis. *Brain* 129, 1438–1445. doi:10.1093/brain/awl107

Siintola, E., Topcu, M., Aula, N., Lohi, H., Minassian, B. A., Paterson, A. D., et al. (2007). The Novel Neuronal Ceroid Lipofuscinosis Gene MFSD8 Encodes a Putative Lysosomal Transporter. *Am. J. Hum. Genet.* 81, 136–146. doi:10.1086/518902

Skowyra, M. L., Schlesinger, P. H., Naismith, T. V., and Hanson, P. I. (2018). Triggered Recruitment of ESCRT Machinery Promotes Endolysosomal Repair. *Science* 360, 5078. doi:10.1126/science.aar5078

Sleat, D. E., Donnelly, R. J., Lackland, H., Liu, C.-G., Sohar, I., Pullarkat, R. K., et al. (1997). Association of Mutations in a Lysosomal Protein with Classical Late-Infantile Neuronal Ceroid Lipofuscinosis. *Science* 277, 1802–1805. doi:10.1126/science.277.5333.1802

Smith, K. R., Dahl, H.-H. M., Canafoglia, L., Andermann, E., Damiano, J., Morbin, M., et al. (2013). Cathepsin F Mutations Cause Type B Kufs Disease, an Adult-Onset Neuronal Ceroid Lipofuscinosis. *Hum. Mol. Genet.* 22, 1417–1423. doi:10.1093/hmg/dds558

Smith, K. R., Damiano, J., Franceschetti, S., Carpenter, S., Canafoglia, L., Morbin, M., et al. (2012). Strikingly Different Clinicopathological Phenotypes Determined by Progranulin-Mutation Dosage. *Am. J. Hum. Genet.* 90, 1102–1107. doi:10.1016/j.ajhg.2012.04.021

Specchio, N., Ferretti, A., Trivisano, M., Pietrafusa, N., Pepi, C., Calabrese, C., et al. (2020). Neuronal Ceroid Lipofuscinosis: Potential for Targeted Therapy. *Drugs* 81, 101–123. doi:10.1007/s40265-020-01440-7

Spillantini, M. G., Schmidt, M. L., Lee, V. M.-Y., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997). α-Synuclein in Lewy Bodies. *Nature* 388, 839–840. doi:10.1038/42166

Staropoli, J. F., Karaa, A., Lim, E. T., Kirby, A., Elbalalesy, N., Romansky, S. G., et al. (2012). A Homozygous Mutation in KCTD7 Links Neuronal Ceroid Lipofuscinosis to the Ubiquitin-Proteasome System. *Am. J. Hum. Genet.* 91, 202–208. doi:10.1016/j.ajhg.2012.05.023

Stefanis, L. (2012). -Synuclein in Parkinson's Disease. *Cold Spring Harb. Perspect. Med.* 2, a009399. doi:10.1101/cshperspect.a009399

Steringer, J. P., Lange, S., Čujová, S., Šachl, R., Poojari, C., Lolicato, F., et al. (2017). Key Steps in Unconventional Secretion of Fibroblast Growth Factor 2 Reconstituted with Purified Components. *Elife* 6, 28985. doi:10.7554/eLife.28985

Stowers, R. S., and Isacoff, E. Y. (2007). Drosophila Huntingtin-Interacting Protein 14 Is a Presynaptic Protein Required for Photoreceptor Synaptic Transmission and Expression of the Palmitoylated Proteins Synaptosome-Associated Protein 25 and Cysteine String Protein. *J. Neurosci.* 27, 12874–12883. doi:10.1523/JNEUROSCI.2464-07.2007

Strating, J. R. P. M., and Martens, G. J. M. (2009). The P24 Family and Selective Transport Processes at the ER-Golgi Interface. *Biol. Cell* 101, 495–509. doi:10.1042/BC20080233

Stutchfield, J., and Cockcroft, S. (1993). Correlation between Secretion and Phospholipase D Activation in Differentiated HL60 Cells. *Biochem. J.* 293 (Pt 3), 649–655. doi:10.1042/bj2930649

Tekirdag, K., and Cuervo, A. M. (2018). Chaperone-mediated Autophagy and Endosomal Microautophagy: Jointed by a Chaperone. *J. Biol. Chem.* 293, 5414–5424. doi:10.1074/jbc.R117.818237

Thiele, D. L., and Lipsky, P. E. (1990). Mechanism of L-Leucyl-L-Leucine Methyl Ester-Mediated Killing of Cytotoxic Lymphocytes: Dependence on a Lysosomal Thiol Protease, Dipeptidyl Peptidase I, that Is Enriched in These Cells. *Proc. Natl. Acad. Sci. U.S.A.* 87, 83–87. doi:10.1073/pnas.87.1.83

Tobaben, S., Thakur, P., Fernández-Chacón, R., Südhof, T. C., Rettig, J., and Stahl, B. (2001). A Trimeric Protein Complex Functions as a Synaptic Chaperone Machine. *Neuron* 31, 987–999. doi:10.1016/s0896-6273(01)00427-5

Umbach, J. A., Zinsmaier, K. E., Eberle, K. K., Buchner, E., Benzer, S., and Gundersen, C. B. (1994). Presynaptic Dysfunction in Drosophila Csp Mutants. *Neuron* 13, 899–907. doi:10.1016/0896-6273(94)90255-0

Uytterhoeven, V., Lauwers, E., Maes, I., Miskiewicz, K., Melo, M. N., Swerts, J., et al. (2015). Hsc70-4 Deforms Membranes to Promote Synaptic Protein Turnover by Endosomal Microautophagy. *Neuron* 88, 735–748. doi:10.1016/j.neuron.2015.10.012

Vaes, G. (1968). On the Mechanisms of Bone Resorption. *J. Cell Biol.* 39, 676–697. doi:10.1083/jcb.39.3.676

van Veen, S., Martin, S., van Den Haute, C., Benoy, V., Lyons, J., Vanhoutte, R., et al. (2020). ATP13A2 Deficiency Disrupts Lysosomal Polyamine Export. *Nature* 578, 419–424. doi:10.1038/s41586-020-1968-7

Vesa, J., Hellsten, E., Verkruyse, L. A., Camp, L. A., Rapola, J., Santavuori, P., et al. (1995). Mutations in the Palmitoyl Protein Thioesterase Gene Causing Infantile Neuronal Ceroid Lipofuscinosis. *Nature* 376, 584–587. doi:10.1038/376584a0

Vietri, M., Radulovic, M., and Stenmark, H. (2020). The Many Functions of ESCRTs. *Nat. Rev. Mol. Cell Biol.* 21, 25–42. doi:10.1038/s41580-019-0177-4

Williams, R. E. (2011). "NCL Incidence and Prevalence Data," in *The Neuronal Ceroid Lipofuscinoses (Batten Disease)*. Editors R. W. Sara Mole and G. O. Hans. second ed. (Oxford: Oxford University Press).

Wu, S., Sirkis, D. W., and Schekman, R. (2022). Unconventional Secretion of α-synuclein Mediated by Palmitoylated DNAJC5 Oligomers. *BioRxiv*. doi:10.1101/2022.01.27.477991

Xu, Y., Cui, L., Dibello, A., Wang, L., Lee, J., Saidi, L., et al. (2018). DNAJC5 Facilitates USP19-Dependent Unconventional Secretion of Misfolded Cytosolic Proteins. *Cell Discov.* 4, 11. doi:10.1038/s41421-018-0012-7

Yoshida, Y., Yasuda, S., Fujita, T., Hamasaki, M., Murakami, A., Kawawaki, J., et al. (2017). Ubiquitination of Exposed Glycoproteins by SCF ^{FBXO27} Directs Damaged Lysosomes for Autophagy. *Proc. Natl. Acad. Sci. U.S.A.* 114, 8574–8579. doi:10.1073/pnas.1702615114

Zhang, M., Liu, L., Lin, X., Wang, Y., Li, Y., Guo, Q., et al. (2020). A Translocation Pathway for Vesicle-Mediated Unconventional Protein Secretion. *Cell* 181, 637–652. e15. doi:10.1016/j.cell.2020.03.031

Zhang, M., and Schekman, R. (2013). Unconventional Secretion, Unconventional Solutions. *Science* 340, 559–561. doi:10.1126/science.1234740

Zhang, W., Yang, X., Chen, L., Liu, Y.-Y., Venkatarangan, V., Reist, L., et al. (2021). A Conserved Ubiquitin- and ESCRT-dependent Pathway Internalizes Human Lysosomal Membrane Proteins for Degradation. *PLoS Biol.* 19, e3001361. doi:10.1371/journal.pbio.3001361

Zhang, Y.-Q., Henderson, M. X., Colangelo, C. M., Ginsberg, S. D., Bruce, C., Wu, T., et al. (2012). Identification of CSPα Clients Reveals a Role in Dynamin 1 Regulation. *Neuron* 74, 136–150. doi:10.1016/j.neuron.2012.01.029

Zinsmaier, K. E. (2010). Cysteine-string Protein's Neuroprotective Role. *J. Neurogenetics* 24, 120–132. doi:10.3109/01677063.2010.489625

Zinsmaier, K. E., Hofbauer, A., Heimbeck, G., Pflugfelder, G. O., Buchner, S., and Buchner, E. (1990). A Cysteine-String Protein Is Expressed in Retina and Brain ofDrosophila. *J. Neurogenetics* 7, 15–29. doi:10.3109/01677069009084150

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 13 May 2022
doi: 10.3389/fcell.2022.863825

Early Bioinformatic Implication of Triacidic Amino Acid Motifs in Autophagy-Dependent Unconventional Secretion of Mammalian Proteins

Malay Ranjan Biswal[1‡], *Sreedevi Padmanabhan*[2‡], *Ravi Manjithaya*[2*] and *Meher K. Prakash*[1*†]

[1]Computational Biology, Theoretical Sciences Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore, India, [2]Autophagy Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore, India

Several proteins are secreted outside the cell, and in many cases, they may be identified by a characteristic signal peptide. However, more and more studies point to the evidence for an "unconventional" secretion, where proteins without a hitherto unknown signal are secreted, possibly in conditions of starvation. In this work, we analyse a set of 202 RNA binding mammalian proteins, whose unconventional secretion has recently been established. Analysis of these proteins secreted by LC3 mediation, the largest unconventionally secreted dataset to our knowledge, identifies the role of KKX motif as well as triacidic amino acid motif in unconventional secretion, the latter being an extension of the recent implicated diacidic amino acid motif. Further data analysis evolves a hypothesis on the sequence or structural proximity of the triacidic or KKX motifs to the LC3 interacting region, and a phosphorylatable amino acid such as serine as a statistically significant feature among these unconventionally secreted proteins. This hypothesis, although needs to be validated in experiments that challenge the specific details of each of these aspects, appears to be one of the early steps in defining what may be a plausible signal for unconventional protein secretion.

Keywords: unconventional protein secretion, autophagy, triacidic motif, LC3 interacting region, mammalian proteins

OPEN ACCESS

Edited by:
Julien Villeneuve, Institute for Functional Genomics (CNRS), France

Reviewed by:
Kai Philip Hussnaetter, Heinrich Heine University of Düsseldorf, Germany
Min Zhang, Tsinghua University, China

****Correspondence:***
Ravi Manjithaya
ravim@jncasr.ac.in
Meher K. Prakash
meherkprakash@gmail.com

†Present Address:
Meher K. Prakash, EssentialTech Centre, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

‡These authors have contributed equally to this work

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *27 January 2022*
Accepted: *11 April 2022*
Published: *13 May 2022*

Citation:
Biswal MR, Padmanabhan S, Manjithaya R and Prakash MK (2022) Early Bioinformatic Implication of Triacidic Amino Acid Motifs in Autophagy-Dependent Unconventional Secretion of Mammalian Proteins. Front. Cell Dev. Biol. 10:863825. doi: 10.3389/fcell.2022.863825

INTRODUCTION

Protein secretion is an essential cellular process. The first step in the translocation of secretory proteins across intracellular membranes and their final localization is the recognition of the "address tags" contained within the amino acid sequences of the proteins. In many cases of protein secretion, a specific configuration of 13–36 amino acids in the N-terminal region acts as a "signal peptide" and helps the translocation across the first membrane on the secretory pathway and thus universally controls the entry of all proteins to the secretory pathway in eukaryotes and prokaryotes. In eukaryotes, the signal peptide of a nascent precursor protein (pre-protein) directs the ribosome to the rough endoplasmic reticulum (ER) membrane and initiates the transport of the growing peptide chain across it (Devillers-Thiery et al., 1975; von Heijne, 1990). The pioneering work done in yeast and mammalian systems elucidated the mechanisms underlying eukaryotic classical secretory

pathway (endoplasmic reticulum (ER)-Golgi-secretory vesicles) and demonstrated that proteins with signal peptides get secreted to the exterior which led to the 2013 Nobel Prize in physiology and medicine (Hata et al., 1993; Sollner et al., 1993; Barlowe et al., 1994; Bonifacino, 2014; Viotti, 2016). However, the conventional protein secretion (CPS) that employs the signal peptide alone is not responsible for the final destination of the mature protein; secretory proteins devoid of further address tags in their sequence are by default secreted to the external environment. Although signal peptides are not highly conserved, they have a common positively charged n-region, a hydrophobic h-region and a neutral, polar c-region (Nakai, 2000). The c-region contains a weakly conserved cleavage site recognized by membrane-bound signal peptidases. Before the translocation of the pre-protein across the ER membrane, a ribonucleoprotein called signal recognition particle (SRP) binds to the signal peptide emerging from the ribosome. Then the SRP-signal peptide-ribosome complex binds to the ER membrane *via* a SRP receptor (Blobel and Dobberstein, 1975).

Alternatively, unconventional protein secretion (UCPS) bypasses the conventional endoplasmic reticulum (ER)-Golgi route. Studies suggest four principal types of UCPS that can be further distinguished into non-vesicular and vesicular pathways (Rabouille et al., 2012; Rabouille, 2017). The non-vesicular pathways are further classified into Type I (e.g., FGF1) and Type II (e.g., yeast MATα). The vesicular pathways are mediated by Type III (e.g., Acb1) and Type IV (e.g., CFTR) mechanisms. Based on a recent classification, Type I is a pore-mediated translocation across the plasma membrane, Type II is an ABC transporter mediated secretion, Type III is an autophagosome/endosome-based secretion and Type IV is a Golgi bypass mechanism (Rabouille, 2017). The type III system has a unique feature as the autophagy process has the ability to form *de novo* vesicles, that have cargo specificity. One such selective form of autophagy that participates in UCPS is known as secretory autophagy (Jiang et al., 2013) wherein the cargo is secreted out instead of being degraded.

Unlike the classical secretory proteins that follow the canonical route of secretion (conventional protein secretion, CPS), the unconventionally secreted protein cargoes follow a plethora of divergent secretory mechanisms. There are no concrete studies on the motif analysis of UCPS. Even the signals that may trigger this UCPS are not clear. One of the early indications for what may be a possible signal in this fascinating unconventional secretion process, has only recently been discovered. The discovery of the diacidic motif, DE as the signal for UCPS of SOD1 (Cruz-Garcia et al., 2017) along with the context dependence of the presence of this motif in proximity with the charged, unstructured amino acids (Padmanabhan et al., 2018) might provide some clues. Similarly, motif-1 of the interleukin family is demonstrated to help in driving the unconventional secretion process (Zhang et al., 2020). On similar lines, the interaction between FGF2 and cell surface heparan sulfate is mediated by basic residues in the C-terminal part of FGF2 with K133 being an essential component of this binding motif (Temmerman et al., 2008; Nickel and Rabouille, 2009, 2008; Steringer et al., 2017).

With the DE motif as a potential UCPS export signal, the LIR containing proteins possess specific membrane associated receptors and the cells might use this in combination for the type III secretion. This can be resonated with the hypothesis that the UCPS cargo containing DE binds to a specific binding partner (Cruz-Garcia et al., 2018).

Predicting whether a protein undergoes a conventional secretion is a relatively well understood phenomenon. Several predictors, such as SecretomeP (Bendtsen et al., 2004) identify the signal peptide with very high accuracy. There are several other newer predictors such as the OutCyte (Zhao et al., 2019) and ExoPred (Ras-Carmona et al., 2021) which are meant to capture the unconventionally secreted proteins as well. These models based on artificial intelligence emphasize the accuracy rather than interpretability in terms of the potential signal-motifs. Further, the quality of the predictions itself may not be reliable as the models are trained on protein secretion data that is highly inhomogeneous. As such, a key to understanding the unconventional secretion signals, and mechanisms is the availability of the relevant high-quality data.

Increasing evidence implicates the role of autophagy proteins (ATGs) in the process of secretion. Indeed, genetic loss-of-function studies have revealed ATGs are required for the efficient secretion of inflammatory cytokines (Stow and Murray, 2013), extracellular release of bactericidal enzymes and tissue repair factors (Bel et al., 2017), extracellular vesicle production (Guo et al., 2017) and unconventional secretion of proteins lacking amino-terminal leader sequences (Rabouille et al., 2012). Some of the unconventional proteins that are shown to be secreted out include Acb1, IL1ß, TGFß (Schotman et al., 2008; Duran et al., 2010; Manjithaya and Subramani, 2010; 2011; Manjithaya et al., 2010; Dupont et al., 2011; Gee et al., 2011; Nilsson et al., 2013; Murrow et al., 2015; Son et al., 2015; 2016; Kortvely et al., 2016; Nuchel et al., 2018). As the process of secretory autophagy (Jiang et al., 2013) has been studied only in a small subset of cargoes the concept of microtubule associated protein Light Chain 3 (LC3) dependent EV loading and secretion (LDELS) from the secretomic studies has opened up more avenues to ponder upon the autophagy mediated secretory protein cargoes in detail (Leidal and Debnath, 2020; Leidal et al., 2020). The recent data on the 202 RNA binding proteins which are unconventionally secreted through an LC3-mediated pathway (Leidal et al., 2020) opens up the possibility of various analyses to understand UCPS. We performed bioinformatic analyses on this largest data set of autophagy mediated unconventionally secreted cargoes (202 RNA binding proteins) known till date to explore the possibility of identifying the signals that trigger unconventional secretion.

METHODS

Sequence Curation

The 202 unconventionally secreted proteins used in the analysis are obtained from the set of proteins proved to be secreted by LC3-mediated mechanism in the analysis of Leidal et al. (Leidal et al., 2020). The set of 1576 conventionally secreted proteins are

obtained from the reference set used for training in SecretomeP (Bendtsen et al., 2004) database (http://119.3.41.228:8080/SPRomeDB/download_enabled.php). For convenience, these data sets are also provided in our Supplementary Data (https://github.com/malayrb/Thesis/tree/main/Ch7).

Discriminatory Motif Analysis

Discriminatory motif (DiMotif) analysis (Asgari et al., 2019) of a set relative to the Swiss-Prot reference was performed using the code: https://github.com/ehsanasgari/dimotif/blob/master/notebook/DiMotif_step_by_step_example.ipynb.

Motif Search Analysis

In addition to the DiMotif analysis, we performed a motif search using our script to analyse the differential occurrence of the motifs. The analyses presented in this work are based on 3 amino acid motifs, and which can result in 4200 combinations or 8000 combinations respectively with and without considering the mirror symmetry of the motifs. The proteins from the conventionally secreted and the LC3-mediated groups were scanned for these motif combinations, and the presence or absence of the motif was noted. Similarly, scripts were also used for analysing 4 amino acid motifs as well as the LIR motif (WXXL) (Noda et al., 2010; Jacomin et al., 2016).

RESULTS

Acidic Motifs Top the Differential Motif Analysis

The presence of a signature signal sequence is common in protein sorting. To identify the presence of a signal sequence in the 202 RNA binding proteins, we performed two different analyses:

Discriminatory Motif Analysis

To capture the unique signature in the 202 LC3 interacting proteins (UCPS-ATG dataset), they were compared against 20,117 proteins from the Swiss-Prot database using the DiMotif server (Asgari et al., 2019). This discriminatory motif analysis is meant to identify the motifs which were significantly represented in a chosen set, relative to all the proteins from the Swiss-Prot database. The most significant motifs identified by this analysis are (details shown in **Supplementary Table S1A**): EEE, DD, DED, DE, AK, KKE, KK, KT, AKK, KE. These discriminatory motifs have two as well as three amino acids.

Custom Motif Analysis

In addition to the above-mentioned analysis from DiMotif server, we also performed a custom motif search comparing the LC3 interacting proteins with the database of conventionally secreted proteins used for training the SecretomeP (Bendtsen et al., 2004). In this analysis, all possible motifs of 2, 3 and 4 amino acids were combinatorially generated and a systematic search for them was performed in the LC3 interacting UCPS-ATG dataset (positive-set), and the conventionally secreted proteins (negative-set) (Bendtsen et al., 2004). The implicit assumption being that the conventionally secreted proteins are not secreted through the LC3-mediated pathway. Comparing the motifs in the positive and the negative sets, the top ten differentiating proteins were identified after imposing a constraint that the motif must occur at least 30% more often among the proteins in the positive-set than in the negative-set. The occurrence of a motif in the protein, rather than the number of its occurrences in the same protein, was considered important. In this differential analysis, three amino acid motifs had the highest difference between the two sets, while two or four amino acid motifs did not appear to differentiate the two sets significantly to appear among the top differentiators. The three amino acid motifs with the highest difference between the two sets are: EEE, KKS, AEK, AKK, KKR, KEL, DEE, KAL, EKL, KER (details in **Supplementary Tables S1B,C**). As may be seen, most of the differentiating motifs are charged, with the triacidic motif at the top.

Acidic Motifs Appear in the Proximity of LIR Motifs

The transport of the specific set of proteins analysed in this work is mediated through the LC3 domains. We identified all the LC3 Interacting Regions (LIR) in each protein by performing a search for WXXL motif (Noda et al., 2010; Jacomin et al., 2016), and studied the frequency of occurrence of the different 3 amino acid motifs in the proximity of LIR. As the structural information of these proteins is sparse, we restricted the primary analysis to sequence-based proximity and wherever the structural information was available, the structural proximity check was subsequently checked for. The most commonly occurring sequences in the proximity of the LIR regions are: KEL, EEL, ALE, KAL, DEE, EKL, AEE, EEE, EEK (details in **Supplementary Table S2**).

Phosphorylatable Amino Acids Occur Preferentially in the UCPS Proteins

Since the unconventional secretion is usually activated under conditions of stress, we explored the possibility that a post-translational modification may be required for its activation. We searched for the presence of serine, threonine, or tyrosine within 3 amino acid positions from the differentiating motifs. For almost all the reference motifs we analysed, the S/T/Y amino acid in the proximity of the motifs occurred preferentially among the proteins from the positive set (**Supplementary Table S3**).

DISCUSSION

Triacidic Motif is Potentially a Signal for UCPS in Mammalian Cells

The discriminatory motifs identified relative to the Swiss-Prot database and the conventional protein secretion dataset were re-grouped to identify the common patterns among them. Two major patterns emerge among these three amino acid motifs: triacidic motifs (EEE/DDD/DEE/etc) occurring in 160 of the 202 from the positive set, and 625 of the 1576 in the negative set, and basic motifs (KKX) occurring in 187 of the 202 from the positive set and 796 of the 1576 in the negative set. Considering either of

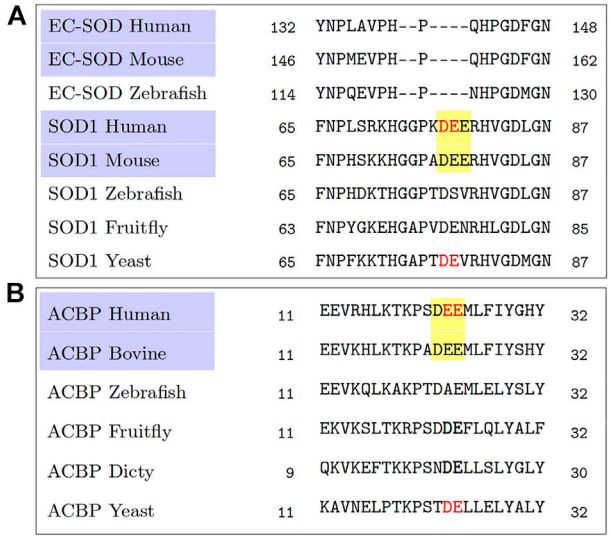


FIGURE 1 | A reanalysis of the multiple sequence alignments obtained from Cruz-Garcia et al. (2017). A comparison among the mammalian sequences, highlighted in purple, shows a common triacidic motif DEE (highlighted in yellow), rather than a diacidic motif (shown in red colored text) when comparing sequences across all species. **(A)** Multiple sequence alignment from homologs of SOD1. **(B)** Multiple sequence alignment from homologs of ACB1.

these triacidic or KKX motifs as a signal, the difference in the proportion between the positive and the negative data sets is statistically significant ($p < 0.0002$ in a Z-test).

Of these two statistically significant observations, the triacidic motif, by coincidence, happens to be an extension of the observation of the diacidic motif (Cruz-Garcia et al., 2017) and our earlier attempt to find the context in which the diacidic motif appears (Padmanabhan et al., 2018). In fact, a quick reanalysis of the multiple sequence alignments from the homologs of SOD1, Acb1 (Cruz-Garcia et al., 2017) by focusing on the mammalian sequences alone shows that they all have a common triacidic motif (**Figure 1**). However, despite the statistical evidence over the 202 proteins for the possibility of KKX as a UCPS signal, it is present neither in SOD1, nor in Acb1. Since there is very limited data on unconventionally secreted proteins, we consider the independent finding of the triacidic motif in an already experimentally validated data set as evidence in support of our finding. Needless to say, the role of which KKX as well as the other features possibly contributing to the signal, as described below require further computational as well as experimental investigations. Further, in the positive set which is derived from LC3-mediated secretion (Leidal et al., 2020), the validation of the triacidic or KKX motifs for other types of unconventional secretion will also require investigation.

Phosphorylation May Be Activating the Signal

Unlike conventional secretion, the UCPS is activated under conditions of stress, suggesting the possibility that post-translational modifications may play a role in activating the signal. In continuation of the hypothesis that the triacidic or KKX motifs may be the "signal", we explored the possibility that the amino acids S/T/Y in the proximity are responsible for activating this signal. S/T/Y amino acids in the proximity of triacidic motifs appeared in 133 of the 202 proteins from the positive-set, and in 422 of the 1576 proteins from the negative set. Similarly, S/T/Y amino acids in the proximity of KKX appeared in 170 of the 202 proteins from the positive set, and in 603 of the 1576 proteins from the negative set. The statistical significance of the difference between the two sets remains high ($p < 0.00001$ in a Z-test).

LIR Motif in the Proximity of Triacidic Motif is Discriminatory

The positive set being analysed here, is about the set of proteins where LC3 conjugation machinery is involved in their secretion (Leidal et al., 2020). However, the LIR motifs are present in abundance in both the positive and the negative sets, making them non-discriminatory. To investigate beyond the statistical averages from the 202 proteins, and to obtain fine-grained insights into the role of LIR and the triacidic motifs, we analysed the 31 class I proteins from the positive-set which were secreted in all three replicates in a statistically significant way. Among them, 6 proteins had LIR motif within 3 amino acids of the triacidic motif (**Figure 2**) along the sequence. From the remaining proteins, structural information was available only for 8 of them and in all of them the LIR region was within a structural proximity (**Figure 3**), if not a sequential proximity of 10 Å from triacidic motifs (**Supplementary Table S4**). Coincidentally, in the cases where the structural proximity between the LIR and the triacidic motifs was not seen, it could be seen with the KKX motifs (**Figure 4**), underscoring the possible complementarity between the triacidic and KKX motifs in signaling the UCPS.

Deriving the Hypothesis for the Signal for UCPS

Given the importance of unconventional protein secretion, it is pivotal to identify the signals that trigger it, if such signals exist.

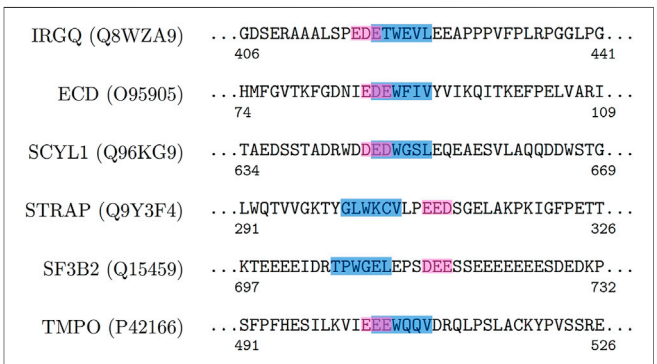


FIGURE 2 | An analysis of the sequence-proximity of the triacidic motif with the LIR motif among some of the proteins from the class I of the UCPS-ATG data set is shown.

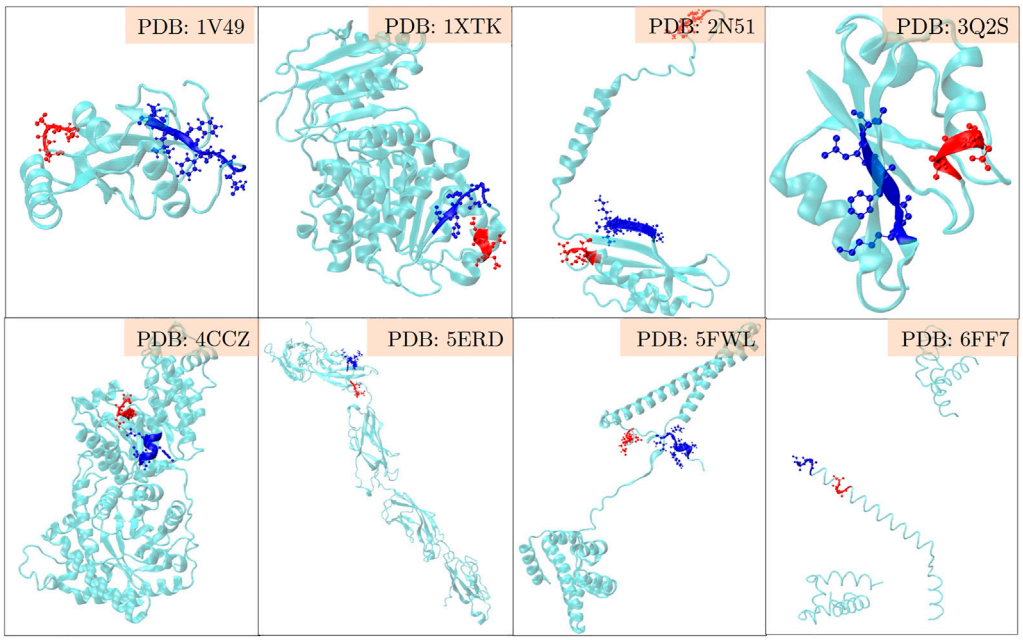


FIGURE 3 | An analysis of the structural-proximity of the triacidic motif with the LIR motif among the proteins from the class I of the UCPS-ATG data set for which structures are known is shown. The blue and red colors indicate the LIR and triacidic motifs. For convenience, only the closest pair is shown and other occurrences of LIR or triacidic motifs are not shown.

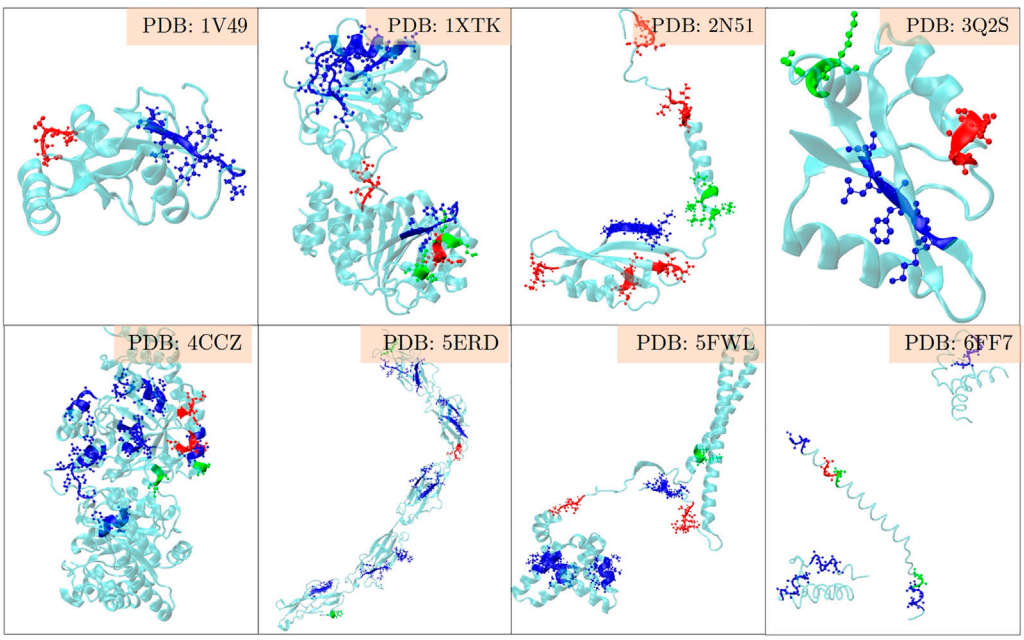


FIGURE 4 | An analysis of the structural-proximity of the triacidic as well as the KKX motifs with all LIR motifs occurring among the proteins from the class I of the UCPS-ATG data set for which structures are known is shown. The blue, green and red colors indicate the LIR, KKX and triacidic motifs. One may notice that in some structures LIR is close to the triacidic motif, and in others to the KKX motif.

The key to building hypotheses is to work with highly reliable data, preferably from fewer sources to avoid any biases in the experimental protocols. In this work, we chose to work with a very specific data set from the LC3 machinery driven protein secretion with 202 proteins, and to build a few hypotheses on what may be the signal for the unconventional secretion. The presence of three amino acid motifs, triacidic or KKX, appears recurrently in the set of 202 UCPS proteins, significantly more than it occurred either in the conventionally secreted proteins or in the Swiss-Prot database. Although the 202 proteins are believed to be secreted by the LC3 dependent pathway, 5 of these proteins do not have an LIR motif that can interact with the LC3 region. Interestingly even in these proteins, triacidic motif in the proximity of a phosphorylatable amino acid is a common occurrence. Among the proteins that had the LIR motif, it was found mostly in the sequence or a structural proximity from the triacidic or the KKX motifs. Thus, it appears that triacidic or KKX amino acid motifs in the proximity of LIR and/or phosphorylatable amino acids may play a significant role in triggering the unconventional secretion. This result was also validated in the independently curated dataset of unconventionally secreted proteins from other mammalian cells (Padmanabhan et al., 2018), where among the 26 mammalian proteins that are secreted unconventionally, 5 of them had triacidic motifs within a 5 amino acid proximity of LIR. 9 of the remaining proteins where there was no sequence proximity, but the structures were available, had LIR motifs within 10 Å of the triacidic motif, and three other structures had them within 15 Å. It will be very interesting to see if this hypothesis can be validated and refined with new experiments in which mutant constructs are designed to challenge each of these aspects of the composite hypothesis–triacidic, KKX, proximity of LIR, proximity of serine amino acid - are developed.

CONCLUSION

In conclusion, we explored the plausible signals for a very fundamental cellular process - unconventional protein secretion. The field is still in its nascent stages compared to conventional protein secretion where the signals as well as the mechanisms are clearly identified. Exploiting the recent experimental findings of a large set of unconventionally secreted proteins, we could perform bioinformatic analyses as well build hypotheses on the potential role of triacidic amino acids or KKX motif in the proximity of LIR region and phosphorylatable amino acids. As the next steps, we will be exploring collaboration with the relevant experimental groups to validate these hypotheses as well as explore the possibility of deciphering the patterns using interpretable deep-learning methods on the same datasets.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

The authors confirm contribution to the paper as follows: study conception and design: SP and MP; data collection: SP; analysis and interpretation of results: MB, SP, MP, and RM; draft manuscript preparation: MB, SP, MP, and RM. All authors reviewed the results and approved the final version of the manuscript.

FUNDING

This study was supported by JNCASR intramural funds, DST–Science and Engineering Research Board (SERB) grant (EMR/2015/001946) to RM, Department of Biotechnology grant in Life Science Research, Education and Training at JNCASR (BT/INF/22/SP27679/2018). The financial support from JNCASR to MB is acknowledged. The financial support from the DBT-RA program in Biotechnology and Life Sciences and DST-SERB NPDF to SP is gratefully acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.863825/full#supplementary-material

REFERENCES

Asgari, E., McHardy, A., and Mofrad, M. R. K. (2019). Probabilistic Variable-Length Segmentation of Protein Sequences for Discriminative Motif Discovery (DiMotif) and Sequence Embedding (ProtVecX). *Sci. Rep.* 9, 3577–3592. doi:10.1038/s41598-019-38746-w

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., et al. (1994). COPII: A Membrane Coat Formed by Sec Proteins that Drive Vesicle Budding from the Endoplasmic Reticulum. *Cell* 77, 895–907. doi:10.1016/0092-8674(94)90138-4

Bel, S., Pendse, M., Wang, Y., Li, Y., Ruhn, K. A., Hassell, B., et al. (2017). Paneth Cells Secrete Lysozyme *via* Secretory Autophagy during Bacterial Infection of the Intestine. *Science* 357, 1047–1052. doi:10.1126/science.aal4677

Bendtsen, J. D., Jensen, L. J., Blom, N., Von Heijne, G., and Brunak, S. (2004). Feature-based Prediction of Non-classical and Leaderless Protein Secretion. *Protein Eng. Des. Selection* 17, 349–356. doi:10.1093/protein/gzh037

Blobel, G., and Dobberstein, B. (1975). Transfer of Proteins across Membranes. I. Presence of Proteolytically Processed and Unprocessed Nascent Immunoglobulin Light Chains on Membrane-Bound Ribosomes of Murine Myeloma. *J. Cel Biol.* 67, 835–851. doi:10.1083/jcb.67.3.835

Bonifacino, J. S. (2014). Vesicular Transport Earns a Nobel. *Trends Cel Biol.* 24, 3–5. doi:10.1016/j.tcb.2013.11.001

Cruz-Garcia, D., Brouwers, N., Duran, J. M., Mora, G., Curwin, A. J., and Malhotra, V. (2017). A Diacidic Motif Determines Unconventional Secretion of Wild-type and Als-Linked Mutant Sod1. *J. Cel Biol.* 216, 2691–2700. doi:10.1083/jcb.201704056

Cruz-Garcia, D., Malhotra, V., and Curwin, A. J. (2018). Unconventional Protein Secretion Triggered by Nutrient Starvation. *Semin. Cel Develop. Biol.* 83, 22–28. doi:10.1016/j.semcdb.2018.02.021

Devillers-Thiery, A., Kindt, T., Scheele, G., and Blobel, G. (1975). Homology in Amino-Terminal Sequence of Precursors to Pancreatic Secretory Proteins. *Proc. Natl. Acad. Sci. U.S.A.* 72, 5016–5020. doi:10.1073/pnas.72.12.5016

Dupont, N., Jiang, S., Pilli, M., Ornatowski, W., Bhattacharya, D., and Deretic, V. (2011). Autophagy-based Unconventional Secretory Pathway for Extracellular Delivery of IL-1β. *EMBO J.* 30, 4701–4711. doi:10.1038/emboj.2011.398

Duran, J. M., Anjard, C., Stefan, C., Loomis, W. F., and Malhotra, V. (2010). Unconventional Secretion of Acb1 Is Mediated by Autophagosomes. *J. Cel Biol.* 188, 527–536. doi:10.1083/jcb.200911154

Gee, H. Y., Noh, S. H., Tang, B. L., Kim, K. H., and Lee, M. G. (2011). Rescue of ΔF508-CFTR Trafficking *via* a GRASP-dependent Unconventional Secretion Pathway. *Cell* 146, 746–760. doi:10.1016/j.cell.2011.07.021

Guo, H., Chitiprolu, M., Roncevic, L., Javalet, C., Hemming, F. J., Trung, M. T., et al. (2017). ATG5 Disassociates the V_1V_0-Atpase to Promote Exosome Production and Tumor Metastasis Independent of Canonical Macroautophagy. *Develop. Cel* 43, 716–730. doi:10.1016/j.devcel.2017.11.018

Hata, Y., Slaughter, C. A., and Südhof, T. C. (1993). Synaptic Vesicle Fusion Complex Contains Unc-18 Homologue Bound to Syntaxin. *Nature* 366, 347–351. doi:10.1038/366347a0

Jacomin, A.-C., Samavedam, S., Promponas, V., and Nezis, I. P. (2016). Ilir Database: A Web Resource for Lir Motif-Containing Proteins in Eukaryotes. *Autophagy* 12, 1945–1953. doi:10.1080/15548627.2016.1207016

Jiang, S., Dupont, N., Castillo, E. F., and Deretic, V. (2013). Secretory versus Degradative Autophagy: Unconventional Secretion of Inflammatory Mediators. *J. Innate Immun.* 5, 471–479. doi:10.1159/000346707

Kortvely, E., Hauck, S. M., Behler, J., Ho, N., and Ueffing, M. (2016). The Unconventional Secretion of ARMS2. *Hum. Mol. Genet.* 25, 3143–3151. doi:10.1093/hmg/ddw162

Leidal, A. M., and Debnath, J. (2020). LC_3-dependent Extracellular Vesicle Loading and Secretion (Ldels). *Autophagy* 16, 1162–1163. doi:10.1080/15548627.2020.1756557

Leidal, A. M., Huang, H. H., Marsh, T., Solvik, T., Zhang, D., Ye, J., et al. (2020). The LC_3-Conjugation Machinery Specifies the Loading of Rna-Binding Proteins into Extracellular Vesicles. *Nat. Cel Biol.* 22, 187–199. doi:10.1038/s41556-019-0450-y

Manjithaya, R., Anjard, C., Loomis, W. F., and Subramani, S. (2010). Unconventional Secretion of Pichia pastoris Acb1 Is Dependent on GRASP Protein, Peroxisomal Functions, and Autophagosome Formation. *J. Cel Biol.* 188, 537–546. doi:10.1083/jcb.200911149

Manjithaya, R., and Subramani, S. (2011). Autophagy: a Broad Role in Unconventional Protein Secretion? *Trends Cel Biol.* 21, 67–73. doi:10.1016/j.tcb.2010.09.009

Manjithaya, R., and Subramani, S. (2010). Role of Autophagy in Unconventional Protein Secretion. *Autophagy* 6, 650–651. doi:10.4161/auto.6.5.12066

Murrow, L., Malhotra, R., and Debnath, J. (2015). ATG_{12}-ATG_3 Interacts with Alix to Promote Basal Autophagic Flux and Late Endosome Function. *Nat. Cel Biol.* 17, 300–310. doi:10.1038/ncb3112

Nakai, K. (2000). Protein Sorting Signals and Prediction of Subcellular Localization. *Adv. Protein Chem.* 54, 277–344. doi:10.1016/S0065-3233(00)54009-1

Nickel, W., and Rabouille, C. (2009). Mechanisms of Regulated Unconventional Protein Secretion. *Nat. Rev. Mol. Cel Biol.* 10, 148–155. doi:10.1038/nrm2617

Nickel, W., and Seedorf, M. (2008). Unconventional Mechanisms of Protein Transport to the Cell Surface of Eukaryotic Cells. *Annu. Rev. Cel Dev. Biol.* 24, 287–308. doi:10.1146/annurev.cellbio.24.110707.175320

Nilsson, P., Loganathan, K., Sekiguchi, M., Matsuba, Y., Hui, K., Tsubuki, S., et al. (2013). Aβ Secretion and Plaque Formation Depend on Autophagy. *Cel Rep.* 5, 61–69. doi:10.1016/j.celrep.2013.08.042

Noda, N. N., Ohsumi, Y., and Inagaki, F. (2010). Atg8-family Interacting Motif Crucial for Selective Autophagy. *FEBS Lett.* 584, 1379–1385. doi:10.1016/j.febslet.2010.01.018

Nüchel, J., Ghatak, S., Zuk, A. V., Illerhaus, A., Mörgelin, M., Schönborn, K., et al. (2018). TGFB1 Is Secreted through an Unconventional Pathway Dependent on the Autophagic Machinery and Cytoskeletal Regulators. *Autophagy* 14, 465–486. doi:10.1080/15548627.2017.1422850

Padmanabhan, S., Biswal, M. R., Manjithaya, R., and Prakash, M. K. (2018). Exploring the context of diacidic motif de as a signal for unconventional protein secretion in eukaryotic proteins. *Wellcome Open Res.* 3, 148. doi:10.12688/2Fwellcomeopenres.14914.1

Rabouille, C., Malhotra, V., and Nickel, W. (2012). Diversity in Unconventional Protein Secretion. *J. Cel Sci.* 125, 5251–5255. doi:10.1242/jcs.103630

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cel Biol.* 27, 230–240. doi:10.1016/j.tcb.2016.11.007

Ras-Carmona, A., Gomez-Perosanz, M., and Reche, P. A. (2021). Prediction of Unconventional Protein Secretion by Exosomes. *BMC Bioinformatics* 22, 1–13. doi:10.1186/s12859-021-04219-z

Schotman, H., Karhinen, L., and Rabouille, C. (2008). dGRASP-mediated Noncanonical Integrin Secretion Is Required for Drosophila Epithelial Remodeling. *Develop. Cel* 14, 171–182. doi:10.1016/j.devcel.2007.12.006

Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., et al. (1993). SNAP Receptors Implicated in Vesicle Targeting and Fusion. *Nature* 362, 318–324. doi:10.1038/362318a0

Son, S. M., Cha, M.-Y., Choi, H., Kang, S., Choi, H., Lee, M.-S., et al. (2016). Insulin-degrading Enzyme Secretion from Astrocytes Is Mediated by an Autophagy-Based Unconventional Secretory Pathway in Alzheimer Disease. *Autophagy* 12, 784–800. doi:10.1080/15548627.2016.1159375

Son, S. M., Kang, S., Choi, H., and Mook-Jung, I. (2015). Statins Induce Insulin-Degrading Enzyme Secretion from Astrocytes via an Autophagy-Based Unconventional Secretory Pathway. *Mol. Neurodegeneration* 10, 56. doi:10.1186/s13024-015-0054-3

Steringer, J. P., Lange, S., Cujova, S., Sachl, R., Poojari, C., Lolicato, F., et al. (2017). Key Steps in Unconventional Secretion of Fibroblast Growth Factor 2 Reconstituted with Purified Components. *eLife* 6, e28985. doi:10.7554/eLife.28985

Stow, J. L., and Murray, R. Z. (2013). Intracellular Trafficking and Secretion of Inflammatory Cytokines. *Cytokine Growth Factor. Rev.* 24 (3), 227–239. doi:10.1016/j.cytogfr.2013.04.001

Temmerman, K., Ebert, A. D., Müller, H.-M., Sinning, I., Tews, I., and Nickel, W. (2008). A Direct Role for Phosphatidylinositol-4,5-Bisphosphate in Unconventional Secretion of Fibroblast Growth Factor 2. *Traffic* 9, 1204–1217. doi:10.1111/j.1600-0854.2008.00749.x

Viotti, C. (2016). ER to Golgi-dependent Protein Secretion: the Conventional Pathway. *Methods Mol. Biol.* 1459, 3–29. doi:10.1007/978-1-4939-3804-9_1

von Heijne, G. (1990). The Signal Peptide. *J. Membrain Biol.* 115, 195–201. doi:10.1007/BF01868635

Zhang, M., Liu, L., Lin, X., Wang, Y., Li, Y., Guo, Q., et al. (2020). A Translocation Pathway for Vesicle-Mediated Unconventional Protein Secretion. *Cell* 181, 637–652. doi:10.1016/j.cell.2020.03.031

Zhao, L., Poschmann, G., Waldera-Lupa, D., Rafiee, N., Kollmann, M., and Stühler, K. (2019). Outcyte: a Novel Tool for Predicting Unconventional Protein Secretion. *Sci. Rep.* 9, 1–9. doi:10.1038/s41598-019-55351-z

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

REVIEW
published: 20 May 2022
doi: 10.3389/fcell.2022.886381

A Journey on Extracellular Vesicles for Matrix Metalloproteinases: A Mechanistic Perspective

Sylvie Thuault[1]*, *Rania Ghossoub*[1], *Guido David*[1,2] and *Pascale Zimmermann*[1,2]*

[1]Centre de Recherche en Cancérologie de Marseille (CRCM), Equipe Labellisée Ligue 2018, CNRS, Inserm, Institut Paoli Calmettes, Aix-Marseille Université, Marseille, France, [2]Department of Human Genetics, KU Leuven, University of Leuven, Leuven, Belgium

Matrix metalloproteinases (MMPs) are key players in matrix remodeling and their function has been particularly investigated in cancer biology. Indeed, through extracellular matrix (ECM) degradation and shedding of diverse cell surface macromolecules, they are implicated in different steps of tumor development, from local expansion by growth to tissue invasion and metastasis. Interestingly, MMPs are also components of extracellular vesicles (EVs). EVs are membrane-limited organelles that cells release in their extracellular environment. These "secreted" vesicles are now well accepted players in cell-to-cell communication. EVs have received a lot of interest in recent years as they are also envisioned as sources of biomarkers and as potentially outperforming vehicles for the delivery of therapeutics. Molecular machineries governing EV biogenesis, cargo loading and delivery to recipient cells are complex and still under intense investigation. In this review, we will summarize the state of the art of our knowledge about the molecular mechanisms implicated in MMP trafficking and secretion. We focus on MT1-MMP, a major effector of invasive cell behavior. We will also discuss how this knowledge is of interest for a better understanding of EV-loading of MMPs. Such knowledge might be of use to engineer novel strategies for cancer treatment. A better understanding of these mechanisms could also be used to design more efficient EV-based therapies.

Keywords: extracellular vesicles, exosomes, invadopodia, matrix metalloproteinases, trafficking

OPEN ACCESS

Edited by:
Julien Villeneuve, Institute for Functional Genomics (IGF) (CNRS), France

Reviewed by:
Marc Coppolino, University of Guelph, Canada
Takanori Eguchi, Okayama University, Japan

***Correspondence:**
Sylvie Thuault
sylvie.thuault@inserm.fr
Pascale Zimmermann
pascale.zimmermann@inserm.fr

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *28 February 2022*
Accepted: *27 April 2022*
Published: *20 May 2022*

Citation:
Thuault S, Ghossoub R, David G and Zimmermann P (2022) A Journey on Extracellular Vesicles for Matrix Metalloproteinases: A Mechanistic Perspective. Front. Cell Dev. Biol. 10:886381. doi: 10.3389/fcell.2022.886381

1 INTRODUCTION

Extracellular matrix (ECM) remodeling plays a crucial role during development and later to maintain tissue homeostasis (Bonnans et al., 2014; Winkler et al., 2020). During cancer progression, tissue matrix is modified to create a microenvironment favoring tumorigenesis and metastasis, supporting tumor growth, migration and invasion, angiogenesis, and immune suppression. Tumor cells, in close collaboration with tumor-associated stromal cells, deposit an ECM that differs from that made by their normal counterparts, altering the biochemical composition of the surrounding microenvironment. By activating enzymes involved in crosslinking ECM components, they also modify the biophysical properties of the ECM. Increased ECM stiffness is correlated to tumor progression in multiple cancer types. Furthermore, tumor cells and stromal cells degrade ECM components, clearing environmental barriers and favoring mobility, but also releasing signaling molecules and activating cell surface receptors.

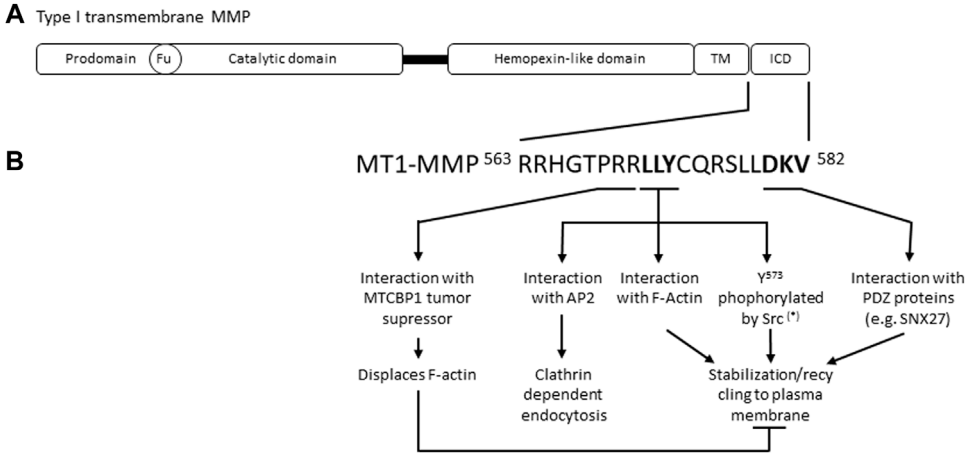


FIGURE 1 | (A) Schematic representation of type I transmembrane MMPs. Fu, Furin cleavage recognition site; TM, transmembrane domain; ICD, intracellular domain. **(B)** Main MT1-MMP intracellular domain molecular features reported to control MT1-MMP endocytic and exocytic cycles. (*) other post-translational modifications affecting MT1-MMP stabilisation and recycling have been reported, please refer to the main text for further details. MTCBP1, MT1-MMP cytoplasmic tail-binding protein-1; AP-2, Adaptor Protein-2; F-Actin, filamentous actin; SNX27, Sorting Nexin 27.

2 MATRIX METALLOPROTEINASES (MMPS)

Matrix metalloproteinases (MMPs) compose a large family of secreted and membrane-associated proteinases essential for ECM remodeling. In total, 23 members are present in humans. Six of them are membrane-associated MMPs (MT-MMP): MT1-, MT2-, MT3-, and MT5-MMP are transmembrane proteinases, whereas MT4- and MT6-MMP are GPI-anchored.

MMPs share a common structure consisting of a pro-domain, a catalytic domain, and a C-terminal hemopexin-like domain (HPX) linked to the catalytic domain by a flexible serine rich region or linker peptide (**Figure 1**). Membrane-associated type I MMPs, such as MT1-MMP, contain a transmembrane domain and a short intracellular domain. MMPs are synthetized as inactive zymogens (pro-MMPs) and their activation requires a proteolytic cleavage that removes the pro-peptide. Indeed, this pro-domain contains a cysteine that interacts with the Zn^{2+} ion present in the catalytic domain, preventing enzymatic proteolytic activity (Van Wart and Birkedal-Hansen, 1990). Pro-domains are generally cleaved by other MMPs or serine proteases outside the cell, except for the transmembrane MMPs (MT-MMP), MMP-11 and MMP-28 which contain a furin recognition motif and are activated by intracellular furin-like serine proteinases. MMP activity can also be activated by oxidative stress, such as ROS, oxidizing the thiol cysteine group. In addition, MMP activity is regulated through 1) regulation of MMP expression, 2) trafficking and subcellular localization (internalization, recycling, secretion), 3) shedding, and 4) association with endogenous inhibitors (e.g., TIMPs, RECK). Regulatory steps depend on dimerization, post-translational modifications (e.g., phosphorylation, ubiquitination), and association with molecular partners. For further details on MMPs structure and activation see Brinckerhoff and Matrisian, 2002; Alaseem et al., 2019.

MMPs have some ECM substrate specificity. Together, MMPs degrade almost all the components of ECM (Bonnans et al., 2014). However, their activity is not limited to ECM components. For example, MT1-MMP is known to degrade ECM factors such as type I, II, III collagen, fibronectin, laminin-1 and -5, vitronectin, and aggrecan (Egeblad and Werb, 2002), but, in collaboration with the tissue inhibitor of matrix metalloproteinase family member TIMP-2, also cleaves the pro-peptide of pro-MMP2 and pro-MMP13, activating these enzymes. MT1-MMP also mediates the shedding of cell surface proteins such as CD44 (Kajita et al., 2001), α_v integrins (Deryugina et al., 2002; Ratnikov et al., 2002) and syndecans (Endo et al., 2003; Barbolina and Stack, 2008). MMPs can also cleave intracellular substrates, such as α-actinin-1 and 4, cofilin-1, filamins (Niland et al., 2021).

As key matrix endopeptidases, MMPs are implicated in diverse physiological processes such as embryogenesis, morphogenesis, and wound healing. Their deregulation is correlated with various pathological conditions, such as fibrotic diseases and cancer (Bonnans et al., 2014; Winkler et al., 2020). They are overexpressed in various types of cancer and are generally defined as bad prognostic factors, their expression increasing with cancer progression. Although generally pro-tumorigenic, some studies show anti-tumorigenic activities for some MMPs (Dufour and Overall, 2013). MMPs are expressed by cancer cells and tumor stromal cells, mainly cancer-associated fibroblasts, and endothelial cells. Their activities remodel the ECM, removing barriers and facilitating cell motility. They induce the production of short ECM fragments from long ECM molecules, called matrikines, acting as cytokines/chemokines. ECM degradation also allows the release and activation of matrix-bound growth factors. Thus, MMPs participate in the production of extracellular signaling molecules, modulating the activities of cell surface receptors, and thereby regulating signaling pathways implicated in cancer

progression (Kessenbrock et al., 2010; Alaseem et al., 2019). MMPs have therefore been envisioned as therapeutical targets for cancer treatments. However, clinical trials are disappointing, due to the fact that inhibitors lack specificity, targeting both pro- and anti-tumorigenic MMPs (Dufour and Overall, 2013; Alaseem et al., 2019), reinforcing the need of a better understanding of the regulation of MMP activities.

3 EXTRACELLULAR VESICLES

Intriguingly, MMPs were identified as extracellular vesicle (EV) cargoes (Shimoda and Khokha, 2017; Sanderson et al., 2019). EVs are membrane-limited organelles secreted by all types of cells in physiological and pathological conditions. EVs contain bioactive materials, such as proteins, nucleic acids and lipids, and enable the release of these materials in the extracellular environment through unconventional secretory pathways. Historically considered as "cell waste", EVs are currently recognized as key actors in cell-to-cell communication (Tkach and Théry, 2016; Sato and Weaver, 2018; Kalluri and LeBleu, 2020). They act locally but also at a distance, circulating in almost all body fluids (e.g., blood, urine, saliva). EVs have received a lot of interest in recent years as they are envisioned as source of biomarkers, but also as promising vehicles for delivering therapeutics.

EVs are heterogeneous in terms of origin and size. Based on biogenesis, EVs can be classified in three major classes of EVs: apoptotic bodies, microvesicles and exosomes. Alternative nomenclatures refer to the method of purification. Apoptotic bodies are released upon cell death and will not be discussed in this review. Microvesicles (150 nm to a few μm), also called ectosomes or microparticles, emerge from outward budding of the plasma membrane. Exosomes (50–150 nm) have an endosomal origin. Intraluminal vesicles (ILV) are formed by an outward/away from the cytosol budding of the endosomal membrane during the maturation of multivesicular endosomes/bodies (MVB). Once ILVs are released in the extracellular microenvironment through fusion of MVBs with the plasma membrane, these are called exosomes. Diverse methods of fractionation allow the enrichment of the different EV subtypes and purification of specific EV subpopulations. For further details, please see Théry et al. (2018), Cocozza et al. (2020).

Molecular mechanisms supporting and regulating EV biogenesis, cargo loading and EV release are multiple and vary between cell types (Colombo et al., 2014; van Niel et al., 2018). These mechanisms, because not fully understood, represent a field of intensive research. The endosomal sorting complex required for transport (ESCRT) machinery is intimately implicated in ILV and MVB biogenesis. ESCRT-0 and ESCRT-I recruit cargoes at the limiting membrane of endosomes and then recruit successively ESCRT-II and ESCRT-III to allow the membrane budding and abscission that generate ILVs (Raiborg and Stenmark, 2009; Schmidt and Teis, 2012). The PDZ protein syntenin, due to its interaction with the accessory ESCRT protein ALIX and together with ESCRT components also regulates ILV biogenesis. The syntenin pathway is responsible for the loading of syndecan heparan sulfate (HS) proteoglycan and cargo bound to syndecan, e.g., FGFR, in exosomes (Baietti et al., 2012; Friand et al., 2015). Heparanase, an enzyme that cleaves HS chains internally, stimulates syntenin-syndecan-ALIX budding in ILVs leading to an increase in exosomal secretion (Roucourt et al., 2015). Interestingly, syntenin was recently proposed as universal exosome biomarker (Kugeratski et al., 2021). Lipids are also important regulators of ILV/exosome biogenesis and secretion (reviewed by (Egea-Jimenez and Zimmermann, 2020)). Indeed, several studies implicate ceramide, or its producing enzyme, neutral sphingomyelinase, in exosome secretion (Trajkovic et al., 2008). Phospholipase D2 and its product Phosphatidic Acid (PA), are also key players in exosome biogenesis and secretion (Ghossoub et al., 2014). Tetraspanins, more specifically CD9, CD63, and CD81, are common exosomal membrane components and can influence exosomal loading by clustering cargoes in specific membrane microdomains (van Niel et al., 2018). Yet tetraspanins can also inhibit exosome production, as illustrated for Tetraspanin-6 that reroutes MVB cargoes to lysosomal degradation (Ghossoub et al., 2020).

Different sub-populations of exosomes have been described to emerge from different endosomal compartments/trafficking routes (Colombo et al., 2014; Blanc and Vidal, 2018). Depending on the cell type, exosomes can emerge from Rab11/35 recycling endosomes, or Rab27 late endosomes. Molecular machineries implicated in MVB fusion with the plasma membrane have been identified. SNARE [Soluble *N*-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptors] molecular machinery is widely implicated in vesicle fusion through formation of a complex between SNAREs present on the vesicles (v-SNAREs) and SNAREs present on the targeted membrane (t-SNAREs). The specific SNAREs involved in MVB fusion to plasma membrane, such as VAMP7 or SNAP23, vary depending on the cell type. Cortactin through its control of actin branching Arp2/3 complex activity and interaction with filamentous actin has also been involved in MVB fusion with plasma membrane (Sinha et al., 2016).

The biogenesis and release of microvesicles from the plasma membrane is influenced by phospholipid membrane constitution and actomyosin contractility (Clancy et al., 2021). In addition, some of the molecular machineries, including ESCRT machinery, used for MVB biogenesis have also been reported to be implicated in microvesicle budding and abscission from the plasma membrane (Hurley, 2015).

In the context of cancer, EVs are implicated in cancer cell growth, adhesion, motility, and invasion. They act on tumor cells but also on cells in the tumor microenvironment, promoting angiogenesis, dampening the immune system, and priming the metastatic niche (Becker et al., 2016; Peinado et al., 2017). Of importance, tumor cells have been shown to release significantly more EVs, compared to non-malignant cells, with numbers that increase with disease progression. Clearly, cancer EV cargoes are also different from normal cell EV cargoes. These alterations are triggered by diverse signals, coming from the tumor itself or from the tumor microenvironment, such as hypoxia or chemotherapeutic drugs (Bebelman et al., 2021). In breast cancer, the stiffness of ECM that is correlated to tumor

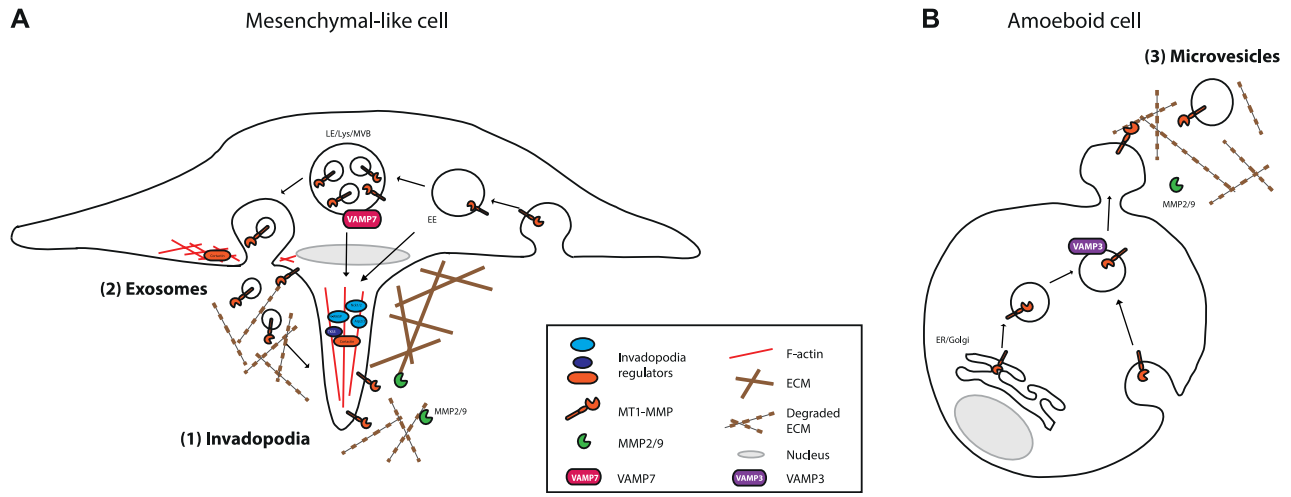


FIGURE 2 | MT1-MMP main vesicular trafficking routes and sites of release/secretion. **(A)** Mesenchymal invading cells deliver MT1-MMP containing vesicles to degradative actin-rich membrane structures, called invadopodia (1), and in the extracellular microenvironment as associated to exosomes (2), after fusion of MVB with the invadopodial plasma membrane. Invadopodia are preferential sites of exosome secretion and exosomes potentiate invadopodia formation and proteolytic activity. **(B)** Amoeboid/blebbing invading cells release MT1-MMP associated to microvesicles (3) shed from the plasma membrane. Newly synthetised (not depicted for mesenchymal cells) and recycled MT1-MMP traffick to these different locations. The endosomal SNARE, VAMP-7 is implicated in the trafficking of MT1-MMP-containing vesicles to invadopodia and associated to exosomes, whereas the endosomal SNARE, VAMP-3 delivers MT1-MMP to microvesicles shed from the plasma membrane. Please refer to the main text for further details. LE, Late Endosome; Lys, Lysosome: MVB, Multi Vesicular Bodies; EE, Early Endosome.

progression has been directly implicated in the increase of EV secretion and cancer cell migration (Patwardhan et al., 2021). Finally, several *in vivo* studies indicate that depletion of EVs reduces tumor progression and metastasis (Peinado et al., 2012; Kosaka et al., 2013; Tickner et al., 2014; Costa-Silva et al., 2015; Nishida-Aoki et al., 2017). EVs have a direct impact on ECM. For example, cancer cells use EVs coated with the ECM component fibronectin as a substrate for directional migration (Sung et al., 2015; Purushothaman et al., 2016). EVs can also carry proteases either sticking at their surface or embedded in their membrane, and therefore have impact on ECM remodeling and cancer cell invasiveness.

4 MMPS IN EXTRACELLULAR VESICLES AND RELATION WITH INVADOPODIA

4.1 EV-Associated MMPs and Their Contribution in ECM Remodeling

MMPs have been identified, among other proteases, as associated with EVs of different tissue origins and in different physiological and pathological conditions (Taraboletti et al., 2002; Hakulinen et al., 2008; Muralidharan-Chari et al., 2009; Rossé et al., 2014). EV-associated MMPs control ECM remodeling and shedding of receptors located either at EV membranes or at the surface of targeted cells (Shimoda and Khokha, 2017; Sanderson et al., 2019; Shimoda, 2019). Furthermore, some MMPs, such as MMP3, have been described to be delivered *via* EV to recipient cell to act intracellularly (Okusha et al., 2020). Compared to the display of MT-MMPs at the cell surface and even the secretion of MMPs in the pericellular environment, EV-associated MMPs are suggested to be more performant at long distance ECM remodeling. EV-associated MMPs have thereby been implicated in activating stromal cells, angiogenesis, and pre-metastatic niche formation (Shimoda and Khokha, 2017). Intriguingly, the amount of EVs and of EV-associated MMPs correlates with the invasive potential of cancer cells (Ginestra et al., 1998; Di Vizio et al., 2012). These observations indicate that MMPs associated with EVs might be used as biomarkers of disease progression and responsiveness to anti-cancer treatments. Of interest, using a nanopatterned microchip, Zhang et al. were able to monitor tumor metastasis through analysis of EV-associated MT1-MMP levels (Zhang et al., 2020).

Molecular machineries delivering MMPs in EVs are poorly understood. Yet, molecular mechanisms implicated in MMP delivery, especially that of MT1-MMP, to the extracellular space has been an intense field of research (Linder, 2007; Poincloux et al., 2009; Frittoli et al., 2011; Castro-Castro et al., 2016; Gifford and Itoh, 2019; Hey et al., 2021). MT1-MMP delivery to the extracellular microenvironment occurs through exocytosis at specialized plasma membrane domains such as lamellipodia and invadopodia, actin-rich cell protrusions with localized proteolytic activity generated by cancer cells (**Figure 2**). An intimate link between invadopodia and exosomes has been described. Below, we develop how these studies might provide a better understanding of MMPs loading in EVs and the biological impact of MMP present in EVs.

4.2 MMP Trafficking to Plasma Membrane and Invadopodia

MT1-MMP is considered as the major protease accounting for invadopodia proteolytic activity and has therefore been the major

MMP studied. Studies of MT1-MMP trafficking to specific plasma membrane domains indicate that the recycling of MT1-MMP is important for MT1-MMP proteolytic activity and thereby its pro-invasive function (Linder, 2007; Poincloux et al., 2009; Frittoli et al., 2011; Castro-Castro et al., 2016; Gifford and Itoh, 2019; Hey et al., 2021).

4.2.1 Importance of MT1-MMP Intracellular Domain in MT1-MMP Trafficking

MT1-MMP internalization, intracellular trafficking, plasma membrane recycling and degradation are mainly dictated by molecular determinants present in the short intracellular domain (20 amino acids) of MT1-MMP (**Figure 1B**). Some studies report that interaction of the extracellular hemopexin like domain of MT1-MMP with specific tetraspanins also regulates MT1-MMP trafficking and activity, positively and negatively, depending on the tetraspanin studied (Takino et al., 2003; Yañez-Mó et al., 2008; Lafleur et al., 2009; Schröder et al., 2013). Tetraspanin-enriched membrane domains act as platforms to selectively load specific cargoes in secretory MVBs (van Niel et al., 2018) and could be implicated in MT1-MMP loading in EVs.

MT1-MMP internalization is abrogated by MT1-MMP intracellular domain deletion (Nakahara et al., 1997; Lehti et al., 2000; Uekita et al., 2001). However, although MT1-MMP cell surface levels are increased and the enzyme is active, cells expressing this mutant MT1-MMP have impaired migratory and invasive capacities, indicating that MT1-MMP endocytosis/recycling/exocytosis cycles are important for MT1-MMP proteolytic activity (Remacle et al., 2003). More precisely, the LLY^{573} motif of MT1-MMP, interacting with the AP-2 clathrin adaptor, is required for MT1-MMP clathrin dependent endocytosis (Uekita et al., 2001). MT1-MMP is also internalized through other endocytic pathways, involving for example caveolae and flotillins, but the molecular features of MT1-MMP required for these types of endocytosis are not known (Remacle et al., 2003; Planchon et al., 2018). The metastasis-suppressor NME1 was recently reported to reduce the rate of MT1-MMP endocytosis in breast cancer cells by direct interaction with the cytoplasmic tail of MT1-MMP (Lodillinsky et al., 2021). Post-translational modifications of the MT1-MMP intracellular domain also influence its endocytosis. Phosphorylation of the Tyr^{573} by the kinases Src or LIMK has been reported to be required for MT1-MMP internalization (Nyalendo et al., 2007; Lagoutte et al., 2016). Phosphorylation of MT1-MMP Thr^{567} by protein kinase C (Moss et al., 2009; Williams and Coppolino, 2011) and palmitoylation of MT1-MMP Cys^{574} (Anilkumar et al., 2005) have also been described to promote MT1-MMP internalization and effects on cell invasion.

Interaction of MT1-MMP intracellular domain with filamentous actin (F-actin) is important for MT1-MMP endosomal trafficking and recycling. The LLY^{573} motif of the MT1-MMP C-terminal tail directly interacts with F-actin stabilizing MT1-MMP at degradative pseudopods of cells embedded in Matrigel (Yu et al., 2012). In contrast, interaction of the tumor suppressor MTCBP-1 (membrane-type 1 matrix metalloproteinase cytoplasmic tail-binding protein-1) with the PRR motif of MT1-MMP intracellular domain, displaces F-actin and inhibits invadopodia formation (Uekita et al., 2004; Qiang et al., 2019). MT1-MMP interaction with endosomal F-actin was also suggested to counteract MT1-MMP lysosomal degradation following the recruitment of the ESCRT-0 subunit Hrs to endosomal MT1-MMP-containing vesicles (MacDonald et al., 2018). The molecular mechanisms controlling the lysosomal degradation of MT1-MMP versus its recycling to plasma membrane/invadopodia deserve further studies.

The extreme C-terminal part of MT1-MMP intracellular domain corresponding to a class III PDZ binding motif (DKV^{582}) plays a major role in MT1-MMP recycling (**Figure 1B**). Pioneer studies indicated that the PDZ binding motif of MT1-MMP was required for MT1-MMP recycling without affecting its internalization (Wang X. et al., 2004). More recently, the PDZ protein Sorting Nexin 27 (SNX27) was reported to interact with MT1-MMP PDZ binding motif allowing the recruitment of the retromer complex to MT1-MMP containing Rab7a-positive endosomes and enabling MT1-MMP recycling to invadopodia (Sharma et al., 2020). Intriguingly, SNX27 does not interact with MT2-MMP although MT2-MMP also contains a class III PDZ binding motif (EWV) (Pei, 1999; Sharma et al., 2020). The PDZ domain containing LIMK kinase also interacts with MT1-MMP PDZ binding motif, this interaction being required for MT1-MMP Tyr^{573} phosphorylation and cortactin accumulation to MT1-MMP endosomal vesicles (Lagoutte et al., 2016). Multiple different PDZ domain containing proteins interact with MT-MMP PDZ binding motifs regulating their activity and trafficking (Wang P. et al., 2004; Roghi et al., 2010). These results suggest that PDZ protein networks could be envisioned as fine tuners of MT-MMPs trafficking. Furthermore, monoubiquitination of MT1-MMP at Lys^{581} was found to depend on Src activity and to be necessary for MT1-MMP recycling to the plasma membrane (Eisenach et al., 2012).

Overall, these studies indicate that the LLY^{573} motif of the MT1-MMP C-terminal tail plays a major role in the regulation of MT1-MMP endocytosis and MT1-MMP stabilization at plasma membrane actin-rich domains, whereas the extreme C-terminal PDZ binding motif (DKV^{582}) of MT1-MMP is mainly involved in its recycling.

4.2.2 Molecular Machineries Implicated in MT1-MMP Endosomal Trafficking

Not surprisingly, Rab GTPases, key players in endosomal trafficking, play crucial roles in MT1-MMP delivery to specialized plasma membrane domains. The late endosome/lysosome (LE/Lys) Rab7- and LAMP1-positive endosomal compartment appears to be acting as a major MT1-MMP reservoir, albeit MT1-MMP recycling to the plasma membrane also occurs from early endosomes. Delivery of these MT1-MMP containing vesicles to invadopodia is dependent on the exocyst complex (Sakurai-Yageta, 2008; Monteiro, 2013), the retromer (Sharma et al., 2020), as well as different SNAREs. VAMP7 (Ti-VAMP) v-SNARE present on LE/Lys vesicles containing MT1-

MMP, in concert with SNAP23 and Syntaxin4, is required for MT1-MMP delivery to invadopodia (Miyata et al., 2004; Steffen et al., 2008; Williams et al., 2014). SNAP23/Syntaxin13/VAMP3 are also involved in MT1-MMP trafficking to the plasma membrane (Kean et al., 2009). In LOX melanoma cells, however, VAMP3 is not required for MT1-MMP delivery to invadopodia, but is for MT1-MMP delivery to microvesicles, i.e., EVs directly shed from the plasma membrane (Clancy et al., 2015). VAMP3-specific loading of MT1-MMP into microvesicles is suggested to depend on the interaction of MT1-MMP with CD9, a tetraspanin implicated in the sorting of specific EV cargoes (Clancy et al., 2015). For more details on the regulation of MT1-MMP trafficking, we refer the readers to seminal reviews on the subject (Poincloux et al., 2009; Frittoli et al., 2011; Castro-Castro et al., 2016; Gifford and Itoh, 2019; Hey et al., 2021).

Why MT1-MMP recycling is important for regulation of MT1-MMP proteolytic activity is not fully understood. Fluorescent recovery after photobleaching (FRAP) experiments indicate that MT1-MMP associated with invadopodia is less mobile than MT1-MMP located in non-invadopodial regions of the plasma membrane (Yu et al., 2012). Thus, polarized recycling of MT1-MMP to invadopodial actin-rich plasma membrane domains would somehow permit MT1-MMP stabilization. We can also surmise that MT1-MMP recycling ultimately also favors MT1-MMP release as an exosome-associated factor.

4.3 Functional Interplay Between Invadopodia and Exosomes

Invadopodia are dynamic degradative actin-rich membrane protrusions elaborated by various cancer cells (Linder et al., 2011; Murphy and Courtneidge, 2011; Eddy et al., 2017). Their physiological counterparts, called podosomes, are elaborated by specialized normal cells, such as macrophages, monocytes, endothelial cells, and osteoclasts. Invadopodia and podosomes allow pericellular ECM proteolysis. In the context of cancer, invadopodia are required for tumor cells to break the basement membrane and to invade through interstitial matrix. They are therefore seen as key players in cancer cell invasiveness and metastasis. Although podosome and invadopodia morphologies differ, they share a common machinery necessary for their degradative function. Indeed, these structures are composed of structural and signaling proteins such as cortactin, cofilin, N-WASP, Arp2/3, Tks4/5 that control the reorganization of the actin cytoskeleton, and the release of proteases involved in matrix degradation (Linder et al., 2011; Murphy and Courtneidge, 2011). Invadopodia formation is a multistep process: 1. initiation, 2. assembly, 3. maturation and 4. disassembly. Firstly, diverse signals, such as growth factors and ECM stiffness, induce actin cytoskeleton reorganization leading to the formation of precursor invadopodia devoid of degradative activity. Then the precursor invadopodia are stabilized and serve as platforms for the recruitment of MMP-containing vesicles, leading to a mature, fully functional invadopodium.

Molecular machineries implicated in secretory MVB fusion with the plasma membrane and in delivery of MT1-MMP-containing vesicles to invadopodia are overlapping. For example, the SNAREs Ti-VAMP/VAMP7 and SNAP23 are necessary for delivery of MT1-MMP-containing vesicles to invadopodia (Steffen et al., 2008; Williams et al., 2014) and are also implicated in secretory MVB fusion with the plasma membrane (Fader et al., 2009; Wei et al., 2017) (**Figure 2**). Another example is cortactin. Cortactin, through its function as an activator of the branched actin nucleator Arp2/3 complex and binder of F-actin, is necessary for the formation of invadopodial membrane protrusions (Artym et al., 2006). In some cell types, cortactin is also required for the recruitment of MT1-MMP containing vesicles to invadopodia to permit their maturation (Clark et al., 2007). Further studies have shown that cortactin is more generally implicated in vesicular trafficking, localizing at the surface of endosomes and at the cell cortex (Kirkbride et al., 2011). In collaboration with Rab27a, cortactin was shown to participate in MVB docking to invadopodia (Sinha et al., 2016).

Furthermore, Hoshino et al. demonstrated that invadopodia and exosomes are intimately linked (Hoshino et al., 2013). Indeed, invadopodia were identified as preferential docking sites for CD63- and Rab27a-positive MVBs. Also, mechanistically, invadopodia formation and exosome secretion are somehow related. Indeed, inhibition of invadopodia formation, by means of Tks5, N-WASP or cortactin depletion, inhibited exosome secretion (Seals et al., 2005; Murphy and Courtneidge, 2011; Hoshino et al., 2013; Sinha et al., 2016). Inversely, invadopodia induction, through expression of a constitutively active form of PI3K (Yamaguchi et al., 2011), enhanced exosome secretion (Hoshino et al., 2013). Impact of the machinery implicated in invadopodia formation on MVB biogenesis was not directly addressed, so we cannot conclude whether the observed effects are reflecting impact on MVB formation or on MVB fusion with invadopodia-specific plasma membrane domains. This also raises the question of what plasma membrane domains compose preferential docking sites for MVBs, if any exist, in cells not forming invadopodia. Could these be secreted at lamellipodia which are also actin-rich structures? Reciprocally, inhibition of exosome production, through Hrs/ESCRT depletion or sphingomyelinase inhibition, the two main pathways implicated in MVB biogenesis, or inhibition of vesicle secretion, through depletion of Rab27a or Synaptotagmin-7, two factors implicated in MVB docking to plasma membrane, inhibited invadopodia formation (Hoshino et al., 2013). Overall, invadopodia seem to be required for exosome secretion, and exosome secretion be required for invadopodia formation in cells forming invadopodia (or to go hand in hand). However, induction of exosome secretion, through overexpression of Rab27b (Ostrowski et al., 2010), does not seem to be sufficient to induce invadopodia formation in cells that do not form invadopodia, such as MCF7 cells (Beghein et al., 2018). Interestingly, exosome-enriched fractions were able to potentiate invadopodia formation and stability (Hoshino et al., 2013). Intriguingly,

ECM stiffness has been shown to increase invadopodia formation (Alexander et al., 2008) and to enhance exosome secretion and modify exosome contents (e.g., MMPs) (Patwardhan et al., 2021), supporting the notion of an intricate relationship between invadopodia and exosomes.

Melanoma cancer cells form invadopodia when seeded on rigid matrix (adopting a mesenchymal-like phenotype) and release less microvesicles (i.e., EVs-enriched in 10 000g pellets) than the same cells adopting an amoeboid-like phenotype when seeded on more compliant matrix (Sedgwick et al., 2015). Exosome release by amoeboid-like cells, however, has not been carefully analyzed. It is worth noticing, that even though amoeboid type of migration seems to be less dependent on ECM proteolysis compared to mesenchymal type of migration (Wolf et al., 2003; Wolf and Friedl, 2011; Orgaz et al., 2014), the degradative potential of microvesicles shed by amoeboid-like cells is high (Muralidharan-Chari et al., 2009; Sedgwick et al., 2015). This suggests that MMPs present on microvesicles might influence the invasive potential of tumor microenvironment cells rather than of the tumor cells themselves.

5 CONCLUDING REMARKS

MMPs are important for ECM remodeling during physiological processes and in pathological conditions, such as cancer. MMPs are exposed to the microenvironment at two main locations: 1) the cell surface, at specialized plasma membrane domains, such as invadopodia of cancer cells, and 2) associated to EVs (**Figure 2**). We can envision that cell surface-associated MMPs and EV-associated MMPs have distinct activities. MMPs associated with cell surfaces, through pericellular ECM remodeling, might obviously have a major autocrine function, whereas EV-associated MMPs might act mainly at distance influencing tumor microenvironment cells activities rather than influencing the producing cell activities. To address this point, it would be necessary to be able to follow EV-associated MMP activity *in vivo*. Furthermore, ECM composition and biophysical properties seem to influence the subtype of EVs released by a cell (i.e., exosomes versus microvesicles) (**Figure 2**). Cells evolving in a compliant matrix would preferentially release microvesicles, whereas cells evolving on stiffer matrix would release exosomes through invadopodia. Thus, cancer cells release MMPs in EVs, in addition to, but independently from, the secretion of MMPs involved in local tissue invasion. This suggests that a prominent role of EV-associated MMPs could be to influence tumor microenvironment at a distance, and, taking advantage of their circulation in body fluids, priming of the pre-metastatic niche. An intimate relationship exists between exosome secretion and invadopodia. This suggests that molecular features of the MT1-MMP intracellular domain implicated in MT1-MMP internalization and trafficking in different endosomal compartments before its release at invadopodia, might also be implicated in MT1-MMP loading in ILVs of MVBs. It would thereby be of interest to analyze the contribution of factors implicated in the loading of specific cargoes in EVs, such as the syntenin/ALIX pathway, and tetraspanin-enriched microdomains, in the loading of MT1-MMP/MMPs in EVs. This knowledge could be used to design molecules that would restrain MT1-MMP presentation at the cell surface or at the surface of EVs with the aim to inhibit the pro-tumorigenic activity of EVs. We could also use such knowledge to engineer EVs with an enhanced capacity to degrade the ECM and thereby EVs with a higher capacity to deliver therapeutics embedded in EVs.

AUTHOR CONTRIBUTIONS

ST, RG, GD, and PZ contributed to manuscript writing. ST designed figures. All authors contributed to the article and approved the submitted version.

FUNDING

The work in the laboratory is supported by the Agence Nationale de la Recherche (ANR-18-CE13-0017, Project SynTEV), the Fund for Scientific Research—Flanders (FWO, G0C5718N) and the internal funds of the KU Leuven (C14/20/105).

REFERENCES

Alaseem, A., Alhazzani, K., Dondapati, P., Alobid, S., Bishayee, A., and Rathinavelu, A. (2019). Matrix Metalloproteinases: A Challenging Paradigm of Cancer Management. *Seminars Cancer Biol.* 56, 100–115. doi:10.1016/j.semcancer.2017.11.008

Alexander, N. R., Branch, K. M., Parekh, A., Clark, E. S., Iwueke, I. C., Guelcher, S. A., et al. (2008). Extracellular Matrix Rigidity Promotes Invadopodia Activity. *Curr. Biol.* 18, 1295–1299. doi:10.1016/j.cub.2008.07.090

Anilkumar, N., Uekita, T., Couchman, J. R., Nagase, H., Seiki, M., and Itoh, Y. (2005). Palmitoylation at Cys574 is Essential for MT1-MMP to Promote Cell Migration. *FASEB J.* 19, 1326–1328. doi:10.1096/fj.04-3651fje

Artym, V. V., Zhang, Y., Seillier-Moiseiwitsch, F., Yamada, K. M., and Mueller, S. C. (2006). Dynamic Interactions of Cortactin and Membrane Type 1 Matrix Metalloproteinase at Invadopodia: Defining the Stages of Invadopodia Formation and Function. *Cancer Res.* 66, 3034–3043. doi:10.1158/0008-5472.CAN-05-2177

Baietti, M. F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., et al. (2012). Syndecan-syntenin-ALIX Regulates the Biogenesis of Exosomes. *Nat. Cell Biol.* 14, 677–685. doi:10.1038/ncb2502

Barbolina, M., and Stack, M. (2008). Membrane Type 1-matrix Metalloproteinase: Substrate Diversity in Pericellular Proteolysis. *Seminars Cell Dev. Biol.* 19, 24–33. doi:10.1016/j.semcdb.2007.06.008

Bebelman, M. P., Janssen, E., Pegtel, D. M., and Crudden, C. (2021). The Forces Driving Cancer Extracellular Vesicle Secretion. *Neoplasia* 23, 149–157. doi:10.1016/j.neo.2020.11.011

Becker, A., Thakur, B. K., Weiss, J. M., Kim, H. S., Peinado, H., and Lyden, D. (2016). Extracellular Vesicles in Cancer: Cell-To-Cell Mediators of Metastasis. *Cancer Cell* 30, 836–848. doi:10.1016/j.ccell.2016.10.009

Beghein, E., Devriese, D., Van Hoey, E., and Gettemans, J. (2018). Cortactin and Fascin-1 Regulate Extracellular Vesicle Release by Controlling Endosomal Trafficking or Invadopodia Formation and Function. *Sci. Rep.* 8, 15606. doi:10.1038/s41598-018-33868-z

Blanc, L., and Vidal, M. (2018). New Insights into the Function of Rab GTPases in the Context of Exosomal Secretion. *Small GTPases* 9, 95–106. doi:10.1080/21541248.2016.1264352

Bonnans, C., Chou, J., and Werb, Z. (2014). Remodelling the Extracellular Matrix in Development and Disease. *Nat. Rev. Mol. Cell Biol.* 15, 786–801. doi:10.1038/nrm3904

Brinckerhoff, C. E., and Matrisian, L. M. (2002). Matrix Metalloproteinases: A Tail of a Frog that Became a Prince. *Nat. Rev. Mol. Cell Biol.* 3, 207–214. doi:10.1038/nrm763

Castro-Castro, A., Marchesin, V., Monteiro, P., Lodillinsky, C., Rossé, C., and Chavrier, P. (2016). Cellular and Molecular Mechanisms of MT1-MMP-Dependent Cancer Cell Invasion. *Annu. Rev. Cell Dev. Biol.* 32, 555–576. doi:10.1146/annurev-cellbio-111315-125227

Clancy, J. W., Sedgwick, A., Rosse, C., Muralidharan-Chari, V., Raposo, G., Method, M., et al. (2015). Regulated Delivery of Molecular Cargo to Invasive Tumour-Derived Microvesicles. *Nat. Commun.* 6, 6919. doi:10.1038/ncomms7919

Clancy, J. W., Schmidtmann, M., and D'Souza-Schorey, C. (2021). The Ins and Outs of Microvesicles. *FASEB BioAdv.* 3, 399–406. doi:10.1096/fba.2020-00127

Clark, E. S., Whigham, A. S., Yarbrough, W. G., and Weaver, A. M. (2007). Cortactin is an Essential Regulator of Matrix Metalloproteinase Secretion and Extracellular Matrix Degradation in Invadopodia. *Cancer Res.* 67, 4227–4235. doi:10.1158/0008-5472.CAN-06-3928

Cocozza, F., Grisard, E., Martin-Jaular, L., Mathieu, M., and Théry, C. (2020). SnapShot: Extracellular Vesicles. *Cell* 182, 262–262. doi:10.1016/j.cell.2020.04.054

Colombo, M., Raposo, G., and Théry, C. (2014). Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles. *Annu. Rev. Cell Dev. Biol.* 30, 255–289. doi:10.1146/annurev-cellbio-101512-122326

Costa-Silva, B., Aiello, N. M., Ocean, A. J., Singh, S., Zhang, H., Thakur, B. K., et al. (2015). Pancreatic Cancer Exosomes Initiate Pre-metastatic Niche Formation in the Liver. *Nat. Cell Biol.* 17, 816–826. doi:10.1038/ncb3169

Deryugina, E. I., Ratnikov, B. I., Postnova, T. I., Rozanov, D. V., and Strongin, A. Y. (2002). Processing of Integrin αv Subunit by Membrane Type 1 Matrix Metalloproteinase Stimulates Migration of Breast Carcinoma Cells on Vitronectin and Enhances Tyrosine Phosphorylation of Focal Adhesion Kinase. *J. Biol. Chem.* 277, 9749–9756. doi:10.1074/jbc.M110269200

Di Vizio, D., Morello, M., Dudley, A. C., Schow, P. W., Adam, R. M., Morley, S., et al. (2012). Large Oncosomes in Human Prostate Cancer Tissues and in the Circulation of Mice with Metastatic Disease. *Am. J. Pathology* 181, 1573–1584. doi:10.1016/j.ajpath.2012.07.030

Dufour, A., and Overall, C. M. (2013). Missing the Target: Matrix Metalloproteinase Antitargets in Inflammation and Cancer. *Trends Pharmacol. Sci.* 34, 233–242. doi:10.1016/j.tips.2013.02.004

Eddy, R. J., Weidmann, M. D., Sharma, V. P., and Condeelis, J. S. (2017). Tumor Cell Invadopodia: Invasive Protrusions that Orchestrate Metastasis. *Trends Cell Biol.* 27, 595–607. doi:10.1016/j.tcb.2017.03.003

Egea-Jimenez, A. L., and Zimmermann, P. (2020). Lipids in Exosome Biology. *Handb. Exp. Pharmacol.* 259, 309–336. doi:10.1007/164_2019_220

Egeblad, M., and Werb, Z. (2002). New Functions for the Matrix Metalloproteinases in Cancer Progression. *Nat. Rev. Cancer* 2, 161–174. doi:10.1038/nrc745

Eisenach, P. A., de Sampaio, P. C., Murphy, G., and Roghi, C. (2012). Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) Ubiquitination at Lys581 Increases Cellular Invasion through Type I Collagen. *J. Biol. Chem.* 287, 11533–11545. doi:10.1074/jbc.M111.306340

Endo, K., Takino, T., Miyamori, H., Kinsen, H., Yoshizaki, T., Furukawa, M., et al. (2003). Cleavage of Syndecan-1 by Membrane Type Matrix Metalloproteinase-1 Stimulates Cell Migration. *J. Biol. Chem.* 278, 40764–40770. doi:10.1074/jbc.M306736200

Fader, C. M., Sánchez, D. G., Mestre, M. B., and Colombo, M. I. (2009). TI-VAMP/VAMP7 and VAMP3/cellubrevin: Two V-SNARE Proteins Involved in Specific Steps of the Autophagy/multivesicular Body Pathways. *Biochim. Biophys. Acta Mol. Cell Res.* 1793, 1901–1916. doi:10.1016/j.bbamcr.2009.09.011

Friand, V., David, G., and Zimmermann, P. (2015). Syntenin and Syndecan in the Biogenesis of Exosomes. *Biol. Cell* 107, 331–341. doi:10.1111/boc.201500010

Frittoli, E., Palamidessi, A., Disanza, A., and Scita, G. (2011). Secretory and Endo/exocytic Trafficking in Invadopodia Formation: The MT1-MMP Paradigm. *Eur. J. Cell Biol.* 90, 108–114. doi:10.1016/j.ejcb.2010.04.007

Ghossoub, R., Lembo, F., Rubio, A., Gaillard, C. B., Bouchet, J., Vitale, N., et al. (2014). Syntenin-ALIX Exosome Biogenesis and Budding into Multivesicular Bodies are Controlled by ARF6 and PLD2. *Nat. Commun.* 5, 3477. doi:10.1038/ncomms4477

Ghossoub, R., Chéry, M., Audebert, S., Leblanc, R., Egea-Jimenez, A. L., Lembo, F., et al. (2020). Tetraspanin-6 Negatively Regulates Exosome Production. *Proc. Natl. Acad. Sci. U.S.A.* 117, 5913–5922. doi:10.1073/pnas.1922447117

Gifford, V., and Itoh, Y. (2019). MT1-MMP-dependent Cell Migration: Proteolytic and Non-proteolytic Mechanisms. *Biochem. Soc. Trans.* 47, 811–826. doi:10.1042/BST20180363

Ginestra, A., La Placa, M. D., Saladino, F., Cassarà, D., Nagase, H., and Vittorelli, M. L. (1998). The Amount and Proteolytic Content of Vesicles Shed by Human Cancer Cell Lines Correlates with Their *in Vitro* Invasiveness. *Anticancer Res.* 18, 3433–3437.

Hakulinen, J., Sankkila, L., Sugiyama, N., Lehti, K., and Keski-Oja, J. (2008). Secretion of Active Membrane Type 1 Matrix Metalloproteinase (MMP-14) into Extracellular Space in Microvesicular Exosomes. *J. Cell. Biochem.* 105, 1211–1218. doi:10.1002/jcb.21923

Hey, S., Ratt, A., and Linder, S. (2021). There and Back Again: Intracellular Trafficking, Release and Recycling of Matrix Metalloproteinases. *Biochim. Biophys. Acta Mol. Cell Res.* 1869, 119189. doi:10.1016/j.bbamcr.2021.119189

Hoshino, D., Kirkbride, K. C., Costello, K., Clark, E. S., Sinha, S., Grega-Larson, N., et al. (2013). Exosome Secretion is Enhanced by Invadopodia and Drives Invasive Behavior. *Cell Rep.* 5, 1159–1168. doi:10.1016/j.celrep.2013.10.050

Hurley, J. H. (2015). ESCRT S are Everywhere. *EMBO J.* 34, 2398–2407. doi:10.15252/embj.201592484

Kajita, M., Itoh, Y., Chiba, T., Mori, H., Okada, A., Kinoh, H., et al. (2001). Membrane-Type 1 Matrix Metalloproteinase Cleaves CD44 and Promotes Cell Migration. *J. Cell Biol.* 153, 893–904. doi:10.1083/jcb.153.5.893

Kalluri, R., and LeBleu, V. S. (2020). The Biology, Function, and Biomedical Applications of Exosomes. *Science* 367, eaau6977. doi:10.1126/science.aau6977

Kean, M. J., Williams, K. C., Skalski, M., Myers, D., Burtnik, A., Foster, D., et al. (2009). VAMP3, Syntaxin-13 and SNAP23 are Involved in Secretion of Matrix Metalloproteinases, Degradation of the Extracellular Matrix and Cell Invasion. *J. Cell Sci.* 122, 4089–4098. doi:10.1242/jcs.052761

Kessenbrock, K., Plaks, V., and Werb, Z. (2010). Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell* 141, 52–67. doi:10.1016/j.cell.2010.03.015

Kirkbride, K. C., Sung, B. H., Sinha, S., and Weaver, A. M. (2011). Cortactin: A Multifunctional Regulator of Cellular Invasiveness. *Cell Adhesion Migr.* 5, 187–198. doi:10.4161/cam.5.2.14773

Kosaka, N., Iguchi, H., Hagiwara, K., Yoshioka, Y., Takeshita, F., and Ochiya, T. (2013). Neutral Sphingomyelinase 2 (nSMase2)-dependent Exosomal Transfer of Angiogenic microRNAs Regulate Cancer Cell Metastasis. *J. Biol. Chem.* 288, 10849–10859. doi:10.1074/jbc.M112.446831

Kugeratski, F. G., Hodge, K., Lilla, S., McAndrews, K. M., Zhou, X., Hwang, R. F., et al. (2021). Quantitative Proteomics Identifies the Core Proteome of Exosomes with Syntenin-1 as the Highest Abundant Protein and a Putative Universal Biomarker. *Nat. Cell Biol.* 23, 631–641. doi:10.1038/s41556-021-00693-y

Lafleur, M. A., Xu, D., and Hemler, M. E. (2009). Tetraspanin Proteins Regulate Membrane Type-1 Matrix Metalloproteinase-dependent Pericellular Proteolysis. *MBoC* 20, 2030–2040. doi:10.1091/mbc.e08-11-1149

Lagoutte, E., Villeneuve, C., Lafanechère, L., Wells, C. M., Jones, G. E., Chavrier, P., et al. (2016). LIMK Regulates Tumor-Cell Invasion and Matrix Degradation Through Tyrosine Phosphorylation of MT1-MMP. *Sci. Rep.* 6, 24925. doi:10.1038/srep24925

Lehti, K., Valtanen, H., Wickström, S., Lohi, J., and Keski-Oja, J. (2000). Regulation of Membrane-Type-1 Matrix Metalloproteinase Activity by its Cytoplasmic Domain. *J. Biol. Chem.* 275, 15006–15013. doi:10.1074/jbc.M910220199

Linder, S., Wiesner, C., and Himmel, M. (2011). Degrading Devices: Invadosomes in Proteolytic Cell Invasion. *Annu. Rev. Cell Dev. Biol.* 27, 185–211. doi:10.1146/annurev-cellbio-092910-154216

Linder, S. (2007). The Matrix Corroded: Podosomes and Invadopodia in Extracellular Matrix Degradation. *Trends Cell Biol.* 17, 107–117. doi:10.1016/j.tcb.2007.01.002

Lodillinsky, C., Fuhrmann, L., Irondelle, M., Pylypenko, O., Li, X.-Y., Bonsang-Kitzis, H., et al. (2021). Metastasis-suppressor NME1 Controls the Invasive Switch of Breast Cancer by Regulating MT1-MMP Surface Clearance. *Oncogene* 40, 4019–4032. doi:10.1038/s41388-021-01826-1

MacDonald, E., Brown, L., Selvais, A., Liu, H., Waring, T., Newman, D., et al. (2018). HRS-WASH axis Governs Actin-Mediated Endosomal Recycling and Cell Invasion. *J. Cell Biol.* 217, 2549–2564. doi:10.1083/jcb.201710051

Miyata, T., Ohnishi, H., Suzuki, J., Yoshikumi, Y., Ohno, H., Mashima, H., et al. (2004). Involvement of Syntaxin 4 in the Transport of Membrane-type 1 Matrix Metalloproteinase to the Plasma Membrane in Human Gastric Epithelial Cells. *Biochem. Biophysical Res. Commun.* 323, 118–124. doi:10.1016/j.bbrc.2004.08.064

Monteiro, P., Rossé, C., Castro-Castro, A., Irondelle, M., Lagoutte, E., Paul-Gilloteaux, P., et al. (2013). Endosomal WASH and Exocyst Complexes Control Exocytosis of MT1-MMP at Invadopodia. *J. Cell Biol.* 203, 1063–1079. doi:10.1083/jcb.201306162

Moss, N. M., Wu, Y. I., Liu, Y., Munshi, H. G., and Stack, M. S. (2009). Modulation of the Membrane Type 1 Matrix Metalloproteinase Cytoplasmic Tail Enhances Tumor Cell Invasion and Proliferation in Three-Dimensional Collagen Matrices. *J. Biol. Chem.* 284, 19791–19799. doi:10.1074/jbc.M109.020362

Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M., Chavrier, P., Raposo, G., et al. (2009). ARF6-regulated Shedding of Tumor Cell-Derived Plasma Membrane Microvesicles. *Curr. Biol.* 19, 1875–1885. doi:10.1016/j.cub.2009.09.059

Murphy, D. A., and Courtneidge, S. A. (2011). The 'ins' and 'outs' of Podosomes and Invadopodia: Characteristics, Formation and Function. *Nat. Rev. Mol. Cell Biol.* 12, 413–426. doi:10.1038/nrm3141

Nakahara, H., Howard, L., Thompson, E. W., Sato, H., Seiki, M., Yeh, Y., et al. (1997). Transmembrane/cytoplasmic Domain-Mediated Membrane Type 1-matrix Metalloprotease Docking to Invadopodia is Required for Cell Invasion. *Proc. Natl. Acad. Sci. U.S.A.* 94, 7959–7964. doi:10.1073/pnas.94.15.7959

Niland, S., Riscanevo, A. X., and Eble, J. A. (2021). Matrix Metalloproteinases Shape the Tumor Microenvironment in Cancer Progression. *Int. J. Mol. Sci.* 23, 146. doi:10.3390/ijms23010146

Nishida-Aoki, N., Tominaga, N., Takeshita, F., Sonoda, H., Yoshioka, Y., and Ochiya, T. (2017). Disruption of Circulating Extracellular Vesicles as a Novel Therapeutic Strategy against Cancer Metastasis. *Mol. Ther.* 25, 181–191. doi:10.1016/j.ymthe.2016.10.009

Nyalendo, C., Michaud, M., Beaulieu, E., Roghi, C., Murphy, G., Gingras, D., et al. (2007). Src-dependent Phosphorylation of Membrane Type I Matrix Metalloproteinase on Cytoplasmic Tyrosine 573. *J. Biol. Chem.* 282, 15690–15699. doi:10.1074/jbc.M608045200

Okusha, Y., Eguchi, T., Tran, M. T., Sogawa, C., Yoshida, K., Itagaki, M., et al. (2020). Extracellular Vesicles Enriched with Moonlighting Metalloproteinase are Highly Transmissive, Pro-Tumorigenic, and Trans-Activates Cellular Communication Network Factor (CCN2/CTGF): CRISPR against Cancer. *Cancers* 12, 881. doi:10.3390/cancers12040881

Orgaz, J. L., Pandya, P., Dalmeida, R., Karagiannis, P., Sanchez-Laorden, B., Viros, A., et al. (2014). Diverse Matrix Metalloproteinase Functions Regulate Cancer Amoeboid Migration. *Nat. Commun.* 5, 4255. doi:10.1038/ncomms5255

Ostrowski, M., Carmo, N. B., Krumeich, S., Fanget, I., Raposo, G., Savina, A., et al. (2010). Rab27a and Rab27b Control Different Steps of the Exosome Secretion Pathway. *Nat. Cell Biol.* 12, 19–30. doi:10.1038/ncb2000

Patwardhan, S., Mahadik, P., Shetty, O., and Sen, S. (2021). ECM Stiffness-Tuned Exosomes Drive Breast Cancer Motility through Thrombospondin-1. *Biomaterials* 279, 121185. doi:10.1016/j.biomaterials.2021.121185

Pei, D. (1999). Identification and Characterization of the Fifth Membrane-type Matrix Metalloproteinase MT5-MMP. *J. Biol. Chem.* 274, 8925–8932. doi:10.1074/jbc.274.13.8925

Peinado, H., Alečković, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., et al. (2012). Melanoma Exosomes Educate Bone Marrow Progenitor Cells toward a Pro-metastatic Phenotype through MET. *Nat. Med.* 18, 883–891. doi:10.1038/nm.2753

Peinado, H., Zhang, H., Matei, I. R., Costa-Silva, B., Hoshino, A., Rodrigues, G., et al. (2017). Pre-metastatic Niches: Organ-specific Homes for Metastases. *Nat. Rev. Cancer* 17, 302–317. doi:10.1038/nrc.2017.6

Planchon, D., Rios Morris, E., Genest, M., Comunale, F., Vacher, S., Bièche, I., et al. (2018). MT1-MMP Targeting to Endolysosomes is Mediated by Flotillin Upregulation of Flotillins. *J. Cell Sci.* 131, jcs218925. doi:10.1242/jcs.218925

Poincloux, R., Lizárraga, F., and Chavrier, P. (2009). Matrix Invasion by Tumour Cells: A Focus on MT1-MMP Trafficking to Invadopodia. *J. Cell Sci.* 122, 3015–3024. doi:10.1242/jcs.034561

Purushothaman, A., Bandari, S. K., Liu, J., Mobley, J. A., Brown, E. E., and Sanderson, R. D. (2016). Fibronectin on the Surface of Myeloma Cell-Derived Exosomes Mediates Exosome-Cell Interactions. *J. Biol. Chem.* 291, 1652–1663. doi:10.1074/jbc.M115.686295

Qiang, L., Cao, H., Chen, J., Weller, S. G., Krueger, E. W., Zhang, L., et al. (2019). Pancreatic Tumor Cell Metastasis is Restricted by MT1-MMP Binding Protein MTCBP-1. *J. Cell Biol.* 218, 317–332. doi:10.1083/jcb.201802032

Raiborg, C., and Stenmark, H. (2009). The ESCRT Machinery in Endosomal Sorting of Ubiquitylated Membrane Proteins. *Nature* 458, 445–452. doi:10.1038/nature07961

Ratnikov, B. I., Rozanov, D. V., Postnova, T. I., Baciu, P. G., Zhang, H., DiScipio, R. G., et al. (2002). An Alternative Processing of Integrin αv Subunit in Tumor Cells by Membrane Type-1 Matrix Metalloproteinase. *J. Biol. Chem.* 277, 7377–7385. doi:10.1074/jbc.M109580200

Remacle, A., Murphy, G., and Roghi, C. (2003). Membrane Type I-Matrix Metalloproteinase (MT1-MMP) is Internalised by Two Different Pathways and is Recycled to the Cell Surface. *J. Cell Sci.* 116, 3905–3916. doi:10.1242/jcs.00710

Roghi, C., Jones, L., Gratian, M., English, W. R., and Murphy, G. (2010). Golgi Reassembly Stacking Protein 55 Interacts with Membrane-type (MT) 1-matrix Metalloprotease (MMP) and Furin and Plays a Role in the Activation of the MT1-MMP Zymogen. *FEBS J.* 277, 3158–3175. doi:10.1111/j.1742-4658.2010.07723.x

Rossé, C., Lodillinsky, C., Fuhrmann, L., Nourieh, M., Monteiro, P., Irondelle, M., et al. (2014). Control of MT1-MMP Transport by Atypical PKC during Breast-Cancer Progression. *Proc. Natl. Acad. Sci. U.S.A.* 111, E1872–E1879. doi:10.1073/pnas.1400749111

Roucourt, B., Meeussen, S., Bao, J., Zimmermann, P., and David, G. (2015). Heparanase Activates the Syndecan-Syntenin-ALIX Exosome Pathway. *Cell Res.* 25, 412–428. doi:10.1038/cr.2015.29

Sanderson, R. D., Bandari, S. K., and Vlodavsky, I. (2019). Proteases and Glycosidases on the Surface of Exosomes: Newly Discovered Mechanisms for Extracellular Remodeling. *Matrix Biol.* 75-76, 160–169. doi:10.1016/j.matbio.2017.10.007

Sato, S., and Weaver, A. M. (2018). Extracellular Vesicles: Important Collaborators in Cancer Progression. *Essays Biochem.* 62, 149–163. doi:10.1042/EBC20170080

Sakurai-Yageta, M., Recchi, C., Le Dez, G., Sibarita, J.-B., Daviet, L., Camonis, J., et al. (2008). The Interaction of IQGAP1 with the Exocyst Complex is Required for Tumor Cell Invasion Downstream of Cdc42 and RhoA. *J. Cell Biol.* 181, 985–998. doi:10.1083/jcb.200709076

Schmidt, O., and Teis, D. (2012). The ESCRT Machinery. *Curr. Biol.* 22, R116–R120. doi:10.1016/j.cub.2012.01.028

Schröder, H. M., Hoffmann, S. C., Hecker, M., Korff, T., and Ludwig, T. (2013). The Tetraspanin Network Modulates MT1-MMP Cell Surface Trafficking. *Int. J. Biochem. Cell Biol.* 45, 1133–1144. doi:10.1016/j.biocel.2013.02.020

Seals, D. F., Azucena, E. F., Pass, I., Tesfay, L., Gordon, R., Woodrow, M., et al. (2005). The Adaptor Protein Tks5/Fish is Required for Podosome Formation and Function, and for the Protease-Driven Invasion of Cancer Cells. *Cancer Cell* 7, 155–165. doi:10.1016/j.ccr.2005.01.006

Sedgwick, A. E., Clancy, J. W., Olivia Balmert, M., and D'Souza-Schorey, C. (2015). Extracellular Microvesicles and Invadopodia Mediate Non-overlapping Modes of Tumor Cell Invasion. *Sci. Rep.* 5, 14748. doi:10.1038/srep14748

Sharma, P., Parveen, S., Shah, L. V., Mukherjee, M., Kalaidzidis, Y., Kozielski, A. J., et al. (2020). SNX27-retromer Assembly Recycles MT1-MMP to Invadopodia and Promotes Breast Cancer Metastasis. *J. Cell Biol.* 219, e201812098. doi:10.1083/jcb.201812098

Shimoda, M., and Khokha, R. (2017). Metalloproteinases in Extracellular Vesicles. *Biochim. Biophys. Acta Mol. Cell Res.* 1864, 1989–2000. doi:10.1016/j.bbamcr.2017.05.027

Shimoda, M. (2019). Extracellular Vesicle-Associated MMPs: A Modulator of the Tissue Microenvironment. *Adv. Clin. Chem.* 88, 35–66. doi:10.1016/bs.acc.2018.10.006

Sinha, S., Hoshino, D., Hong, N. H., Kirkbride, K. C., Grega-Larson, N. E., Seiki, M., et al. (2016). Cortactin Promotes Exosome Secretion by Controlling Branched Actin Dynamics. *J. Cell Biol.* 214, 197–213. doi:10.1083/jcb.201601025

Steffen, A., Le Dez, G., Poincloux, R., Recchi, C., Nassoy, P., Rottner, K., et al. (2008). MT1-MMP-dependent Invasion is Regulated by TI-VAMP/VAMP7. *Curr. Biol.* 18, 926–931. doi:10.1016/j.cub.2008.05.044

Sung, B. H., Ketova, T., Hoshino, D., Zijlstra, A., and Weaver, A. M. (2015). Directional Cell Movement Through Tissues is Controlled by Exosome Secretion. *Nat. Commun.* 6, 7164. doi:10.1038/ncomms8164

Takino, T., Miyamori, H., Kawaguchi, N., Uekita, T., Seiki, M., and Sato, H. (2003). Tetraspanin CD63 Promotes Targeting and Lysosomal Proteolysis of Membrane-type 1 Matrix Metalloproteinase. *Biochem. Biophys. Res. Commun.* 304, 160–166. doi:10.1016/s0006-291x(03)00544-8

Taraboletti, G., D'Ascenzo, S., Borsotti, P., Giavazzi, R., Pavan, A., and Dolo, V. (2002). Shedding of the Matrix Metalloproteinases MMP-2, MMP-9, and MT1-MMP as Membrane Vesicle-Associated Components by Endothelial Cells. *Am. J. Pathol.* 160, 673–680. doi:10.1016/S0002-9440(10)64887-0

Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2018). Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): A Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracell. Vesicles* 7, 1535750. doi:10.1080/20013078.2018.1535750

Tickner, J. A., Urquhart, A. J., Stephenson, S.-A., Richard, D. J., and O'Byrne, K. J. (2014). Functions and Therapeutic Roles of Exosomes in Cancer. *Front. Oncol.* 4, 127. doi:10.3389/fonc.2014.00127

Tkach, M., and Théry, C. (2016). Communication by Extracellular Vesicles: Where We are and Where We Need to Go. *Cell* 164, 1226–1232. doi:10.1016/j.cell.2016.01.043

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., et al. (2008). Ceramide Triggers Budding of Exosome Vesicles into Multivesicular Endosomes. *Science* 319, 1244–1247. doi:10.1126/science.1153124

Uekita, T., Itoh, Y., Yana, I., Ohno, H., and Seiki, M. (2001). Cytoplasmic Tail-dependent Internalization of Membrane-type 1 Matrix Metalloproteinase is Important for its Invasion-Promoting Activity. *J. Cell Biol.* 155, 1345–1356. doi:10.1083/jcb.200108112

Uekita, T., Gotoh, I., Kinoshita, T., Itoh, Y., Sato, H., Shiomi, T., et al. (2004). Membrane-type 1 Matrix Metalloproteinase Cytoplasmic Tail-Binding Protein-1 is a New Member of the Cupin Superfamily. *J. Biol. Chem.* 279, 12734–12743. doi:10.1074/jbc.M309957200

van Niel, G., D'Angelo, G., and Raposo, G. (2018). Shedding Light on the Cell Biology of Extracellular Vesicles. *Nat. Rev. Mol. Cell Biol.* 19, 213–228. doi:10.1038/nrm.2017.125

Van Wart, H. E., and Birkedal-Hansen, H. (1990). The Cysteine Switch: A Principle of Regulation of Metalloproteinase Activity with Potential Applicability to the Entire Matrix Metalloproteinase Gene Family. *Proc. Natl. Acad. Sci. U.S.A.* 87, 5578–5582. doi:10.1073/pnas.87.14.5578

Wang, P., Wang, X., and Pei, D. (2004). Mint-3 Regulates the Retrieval of the Internalized Membrane-type Matrix Metalloproteinase, MT5-MMP, to the Plasma Membrane by Binding to its Carboxyl End Motif EWV. *J. Biol. Chem.* 279, 20461–20470. doi:10.1074/jbc.M400264200

Wang, X., Ma, D., Keski-Oja, J., and Pei, D. (2004). Co-recycling of MT1-MMP and MT3-MMP through the Trans-Golgi Network. *J. Biol. Chem.* 279, 9331–9336. doi:10.1074/jbc.M312369200

Wei, Y., Wang, D., Jin, F., Bian, Z., Li, L., Liang, H., et al. (2017). Pyruvate Kinase Type M2 Promotes Tumour Cell Exosome Release via Phosphorylating Synaptosome-Associated Protein 23. *Nat. Commun.* 8, 14041. doi:10.1038/ncomms14041

Williams, K. C., and Coppolino, M. G. (2011). Phosphorylation of Membrane Type 1-matrix Metalloproteinase (MT1-MMP) and its Vesicle-Associated Membrane Protein 7 (VAMP7)-dependent Trafficking Facilitate Cell Invasion and Migration. *J. Biol. Chem.* 286, 43405–43416. doi:10.1074/jbc.M111.297069

Williams, K. C., McNeilly, R. E., and Coppolino, M. G. (2014). SNAP23, Syntaxin4, and Vesicle-Associated Membrane Protein 7 (VAMP7) Mediate Trafficking of Membrane Type 1-matrix Metalloproteinase (MT1-MMP) during Invadopodium Formation and Tumor Cell Invasion. *MBoC* 25, 2061–2070. doi:10.1091/mbc.E13-10-0582

Winkler, J., Abisoye-Ogunniyan, A., Metcalf, K. J., and Werb, Z. (2020). Concepts of Extracellular Matrix Remodelling in Tumour Progression and Metastasis. *Nat. Commun.* 11, 5120. doi:10.1038/s41467-020-18794-x

Wolf, K., and Friedl, P. (2011). Extracellular Matrix Determinants of Proteolytic and Non-proteolytic Cell Migration. *Trends Cell Biol.* 21, 736–744. doi:10.1016/j.tcb.2011.09.006

Wolf, K., Mazo, I., Leung, H., Engelke, K., von Andrian, U. H., Deryugina, E. I., et al. (2003). Compensation Mechanism in Tumor Cell Migration: Mesenchymal-Amoeboid Transition after Blocking of Pericellular Proteolysis. *J. Cell Biol.* 160, 267–277. doi:10.1083/jcb.200209006

Yamaguchi, H., Yoshida, S., Muroi, E., Yoshida, N., Kawamura, M., Kouchi, Z., et al. (2011). Phosphoinositide 3-kinase Signaling Pathway Mediated by P110α Regulates Invadopodia Formation. *J. Cell Biol.* 193, 1275–1288. doi:10.1083/jcb.201009126

Yañez-Mó, M., Barreiro, O., Gonzalo, P., Batista, A., Megías, D., Genís, L., et al. (2008). MT1-MMP Collagenolytic Activity Is Regulated through Association with Tetraspanin CD151 in Primary Endothelial Cells. *Blood* 112, 3217–3226. doi:10.1182/blood-2008-02-139394

Yu, X., Zech, T., McDonald, L., Gonzalez, E. G., Li, A., Macpherson, I., et al. (2012). N-WASP Coordinates the Delivery and F-Actin-Mediated Capture of MT1-MMP at Invasive Pseudopods. *J. Cell Biol.* 199, 527–544. doi:10.1083/jcb.201203025

Zhang, P., Wu, X., Gardashova, G., Yang, Y., Zhang, Y., Xu, L., et al. (2020). Molecular and Functional Extracellular Vesicle Analysis Using Nanopatterned Microchips Monitors Tumor Progression and Metastasis. *Sci. Transl. Med.* 12, eaaz2878. doi:10.1126/scitranslmed.aaz2878

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

MINI REVIEW
published: 01 June 2022
doi: 10.3389/fcell.2022.883841

Unconventional Secretion of Plant Extracellular Vesicles and Their Benefits to Human Health: A Mini Review

Joshua T. Farley[1†], *Mahmoud K. Eldahshoury*[1†] *and Carine de Marcos Lousa*[1,2*]

[1]Biomedical Sciences, School of Health, Leeds Beckett University, Leeds, United Kingdom, [2]Centre for Plant Sciences, University of Leeds, Leeds, United Kingdom

Mechanisms devoted to the secretion of proteins via extracellular vesicles (EVs) have been found in mammals, yeasts, and plants. Since they transport a number of leader-less proteins to the plasma membrane or the extracellular space, EVs are considered part of Unconventional protein secretion (UPS) routes. UPS involving EVs are a relatively new field in plants. Aside from their role in plant physiology and immunity, plant extracts containing EVs have also been shown to be beneficial for human health. Therefore, exploring the use of plant EVs in biomedicine and their potential as drug delivery tools is an exciting avenue. Here we give a summary of the state of knowledge on plant EVs, their crosstalk with mammalian systems and potential research routes that could lead to practical applications in therapeutic drug delivery.

Keywords: extracellular vesicles, unconventional protein secretion (UPS), plant EVs, biomedicine, biopharming, exosomes

OPEN ACCESS

Edited by:
Marioara Chiritoiu-Butnaru, Institute of Biochemistry of the Romanian Academy, Romania

Reviewed by:
Hailing Jin, University of California, Riverside, United States
Gian Pietro Di Sansebastiano, University of Salento, Italy

***Correspondence:**
Carine de Marcos Lousa
c.de-marcos-lousa@leedsbeckett.ac.uk; fbscd@leeds.ac.uk

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *25 February 2022*
Accepted: *09 May 2022*
Published: *01 June 2022*

Citation:
Farley JT, Eldahshoury MK and de Marcos Lousa C (2022) Unconventional Secretion of Plant Extracellular Vesicles and Their Benefits to Human Health: A Mini Review. Front. Cell Dev. Biol. 10:883841. doi: 10.3389/fcell.2022.883841

1 INTRODUCTION

Extracellular vesicles (EVs) are a collection of vesicles with different origins, size ranges, and molecular composition. Originally considered as cellular waste, their discovery has revolutionised our understanding of cell-cell communications and transfer of biological information from 1 cell to another. Since leaderless proteins loaded in these vesicles bypass the Golgi and are recruited in EVs from the cytosol, most EVs are considered part of the unconventional secretion pathway (UPS). Exosomes, a particular type of EV, are particularly interesting in this context for the following reasons: the mechanism of cargo loading *in vivo* and *in vitro* are being better understood in human cells (Xu et al., 2020), exosomes have the ability to cross natural barriers (Blood brain barrier and placenta) and are described as safe and stable nanoparticles (Banks et al., 2020; Elliott and He, 2021). Consequently, mammalian exosomes are being investigated for their potential in drug delivery (Xu et al., 2020; Choi et al., 2021). Plants also secrete extracellular vesicles, and exosomes have been identified (He et al., 2021). While keeping the benefits of human exosomes, the use of plant exosomes as drug delivery tools in biomedicine might offer various additional advantages such as lower production costs involved in biopharming and reduced cross-human contaminations. In this mini-review, we are summarising the current knowledge on plant UPS specifically focusing on EVs and

Abbreviations: EVs, extracellular vesicles; ER, endoplasmic reticulum; PM, plasma membrane; MVB, multivesicular body; TGN, trans-Golgi network; EE, Early endosome; ILVs, Intraluminal vesicles

exosomes. We are then clarifying the extraction procedures of various plant EVs and finally we are proposing a view on the potential benefits of using plant EVs as drug delivery tools in human health.

2 Linking UPS and EVs in Mammals and Plants

2.1 Mammalian UPS and EVs

Unconventional protein secretion (UPS) involves a range of mechanisms that allow proteins to reach the extracellular medium, bypassing at least part of the conventional ER-Golgi-PM secretory pathway. While this conventional pathway usually involves the presence of signal peptides at the N-terminus of proteins, UPS leads to the secretion of leaderless soluble proteins in the extracellular medium or trafficking of membrane proteins via an alternative route than through the Golgi (Rabouille et al., 2012; Rabouille, 2017). These mechanisms are being intensively studied in mammals and yeasts because they are often associated with stress and pathologies such as inflammatory diseases or cancer (Kim et al., 2018; Cohen et al., 2020). Therefore, understanding the mechanisms of UPS is a promising new route into identifying new therapeutic targets. Extracellular vesicles, in particular, represent a specific type of vesicular UPS that has been extensively studied since their discovery 40 years ago (Harding et al., 2013). Their ability to pack biological information which is then transmitted to adjacent or long-distance cells have triggered extensive research into their use as a drug delivery system. There are various types of extracellular vesicles that can be classified depending on their origin and content (Théry et al., 2018). This classification is constantly updated with new knowledge. Exosomes, a specific class of small EVs (sEVs) released by the fusion of MVBs with the membrane, are of particular interest for targeted drug delivery since they have been shown to cross natural barriers such as the Blood brain barrier and placenta (for review Elliott and He, 2021). The use of mammalian exosomes in drug delivery presents various advantages described above but also some challenges (Meng et al., 2020; Chen et al., 2021). Three of these challenges are the lack of homogeneity, the lack of large-scale cost-effective production, and ethical issues linked with transferring human material.

2.2 Plant UPS and EVs

To address some of these challenges in terms of cost-effective production and lack of ethical issues, plants might offer an alternative source of exosomes and EVs. As a result, a growing number of studies are looking into their potential health benefits. For example, the effect of plant extracellular vesicles loaded with curcumin are currently being tested in clinical trials (NCT01294072) to evaluate their impact on surgery of newly diagnosed colon cancer patients (https://clinicaltrials.gov/ct2/show/NCT01294072).

Unfortunately, plant unconventional protein secretion pathways have attracted only late interests and our current knowledge of plant UPS and EVs is growing but still limited (Ding et al., 2014a; Robinson et al., 2016; Hansen and Nielsen 2017; Cui et al., 2019). The presence of leaderless proteins in apoplastic extracellular vesicles has confirmed that these EVs represent genuine plant UPS pathways involved in cell wall remodelling and resistance to infection (Delaunois et al., 2013, 2014). Investigations around these vesicular mechanisms have uncovered the existence of at least three pathways that result in the release of extracellular vesicles in plants: exocyst-positive organelle mediated secretion (EXPO), vesicle budding from the PM (including microvesicles), and multivesicular body (MVB)-PM fusion (Wang et al., 2010; Regente et al., 2012; Cui et al., 2019). A growing number of studies report the beneficial effect of crude and pure extracts of plant EVs on human health (Akuma et al., 2019; Alfieri et al., 2021; Urzì et al., 2021). To evaluate their potential as drug delivery tools, the current state of the field in terms of plant EVs classification, purification, and biomedical applications is presented below.

3 Plant EV Classification and Isolation

3.1 Plant EV Subtypes and Biogenesis

The term "plant extracellular vesicles" generally refers to apoplastic vesicles. Plant-derived nanovesicles (PDNVs) or exosomes-like nanoparticles (ELNs) are terms used to refer to vesicles that have been isolated from total plant extracts and usually contain a mix of EVs and other cellular microvesicles (Pinedo et al., 2021). Since the identification of specific markers for different EV subclasses is only recent, the classification of plant EVs is not well established, but three main classes have been described (Cai et al., 2021). One class involves EXPO vesicles secreted into the apoplast after the fusion of EXPO double membrane organelles with the plasma membrane. The second class includes microvesicles (or ectosomes), suggested to be smaller (150nm-1um) and originate by budding from the plasma membrane. Finally, exosomes (30–150 nm) are the third class of plant EVs and are released by fusion of MVBs (containing intraluminal vesicle) with the plasma membrane (**Figure 1**). The mechanisms by which all these fusions and releases in the extracellular space occur are not well understood in plants.

While Exo70E2 protein has been identified as a marker of EXPO vesicles, it has been reported that exosomes specifically contain TET8, a tetraspanin protein (Wang et al., 2010; Cai et al., 2018). This assumption is supported by the fact that TET8 is a plant orthologue for the human exosomal marker CD63 (Théry et al., 2018). In addition, the density of TET8 fraction (1.12–1.19 g/ml) isolated at 100,000 g correlates with the density of human exosomes, and TET8 is found to colocalize with MVB markers (He et al., 2021). Microvesicles, on the other hand, appear to be positive for the syntaxin SYP121, which has often been referred to as PEN1 (Ding et al., 2014; Rutter and Innes, 2017; He et al., 2021). The SYP121/PEN1-positive fraction appears to be slightly less dense (1.029–1.056 g/ml), and contains larger vesicles ranging from 50 to 300 nm that can be pelleted at 40 000 g (Rutter and Innes, 2017). SYP121/PEN1 has also been reported to be involved in Golgi-PM trafficking, reinforcing the fact that SYP121/PEN1 positive vesicles might not be of MVB origin (Nielsen et al., 2012; He et al., 2021).

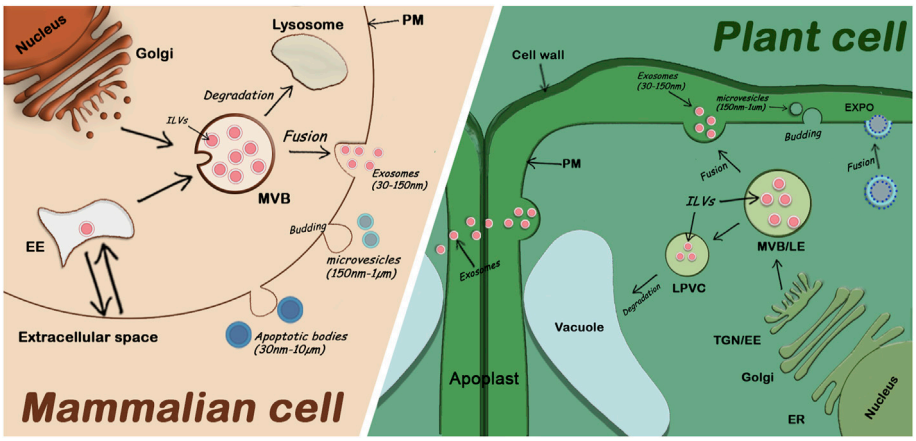


FIGURE 1 | Comparison of extracellular vesicle secretion in mammalian cells and plant cells. Mammalian EVs including apoptotic bodies, microvesicles, and exosomes are secreted in the extracellular medium. Plant EVs are also secreted in the extracellular medium (the apoplast). Exosomes are secreted by fusion of MVBs with the PM, EXPO vesicles are also secreted by fusion with the PM while microvesicles and apoptotic bodies are released through budding of the PM. EE: Early Endosome; ER: Endoplasmic Reticulum; ILVs: Intraluminal Vesicles; LE: Late Endosome; LPVC: Late Pre-vacuolar Compartment; MVB: Multivesicular Body; PM: Plasma Membrane; TGN: Trans Golgi Network; (proportions of organelle sizes not conserved).

3.2 Plant EV Isolations for Drug Delivery

The processes described to isolate plant EVs depend on the nature of the plant material. Apoplastic fluids are usually extracted from leaves, while blending/juicing is performed on fruits or roots. EVs can also be isolated from liquid plant exudates (Araya et al., 2015). Although the purities of different EV fractions will vary, they have all been found to have therapeutic potential in biomedicine.

3.2.1 Apoplastic Washing

The apoplast is the space outside the plasma membrane of plant cells where material can freely move (Sattelmacher, 2001). Although it is unknown how EVs cross the cell wall, their presence in the apoplast has been confirmed (Regente et al., 2012; Rutter et al., 2017; He et al., 2021). To recover these vesicles, a standard technique based on vacuum-infiltration and ultracentrifugation is performed (O'Leary et al., 2014). Applying sequential rounds of negative and atmospheric pressure onto leaves forces a buffer into the apoplastic space that can be recovered after centrifugation of the leaf. This method ensures that plant cells remain mostly undamaged and results in a relatively pure fraction containing EVs but depleted of intracellular components. It has been mostly used to purify EVs from leaf material (*Arabidopsis thaliana*, Nicotiana benthamiana) or seeds (sunflower) (Regente et al., 2009; Rutter and Innes, 2017; Zhang et al., 2020). Additional purification steps will allow further isolation of different types of EVs as described above (Regente et al., 2009; Rutter and Innes, 2017; He et al., 2021). Recently, a comparative analysis of two major methods for isolating EVs from apoplastic wash fluids has provided a guide into the selection of the right method adapted to the type of downstream applications desired (Huang et al., 2021).

3.2.2 Blending or Juice Extraction

Enriched EV fractions have been obtained through blending plant matter such as ginger roots, herbs, wheat, and dandelion (Mu et al., 2014; Xiao et al., 2018; Chen et al., 2019). Juicing of citrus fruits, pears, grapefruit, watermelons, and coconut water has also been used to prepare EV extracts (Liang et al., 2015; Raimondo et al., 2015; Xiao et al., 2018; Zhao et al., 2018). However, unless they are subjected to further purification steps, these methods often result in a mix of EVs and intracellular content (vesicles, organelles, membranes), meaning they are not solely products of UPS (Pinedo et al., 2021). They are, therefore, referred to as Plant-derived nanovesicles (PDNVs) or Exosome-like nanovesicles (ELNs) rather than EVs which refer to the purer fractions. There is increasing evidence that these PDNVs have significant biological effects on human cells and have brought new hope into novel forms of natural drug delivery systems (Di Gioia et al., 2020; Alfieri et al., 2021; Urzì et al., 2021).

3.2.3 Plant Exudates

Plant exudates are substances excreted from plants that include liquids flowing through and out of plants. This includes sap, gum, resins or root exudates. They have been used for many years in traditional medicine. Exudates contain many bioactive compounds, amongst them peptides, with beneficial effects on human health such as reduction of oedema and inflammation (Licá et al., 2018). Plant EVs derived from exudates are a relatively new research topic. EVs isolated from the sap of two plants (namely Dendropanax morbifera, and Pinus densiflora) have shown cytotoxic and anti-metastatic effects on human tumour cells (Kim et al., 2020a; Kim et al., 2020b). Furthermore, EVs from a hydroponic solution containing tomato (Solanum lycopersicum L.) root exudates were shown to inhibit the spore germination of three fungal phytopathogens (*Fusarium* oxysporum, Botrytis cinerea and *Alternaria alternata*) suggesting an antifungal activity in plants (De Palma et al., 2020). Whether this activity can be applied to mammalian fungal pathogens has not been tested. More research is needed to understand if exudates EVs could hold promising therapeutic applications.

4 Plant EVs as a Drug Delivery Tool

Plants have been known for centuries to be beneficial for human health. Yet the identification of extracellular vesicles and their molecular content shed a new light on our understanding of cross-kingdom interaction and transfer of bioactive molecules.

4.1 Benefits of Plant PDNVs Bioactive Compounds

In the past decade, numerous reports have described the beneficial effects of plant PDNVs/EVs in mammalian health. While PDNV proteomes from various plant origins have been characterised and some common proteins frequently identified in these vesicles, the variety of PDNVs and the lack of specific protein markers limits their classification which may prove problematic for large scale good manufacturing practices (GMPs). Nevertheless, PDNVs contain a range of bioactive molecules such as proteins, lipids, or metabolites with therapeutic effects summarised in (Woith et al., 2019; Di Gioia et al., 2020; Kocak et al., 2020; Alfieri et al., 2021; Urzì et al., 2021). Amongst the most studied plant PDNVs are those originating from ginger. These EV-containing PDNV isolates have many natural therapeutic potentials and can induce physiological changes in mammals. They were shown to influence the human gut microbiota (Teng et al., 2018), inhibit inflammasome activation (Chen, Zhou and Yu, 2019), and found to have a positive effect on inflammatory bowel disease and colitis-associated cancer (Zhang et al., 2016). They have also been shown to be taken up by, and inhibit the pathogenicity of, the periodontitis-causing Porphyromonas gingivalis (Sundaram et al., 2019). In parallel, wheat derived nanovesicles have been shown to aid *in vitro* wound healing by promoting proliferation and migration of dermal fibroblasts, endothelial, and epithelial cells (Şahin et al., 2018). Nanovesicles derived from various fruits and vegetables were also shown to inhibit cancer cell growth (Kameli et al., 2021). Despite their numerous health benefits, it is unclear however, if this positive impact is attributable to the combined action of various bioactive components in the crude fraction or to particular compounds that may be isolated from purer EVs preparations.

4.2 Plant EV Engineering and Biopharming

Research on EVs (obtained from the apoplast of plants) as potential drug delivery systems is far more restricted than those on PDNVs. So far, to our knowledge, only one study has shown that purified apoplastic small EVs (sEVs) are efficiently taken up by human ovarian cancer cells OVAR5 (Liu et al., 2020). This paper compared the uptake of apoplastic sEVs (purified from the apoplast of Arabidopsis leaves) and nanovesicles (obtained from disrupted leaf material). OVAR5 cells were found to be significantly more susceptible to apoplastic sEV uptake than leaf nanovesicle uptake, based on elevated numbers of fluorescent cells. These results suggest that pure EV samples have the same, if not greater, drug delivery potentials than PDNV isolates have, and that EVs may be the contributing factor to PDNV success. Unfortunately, to our knowledge, this is the only study that uses purified apoplastic EVs in human cells and more data is required to conclude. In addition, an assessment of immunogenicity and toxicity should be undertaken to validate pure plant EVs as a drug delivery system.

Based on the successes of PDNVs, efficient uptake of sEVs, and the potential of engineering exosomes in plants, biopharming is an attractive solution to produce cheap pharmaceuticals with a rapid turnover. Biopharming, or plant molecular farming, refers to the use of genetic tools to produce a wide range of pharmaceuticals. Plants have already been used to produce antibodies and vaccines for humans, animals, and aquaculture (Shoji et al., 2012; Takeyama et al., 2015; Yao et al., 2015; Lefebvre and Lécuyer, 2017; Zahara et al., 2017; Su et al., 2021). Recently, plants have been explored as a rapid alternative biofactory for the production of COVID vaccines through the expression of Virus-like particles exposing an immunogenic part of the Spike S protein (Dhama et al., 2020; Maharjan and Choe, 2021). Regarding clinical trials, intravenous administration of β-glucocerebrosidase protein expressed in carrots has been approved as being safe and efficient and successfully used for 2 decades (Shaaltiel et al., 2015). The advantages of using plants as Biofactories include their ability to produce functional proteins in large amounts, and at lower costs (Shaaltiel et al., 2007). One additional advantage is the possibility of relatively simple engineering associated with plants, potentially allowing *in vivo* packaging of exogenous cargo into EVs, ready for extraction. More data on the mechanisms of loading into plant EVs is still required, but with this possibility in mind, and given that delivery of therapeutic molecules by mammalian EVs has already been demonstrated by several studies (Alvarez-Erviti et al., 2011; Batrakova and Kim, 2016; Elsharkasy et al., 2020), biopharming plants to isolate therapeutic pure EVs is a very exciting avenue that needs to be explored.

4.3 Administration and Bioavailability

If plant EVs are to be potential drug delivery systems, their administration and bioavailability must be considered. The first strong evidence of cross kingdom effects was provided when isolated PDNVs were fed to mice and found to reach intestinal macrophages. The vesicle uptake in these cells increased the expression of interleukins and alleviated colitis symptoms (Ju et al., 2013; Mu et al., 2014). This study has demonstrated that PDNVs are able to resist gastric and intestinal digestion, suggesting oral administration methods of plant nanoparticles are suitable for targeting these organs. In order to reach other organs, alternative administration methods have been investigated. In particular, intravenous injection is normally considered to have the advantage of avoiding the first-pass effect of hepatic metabolism, producing the highest bioavailability. When intravenous administration of edible tea flower nanoparticles was compared to oral administration, no difference was noted in terms of body weight and main pro-inflammatory cytokines levels. However, a sharp increase of complement C3 concentrations was detected, suggesting a slight immune reaction induced by these nanoparticles when they are administered intravenously (IV) (Chen et al., 2022). Other studies have suggested that IV administration of ginger derived exosome-like nanovesicles (GDELN) did not promote an immune reaction, though only body weight was examined (Li et al., 2018). The slight

immune reaction induced by repetitive intravenous injection of EVs appears non-specific to plant EVs since a mild immune response has also been reported for human EVs (Saleh et al., 2019). The authors found that EVs purified from different sources could induce different responses. Therefore, this could also be the case for plant EVs, and more information needs to be collected before a conclusion could be drawn on intravenous injections of plant EVs. In parallel, one study has reported that intranasal administration of engineered grapefruit-derived nanovectors (GNVs) could slow down tumour brain progression in mice (Zhuang et al., 2016). This brings hope for the use of plant EVs as therapeutic tools in neurodegenerative diseases. It is noticeable that EV biodistribution changes with the administration method. While intravenous injection of mammalian and plant EVs results in the wide uptake by various organs (including spleen, liver, kidney, lung, heart, and brain) (Lai et al., 2014; Garaeva et al., 2021), the gut is more specifically targeted in oral administration of edible EVs (Ju et al., 2013; Mu et al., 2014; Zhang et al., 2016; Deng et al., 2017; Teng et al., 2018). In addition, plant EVs have been shown to penetrate a human skin model, which encourages their consideration for skin care treatments (Lee et al., 2020). Altogether, the data accumulated suggests that specific administration methods would have to be developed depending on the pathology targeted and that plant EVs present a lot of potential in therapeutic processes.

5 CONCLUSION

Extracellular vesicles (EVs) are associated with Unconventional protein secretion (UPS) routes. They are released in the extracellular space through mechanisms that are still poorly understood. The field of plant EVs is relatively new but is proving to have great prospects in biomedicine. The potential to produce pure plant EV subtypes such as exosomes through biopharming and be able to deliver therapeutic molecules is very appealing. Additional advantages include the engineering capability of *in vivo* cargo loading associated with low production costs and easy extraction procedures. Before validating plant EVs as putative drug delivery tools, further research investigating their toxicity and immunogenicity needs to be undertaken. In addition, a more robust composition and characterization of plant EVs is also essential in order to standardise production for good manufacturing practice (GMPs). Nevertheless, preliminary data seem very promising such as the efficient uptake of plant EVs by human cells, their expected low immunogenic character (associated with nutrition) and their positive effect on human health. As a consequence, using plant EVs as a drug delivery tool might represent a powerful future alternative to classical therapeutic systems.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

JF and ME are supported by a PhD scholarship from Leeds Beckett University.

REFERENCES

Akuma, P., Okagu, O. D., and Udenigwe, C. C. (2019). Naturally Occurring Exosome Vesicles as Potential Delivery Vehicle for Bioactive Compounds. *Front. Sustain. Food Syst.* 3, 23.doi:10.3389/fsufs.2019.00023

Alfieri, M., Leone, A., and Ambrosone, A. (2021). Plant-Derived Nano and Microvesicles for Human Health and Therapeutic Potential in Nanomedicine. *Pharmaceutics* 13 (4), 498. doi:10.3390/pharmaceutics13040498

Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S., and Wood, M. J. A. (2011). Delivery of siRNA to the Mouse Brain by Systemic Injection of Targeted Exosomes. *Nat. Biotechnol.* 29, 341–345. doi:10.1038/nbt.1807

Araya, T., Bohner, A., and von Wirén, N. (2015). Extraction of Apoplastic Wash Fluids and Leaf Petiole Exudates from Leaves of *Arabidopsis thaliana*. *Bio-protocol* 5 (24), e1691. doi:10.21769/bioprotoc.1691

BanksSharma, W. A. P., Sharma, P., Bullock, K. M., Hansen, K. M., Ludwig, N., and Whiteside, T. L. (2020). Transport of Extracellular Vesicles across the Blood-Brain Barrier: Brain Pharmacokinetics and Effects of Inflammation. *Ijms* 21 (12), 4407. doi:10.3390/ijms21124407

Batrakova, E. V., and Kim, M. S. (2016). Development and Regulation of Exosome-based Therapy Products. *WIREs Nanomed Nanobiotechnol* 8 (5), 744–757. doi:10.1002/wnan.1395

Cai, Q., He, B., Wang, S., Fletcher, S., Niu, D., Mitter, N., et al. (2021). Message in a Bubble: Shuttling Small RNAs and Proteins between Cells and Interacting Organisms Using Extracellular Vesicles. *Annu. Rev. Plant Biol.* 72 (June), 497–524. doi:10.1146/annurev-arplant-081720-010616

Cai, Q., Qiao, L., Wang, M., He, B., Lin, F.-M., Palmquist, J., et al. (2018). Plants Send Small RNAs in Extracellular Vesicles to Fungal Pathogen to Silence Virulence Genes. *Science* 360, 1126–1129. doi:10.1126/science.aar4142

Chen, H., Wang, L., Zeng, X., Schwarz, H., Nanda, H. S., Peng, X., et al. (2021). Exosomes, a New Star for Targeted Delivery. *Front. Cell. Dev. Biol.* 9, 751079. doi:10.3389/fcell.2021.751079

Chen, Q., Li, Q., Liang, Y., Zu, M., Chen, N., Canup, B. S. B., et al. (2022). Natural Exosome-like Nanovesicles from Edible Tea Flowers Suppress Metastatic Breast Cancer via ROS Generation and Microbiota Modulation. *Acta Pharm. Sin. B* 12, 907–923. doi:10.1016/j.apsb.2021.08.016

Chen, X., Zhou, Y., and Yu, J. (2019). Exosome-like Nanoparticles from Ginger Rhizomes Inhibited NLRP3 Inflammasome Activation. *Mol. Pharm.* 16 (6), 2690–2699. doi:10.1021/acs.molpharmaceut.9b00246

Choi, H., Choi, Y., Yim, H. Y., Mirzaaghasi, A., Yoo, J.-K., and Choi, C. (2021). Biodistribution of Exosomes and Engineering Strategies for Targeted Delivery of Therapeutic Exosomes. *Tissue Eng. Regen. Med.* 18 (4), 499–511. doi:10.1007/s13770-021-00361-0

Cohen, M. J., Chirico, W. J., and Lipke, P. N. (2020). Through the Back Door: Unconventional Protein Secretion. *Cell. Surf.* 6, 100045. doi:10.1016/j.tcsw.2020.100045

Cui, Y., Gao, J., He, Y., and Jiang, L. (2019). Plant Extracellular Vesicles. *Protoplasma* 257, 3–12. doi:10.1007/s00709-019-01435-6

De Palma, M., Ambrosone, A., Leone, A., Del Gaudio, P., Ruocco, M., Turiák, L., et al. (2020). Plant Roots Release Small Extracellular Vesicles with Antifungal Activity. *Plants* 9 (12), 1777. doi:10.3390/plants9121777

Delaunois, B., Colby, T., Belloy, N., Conreux, A., Harzen, A., Baillieul, F., et al. (2013). Large-scale Proteomic Analysis of the Grapevine Leaf Apoplastic Fluid Reveals Mainly Stress-Related Proteins and Cell Wall Modifying Enzymes. *BMC Plant Biol.* 13, 24. doi:10.1186/1471-2229-13-24

Delaunois, B., Jeandet, P., ClÃ©ment, C., Baillieul, F., Dorey, S. p., and Cordelier, S. (2014). Uncovering Plant-Pathogen Crosstalk through Apoplastic Proteomic Studies. *Front. Plant Sci.* 5, 249. doi:10.3389/fpls.2014.00249

Deng, Z., Rong, Y., Teng, Y., Mu, J., Zhuang, X., Tseng, M., et al. (2017). Broccoli-Derived Nanoparticle Inhibits Mouse Colitis by Activating Dendritic Cell AMP-Activated Protein Kinase. *Mol. Ther.* 25 (7), 1641–1654. doi:10.1016/j.ymthe.2017.01.025

Dhama, K., Natesan, S., Iqbal Yatoo, M., Patel, S. K., Tiwari, R., Saxena, S. K., et al. (2020). Plant-based Vaccines and Antibodies to Combat COVID-19: Current Status and Prospects. *Hum. vaccines Immunother.* 16 (12), 2913–2920. doi:10.1080/21645515.2020.1842034

Di Gioia, S., Hossain, M. N., and Conese, M. (2020). Biological Properties and Therapeutic Effects of Plant-Derived Nanovesicles. *open-access J.* 15 (1), 1096–1122. doi:10.1515/med-2020-0160

Ding, Y., Robinson, D. G., and Jiang, L. (2014a). Unconventional Protein Secretion (UPS) Pathways in Plants. *Curr. Opin. Cell. Biol.* 29 (August), 107–115. doi:10.1016/j.ceb.2014.05.008

Ding, Y., Wang, J., Chun Lai, J. H., Ling Chan, V. H., Wang, X., Cai, Y., et al. (2014b). Exo70E2 Is Essential for Exocyst Subunit Recruitment and EXPO Formation in Both Plants and Animals. *MBoC* 25 (3), 412–426. doi:10.1091/mbc.e13-10-0586

Elliott, R. O., and He, M. (2021). Unlocking the Power of Exosomes for Crossing Biological Barriers in Drug Delivery. *Pharmaceutics* 13 (1), 122. doi:10.3390/pharmaceutics13010122

Elsharkasy, O. M., Nordin, J. Z., Hagey, D. W., de Jong, O. G., Schiffelers, R. M., Andaloussi, S. E., et al. (2020). Extracellular Vesicles as Drug Delivery Systems: Why and How? *Adv. drug Deliv. Rev.* 159, 332–343. doi:10.1016/j.addr.2020.04.004

Garaeva, L., Kamyshinsky, R., Kil, Y., Varfolomeeva, E., Verlov, N., Komarova, E., et al. (2021). Delivery of Functional Exogenous Proteins by Plant-Derived Vesicles to Human Cells *In Vitro*. *Sci. Rep.* 11 (1), 1–12. doi:10.1038/s41598-021-85833-y

Hansen, L. L., and Nielsen, M. E. (2017). Plant Exosomes: Using an Unconventional Exit to Prevent Pathogen Entry? *J. Exp. Bot.* 69 (1), 59–68. doi:10.1093/jxb/erx319

Harding, C. V., Heuser, J. E., and Stahl, P. D. (2013). Exosomes: Looking Back Three Decades and into the Future. *J. Cell Biol.* 200, 367–371. doi:10.1083/jcb.201212113

He, B., Cai, Q., Qiao, L., Huang, C. Y., Wang, S., Miao, W., et al. (2021). RNA-binding Proteins Contribute to Small RNA Loading in Plant Extracellular Vesicles. *Nat. Plants* 7 (3), 342–352. doi:10.1038/s41477-021-00863-8

Huang, Y., Wang, S., Cai, Q., and Jin, H. (2021). Effective Methods for Isolation and Purification of Extracellular Vesicles from Plants. *J. Integr. Plant Biol.* 63 (12), 2020–2030. doi:10.1111/jipb.13181

Ju, S., Mu, J., Dokland, T., Zhuang, X., Wang, Q., Jiang, H., et al. (2013). Grape Exosome-like Nanoparticles Induce Intestinal Stem Cells and Protect Mice from DSS-Induced Colitis. *Mol. Ther.* 21 (7), 1345–1357. doi:10.1038/mt.2013.64

Kameli, N., Dragojlovic-Kerkache, A., Savelkoul, P., and Stassen, F. R. (2021). Plant-Derived Extracellular Vesicles: Current Findings, Challenges, and Future Applications. *Membranes* 11 (6), 411. doi:10.3390/membranes11060411

Kim, J., Gee, H. Y., and Lee, M. G. (2018). Unconventional Protein Secretion - New Insights into the Pathogenesis and Therapeutic Targets of Human Diseases. *J. Cell Sci.* 131 (12), jcs213686. doi:10.1242/jcs.213686

Kim, K., Jung, J.-H., Yoo, H. J., Hyun, J.-K., Park, J.-H., Na, D., et al. (2020). Anti-Metastatic Effects of Plant Sap-Derived Extracellular Vesicles in a 3D Microfluidic Cancer Metastasis Model. *Jfb* 11 (3), 49. doi:10.3390/jfb11030049

Kim, K., Yoo, H. J., Jung, J.-H., Lee, R., Hyun, J.-K., Park, J.-H., et al. (2020). Cytotoxic Effects of Plant Sap-Derived Extracellular Vesicles on Various Tumor Cell Types. *Jfb* 11 (2), 22. doi:10.3390/jfb11020022

Kocak, P., Kala, E. Y., Gunes, M., Unsal, N., Yilmaz, H., Metin, B., et al. (2020). Edible Plant-Derived Exosomes and Their Therapeutic Applicatons. *J. Biomed. Imag. Bioeng.* 4 (1), 130–135. Available at: https://www.alliedacademies.org/articles/edible-plantderived-exosomes-and-their-therapeutic-applicatons.pdf

Lai, C. P., Mardini, O., Ericsson, M., Prabhakar, S., Maguire, C. A., Chen, J. W., et al. (2014). Dynamic Biodistribution of Extracellular Vesicles *In Vivo* Using a Multimodal Imaging Reporter. *ACS Nano* 8 (1), 483–494. doi:10.1021/nn404945r

Lee, R., Ko, H. J., Kim, K., Sohn, Y., Min, S. Y., Kim, J. A., et al. (2020). Anti-melanogenic Effects of Extracellular Vesicles Derived from Plant Leaves and Stems in Mouse Melanoma Cells and Human Healthy Skin. *J. Extracell. Vesicles* 9 (1), 1703480. doi:10.1080/20013078.2019.1703480

Lefebvre, F. A., and Lécuyer, E. (2017). Small Luggage for a Long Journey: Transfer of Vesicle-Enclosed Small RNA in Interspecies Communication. *Front. Microbiol.* 8, 377. doi:10.3389/fmicb.2017.00377

Li, Z., Wang, H., Yin, H., Bennett, C., Zhang, H. G., Guo, P., et al. (2018). Arrowtail RNA for Ligand Display on Ginger Exosome-like Nanovesicles to Systemic Deliver siRNA for Cancer Suppression. *Sci. Rep.* 8 (1), 14644. doi:10.1038/s41598-018-32953-7

Liang, H., Zhang, S., Fu, Z., Wang, Y., Wang, N., Liu, Y., et al. (2015). Effective Detection and Quantification of Dietetically Absorbed Plant microRNAs in Human Plasma. *J. Nutr. Biochem.* 26 (5), 505–512. doi:10.1016/j.jnutbio.2014.12.002

Licá, I. C. L., Soares, A. M. d. S., de Mesquita, L. S. S., and Malik, S. (2018). Biological Properties and Pharmacological Potential of Plant Exudates. *Food Res. Int.* 105, 1039–1053. doi:10.1016/j.foodres.2017.11.051

Liu, Y., Wu, S., Koo, Y., Yang, A., Dai, Y., Khant, H., et al. (2020). Characterization of and Isolation Methods for Plant Leaf Nanovesicles and Small Extracellular Vesicles. *Nanomedicine* 29, 102271. doi:10.1016/j.nano.2020.102271

Maharjan, P. M., and Choe, S. (2021). Plant-Based COVID-19 Vaccines: Current Status, Design, and Development Strategies of Candidate Vaccines. *Vaccines* 9 (9), 992. doi:10.3390/vaccines9090992

Meng, W., He, C., Hao, Y., Wang, L., Li, L., and Zhu, G. (2020). Prospects and Challenges of Extracellular Vesicle-Based Drug Delivery System: Considering Cell Source. *Drug Deliv.* 27 (1), 585–598. doi:10.1080/10717544.2020.1748758

Mu, J., Zhuang, X., Wang, Q., Jiang, H., Deng, Z. B., Wang, B., et al. (2014). Interspecies Communication between Plant and Mouse Gut Host Cells through Edible Plant Derived Exosome-like Nanoparticles. *Mol. Nutr. Food Res.* 58 (7), 1561–1573. doi:10.1002/mnfr.201300729

Nielsen, M. E., Feechan, A., Böhlenius, H., Ueda, T., and Thordal-Christensen, H. (2012). Arabidopsis ARF-GTP Exchange Factor, GNOM, Mediates Transport Required for Innate Immunity and Focal Accumulation of Syntaxin PEN1. *Proc. Natl. Acad. Sci. U.S.A.* 109 (28), 11443–11448. doi:10.1073/pnas.1117596109

O'Leary, B. M., Rico, A., McCraw, S., Fones, H. N., and Preston, G. M. (2014). The Infiltration-Centrifugation Technique for Extraction of Apoplastic Fluid from Plant Leaves Using Phaseolus vulgaris as an Example. *JoVE* (94), e52113. doi:10.3791/52113

Pinedo, M., de la Canal, L., and de Marcos Lousa, C. (2021). A Call for Rigor and Standardization in Plant Extracellular Vesicle Research. *J. Extracell. Vesicles* 10 (6), e12048. doi:10.1002/jev2.12048

Rabouille, C., Malhotra, V., and Nickel, W. (2012). Diversity in Unconventional Protein Secretion. *J. Cell. Sci.* 125 (Pt 22), 5251–5255. doi:10.1242/jcs.103630

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cell. Biol.* 27 (3), 230–240. doi:10.1016/j.tcb.2016.11.007

Raimondo, S., Naselli, F., Fontana, S., Monteleone, F., Lo Dico, A., Saieva, L., et al. (2015). Citrus Limon-Derived Nanovesicles Inhibit Cancer Cell Proliferation and Suppress CML Xenograft Growth by Inducing TRAIL-Mediated Cell Death. *Oncotarget* 6 (23), 19514–19527. doi:10.18632/oncotarget.4004

Regente, M., Corti-Monzón, G., Maldonado, A. M., Pinedo, M., Jorrín, J., and De la Canal, L. (2009). Vesicular Fractions of Sunflower Apoplastic Fluids Are Associated with Potential Exosome Marker Proteins. *FEBS Lett.* 583 (20), 3363–3366. doi:10.1016/j.febslet.2009.09.041

Regente, M., Pinedo, M., Elizalde, M., and de la Canal, L. (2012). Apoplastic Exosome-like Vesicles: a New Way of Protein Secretion in Plants? *Plant Signal. Behav.* 7 (5), 544–546. doi:10.4161/psb.19675

Robinson, D. G., Ding, Y., and Jiang, L. (2016). Unconventional Protein Secretion in Plants: A Critical Assessment. *Protoplasma* 253 (1), 31–43. doi:10.1007/s00709-015-0887-1

Rutter, B. D., and Innes, R. W. (2017). Extracellular Vesicles Isolated from the Leaf Apoplast Carry Stress-Response Proteins. *Plant Physiol.* 173 (1), 728–741. doi:10.1104/pp.16.01253

Şahin, F., Koçak, P., Güneş, M. Y., Özkan, İ., Yıldırım, E., and Kala, E. Y. (2018). *In Vitro* Wound Healing Activity of Wheat-Derived Nanovesicles. *Appl. Biochem. Biotechnol.* 188 (2), 381–394. doi:10.1007/s12010-018-2913-1

Saleh, A. F., Lázaro-Ibáñez, E., Forsgard, M. A.-M., Shatnyeva, O., Osteikoetxea, X., Karlsson, F., et al. (2019). Extracellular Vesicles Induce Minimal Hepatotoxicity and Immunogenicity. *Nanoscale* 11 (14), 6990–7001. doi:10.1039/c8nr08720b

Sattelmacher, B. (2001). The Apoplast and its Significance for Plant Mineral Nutrition. *New Phytol.* 149 (2), 167–192. doi:10.1046/j.1469-8137.2001.00034.x

Shaaltiel, Y., Bartfeld, D., Hashmueli, S., Baum, G., Brill-Almon, E., Galili, G., et al. (2007). Production of Glucocerebrosidase with Terminal Mannose Glycans for Enzyme Replacement Therapy of Gaucher's Disease Using a Plant Cell System. *Plant Biotechnol. J.* 5 (5), 579–590. doi:10.1111/j.1467-7652.2007.00263.x

Shaaltiel, Y., Gingis-Velitski, S., Tzaban, S., Fiks, N., Tekoah, Y., and Aviezer, D. (2015). Plant-based Oral Delivery of β-glucocerebrosidase as an Enzyme Replacement Therapy for Gaucher's Disease. *Plant Biotechnol. J.* 13 (8), 1033–1040. doi:10.1111/pbi.12366

Shoji, Y., Farrance, C. E., Bautista, J., Bi, H., Musiychuk, K., Horsey, A., et al. (2012). A Plant-Based System for Rapid Production of Influenza Vaccine Antigens. *Influenza other Respir. viruses* 6 (3), 204–210. doi:10.1111/j.1750-2659.2011.00295.x

Su, H., Yakovlev, I. A., van Eerde, A., Su, J., and Clarke, J. L. (2021). Plant-Produced Vaccines: Future Applications in Aquaculture. *Front. Plant Sci.* 12, 718775. doi:10.3389/fpls.2021.718775

Sundaram, K., Miller, D. P., Kumar, A., Teng, Y., Sayed, M., Mu, J., et al. (2019). Plant-Derived Exosomal Nanoparticles Inhibit Pathogenicity of Porphyromonas Gingivalis. *iScience* 21, 308–327. doi:10.1016/j.isci.2019.10.032

Takeyama, N., Kiyono, H., and Yuki, Y. (2015). Plant-based Vaccines for Animals and Humans: Recent Advances in Technology and Clinical Trials. *Ther. Adv. Vaccines* 3 (5-6), 139–154. doi:10.1177/2051013615613272

Teng, Y., Ren, Y., Sayed, M., Hu, X., Lei, C., Kumar, A., et al. (2018). Plant-Derived Exosomal MicroRNAs Shape the Gut Microbiota. *Cell. Host Microbe* 24 (5), 637–e8. e8. doi:10.1016/j.chom.2018.10.001

Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., et al. (2018). Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): a Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracell. Vesicles* 7 (1), 1535750. doi:10.1080/20013078.2018.1535750

Urzì, O., Raimondo, S., and Alessandro, R. (2021). Extracellular Vesicles from Plants: Current Knowledge and Open Questions. *Ijms* 22 (10), 5366. doi:10.3390/ijms22105366

Wang, J., Ding, Y., Wang, J., Hillmer, S., Miao, Y., Lo, S. W., et al. (2010). EXPO, an Exocyst-Positive Organelle Distinct from Multivesicular Endosomes and Autophagosomes, Mediates Cytosol to Cell Wall Exocytosis inArabidopsisand Tobacco Cells. *Plant Cell* 22 (12), 4009–4030. doi:10.1105/tpc.110.080697

Woith, E., Fuhrmann, G., and Melzig, M. F. (2019). Extracellular Vesicles-Connecting Kingdoms. *Ijms* 20 (22), 5695. doi:10.3390/ijms20225695

Xiao, J., Feng, S., Wang, X., Long, K., Luo, Y., Wang, Y., et al. (2018). Identification of Exosome-like Nanoparticle-Derived microRNAs from 11 Edible Fruits and Vegetables. *PeerJ* 6, e5186. doi:10.7717/peerj.5186

Xu, M., Yang, Q., Sun, X., and Wang, Y. (2020). Recent Advancements in the Loading and Modification of Therapeutic Exosomes. *Front. Bioeng. Biotechnol.* 8, 586130. doi:10.3389/fbioe.2020.586130

Yao, J., Weng, Y., Dickey, A., and Wang, K. (2015). Plants as Factories for Human Pharmaceuticals: Applications and Challenges. *Ijms* 16 (12), 28549–28565. doi:10.3390/ijms161226122

Zahara, K., Bibi, Y., Bibi, Y., Ajmal, M., Sadaf, H. M., Bibi, F., et al. (2017). Tobacco Plant: A Possible Key to Ebola Vaccine. *Jclm* 5 (5), 206–211. doi:10.12980/jclm.5.2017j7-9

Zhang, J., Qiu, Y., and Xu, K. (2020). Characterization of GFP-AtPEN1 as a Marker Protein for Extracellular Vesicles Isolated from Nicotiana Benthamiana Leaves. *Plant Signal. Behav.* 15 (9), 1791519. doi:10.1080/15592324.2020.1791519

Zhang, M., Viennois, E., Prasad, M., Zhang, Y., Wang, L., Zhang, Z., et al. (2016). Edible Ginger-Derived Nanoparticles: A Novel Therapeutic Approach for the Prevention and Treatment of Inflammatory Bowel Disease and Colitis-Associated Cancer. *Biomaterials* 101, 321–340. doi:10.1016/j.biomaterials.2016.06.018

Zhao, Z., Yu, S., Li, M., Gui, X., and Li, P. (2018). Isolation of Exosome-like Nanoparticles and Analysis of MicroRNAs Derived from Coconut Water Based on Small RNA High-Throughput Sequencing. *J. Agric. Food Chem.* 66 (11), 2749–2757. doi:10.1021/acs.jafc.7b05614

Zhuang, X., Teng, Y., Samykutty, A., Mu, J., Deng, Z., Zhang, L., et al. (2016). Grapefruit-derived Nanovectors Delivering Therapeutic miR17 through an Intranasal Route Inhibit Brain Tumor Progression. *Mol. Ther.* 24 (1), 96–105. doi:10.1038/mt.2015.188

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

REVIEW
published: 09 June 2022
doi: 10.3389/fcell.2022.877344

Unconventional Protein Secretion Dependent on Two Extracellular Vesicles: Exosomes and Ectosomes

Jacopo Meldolesi [1,2]*

[1]The San Raffaele Institute, Vita-Salute San Raffaele University, Milan, Italy, [2]The CNR Institute of Neuroscience at Milano-Bicocca University, Milan, Italy

In addition to conventional protein secretion, dependent on the specific cleavage of signal sequences, proteins are secreted by other processes, all together called unconventional. Among the mechanisms operative in unconventional secretion, some are based on two families of extracellular vesicle (EVs), expressed by all types of cells: the exosomes (before secretion called ILVs) and ectosomes (average diameters ~70 and ~250 nm). The two types of EVs have been largely characterized by extensive studies. ILVs are assembled within endocytic vacuoles by inward budding of small membrane microdomains associated to cytosolic cargos including unconventional secretory proteins. The vacuoles containing ILVs are called multivesicular bodies (MVBs). Upon their possible molecular exchange with autophagosomes, MVBs undergo two alternative forms of fusion: 1. with lysosomes, followed by large digestion of their cargo molecules; and 2. with plasma membrane (called exocytosis), followed by extracellular diffusion of exosomes. The vesicles of the other type, the ectosomes, are differently assembled. Distinct plasma membrane rafts undergo rapid outward budding accompanied by accumulation of cytosolic/secretory cargo molecules, up to their sewing and pinching off. Both types of EV, released to the extracellular fluid in their complete forms including both membrane and cargo, start navigation for various times and distances, until their fusion with target cells. Release/navigation/fusion of EVs establish continuous tridimensional networks exchanging molecules, signals and information among cells. The proteins unconventionally secreted *via* EVs are a few hundreds. Some of them are functionally relevant (examples FADD, TNF, TACE), governing physiological processes and important diseases. Such proteins, at present intensely investigated, predict future discoveries and innovative developments, relevant for basic research and clinical practice.

Keywords: endocytosis, multivesicular body, exocytosis, pinching off, navigation, vesicle fusion, interconnected networks

OPEN ACCESS

Edited by:
Chiou-Feng Lin,
Taipei Medical University, Taiwan

Reviewed by:
Thierry Galli,
Institut National de la Santé et de la Recherche Médicale (INSERM), France
Sangyong Jung,
*Institute of Molecular and Cell Biology (A*STAR), Singapore*

***Correspondence:**
Jacopo Meldolesi
meldolesi.jacopo@hsr.it

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *16 February 2022*
Accepted: *03 May 2022*
Published: *09 June 2022*

Citation:
Meldolesi J (2022) Unconventional Protein Secretion Dependent on Two Extracellular Vesicles: Exosomes and Ectosomes. Front. Cell Dev. Biol. 10:877344. doi: 10.3389/fcell.2022.877344

1 DISCOVERY OF UNCONVENTIONAL PROTEIN SECRETION

The existence of specific protein secretion, a property of all types of cells, was already known at the beginning of the last century. At that time, however, the mechanisms of the process were unknown and remained so for decades. Information started to emerge at the beginning of 1960. Digestive enzymes of pancreatic acinar cells, in the course of their synthesis by bound polyribosomes, are transported to the lumen of the endoplasmic reticulum (ER). Segregated enzymes were found to

move to the Golgi complex (GC), and then concentrate in the cargo of secretory granules, which accumulate in the cytoplasm during jejunum. Upon food intake or cell stimulation the granules were found to undergo exocytosis by fusion of their membrane to the plasma membrane, followed by extracellular discharge of their cargos (Palade et al., 1962; Caro and Palade, 1964). Subsequent studies demonstrated that mechanisms analogous to those of the pancreas operate also in other cell types (Schramm, 1967; Meldolesi et al., 1978). In addition, the general processes governing the various steps of the secretory pathways started to be discovered. The first step, concerning the signal recognition particles of the ER membrane surface, was shown to induce translocation of pre-secretory proteins into the corresponding ER lumen. For years, cleavage of signal sequences (Walter and Blobel, 1981; Müller et al., 1982; Walter et al., 1984) was considered necessary for the development of a secretion now called conventional or canonical.

For over 2 decades, progress about secretion concerned only the conventional pathway. Around 1990, however, evidence incompatible with that interpretation begun to emerge. Cytosolic proteins lacking a signal sequence in their gene, such as interleukin-β1, bacterial enzymes and growing numbers of proteins and factors, were shown to be discharged by unconventional secretion (Rubartelli et al., 1990; Rubartelli et al., 1993; Akatsuka et al., 1995; Nickel and Rabouille, 2009). Initially these processes were proposed to activate, in the plasma membrane, various types of pore permeable to secretory proteins (Rubartelli et al., 1990; Nickel and Rabouille, 2009). Plasma membrane pores, together with a channel in the ER/Golgi membranes, are still considered of relevance in the trans-membrane transport of proteins lacking signal peptides (Rabouille, 2017; Zhang et al., 2020). At present, however, the major pathways of unconventional protein secretion appear based on the participation of various types of organelles (Rabouille, 2017; Gruenberg, 2020).

In the cytoplasm, at least three types of organelles, involved also in other important functions, are known to participate in unconventional secretion. These organelles include: lysosomes, with many enzymes necessary for catabolism; autophagosomes, that in their journey from ER to lysosomes fuse with vesicles and integrate cytosolic molecules and nutrients (Zhao and Zhang, 2019); and multivesicular bodies (MVBs), the only endocytic vacuoles known to accumulate large numbers of small intraluminal vesicles, the ILVs (Karim et al., 2018) (**Figure 1**). This review is focused on two types of extracellular vesicles (EVs), expressed by all types of cells and active in unconventional secretion: ILVs (called exosomes upon their release to the extracellular space) and the larger ectosomes (also known as microvesicles and microparticles). The exosomes are released upon exocytosis of MVBs; the ectosomes, independent of MVBs, are generated and released by shedding from the plasma membrane.

2 PRESENTATION OF ILV/EXOSOMES AND ECTOSOMES

Before the generation and function of ILV/exosomes and ectosomes, illustrated in the next two Sections, the vesicles are presented in their general properties. Let's start with MVBs and ILVs. Upon its ILV accumulation, MVBs undergo their maturation. Their destiny is two fold. Upon interaction with the Rab7 ortolog Ypt2 and the multisubunit tethering complex HOPS, a fraction of MVBs proceed to specific fusion with lysosomes by a process including the Qa-SNARE Pep12. The ILVs discharged to the lysosomal lumen are thus exposed to hydrolases for catabolism (Karim et al., 2018). Other, apparently distinct MVBs, by interacting with actin and microtubule cytoskeleton move towards the microtubule-organizing center and then to the plasma membrane. From such location they undergo exocytosis in response to appropriate cell stimulation (Hessvik and Llorente, 2018; Raudenska et al., 2021). Details about their discharge are illustrated in the following **Section 3.1**. New aspects of the processes involving MVBs include a co-operation with autophagosomes (**Figure 1**). Comparative studies of exocytosis with and without autophagy inhibitors have revealed that, during their intracellular traffic, MVBs and autophagosomes interact with each other with exchange of their cargo proteins. When autophagosomes are not available, exocytosis of MVBs is greatly reduced (Bebelman et al., 2020). The interaction of the two organelles plays therefore an integrated form of unconventional secretion.

Stimulation of the MVB exocytosis discussed so far is followed by the diffusion of many ILV/exosome vesicles, from the lumen to the extracellular space (**Figure 1**) (Rabouille, 2017; Bebelman et al., 2020; Gruenberg, 2020; Zhang et al., 2020; Gurung et al., 2021; Ras-Carmona et al., 2021; Raudenska et al., 2021). In many cases such process is accompanied by the release of ectosomes, the other type of EVs, generated not within the cytoplasm but at the plasma membrane. Being a single process, ectosome generation and release are not presented here. They will be illustrated in the subsequent **Section 3.2**, i.e. just after 3.1, the Section dedicated to the nature and generation of exosomes.

In order to complete the presentation of the two EV types, I intend to emphasize three properties of their research. For many years, simple procedures such as ultracentrifugation, precipitation, filtration, chromatography, and immune-affinity-based approaches, have been employed to separate the two types of vesicle present in extracellular fluids. However, these approaches yielded poor results (Shtam et al., 2020). As a consequence, research interests were primarily focused on only one type, the exosomes. Results about ectosomes remained marginal. Among reported differences only one was conclusive, concerning the different size of the two types of vesicles, with diameters between 30 and 150 nm for exosomes, between 100 and 400 (or more) nm for ectosomes. Other differential properties, including protein and RNA composition, fusion to cell targets, and functional effects, became convincing only upon development of better isolation procedures (see for example Meldolesi, 2018; Van Niel et al., 2018; Royo et al., 2020). The results obtained by such procedures have shown the two types of vesicles to differ considerably in some cells, and not so much in other cells (Pizzirani et al., 2007; Kowal et al., 2016; Meldolesi, 2018; Van Niel et al., 2018; Cocozza et al., 2020; Royo et al., 2020; Lim et al., 2021; Mathieu et al., 2021).

At variance with granules and vesicles of conventional secretion, of which only cargos are discharged upon cell activation, the discharge to the extracellular space occurs by whole EVs, composed by cargos bound by their membrane. In fact, membranes are essential for the EV navigation and for its specific binding and fusion to their target cells. As long as the EVs are intact their cargo components: many proteins, various types of RNAs, short DNA sequences, a few types of lipids, metabolic molecules and various ions, remain largely assembled.

Exosomes and ectosomes are unconventional secretory vesicles, released by all types of cells. Their heterogeneity depends on their distinct differentiation and also on their cells of origin. At variance with the other cellular organelles, discharged exosomes and ectosomes are very resistant, they withstand harsh conditions such as those of the human stomach. Based on these properties, their origin has been hypothesized more ancient than that of intracellular organelles (Askenase, 2021). For these unique natural properties, and also for their engineered treatments of biotechnological relevance, EVs are considered of interest for a number of pathologies, including cancers, neurodegenerative and viral diseases. These properties deal not only with unconventional secretion, but also with other functions that will not be presented here. Nevertheless, they are of high medical relevance, already presented by a vast literature.

3 DEVELOPMENT OF SECRETORY VESICLES WITHIN THE CELL

Before detailed illustration of EVs, let's consider the nomenclatures valid for the two secretory vesicles, ILVs and ectosomes within the cell, exosomes and ectosomes, upon their release. Relevant studies have emphasized the heterogeneity of single EV populations (Pizzirani et al., 2007). In the future, therefore, unexpected results dependent on the coexistence of vesicle subtypes cannot be excluded. In various articles the EVs, in addition to exosomes and etosomes, have been reported to include larger structures, with diameters of one um and more. Most of these structures, however, are membrane fragments and cell debris. Because of their non-vesicular nature they will not be considered in the present review.

The initial life of the two EVs is profoundly different. They are not only generated at different sites and by different processes (**Figure 1**), they differ also in their subsequent intracellular life: long lasting for ILVs segregated within MVBs; very short for ectosomes. In view of these differences, the intracellular life of the two vesicles, focused on the mechanisms of their occurrence, are presented separately.

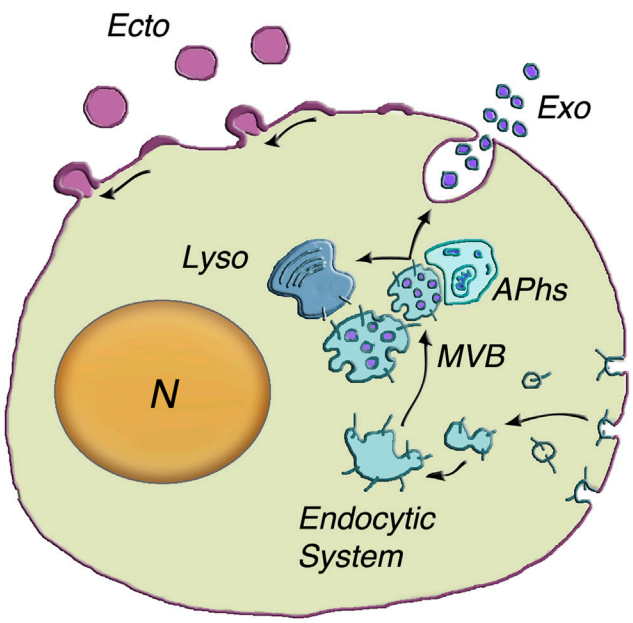


FIGURE 1 | Generation and release of both exosomes and ectosomes. The dependence of exosome generation on the Endocytic System is illustrated at the center of the figure. The multivesicular body (MVB) is an endocytic vacuole occupied by vesicles corresponding to distinct intraluminal vesicles (ILVs) filled by specific cargo accumulated from the cytosol. The inward budding of the vesicles is followed by their fission and the release of ILVs into the MVB lumen (50–150 nm diameter). Upon their generation, the MVBs can proceed in two alternative directions (arrows): towards lysosomes (Lyso) or towards the plasma membrane. Their close interaction with autophagosomes) can induce reciprocal exchange of cargo components and ensuing increased fusion in response to stimulation. MVB exocytosis is followed by prompt extracellular release of vesicles, now named exosomes (Exo). The assembly and release of ectosomes, on top left of the cell surface, take place at the plasma membrane. The initial step is the assembly of membrane larger microdomains (100–400 nm in ectosome diameter). Their composition is distinct from that of the plasma membrane of origin and partially similar to that of ILV membranes. Concomitantly specific cargos, composed of proteins, lipids and nucleic acids, accumulate in the vesicle lumen. Upon their rapid outward curvature and budding, the ectosome vesicles (Ecto) undergo pinching off and shedding to the extracellular space. N = nucleus. Modification of the image reproduced with permission from Meldolesi (2018).

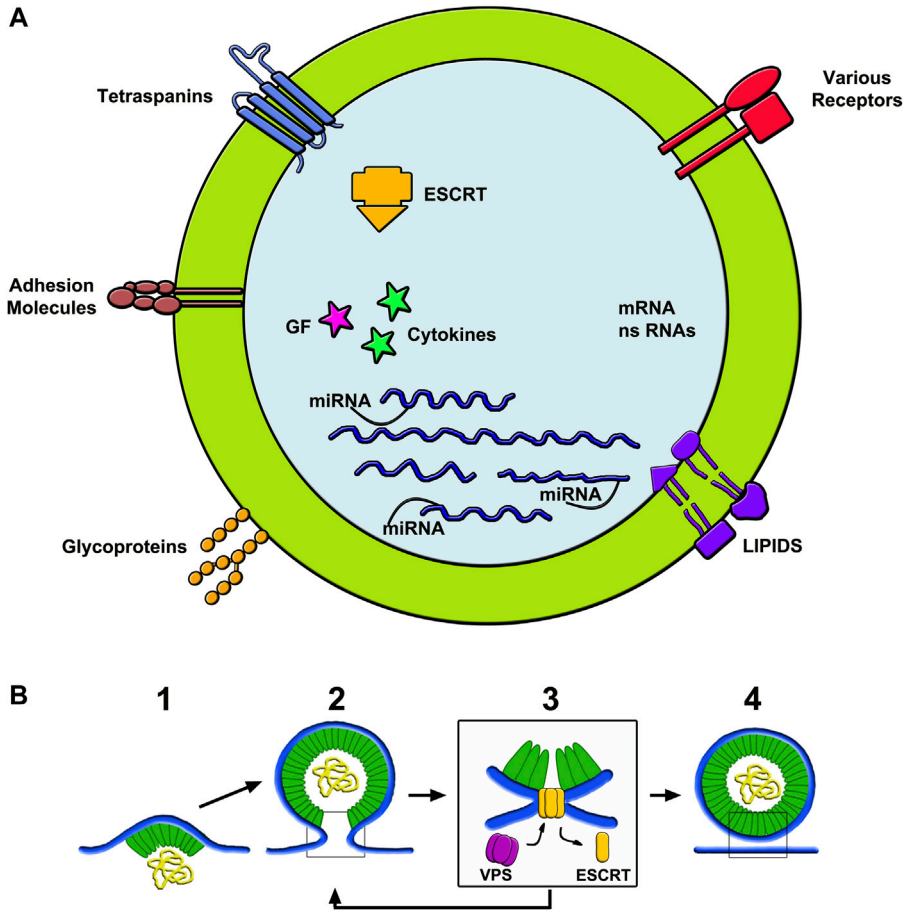


FIGURE 2 | Structure of exosomes **(A)** and assembly of ectosomes **(B)**. **(A)** shows the composition of the membrane and luminal cargos of an exosome. The components shown in the membrane are among the most abundant in these vesicles. The content shows numerous proteins, some of which bound to miRNAs. Other components are fluid factors such as cytokines and growth factors shown as arrows. ESCRT complexes participate of membrane growth. mRNAs and ns-RNAs are other nucleotides accumulated here from the cytosol. **(B)** illustrates an example of ectosome rapidly assembled from the initial microdomain of the plasma membrane. To the left, 1) shows an initial curvature, already associated to a first cargo, followed by its budding 2) and then by its fission dependent on ESCRT-III interaction with VPS 3). To the right 4) the ectosome is free, ready to navigate in the extracellular fluid. The ectosome yellow sequences shown in the **(B)** lumen correspond to those drown in the exosome lumen of **(A)**.

3.1 ILVs/Exosomes

As already mentioned in **Section 1**, generation of ILVs induces the conversion of normal endosomal vacuoles into peculiar MVBs. The conversion starts with two processes: budding inside the vacuoles of the small membrane protrusions concomitantly loaded with their cargo content.

Membrane. The generation of ILV membrane processes are mostly governed by the Endosomal Sorting Complexes Required for Transport proteins, i.e. by ESCRT-0, -I and -II, working together with their associated protein factors to induce membrane curvature (Gruenberg, 2020; Pavlin and Hurley, 2020; Juan and Fürthauer, 2018a). ESCRT-III subunits operate assembled with Alix and other proteins. Their interaction with helical filaments of various forms of vacuolar protein sorting (VPS) including an ATPase, mediates membrane remodeling (Huber et al., 2020). Fission occurs when ESCRT-III is removed, leading to scission of the ILVs neck (**Figure 2B**) (Huber et al., 2020; Johnson et al., 2018; Tseng et al., 2021). The membrane of ILVs contain a peculiar lipid, LBPA, present together with many specific proteins in the unique MBV endocytic vacuoles (Gruenberg, 2020). Abundant are the small tetraspanins (predominant CD63), critically important during vesicle assembly with membrane and cargo protein trafficking. Additional proteins of lower concentration, including adhesion proteins, receptors, glycoproteins and metalloproteases, are present in ILV membranes (**Figure 2A**) (Meldolesi et al., 1978; Pizzirani et al., 2007). In a variety of cell types ILV loading within MVBs and the ensuing release of exosomes depend on sirtuin2, a deacetylase enzyme known to participate also in several other processes (Lee et al., 2019).

Cargos. Luminal cargos begin their accumulation in the initial ILV membrane protrusions. Among proteins, those concentrated in the lumen are typical of growing vesicles. Unconventional secretory proteins are also present, however at concentration

lower than that of tetraspanins (Meldolesi, 2018). Small cytosolic proteins are also trapped within ILVs. In addition to proteins, cargos contain molecules of different nature: various types of RNA (mostly microRNAs, miRs, together with messenger RNAs, mRNAs, long non-coding RNAs, lncRNAs, and ribosome RNAs, rRNAs), small sequences of DNA, lipids and metabolic molecules (**Figure 2A**) (Yang and Gould, 2013; Ras-Carmona et al., 2021). Recent studies have shown ubiquitin and ubiquitin-like proteins to participate in protein post-translational modifications and in the control of protein complex composition (Chen et al., 2021; Padovani et al., 2022), thus contributing to their accumulation within ILVs (Yang and Gould, 2013; Chen et al., 2021; Ras-Carmona et al., 2021). Surface proteins of cargo are often anchored to the ILV membrane by myristoylation, palmitoylation or other sequences. The mechanisms of cargo accumulation remain unclear. Discoveries of a few years ago demonstrated the targeting and association of single proteins to the growing ILV lumen (Chen et al., 2021). Details have been clarified by the identification of proteins, such as endofin and arrestin-domain containing protein (ARRDC1), in the assembly of cargos (Ageta and Tsuchida, 2019; Kazan et al., 2021). Condensates of another protein of ample specificity, YBX1, have been shown to induce abundant liquid-liquid phase separations selectively recruiting a single miRNA, miR223 (Liu H. et al., 2021). The present hypothesis is that, in cargo development, other RNA-binding proteins undergo condensation. By such mechanism various proteins, together with important factors such as IL-1β and TNF-α, undergo a selective engulfment within ILVs before release by unconventional secretion. (Bello - Gamboa et al., 2020).

Journey and exocytosis of MVBs. Once established, MVBs travel within the cell. In immune and other cells, actin reorganization induces convergence of the non-lysosome binding fraction to the microtubule-organizing center (Bello - Gamboa et al., 2020). In response to various types of stimulation MVBs move, approaching the plasma membrane (**Figure 1**) (Calvo and Izquierdo, 2020). Understanding of these processes has been strengthened by the use of optical reporters associated with ILV markers (Verweij et al., 2018; Liu X.-M. et al., 2021). Upon tethering to specific sites (Davis et al., 2021), some heterogeneity in the molecules participating in MVB exocytosis has been reported among various cell types. The first Ras GTPase reported in the process has been Ras 11. Additional forms, such as Rab27a and Rab 27b, as well as the Rho, Rac, cdc42 family, have been reported to operate in many, but not in all types of cells (Hessvik and Llorente, 2018; Hyenne et al., 2018; Colombo et al., 2021). Concerning the fusion complex, the factor most frequently involved in various tissues and also in cancers (Peng et al., 2021) is the R-SNARE VAMP7 (vesicle-associated protein 7) together with the Q-SNARE SNAP23. Other R-SNAREs, such as VAMP3 and VAMP8, are also effective, however with lower frequency (Verweij et al., 2018; Zhao et al., 2022). Their ternary complex, established with SNAP23 associated to Syntaxin-4, induces the generation of enlarging pores, called invadopodia, key sites for MVB fusion with the plasma membrane and for the ensuing ILV release (Puri and Roche, 2006; Verweij et al., 2018; Colombo et al., 2021; Peng et al., 2021; Zhao et al., 2022). Q-SNAREs analogous to SNAP23, such as SNAP25, are ineffective in such fusion. Additional small GTPases involved include Ral, Rab (especially Rab35) and other Ras (Zhu et al., 2015; Yang et al., 2019). The integrated analysis of the various participants has revealed the role of non-coding RNAs (Yang et al., 2019) and G protein-coupled receptors. The latter, via their cAMP effect, promote the fusion via a SNAP23 phosphorylation occurring at the Ser 110 position (Verweij et al., 2018). SNARE dependence participates also in the unconventional secretion of important proteins such as α-synuclein (Zhao et al., 2022). The latter aspect will be presented in the following **Section 5**.

During the last few years, exocytosis and exosome secretion have been intensely investigated by a variety of techniques. The recent development of pH-dependent fluorescence microscopy has introduced the direct revelation of the processes. Fluorescent proteins of exosome membranes, such as CD63-pHfluorin, start emitting fluorescence upon exocytosis. The steps revealed by these approaches are numerous, from the efficacy and intracellular signaling of exogenous stimuli to the frequency, localization and machinery of exocytosis, up to the navigation of the released vesicles (Liu X.-M. et al., 2021; Gurung et al., 2021). The exosome localization studies have revealed various unexpected results. In lymphocytes the site of exocytosis is redistributed upon the establishment of immune synapses (Bello - Gamboa et al., 2020). In epithelial cells, where the plasma membrane includes two distinct areas, MVB exocytosis addressed to the baso-lateral area are different from those addressed to the apical area. Differences have been demonstrated also between the two corresponding families of released exosomes (Colombo et al., 2021; Matsui et al., 2021). Heterogeneity is therefore a common property even of exosomes secreted by single cells.

3.2 Ectosomes

As already mentioned knowledge of ectosomes, more limited than that of ILV/exosomes, has been questioned for many years. This because the preparations employed were widely contaminated by membrane fragments of other origin; and because the studies of ectosomes were less numerous and less detailed compared to those of ILV/exosomes. Nevertheless, the information about ectosomes has grown, dependent in many cases on parallel studies about both exosomes and ectosomes (Van Niel et al., 2018; Meldolesi, 2018). Compared to exosomes, the intracellular life of ectosomes is much shorter. In cells stimulated by a variety of agents, such as ATP, ectosome generation by outward budding and pinching off of small plasma membrane microdomains starts within a few minutes (Van Niel et al., 2018) (**Figure 2B**). Regulation of ectosome generation depends on various factors. Cdc42, a small G protein of the Rho family, is a convergent node of multiple regulatory signals. The binding to its downstream effector, Ras GTPase-activating-like protein 1 (IQGAP1), is required for ectosome shedding (Wang et al., 2021; Dai et al., 2019). Additional stimulatory events are the up-regulation of RhoA, Rock and phosphorylated LINK1, a kinase that controls actin cytoskeleton dynamics. RhoA inhibitors suppress the

production of ectosomes (Sun et al., 2021). Subsequent developments are supported by involvement of ESCRTs and their associated proteins analogous, but not identical, to those of ILVs (Gruenberg, 2020; Pavlin and Hurley, 2020; Juan and Fürthauer, 2018a; Huber et al., 2020). At least two complexes activate typical processes, i.e. their membrane dynamics involves outward budding and fission of corresponding plasma membrane microdomains. (Askenase, 2021; Pavlin and Hurley, 2020). ESCRT-III, followed by appropriate ATPase, governs the increased curvature, with ensuing narrowing of the neck followed by final scission (**Figure 2B**) (Johnson et al., 2018; Huber et al., 2020; Tseng et al., 2021; Wang et al., 2021). Regulation of further processes depends also on protein phosphorylation and calmodulin activation (Ni et al., 2020). Based on their properties, ectosomes and their molecules play a critical role in the regulation of cellular biology (Lv et al., 2019).

Membranes, Cargo, Release. The microdomains involved in the generations of ectosomes exhibit differences with respect to the rest of the plasma membrane. Their asymmetric phospholipid layers are rapidly rearranged. Several membrane proteins are analogous, but not identical to those of ILVs. For example, the most abundant tetraspanin in ectosomes is not the CD63 of ILVs but CD9 (Mathieu et al., 2021). Proteins of plasma membrane are present in ectosome membranes during and after their generation, however at low concentration.

Knowledge about ectosome cargo is limited. Accumulation of proteins by high affinity binding to miRNAs are processes known within exosomes (Yang and Gould, 2013; Chen et al., 2021). They might occur also within the ectosome lumen. Among such proteins is ARRDC1, an adaptor of ubiquitin ligases, involved in the regulation of ectosome generation and release (Anand et al., 2018). Loading of ectosome cargos with both RNA-binding proteins and miRNAs are supported by the LC3-conjugated machinery, an example of vesicle/autophagy interaction (Leidal et al., 2020). The ectosome cargo formation is regulated by caveolin-1, a structural protein as abundant as in plasma membrane caveolae. In contrast in exosome cargos caveolin-1 is not abundant (Ni et al., 2020) In addition, the ectosome cargos contain IL-1β, various cytokines and factors of the TNF family, together with other proteins of unconventional secretion (Cohen et al., 2020). Upon release from the plasma membrane, ectosomes coincide to EVs characterized by their large size and specific extracellular properties. In epithelial cells, plasma membrane areas induce two distinct types of such EVs, characterized by different functional roles (Colombo et al., 2021).

4 EVS: ORIGIN, NAVIGATION AND FUSION WITH TARGET CELLS

Contrary to some conventional beliefs, EVs are not membrane fragments released as a result of cell leakage. They are two types of extracellular vesicles, secreted by all cells and providing routes of intercellular communication. In fact, they transmit *in vitro* and *in vivo* biological messages between cells, with activation of specific signal transductions in their target cells. As a consequence, EVs are important tools active for unconventional secretion. Upon reaching their extracellular space, EVs start their navigation. An unexpected property of such activity is the vastness of its traffic, which is not restricted to the space adjacent to their cells of origin but is almost unlimited in the whole body. EVs are in fact the only membrane-bound structures to which the blood-brain barrier and various types of intercellular junctions are largely permeable. This property explains the EV circulation among organs and in the fluid spaces of the body, most important being the central spinal fluid and the blood plasma. Summing up, the wide navigation is essential for EV interaction and fusion with target cells. Their signal transduction cascades are activated at all distances of intercellular communications.

The specific properties of the various EVs are mostly due to the elaborate assembly of their precursors. During their biogenesis, the bending away and the ensuing budding and fission of membranes are established with the contribution of ESCRT complexes (Juan and Fürthauer, 2018; Cocozza et al., 2020; Pavlin and Hurley, 2020; Askenase 2021; Lim et al., 2021; Sun et al., 2021). Recent evidence has demonstrated that ESCRT complexes contribute also to a variety of EV processes, including their membrane specificity and the integrated structure of their cargos (Juan and Fürthauer, 2018). The membrane composition is complex. In addition to members of the teraspanin family, tthey include glycoproteins, adhesion molecules, various lipids and also receptors (**Figure 2A**), important for binding to other vesicles and target cells. Their cargos include long-term proteins often bound to miRNAs, which are known to possess sorting sequences involved in the regulation of their secretion (Garcia-Martin et al., 2021). Additional components include other types of non-coding RNAs and coding mRNAs together with lipids, enzymes, cytokines, growth factors and various proteins of unconventional secretion, most often originated from the cytoplasm (Boilard, 2018; Greening and Simpson, 2018; Garcia-Martin et al., 2021). Among phospholipid components, abundant are the arachidonic acids and other polyunsaturated eicosanoids (Juan and Fürthauer, 2018). Release of EVs to the extracellular space depends on the functional state of their cells of origin (Hessvik and Llorente, 2018; Cohen et al., 2020; Sun et al., 2021). Additional mechanisms known to regulate the EV generation and function depend on the media of their culture. For example, addition of fetal bovine serum, widely used commercially, induces negative effects, whereas with various growth factors and also with glucose the induced effects are positive (Bost et al., 2021; Guan et al., 2021).

Comparative analysis of the EVs derived from exosomes and ectosomes have revealed interesting aspects of heterogeneity. Proteins of important families, from their membranes (tetraspanins) and lumena (proteins associated to transport and fusion) are present in both types of EVs, however with differences in their levels and components (Pizzirani et al., 2007; Van Niel et al., 2018; Bost et al., 2021; Guan et al., 2021; Mathieu et al., 2021). Differences have been reported also for weakly expressed proteins, some of which heterogeneous also among EVs from distinct subtypes of cellular vesicles (Greening and Simpson, 2018; Gurunathan et al., 2021). Exosomes and

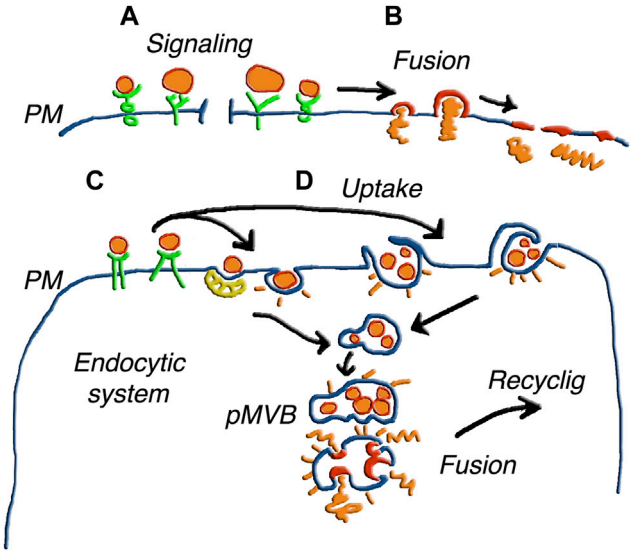


FIGURE 3 | Various forms of EV fusion with target cells. The different size of the EVs illustrates the parallel processes of ectosomes and exosomes. **(A)** shows vesicles inducing only signaling upon binding to receptors. In **(B)** the fusion of single vesicles followed by the integration of the membrane the plasma membrane (PM), induces release of cargo to the cytoplasm. In **(C)** the EVs, bound to receptors such as those in **(A)**, are internalized in an endosomal cisterna, and the same occurs in **(D)** for EV groups internalized by phagocytosis or macro-pinocytosis. Upon internalization, the EVs of **(C,D)** are accumulated within pseudo multi-vesicular bodies (pMVB). Elimination of these structures by ectosomal block and possible fusion with lysosomes do not appear in this Figure. Alternatively, the EV membranes fuse with endosome membrane and the cargos are released in the depth of the cytoplasm. The possible integration of molecules from fused EVs with molecules generated locally to participate in a new generation of EVs is suggested by the word Recycling. Figure reproduced with permission from Meldolesi (2018).

ectosomes differ also in metabolites, a property relevant for navigation fluids, including blood plasma (Bost et al., 2021; Gurunathan et al., 2021; Lim et al., 2021). In addition, recent evidence has demonstrated the ability of EVs to establish surface protein-protein interactions. It appears, therefore, that at least a fraction of EVs tends to show functionally integrated complexes (Leidal et al., 2020; Levy et al., 2021; Nikoloff et al., 2021; Razzauti and Laurent, 2021). At present, populations of single EV type can be isolated by various techniques based on distinct approaches, including monoclonal antibodies (Levy et al., 2021; Lim et al., 2021). The state of knowledge and techniques are already advanced. Additional developments are expected for the future including their markers and signatures, useful for the identification of subtype-specific EVs and the unconventional secretion of their proteins (Garcia-Martin et al., 2021; Levy et al., 2021; Nikoloff et al., 2021; Razzauti and Laurent, 2021).

After navigation in their extracellular fluid, EVs can undergo fusion with target cells, not only in the proximity but also at large distances from their cells of origin. Such fusions need to be efficient and specific, inducing transfer of their cargos to target cells. The first step is a tethering, which is essential, established between vesicles and the surface of target cells. In fact, block of tethering results in the prevention of all fusions (Guan et al., 2021; Gurunathan et al., 2021). On the other hand, tethering is not followed by fusion in all cases. The ensuing interactions are not always of the same type. The specific binding of a vesicle agonist to its receptor at the cell surface can be transient followed by generation of intracellular signals. In many cases, however, binding is followed by insertion of the vesicle membrane in the plasma membrane, with enlargement of the cell surface area and cargo discharged into the cell cytoplasm (Levy et al., 2021; An et al., 2021). Upon the introduction of new techniques to reveal the progressive changes of the EV protein distribution, the membrane and cargo events have been intensively investigated (Lim et al., 2021; Bost et al., 2021). Where does the EV fusion occur in target cells? In only a fraction of cases the process occurs at the surface, and cargos are discharged through the plasma membrane (**Figure 3**) (Somiya and Kuroda, 2021; Perissinotto et al., 2021; Song et al., 2021; Hung and Leonard, 2016). In many other cases fusion, preceded by the internalization of EV in the endocytic system, occurs in a moderately acidic environment (**Figure 3**) (Joshi et al., 2020; Somiya, 2020). A possible consequence of this pathway is the capture of EVs by the endo/lyso system, with limited escape of their cargos to the cytoplasm. However, the disruption of discharged cargo molecules is not always the case. Rather, many proteins remain intact and functional, as it happens with receptors still active upon their discharge (Hung and Leonard, 2016; Somiya, 2020; Levy et al., 2021). During and after such fusions also target cells release vesicles of their origin, containing at least part of the components received by their EV fusion. A process of this type is usually called recycling (Somiya, 2020). In conclusion, secretion pathways of EVs and their components appear more complex than previously expected. The problem is further discussed in the following Section focused exclusively on unconventional secretion.

5 RELEVANT EXAMPLES OF UNCONVENTIONAL PROTEIN SECRETION

Interest about EVs is growing depending on their properties such heterogeneity, navigation, fusion and recycling. In previous Sections unconventional secretion of proteins has been described, however only marginally. Here their presentation is expanded, focusing on the physiology of well-know examples. At present, in fact, unconventional protein secretion is not interesting only for basic physiology. It is relevant in diagnoses, therapies and clinical applications of diseases in the brain, heart and vessels, bones, and other tissues (Ng and Tang, 2016; Joshi et al., 2020; Gurunathan et al., 2021; He et al., 2021). In the present review, however, no space is available to deal with the EV role in these diseases. News about them will be reported in a future review to be published elsewhere.

Mechanisms underlying unconventional secretion have been deciphered during the last several years (Ng and Tang, 2016; Cohen et al., 2020). Recent developments about immune modulations, cell signaling, growth, redox control, as well as moonlighting activities, have started to be identified and

characterized (Cohen et al., 2020; Sitia and Rubartelli, 2020). High interest for EV crosstalk has been demonstrated with other structures and functions, such as autophagy and inflammasomes. Autophagy is best known for its role in organelle and protein turnover. In addition their machinery, together with MVBs, participates in ILV assembly (Bebelman et al., 2020; Raudenska et al., 2021). Machinery and MVBs often fuse with lysosomes, and in these cases they are often digested. In other cases, however, changes occur during MVB traffic towards the plasma membrane. Upon their integration in EV structure, autophagy components follow the EV pathway, from their exocytosis to navigation and cell fusion, activating the unconventional accumulation of important proteins in target cells (Hassanpour et al., 2020; Raudenska et al., 2021). Among such proteins is tau, known to play a critical role in brain diseases such as Alzheimer's and tau diseases. Its autophagy crosstalk with the two types of EV appears relevant for unconventional secretion involving neurons, astrocytes and microglia (Brunello et al., 2020; Jiang and Bhaskar, 2020). Another example of crosstalk concerns SCAMP5 (Secretory Carrier Membrane Protein 5), an inhibitor of autophagosome fusion with lysosomes. Its increased activity promotes a fragmentation of GC with block of its conventional and increased unconventional secretion (Yang et al., 2017). This unexpected switch of the secretory processes is relevant because it results in physiological changes including the metabolism of important proteins such as α-synuclein (Yang et al., 2017; Zhao et al., 2022). Interestingly, once α-synuclein metabolism is switched away from conventional secretion, it can be addressed not only to EVs but also to other unconventional pathways including autophagosomes and lysosomes (Zhao et al., 2022). In addition to brain physiology, unconventional secretion is important for inter-neuronal transmission (Yang et al., 2017; Bieri et al., 2018). Moreover, autophagic crosstalk concerns two proteins unconventionally secreted by teratocyte cells of insects upon their accumulation within exosomes (Salvia et al., 2019).

The most important form of vesicle crosstalk deals however with inflammasomes. The latter are large protein complexes assembled by variously recognized receptors together with pathogen-associated or damage-associated protein patterns, both activated by caspase-1 (Dai et al., 2019). Generation of various exosomes by inflammations results in the activation of several tasks including unconventional secretion of IL-1β, IL-18 and many other proteins. Moreover, exosomes can induce promotion or inhibition of various types of inflammasome (Bieri et al., 2018; Salvia et al., 2019). Finally, crosstalk of a peculiar inflammation with ectosomes, rather than exosomes, regulates the expression and unconventional secretion of an important protein, FADD, involved in a number of important processes: cell death, proliferation, immunity and inflammation (Cypryk et al., 2018; Noonin and Thongboonkerd, 2021). Thus, EVs of the two families, combined to various inflammasomes, induce distinct effects of critical importance for unconventional secretion (Bieri et al., 2018; Cypryk et al., 2018; Salvia et al., 2019; Noonin and Thongboonkerd, 2021).

Secretion of other proteins can occur by either conventional or unconventional pathways depending on their structure or binding to other component. This is the case of the cytokine TNF, which is switched off by the conventional secretion of ATP. In contrast, ATP induces TNF accumulation within ectosomes followed by exocytosis, navigation and fusion, thus establishing cell-to-cell connections (Noonin and Thongboonkerd, 2021). Another protein, the proinflammatory transmembrane protease TACE, when expressed upon its tyrosine phosphorylation, induces loss of a trafficking factor followed by its translocation into EVs and secretion via their unconventional pathway (Zhao et al., 2019). On the other hand, galectin-3 was found to include in its amino terminus a highly conserved tetrapeptide motif necessary for its direct binding to Tsg101, an associated protein of the ESCRT complex. Such motif is necessary for galectin to undergo unconventional secretion with exosomes. Mutations of its tetrapeptide prevents secretion of galectin-3 (Bänfer et al., 2018).

6 CONCLUSION

The unconventional secretion emerging from the present review is unique. As emphasized in **Section 1**, its discovery was based on the recognition of properties distinct from those of conventional secretion. Here attention has been focused on the two unconventional secretory EVs, the small exosomes and the larger ectosomes. In the last Section the properties of the two EVs are summarized together, including processes that in previous Sections are presented separately.

The membranes and cargos of the two types of EV are largely, but not completely, different from the membranes and cytoplasm of their original cells. Various distinctions exist between these two types of vesicles, concerning not only their size but also their molecular composition (Meldolesi, 2018; Van Niel et al., 2018; Royo et al., 2020; Raudenska et al., 2021), intracellular life (Rabouille, 2017; Van Niel et al., 2018; Gurung et al., 2021; Raudenska et al., 2021), and release processes (Van Niel et al., 2018; Shtam et al., 2020; Gurung et al., 2021; Ras-Carmona et al., 2021; Raudenska et al., 2021). A major distinction of EVs, however, exists with respect to the vesicles and granules of conventional secretion. From such organelles only cargos are secreted, most often into the extracellular space. During their release the membranes are first integrated in the plasma membrane and then recycled by endocytosis to the cytoplasm of secretory cells. In contrast the EVs, upon their release and during navigation, maintain their whole structure, composed by both membranes and cargos. Upon their fusion to specific target cells, their membranes are integrated into the plasma or endocytic membranes, while the cargos are discharged and diffuse in the cytoplasm (Bost et al., 2021; Guan et al., 2021). Depending on their properties, the intracellular distribution of the received cargo proteins varies: a l fraction goes to the nucleus, others to the plasma membrane/intracellular membranes, or remain in the cytosol (Somiya and Kuroda, 2021). In each animal, received cargo proteins recycle within re-assembled EVs, circulating along their pathways distributed following dynamic, possibly interconnected networks. Based on the present knowledge, the distinction of the two EVs with respect to conventional secretory

organelles has been hypothesized to depend on their ancient evolutionary origin (Askenase, 2021).

The results of EV fusions induce many operational effects, distinct from those of conventional secretion, including epigenetic mechanisms, widely spread among cells. Unconventionally secreted proteins presented in the review include tau, α-synuclein, SCAMP, FADD, interleukins (Yang et al., 2017; Bieri et al., 2018; Cypryk et al., 2018; Salvia et al., 2019; Brunello et al., 2020; Hassanpour et al., 2020; Jiang and Bhaskar, 2020; Sitia and Rubartelli, 2020). A list of highly relevant protein secretion can switch from conventional to unconventional and vice-versa, depending on the cells involved and their specific environmental conditions (Yang et al., 2017; Bieri et al., 2018). Moreover, interaction with autophagy can lead to fusion including machinery molecules (Hassanpour et al., 2020; Raudenska et al., 2021); and crosstalk of EVs with inflammasomes induces unconventional secretory proteins dependent on immune responses (Cypryk et al., 2018; Mouasni et al., 2019; Noonin and Thongboonkerd, 2021). In conclusion, the wide circulation of EVs can operate by biological and also medical processes dependent on critical molecules that do not diffuse independently through intercellular fluids but exchanges by reciprocal fusion of EVs between cells.

Compared to conventional secretion, knowledge of unconventional secretion is still limited, however it is growing, especially in the disease areas already intensely investigated (see for example the reviews Gonzalez et al., 2020; Raffaele et al., 2020; Ganesan and Cai, 2021). In the near future, therefore, the role of unconventional protein secretion will become more and more relevant in key areas, from physiology to medicine and especially to clinical practice.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

Thanks to Gabriella Racchetti for her support with the Figures of this work.

REFERENCES

Ageta, H., and Tsuchida, K. (2019). Post-translational Modification and Protein Sorting to Small Extracellular Vesicles Including Exosomes by Ubiquitin and UBLs. *Cell Mol. Life Sci.* 76, 4829–4848. doi:10.1007/s00018-019-03246-7

Akatsuka, H., Kawai, E., Omori, K., and Shibatani, T. (1995). The Three Genes lipB, lipC, and lipD Involved in the Extracellular Secretion of the Serratia marcescens Lipase Which Lacks an N-Terminal Signal Peptide. *J. Bacteriol.* 177, 6381–6389. doi:10.1128/jb.177.22.6381-6389.1995

An, S. J., Rivera-Molina, F., Anneken, A., Xi, Z., McNellis, B., Polejaev, V. I., and Toomre, D. (2021). An Active Tethering Mechanism Controls the Fate of Vesicles. *Nat. Commun.* 12, 5434. doi:10.1038/s41467-021-25465-y

Anand, S., Foot, N., Ang, C. S., Gembus, K. M., Keerthikumar, S., Adda, C. G., Mathivanan, S., and Kumar, S. (2018). Arrestin-Domain Containing Protein 1 (Arrdc1) Regulates the Protein Cargo and Release of Extracellular Vesicles. *Proteomics* 18, 1800266. doi:10.1002/pmic.201800266

Askenase, P. W. (2021). Ancient Evolutionary Origin and Properties of Universally Produced Natural Exosomes Contribute to Their Therapeutic Superiority Compared to Artificial Nanoparticles. *Ijms* 22, 1429. doi:10.3390/ijms22031429

Bänfer, S., Schneider, D., Dewes, J., Strauss, M. T., Freibert, S.-A., Heimerl, T., Maier, U. G., Elsässer, H.-P., Jungmann, R., and Jacob, R. (2018). Molecular Mechanism to Recruit Galectin-3 into Multivesicular Bodies for Polarized Exosomal Secretion. *Proc. Natl. Acad. Sci. U.S.A.* 115, E4396–E4405. doi:10.1073/pnas.1718921115

Bebelman, M. P., Bun, P., Huveneers, S., van Niel, G., Pegtel, D. M., and Verweij, F. J. (2020). Real-time Imaging of Multivesicular Body-Plasma Membrane Fusion to Quantify Exosome Release from Single Cells. *Nat. Protoc.* 15, 102–121. doi:10.1038/s41596-019-0245-4

Bello-Gamboa, A., Velasco, M., Moreno, S., Herranz, G., Ilie, R., Huetos, S., Dávila, S., Sánchez, A., Bernardino De La Serna, J., Calvo, V., and Izquierdo, M. (2020). Actin Reorganization at the Centrosomal Area and the Immune Synapse Regulates Polarized Secretory Traffic of Multivesicular Bodies in T Lymphocytes. *J. Extracell. Vesicles* 9, 1759926. doi:10.1080/20013078.2020.1759926

Bieri, G., Gitler, A. D., and Brahic, M. (2018). Internalization, Axonal Transport and Release of Fibrillar Forms of Alpha-Synuclein. *Neurobiol. Dis.* 109, 219–225. doi:10.1016/j.nbd.2017.03.007

Boilard, E. (2018). Thematic Review Series: Exosomes and Microvesicles: Lipids as Key Components of Their Biogenesis and Functions Extracellular Vesicles and Their Content in Bioactive Lipid Mediators: More Than a Sack of microRNA. *J. Lipid Res.* 59, 2037–2046. doi:10.1194/jlr.R084640

Bost, J. P., Saher, O., Hagey, D., Mamand, D. R., Liang, X., Zheng, W., Corso, G., Gustafsson, O., Görgens, A., Smith, C. E., Zain, R., El Andaloussi, S., and Gupta, D. (2021). Growth Media Conditions Influence the Secretion Route and Release Levels of Engineered Extracellular Vesicles. *Adv. Healthc. Mater.* 11, 2101658. doi:10.1002/adhm.202101658

Brunello, C. A., Merezhko, M., Uronen, R.-L., and Huttunen, H. J. (2020). Mechanisms of Secretion and Spreading of Pathological Tau Protein. *Cell. Mol. Life Sci.* 77, 1721–1744. doi:10.1007/s00018-019-03349-1

Calvo, V., and Izquierdo, M. (2020). Inducible Polarized Secretion of Exosomes in T and B Lymphocytes. *Ijms* 21, 2631. doi:10.3390/ijms21072631

Caro, L. G., and Palade, G. E. (1964). Protein Synthesis, Storage, and Discharge in the Pancreatic Exocrine Cell. *J. Cell Biol.* 20, 473–495. doi:10.1083/jcb.20.3.473

Chen, Y., Zhao, Y., Yin, Y., Jia, X., and Mao, L. (2021). Mechanism of Cargo Sorting into Small Extracellular Vesicles. *Bioengineered* 12, 8186–8201. doi:10.1080/21655979.2021.1977767

Cocozza, F., Grisard, E., Martin-Jaular, L., Mathieu, M., and Théry, C. (2020). SnapShot: Extracellular Vesicles. *Cell* 182, 262–262e1. doi:10.1016/j.cell.2020.04.054

Cohen, M. J., Chirico, W. J., and Lipke, P. N. (2020). Through the Back Door: Unconventional Protein Secretion. *Cell Surf.* 6, 100045. doi:10.1016/j.tcsw.2020.100045

Colombo, F., Casella, G., Podini, P., Finardi, A., Racchetti, G., Norton, E. G., Cocucci, E., and Furlan, R. (2021). Polarized Cells Display Asymmetric Release of Extracellular Vesicles. *Traffic* 22, 98–110. doi:10.1111/tra.12775

Corbeil, D., Santos, M. F., Karbanová, J., Kurth, T., Rappa, G., and Lorico, A. (2020). Uptake and Fate of Extracellular Membrane Vesicles: Nucleoplasmic Reticulum-Associated Late Endosomes as a New Gate to Intercellular Communication. *Cells* 9, 1931. doi:10.3390/cells9091931

Cypryk, W., Nyman, T. A., and Matikainen, S. (2018). From Inflammasome to Exosome-Does Extracellular Vesicle Secretion Constitute an Inflammasome-dependent Immune Response? *Front. Immunol.* 9, 2188. doi:10.3389/fimmu.2018.02188

Dai, H., Zhang, S., Du, X., Zhang, W., Jing, R., Wang, X., and Pan, L. (2019). RhoA Inhibitor Suppresses the Production of Microvesicles and Rescues High Ventilation Induced Lung Injury. *Int. Immunopharmacol.* 72, 74–81. doi:10.1016/j.intimp.2019.03.059

Davis, L. J., Bright, N. A., Edgar, J. R., Parkinson, M. D. J., Wartosch, L., Mantell, J., Peden, A. A., and Luzio, J. P. (2021). Organelle Tethering, Pore Formation and SNARE Compensation in the Late Endocytic Pathway. *J. Cell Sci.* 134, jcs255463. doi:10.1242/jcs.255463

Ganesan, D., and Cai, Q. (2021). Understanding Amphisomes. *Biochem. J.* 478, 1959–1976. doi:10.1042/BCJ20200917

Garcia-Martin, R., Wang, G., Brandão, B. B., Zanotto, T. M., Shah, S., Kumar Patel, S., Schilling, B., and Kahn, C. R. (2021). MicroRNA Sequence Codes for Small Extracellular Vesicle Release and Cellular Retention. *Nature* 601, 446–451. doi:10.1038/s41586-021-04234-3

Gonzalez, C. D., Resnik, R., and Vaccaro, M. I. (2020). Secretory Autophagy and its Relevance in Metabolic and Degenerative Disease. *Front. Endocrinol.* 11, 266. doi:10.3389/fendo.2020.00266

Greening, D. W., and Simpson, R. J. (2018). Understanding Extracellular Vesicle Diversity - Current Status. *Expert Rev. Proteomics* 15, 887–910. doi:10.1080/14789450.2018.1537788

Gruenberg, J. (2020). Life in the Lumen: The Multivesicular Endosome. *Traffic* 21, 76–93. doi:10.1111/tra.12715

Guan, F., Xiang, X., Xie, Y., Li, H., Zhang, W., Shu, Y., Wang, J., and Qin, W. (2021). Simultaneous Metabolomics and Proteomics Analysis of Plasma-Derived Extracellular Vesicles. *Anal. Methods* 13, 1930–1938. doi:10.1039/d1ay00060h

Gurunathan, S., Kang, M.-H., Qasim, M., Khan, K., and Kim, J.-H. (2021). Biogenesis, Membrane Trafficking, Functions, and Next Generation Nanotherapeutics Medicine of Extracellular Vesicles. *Ijn* 16, 3357–3383. doi:10.2147/IJN.S310357

Gurung, S., Perocheau, D., Touramanidou, L., and Baruteau, J. (2021). The Exosome Journey: from Biogenesis to Uptake and Intracellular Signalling. *Cell Commun. Signal* 19, 47. doi:10.1186/s12964-021-00730-1

Hassanpour, M., Rezabakhsh, A., Rezaie, J., Nouri, M., and Rahbarghazi, R. (2020). Exosomal Cargos Modulate Autophagy in Recipient Cells via Different Signaling Pathways. *Cell Biosci.* 10, 92. doi:10.1186/s13578-020-00455-7

He, J., Ren, W., Wang, W., Han, W., Jiang, L., Zhang, D., and Guo, M. (2021). Exosomal Targeting and its Potential Clinical Application. *Drug Deliv. Transl. Res.* doi:10.1007/s13346-021-01087-1

Hessvik, N. P., and Llorente, A. (2018). Current Knowledge on Exosome Biogenesis and Release. *Cell. Mol. Life Sci.* 75, 193–208. doi:10.1007/s00018-017-2595-9

Huber, S. T., Mostafavi, S., Mortensen, S. A., and Sachse, C. (2020). Structure and Assembly of ESCRT-III Helical Vps24 Filaments. *Sci. Adv.* 6, eaba4897. doi:10.1126/sciadv.aba4897

Hung, M. E., and Leonard, J. N. (2016). A Platform for Actively Loading Cargo RNA to Elucidate Limiting Steps in EV-Mediated Delivery. *J. Extracell. Vesicles* 5, 31027. doi:10.3402/jev.v5.31027

Hyenne, V., Labouesse, M., and Goetz, J. G. (2018). The Small GTPase Ral Orchestrates MVB Biogenesis and Exosome Secretion. *Small GTPases* 9, 445–451. doi:10.1080/21541248.2016.1251378

Jiang, S., and Bhaskar, K. (2020). Degradation and Transmission of Tau by Autophagic-Endolysosomal Networks and Potential Therapeutic Targets for Tauopathy. *Front. Mol. Neurosci.* 13, 586731. doi:10.3389/fnmol.2020.586731

Johnson, D. S., Bleck, M., and Simon, S. M. (2018). Timing of ESCRT-III Protein Recruitment and Membrane Scission during HIV-1 Assembly. *Elife* 7, e36221. doi:10.7554/eLife.36221

Joshi, B. S., de Beer, M. A., Giepmans, B. N. G., and Zuhorn, I. S. (2020). Endocytosis of Extracellular Vesicles and Release of Their Cargo from Endosomes. *ACS Nano* 14, 4444–4455. doi:10.1021/acsnano.9b10033

Juan, T., and Fürthauer, M. (2018). Biogenesis and Function of ESCRT-dependent Extracellular Vesicles. *Seminars Cell & Dev. Biol.* 74, 66–77. doi:10.1016/j.semcdb.2017.08.022

Karim, M. A., Samyn, D. R., Mattie, S., and Brett, C. L. (2018). Distinct Features of Multivesicular Body-Lysosome Fusion Revealed by a New Cell-free Content-Mixing Assay. *Traffic* 19, 138–149. doi:10.1111/tra.12543

Kazan, J. M., Desrochers, G., Martin, C. E., Jeong, H., Kharitidi, D., Apaja, P. M., Roldan, A., St. Denis, N., Gingras, A.-C., Lukacs, G. L., and Pause, A. (2021). Endofin Is Required for HD-PTP and ESCRT-0 Interdependent Endosomal Sorting of Ubiquitinated Transmembrane Cargoes. *iScience* 24, 103274. doi:10.1016/j.isci.2021.103274

Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J. P., Primdal-Bengtson, B., Dingli, F., Loew, D., Tkach, M., and Théry, C. (2016). Proteomic Comparison Defines Novel Markers to Characterize Heterogeneous Populations of Extracellular Vesicle Subtypes. *Proc. Natl. Acad. Sci. U.S.A.* 113, E968–E977. doi:10.1073/pnas.1521230113

Lee, B. R., Sanstrum, B. J., Liu, Y., and Kwon, S.-H. (2019). Distinct Role of Sirtuin 1 (SIRT1) and Sirtuin 2 (SIRT2) in Inhibiting Cargo-Loading and Release of Extracellular Vesicles. *Sci. Rep.* 9, 20049. doi:10.1038/s41598-019-56635-0

Leidal, A. M., Huang, H. H., Marsh, T., Solvik, T., Zhang, D., Ye, J., Kai, F., Goldsmith, J., Liu, J. Y., Huang, Y.-H., Monkkonen, T., Vlahakis, A., Huang, E. J., Goodarzi, H., Yu, L., Wiita, A. P., and Debnath, J. (2020). The LC3-Conjugation Machinery Specifies the Loading of RNA-Binding Proteins into Extracellular Vesicles. *Nat. Cell Biol.* 22, 187–199. doi:10.1038/s41556-019-0450-y

Levy, D., Do, M. A., Zhang, J., Brown, A., and Lu, B. (2021). Orchestrating Extracellular Vesicle with Dual Reporters for Imaging and Capturing in Mammalian Cell Culture. *Front. Mol. Biosci.* 8, 680580. doi:10.3389/fmolb.2021.680580

Lim, H. J., Yoon, H., Kim, H., Kang, Y.-W., Kim, J.-E., Kim, O. Y., Lee, E.-Y., Twizere, J.-C., Rak, J., and Kim, D.-K. (2021). Extracellular Vesicle Proteomes Shed Light on the Evolutionary, Interactive, and Functional Divergence of Their Biogenesis Mechanisms. *Front. Cell Dev. Biol.* 9, 734950. doi:10.3389/fcell.2021.734950

Liu, H., Liu, S., Xiao, Y., Song, W., Li, H., Ho, L. W. C., Shen, Z., and Choi, C. H. J. (2021). A pH-Reversible Fluorescent Probe for *In Situ* Imaging of Extracellular Vesicles and Their Secretion from Living Cells. *Nano Lett.* 21, 9224–9232. doi:10.1021/acs.nanolett.1c03110

Liu, X.-M., Ma, L., and Schekman, R. (2021). Selective Sorting of microRNAs into Exosomes by Phase-Separated YBX1 Condensates. *Elife* 10, e71982. doi:10.7554/eLife.71982

Lv, Y., Tan, J., Miao, Y., and Zhang, Q. (2019). The Role of Microvesicles and its Active Molecules in Regulating Cellular Biology. *J. Cell Mol. Med.* 23, 7894–7904. doi:10.1111/jcmm.14667

Mathieu, M., Névo, N., Jouve, M., Valenzuela, J. I., Maurin, M., Verweij, F. J., Palmulli, R., Lankar, D., Dingli, F., Loew, D., Rubinstein, E., Boncompain, G., Perez, F., and Théry, C. (2021). Specificities of Exosome versus Small Ectosome Secretion Revealed by Live Intracellular Tracking of CD63 and CD9. *Nat. Commun.* 12, 4389. doi:10.1038/s41467-021-24384-2

Matsui, T., Osaki, F., Hiragi, S., Sakamaki, Y., and Fukuda, M. (2021). ALIX and Ceramide Differentially Control Polarized Small Extracellular Vesicle Release from Epithelial Cells. *EMBO Rep.* 2222, e51475. doi:10.15252/embr.202051475

Meldolesi, J., Borgese, N., De Camilli, P., and Ceccarelli, B. (1978). "Cytoplasmic Membranes and the Secretory Process," in *Membrane Fusion*. Editors G. Poste and G.L. Nicolson (Amsterdam: Elsevier/North-Holland Biomed Press), 509–627.

Meldolesi, J. (2018). Exosomes and Ectosomes in Intercellular Communication. *Curr. Biol.* 28, R435–R444. doi:10.1016/j.cub.2018.01.059

Mouasni, S., Gonzalez, V., Schmitt, A., Bennana, E., Guillonneau, F., Mistou, S., Avouac, J., Ea, H. K., Devauchelle, V., Gottenberg, J.-E., Chiocchia, G., and Tourneur, L. (2019). The Classical NLRP3 Inflammasome Controls FADD Unconventional Secretion through Microvesicle Shedding. *Cell Death Dis.* 10, 190. doi:10.1038/s41419-019-1412-9

Müller, M., Ibrahimi, I., Chang, C. N., Walter, P., and Blobel, G. (1982). A Bacterial Secretory Protein Requires Signal Recognition Particle for Translocation across Mammalian Endoplasmic Reticulum. *J. Biol. Chem.* 257, 11860–11863. doi:10.1016/s0021-9258(18)33642-1

Ng, F., and Tang, B. L. (2016). Unconventional Protein Secretion in Animal Cells. *Methods Mol. Biol.* 1459, 31–46. doi:10.1007/978-1-4939-3804-9_2

Ni, K., Wang, C., Carnino, J. M., and Jin, Y. (2020). The Evolving Role of Caveolin-1: a Critical Regulator of Extracellular Vesicles. *Med. Sci.* 8, 46. doi:10.3390/medsci8040046

Nickel, W., and Rabouille, C. (2009). Mechanisms of Regulated Unconventional Protein Secretion. *Nat. Rev. Mol. Cell Biol.* 10, 148–155. doi:10.1038/nrm2617

Nikoloff, J. M., Saucedo-Espinosa, M. A., Kling, A., and Dittrich, P. S. (2021). Identifying Extracellular Vesicle Populations from Single Cells. *Proc. Natl. Acad. Sci. U.S.A.* 118, e2106630118. doi:10.1073/pnas.2106630118

Noonin, C., and Thongboonkerd, V. (2021). Exosome-inflammasome Crosstalk and Their Roles in Inflammatory Responses. *Theranostics* 11, 4436–4451. doi:10.7150/thno.54004

Padovani, C., Jevtić, P., and Rapé, M. (2022). Quality Control of Protein Complex Composition. *Mol. Cell* 82, 1439–1450. doi:10.1016/j.molcel.2022.02.029

Palade, G. E., Siekevitz, P., and Caro, L. G. (1962). "Structure, Chemistry and Function of the Pancreatic Acinar Cell CIBA Fdn. Symposium," in *The Exocrine Pancreas, Normal and Abnormal Function*. Editors A. V. S. de Reuck, M.P.J. Cameron, and A. Cameron(United Kingdom: Churchill), 23–55.

Pavlin, M. R, and Hurley, J. H. (2020). The ESCRTs - Converging on Mechanism. *J. Cell Sci.* 133, jcs24333. doi:10.1242/jcs.240333

Peng, X., Li, X., Yang, S., Huang, M., Wei, S., Ma, Y., Li, Y., Wu, B., Jin, H., Li, B., Tang, S., Fan, Q., Liu, J., Yang, L., and Li, H. (2021). LINC00511 Drives Invasive Behavior in Hepatocellular Carcinoma by Regulating Exosome Secretion and Invadopodia Formation. *J. Exp. Clin. Cancer Res.* 40, 183. doi:10.1186/s13046-021-01990-y

Perissinotto, F., Rondelli, V., Senigagliesi, B., Brocca, P., Almásy, L., Bottyán, L., Merkel, D. G., Amenitsch, H., Sartori, B., Pachler, K., Mayr, M., Gimona, M., Rohde, E., Casalis, L., and Parisse, P. (2021). Structural Insights into Fusion Mechanisms of Small Extracellular Vesicles with Model Plasma Membranes. *Nanoscale* 13, 5224–5233. doi:10.1039/d0nr09075a

Pizzirani, C., Ferrari, D., Chiozzi, P., Adinolfi, E., Sandonà, D., Savaglio, E., and Di Virgilio, F. (2007). Stimulation of P2 Receptors Causes Release of IL-1β -loaded Microvesicles from Human Dendritic Cells. *Blood* 109, 3856–3864. doi:10.1182/blood-2005-06-031377

Puri, N., and Roche, P. A. (2006). Ternary SNARE Complexes Are Enriched in Lipid Rafts during Mast Cell Exocytosis. *Traffic* 7, 1482–1494. doi:10.1111/j.1600-0854.2006.00490.x

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cell Biol.* 27, 230–240. doi:10.1016/j.tcb.2016.11.007

Raffaele, S., Lombardi, M., Verderio, C., and Fumagalli, M. (2020). TNF Production and Release from Microglia via Extracellular Vesicles: Impact on Brain Functions. *Cells* 9, 2145. doi:10.3390/cells9102145

Ras-Carmona, A., Gomez-Perosanz, M., and Reche, P. A. (2021). Prediction of Unconventional Protein Secretion by Exosomes. *BMC Bioinforma.* 22, 333. doi:10.1186/s12859-021-04219-z

Raudenska, M., Balvan, J., and Masarik, M. (2021). Crosstalk between Autophagy Inhibitors and Endosome-Related Secretory Pathways: a Challenge for Autophagy-Based Treatment of Solid Cancers. *Mol. Cancer* 20, 140. doi:10.1186/s12943-021-01423-6

Razzauti, A., and Laurent, P. (2021). Ectocytosis Prevents Accumulation of Ciliary Cargo in *C. elegans* Sensory Neurons. *Elife* 10, e67670. doi:10.7554/eLife.67670

Royo, F., Théry, C., Falcón-Pérez, J. M., Nieuwland, R., and Witwer, K. W. (2020). Methods for Separation and Characterization of Extracellular Vesicles: Results of a Worldwide Survey Performed by the ISEV Rigor and Standardization Subcommittee. *Cells* 9, 1955. doi:10.3390/cells9091955

Rubartelli, A., Bajetto, A., Allavena, G., Cozzolino, F., and Sitia, R. (1993). Post-translational Regulation of Interleukin 1β Secretion. *Cytokine* 5, 117–124. doi:10.1016/1043-4666(93)90050-F

Rubartelli, A., Cozzolino, F., Talio, M., and Sitia, R. (1990). A Novel Secretory Pathway for Interleukin-1 Beta, a Protein Lacking a Signal Sequence. *EMBO J.* 9, 1503–1510. doi:10.1002/j.1460-2075.1990.tb08268.x

Salvia, R., Grimaldi, A., Girardello, R., Scieuzo, C., Scala, A., Bufo, S. A., Vogel, H., and Falabella, P. (2019). Aphidius Ervi Teratocytes Release Enolase and Fatty Acid Binding Protein through Exosomal Vesicles. *Front. Physiol.* 10, 715. doi:10.3389/fphys.2019.00715

Schramm, M. (1967). Secretion of Enzymes and Other Macromolecules. *Annu. Rev. Biochem.* 36, 307–320. doi:10.1146/annurev.bi.36.070167.001515

Shtam, T., Evtushenko, V., Samsonov, R., Zabrodskaya, Y., Kamyshinsky, R., Zabegina, L., Verlov, N., Burdakov, V., Garaeva, L., Slyusarenko, M., Nikiforova, N., Konevega, A., and Malek, A. (2020). Evaluation of Immune and Chemical Precipitation Methods for Plasma Exosome Isolation. *PLoS One* 15, e0242732. doi:10.1371/journal.pone.0242732

Sitia, R., and Rubartelli, A. (2020). Evolution, Role in Inflammation, and Redox Control of Leaderless Secretory Proteins. *J. Biol. Chem.* 295, 7799–7811. doi:10.1074/jbc.REV119.008907

Somiya, M., and Kuroda, S. i. (2021). Reporter Gene Assay for Membrane Fusion of Extracellular Vesicles. *J. Extracell. Vesicles* 10, e12171. doi:10.1002/jev2.12171

Somiya, M. (2020). Where Does the Cargo Go?: Solutions to Provide Experimental Support for the "extracellular Vesicle Cargo Transfer Hypothesis". *J. Cell Commun. Signal.* 14, 135–146. doi:10.1007/s12079-020-00552-9

Song, L., Chen, J., Sun, A., and Schekman, R. (2021). APEX-mediated Proximity Labeling of Proteins in Cells Targeted by Extracellular Vesicles. *Bio-protocol* 11, e4213. doi:10.21769/BioProtoc.4213

Sun, M., Xue, X., Li, L., Xu, D., Li, S., Li, S. C., and Su, Q. (2021). Ectosome Biogenesis and Release Processes Observed by Using Live-Cell Dynamic Imaging in Mammalian Glial Cells. *Quant. Imaging Med. Surg.* 11, 4604–4616. doi:10.21037/qims-20-1015

Tseng, C.-C., Dean, S., Davies, B. A., Azmi, I. F., Pashkova, N., Payne, J. A., Staffenhagen, J., West, M., Piper, R. C., Odorizzi, G., and Katzmann, D. J. (2021). Bro1 Stimulates Vps4 to Promote Intralumenal Vesicle Formation during Multivesicular Body Biogenesis. *J. Cell Biol.* 220, e202102070. doi:10.1083/jcb.202102070

Van Niel, G., D'Angelo, G., and Raposo, G. (2018). Shedding Light on the Cell Biology of Extracellular Vesicles. *Nat. Rev. Mol. Cell Biol.* 19, 213–228. doi:10.1038/nrm.2017.125

Verweij, F. J., Bebelman, M. P., Jimenez, C. R., Garcia-Vallejo, J. J., Janssen, H., Neefjes, J., Knol, J. C., de Goeij-de Haas, R., Piersma, S. R., Baglio, S. R., Verhage, M., Middeldorp, J. M., Zomer, A., van Rheenen, J., Coppolino, M. G., Hurbain, I., Raposo, G., Smit, M. J., Toonen, R. F. G., van Niel, G., and Pegtel, D. M. (2018). Quantifying Exosome Secretion from Single Cells Reveals a Modulatory Role for GPCR Signaling. *J. Cell Biol.* 217, 1129–1142. doi:10.1083/jcb.201703206

Walter, P., and Blobel, G. (1981). Translocation of Proteins across the Endoplasmic Reticulum. II. Signal Recognition Protein (SRP) Mediates the Selective Binding to Microsomal Membranes of In-Vitro-Assembled Polysomes Synthesizing Secretory Protein. *J. Cell Biol.* 91, 551–556. doi:10.1083/jcb.91.2.551

Walter, P., Gilmore, R., and Blobel, G. (1984). Protein Translocation across the Endoplasmic Reticulum. *Cell* 38, 5–8. doi:10.1016/0092-8674(84)90520-8

Wang, J., Zhuang, X., Greene, K. S., Si, H., Antonyak, M. A., Druso, J. E., Wilson, K. F., Cerione, R. A., Feng, Q., and Wang, H. (2021). Cdc42 Functions as a Regulatory Node for Tumour-derived Microvesicle Biogenesis. *J. Extracell. Vesicles* 10, e12051. doi:10.1002/jev2.12051

Yang, J.-M., and Gould, S. J. (2013). The Cis-Acting Signals that Target Proteins to Exosomes and Microvesicles. *Biochem. Soc. Trans.* 41, 277–282. doi:10.1042/BST20120275

Yang, L., Peng, X., Li, Y., Zhang, X., Ma, Y., Wu, C., Fan, Q., Wei, S., Li, H., and Liu, J. (2019). Long Non-coding RNA HOTAIR Promotes Exosome Secretion by Regulating RAB35 and SNAP23 in Hepatocellular Carcinoma. *Mol. Cancer* 18, 78. doi:10.1186/s12943-019-0990-6

Yang, Y., Qin, M., Bao, P., Xu, W., and Xu, J. (2017). Secretory Carrier Membrane Protein 5 Is an Autophagy Inhibitor that Promotes the Secretion of α-Synuclein via Exosome. *PLoS One* 12, e0180892. doi:10.1371/journal.pone.0180892

Zhang, M., Liu, L., Lin, X., Wang, Y., Li, Y., Guo, Q., Li, S., Sun, Y., Tao, X., Zhang, D., Lv, X., Zheng, L., and Ge, L. (2020). A Translocation Pathway for Vesicle-Mediated Unconventional Protein Secretion. *Cell* 181, 637–652.e15. doi:10.1016/j.cell.2020.03.031

Zhao, X., Guan, Y., Liu, F., Yan, S., Wang, Y., Hu, M., Li, Y., Li, R., and Zhang, C. X. (2022). SNARE Proteins Mediate α-Synuclein Secretion via Multiple Vesicular Pathways. *Mol. Neurobiol.* 59, 405–419. doi:10.1007/s12035-021-02599-0

Zhao, Y. G., and Zhang, H. (2019). Autophagosome Maturation: An Epic Journey from the ER to Lysosomes. *J. Cell Biol.* 218, 757–770. doi:10.1083/jcb.201810099

Zhao, Z., Kesti, T., Uğurlu, H., Baur, A. S., Fagerlund, R., and Saksela, K. (2019). Tyrosine Phosphorylation Directs TACE into Extracellular Vesicles via Unconventional Secretion. *Traffic* 20, 202–212. doi:10.1111/tra.12630

Zhu, Q., Yamakuchi, M., and Lowenstein, C. J. (2015). SNAP23 regulates endothelial exocytosis of von Willebrand factor. *PLoS One* 10, e0118737. doi:10.1371/journal.pone.0118737

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

REVIEW
published: 14 June 2022
doi: 10.3389/fcell.2022.892450

Autophagy-Related Pathways in Vesicular Unconventional Protein Secretion

Shin Hye Noh[1]*, *Ye Jin Kim*[2] *and Min Goo Lee*[1,2]*

[1]Severance Biomedical Science Institute, Yonsei University College of Medicine, Seoul, South Korea, [2]Department of Pharmacology, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, South Korea

Cellular proteins directed to the plasma membrane or released into the extracellular space can undergo a number of different pathways. Whereas the molecular mechanisms that underlie conventional ER-to-Golgi trafficking are well established, those associated with the unconventional protein secretion (UPS) pathways remain largely elusive. A pathway with an emerging role in UPS is autophagy. Although originally known as a degradative process for maintaining intracellular homeostasis, recent studies suggest that autophagy has diverse biological roles besides its disposal function and that it is mechanistically involved in the UPS of various secretory cargos including both leaderless soluble and Golgi-bypassing transmembrane proteins. Here, we summarize current knowledge of the autophagy-related UPS pathways, describing and comparing diverse features in the autophagy-related UPS cargos and autophagy machineries utilized in UPS. Additionally, we also suggest potential directions that further research in this field can take.

Keywords: unconventional protein secretion (UPS), autophagy, multi-vesicular body, GRASP, golgi bypass

OPEN ACCESS

Edited by:
Julien Villeneuve,
Institute for Functional Genomics (IGF) (CNRS), France

Reviewed by:
Sungmin Son,
University of Cambridge, United Kingdom
Yanzhuang Wang,
University of Michigan, United States

***Correspondence:**
Shin Hye Noh
alley282@yuhs.ac
Min Goo Lee
mlee@yuhs.ac

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *09 March 2022*
Accepted: *26 May 2022*
Published: *14 June 2022*

Citation:
Noh SH, Kim YJ and Lee MG (2022) Autophagy-Related Pathways in Vesicular Unconventional Protein Secretion. Front. Cell Dev. Biol. 10:892450. doi: 10.3389/fcell.2022.892450

INTRODUCTION

Since the processes underpinning secretory protein synthesis and the intracellular trafficking pathway were initially identified over 50 years ago, it has been believed that the majority of proteins secreted into the extracellular space or to be anchored on the plasma membrane move along the classical trafficking pathway (Blobel and Sabatini, 1971; Palade, 1975; Rothman, 1994). Secretory proteins bearing a signal sequence (also known as the leader sequence) or transmembrane domain are targeted to the endoplasmic reticulum (ER) where they undergo N-glycosylation, folding, quality control, and oligomerization, followed by sorting into COPII-coated vesicles before transport to the Golgi apparatus en route to their final destinations (Ferro-Novick and Brose, 2013). In addition, it has also been noted that signal peptide-lacking proteins (leaderless proteins) and some integral membrane proteins can be secreted or reach the plasma membrane via one of several alternative routes collectively known as the unconventional protein secretion (UPS) pathway (Rabouille et al., 2012). To date, it has been revealed that a considerable number of proteins can be transported via UPS and that diverse machineries and mechanisms are involved.

The UPS pathways can be classified into four types according to the distinct features of the cargo proteins and mediating mechanisms involved: type I, pore-mediated translocation across the plasma membrane; type II, ABC transporter-mediated secretion; type III, membrane-bound intermediates; and type IV, Golgi bypass of transmembrane proteins (**Figure 1**) (Nickel and Rabouille, 2009; Rabouille et al., 2012; Rabouille, 2017). The type I, II and III UPS cargos are mostly leaderless soluble proteins, lacking a signal peptide, and are synthesized in the cytosol. On the other hand, type IV UPS

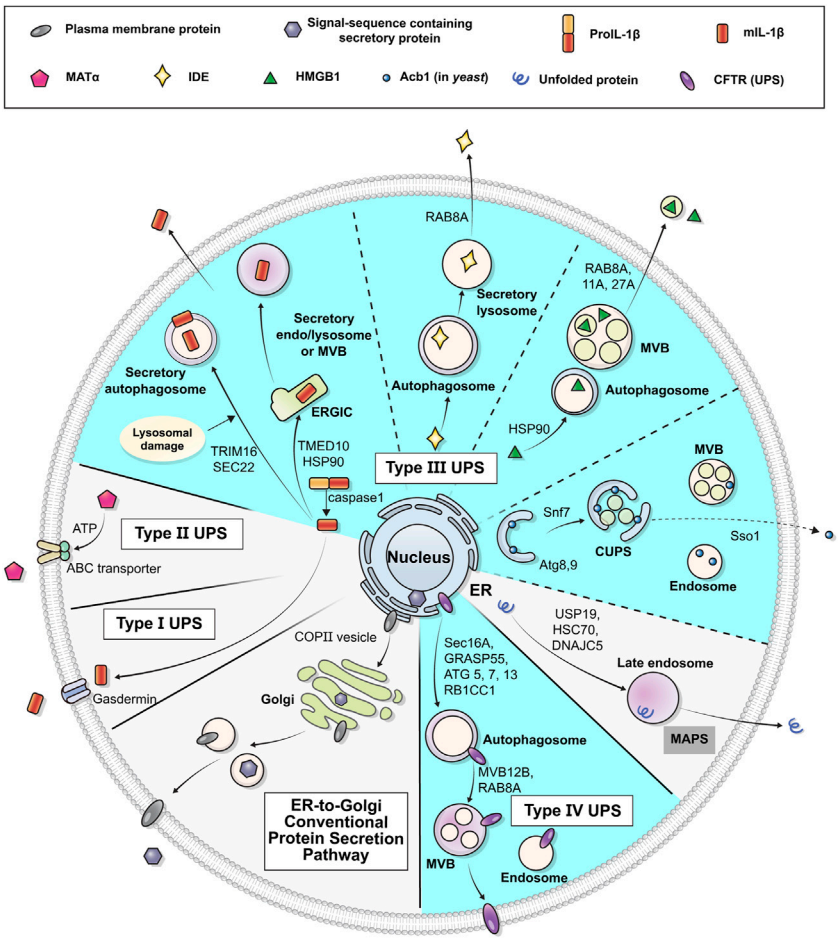


FIGURE 1 | Schematic representation of unconventional protein secretion. Transmembrane proteins and ER luminal cytosolic proteins in general pass through the ER and the Golgi apparatus in their route to their final destinations (conventional secretion). Some leaderless cytosolic proteins can be secreted to extracellular space from cytosol via the membrane pore (Type I UPS), ABC-transporter (Type II UPS), or vesicles (Type III UPS). Some transmembrane proteins may alternatively be transported to the plasma membrane via an unconventional secretory pathway that bypasses the Golgi (Type IV UPS). Diverse vesicular systems, including autophagosomes, multivesicular bodies (MVBs) and CUPS, are involved in the type III and IV UPS pathways. The UPS cargos utilizing autophagy machineries are marked in cyan (see text for details).

cargos are originally synthesized in the ER and bypass the Golgi apparatus under certain circumstances, such as ER stress or ER-Golgi blockade, or at specific cellular localizations such as cilia and neuronal axons (Gee et al., 2018; Gonzalez et al., 2018). Whereas type I and II cargos are secreted into the extracellular space across the plasma membrane directly, type III and IV pathways utilize vesicular intermediates. These vesicular transport pathways are mediated by membrane-bound compartments, in which hydrophilic/cytosolic proteins are carried enclosed in the lumen of vesicles while integral membrane proteins are delivered via insertion into the vesicular membranes. Secretory proteins following the conventional pathway mostly use COPI, COPII, and clathrin-coated vesicles for their movement to the next stop or destination (Bard and Malhotra, 2006; Béthune and Wieland, 2018); however, the molecular nature of the vesicular machineries that mediate UPS appears to be complicated rather than composed of a single unified route. Many researchers have suggested a number of components and mechanisms that could modulate vesicular UPS. Notably, mounting evidence highlights the importance of autophagy-related proteins in the vesicular UPS.

Autophagy is an evolutionarily conserved mechanism originally defined as a degradative process which, upon starvation or cellular signal, is induced to recycle and supplement nutrients. This process involves the degradation of cytoplasmic materials (damaged organelles or dispensable components) to eliminate toxic and superfluous cytosolic substances via engulfment into the double-membranous vesicle (or so-called autophagosome), which is the most characteristic feature of this process, followed by degradation through fusion with a lysosome to ultimately maintain intracellular homeostasis (Glick et al., 2010). However, elimination of the captured material is not the only function of autophagy. Recent results show that autophagy components also play diverse roles in non-degradative processes including those occurring in the protein secretory pathways (Dupont et al., 2011; Bestebroer et al., 2013; Cadwell

TABLE 1 | Autophagy-dependency of UPS cargo proteins.

UPS type			UPS cargo	References
Non-vesicular	Type I		FGF2, HIV-TAT, IL-1β[a], PfCDPK1, tau[a]	Chang et al. (1997); Möskes et al. (2004); Schäfer et al. (2004); Evavold et al. (2018); Merezhko et al. (2018)
	Type II		MATα, HASPB	McGrath and Varshavsky, (1989); Denny et al. (2000)
Vesicular	Type III	Autophagy-related	AcbA, Acb1, IL-1β[a], ferritin, IL18, HMGB1, MMP2, TGM2, α-synuclein[b], IDE, FABP4[b], α-crystallin-B/HSPB5, TGFβ1, PARK7/DJ-1, annexin-A2, tau[a], SOD1	Kinseth et al. (2007); Duran et al. (2010); Manjithaya et al. (2010); Dupont et al. (2011); Poehler et al. (2014); Son et al. (2016); Chen et al. (2017); Kimura et al. (2017); Nüchel et al. (2018); Urano et al. (2018); Josephrajan et al. (2019); Kang et al. (2019); D'Agostino et al. (2019); Nüchel et al. (2021)
		Not-defined	tau[a], LIF, FAM3C, DKK3, AIM2, ARMS2, BAG3	
	Type IV	Autophagy-related	CFTR, Mpl	George et al. (1999); Baldwin and Ostergaard, (2002); Hasdemir et al. (2005); Penuela et al. (2007); Schotman et al. (2008); Merregaert et al. (2010); Hoffmeister et al. (2011); Gee et al. (2011); Tian et al. (2014); Cleyrat et al. (2014); Jung et al. (2016); Gee et al. (2018)
		Not-defined	αPS1 integrin in *drosophila*, polycystin2, M2 mutant of smoothened, Peripherin/rds,CD45, Connexin26 and 30, Kv4, pannexin1 and 3, phospholipid scramblase1, pendrin	

[a]*Following diverse routes.*
[b]*Existing contrary results.*

and Debnath, 2018; Cruz-Garcia et al., 2018; Galluzzi and Green, 2019).

The UPS process is generally activated under cellular stress conditions and is considered part of the cellular stress response (Kim et al., 2018; Park et al., 2020). Similarly, autophagy is also triggered by various cellular stress-inducing stimuli, so that the UPS and autophagy processes may share many common traits with significant overlap. There are some conceptual overlaps among UPS, secretory autophagy, and extracellular vesicle (EV) secretion. The EVs are small, membrane-bound particles secreted from cells, which can range from ~30–1,000 nm in diameter, and the smallest type of EV is the exosome, which is ~30–150 nm in size. While protein secretion via UPS includes the cell-surface translocation of transmembrane proteins, the secretory autophagy and exosome secretion entail the EV-mediated extracellular secretion of non-protein cargos such as RNAs and DNAs. Recent advances in our understanding of secretory autophagy and its role in EV secretion have been summarized in several reviews (Ponpuak et al., 2015; Leidal and Debnath, 2021). Here, we summarize the recent discoveries, showing where UPS and autophagy overlap and in particular focusing on the role of autophagy machineries in the vesicular type III and IV UPS pathways.

AUTOPHAGY-RELATED UPS CARGOS

Due to the vesicular nature of autophagy, unlike in the case of non-vesicular type I (e.g., FGF2, HIV-TAT) and type II (e.g., yeast MATα) UPS, the vesicular UPS cargos such as hydrophilic cytosolic proteins in type III UPS and transmembrane proteins in type IV UPS are subject to autophagy-related UPS cargos (**Table 1**).

Cytokines

It has been shown that several cytokines that do not bear the leader sequence are secreted in an autophagy-dependent manner. For example, interleukin-1 (IL-1) family members are secreted without entering the ER-Golgi conventional pathway after processed from their precursors upon inflammasome activation (Dupont et al., 2011). Secretion of IL-1β is enhanced upon stimulation of autophagy by the starvation and nigericin co-treatment, which depends on ATG5, GRASP55, and Rab8a (Dupont et al., 2011). High mobility group box 1 (HMGB1) and IL-18 also are released in a similar manner. HMGB1 is a nuclear protein whose secretion is mediated by inflammasome in response to various stimuli. Secretion of HMGB1 is inhibited by an early autophagy inhibitor, wortmannin or 3-methyladenine (3-MA), and reduced in ATG5-deficient cells (Kim et al., 2021; Wang et al., 2021).

Moreover, signal peptide-bearing proteins can be released via UPS under certain conditions. For example, transforming growth factor beta 1 (TGFβ1), a multifunctional cytokine possessing a signal peptide and playing a role in apoptosis, differentiation and development, was proposed to be secreted via different pathways independent of the conventional route while also involving the autophagic components (Nüchel et al., 2018). In addition, it has been indicated in a proteome analysis that approximately 40–50% of mTORC1- and GRASP55-dependent secretome and surfactome proteins harbor a signal peptide, implying that many signal peptide-bearing cargos can use UPS pathways for their delivery to the surface under certain cellular conditions (Nüchel et al., 2021). Further investigation is required to confirm whether all of these are exclusively UPS cargos, since it is possible that some mTORC1- and GRASP55-dependent secretory proteins may also undergo conventional pathways.

Neurodegenerative Disease-Associated Proteins

A number of neurodegenerative disease-associated proteins can be delivered to the plasma membrane through autophagy-mediated UPS pathways (Ejlerskov et al., 2013; Nilsson et al., 2013). α-Synuclein, particularly in its aggregated forms, has been implicated in the pathogenesis of Parkinson's disease (PD) and other neurological disorders (Polymeropoulos et al., 1997; Kahle, 2008). The extracellular secretion of α-synuclein may result in a cell-to-cell transmission of protein aggregates, which occurs in

many neurodegenerative disorders (Lee et al., 2005; Lee et al., 2010). Several studies have indicated that α-synuclein undergoes an autophagy-related UPS pathway (Ejlerskov et al., 2013; Cleyrat et al., 2014; Poehler et al., 2014; Yang et al., 2017). However, the role of autophagy in α-synuclein secretion has also been challenged by other researchers whose studies suggest that autophagy inhibition could promote α-synuclein secretion (Lee et al., 2013; Lee and Lee, 2016), and thus further investigation is required for clarification of specifically which autophagy components mediate α-synuclein secretion and how they do so. Accumulation of misfolded aggregated tau proteins in the nervous system is a hallmark of tauopathies such as Alzheimer's disease (AD). The tau secretion through the UPS pathway involves both non-vesicular and vesicular mechanisms (Brunello et al., 2020); and several recent studies suggest that autophagy machinery participates in the vesicular route-mediated tau secretion (Mohamed et al., 2014; Kang et al., 2019; Chen et al., 2020).

It is noteworthy that proteins which inhibit the aggregation of misfolded proteins, by promoting degradation or assisting the proper folding of aggregation-prone proteins, can also be secreted via an autophagy-associated UPS route. PARK7/DJ-1 (Parkinsonism associated deglycase) is a PD- and cancer-associated protein devoid of the leader sequence, functioning as a redox-sensitive chaperone and protein deglycase that inhibits the aggregation of α-synuclein and protects neurons against oxidative stress and cell death. Autophagy-related proteins ATG5, ATG9, and ATG16L1 were shown to be required for PARK7 secretion (Urano et al., 2018). Insulin-degrading enzyme (IDE) is a ubiquitously expressed protease with the ability to degrade pathological extracellular substrates such as an amyloid-beta peptide (Aβ), whose accumulation is associated with AD. IDE secretion has been reported to be unaffected by brefeldin A (BFA), an inhibitor of the conventional secretion pathway (Zhao et al., 2009), suggesting relevance to an unconventional secretion pathway; and other results, showing that a deficiency of ATG7 in mice decreases IDE activity, provide further validation of the role of IDE as a cargo of the autophagy-based UPS pathway (Son et al., 2016). α-Crystallin B (or HspB5) is a small heat-shock chaperone preventing aggregation and unfolding of target proteins under conditions of cellular stress and requires the autophagy-related unconventional pathway (D'Agostino et al., 2019). Mutant huntingtin (mHTT), causing Huntington's disease, has also been suggested as a cargo for autophagy-dependent UPS (Ahat et al., 2021). Mutant HTT secretion is enhanced under cellular stress conditions which involve autophagy activations including ATG7. Lastly, the mutant form of superoxide dismutase 1 (SOD1), linked to amyotrophic lateral sclerosis (ALS), has been shown to be secreted via a nutrient starvation-induced UPS pathway in yeast and HeLa cells (Cruz-Garcia et al., 2017).

Lipid Binding Proteins

Several lipid-binding proteins are shown to be secreted via an autophagy-associated UPS pathway. Annexin A2 (ANXA2), a phospholipid binding protein, was observed in autophagosomes and multivesicular bodies (MVBs), followed by exosomal release, but not in ATG5 knockdown cells (Chen et al., 2017). Fatty acid binding protein 4 (FABP4) is a signal sequence-lacking protein and mainly expressed in adipocytes to regulate the transport of lipids. FABP4 has been proposed to be a UPS cargo, but was initially described as an autophagy-independent cargo because FABP4 secretion was not affected in ATG5 deficient cells (Villeneuve et al., 2018). However, a later study showed that early autophagic genes such as Ulk1/2, Fip200 or Beclin-1 were required for FABP4 secretion even though knockdown of ATG5 enhanced the FABP4 release (Josephrajan et al., 2019).

Proteins in Yeast

Autophagy has been extensively investigated in yeast (Takeshige et al., 1992; Ohsumi, 2014), with early reports attempting to delineate the autophagy-related UPS cargos in yeast (Duran et al., 2010; Manjithaya et al., 2010). Secretion of Acb1 in yeast is induced by rapamycin, an autophagy activator, and is mediated by core autophagy machinery proteins which are necessary for autophagosome formation. Malhotra et al. identified a membrane structure in yeast, known as the compartment for unconventional protein secretion (CUPS), as being the source of organelles or trafficking intermediates for autophagy-based unconventional secretion of Acb1 (Bruns et al., 2011). It has been suggested that the CUPS is built during starvation and formed by COPI-independent extraction of membranes from the early Golgi cisternae, requiring PI4P for its biogenesis and PI3P for stability (Cruz-Garcia et al., 2014).

Transmembrane Proteins

Another important class of autophagy-related UPS is the type IV UPS whose substrates are transmembrane proteins. Despite harboring a signal peptide, a number of transmembrane proteins (e.g., CFTR, Mpl, Pendrin, αPS1 integrin) have been found to bypass the Golgi when reaching the plasma membrane under specific conditions (Schotman et al., 2008; Hoffmeister et al., 2011; Tian et al., 2014; Jung et al., 2016; Gee et al., 2018). Among these, two cargos have hitherto been proven to be associated with autophagy: cystic fibrosis transmembrane regulator (CFTR) and myeloproliferative leukemia protein (Mpl) (**Table 1**).

N-glycosylated membrane proteins acquire their complex glycosylation pattern while they travel through the Golgi (Reily et al., 2019). Golgi-mediated complex-glycosylation is thus considered to be a feature of proteins undergoing the conventional pathway, whereas proteins bypassing the Golgi demonstrate only an immature form of ER core-glycosylation. Therefore, the cell-surface expression of ER-core glycosylated proteins can be a sign of Golgi-bypass for those transmembrane proteins for which Golgi-mediated glycosylation patterns are defined, including CFTR, pendrin, and Mpl. CFTR is an anion channel transporting Cl^- and HCO_3^- across the apical membrane of epithelial cells and its mutations cause cystic fibrosis (CF) and chronic pancreatitis (Lee et al., 2012; Kim et al., 2020). The most common CFTR mutation among CF patients is the deletion of phenylalanine at the amino acid position 508 (ΔF508-CFTR), resulting in folding defects followed by ER-associated degradation (ERAD). Therefore, the folding defective ΔF508-CFTR neither exits the ER nor reaches the plasma membrane via

the conventional secretory pathway. Gee et al. (2011) have shown that the ER core-glycosylated ΔF508-CFTR can be rescued to the cell surface via a UPS pathway bypassing the Golgi, this process being induced by ER stress or GRASP55 overexpression in both mammalian cells and mouse models. Later studies have indicated that the early and core machineries of autophagy are required for the transport of ΔF508-CFTR to the plasma membrane via the UPS route (Noh et al., 2018). The core-glycosylated ER form of Mpl (a.k.a. the thrombopoietin receptor) has similarly been shown to reach the cell surface via a non-canonical pathway, involving the mechanism taken up into LC3-positive autophagic structures (Cleyrat et al., 2014). The UPS both of CFTR and Mpl utilizes GRASP55 for translocation of the proteins to the plasma membrane in addition to the autophagy machineries (Cleyrat et al., 2014; Noh et al., 2018).

AUTOPHAGY MACHINERIES UTILIZED IN UPS

Autophagy-Related Genes and Proteins

Autophagy can be subclassified into three types, depending on the manner of cargo delivery to the lysosome: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Mizushima et al., 2011). Among them, macroautophagy is the major type of autophagy and the most extensively researched. Macroautophagy is defined as a mechanism reliant on the ordered processing of ATG proteins congregated at the phagophore assembly site (PAS) to construct a double membranous organelle autophagosome, which will fuse with a lysosome. The overall macroautophagy (hereafter referred to as autophagy) process is composed of multiple steps: 1) initiation by stimuli inducing autophagy; 2) nucleation of the membrane, formed from diverse membrane sources, at the PAS; 3) expansion and formation of the double membrane structure with engulfment substrates; 4) fusion with lysosomal compartments; and 5) degradation of the captured materials.

The Autophagy-related genes (ATGs), evolutionarily conserved in mammals (Mizushima, 2018), were initially discovered in yeast and are known to be required for nucleation and elongation during the biogenesis of autophagosome (Klionsky, 2007). Recent evidence indicates that many ATG proteins perform a role not only in classical degradative autophagy but also in membrane trafficking, exosome secretion, and UPS (Galluzzi and Green, 2019). ATG5 is a representative protein participating in the early stages of the autophagy process. A knockdown or knockout ATG5 model has been utilized in most articles suggesting the association of UPS with autophagy (Dupont et al., 2011; Gee et al., 2011; Cleyrat et al., 2014; Urano et al., 2018; Kim et al., 2021). However, ATG5 does not seem to be involved in the UPS processes of some cargo proteins such as ANXA2 and FABP4. For example, as aforementioned, secretion of FABP4 from white adipose tissue upon lipolytic stimulation requires components of the ULK complex and PI3K class III that are necessary for autophagy initiation, while ATG5 inhibition enhances the FABP4 release (Josephrajan et al., 2019).

LC3B, an ATG8 family member, is most widely used as an autophagy marker. The conversion of LC3B-I into LC3B-II represents autophagosome maturation, since ATG8 proteins should become lipidated to be anchored in the autophagosome membranes and lipidated ATG8 proteins can recruit LIR-containing substrates (Klionsky et al., 2021). Deletion or knockdown of ATG8 genes have been shown to result in a reduced secretion of Acb1 and CFTR (Manjithaya et al., 2010; Gee et al., 2011). Quantitative measurements of lipidated LC3B-II relative to LC3B-I have been examined in several studies to show the relevance of autophagy in the UPS of diverse cargo proteins (Son et al., 2016; Noh et al., 2018; Urano et al., 2018; Josephrajan et al., 2019; Wang et al., 2021).

Not only ATG5 and LC3, but also multiple ATG proteins are involved in UPS. In yeast, Atg1, Atg6, Atg8, Atg9, Atg11, and Atg17 are necessary for Acb1 secretion in P. *pastoris* (Manjithaya et al., 2010). Additionally, Atg5, Atg7, Atg8 and Atg12 have been shown to be required for Acb1 secretion in S. *cerevisiae* (Duran et al., 2010). ATG16L1, interacting with the ATG12-ATG5 conjugate, also participates in the UPS of IL-1β, PARK7/DJ-1, and α-synuclein in mammalian cells (Kimura et al., 2017; Urano et al., 2018; Burbidge et al., 2021). The respective secretions of chemokine C-X-C motif ligand 8 (CXCL8), leukemia inhibitory factor (LIF), and family with sequence similarity 3 member C (FAM3C) were each enhanced when low-autophagy melanoma cells were treated with the autophagy-inducing tat-BECN1 peptide and reduced when ATG7 was silenced in high-autophagy cells (Kraya et al., 2015). The only autophagy related integral membrane protein, ATG9 (yeast Atg9), is proposed to be a supplier of autophagic membrane sources for the UPS of Acb1 and PARK7/DJ-1 (Manjithaya et al., 2010; Bruns et al., 2011).

Since phosphoinositide 3-kinase class 3 (PI3KC3) plays a key role in regulating autophagosome formation, the decrease in cargo secretion resulting from inhibition of PI3KC3 is also indicative of the relationship between autophagy and UPS. Not only genetic silencing of PI3KC3 itself, but also pharmacological inhibitory reagents (e.g., 3-MA, wortmannin, and Vps34-IN136) were utilized in an effort to establish the autophagic dependency of various UPS processes (Son et al., 2016; Noh et al., 2018; Nüchel et al., 2018; D'Agostino et al., 2019; Josephrajan et al., 2019; Kim et al., 2021).

Autophagy Receptors and Adaptors

Although classical autophagy degrades cytoplasmic materials non-selectively, each UPS cargo seems to use different autophagy machineries. An explanation of precisely how cargo selection occurs, mechanistically, could perhaps be found in the cargo receptors involved in selective autophagy (Kirkin and Rogov, 2019). During selective autophagy, the cargo proteins, subsequent to being labeled with ubiquitin or galectins, are recognized by autophagic receptors such as SQSTM1, NBR1 and CALCOCO2 and in turn link to autophagosomal membranes (Johansen and Lamark, 2020). It has been shown that the secretory autophagy cargo IL-1β is recognized by the autophagy cargo receptor TRIM16 before interacting with the Sec22b to facilitate the delivery of IL-1β to the LC3B-II positive

membrane (Kimura et al., 2017). TRIM16 is known to interact with GABARAP yet not with sequestosome-1/p62 (Mandell et al., 2014), a classical degradative autophagy receptor (Bjørkøy et al., 2005), which suggests that certain receptor/adaptor proteins potentially guide cargos to the secretory route avoiding disposal mechanism. A recent study additionally shows that the galectin3-mediated UPS of α-synuclein is dependent on TRIM16 (Burbidge et al., 2021).

Because the ER membrane is regarded as the prime source of autophagy-associated membrane structures, adaptor proteins in the ER-phagy (e.g., FAM134B, TRN3L, and ATL3) (Chino and Mizushima, 2020) may play a major role in the membrane vesicle formation and cargo selection in autophagy-associated UPS. Further investigation into precisely which autophagy receptors and adaptors facilitate the secretion for each cargo will shed more light on the precise mechanisms underpinning the UPS.

SNAREs and Rab Proteins

The primary role of SNARE [soluble N-ethylmaleimide-sensitive fusion (NSF) attachment protein receptor] proteins is to mediate the fusion of vesicles with the target membrane and other membrane-bound compartments. A number of reports have indicated that SNAREs also mediate membrane fusion in autophagy-related vesicular trafficking (Moreau et al., 2013). In yeast, the plasma membrane target SNARE (t-SNARE) Sso1 and a phospholipase D (Spo14) have been shown to be required for translocation of Acb1 onto the plasma membrane via UPS (Duran et al., 2010; Manjithaya et al., 2010). The vesicular R-SNARE Sec22b and the plasma membrane Q-SNAREs syntaxin 3 and syntaxin 4 cooperate in IL-1β secretion (Kimura et al., 2017). On the other hand, VAMP7, an R-SNARE required for heterotypic fusion of autophagosomes with lysosomes, shows pleiotropic aspects in autophagy-related UPS (Gee et al., 2011; Villeneuve et al., 2018) and have a relevance linked to that of lysosome-dependency (see below).

The Rab family of proteins, moreover, which are part of the Ras superfamily of small G proteins regulating intracellular vesicular transport, are involved in autophagy-associated UPS. Examples of this are that secretory FABP4 has been localized to structures that are positive for Rab7 (Josephrajan et al., 2019) and that the UPS of TGFβ1, ANXA2 and CFTR has been shown to require RAB8A yet not RAB8B, the latter being involved in the maturation of degradative autophagosomes (Noh et al., 2018; Nüchel et al., 2018). RAB27A is a necessary requirement for the autophagy-related secretion of ANXA2, while RAB27B is not (Chen et al., 2017). Further investigations into SNAREs and Rabs may contribute to our understanding of the detailed mechanisms involved in autophagy-related UPS.

STRUCTURAL AND FUNCTIONAL ASSOCIATIONS BETWEEN AUTOPHAGY AND UPS

Autophagosome

Autophagosomes, which are double-membraned sequestering vesicles formed by distinct molecular components, constitute a representative morphological hallmark of macroautophagy. Therefore, the presence of UPS cargos in autophagosome-like structures has frequently been highlighted with the aim of demonstrating autophagy dependency in the UPS. Since an autophagosome can be recognized by its morphological features, an electron microscopic examination is one of the best approaches to analyze whether the UPS cargo is intermediated via autophagosomes. In practice, however, this can prove rather difficult because the autophagosome is a transient organelle in the autophagic process (Klionsky et al., 2021). The Mpl protein has been shown to be located in autophagosome-like structures during its UPS (Cleyrat et al., 2014). It has additionally been proposed that there is an association between autophagosomes and UPS-mediated CFTR trafficking to the plasma membrane via the Golgi-bypassing pathway by showing that GRASP55, a binding partner of CFTR upon UPS, has been captured in autophagosomes under UPS-evoking conditions (Noh et al., 2018).

LC3, a mammalian homolog of yeast Atg8, is abundant at both the inner and outer membranes of autophagosomes and is regarded as a reliable indicator of autophagosome presence (Tanida et al., 2008). Images showing colocalizations of LC3 and UPS cargo, via immunocytochemistry or co-fractionation on the same membrane compartments, have been described as evidence for an autophagosome-mediated process in UPS (Duran et al., 2010; Dupont et al., 2011; Zhang et al., 2015; Son et al., 2016; Chen et al., 2017; Nüchel et al., 2018; Urano et al., 2018; D'Agostino et al., 2019; Wang et al., 2021; Kim et al., 2021). However, a degree of caution is needed when employing LC3 as an indicator of autophagosomes since it can also be a component of phagophore structures other than autophagosomes (Runwal et al., 2019).

MVB and Endosome

There is abundant evidence that autophagosomes fuse with endosomes, after the formation of MVBs and further amphisomes in mammalian cells (Liou et al., 1997; Jäger et al., 2004; Morvan et al., 2009; Razi et al., 2009). The biogenesis of MVBs via invagination of the endosomal membrane is catalyzed by the endosomal sorting complexes required for transport (ESCRT) proteins (Hurley, 2008). The MVBs and amphisomes are known to be directed to the disposal pathway by fusion with lysosomes; but recent discoveries highlight that they also play a role in the delivery of vesicular cargos to the plasma membrane, as in the case of exosome secretion (Hessvik and Llorente, 2018; Ganesan and Cai, 2021). Some UPS cargos such as IL-1β (Zhang et al., 2015) and CFTR (Noh et al., 2018) have likewise been shown to be present in MVBs interlinked with autophagy, which are routed to the extracellular space or the plasma membrane. MVB12B, a component of the ESCRT-I complex, participates in the MVB-associated Type IV UPS of CFTR (Noh et al., 2018). In yeast, Acb1 secretion also requires Vps23 and several ESCRT-I, -II, and –III, but not Vps4 for the biogenesis of CUPS (Curwin et al., 2016).

Endosomes have also been shown to be involved in the vesicular UPS. For example, the endosome-specific t-SNARE Tlg2 is required for Acb1 secretion (Duran et al., 2010),

suggesting that a certain endosomal compartment serves as an intermediate structure in the UPS of Acb1. Therefore, it is plausible that vesicular structures originating from autophagosomes and MVBs may fuse with the recycling endosomes at a later stage of the UPS for the final delivery of cargo proteins to the plasma membrane. Interestingly, endosomes can also directly mediate UPS unrelated to autophagy. In a study suggesting that FABP4 secretion is independent of autophagy and MVB, endosomal compartments are required for the UPS of FABP4 (Villeneuve et al., 2018).

Interestingly, certain cytosolic misfolded proteins have been shown to be secreted via late endosomes in a process termed MAPS (misfolding-associated protein secretion), in which the USP19-DNAJC5 chaperone cascade appears to play an important role (Lee et al., 2016). In addition, it has been reported that secretion of some neurodegenerative disease-associated proteins such as Tau, SOD1, spinocerebellar ataxia 3 (SCA3), TDP43 and α-synuclein are also engaged in USP19-dependent MAPS (Xu et al., 2018). Despite MAPS and CMA being similar in that both involve HSC70 and endo-lysosomes, the latter process is enhanced upon serum starvation while the former is inhibited under the same serum deprived condition (Lee and Ye, 2018).

CUPS

Another interesting structural compartment related to uncanonical secretion is CUPS (Bruns et al., 2011). Unconventional secretion of Acb1 in yeast is mediated by CUPS requiring Atg and MVB components, such as Sso1, Grh1, Vps23, Atg8, and Atg9. Interestingly, the CUPS formation is induced by starvation but not by rapamycin, a classical autophagy inducer. The formation of the CUPS structure requires ESCRT-I, -II and -III components, but not those of Vps4. An ESCRT-III component, Snf7, localizes to CUPS containing Grh1 (yeast GRASP) and plays a key role instead of Vps4 in the formation and stabilization of functional CUPS (Curwin et al., 2016). CUPS has hitherto only been demonstrated in yeast, and not in mammalian cells.

Lysosome

Canonical autophagy drives autophagosome–lysosome fusion for substrate clearance. However, proteins to be secreted to the exterior of the cell via UPS need to maintain an intact form and avoid degradative processes. In order to do so, in their route to the plasma membrane, the UPS cargos are required to circumvent lysosomes, or to escape the lysosome-mediated degradation. It has been demonstrated that Acb1 in yeast is sorted for packing into autophagosomes, the outcome being extracellular release rather than degradation in lysosomes/vacuoles (Duran et al., 2010; Manjithaya et al., 2010). The plasma membrane rescue of CFTR-ΔF508 via UPS is evidently unaffected by treatment of the lysosomal vacuolar H^+ ATPase (V-ATPase) inhibitor bafilomycin A1 (Noh et al., 2018), or depletion of the vesicular SNARE for lysosomal membrane fusion VAMP7 (Gee et al., 2011). It has been shown that IL-1β secretion is not diminished by knockdown of syntaxin 17, a SNARE for autophagosome membrane fusion with the lysosome membrane (Kimura et al., 2017). In studies on α-Crystallin B UPS, the cargo-containing LC3-positive compartments were not colocalized with LAMP1, an endolysosomal marker, suggesting that they bypass lysosomes (D'Agostino et al., 2019). The bafilomycin A1 and chloroquine treatments to inhibit autolysosome formation have been shown to promote HMGB1 secretion (Kim et al., 2021). Considered collectively, these results indicate that the abovementioned cargos undergo UPS independent of lysosomes.

Conversely, the UPS of certain cargos requires structural or functional lysosome integrity. Studies have shown that when lysosomes are disrupted by bafilomycin A1, IDE (Son et al., 2016), TGFβ1 (Nüchel et al., 2018) and mHTT (Ahat et al., 2021) secretions are blocked or reduced. Interestingly, bafilomycin A1 diminished IL-1β secretion in cells stimulated for autophagy via starvation, whereas no change was observed in cells undergoing basal autophagy (Dupont et al., 2011). Mpl was shown to be colocalized with the lysosomal marker LAMP1 as well as the autophagy marker LC3, and bafilomycin A1 led to a decreased cell-surface level of immature core-glycosylated Mpl (Cleyrat et al., 2014). The knockout of VAMP7 and syntaxin 7, SNAREs involved in lysosomal membrane fusion, has been shown to reduce FABP4 secretion, suggesting the involvement of lysosomal exocytosis in the FABP4 release to the extracellular space (Villeneuve et al., 2018). These cargos may have specific mechanisms to avoid degradation during their stay at the lysosome or lysosome-like structures. For example, it has been suggested that the SlyX domain (EKPPHY) of IDE contributes to the Aβ-induced IDE secretion by preventing lysosomal degradation (Son et al., 2016).

ERGIC and TMEDs

In addition to several cellular organelles that have been implicated as the membrane sources for autophagy (Nakatogawa, 2020), such as the ER, mitochondria, plasma membrane and Golgi, the ER-Golgi intermediate compartment (ERGIC) has also been suggested as a membrane source for autophagosome biogenesis (Ge et al., 2013) with articles suggesting associations between ERGIC and secretory pathways including autophagy-derived UPS (Bernard and Klionsky, 2014; Ge et al., 2015; Zhang et al., 2020).

The transmembrane emp24 domain-containing proteins (TMEDs, also known as p24 proteins) are a family of type I membrane proteins distributed in the membranes of the early secretory pathway. Although TMED proteins have been suggested to function as cargo receptors for the anterograde transport of certain secretory cargos (Schimmoller et al., 1995) or as primary receptors for the small GTPase of COPI-vesicle formation (Contreras et al., 2004), most of their functions remain elusive. Recently, TMED10 was identified as a translocator for IL-1β into the ERGIC, potentially via the formation of a homomultimeric channel during the UPS process (Zhang et al., 2020). However, this result is somewhat in conflict with the claim in a previous report that IL-1β is captured by the secretory autophagy receptor TRIM16 then translocated directly to the autophagosomal vesicles (Kimura et al., 2017). In addition, it has been suggested that TMED proteins in general function as

stable heteromeric complexes rather than possessing a homomultimeric form (Pastor-Cantizano et al., 2016). In fact, we recently found that the heteromultimeric TMED complex cargo recruitment in the ER stress-associated type IV UPS of CFTR, pendrin, and SARS-CoV-2 Spike. More specifically, TMED3 initially recognizes the ER core-glycosylated transmembrane protein cargos; and the TMED2/3/9/10 heteromultimeric complex facilitates the UPS of these membrane cargos (Park et al., (2022)). However, the study of Zhang et al., (2020) examined neither the relationship between TMED10 and other TMEDs nor the role of other TMED proteins in IL-1β UPS. Further investigations are required to identify the mechanistic details underlying TMED10-mediated IL-1β UPS as well as the role of ERGIC as a membrane source in autophagy-associated UPS.

mTOR and Rapamycin

The mammalian target of rapamycin (mTOR), a serine/threonine kinase, regulates autophagy by sensing cellular stresses and growth factor signals. The inhibition of the mTOR signaling pathway in general induces autophagy processes (Kim and Guan, 2015). The mTOR inhibitor rapamycin is a well-known classical autophagy inducer and is widely used to examine the autophagic dependency of cellular events. Interestingly, treatments with rapamycin have evoked variable results in the autophagy-associated UPS of different cargo proteins. The rapamycin application to activate autophagy has resulted in an increased IDE secretion from astrocytes (Son et al., 2016) and α-Crystallin B secretion from the COS-7 monkey kidney fibroblast cells (D'Agostino et al., 2019), indicating that the UPS of these cargos is dependent on the mTOR pathway. It has been demonstrated that the inactivation of the mTOR complex C1 (mTORC1) by cellular stress also affects the composition of extracellular secretome mediated via a GRASP55-dependent UPS (Nüchel et al., 2021).

In contrast, rapamycin treatments neither stimulated PARK7 release (Urano et al., 2018) nor affected CFTR UPS (Noh et al., 2018). Since the mTOR senses cellular nutritional status and is primarily involved in the induction of degradative autophagy, it is plausible that the mTOR-independent signaling pathway is more responsible for secretory autophagy. Interestingly, while rapamycin triggered Acb1 secretion in yeast (Manjithaya et al., 2010), rapamycin alone was not sufficient to induce the biogenesis of CUPS believed to play a critical role in Acb1 secretion (Bruns et al., 2011). Further investigation is needed to dissect the role of mTOR pathways in autophagy-related UPS.

GRASP

Despite the Golgi reassembly stacking proteins (GRASPs) 55 and 65 initially being identified as components of the Golgi-stacking machinery binding to the vesicle docking protein receptor GM130 (Barr et al., 1998; Shorter et al., 1999), later research has revealed that GRASPs (Grh1 in yeast, dGRASP in *Drosophila*) are required for Golgi-independent UPS. This can be seen, for example, in the cases of the starvation-induced secretion of Acb1 (or AcbA) in yeast (Kinseth et al., 2007; Duran et al., 2010; Manjithaya et al., 2010) and the mechanical stress-induced secretion of *Drosophila* integrins (Schotman et al., 2008). Thereafter, in mammalian cells, diverse proteins following UPS, including transmembrane proteins as well as leaderless proteins, were indicated to be dependent on GRASPs for secretion (Gee et al., 2011; Son et al., 2016; Kim et al., 2020; Kim et al., 2021; Nüchel et al., 2021). In these results, GRASP55/GORASP2 was commonly identified in CUPS and autophagosome-like structures; and this observation is corroborated by recent studies determining that GRASP55 can interact with LC3 via a LIR motif in the PDZ2 domain of GRASP55 (Nüchel et al., 2018; Zhang et al., 2018).

GRASPs are known to localize at the Golgi in the basal state, but changes in the phosphorylation (i.e., phosphorylation at S441 and dephosphorylation at T264 of GRASP55) and glycosylation (de-O-GlcNAcylation at the C-terminal region of GRASP55) status by certain stimuli can redirect their location to other sites such as the ER, autophagosomes, or MVBs, which leads GRASPs to perform other functions, particularly evident in UPS (**Figure 2**) (Kim et al., 2016; Noh et al., 2018; Zhang et al., 2018; Nüchel et al., 2021). It has been shown that phosphorylation at the C-terminal end region of GRASP55 by ER stress signals, particularly at S441, induces the ER redistribution of GRASP55 to facilitate the UPS of transmembrane proteins (Gee et al., 2011; Kim et al., 2016). In contrast, a recent study suggests that dephosphorylation at T264 by mTORC1 inhibition facilitates the movement of GRASP55 to autophagosomes and MVBs, enabling the UPS of selected cargos to reshape the extracellular proteome upon stress (Nüchel et al., 2021). Precisely how the phosphorylation at S441 and the dephosphorylation at T264 of GRASP55 evoked the same outcome of re-localizing GRASP55 into autophagy-associated structures needs to be further investigated.

An interesting observation in the electron microscopic analysis is that GRASP55 appears to be also localized on the inner vesicle membranes of autophagosomes and MVBs upon ER stress and mTORC1-inhibition (Noh et al., 2018; Nüchel et al., 2021) (**Figure 2**). According to the classical concept of the vesicle fusion process, cargos on the outer membrane of the MVBs, rather than on the membranes of the inner vesicles, would be suitably placed for localization to the plasma membrane, particularly for the type IV UPS of transmembrane proteins. In fact, a recent report has suggested that GRASP55 stays on the outer membrane of autophagosomes and surface of lysosomes under the starvation-induced conditions (Zhang et al., 2018). Further research is required to elucidate whether the outer membrane localization of GRASP55 is specific to starvation-induced UPS and how cargos on the membranes of the inner vesicles might reach the cell surface.

Although a number of studies have indicated the involvement of GRASPs in UPS, no consensus has yet been reached on a unified mechanistic role that they play in the process, with diverse features being reported depending on the experimental conditions (Dupont et al., 2011; Son et al., 2016; Zhang et al., 2018; Zhang et al., 2019; Liu et al., 2021). For example, it has been suggested that GRASP55 activates the early autophagy process but not its maturation, by showing that GRASP55 knockdown reduced the LC3-II levels and autophagosome formation in

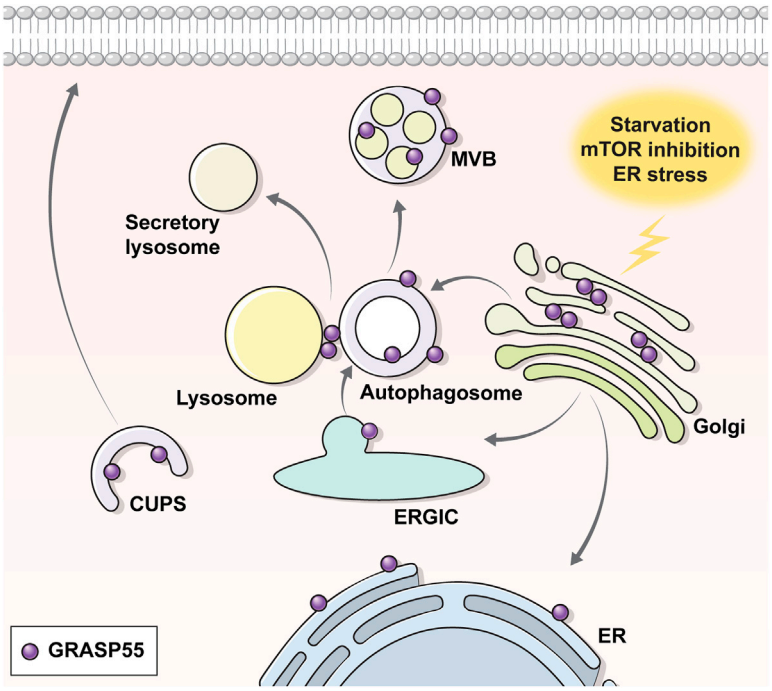


FIGURE 2 | Translocation of GRASP55 upon UPS. Under normal conditions, the Golgi peripheral protein GRASP55 resides at the Golgi. Under UPS-inducing conditions (e.g., starvation, ER stress, ER-to-Golgi block etc.), GRASP55 delocalizes to the ER, autophagosome, multivesicular body or CUPS. Transmission electron microscopic images show that GRASP55 is localized at autophagosomes and multivesicular bodies under ER stress, mTOR inhibition, and starvation conditions (see text for details).

inflammation-associated IL-1β secretion (Dupont et al., 2011). In contrast, GRASP55 has been suggested to play a role in autophagosome maturation including fusion with lysosome via the physical interactions of GRASP55 with LC3 on the autophagosomes and with LAMP2 on the lysosomes under the starvation conditions (Zhang et al., 2018; Zhang et al., 2019; Liu et al., 2021). Outside these studies, there have been suggestions that GRASP55 principally mediates CFTR UPS as a cargo recruiting factor via a PDZ-based direct interaction with the cargo molecule under the ER stress-associated UPS conditions (Gee et al., 2011; Kim et al., 2016). In addition, GRASP55 knockout mice did not display discernible autophagy-related phenotypes in a study (Kim et al., 2020). Though precise mechanistic details are unknown at present, a common feature in these studies is that early autophagy components, but not those of late autophagy, contribute to the inflammation- or ER stress-associated UPS, while the late autophagy components as well as the early ones are involved in the starvation-related UPS.

Ubiquitin

Another important molecule to be considered in autophagy-associated UPS is ubiquitin. Most proteins to be degraded via autophagy are ubiquitinated. Interestingly, several reports imply that deubiquitinated cargos are more prone to UPS as a means to evade autolysosomal or proteosomal degradation. For example, the ER-associated deubiquitinase USP19 (ubiquitin specific peptidase 19), which rescues the ERAD substrates ΔF508-CFTR and T-cell receptor-α (TCRα) from proteasomal degradation (Hassink et al., 2009), has been shown to play a critical role in the UPS of misfolded cytosolic proteins (Lee et al., 2016). In general, ESCRT-I components mediate the sorting of ubiquitinated cargo proteins to MVBs. MVB12B, an ESCRT-I component that does not have a ubiquitin-binding domain being different from other MVB12 proteins (Tsunematsu et al., 2010), has been shown to be indispensable in the rescue of ΔF508-CFTR via UPS (Noh et al., 2018). In light of the above, it is plausible that non-ubiquitinated substrates could be redirected to UPS with the assistance of ubiquitin-independent secretory structures while ubiquitinated ones go to disposal pathways via the canonical degradative process.

CONCLUSION

It is evident that the UPS pathway intersects with autophagy-associated cellular mechanisms. For example, a number of ATG proteins and autophagy receptors/adaptors have been shown to be involved in the UPS of diverse cargo proteins. There is considerable diversity in each UPS process, however, depending on the cargo proteins involved. This begs several questions, requiring further investigation: 1) How are certain cytosolic and membrane proteins selectively incorporated into the UPS vesicles? 2) How are some proteins incorporated into autophagy-associated vesicles transported to the secretory

pathway, with others following the degradation pathway? 3) Is deubiquitination required for cargos recruited to the UPS pathway evading the degradative route? 4) Are there mammalian structures corresponding to the yeast CUPS? 5) What could be regarded as the unified and prime role of GRASPs in UPS? and 6) Are intact lysosomes indeed required for the UPS of some cargo proteins?

Though the mechanisms of autophagy-related UPS are yet to be elucidated, there is mounting evidence of its applicability as a therapeutic target for human diseases. As abovementioned, autophagy-related UPS is largely involved in human diseases including neurodegenerative and metabolic ones (Kim et al., 2018; Gonzalez et al., 2020) and its clinical applications should be viewed as dependent on the specific disease-associated mechanisms involved. In the case of the UPS cargos whose excessive release provokes pathologies, such as IL-1β in diabetes and AD, α-synuclein in PD, and HMGB1 in sepsis, downregulation of their releases would be beneficial for disease control. On the contrary, activations of the UPS process could be employed as a therapeutic measure in some diseases, such as for FGF2 in AD and PD, IDE in AD, trafficking-deficient CFTR in cystic fibrosis, and trafficking-deficient pendrin in Pendred syndrome. For example, statins have yielded potential therapeutic results by inducing the autophagy-related UPS of IDE to relieve Aβ-induced pathologies in AD (Glebov and Walter, 2012; Son et al., 2015). Further investigation of the mechanistic details and pharmacological implications of UPS will provide the requisite knowledge to pave the way for the treatment of related diseases.

Each circumstance inducing UPS (e.g., starvation, ER stress, mechanical stress, developmental signals, etc.) should lead to varied cellular environments and structural organizations. Accordingly, it would be natural for each UPS substrate to be located under different conditions and its cell surface delivery to be engaged in different autophagy-related features. Furthermore, it is plausible that the same cargo protein could be delivered to the cell surface through dissimilar pathways dependent on the cellular circumstances as in the case of IL-1β and tau secretions (Merezhko et al., 2020). Despite substantial research being conducted, UPS still appears convoluted and requires future investigation for a more precise understanding of the diverse trafficking mechanisms which should, in turn, help the scientific community to discover potential ways to modulate the export of UPS cargos involved in human health and diseases.

AUTHOR CONTRIBUTIONS

SN designed and wrote the initial draft of the manuscript. YK assisted with manuscript writing. ML designed, re-drafted, and edited the manuscript.

FUNDING

This work was supported by grants 2022R1A2C3002917, 2013R1A3A2042197, and 2022R1A4A1031336 (to ML) and a grant 2021R1I1A1A01049255 (to SN) from the National Research Foundation (NRF), funded by the Ministry of Education, South Korea.

ACKNOWLEDGMENTS

We thank Dong Soo Chang for contributing medical illustrations and Benjamin Latimer for providing editorial assistance.

REFERENCES

Ahat, E., Bui, S., Zhang, J., Leprevost, F. V., Sharkey, L., Reid, W., et al. (2021). GRASP55 Regulates Mutant Huntingtin Unconventional Secretion and Aggregation. *bioRxiv*. doi:10.1101/2021.10.05.463252

Baldwin, T. A., and Ostergaard, H. L. (2002). The Protein-Tyrosine Phosphatase CD45 Reaches the Cell Surface via Golgi-dependent and -independent Pathways. *J. Biol. Chem.* 277, 50333–50340. doi:10.1074/jbc.M209075200

Bard, F., and Malhotra, V. (2006). The Formation of TGN-To-Plasma-Membrane Transport Carriers. *Annu. Rev. Cell Dev. Biol.* 22, 439–455. doi:10.1146/annurev.cellbio.21.012704.133126

Barr, F. A., Nakamura, N., and Warren, G. (1998). Mapping the Interaction between GRASP65 and GM130, Components of a Protein Complex Involved in the Stacking of Golgi Cisternae. *Embo J.* 17, 3258–3268. doi:10.1093/emboj/17.12.3258

Bernard, A., and Klionsky, D. J. (2014). Defining the Membrane Precursor Supporting the Nucleation of the Phagophore. *Autophagy* 10, 1–2. doi:10.4161/auto.27242

Bestebroer, J., V'Kovski, P., Mauthe, M., and Reggiori, F. (2013). Hidden behind Autophagy: the Unconventional Roles of ATG Proteins. *Traffic* 14, 1029–1041. doi:10.1111/tra.12091

Béthune, J., and Wieland, F. T. (2018). Assembly of COPI and COPII Vesicular Coat Proteins on Membranes. *Annu. Rev. Biophys.* 47, 63–83. doi:10.1146/annurev-biophys-070317-033259

Bjørkøy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Overvatn, A., et al. (2005). p62/SQSTM1 Forms Protein Aggregates Degraded by Autophagy and Has a Protective Effect on Huntingtin-Induced Cell Death. *J. Cell Biol.* 171, 603–614. doi:10.1083/jcb.200507002

Blobel, G., and Sabatini, D. D. (1971). "Ribosome-Membrane Interaction in Eukaryotic Cells," in *Biomembranes*. Editor L. A. Manson (Boston, MA: Springer US), 2, 193–195. doi:10.1007/978-1-4684-3330-2_16

Brunello, C. A., Merezhko, M., Uronen, R. L., and Huttunen, H. J. (2020). Mechanisms of Secretion and Spreading of Pathological Tau Protein. *Cell Mol. Life Sci.* 77, 1721–1744. doi:10.1007/s00018-019-03349-1

Bruns, C., McCaffery, J. M., Curwin, A. J., Duran, J. M., and Malhotra, V. (2011). Biogenesis of a Novel Compartment for Autophagosome-Mediated Unconventional Protein Secretion. *J. Cell Biol.* 195, 979–992. doi:10.1083/jcb.201106098

Burbidge, K., Rademacher, D. J., Mattick, J., Zack, S., Grillini, A., Bousset, L., et al. (2021). LGALS3 (Galectin 3) Mediates an Unconventional Secretion of SNCA/α-synuclein in Response to Lysosomal Membrane Damage by the Autophagic-Lysosomal Pathway in Human Midbrain Dopamine Neurons. *Autophagy*, 1–29. doi:10.1080/15548627.2021.1967615

Cadwell, K., and Debnath, J. (2018). Beyond Self-Eating: The Control of Nonautophagic Functions and Signaling Pathways by Autophagy-Related Proteins. *J. Cell Biol.* 217, 813–822. doi:10.1083/jcb.201706157

Chang, H. C., Samaniego, F., Nair, B. C., Buonaguro, L., and Ensoli, B. (1997). HIV-1 Tat Protein Exits from Cells via a Leaderless Secretory Pathway and Binds to Extracellular Matrix-Associated Heparan Sulfate Proteoglycans through its Basic Region. *Aids* 11, 1421–1431. doi:10.1097/00002030-199712000-00006

Chen, X., Li, Y., Wang, C., Tang, Y., Mok, S. A., Tsai, R. M., et al. (2020). Promoting Tau Secretion and Propagation by Hyperactive P300/CBP via Autophagy-Lysosomal Pathway in Tauopathy. *Mol. Neurodegener.* 15, 2. doi:10.1186/s13024-019-0354-0

Chen, Y. D., Fang, Y. T., Cheng, Y. L., Lin, C. F., Hsu, L. J., Wang, S. Y., et al. (2017). Exophagy of Annexin A2 via RAB11, RAB8A and RAB27A in IFN-γ-Stimulated Lung Epithelial Cells. *Sci. Rep.* 7, 5676. doi:10.1038/s41598-017-06076-4

Chino, H., and Mizushima, N. (2020). ER-phagy: Quality Control and Turnover of Endoplasmic Reticulum. *Trends Cell Biol.* 30, 384–398. doi:10.1016/j.tcb.2020.02.001

Cleyrat, C., Darehshouri, A., Steinkamp, M. P., Vilaine, M., Boassa, D., Ellisman, M. H., et al. (2014). Mpl Traffics to the Cell Surface through Conventional and Unconventional Routes. *Traffic* 15, 961–982. doi:10.1111/tra.12185

Contreras, I., Ortiz-Zapater, E., and Aniento, F. (2004). Sorting Signals in the Cytosolic Tail of Membrane Proteins Involved in the Interaction with Plant ARF1 and Coatomer. *Plant J.* 38, 685–698. doi:10.1111/j.1365-313X.2004.02075.x

Cruz-Garcia, D., Brouwers, N., Duran, J. M., Mora, G., Curwin, A. J., and Malhotra, V. (2017). A Diacidic Motif Determines Unconventional Secretion of Wild-type and ALS-Linked Mutant SOD1. *J. Cell Biol.* 216, 2691–2700. doi:10.1083/jcb.201704056

Cruz-Garcia, D., Curwin, A. J., Popoff, J. F., Bruns, C., Duran, J. M., and Malhotra, V. (2014). Remodeling of Secretory Compartments Creates CUPS during Nutrient Starvation. *J. Cell Biol.* 207, 695–703. doi:10.1083/jcb.201407119

Cruz-Garcia, D., Malhotra, V., and Curwin, A. J. (2018). Unconventional Protein Secretion Triggered by Nutrient Starvation. *Semin. Cell Dev. Biol.* 83, 22–28. doi:10.1016/j.semcdb.2018.02.021

Curwin, A. J., Brouwers, N., Alonso, Y. A. M., Teis, D., Turacchio, G., Parashuraman, S., et al. (2016). ESCRT-III Drives the Final Stages of CUPS Maturation for Unconventional Protein Secretion. *Elife* 5, e16299. doi:10.7554/eLife.16299

D'Agostino, M., Scerra, G., Cannata Serio, M., Caporaso, M. G., Bonatti, S., and Renna, M. (2019). Unconventional Secretion of α-Crystallin B Requires the Autophagic Pathway and Is Controlled by Phosphorylation of its Serine 59 Residue. *Sci. Rep.* 9, 16892. doi:10.1038/s41598-019-53226-x

Denny, P. W., Gokool, S., Russell, D. G., Field, M. C., and Smith, D. F. (2000). Acylation-dependent Protein Export in Leishmania. *J. Biol. Chem.* 275, 11017–11025. doi:10.1074/jbc.275.15.11017

Dupont, N., Jiang, S., Pilli, M., Ornatowski, W., Bhattacharya, D., and Deretic, V. (2011). Autophagy-based Unconventional Secretory Pathway for Extracellular Delivery of IL-1β. *Embo J.* 30, 4701–4711. doi:10.1038/emboj.2011.398

Duran, J. M., Anjard, C., Stefan, C., Loomis, W. F., and Malhotra, V. (2010). Unconventional Secretion of Acb1 Is Mediated by Autophagosomes. *J. Cell Biol.* 188, 527–536. doi:10.1083/jcb.200911154

Ejlerskov, P., Rasmussen, I., Nielsen, T. T., Bergström, A. L., Tohyama, Y., Jensen, P. H., et al. (2013). Tubulin Polymerization-Promoting Protein (TPPP/p25α) Promotes Unconventional Secretion of α-synuclein through Exophagy by Impairing Autophagosome-Lysosome Fusion. *J. Biol. Chem.* 288, 17313–17335. doi:10.1074/jbc.M112.401174

Evavold, C. L., Ruan, J., Tan, Y., Xia, S., Wu, H., and Kagan, J. C. (2018). The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages. *Immunity* 48, 35–44. e36. doi:10.1016/j.immuni.2017.11.013

Ferro-Novick, S., and Brose, N. (2013). Nobel 2013 Physiology or Medicine: Traffic Control System within Cells. *Nature* 504, 98. doi:10.1038/504098a

Galluzzi, L., and Green, D. R. (2019). Autophagy-Independent Functions of the Autophagy Machinery. *Cell* 177, 1682–1699. doi:10.1016/j.cell.2019.05.026

Ganesan, D., and Cai, Q. (2021). Understanding Amphisomes. *Biochem. J.* 478, 1959–1976. doi:10.1042/bcj20200917

Ge, L., Melville, D., Zhang, M., and Schekman, R. (2013). The ER-Golgi Intermediate Compartment Is a Key Membrane Source for the LC3 Lipidation Step of Autophagosome Biogenesis. *Elife* 2, e00947. doi:10.7554/eLife.00947

Ge, L., Wilz, L., and Schekman, R. (2015). Biogenesis of Autophagosomal Precursors for LC3 Lipidation from the ER-Golgi Intermediate Compartment. *Autophagy* 11, 2372–2374. doi:10.1080/15548627.2015.1105422

Gee, H. Y., Kim, J., and Lee, M. G. (2018). Unconventional Secretion of Transmembrane Proteins. *Semin. Cell Dev. Biol.* 83, 59–66. doi:10.1016/j.semcdb.2018.03.016

Gee, H. Y., Noh, S. H., Tang, B. L., Kim, K. H., and Lee, M. G. (2011). Rescue of ΔF508-CFTR Trafficking via a GRASP-dependent Unconventional Secretion Pathway. *Cell* 146, 746–760. doi:10.1016/j.cell.2011.07.021

George, C. H., Kendall, J. M., and Evans, W. H. (1999). Intracellular Trafficking Pathways in the Assembly of Connexins into Gap Junctions. *J. Biol. Chem.* 274, 8678–8685. doi:10.1074/jbc.274.13.8678

Glebov, K., and Walter, J. (2012). Statins in Unconventional Secretion of Insulin-Degrading Enzyme and Degradation of the Amyloid-β Peptide. *Neurodegener. Dis.* 10, 309–312. doi:10.1159/000332595

Glick, D., Barth, S., and Macleod, K. F. (2010). Autophagy: Cellular and Molecular Mechanisms. *J. Pathol.* 221, 3–12. doi:10.1002/path.2697

Gonzalez, C., Cornejo, V. H., and Couve, A. (2018). Golgi Bypass for Local Delivery of Axonal Proteins, Fact or Fiction? *Curr. Opin. Cell Biol.* 53, 9–14. doi:10.1016/j.ceb.2018.03.010

Gonzalez, C. D., Resnik, R., and Vaccaro, M. I. (2020). Secretory Autophagy and its Relevance in Metabolic and Degenerative Disease. *Front. Endocrinol. (Lausanne)* 11, 266. doi:10.3389/fendo.2020.00266

Hasdemir, B., Fitzgerald, D. J., Prior, I. A., Tepikin, A. V., and Burgoyne, R. D. (2005). Traffic of Kv4 K+ Channels Mediated by KChIP1 Is via a Novel Post-ER Vesicular Pathway. *J. Cell Biol.* 171, 459–469. doi:10.1083/jcb.200506005

Hassink, G. C., Zhao, B., Sompallae, R., Altun, M., Gastaldello, S., Zinin, N. V., et al. (2009). The ER-Resident Ubiquitin-specific Protease 19 Participates in the UPR and Rescues ERAD Substrates. *EMBO Rep.* 10, 755–761. doi:10.1038/embor.2009.69

Hessvik, N. P., and Llorente, A. (2018). Current Knowledge on Exosome Biogenesis and Release. *Cell Mol. Life Sci.* 75, 193–208. doi:10.1007/s00018-017-2595-9

Hoffmeister, H., Babinger, K., Gürster, S., Cedzich, A., Meese, C., Schadendorf, K., et al. (2011). Polycystin-2 Takes Different Routes to the Somatic and Ciliary Plasma Membrane. *J. Cell Biol.* 192, 631–645. doi:10.1083/jcb.201007050

Hurley, J. H. (2008). ESCRT Complexes and the Biogenesis of Multivesicular Bodies. *Curr. Opin. Cell Biol.* 20, 4–11. doi:10.1016/j.ceb.2007.12.002

Jäger, S., Bucci, C., Tanida, I., Ueno, T., Kominami, E., Saftig, P., et al. (2004). Role for Rab7 in Maturation of Late Autophagic Vacuoles. *J. Cell Sci.* 117, 4837–4848. doi:10.1242/jcs.01370

Johansen, T., and Lamark, T. (2020). Selective Autophagy: ATG8 Family Proteins, LIR Motifs and Cargo Receptors. *J. Mol. Biol.* 432, 80–103. doi:10.1016/j.jmb.2019.07.016

Josephrajan, A., Hertzel, A. V., Bohm, E. K., McBurney, M. W., Imai, S. I., Mashek, D. G., et al. (2019). Unconventional Secretion of Adipocyte Fatty Acid Binding Protein 4 Is Mediated by Autophagic Proteins in a Sirtuin-1-dependent Manner. *Diabetes* 68, 1767–1777. doi:10.2337/db18-1367

Jung, J., Kim, J., Roh, S. H., Jun, I., Sampson, R. D., Gee, H. Y., et al. (2016). The HSP70 Co-chaperone DNAJC14 Targets Misfolded Pendrin for Unconventional Protein Secretion. *Nat. Commun.* 7, 11386. doi:10.1038/ncomms11386

Kahle, P. J. (2008). Alpha-Synucleinopathy Models and Human Neuropathology: Similarities and Differences. *Acta Neuropathol.* 115, 87–95. doi:10.1007/s00401-007-0302-x

Kang, S., Son, S. M., Baik, S. H., Yang, J., and Mook-Jung, I. (2019). Autophagy-Mediated Secretory Pathway Is Responsible for Both Normal and Pathological Tau in Neurons. *J. Alzheimers Dis.* 70, 667–680. doi:10.3233/jad-190180

Kim, J., Gee, H. Y., and Lee, M. G. (2018). Unconventional Protein Secretion - New Insights into the Pathogenesis and Therapeutic Targets of Human Diseases. *J. Cell Sci.* 131, jcs213686. doi:10.1242/jcs.213686

Kim, J., Kim, H., Noh, S. H., Jang, D. G., Park, S. Y., Min, D., et al. (2020). Grasp55(-/-) Mice Display Impaired Fat Absorption and Resistance to High-Fat Diet-Induced Obesity. *Nat. Commun.* 11, 1418. doi:10.1038/s41467-020-14912-x

Kim, J., Noh, S. H., Piao, H., Kim, D. H., Kim, K., Cha, J. S., et al. (2016). Monomerization and ER Relocalization of GRASP Is a Requisite for Unconventional Secretion of CFTR. *Traffic* 17, 733–753. doi:10.1111/tra.12403

Kim, Y. C., and Guan, K. L. (2015). mTOR: a Pharmacologic Target for Autophagy Regulation. *J. Clin. Invest.* 125, 25–32. doi:10.1172/jci73939

Kim, Y. H., Kwak, M. S., Lee, B., Shin, J. M., Aum, S., Park, I. H., et al. (2021). Secretory Autophagy Machinery and Vesicular Trafficking Are Involved in

HMGB1 Secretion. *Autophagy* 17, 2345–2362. doi:10.1080/15548627.2020.1826690

Kim, Y., Jun, I., Shin, D. H., Yoon, J. G., Piao, H., Jung, J., et al. (2020). Regulation of CFTR Bicarbonate Channel Activity by WNK1: Implications for Pancreatitis and CFTR-Related Disorders. *Cell Mol. Gastroenterol. Hepatol.* 9, 79–103. doi:10.1016/j.jcmgh.2019.09.003

Kimura, T., Jia, J., Kumar, S., Choi, S. W., Gu, Y., Mudd, M., et al. (2017). Dedicated SNAREs and Specialized TRIM Cargo Receptors Mediate Secretory Autophagy. *Embo J.* 36, 42–60. doi:10.15252/embj.201695081

Kinseth, M. A., Anjard, C., Fuller, D., Guizzunti, G., Loomis, W. F., and Malhotra, V. (2007). The Golgi-Associated Protein GRASP Is Required for Unconventional Protein Secretion during Development. *Cell* 130, 524–534. doi:10.1016/j.cell.2007.06.029

Kirkin, V., and Rogov, V. V. (2019). A Diversity of Selective Autophagy Receptors Determines the Specificity of the Autophagy Pathway. *Mol. Cell* 76, 268–285. doi:10.1016/j.molcel.2019.09.005

Klionsky, D. J., Abdel-Aziz, A. K., Abdelfatah, S., Abdellatif, M., Abdoli, A., Abel, S., et al. (2021). Guidelines for the Use and Interpretation of Assays for Monitoring Autophagy (4th Edition)[1]. *Autophagy* 17, 1–382. doi:10.1080/15548627.2020.179728010.1080/15548627.2015.1100356

Klionsky, D. J. (2007). Autophagy: from Phenomenology to Molecular Understanding in Less Than a Decade. *Nat. Rev. Mol. Cell Biol.* 8, 931–937. doi:10.1038/nrm2245

Kraya, A. A., Piao, S., Xu, X., Zhang, G., Herlyn, M., Gimotty, P., et al. (2015). Identification of Secreted Proteins that Reflect Autophagy Dynamics within Tumor Cells. *Autophagy* 11, 60–74. doi:10.4161/15548627.2014.984273

Lee, H. J., Cho, E. D., Lee, K. W., Kim, J. H., Cho, S. G., and Lee, S. J. (2013). Autophagic Failure Promotes the Exocytosis and Intercellular Transfer of Alpha-Synuclein. *Exp. Mol. Med.* 45, e22. doi:10.1038/emm.2013.45

Lee, H. J., Patel, S., and Lee, S. J. (2005). Intravesicular Localization and Exocytosis of Alpha-Synuclein and its Aggregates. *J. Neurosci.* 25, 6016–6024. doi:10.1523/JNEUROSCI.0692-05.2005

Lee, J. G., Takahama, S., Zhang, G., Tomarev, S. I., and Ye, Y. (2016). Unconventional Secretion of Misfolded Proteins Promotes Adaptation to Proteasome Dysfunction in Mammalian Cells. *Nat. Cell Biol.* 18, 765–776. doi:10.1038/ncb3372

Lee, J. S., and Lee, S. J. (2016). Mechanism of Anti-alpha-synuclein Immunotherapy. *J. Mov. Disord.* 9, 14–19. doi:10.14802/jmd.15059

Lee, J., and Ye, Y. (2018). The Roles of Endo-Lysosomes in Unconventional Protein Secretion. *Cells* 7, 198. doi:10.3390/cells7110198

Lee, M. G., Ohana, E., Park, H. W., Yang, D., and Muallem, S. (2012). Molecular Mechanism of Pancreatic and Salivary Gland Fluid and HCO3 Secretion. *Physiol. Rev.* 92, 39–74. doi:10.1152/physrev.00011.2011

Lee, S. J., Desplats, P., Sigurdson, C., Tsigelny, I., and Masliah, E. (2010). Cell-to-cell Transmission of Non-prion Protein Aggregates. *Nat. Rev. Neurol.* 6, 702–706. doi:10.1038/nrneurol.2010.145

Leidal, A. M., and Debnath, J. (2021). Emerging Roles for the Autophagy Machinery in Extracellular Vesicle Biogenesis and Secretion. *FASEB Bioadv* 3, 377–386. doi:10.1096/fba.2020-00138

Liou, W., Geuze, H. J., Geelen, M. J., and Slot, J. W. (1997). The Autophagic and Endocytic Pathways Converge at the Nascent Autophagic Vacuoles. *J. Cell Biol.* 136, 61–70. doi:10.1083/jcb.136.1.61

Liu, J. Y., Lin, Y. T., Leidal, A. M., Huang, H. H., Ye, J., Wiita, A. P., et al. (2021). GRASP55 Restricts Early-Stage Autophagy and Regulates Spatial Organization of the Early Secretory Network. *Biol. Open* 10, bio058736. doi:10.1242/bio.058736

Mandell, M. A., Jain, A., Arko-Mensah, J., Chauhan, S., Kimura, T., Dinkins, C., et al. (2014). TRIM Proteins Regulate Autophagy and Can Target Autophagic Substrates by Direct Recognition. *Dev. Cell* 30, 394–409. doi:10.1016/j.devcel.2014.06.013

Manjithaya, R., Anjard, C., Loomis, W. F., and Subramani, S. (2010). Unconventional Secretion of Pichia pastoris Acb1 Is Dependent on GRASP Protein, Peroxisomal Functions, and Autophagosome Formation. *J. Cell Biol.* 188, 537–546. doi:10.1083/jcb.200911149

McGrath, J. P., and Varshavsky, A. (1989). The Yeast STE6 Gene Encodes a Homologue of the Mammalian Multidrug Resistance P-Glycoprotein. *Nature* 340, 400–404. doi:10.1038/340400a0

Merezhko, M., Brunello, C. A., Yan, X., Vihinen, H., Jokitalo, E., Uronen, R. L., et al. (2018). Secretion of Tau via an Unconventional Non-vesicular Mechanism. *Cell Rep.* 25, 2027–2035. e2024. doi:10.1016/j.celrep.2018.10.078

Merezhko, M., Uronen, R. L., and Huttunen, H. J. (2020). The Cell Biology of Tau Secretion. *Front. Mol. Neurosci.* 13, 569818. doi:10.3389/fnmol.2020.569818

Merregaert, J., Van Langen, J., Hansen, U., Ponsaerts, P., El Ghalbzouri, A., Steenackers, E., et al. (2010). Phospholipid Scramblase 1 Is Secreted by a Lipid Raft-dependent Pathway and Interacts with the Extracellular Matrix Protein 1 in the Dermal Epidermal Junction Zone of Human Skin. *J. Biol. Chem.* 285, 37823–37837. doi:10.1074/jbc.M110.136408

Mizushima, N. (2018). A Brief History of Autophagy from Cell Biology to Physiology and Disease. *Nat. Cell Biol.* 20, 521–527. doi:10.1038/s41556-018-0092-5

Mizushima, N., Yoshimori, T., and Ohsumi, Y. (2011). The Role of Atg Proteins in Autophagosome Formation. *Annu. Rev. Cell Dev. Biol.* 27, 107–132. doi:10.1146/annurev-cellbio-092910-154005

Mohamed, N. V., Plouffe, V., Rémillard-Labrosse, G., Planel, E., and Leclerc, N. (2014). Starvation and Inhibition of Lysosomal Function Increased Tau Secretion by Primary Cortical Neurons. *Sci. Rep.* 4, 5715. doi:10.1038/srep05715

Moreau, K., Renna, M., and Rubinsztein, D. C. (2013). Connections between SNAREs and Autophagy. *Trends Biochem. Sci.* 38, 57–63. doi:10.1016/j.tibs.2012.11.004

Morvan, J., Köchl, R., Watson, R., Collinson, L. M., Jefferies, H. B., and Tooze, S. A. (2009). *In Vitro* reconstitution of Fusion between Immature Autophagosomes and Endosomes. *Autophagy* 5, 676–689. doi:10.4161/auto.5.5.8378

Möskes, C., Burghaus, P. A., Wernli, B., Sauder, U., Dürrenberger, M., and Kappes, B. (2004). Export of Plasmodium Falciparum Calcium-dependent Protein Kinase 1 to the Parasitophorous Vacuole Is Dependent on Three N-Terminal Membrane Anchor Motifs. *Mol. Microbiol.* 54, 676–691. doi:10.1111/j.1365-2958.2004.04313.x

Nakatogawa, H. (2020). Mechanisms Governing Autophagosome Biogenesis. *Nat. Rev. Mol. Cell Biol.* 21, 439–458. doi:10.1038/s41580-020-0241-0

Nickel, W., and Rabouille, C. (2009). Mechanisms of Regulated Unconventional Protein Secretion. *Nat. Rev. Mol. Cell Biol.* 10, 148–155. doi:10.1038/nrm2617

Nilsson, P., Loganathan, K., Sekiguchi, M., Matsuba, Y., Hui, K., Tsubuki, S., et al. (2013). Abeta Secretion and Plaque Formation Depend on Autophagy. *Cell Rep.* 5, 61–69. doi:10.1016/j.celrep.2013.08.042

Noh, S. H., Gee, H. Y., Kim, Y., Piao, H., Kim, J., Kang, C. M., et al. (2018). Specific Autophagy and ESCRT Components Participate in the Unconventional Secretion of CFTR. *Autophagy* 14, 1761–1778. doi:10.1080/15548627.2018.1489479

Nüchel, J., Ghatak, S., Zuk, A. V., Illerhaus, A., Mörgelin, M., Schönborn, K., et al. (2018). TGFB1 Is Secreted through an Unconventional Pathway Dependent on the Autophagic Machinery and Cytoskeletal Regulators. *Autophagy* 14, 465–486. doi:10.1080/15548627.2017.1422850

Nüchel, J., Tauber, M., Nolte, J. L., Mörgelin, M., Türk, C., Eckes, B., et al. (2021). An mTORC1-GRASP55 Signaling axis Controls Unconventional Secretion to Reshape the Extracellular Proteome upon Stress. *Mol. Cell* 81, 3275–3293. e3212. doi:10.1016/j.molcel.2021.06.017

Ohsumi, Y. (2014). Historical Landmarks of Autophagy Research. *Cell Res.* 24, 9–23. doi:10.1038/cr.2013.169

Palade, G. (1975). Intracellular Aspects of the Process of Protein Synthesis. *Science* 189, 347–358. doi:10.1126/science.1096303

Park, H., Shin, D. H., Sim, J. R., Aum, S., and Lee, M. G. (2020). IRE1alpha Kinase-Mediated Unconventional Protein Secretion Rescues Misfolded CFTR and Pendrin. *Sci. Adv.* 6, eaax9914. doi:10.1126/sciadv.aax9914

Park, H., Seo, S. K., Sim, J. R., Hwang, S. J., Kim, Y. J., et al. (2022). $TMED_3$ Complex Mediates ER Stress-Associated Secretion of CFTR, Pendrin, and SARS-CoV-2 Spike. *Adv. Sci.*, 2105320. doi:10.1002/advs.202105320

Pastor-Cantizano, N., Montesinos, J. C., Bernat-Silvestre, C., Marcote, M. J., and Aniento, F. (2016). p24 Family Proteins: Key Players in the Regulation of Trafficking along the Secretory Pathway. *Protoplasma* 253, 967–985. doi:10.1007/s00709-015-0858-6

Penuela, S., Bhalla, R., Gong, X. Q., Cowan, K. N., Celetti, S. J., Cowan, B. J., et al. (2007). Pannexin 1 and Pannexin 3 Are Glycoproteins that Exhibit Many Distinct Characteristics from the Connexin Family of Gap Junction Proteins. *J. Cell Sci.* 120, 3772–3783. doi:10.1242/jcs.009514

Poehler, A. M., Xiang, W., Spitzer, P., May, V. E., Meixner, H., Rockenstein, E., et al. (2014). Autophagy Modulates SNCA/α-synuclein Release, Thereby Generating a Hostile Microenvironment. *Autophagy* 10, 2171–2192. doi:10.4161/auto.36436

Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., et al. (1997). Mutation in the Alpha-Synuclein Gene Identified in Families with Parkinson's Disease. *Science* 276, 2045–2047. doi:10.1126/science.276.5321.2045

Ponpuak, M., Mandell, M. A., Kimura, T., Chauhan, S., Cleyrat, C., and Deretic, V. (2015). Secretory Autophagy. *Curr. Opin. Cell Biol.* 35, 106–116. doi:10.1016/j.ceb.2015.04.016

Rabouille, C., Malhotra, V., and Nickel, W. (2012). Diversity in Unconventional Protein Secretion. *J. Cell Sci.* 125, 5251–5255. doi:10.1242/jcs.103630

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cell Biol.* 27, 230–240. doi:10.1016/j.tcb.2016.11.007

Razi, M., Chan, E. Y., and Tooze, S. A. (2009). Early Endosomes and Endosomal Coatomer Are Required for Autophagy. *J. Cell Biol.* 185, 305–321. doi:10.1083/jcb.200810098

Reily, C., Stewart, T. J., Renfrow, M. B., and Novak, J. (2019). Glycosylation in Health and Disease. *Nat. Rev. Nephrol.* 15, 346–366. doi:10.1038/s41581-019-0129-4

Rothman, J. E. (1994). Mechanisms of Intracellular Protein Transport. *Nature* 372, 55–63. doi:10.1038/372055a0

Runwal, G., Stamatakou, E., Siddiqi, F. H., Puri, C., Zhu, Y., and Rubinsztein, D. C. (2019). LC3-positive Structures Are Prominent in Autophagy-Deficient Cells. *Sci. Rep.* 9, 10147. doi:10.1038/s41598-019-46657-z

Schäfer, T., Zentgraf, H., Zehe, C., Brügger, B., Bernhagen, J., and Nickel, W. (2004). Unconventional Secretion of Fibroblast Growth Factor 2 Is Mediated by Direct Translocation across the Plasma Membrane of Mammalian Cells. *J. Biol. Chem.* 279, 6244–6251. doi:10.1074/jbc.M310500200

Schimmoller, F., Singer-Kruger, B., Schroder, S., Kruger, U., Barlowe, C., and Riezman, H. (1995). The Absence of Emp24p, a Component of ER-Derived COPII-Coated Vesicles, Causes a Defect in Transport of Selected Proteins to the Golgi. *EMBO J.* 14, 1329–1339. doi:10.1002/j.1460-2075.1995.tb07119.x

Schotman, H., Karhinen, L., and Rabouille, C. (2008). dGRASP-mediated Noncanonical Integrin Secretion Is Required for Drosophila Epithelial Remodeling. *Dev. Cell* 14, 171–182. doi:10.1016/j.devcel.2007.12.006

Shorter, J., Watson, R., Giannakou, M. E., Clarke, M., Warren, G., and Barr, F. A. (1999). GRASP55, a Second Mammalian GRASP Protein Involved in the Stacking of Golgi Cisternae in a Cell-free System. *Embo J.* 18, 4949–4960. doi:10.1093/emboj/18.18.4949

Son, S. M., Cha, M. Y., Choi, H., Kang, S., Choi, H., Lee, M. S., et al. (2016). Insulin-degrading Enzyme Secretion from Astrocytes Is Mediated by an Autophagy-Based Unconventional Secretory Pathway in Alzheimer Disease. *Autophagy* 12, 784–800. doi:10.1080/15548627.2016.1159375

Son, S. M., Kang, S., Choi, H., and Mook-Jung, I. (2015). Statins Induce Insulin-Degrading Enzyme Secretion from Astrocytes via an Autophagy-Based Unconventional Secretory Pathway. *Mol. Neurodegener.* 10, 56. doi:10.1186/s13024-015-0054-3

Takeshige, K., Baba, M., Tsuboi, S., Noda, T., and Ohsumi, Y. (1992). Autophagy in Yeast Demonstrated with Proteinase-Deficient Mutants and Conditions for its Induction. *J. Cell Biol.* 119, 301–311. doi:10.1083/jcb.119.2.301

Tanida, I., Ueno, T., and Kominami, E. (2008). LC3 and Autophagy. *Methods Mol. Biol.* 445, 77–88. doi:10.1007/978-1-59745-157-4_4

Tian, G., Ropelewski, P., Nemet, I., Lee, R., Lodowski, K. H., and Imanishi, Y. (2014). An Unconventional Secretory Pathway Mediates the Cilia Targeting of Peripherin/rds. *J. Neurosci.* 34, 992–1006. doi:10.1523/jneurosci.3437-13.2014

Tsunematsu, T., Yamauchi, E., Shibata, H., Maki, M., Ohta, T., and Konishi, H. (2010). Distinct Functions of Human MVB12A and MVB12B in the ESCRT-I Dependent on Their Posttranslational Modifications. *Biochem. Biophys. Res. Commun.* 399, 232–237. doi:10.1016/j.bbrc.2010.07.060

Urano, Y., Mori, C., Fuji, A., Konno, K., Yamamoto, T., Yashirogi, S., et al. (2018). 6-Hydroxydopamine Induces Secretion of PARK7/DJ-1 via Autophagy-Based Unconventional Secretory Pathway. *Autophagy* 14, 1943–1958. doi:10.1080/15548627.2018.1493043

Villeneuve, J., Bassaganyas, L., Lepreux, S., Chiritoiu, M., Costet, P., Ripoche, J., et al. (2018). Unconventional Secretion of FABP4 by Endosomes and Secretory Lysosomes. *J. Cell Biol.* 217, 649–665. doi:10.1083/jcb.201705047

Wang, Z., Zhou, H., Zheng, H., Zhou, X., Shen, G., Teng, X., et al. (2021). Autophagy-based Unconventional Secretion of HMGB1 by Keratinocytes Plays a Pivotal Role in Psoriatic Skin Inflammation. *Autophagy* 17, 529–552. doi:10.1080/15548627.2020.1725381

Xu, Y., Cui, L., Dibello, A., Wang, L., Lee, J., Saidi, L., et al. (2018). DNAJC5 Facilitates USP19-dependent Unconventional Secretion of Misfolded Cytosolic Proteins. *Cell Discov.* 4, 11. doi:10.1038/s41421-018-0012-7

Yang, Y., Qin, M., Bao, P., Xu, W., and Xu, J. (2017). Secretory Carrier Membrane Protein 5 Is an Autophagy Inhibitor that Promotes the Secretion of α-synuclein via Exosome. *PLoS One* 12, e0180892. doi:10.1371/journal.pone.0180892

Zhang, M., Kenny, S. J., Ge, L., Xu, K., and Schekman, R. (2015). Translocation of Interleukin-1β into a Vesicle Intermediate in Autophagy-Mediated Secretion. *Elife* 4, e11205. doi:10.7554/eLife.11205

Zhang, M., Liu, L., Lin, X., Wang, Y., Li, Y., Guo, Q., et al. (2020). A Translocation Pathway for Vesicle-Mediated Unconventional Protein Secretion. *Cell* 181, 637–652. e615. doi:10.1016/j.cell.2020.03.031

Zhang, X., Wang, L., Ireland, S. C., Ahat, E., Li, J., Bekier, M. E., 2nd, et al. (2019). GORASP2/GRASP55 Collaborates with the PtdIns3K UVRAG Complex to Facilitate Autophagosome-Lysosome Fusion. *Autophagy* 15, 1787–1800. doi:10.1080/15548627.2019.1596480

Zhang, X., Wang, L., Lak, B., Li, J., Jokitalo, E., and Wang, Y. (2018). GRASP55 Senses Glucose Deprivation through O-GlcNAcylation to Promote Autophagosome-Lysosome Fusion. *Dev. Cell* 45, 245–261. e246. doi:10.1016/j.devcel.2018.03.023

Zhao, J., Li, L., and Leissring, M. A. (2009). Insulin-degrading Enzyme Is Exported via an Unconventional Protein Secretion Pathway. *Mol. Neurodegener.* 4, 4. doi:10.1186/1750-1326-4-4

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

REVIEW
published: 15 June 2022
doi: 10.3389/fcell.2022.907423

Unconventional Protein Secretion in Brain Tumors Biology: Enlightening the Mechanisms for Tumor Survival and Progression

*Rebeca Piatniczka Iglesia[1,2†], Mariana Brandão Prado[1†], Rodrigo Nunes Alves[1†], Maria Isabel Melo Escobar[1], Camila Felix de Lima Fernandes[1], Ailine Cibele dos Santos Fortes[1], Maria Clara da Silva Souza[1], Jacqueline Marcia Boccacino[1], Giovanni Cangiano[1], Samuel Ribeiro Soares[1], João Pedro Alves de Araújo[1], Deanna Marie Tiek[2], Anshika Goenka[2], Xiao Song[2], Jack Ryan Keady[2], Bo Hu[2], Shi Yuan Cheng[2] and Marilene Hohmuth Lopes[1]**

[1]Laboratory of Neurobiology and Stem Cells, Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil, [2]The Robert H. Lurie Comprehensive Cancer Center, The Ken and Ruth Davee Department of Neurology, Lou and Jean Malnati Brain Tumor Institute at Northwestern Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL, United States

Non-canonical secretion pathways, collectively known as unconventional protein secretion (UPS), are alternative secretory mechanisms usually associated with stress-inducing conditions. UPS allows proteins that lack a signal peptide to be secreted, avoiding the conventional endoplasmic reticulum-Golgi complex secretory pathway. Molecules that generally rely on the canonical pathway to be secreted may also use the Golgi bypass, one of the unconventional routes, to reach the extracellular space. UPS studies have been increasingly growing in the literature, including its implication in the biology of several diseases. Intercellular communication between brain tumor cells and the tumor microenvironment is orchestrated by various molecules, including canonical and non-canonical secreted proteins that modulate tumor growth, proliferation, and invasion. Adult brain tumors such as gliomas, which are aggressive and fatal cancers with a dismal prognosis, could exploit UPS mechanisms to communicate with their microenvironment. Herein, we provide functional insights into the UPS machinery in the context of tumor biology, with a particular focus on the secreted proteins by alternative routes as key regulators in the maintenance of brain tumors.

Keywords: secretion, brain, cancer, ER stress, leaderless, glioma, glioblastoma

OPEN ACCESS

Edited by:
Julien Villeneuve, Institute for Functional Genomics (IGF), France

Reviewed by:
Thomas Daubon, UMR5095 Institut de Biochimie et Génétique Cellulaires (IBGC), France
Luciana N. S. Andrade, University of São Paulo, Brazil
Marioara Chiritoiu-Butnaru, Institute of Biochemistry of the Romanian Academy, Romania

****Correspondence:***
Marilene Hohmuth Lopes
marilenehl@usp.br

[†]These authors contributed equally to this work

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *29 March 2022*
Accepted: *26 May 2022*
Published: *15 June 2022*

Citation:
Iglesia RP, Prado MB, Alves RN, Escobar MIM, Fernandes CFL, Fortes ACdS, Souza MCdS, Boccacino JM, Cangiano G, Soares SR, Araújo JPAd, Tiek DM, Goenka A, Song X, Keady JR, Hu B, Cheng SY and Lopes MH (2022) Unconventional Protein Secretion in Brain Tumors Biology: Enlightening the Mechanisms for Tumor Survival and Progression. Front. Cell Dev. Biol. 10:907423. doi: 10.3389/fcell.2022.907423

INTRODUCTION

Eukaryotic cells have developed an array of mechanisms involved in protein secretion, which plays a crucial role in cellular homeostasis and cell-to-cell communication (Sicari et al., 2019). Proteins destined for secretion to the extracellular environment are initially synthesized on ribosomes in the cytoplasm and then transported to the endoplasmic reticulum (ER) (Cavalli and Cenci, 2020) in the presence of signal peptide sequences, which have the utmost importance to direct the newly produced proteins to the ER (Rehm et al., 2001). At the beginning of protein synthesis, the 7S RNA from the signal recognition particle binds to the extremity of the polypeptide chain, which pauses the

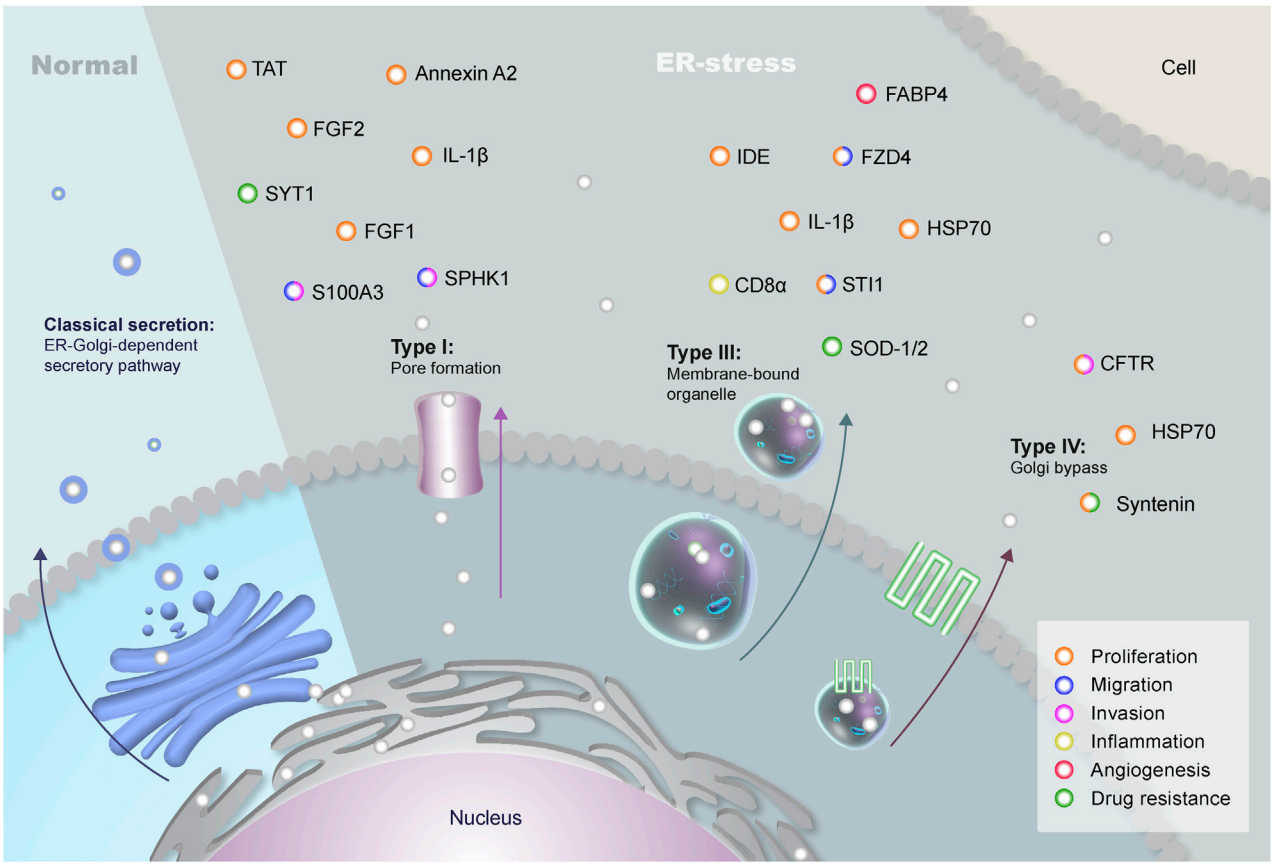


FIGURE 1 | Types of Unconventional Protein Secretion in Eukaryotes. The classical secretion of proteins containing signal peptides involves the endoplasmic reticulum (ER) and the Golgi complex during normal conditions. These proteins are transported through vesicles that bud off the Golgi complex and fuse with the plasma membrane. However, leaderless proteins can be secreted through an unconventional pathway (UPS) that bypasses the Golgi during stress conditions. There are four different USPs in eukaryotes: Type I, in which proteins are secreted through a pore in the plasma membrane; Type II, with the transport of proteins through the superfamily of ATP-binding cassette (ABC) transporters (not shown in the figure); Type III, which uses autophagosomes/endosomes to transport proteins to the extracellular; and type IV, in which proteins containing a signal peptide are secreted bypassing the Golgi. Brain tumors can make use of three different types of UPS: type I (membrane pores), type III (differential vesicles), and type IV (Golgi bypass). The plethora of proteins secreted through UPS can interact with neighboring cells, promoting distinct pathways of key importance in GBM biology, such as proliferation (orange), migration (blue), invasion (pink), inflammation (yellow), angiogenesis (red) and drug resistance (green).

translation and transports the complex (mRNA and ribosome) to ER anchorage points (Hebert and Molinari, 2007). The translation is then restarted, and, as the polypeptide chain is extended, the chaperones that reside in the ER lumen assist the newly synthesized proteins in achieving their native conformations. Alternatively, translation can occur entirely in the cytoplasm, where after synthesis, the Sec62-Sec63 complex orchestrates protein translocation to the ER lumen along with additional chaperones (Cohen et al., 2020). In the ER, proteins may undergo modifications with the support of local chaperones when necessary, being encapsulated into transport vesicles formed by COPII and addressed to the Golgi complex (Cavalli and Cenci, 2020). Once in the Golgi apparatus, these proteins undergo additional modifications and will finally be selected for transport vesicles, which bud off from the Golgi complex. Motor proteins then carry these vesicles to fuse with different portions across the plasma membrane to release their content, which is dictated by specific destination domains (Cohen et al., 2020).

Therefore, the classical secretory pathway consists of the secretion of proteins containing a signal peptide and/or transmembrane domain, which leads them to the ER where COPII-coated vesicles bud to transport secretory proteins through the Golgi apparatus, reaching the plasma membrane where they are released into the extracellular milieu (Palade, 1975; Rabouille, 2017). However, during a stress response, cells present distinguished manners to express and secrete proteins to promote survival (Ferro-Novick and Brose, 2013). Under stressful conditions, the facilitated transport of proteins across the membranes of vesicles and the fast response in protein secretion along with signaling activation led to alternative pathways of secretion. It has been experimentally shown that only a limited number of proteins enter the non-classical secretory pathway (Nickel and Rabouille, 2009), including

primarily fibroblast growth factors, interleukins, and galectins found in the extracellular matrix (Hughes, 1999; Nickel, 2003). These leaderless proteins lack a classical N-terminal signal peptide and function independently of the ER-Golgi network (Bendtsen et al., 2004). Additionally, their export from cells is not affected by the classical secretion inhibitors brefeldin A (BFA) (Fujiwara et al., 1988) and monensin (Schuerwegh et al., 2001; Wesche et al., 2006; Zhao et al., 2009). Recently, studies have described the cell trafficking mechanisms that avoid the conventional ER-Golgi system and comprise unconventional protein secretion (UPS) (Nickel and Rabouille, 2009; Ferro-Novick and Brose, 2013) (**Figure 1**). While the UPS system mainly promotes the secretion of proteins lacking the signal peptide sequences and transmembrane domains - namely leaderless proteins - it may also cause conventional proteins to be alternatively secreted via Golgi bypass (Nickel and Rabouille, 2009; Rabouille, 2017).

UPS comprises types I to IV, and the molecules secreted via non-canonical routes include cytoplasmic proteins with a central role in cell biology and its microenvironment. Briefly, type I UPS is related to the translocation of leaderless proteins across the membrane through pores. Type II is associated with ABC transporter-dependent secretion, while type III uses intracellular intermediates including endosomes, autophagosomes and lysosomes for secretion (Nickel and Rabouille, 2009; Rabouille, 2017). Finally, type IV comprises proteins that, albeit having a signal peptide or transmembrane domain bypass the Golgi apparatus, being transported from the ER to the plasma membrane. Interestingly, the family of peripheral Golgi proteins named Golgi Reassembly and Stacking Proteins (GRASPs) can participate in the Golgi bypass and in type III endosomal transport (Giuliani et al., 2011; Rabouille, 2017). These mechanisms will be better discussed through this study in a tumoral context, focusing on the role of UPS in brain tumors maintenance and progression (**Figure 1**).

UNCONVENTIONAL PROTEIN SECRETION IN BRAIN TUMORS

Protein secretion is a fundamental process in both health and disease, playing pivotal roles in intercellular communication, which is a critical aspect in tumor progression and metastasis (Peinado et al., 2017). The tumor microenvironment (TME) is composed of blood vessels, extracellular matrix components, tumor-associated immune cells, fibroblasts, neural cells including astrocytes and neurons, and a plethora of different signaling molecules and cytokines derived from the TME (Spill et al., 2016; Greten and Grivennikov, 2019). Cancer cells require active communication with neighboring cells and the local microenvironment during tumor initiation and progression. Indeed, protein secretion has been broadly described as an essential mechanism for tumor initiation and progression, including in central nervous system (CNS) tumors such as glioblastoma (GBM) (Kucharzewska et al., 2013; Broekman et al., 2018). GBM, a grade IV astrocytoma, is an incurable malignancy and extremely aggressive neoplasm in adults characterized by microvascular proliferation, necrosis, and inter- and intratumoral heterogeneity, which may contribute to therapy resistance. Even with recent advances in GBM therapy, the overall patient survival is 15 months with few long-term survivors. Glioblastomas are characterized by presenting Isocitrate dehydrogenase (IDH) wildtype profile, usually associated with worst prognosis compared to mutant, present amplification in the epidermal growth factor receptor (EGFR), and Telomerase reverse transcriptase (TERT) promoter mutation that lead to lengthened telomeres (Louis et al., 2021). Finally, GBM also present frequently alterations in gain or loss of chromosome copy numbers (+7/−10) (Parsons et al., 2008; Louis et al., 2021). The TME exerts great influence in tumor development and secreted molecules involved in cell-to-cell communication is crucial to promoting tumor maintenance (Zhou et al., 2015). Proteins and molecules secreted by the tumor and its associated cells seem to play a crucial role in chemo and radiotherapy resistance, assisting in the poor prognosis of patients with GBM (Ou et al., 2020). It is also important to highlight that under stress conditions - such as hypoxia, which is relatively common in brain tumors - there is an increase in chemotherapy-resistant cells (Goenka et al., 2021; Singh et al., 2021). Hence, under such conditions, the tumor cells might use UPS to release proteins and molecules to modulate the TME (**Figure 1**).

As we will discuss in this review, the UPS routes are used by many proteins with key roles in promoting tumor chemoresistance, such as HSP70 family-like glucose-regulated protein 78 (GRP78) (Lee et al., 2008) and ATF6 (Dadey et al., 2016). Therefore, in the following sections, we will describe UPS types and address their specific roles in the context of brain tumors, focusing on the contributions of each non-canonical secretion route to tumor progression and resistance to treatment.

Types I and II UPS—Translocation of Leaderless Proteins Through Membrane Pores

Type I UPS is characterized by the formation of plasma membrane pores that induce the translocation of cytoplasmic proteins without the participation of vesicular intermediates (Rabouille, 2017). Leaderless proteins can be translocated across the plasma membrane through pores that allow the traffic of cytoplasmic cargoes (Rabouille, 2017). Pore formation is, however, a complex process that can either be self-dependent or driven by inflammation, two pivotal mechanisms when it comes to protein release to the extracellular space (Rabouille, 2017). Regulated pore formation for UPS requires the recruitment of leaderless proteins by acidic membrane lipids at the inner leaflet of the plasma membrane, followed by oligomerization-induced membrane insertion and tyrosine phosphorylation (Rabouille, 2017). A classic example of this mechanism is the constitutive export of fibroblast growth factor 2 (FGF2). This process depends on sequential interactions of FGF2 with the phosphoinositide PI(4,5)P2 at the inner leaflet and heparan sulfate proteoglycans (HPSG) at the outer leaflet of the plasma membrane (Dimou and Nickel, 2018). Eventually,

PI(4,5)P2-induced self-oligomerization stimulates membrane insertion, aided by Tec kinase-mediated phosphorylation (Steringer et al., 2015). Furthermore, FGF2 secretion is related to cell-surface ligands such as HPSG, as shown by Zehe and co-workers in a study that reported inhibition of FGF2 secretion under pharmacological inhibition of HPSG biosynthesis (Zehe et al., 2006). This data indicates that HPSG drives the translocation of FGF2 across the membrane through a molecular trap (Zehe et al., 2006). In detail, several cis-elements participate in FGF2 secretion, namely: K127/R128/K133 forming the PI(4,5)P2 binding pocket, Y81 being the target of Tec kinase, and two cysteine residues C77/C95 promoting FGF2 oligomerization, as well as four trans-acting factors: the aforementioned PI(4,5)P2, ATP1A1, Tec kinase, and HPSGs (Steringer et al., 2017). Interestingly, FGF1 and FGF2 are soluble molecules well described in the brain TME. FGF1 is a 140 amino-acid polypeptide belonging to the fibroblast growth factor family (Jaye et al., 1986; Di Serio et al., 2008) that binds to FGF receptors (FGFR), as well as other membrane receptors, such as integrin. FGF1 receptor binding stimulates a plethora of biological processes related to tumor progression, such as cell survival, proliferation, angiogenesis, differentiation, and migration (Mori et al., 2008; Yamaji et al., 2010). In brain tumors, such as gliomas, FGF1 is involved in chemotaxis and migration of tumor cells (Brockmann et al., 2003) which primarily express the FGF1B and FGF1D isoforms (Myers et al., 1995). This protein has also been considered a therapeutic target in glioma, in which the inhibition of its receptor FGFR1 decreased tumor growth (He et al., 2018).

The FGF2 is either located in the nucleus and the cytosol or released in the extracellular milieu through UPS (Akl et al., 2016). While most of its physiological functions are shared with FGF1 (Mori et al., 2008; Yamaji et al., 2010), FGF2 plays a vital role in tumor-induced angiogenesis, contributing to tumor growth. FGF2 is overexpressed in human cancers, including gliomas, and acts as an autocrine and paracrine angiogenic factor (Takahashi et al., 1992; Akl et al., 2016). In gliomas, both FGF2 and VEGF seem to have an essential role in regulating tumor growth and angiogenesis, indicating that their inhibition could be implemented as an antitumoral treatment (Bian et al., 2000). In addition, FGF2 can promote proliferation and cell survival through the activation of the Akt signaling pathway (Wang et al., 2015), which corroborates the fact that anti-FGF2 antibodies inhibited both anchorage-dependent and independent tumor growth of glioma U87MG and T98G cells (Takahashi et al., 1992). For instance, FGF2 membrane translocation through the membrane pore occurs in a fully folded conformation that requires an interaction with PIP2, which causes FGF2 to oligomerize (Torrado et al., 2009). Only then this complex can achieve membrane insertion, highlighting the need for an internal quality control mechanism that ensures the secretion of fully folded and biologically active FGF2 proteins (Torrado et al., 2009).

Interleukin-1β (IL-1β) secretion also follows the type I UPS pathway upon inflammatory cues in monocytes, macrophages, and dendritic cells (Rabouille, 2017). IL-1β is a polypeptide related to host defense and homeostasis and has been shown as one of the many mediators of infection, inflammation, and autoimmune diseases (di Giovine et al., 1991). Although IL-1β does not directly bind to PI(4,5)P2, it has been shown that inflammasome activation induces pores in the plasma membrane that allows IL-1β to reach the extracellular space (Cavalli and Cenci, 2020). Direct IL-1β secretion depends on the activation of caspase-11 in mice or caspase-4 and caspase-5 in humans, which activates gasdermin-D, a cytosolic protein containing two domains separated by a linker peptide (Kayagaki et al., 2015). Gasdermin-D undergoes a conformational change in its annular shape and drives membrane pore formation with its active amino-terminal fragment in a PI(4,5)P2-dependent manner (Liu et al., 2016). IL-1β is secreted by activated monocytes in a process related to the translocation of intracellular membranes, mostly during cell stress (Rubartelli et al., 1990). IL-1β secretion by tumor-associated macrophages in gliomas presents an essential role in tumor maintenance (Lu et al., 2020). Data from the literature demonstrate that tumor-infiltrating macrophages can help metabolism reprogramming for glioma cell survival. This effect occurs through the secretion of IL-1β since it triggers a shift in energy metabolism (from oxidative phosphorylation to aerobic glycolysis) and induces tumorigenesis and cell proliferation (Lu et al., 2020). It is noteworthy that along with tumor necrosis factor (TNF), IL-1β is one of the most critical neuro-pro-inflammatory molecules in both health and disease (Rizzo et al., 2018).

Other examples of leaderless proteins that follow the type I UPS mechanism are sphingosine kinase 1 (SPHK1), annexin A2, synaptotagmin 1 (SYT1), small calcium protein (S100A3), and TAT, among others (Rubartelli and Sitia, 1991; Kim, 2006; Rabouille, 2017; Steringer et al., 2017; Cruz-Garcia et al., 2018; Popa et al., 2018; Ye, 2018; Aliyu et al., 2019; Cavalli and Cenci, 2020; Cohen et al., 2020).

Specifically, SPHK1 is an enzyme with multiple functions, one of which catalyzes the phosphorylation of sphingosine to S1P, a lipid that regulates processes at both the intra- and extracellular levels (Wang et al., 2013). Furthermore, this enzyme is related to ceramide biosynthesis, decreasing its production, and acting as an anti-apoptotic factor (Maceyka et al., 2005). SPHK1 also regulates the inflammatory response in the nervous system due to S1P, which stimulates the TRAF2 E3 ubiquitin ligase activity and promotes the activation of the NF-κB signaling pathway (Alvarez et al., 2010; Adada et al., 2013). Loss-of-function studies have also shown that SPHK1 takes part in endocytic membrane trafficking and recycling, and is enriched in the nerve terminus, which is essential for neurotransmission (Shen et al., 2014; Lima et al., 2017). A higher expression of SPHK1 has also been shown to correlate to a poor prognosis in GBM, elevating both migration and invasion rates (Paugh et al., 2009). In addition to IL-1, EGFR, a well-described oncogenic driver in GBM, has also been described as a modulator of SPHK1 activity in glioma spheres since EGFR inhibition leads to a decrease in angiogenesis, cell viability and increases apoptosis in GBM9 cell lines (Estrada-Bernal et al., 2011), while also increasing ceramide levels (SPHK1's precursor molecule) (Kapitonov et al., 2009; Abuhusain et al., 2013).

Moreover, annexin A2, a type I UPS protein (Rabouille, 2017), is localized to the basement membrane of epithelial cells, endothelial cells, and keratinocytes (Waisman et al., 1995), belonging to a family of calcium-dependent proteins that bind to the membrane and phospholipids (Mayer et al., 2008). In the microenvironment, annexin A2 acts as a co-receptor for plasminogen and plasminogen tissue activators, promoting vascular fibrinolysis (Seidah et al., 2012). Annexin A2 also plays an important role in cholesterol homeostasis by interacting with PCSK9, a convertase that regulates the degradation of the LDL receptor (Ly et al., 2014). In tumors, annexin A2 pseudogene 2 (A2P2) is highly expressed in tumor tissues and cell lines, indicating its potential role as a prognostic biomarker (Du et al., 2020). In addition, A2P2 inhibition in glioma cells decreased cell proliferation and aerobic glycolysis, showing a correlation with the Warburg effect in which cells shift to anaerobic glucose metabolism (Du et al., 2020). In gliomas, annexin A2 is overexpressed and associated with a mesenchymal and invasive phenotype due to its interaction with transcription factors involved in the epithelial-mesenchymal transition (EMT), such as RUNX1, FOSL2, and BHLHB2 (Kling et al., 2016; Maule et al., 2016). These data indicate the valuable role of annexin A2 as a potential therapeutic target for treating gliomas (Kling et al., 2016; Maule et al., 2016). Annexin A2 is also found in extracellular vesicles (EVs) derived from GBM cells, contributing to an increase in aggressiveness, being a direct target of microRNAs (miR) such as miR-1 and mi-R155HG (Bronisz et al., 2014; Wu et al., 2019).

SYT1 is a known gatekeeper of neurotransmitter release sensitive to calcium (Fernandez-Chacon et al., 2001), that has been marked as a differentially expressed gene in GBM and other types of human cancers, and its expression is inversely correlated with the survival of patients with cancer (Yang and Yang, 2020). In addition, this protein has also been shown to be a potential target of tumor suppressor miR-34c, which plays a key role in inhibiting cell growth and inducing apoptosis (Shi et al., 2020). On the other hand, S100A3 is a protein from the S100 family involved in epithelial cell differentiation (Kizawa et al., 2008) which has also been identified as a differentially expressed protein from grades II-IV of astrocytomas, differing according to the tumor malignancy (Camby et al., 1999). Fewer studies have also indicated that S100A3 might be related to glioma immunity, even though the mechanism is still not fully understood (Zhang et al., 2021). Lastly, TAT (or HIV-1 TAT Stimulatory Factor) is a small protein essential for HIV replication (De Marco et al., 2010) that shares a similar secretion mechanism with FGF2 (Steringer et al., 2017). Taking a closer look into the TAT's non-conventional roles, data have shown that this molecule has a neurotoxic activity affecting cell a composite peptide containing permeabilization and membrane depolarization in neuroblastoma cells and decreasing cell growth of gliomas (Sabatier et al., 1991; Daniel et al., 2004). Interestingly, TAT (BRBP1-TAT-KLA) has also been used as a therapeutic target against metastatic brain tumor cells, inducing mitochondrial damage and apoptosis (Fu et al., 2015).

The need for alternative mechanisms of protein secretion protein secretion in cancer cells is still not fully understood. However, it might be related to cellular strategies for protein quality control, as well as to cope with the quantity and speed of protein secretion needed to respond to essential processes such as inflammation triggered by tumors. Additionally, this rapid response is very characteristic of survival mechanisms that in cancer are related to tumor progression, resistance, and recurrence processes. Despite these data describing the function of type I UPS proteins in brain tumors, the specific path of secretion of these proteins in the tumor context still requires further investigation.

Regarding type II UPS, it comprises specifically the transport through the superfamily of ATP-binding cassette (ABC) transporters, which are integral membrane proteins that bind and translocate a substrate in an ATP-dependent manner, modulating the uptake and export of macromolecules or ions (Rees et al., 2009; Wilkens, 2015; Locher, 2016; Stefan, 2019). The UPS mechanism modulated by ABC transporters was studied essentially in non-eukaryotic models. Thus, this specific model will not be further discussed in this review. However, it is noteworthy that ABC transporters are related to the unconventional secretion of heat shock Protein 70 (HSP70) in mammalian cells since they modulate the entrance of HSP70 to endolysosomal vesicles prior to secretion after the heat shock stimuli (Mambula and Calderwood, 2006; Cohen et al., 2020). The role of HSP70 in tumors is well established, and it will be further discussed in the following sections.

Type III UPS—Vesicular Transportation of Leaderless Proteins

Type III UPS, also known as autophagosome/endosome-based secretion, is a stress-induced pathway characterized by the recruitment of membrane-bound organelles that are co-opted for secretion (Rabouille, 2017). Leaderless proteins cross the membrane of endosomes and autophagosomes and are later secreted after the organelle fuses with the cell membrane (Duran et al., 2010). Although the role of exosome-mediated secretion is well known, what might distinguish it from type III UPS is their different strategies in recruiting cargo (Ye, 2018). As an example, mammalian misfolded proteins might be secreted using type III UPS (Misfolding-Associated Protein Secretion, or MAPS), being translocated from ER to the lumen of late endosomes afterward secreted through fusion with the plasma membrane (Lee et al., 2016). In this way, there are no extracellular vesicles released. HSP70 and its co-chaperone DNAJC5 are also involved in MAPS (Xu et al., 2018). This mechanism consists of the recruitment of misfolded proteins to the surface of the ER by an associated deubiquitinase (DUB) named USP19 (Xu et al., 2018). Cargo proteins enter the lumen of late endosomes, and secretion occurs when vesicles released from endosomes fuse directly with the plasma membrane. Interestingly, this process has been associated with several key proteins in neurodegenerative diseases such as TDP-43 and α-synuclein (Fontaine et al., 2016; Lee et al., 2016).

In certain eukaryotes, type III UPS promotes the formation of Compartments for Unconventional Protein Secretion (CUPS), which were first described in yeast and are characterized by the involvement of a cup-shaped collection of tubulo-vesicular membranes that act as transport intermediates for secretion

(Rabouille, 2017). The biogenesis of CUPS can be traced by the expression of the Grh1 protein (the yeast ortholog of GRASP), which migrates to distinct membrane foci of cells undergoing stress (Bruns et al., 2011) or starvation-induced autophagy (Yang and Klionsky, 2010). However, CUPS biogenesis is not triggered by rapamycin as observed in conventional pathways, and it involves proteins that are not required for classical autophagy, such as Bug1 and endosomal sorting complex required for transport (ESCRT)-II and -III (Bruns et al., 2011). CUPS can form initially from pre-existing Golgi complex membranes that mature by the contribution of endosomal membranes, depending on the activity of PI-3 kinase for its maintenance (Cruz-Garcia et al., 2014).

The involvement of autophagy-related proteins (ATG-related ATG8 and ATG9) in CUPS led to the hypothesis that a secretory and non-degrading autophagosome-like vesicle forms in UPS (Duran et al., 2010; Manjithaya et al., 2010; Bruns et al., 2011). Interestingly, ATG8-mediated autophagy in glioma cells modulates radiotherapy resistance and malignancy (Huang et al., 2017). On the other hand, ATG9A modulates an alternative lysosomal transport of ferritin in glioma cells (Goodwin et al., 2017), as well as regulates hypoxia in GBM cells, with its silencing leading to inhibition of cell proliferation and tumor growth (Abdul Rahim et al., 2017). The unconventional secretion related to autophagy was recently described in GBM modulating TMZ sensitivity through HMGB1 which, in turn, enhances M1-like polarization of tumor-associated macrophages (TAMs) (Li et al., 2022). Indeed, autophagy has been broadly studied for developing potential therapies for GBM, presenting controversial roles in the tumor's biology since different studies have described both the induction and repression of autophagy as potential strategies for therapy (Manea and Ray, 2021).

In addition, heat shock proteins are also implicated in the transport of some cargoes in type III UPS, as transport by membrane fusion is restricted to unfolded proteins. This mechanism requires the two members of the mammalian GRASP family: GRASP55 (Dupont et al., 2011) and GRASP65 (Zhang et al., 2015), with a role for GRASP55 in the formation of secretory autophagosomes (Dupont et al., 2011).

GRASPs are comprised of a range of proteins related to Golgi reassembly and cisternae stacking. These molecules exist in homologous forms across different organisms: GRASP55 and GRASP65 in mammals; dGRASP in *Drosophila*; Grh1 in yeast; and GrpA in *Dictyostelium* (Deretic et al., 2012). The yeast GRASP Grh1 was demonstrated to colocalize with COPII in the transitional endoplasmic reticulum, and it was suggested to play roles in the early secretory process, albeit it was shown to be unessential in the organization of secretory compartments (Levi et al., 2010). In this case, the currently proposed mechanism consists of the formation of a collection of small vesicles and tubules that mature and get surrounded by flat saccules of an unknown nature that will fuse with the plasma membrane (Curwin et al., 2016). Therefore, in type III UPS, loads translocate through the membrane of the "secretory" organelle, with different structures such as a saccule, an early autophagosome, and a late endosome being reported.

Mammalian GRASP55 and GRASP65 were reported to play essential roles in the maintenance of Golgi architecture (Barr et al., 1997; Shorter et al., 1999). Despite GRASP55 and GRASP65 being homologous to each other and exhibiting similar functions, they present their own specific characteristics. The 65 kDa GRASP may be found in the cis-Golgi cistern and assembles into a complex with GM130 (a protein that has been characterized as a component and regulator of cis-Golgi structure (Nakamura et al., 1995) and p115, a membrane tethering molecule that is related to Golgi maintenance (Radulescu et al., 2011). On the other hand, the 55 KDa GRASP is localized to the medial- and trans-Golgi cisternae and does not interact significantly with the same proteins as GRASP65 (Shorter et al., 1999; Zhang et al., 2018). Since their discovery and initial characterization more than 20 years ago, GRASP55 and GRASP65 have been extensively studied by several groups. Of note, mTORC1 has been described as a phosphorylating agent of GRASP55, which consequently stacks GRASP55 within the Golgi complex (Nuchel et al., 2021). Remarkably, the lack of mTORC1 activity promotes the dephosphorylation of the GRASP protein, which, in turn, leads to a change in its localization within the cell and can consequently cause the secretion of extracellular matrix proteins via UPS (Nuchel et al., 2021). Interestingly, not only has mTORC1 surfaced as a potential therapeutic target in GBM (Ronellenfitsch et al., 2018), but studies showed that the use of mTORC1 inhibitor everolimus has great therapeutic potential against pediatric low-grade gliomas (Poore et al., 2019; Cacchione et al., 2020). GRASPs are closely related to UPS mechanisms such as type III and IV UPS (Giuliani et al., 2011), and GRASP55 is considered an unconventional secretion factor (van Ziel et al., 2019).

GRASP55 and GRASP65 have been shown to control the transport of proteins such as CD8α - a dendritic cell marker with increased expression in pro-inflammatory niches of brain tumors (Pituch et al., 2018) - and Frizzled-4 (FZD4), both containing valine residues at the C-terminal during Golgi trafficking (D'Angelo et al., 2009). In addition, proteins of the Frizzled family, such as FZD4 and FZD5, participate in the WNT signaling pathway and inflammatory processes in nervous tissue (Zhao et al., 2015) and are related to tumor initiation and cell proliferation of glioma cells (Sarkar et al., 2020), respectively, and can modulate tumor progression. Additionally, soluble Frizzled-related proteins, or sFRPs, also have an important role in glioma maintenance, modulating tumor growth and migration through MMP-2 and tyrosine phosphorylation of beta-catenin (Roth et al., 2000). Altogether, these features place Frizzled proteins as a potential therapeutic target for specific subtypes of GBM (El-Sehemy et al., 2020).

IL-1β is one of the most intensively investigated unconventional secretion loads, with several non-conventional mechanisms involved in its secretion (Andrei et al., 1999; MacKenzie et al., 2001; Brough et al., 2003; Qu et al., 2007; Lopez-Castejon and Brough, 2011). The translocation through pores was described above in this review. Moreover, when lipopolysaccharide (LPS) is the trigger, IL-1β is secreted in vesicles containing cathepsin D and Lamp-1, indicating a

secretion pathway of endolysosomal origin (Andrei et al., 1999). According to this model in human monocytes, upon reaching the endolysosomes, the pro-IL-1β polypeptide is cleaved by caspase-1 and converted into a mature IL-1β protein, which is released into the extracellular space by fusion of the compartment with the plasma membrane (Piccini et al., 2008; Kimura et al., 2017). This process is mediated by the HSP90 chaperone, which interacts with a signal peptide in the mature region of IL-1β, with the participation of GRASPs, to deliver the charge to a phagophore, a precursor of the autophagosome that, when mature, transports IL-1β to the cell surface (Zhang et al., 2015). Interestingly, not only can IL-1β promote hypoxia-induced apoptosis in GBM through the inhibition of the HIF-1/AM axis (Sun et al., 2014), but it also induces tumorigenicity and promotes the formation of glioma spheres in LN-229 glioma cells (Wang et al., 2012).

The fatty acid-binding protein 4 (FABP4) is a cytoplasmic adipokine with chaperone functions whose secretion relies on UPS. Since FABP4 lacks a peptide signal sequence (Schlottmann et al., 2014), it is secreted in a GRASP-independent manner via endosomes and secretory lysosomes (Villeneuve et al., 2018). FABP4 secretion was also shown to be calcium-dependent in adipocytes (Schlottmann et al., 2014). FABP4 is upregulated in normal and low-grade gliomas, mainly related to angiogenesis (Cataltepe et al., 2012), and presents an essential role in GBM, contributing to tumor growth through the activation of WNT signaling (Li et al., 2018). FABP4 expression is observed in grade III anaplastic meningiomas, is highly expressed in vascular endothelial cells, and functions as a potential biomarker for this type of brain tumor. Additionally, other protein from the fatty acid-binding protein family, FABP7, has also been implicated as a glioma prognostic marker, and was correlated with the recurrence of several types of gliomas (Elsherbiny et al., 2013).

Like FABP4, the insulin-degrading enzyme (IDE) does not have a peptide signal sequence, relying on UPS to be transported to the extracellular space (Son et al., 2016). In HeLa cells and murine hepatocytes, IDE secretion was insensitive to inhibitors of the classical secretory pathway and conventional stimulators of protein secretion, which indicated the role of UPS in the transport and release of this protein (Zhao et al., 2009). This amyloid β protease has been investigated in Alzheimer's disease and was shown to be secreted by astrocytes via the autophagic pathway and RAB8A, where GRASP activity was necessary for this process to occur (Son et al., 2016). Additionally, statins have been demonstrated to induce the autophagy-mediated secretion of IDE (Son et al., 2015). In N2a cells, it was shown that IDE might be transported into multivesicular bodies, which is followed by sorting into exosomes (Bulloj et al., 2010). Furthermore, the overexpression of IDE is associated with tumor progression, with its silencing inhibiting cell proliferation and promoting cell death in neuroblastoma (Tundo et al., 2013).

An interesting protein described in the literature that has been differentially secreted is the heat shock organizing protein (HOP), the human ortholog of stress-inducible protein one (STI1), which does not present a signal peptide for secretion, but it is found in the extracellular environment associated with vesicles (Hajj et al., 2013; Cruz et al., 2018). HOP is an adaptor molecule that assists the chaperones HSP70 and HSP90 in protein folding in several species, including humans (Song and Masison, 2005). Furthermore, in GBM, HOP modulates cell proliferation *in vitro* and tumor growth *in vivo* in its soluble secreted form, which interacts specifically with the cellular prion protein (PrP^C) on the cell surface (Lopes et al., 2015; Iglesia et al., 2019). Additionally, secreted HOP binding to PrP^C in glioma stem-like cells (GSC) leads to an increase in self-renewal, proliferation, and migration (Iglesia et al., 2017), and the blockage of this interaction has presented a therapeutic potential in some studies (Lopes et al., 2015; Iglesia et al., 2017).

Superoxide scavenger enzyme or superoxide dismutase 1 (SOD1) is another protein that does not have a signal sequence but shows a conserved diacidic motif that determines its UPS fate (Cruz-Garcia et al., 2017). Pathologically, this motif is also present in a mutated form of SOD1 that is related to amyotrophic lateral sclerosis (Cruz-Garcia et al., 2017). SOD2, a second family member, was related to resistance to temozolomide (TMZ) in GSCs and GBM recurrence (Chien et al., 2019). In brain tumors, recombinant SOD1 and two associated with manganese (r-hMnSOD) exhibit a therapeutic potential since they can attenuate edemas by combating the oxygen-free radicals produced during the inflammatory response (Shoshan and Siegal, 1996). Indeed, the expression of several SODs and other antioxidants are inversely correlated with glioma malignancy and prognosis (Aggarwal et al., 2006), presenting low activity in tumors compared to normal tissues (Popov et al., 2003), thus supporting their anti-tumor activity. Furthermore, the transcription factor SP1 was shown to regulate SOD2 expression, which is related to TMZ resistance and recurrence in an MGMT-independent manner (Chang et al., 2017).

It is noteworthy that many leaderless proteins in the brain tumor context are related to cell survival, especially regulated by stress response regulators such as chaperones and associated molecules, inflammatory response, antioxidants, and proteins that participate in autophagy, which support the participation of UPS mechanisms in tumor progression and resistance to therapy.

Type IV UPS—Golgi Bypass

While leaderless proteins can be secreted via unconventional routes, proteins with a signal peptide and/or a transmembrane domain can also deviate from the conventional secretory pathway. If these proteins are not directed to the Golgi apparatus on their way to vesicular organelles, the plasma membrane, or the extracellular environment, they undergo UPS via Golgi bypass, whose mechanism harbors many similarities with the other UPS types, despite certain exclusive features (Grieve and Rabouille, 2011; Rabouille, 2017). Importantly, the Golgi bypass has been a research topic of increasing interest that remains poorly understood. Although several studies point to type IV UPS being triggered by stress (ER and mechanical) (Giuliani et al., 2011), emerging evidence shows that different proteins can be constitutively secreted by both the

conventional mechanism and Golgi bypass (Baldwin and Ostergaard, 2002).

The first example of proteins "skipping" the Golgi comes from a study in 1980 by Bergfeld et al., who observed this phenomenon in the formation of storage protein bodies and accumulation of proteins in the vacuole of *Sinapis alba* through electron microscopy (Bergfeld et al., 1980). Since then, the process has been observed in different organisms, including plants, fungi, *Drosophila*, and mammalian cells (Bergfeld et al., 1980; Morre, 1981; Sluiman, 1984; Schotman et al., 2008; Davis et al., 2016; Ng and Tang, 2016; Dimou et al., 2020)), indicating that this process is a conserved mechanism throughout evolution. Furthermore, the Golgi apparatus is the central organelle for protein processing, in which many resident proteases change protein composition through post-translational modification (Kulkarni-Gosavi et al., 2019; Frappaolo et al., 2020). If proteins bypass the Golgi, their structural composition is maintained as it was initially synthesized in the ER. These proteins will present the commonly high-mannose oligosaccharide N-linked core but will not be processed in Golgi, where sugar would be added to this core by resident proteases (Roth, 2002; Ito and Takeda, 2012; Fujikawa et al., 2016). Therefore, the Golgi bypass could represent a mechanism that modulates protein composition, function, and affinity with other molecules through its structural composition (i.e., glycosylation state).

Proteins that undergo the Golgi bypass can have different functions (Baldwin and Ostergaard, 2002; Gonzalez et al., 2018; Witzgall, 2018; Van Krieken et al., 2021), but all of these proteins show similar characteristics that are utilized for their identification (Grieve and Rabouille, 2011), such as resistance to BFA, which inhibits the formation of COPI coats in Golgi membranes through Arf1 activation (Zeghouf et al., 2005; Langhans et al., 2007). Thus, only proteins sorted to the Golgi bypass, and consequently do not require COPI or COPII-coated vesicles to reach the plasma membrane or the extracellular medium, are BFA-resistant (Rabouille et al., 2012). Proteins are also found to be independent of specific SNAREs involved in the ER to Golgi transport and beyond (Yoo et al., 2002). Specifically, Syntaxin 5 (STX5) is known to be extremely important to Golgi transport (Dascher et al., 1994), and protein secretion in its absence suggests the independence of these groups of proteins to reach their proper localization (Grieve and Rabouille, 2011; Kim et al., 2016). Furthermore, SNAREs are quite relevant to the biology of brain tumors. For example, Syntaxin 1 (STX1) expression supports tumor growth and invasiveness in GBM models (Ulloa et al., 2015), and several genes from the SNARE family are enriched in pediatric medulloblastoma (Huang et al., 2020b). Thus, the correlation of SNARE-independent transport with brain tumors warrants further investigation.

Another important aspect is that proteins that can bypass the Golgi appear to have one or more Postsynaptic density-95, disks-large, and zonula occludens-1 (PDZ) domains, a protein interaction module responsible for target recognition (Gee et al., 2011; Vinke et al., 2011; Liu and Fuentes, 2019). Previous studies described some of these molecules related to brain tumors, although their secretion mechanism is not fully understood. For example, the scaffold protein called syntenin, which contains two postsynaptic density protein-95/discs-large/PDZ domains, also presents as a potential new therapeutic target in GBM (Haugaard-Kedstrom et al., 2021). The highly selective inhibitor of syntenin KSL-128114 can bind to the PDZ1 domain of syntenin and demonstrates a decrease in cell viability of primary GBM cells and significantly increases survival in patient-derived xenograft mouse models (Haugaard-Kedstrom et al., 2021). Additionally, specific inhibition of syntenin activity by the PDZ1 inhibitor decreases radioresistance of human GBM cells and decreases invasion post-radiotherapy (Kegelman et al., 2017). Indeed, syntenin is a scaffold protein that acts at the cell surface, and its expression is more evident in high-grade gliomas compared to its counterparts. Syntenin also increases cell migration and invasion, and its silencing decreases tumor growth and therapy resistance (Kegelman et al., 2014; Kegelman et al., 2017). The transcriptional coactivator with PDZ-binding motif (TAZ) participates in the Hippo pathway and modulates glioma cell EMT, proliferation, invasion, differentiation, and patient survival (Bhat et al., 2011; Li et al., 2016). Other examples of PDZ-containing proteins that are essential for brain tumor biology include the Tax-interacting protein (TIP)-1 related to GBM motility (Wang et al., 2014), membrane-associated guanylate kinase inverted 3 (MAGI3), and Protein interacting with C kinase 1 (PICK1), which are inversely correlated with glioma malignancy and progression (Cockbill et al., 2015; Ma et al., 2015). However, the specific mechanisms of translocation of these proteins to the membrane of brain tumors have not been fully explored, and more research is required to confirm their association with the UPS.

The Golgi bypass could be a strategy for cells to deliver proteins to the plasma membrane and extracellular space faster than the canonical secretory pathway (Baldwin and Ostergaard, 2002; Grieve and Rabouille, 2011). The first sorting mechanism described for the Golgi bypass was discovered by observing the secretion of the cystic fibrosis transmembrane conductance regulator (CFTR). Mutated CFTR is known for its role in cystic fibrosis disease, and its most common mutation is associated with its cell surface expression (Elborn, 2016). Despite wild-type CFTR being conventionally secreted from ER exit sites using COPII-coated vesicles, wild-type and mutated CFTR also present unconventional secretion mediated by GRASP55 (Gee et al., 2011). GRASP55 can form a homodimer through their PDZ domains in the Golgi, which is important for Golgi structural assembly (Wu et al., 2020). Upon ER stress, GRASP55 is phosphorylated at serine 441 residue by a yet unidentified kinase, leading GRASP55 back to the ER as a monomer (Kim et al., 2016). Monomeric GRASP55, *via* its PDZ domain, can recognize other PDZ domains of proteins that undergo the Golgi bypass (Gee et al., 2011; Kim et al., 2016). Mouse models carrying mutations in the CFTR promoter develop ependymoma tumors and hydrocephalus, with no other alterations in vital organs such as the lungs and pancreas (Perraud et al., 1992). On the other hand, the expression of CFTR in human GBM cells is less evident when compared to normal tissue, and it abrogates GBM cell proliferation and invasion through the inhibition of the JAK2/STAT3 signaling

pathway (Zhong et al., 2019). This demonstrates that the mutated CFTR may present an opposite role to its wild-type counterpart in tumors, thus suggesting a role for UPS in this process.

More recently, additional sorting machinery was proposed involving HSP70, a protein that is an essential molecular chaperone in health and disease and displays constitutive expression despite being highly induced by different stress stimuli (Rosenzweig et al., 2019). Additionally, the HSP70 family and other chaperones present significant participation in brain tumor biology, including GBMs (Iglesia et al., 2019). In the context of ER stress and UPS activation, the heat shock cognate Hsc70 (a constitutive human isoform of HSP70) associated with its co-chaperone DNAJC14 directly interacts with cargo proteins selected to the Golgi bypass, directing the cargo to the plasma membrane instead of directing it to refold or to the ER-associated degradation (ERAD) system (Jung et al., 2016). Furthermore, Hsc70 is highly expressed in tumor tissues, including gliomas, and is directly related to the poor prognosis of high-grade gliomas (HGG), where its silencing decreases tumor proliferation and survival (Sun et al., 2019).

Interestingly, in insulin-positive alpha and beta cells of patients with and without type 1 diabetes, PrP^C was found in the plasma membrane and the ER but not in Golgi, possibly indicating UPS by the Golgi bypass. In this work, the authors suggest that the PrP^C's Golgi bypass observed in the human pancreas could be through HSP70/DNAJC14 or GRASP55 (Hiller et al., 2021). As aforementioned, PrP^C associates with the HSP70/90 co-chaperone STI1/HOP in the cell surface (Lopes et al., 2005; Rosenzweig et al., 2019), which could indicate a greater tendency of secretion to be via HSP70/DNAJC14, although this hypothesis must be tested and the mechanism for PrP^C UPS needs to be clarified. As previously mentioned, the interaction of PrP^C and STI1 in GBM cells promotes the self-renewal and migration of GSCs, as well as proliferation and survival (Iglesia et al., 2017) of heterogeneous tumors (Lopes et al., 2015).

In addition, the HSP70 co-chaperone, heat shock protein 70-binding protein (HspBP), is usually found overexpressed in brain tumors and presents diverse cellular sub-localizations, including in the extracellular media when compared to normal tissue (Graner et al., 2009). Furthermore, HspBP interacts with several members of the HSP70 family-like glucose-regulated proteins 75 and 78 (GRP75 and GRP78, respectively) and Hsp110, among others, including cell surface receptors. However, in normal conditions, HspBP binds only Hsc70, GRP75, and HSP110 (Graner et al., 2009), demonstrating a different stress response in tumor conditions that includes its secretion. Notably, GRP78 was associated with ER stress in another mechanism broadly described in the literature, called the unfolded protein response (UPR) (Markouli et al., 2020).

The UPR consists of an adaptive response to ER stress usually caused by by the accumulation of unfolded proteins (Le Reste et al., 2016). This mechanism involves the inhibition of broad protein translation while increasing the translation of chaperones to enhance the folding capacity and the degradation of unfolded proteins to clear the ER (Mann and Hendershot, 2006). A single chaperone, GRP78, controls these processes. GRP78 acts through the release of its binding to three proteins: Activating Transcription Factor 6α (ATF6) (Haze et al., 1999), Inositol Requiring Enzyme 1 (IRE1α) (Tirasophon et al., 1998), and PKR-like endoplasmic reticulum kinase (PERK) (Harding et al., 1999). Once GRP78 dissociates from the binding proteins, it associates with the hydrophobic domains of unfolded proteins, leading to the phosphorylation of the primary binding proteins and consequent activation of signaling to mediate the stress response. Moreover, ATF6 modulates the transcription of genes related to protein folding and ERAD. IRE1α also modulates protein folding and ERAD, lipid synthesis and secretion, and PERK mediates amino acid metabolism, folding, autophagy processes, and apoptosis (Bertolotti et al., 2000; Acosta-Alvear et al., 2007; Yamamoto et al., 2007; Hetz et al., 2009; Scriven et al., 2009; Ye and Koumenis, 2009; Chevet et al., 2015; Dejeans et al., 2015). It is broadly discussed in the literature that tumors secrete specific cores of molecules to promote angiogenesis, proliferation, invasion, survival, and even reprogramming and EMT (Le Reste et al., 2016; Markouli et al., 2020). Since UPR mechanisms can remodel the cascade of activated signaling to respond to ER stress, it is natural to associate this process with the ER stress-mediated UPS.

Several studies associate ER stress and the central molecules of UPR modulation, ATF6, IRE1α, and PERK with brain tumor biology (Markouli et al., 2020). For example, ATF6 was associated with GBM resistance to radiotherapy (Dadey et al., 2016) and the formation of a pro-angiogenic GBM TME since it responds to VEGF secretion (Karali et al., 2014). ATF6 signaling was described as modulating NOTCH signaling in gliomas in hypoxia conditions, leading to radiotherapy resistance of GSCs (Dadey et al., 2016). In meningiomas, ATF6 expression levels were associated with tumor aggressiveness (Iglesias Gomez and Mosquera Orgueira, 2014). IRE1α was related to glioma growth, angiogenesis, and invasion (Drogat et al., 2007; Dejeans et al., 2012; Auf et al., 2013; Pluquet et al., 2013; Jabouille et al., 2015; Minchenko et al., 2020). Gliomas expressing low levels of IRE1α present impaired growth and angiogenesis ability and increased survival of glioma xenograft-bearing animals (Auf et al., 2010). IRE1α can also modulate the expression of hypoxia-related genes in GBM (Minchenko et al., 2016), hypoxia-induced cell death (Romero-Ramirez et al., 2004; Minchenko, et al., 2020), and the neuroinflammation associated with gliomas through the secretion of interleukins and activation of NF-κB (Hu et al., 2006; Auf et al., 2010). IRE1α activation in ER stress of gliomas caused by nutrient starvation or hypoxia leads to VEGF-mediated angiogenesis (Drogat et al., 2007), and IRE1α signaling activation was correlated with the increase of invasion markers expression and tumor infiltration by immune cells (Lhomond et al., 2018).

PERK is related to tumor metabolism and therapy resistance of GBM (Hamed et al., 2010; Yacoub et al., 2010; Hou et al., 2015). Indeed, gliomas do present high levels of glycolysis, also due to the hypoxia, which supports tumor growth, and this mechanism may be regulated by PERK and the activation of Akt signaling (Hou et al., 2015). The inhibition of upstream effectors of PERK sensitizes GSCs to radiotherapy and decreases recurrence (Yang et al., 2020). Furthermore, PERK modulates angiogenesis in GBM

in hypoxic conditions (Soni et al., 2020), and it is correlated with the stem-like cell phenotype through the modulation of SOX2 expression (Penaranda-Fajardo et al., 2019). In medulloblastomas, PERK activation is associated with cerebellar dysplasia (Lin et al., 2011), angiogenesis, cell migration (Jamison et al., 2015), and tumorigenesis (Ho et al., 2016).

Furthermore, GRP78 is highly expressed in gliomas, assisting tumor initiation and protection against cell damage and death mediated by reactive oxygen species (Suyama et al., 2014). In GBM, this protein is also overexpressed, especially in recurrent GBM, and correlates with tumor progression (Wen et al., 2020) and therapy resistance to TMZ (Pyrko et al., 2007; Lee et al., 2008) and radiation (Lee et al., 2008; Dadey et al., 2016). GRP78 expression is increased in endothelial cells derived from clinical gliomas as compared to endothelial cells from healthy tissues. Interestingly, these patient gliomas-derived endothelial cells are highly resistant to apoptosis, and GRP78 expression in these cells was recently associated with the resistance to chemotherapist agents (Virrey et al., 2008). The expression of GRP78 was evaluated in GBM treated with the UPR inducer TAK-243, a ubiquitin-activating enzyme 1 (UBA1) inhibitor, to inhibit tumor cell viability and, interestingly, the expression of GRP78 was related to the stem-like phenotype and increased sensitivity of these cells to the treatment (Liu et al., 2021). Another enzyme, the Ubiquitin-conjugating enzyme E2T (UBE2T), is correlated with tumor recurrence, highly expressed in GBM, and associated with poor prognosis, EMT regulation, and invasion of GBM cells through GRP78 (Huang et al., 2020a). Also, in recurrent GBM, it was demonstrated that overexpression of GRP78 in patient-derived samples correlated with poor survival and tumor progression (Dadey et al., 2016). Data from the literature demonstrated that a recurrent glioma sample that was subjected to the Stupp protocol, which consists of a combination of TMZ with fractionated radiation, presented a higher level of GRP78 compared to primary samples and was correlated to ER stress and therapy resistance (Shah et al., 2019). Regarding therapeutic possibilities using ER-stress as a target against brain tumors, the treatment with betulinic acid (BA) inhibited GBM primary and recurrent tumor cells growth through the activation of UPR by the PERK axis (Lo et al., 2020).

Indeed, therapeutic possibilities have been studied using ER stress as a target against brain tumors. For example, the use of ursodeoxycholic acid (UDCA) alone or associated with the proteasome inhibitor bortezomib (BTZ) leads to G1 cell cycle arrest and consequent decrease in cell viability by apoptosis in GBM, triggering ER stress through the ATF6-IRE1-PERK axis (Yao et al., 2020). Another example is the combination of TMZ with Fluoxetine (FLT), which activates ER stress through the ATF6-IRE1α-PERK cascade, causing an increase in early apoptosis levels and inhibition of cell proliferation in glioma (Ma et al., 2016). The combination treatment of TMZ and simvastatin (Simva) also effectively triggers UPR and leads to apoptosis. The use of inhibitors such as MKC8866 (IRE) and GSK-2606414 (PERKi) led to an impairment in the viability of GBM cells (Dastghaib et al., 2020; Le Reste et al., 2020). Additionally, the stimulation of UPR with 2-Deoxy-D-Glucose (2-DG) enhanced the radiotherapy effects in GSCs by increasing apoptosis (Shah et al., 2019).

CONCLUSION AND FUTURE PERSPECTIVES

Herein, we described the mechanisms of UPS and their participation in brain tumor maintenance. The UPS system is related to survival mechanisms since it allows the activation of alternative paths that promote the stress response and rapid turnover of cell behavior, either through the secretion of leaderless proteins or the fast release of proteins across the membrane, some bypassing the Golgi. On the other hand, the biology of cancer cells are remarkable, given that they present an outstanding ability to survive and proliferate in adverse environments. Some of these behaviors are sustained by substantial expression and secretion of factors related to stress response by those cells, as their microenvironment is enriched in and has a high activation of multiple signaling pathways (Rabouille, 2017; Dimou and Nickel, 2018) (**Supplementary Table S1**).

In this context, the UPS system can actively promote cancer survival and response to the TME, including the ability of the cells to resist therapy. Brain tumors are highly lethal and present several attributes that compromise treatment efficacy, such as the location of the tumor, the invasive capacity, therapy resistance, and quiescence ability. It is widely described in the literature that the role of the TME in the survival of brain tumors, and many secreted proteins, autocrine or paracrine, were correlated with key features related to the prognostic of patients with brain tumors (Quail and Joyce, 2017). Furthermore, the recent identification of UPS mechanisms and their study could bring together the significant correlation of non-canonical protein secretion with cancer cell survival and present a new field of study for therapy development. Indeed, the hypothesis of non-canonical pathways of secretion assisting tumor evasion override and overtake the options for inhibitors targeting classical secretion pathways. Nevertheless, very little is currently understood about the regulation of UPS in brain tumors, as this is a new and emerging research subject. A greater comprehension of the mechanisms underlying the processes involved in the activation and maintenance of UPS pathways is essential for developing new inhibitory drugs for the treatment of brain tumors and the advancement of cancer therapeutics.

AUTHOR CONTRIBUTIONS

RI, MP, and RA conceived the presented idea and proof outline, organized the table, wrote the manuscript; CF, AF, MS, JB, GC, SS, and JA wrote/edited manuscript, ME wrote the manuscript and designed the figures and S-YC, DT, AG, XS, JK, BH, ML, RI, MP, JB, CF, and RA reviewed and edited the manuscript.

FUNDING

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Processes numbers: RI: 2019/12710-9, 2020/03714-8; MP: 2017/26158-0; RA: 2020/04687-4; ME: 2019/11097-1; CF: 2019/14741-9, 2021/13114-0; MS: 2019/06971-4; 2021/13070-3; SS: 2020/05443-1; JA: 2021/05287-2; AF: 135731/2020-8; JMB: 2020/07450-5; ML: 2018/15557-4 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Processes numbers: JB: 101796/2020-0; ML: 409941/2021-2). DT. US NIH CA234799, S-YC. US NIH NS115403 and Lou and Jean Malnati Brain Tumor Institute at Northwestern Medicine.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.907423/full#supplementary-material

REFERENCES

Abdul Rahim, S. A., Dirkse, A., Oudin, A., Schuster, A., Bohler, J., Barthelemy, V., et al. (2017). Regulation of Hypoxia-Induced Autophagy in Glioblastoma Involves ATG9A. *Br. J. Cancer* 117 (6), 813–825. doi:10.1038/bjc.2017.263

Abuhusain, H. J., Matin, A., Qiao, Q., Shen, H., Kain, N., Day, B. W., et al. (2013). A Metabolic Shift Favoring Sphingosine 1-phosphate at the Expense of Ceramide Controls Glioblastoma Angiogenesis. *J. Biol. Chem.* 288, 37355–37364. doi:10.1074/jbc.m113.494740

Acosta-Alvear, D., Zhou, Y., Blais, A., Tsikitis, M., Lents, N. H., Arias, C., et al. (2007). XBP1 Controls Diverse Cell Type- and Condition-specific Transcriptional Regulatory Networks. *Mol. Cell* 27, 53–66. doi:10.1016/j.molcel.2007.06.011

Adada, M. M., Orr-Gandy, K. A., Snider, A. J., Canals, D., Hannun, Y. A., Obeid, L. M., et al. (2013). Sphingosine Kinase 1 Regulates Tumor Necrosis Factor-Mediated RANTES Induction through P38 Mitogen-Activated Protein Kinase but Independently of Nuclear Factor κB Activation. *J. Biol. Chem.* 288, 27667–27679. doi:10.1074/jbc.m113.489443

Aggarwal, S., Subberwal, M., Kumar, S., and Sharma, M. (2006). Brain Tumor and Role of Beta-Carotene, A-Tocopherol, Superoxide Dismutase and Glutathione Peroxidase. *J. Cancer Res. Ther.* 2, 24–27. doi:10.4103/0973-1482.19771

Akl, M. R., Nagpal, P., Ayoub, N. M., Tai, B., Prabhu, S. A., Capac, C. M., et al. (2016). Molecular and Clinical Significance of Fibroblast Growth Factor 2 (FGF2/bFGF) in Malignancies of Solid and Hematological Cancers for Personalized Therapies. *Oncotarget* 7, 44735–44762. doi:10.18632/oncotarget.8203

Aliyu, I. A., Ling, K. H., Md Hashim, N., and Chee, H. Y. (2019). Annexin A2 Extracellular Translocation and Virus Interaction: A Potential Target for Antivirus-drug Discovery. *Rev. Med. Virol.* 29, e2038. doi:10.1002/rmv.2038

Alvarez, S. E., Harikumar, K. B., Hait, N. C., Allegood, J., Strub, G. M., Kim, E. Y., et al. (2010). Sphingosine-1-phosphate Is a Missing Cofactor for the E3 Ubiquitin Ligase TRAF2. *Nature* 465, 1084–1088. doi:10.1038/nature09128

Andrei, C., Dazzi, C., Lotti, L., Torrisi, M. R., Chimini, G., and Rubartelli, A. (1999). The Secretory Route of the Leaderless Protein Interleukin 1β Involves Exocytosis of Endolysosome-Related Vesicles. *MBoC* 10, 1463–1475. doi:10.1091/mbc.10.5.1463

Auf, G., Jabouille, A., Delugin, M., Guérit, S., Pineau, R., North, S., et al. (2013). High Epiregulin Expression in Human U87 Glioma Cells Relies on IRE1α and Promotes Autocrine Growth through EGF Receptor. *BMC Cancer* 13, 597. doi:10.1186/1471-2407-13-597

Auf, G., Jabouille, A., Guérit, S., Pineau, R., Delugin, M., Bouchecareilh, M., et al. (2010). Inositol-requiring Enzyme 1α Is a Key Regulator of Angiogenesis and Invasion in Malignant Glioma. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15553–15558. doi:10.1073/pnas.0914072107

Baldwin, T. A., and Ostergaard, H. L. (2002). The Protein-Tyrosine Phosphatase CD45 Reaches the Cell Surface via Golgi-dependent and -independent Pathways. *J. Biol. Chem.* 277, 50333–50340. doi:10.1074/jbc.m209075200

Barr, F. A., Puype, M., Vandekerckhove, J., and Warren, G. (1997). GRASP65, a Protein Involved in the Stacking of Golgi Cisternae. *Cell* 91, 253–262. doi:10.1016/s0092-8674(00)80407-9

Bendtsen, J. D., Jensen, L. J., Blom, N., Von Heijne, G., and Brunak, S. (2004). Feature-based Prediction of Non-classical and Leaderless Protein Secretion. *Protein Eng. Des. Sel.* 17, 349–356. doi:10.1093/protein/gzh037

Bergfeld, R., Kühnl, T., and Schopfer, P. (1980). Formation of Protein Storage Bodies during Embryogenesis in Cotyledons of Sinapis Alba L. *Planta* 148, 146–156. doi:10.1007/bf00386415

Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., and Ron, D. (2000). Dynamic Interaction of BiP and ER Stress Transducers in the Unfolded-Protein Response. *Nat. Cell Biol.* 2, 326–332. doi:10.1038/35014014

Bhat, K. P. L., Salazar, K. L., Balasubramaniyan, V., Wani, K., Heathcock, L., Hollingsworth, F., et al. (2011). The Transcriptional Coactivator TAZ Regulates Mesenchymal Differentiation in Malignant Glioma. *Genes Dev.* 25, 2594–2609. doi:10.1101/gad.176800.111

Bian, X. W., Du, L. L., Shi, J. Q., Cheng, Y. S., and Liu, F. X. (2000). Correlation of bFGF, FGFR-1 and VEGF Expression with Vascularity and Malignancy of Human Astrocytomas. *Anal. Quant. Cytol. Histol.* 22, 267–274.

Brockmann, M.-A., Ulbricht, U., Grüner, K., Fillbrandt, R., Westphal, M., and Lamszus, K. (2003). Glioblastoma and Cerebral Microvascular Endothelial Cell Migration in Response to Tumor-Associated Growth Factors. *Neurosurgery* 52, 1391–1399. doi:10.1227/01.neu.0000064806.87785.ab

Broekman, M. L., Maas, S. L. N., Abels, E. R., Mempel, T. R., Krichevsky, A. M., and Breakefield, X. O. (2018). Multidimensional Communication in the Microenvirons of Glioblastoma. *Nat. Rev. Neurol.* 14, 482–495. doi:10.1038/s41582-018-0025-8

Bronisz, A., Wang, Y., Nowicki, M. O., Peruzzi, P., Ansari, K. I., Ogawa, D., et al. (2014). Extracellular Vesicles Modulate the Glioblastoma Microenvironment via a Tumor Suppression Signaling Network Directed by miR-1. *Cancer Res.* 74, 738–750. doi:10.1158/0008-5472.can-13-2650

Brough, D., Le Feuvre, R. A., Wheeler, R. D., Solovyova, N., Hilfiker, S., Rothwell, N. J., et al. (2003). Ca2+Stores and Ca2+Entry Differentially Contribute to the Release of IL-1β and IL-1α from Murine Macrophages. *J. Immunol.* 170, 3029–3036. doi:10.4049/jimmunol.170.6.3029

Bruns, C., McCaffery, J. M., Curwin, A. J., Duran, J. M., and Malhotra, V. (2011). Biogenesis of a Novel Compartment for Autophagosome-Mediated Unconventional Protein Secretion. *J. Cell Biol.* 195, 979–992. doi:10.1083/jcb.201106098

Bulloj, A., Leal, M. C., Xu, H., Castaño, E. M., and Morelli, L. (2010). Insulin-Degrading Enzyme Sorting in Exosomes: A Secretory Pathway for a Key Brain Amyloid-β Degrading Protease. *Jad* 19, 79–95. doi:10.3233/jad-2010-1206

Cacchione, A., Lodi, M., Carai, A., Miele, E., Tartaglia, M., Megaro, G., et al. (2020). Upfront Treatment with mTOR Inhibitor Everolimus in Pediatric Low-Grade Gliomas: A Single-Center Experience. *Int. J. Cancer* 2020. doi:10.1002/ijc.33438

Camby, I., Nagy, N., Lopes, M. B., Schäfer, B. W., Maurage, C. A., Ruchoux, M. M., et al. (1999). Supratentorial Pilocytic Astrocytomas, Astrocytomas, Anaplastic Astrocytomas and Glioblastomas Are Characterized by a Differential Expression of S100 Proteins. *Brain Pathol.* 9, 1–19. doi:10.1111/j.1750-3639.1999.tb00205.x

Cataltepe, O., Arikan, M. C., Ghelfi, E., Karaaslan, C., Ozsurekci, Y., Dresser, K., et al. (2012). Fatty Acid Binding Protein 4 Is Expressed in Distinct Endothelial and Non-endothelial Cell Populations in Glioblastoma. *Neuropathol. Appl. Neurobiol.* 38, 400–410. doi:10.1111/j.1365-2990.2011.01237.x

Cavalli, G., and Cenci, S. (2020). Autophagy and Protein Secretion. *J. Mol. Biol.* 432, 2525–2545. doi:10.1016/j.jmb.2020.01.015

Chang, K.-Y., Hsu, T.-I., Hsu, C.-C., Tsai, S.-Y., Liu, J.-J., Chou, S.-W., et al. (2017). Specificity Protein 1-modulated Superoxide Dismutase 2 Enhances Temozolomide Resistance in Glioblastoma, Which Is Independent of O6-

Methylguanine-DNA Methyltransferase. *Redox Biol.* 13, 655–664. doi:10.1016/j.redox.2017.08.005

Chevet, E., Hetz, C., and Samali, A. (2015). Endoplasmic Reticulum Stress-Activated Cell Reprogramming in Oncogenesis. *Cancer Discov.* 5, 586–597. doi:10.1158/2159-8290.cd-14-1490

Chien, C.-H., Chuang, J.-Y., Yang, S.-T., Yang, W.-B., Chen, P.-Y., Hsu, T.-I., et al. (2019). Enrichment of Superoxide Dismutase 2 in Glioblastoma Confers to Acquisition of Temozolomide Resistance that Is Associated with Tumor-Initiating Cell Subsets. *J. Biomed. Sci.* 26, 77. doi:10.1186/s12929-019-0565-2

Cockbill, L. M. R., Murk, K., Love, S., and Hanley, J. G. (2015). Protein Interacting with C Kinase 1 Suppresses Invasion and Anchorage-independent Growth of Astrocytic Tumor Cells. *MBoC* 26, 4552–4561. doi:10.1091/mbc.e15-05-0270

Cohen, M. J., Chirico, W. J., and Lipke, P. N. (2020). Through the Back Door: Unconventional Protein Secretion. *Cell Surf.* 6, 100045. doi:10.1016/j.tcsw.2020.100045

Cruz, L., Arevalo Romero, J. A., Brandão Prado, M., Santos, T. G., and Hohmuth Lopes, M. (2018). Evidence of Extracellular Vesicles Biogenesis and Release in Mouse Embryonic Stem Cells. *Stem Cell Rev Rep* 14 (2), 262–276. doi:10.1007/s12015-017-9776-7

Cruz-Garcia, D., Brouwers, N., Duran, J. M., Mora, G., Curwin, A. J., and Malhotra, V. (2017). A Diacidic Motif Determines Unconventional Secretion of Wild-type and ALS-Linked Mutant SOD1. *J. Cell Biol.* 216, 2691–2700. doi:10.1083/jcb.201704056

Cruz-Garcia, D., Curwin, A. J., Popoff, J.-F., Bruns, C., Duran, J. M., and Malhotra, V. (2014). Remodeling of Secretory Compartments Creates CUPS during Nutrient Starvation. *J. Cell Biol.* 207, 695–703. doi:10.1083/jcb.201407119

Cruz-Garcia, D., Malhotra, V., and Curwin, A. J. (2018). Unconventional Protein Secretion Triggered by Nutrient Starvation. *Seminars Cell & Dev. Biol.* 83, 22–28. doi:10.1016/j.semcdb.2018.02.021

Curwin, A. J., Brouwers, N., Alonso Y Adell, M., Teis, D., Turacchio, G., Parashuraman, S., et al. (2016). ESCRT-III Drives the Final Stages of CUPS Maturation for Unconventional Protein Secretion. *Elife* 5, e16299. doi:10.7554/eLife.16299

D'Angelo, G., Prencipe, L., Iodice, L., Beznoussenko, G., Savarese, M., Marra, P., et al. (2009). GRASP65 and GRASP55 Sequentially Promote the Transport of C-Terminal Valine-Bearing Cargos to and through the Golgi Complex. *J. Biol. Chem.* 284, 34849–34860. doi:10.1074/jbc.m109.068403

Dadey, D. Y. A., Kapoor, V., Khudanyan, A., Urano, F., Kim, A. H., Thotala, D., et al. (2016). The ATF6 Pathway of the ER Stress Response Contributes to Enhanced Viability in Glioblastoma. *Oncotarget* 7, 2080–2092. doi:10.18632/oncotarget.6712

Daniel, D. C., Kinoshita, Y., Khan, M. A., Valle, L. D., Khalili, K., Rappaport, J., et al. (2004). Internalization of Exogenous Human Immunodeficiency Virus-1 Protein, Tat, by KG-1 Oligodendroglioma Cells Followed by Stimulation of DNA Replication Initiated at the JC Virus Origin. *DNA Cell Biol.* 23, 858–867. doi:10.1089/dna.2004.23.858

Dascher, C., Matteson, J., and Balch, W. E. (1994). Syntaxin 5 Regulates Endoplasmic Reticulum to Golgi Transport. *J. Biol. Chem.* 269 (47), 29363–29366. doi:10.1016/s0021-9258(18)43884-7

Dastghaib, S., Shojaei, S., Mostafavi-Pour, Z., Sharma, P., Patterson, J. B., Samali, A., et al. (2020). Simvastatin Induces Unfolded Protein Response and Enhances Temozolomide-Induced Cell Death in Glioblastoma Cells. *Cells* 9, 2339. doi:10.3390/cells9112339

Davis, D. J., Kang, B.-H., Heringer, A. S., Wilkop, T. E., and Drakakaki, G. (2016). Unconventional Protein Secretion in Plants. *Methods Mol. Biol.* 1459, 47–63. doi:10.1007/978-1-4939-3804-9_3

De Marco, A., Dans, P. D., Knezevich, A., Maiuri, P., Pantano, S., and Marcello, A. (2010). Subcellular Localization of the Interaction between the Human Immunodeficiency Virus Transactivator Tat and the Nucleosome Assembly Protein 1. *Amino Acids* 38, 1583–1593. doi:10.1007/s00726-009-0378-9

Dejeans, N., Pluquet, O., Lhomond, S., Grise, F., Bouchecareilh, M., Juin, A., et al. (2012). Autocrine Control of Glioma Cells Adhesion and Migration through IRE1α-Mediated Cleavage of SPARC mRNA. *J. Cell Sci.* 125, 4278–4287. doi:10.1242/jcs.099291

Dejeans, N., Barroso, K., Fernandez-Zapico, M. E., Samali, A., and Chevet, E. (2015). Novel Roles of the Unfolded Protein Response in the Control of Tumor Development and Aggressiveness. *Seminars Cancer Biol.* 33, 67–73. doi:10.1016/j.semcancer.2015.04.007

Deretic, V., Jiang, S., and Dupont, N. (2012). Autophagy Intersections with Conventional and Unconventional Secretion in Tissue Development, Remodeling and Inflammation. *Trends Cell Biol.* 22, 397–406. doi:10.1016/j.tcb.2012.04.008

di Giovine, F. S., Symons, J. A., and Duff, G. W. (1991). Kinetics of IL1 Beta mRNA and Protein Accumulation in Human Mononuclear Cells. *Immunol. Lett.* 29, 211–218. doi:10.1016/0165-2478(91)90172-7

Di Serio, C., Doria, L., Pellerito, S., Prudovsky, I., Micucci, I., Massi, D., et al. (2008). The Release of Fibroblast Growth Factor-1 from Melanoma Cells Requires Copper Ions and Is Mediated by Phosphatidylinositol 3-kinase/Akt Intracellular Signaling Pathway. *Cancer Lett.* 267, 67–74. doi:10.1016/j.canlet.2008.03.001

Dimou, E., and Nickel, W. (2018). Unconventional Mechanisms of Eukaryotic Protein Secretion. *Curr. Biol.* 28, R406–R410. doi:10.1016/j.cub.2017.11.074

Dimou, S., Martzoukou, O., Dionysopoulou, M., Bouris, V., Amillis, S., and Diallinas, G. (2020). Translocation of Nutrient Transporters to Cell Membrane via Golgi Bypass in Aspergillus nidulans. *EMBO Rep.* 21, e49929. doi:10.15252/embr.201949929

Drogat, B., Auguste, P., Nguyen, D. T., Bouchecareilh, M., Pineau, R., Nalbantoglu, J., et al. (2007). IRE1 Signaling Is Essential for Ischemia-Induced Vascular Endothelial Growth Factor-A Expression and Contributes to Angiogenesis and Tumor Growth *In Vivo*. *Cancer Res.* 67, 6700–6707. doi:10.1158/0008-5472.can-06-3235

Du, P., Liao, Y., Zhao, H., Zhang, J., Muyiti, Keremu., Keremu, K., et al. (2020). ANXA2P2/miR-9/LDHA axis Regulates Warburg Effect and Affects Glioblastoma Proliferation and Apoptosis. *Cell. Signal.* 74, 109718. doi:10.1016/j.cellsig.2020.109718

Dupont, N., Jiang, S., Pilli, M., Ornatowski, W., Bhattacharya, D., and Deretic, V. (2011). Autophagy-based Unconventional Secretory Pathway for Extracellular Delivery of IL-1β. *EMBO J.* 30, 4701–4711. doi:10.1038/emboj.2011.398

Duran, J. M., Anjard, C., Stefan, C., Loomis, W. F., and Malhotra, V. (2010). Unconventional Secretion of Acb1 Is Mediated by Autophagosomes. *J. Cell Biol.* 188, 527–536. doi:10.1083/jcb.200911154

El-Sehemy, A., Selvadurai, H., Ortin-Martinez, A., Pokrajac, N., Mamatjan, Y., Tachibana, N., et al. (2020). Norrin Mediates Tumor-Promoting and -suppressive Effects in Glioblastoma via Notch and Wnt. *J. Clin. Invest* 130, 3069–3086. doi:10.1172/jci128994

Elborn, J. S. (2016). Cystic Fibrosis. *Lancet* 388, 2519–2531. doi:10.1016/s0140-6736(16)00576-6

Elsherbiny, M. E., Emara, M., and Godbout, R. (2013). Interaction of Brain Fatty Acid-Binding Protein with the Polyunsaturated Fatty Acid Environment as a Potential Determinant of Poor Prognosis in Malignant Glioma. *Prog. Lipid Res.* 52, 562–570. doi:10.1016/j.plipres.2013.08.004

Estrada-Bernal, A., Lawler, S. E., Nowicki, M. O., Ray Chaudhury, A., and Van Brocklyn, J. R. (2011). The Role of Sphingosine Kinase-1 in EGFRvIII-Regulated Growth and Survival of Glioblastoma Cells. *J. Neurooncol* 102, 353–366. doi:10.1007/s11060-010-0345-z

Fernández-Chacón, R., Königstorfer, A., Gerber, S. H., García, J., Matos, M. F., Stevens, C. F., et al. (2001). Synaptotagmin I Functions as a Calcium Regulator of Release Probability. *Nature* 410, 41–49. doi:10.1038/35065004

Ferro-Novick, S., and Brose, N. (2013). Traffic Control System within Cells. *Nature* 504, 98. doi:10.1038/504098a

Fontaine, S. N., Zheng, D., Sabbagh, J. J., Martin, M. D., Chaput, D., Darling, A., et al. (2016). DnaJ/Hsc70 Chaperone Complexes Control the Extracellular Release of Neurodegenerative-associated Proteins. *EMBO J.* 35, 1537–1549. doi:10.15252/embj.201593489

Frappaolo, A., Karimpour-Ghahnavieh, A., Sechi, S., and Giansanti, M. G. (2020). The Close Relationship between the Golgi Trafficking Machinery and Protein Glycosylation. *Cells* 9, 2652. doi:10.3390/cells9122652

Fu, B., Long, W., Zhang, Y., Zhang, A., Miao, F., Shen, Y., et al. (2015). Enhanced Antitumor Effects of the BRBP1 Compound Peptide BRBP1-TAT-KLA on Human Brain Metastatic Breast Cancer. *Sci. Rep.* 5, 8029. doi:10.1038/srep08029

Fujikawa, K., Seko, A., Takeda, Y., and Ito, Y. (2016). Approaches toward High-mannose-type Glycan Libraries. *Chem. Rec.* 16, 35–46. doi:10.1002/tcr.201500222

Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., and Ikehara, Y. (1988). Brefeldin A Causes Disassembly of the Golgi Complex and Accumulation of Secretory Proteins in the Endoplasmic Reticulum. *J. Biol. Chem.* 263, 18545–18552. doi:10.1016/s0021-9258(19)81393-5

Gee, H. Y., Noh, S. H., Tang, B. L., Kim, K. H., and Lee, M. G. (2011). Rescue of ΔF508-CFTR Trafficking via a GRASP-dependent Unconventional Secretion Pathway. *Cell* 146, 746–760. doi:10.1016/j.cell.2011.07.021

Giuliani, F., Grieve, A., and Rabouille, C. (2011). Unconventional Secretion: a Stress on GRASP. *Curr. Opin. Cell Biol.* 23, 498–504. doi:10.1016/j.ceb.2011.04.005

Goenka, A., Tiek, D., Song, X., Huang, T., Hu, B., and Cheng, S. Y. (2021). The Many Facets of Therapy Resistance and Tumor Recurrence in Glioblastoma. *Cells* 10, 5794. doi:10.3390/cells10030484

González, C., Cornejo, V. H., and Couve, A. (2018). Golgi Bypass for Local Delivery of Axonal Proteins, Fact or Fiction? *Curr. Opin. Cell Biol.* 53, 9–14. doi:10.1016/j.ceb.2018.03.010

Goodwin, J. M., Dowdle, W. E., DeJesus, R., Wang, Z., Bergman, P., Kobylarz, M., et al. (2017). Autophagy-Independent Lysosomal Targeting Regulated by ULK1/2-FIP200 and ATG9. *Cell Rep.* 20, 2341–2356. doi:10.1016/j.celrep.2017.08.034

Graner, M. W., Raynes, D. A., Bigner, D. D., and Guerriero, V. (2009). Heat Shock Protein 70-binding Protein 1 Is Highly Expressed in High-Grade Gliomas, Interacts with Multiple Heat Shock Protein 70 Family Members, and Specifically Binds Brain Tumor Cell Surfaces. *Cancer Sci.* 100, 1870–1879. doi:10.1111/j.1349-7006.2009.01269.x

Greten, F. R., and Grivennikov, S. I. (2019). Inflammation and Cancer: Triggers, Mechanisms, and Consequences. *Immunity* 51, 27–41. doi:10.1016/j.immuni.2019.06.025

Grieve, A. G., and Rabouille, C. (2011). Golgi Bypass: Skirting Around the Heart of Classical Secretion. *Cold Spring Harb. Perspect. Biol.* 3. doi:10.1101/cshperspect.a005298

Hajj, G. N. M., Arantes, C. P., Dias, M. V. S., Roffé, M., Costa-Silva, B., Lopes, M. H., et al. (2013). The Unconventional Secretion of Stress-Inducible Protein 1 by a Heterogeneous Population of Extracellular Vesicles. *Cell. Mol. Life Sci.* 70 (17), 3211–3227. doi:10.1007/s00018-013-1328-y

Hamed, H. A., Yacoub, A., Park, M. A., Eulitt, P. J., Sarkar, D., Dimitrie, I. P., et al. (2010). OSU-03012 Enhances Ad.7-induced GBM Cell Killing via ER Stress and Autophagy and by Decreasing Expression of Mitochondrial Protective Proteins. *Cancer Biol. Ther.* 9, 526–536. doi:10.4161/cbt.9.7.11116

Harding, H. P., Zhang, Y., and Ron, D. (1999). Protein Translation and Folding Are Coupled by an Endoplasmic-Reticulum-Resident Kinase. *Nature* 397, 271–274. doi:10.1038/16729

Haugaard-Kedström, L. M., Clemmensen, L. S., Sereikaite, V., Jin, Z., Fernandes, E. F. A., Wind, B., et al. (2021). A High-Affinity Peptide Ligand Targeting Syntenin Inhibits Glioblastoma. *J. Med. Chem.* 64, 1423–1434. doi:10.1021/acs.jmedchem.0c00382

Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999). Mammalian Transcription Factor ATF6 Is Synthesized as a Transmembrane Protein and Activated by Proteolysis in Response to Endoplasmic Reticulum Stress. *MBoC* 10, 3787–3799. doi:10.1091/mbc.10.11.3787

He, X. X., Du, S., Gao, S. Q., Chen, J. Y., Cao, R. J., Xing, Z. K., et al. (2018). Humanization of Fibroblast Growth Factor 1 Single-chain Antibody and Validation for its Antitumorigenic Efficacy in Breast Cancer and Glioma Cells. *J. Cell. Mol. Medi* 22, 3259–3263. doi:10.1111/jcmm.13547

Hebert, D. N., and Molinari, M. (2007). In and Out of the ER: Protein Folding, Quality Control, Degradation, and Related Human Diseases. *Physiol. Rev.* 87, 1377–1408. doi:10.1152/physrev.00050.2006

Hetz, C., Thielen, P., Matus, S., Nassif, M., Court, F., Kiffin, R., et al. (2009). XBP-1 Deficiency in the Nervous System Protects against Amyotrophic Lateral Sclerosis by Increasing Autophagy. *Genes Dev.* 23, 2294–2306. doi:10.1101/gad.1830709

Hiller, H., Yang, C., Beachy, D. E., Kusmartseva, I., Candelario-Jalil, E., Posgai, A. L., et al. (2021). Altered Cellular Localisation and Expression, Together with Unconventional Protein Trafficking, of Prion Protein, PrPC, in Type 1 Diabetes. *Diabetologia* 64, 2279–2291. doi:10.1007/s00125-021-05501-8

Ho, Y., Li, X., Jamison, S., Harding, H. P., McKinnon, P. J., Ron, D., et al. (2016). PERK Activation Promotes Medulloblastoma Tumorigenesis by Attenuating Premalignant Granule Cell Precursor Apoptosis. *Am. J. Pathology* 186, 1939–1951. doi:10.1016/j.ajpath.2016.03.004

Hou, X., Liu, Y., Liu, H., Chen, X., Liu, M., Che, H., et al. (2015). PERK Silence Inhibits Glioma Cell Growth under Low Glucose Stress by Blockage of P-AKT and Subsequent HK2's Mitochondria Translocation. *Sci. Rep.* 5, 9065. doi:10.1038/srep09065

Hu, P., Han, Z., Couvillon, A. D., Kaufman, R. J., and Exton, J. H. (2006). Autocrine Tumor Necrosis Factor Alpha Links Endoplasmic Reticulum Stress to the Membrane Death Receptor Pathway through IRE1α-Mediated NF-κB Activation and Down-Regulation of TRAF2 Expression. *Mol. Cell Biol.* 26, 3071–3084. doi:10.1128/mcb.26.8.3071-3084.2006

Huang, P., Guo, Y.-D., and Zhang, H.-W. (2020b). Identification of Hub Genes in Pediatric Medulloblastoma by Multiple-Microarray Analysis. *J. Mol. Neurosci.* 70, 522–531. doi:10.1007/s12031-019-01451-4

Huang, P., Guo, Y., Zhao, Z., Ning, W., Wang, H., Gu, C., et al. (2020a). UBE2T Promotes Glioblastoma Invasion and Migration via Stabilizing GRP78 and Regulating EMT. *Aging* 12, 10275–10289. doi:10.18632/aging.103239

Huang, T., Kim, C. K., Alvarez, A. A., Pangeni, R. P., Wan, X., Song, X., et al. (2017). MST4 Phosphorylation of ATG4B Regulates Autophagic Activity, Tumorigenicity, and Radioresistance in Glioblastoma. *Cancer Cell* 32, 840–855. doi:10.1016/j.ccell.2017.11.005

Hughes, R. (1999). Secretion of the Galectin Family of Mammalian Carbohydrate-Binding Proteins. *Biochim. Biophys. Acta* 1473, 172–185. doi:10.1016/s0304-4165(99)00177-4

Iglesia, R. P., Fernandes, C. F. L., Coelho, B. P., Prado, M. B., Melo Escobar, M. I., Almeida, G. H. D. R., et al. (2019). Heat Shock Proteins in Glioblastoma Biology: Where Do We Stand? *Int. J. Mol. Sci.* 20, 5794. doi:10.3390/ijms20225794

Iglesia, R. P., Prado, M. B., Cruz, L., Martins, V. R., Santos, T. G., and Lopes, M. H. (2017). Engagement of Cellular Prion Protein with the Co-chaperone Hsp70/90 Organizing Protein Regulates the Proliferation of Glioblastoma Stem-like Cells. *Stem Cell Res. Ther.* 8, 76. doi:10.1186/s13287-017-0518-1

Iglesias Gómez, J. C., and Mosquera Orgueira, A. (2014). An Integrative Analysis of Meningioma Tumors Reveals the Determinant Genes and Pathways of Malignant Transformation. *Front. Oncol.* 4, 147. doi:10.3389/fonc.2014.00147

Ito, Y., and Takeda, Y. (2012). Analysis of Glycoprotein Processing in the Endoplasmic Reticulum Using Synthetic Oligosaccharides. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* 88, 31–40. doi:10.2183/pjab.88.31

Jabouille, A., Delugin, M., Pineau, R., Dubrac, A., Soulet, F., Lhomond, S., et al. (2015). Glioblastoma Invasion and Cooption Depend on IRE1α Endoribonuclease Activity. *Oncotarget* 6, 24922–24934. doi:10.18632/oncotarget.4679

Jamison, S., Lin, Y., and Lin, W. (2015). Pancreatic Endoplasmic Reticulum Kinase Activation Promotes Medulloblastoma Cell Migration and Invasion through Induction of Vascular Endothelial Growth Factor A. *PLoS One* 10, e0120252. doi:10.1371/journal.pone.0120252

Jaye, M., Howk, R., Burgess, W., Ricca, G. A., Chiu, I.-M., Ravera, M. W., et al. (1986). Human Endothelial Cell Growth Factor: Cloning, Nucleotide Sequence, and Chromosome Localization. *Science* 233, 541–545. doi:10.1126/science.3523756

Jung, J., Kim, J., Roh, S. H., Jun, I., Sampson, R. D., Gee, H. Y., et al. (2016). The HSP70 Co-chaperone DNAJC14 Targets Misfolded Pendrin for Unconventional Protein Secretion. *Nat. Commun.* 7, 11386. doi:10.1038/ncomms11386

Kapitonov, D., Allegood, J. C., Mitchell, C., Hait, N. C., Almenara, J. A., Adams, J. K., et al. (2009). Targeting Sphingosine Kinase 1 Inhibits Akt Signaling, Induces Apoptosis, and Suppresses Growth of Human Glioblastoma Cells and Xenografts. *Cancer Res.* 69, 6915–6923. doi:10.1158/0008-5472.can-09-0664

Karali, E., Bellou, S., Stellas, D., Klinakis, A., Murphy, C., and Fotsis, T. (2014). VEGF Signals through ATF6 and PERK to Promote Endothelial Cell Survival and Angiogenesis in the Absence of ER Stress. *Mol. Cell* 54, 559–572. doi:10.1016/j.molcel.2014.03.022

Kayagaki, N., Stowe, I. B., Lee, B. L., O'Rourke, K., Anderson, K., Warming, S., et al. (2015). Caspase-11 Cleaves Gasdermin D for Non-canonical Inflammasome Signalling. *Nature* 526, 666–671. doi:10.1038/nature15541

Kegelman, T. P., Das, S. K., Hu, B., Bacolod, M. D., Fuller, C. E., Menezes, M. E., et al. (2014). MDA-9/syntenin Is a Key Regulator of Glioma Pathogenesis. *Neuro Oncol.* 16, 50–61. doi:10.1093/neuonc/not157

Kegelman, T. P., Wu, B., Das, S. K., Talukdar, S., Beckta, J. M., Hu, B., et al. (2017). Inhibition of Radiation-Induced Glioblastoma Invasion by Genetic and Pharmacological Targeting of MDA-9/Syntenin. *Proc. Natl. Acad. Sci. U.S.A.* 114, 370–375. doi:10.1073/pnas.1616100114

Kim, J., Noh, S. H., Piao, H., Kim, D. H., Kim, K., Cha, J. S., et al. (2016). Monomerization and ER Relocalization of GRASP Is a Requisite for Unconventional Secretion of CFTR. *Traffic* 17, 733–753. doi:10.1111/tra.12403

Kim, S.-Y. (2006). Transglutaminase 2 in Inflammation. *Front. Biosci.* 11, 3026–3035. doi:10.2741/2030

Kimura, T., Jia, J., Kumar, S., Choi, S. W., Gu, Y., Mudd, M., et al. (2017). Dedicated SNARE S and Specialized TRIM Cargo Receptors Mediate Secretory Autophagy. *EMBO J.* 36, 42–60. doi:10.15252/embj.201695081

Kizawa, K., Takahara, H., Troxler, H., Kleinert, P., Mochida, U., and Heizmann, C. W. (2008). Specific Citrullination Causes Assembly of a Globular S100A3 Homotetramer. *J. Biol. Chem.* 283, 5004–5013. doi:10.1074/jbc.m709357200

Kling, T., Ferrarese, R., Ó hAilín, D., Johansson, P., Heiland, D. H., Dai, F., et al. (2016). Integrative Modeling Reveals Annexin A2-Mediated Epigenetic Control of Mesenchymal Glioblastoma. *EBioMedicine* 12, 72–85. doi:10.1016/j.ebiom.2016.08.050

Kucharzewska, P., Christianson, H. C., Welch, J. E., Svensson, K. J., Fredlund, E., Ringnér, M., et al. (2013). Exosomes Reflect the Hypoxic Status of Glioma Cells and Mediate Hypoxia-dependent Activation of Vascular Cells during Tumor Development. *Proc. Natl. Acad. Sci. U.S.A.* 110, 7312–7317. doi:10.1073/pnas.1220998110

Kulkarni-Gosavi, P., Makhoul, C., and Gleeson, P. A. (2019). Form and Function of the Golgi Apparatus: Scaffolds, Cytoskeleton and Signalling. *FEBS Lett.* 593, 2289–2305. doi:10.1002/1873-3468.13567

Langhans, M., Hawes, C., Hillmer, S., Hummel, E., and Robinson, D. G. (2007). Golgi Regeneration after Brefeldin A Treatment in BY-2 Cells Entails Stack Enlargement and Cisternal Growth Followed by Division. *Plant Physiol.* 145 (2), 527–538. doi:10.1104/pp.107.104919

Le Reste, P.-J., Avril, T., Quillien, V., Morandi, X., and Chevet, E. (2016). Signaling the Unfolded Protein Response in Primary Brain Cancers. *Brain Res.* 1642, 59–69. doi:10.1016/j.brainres.2016.03.015

Le Reste, P. J., Pineau, R., Voutetakis, K., Samal, J., Jégou, G., Lhomond, S., et al. (2020). Local Intracerebral Inhibition of IRE1 by MKC8866 Sensitizes Glioblastoma to Irradiation/chemotherapy *In Vivo*. *Cancer Lett.* 494, 73–83. doi:10.1016/j.canlet.2020.08.028

Lee, H. K., Xiang, C., Cazacu, S., Finniss, S., Kazimirsky, G., Lemke, N., et al. (2008). GRP78 Is Overexpressed in Glioblastomas and Regulates Glioma Cell Growth and Apoptosis. *Neuro Oncol.* 10, 236–243. doi:10.1215/15228517-2008-006

Lee, J.-G., Takahama, S., Zhang, G., Tomarev, S. I., and Ye, Y. (2016). Unconventional Secretion of Misfolded Proteins Promotes Adaptation to Proteasome Dysfunction in Mammalian Cells. *Nat. Cell Biol.* 18, 765–776. doi:10.1038/ncb3372

Leinartaite, L., and Johansson, A. S. (2013). Disulfide Scrambling in Superoxide Dismutase 1 Reduces its Cytotoxic Effect in Cultured Cells and Promotes Protein Aggregation. *PLoS One* 8, e78060. doi:10.1371/journal.pone.0078060

Levi, S. K., Bhattacharyya, D., Strack, R. L., Austin, J. R., 2nd, and Glick, B. S. (2010). The Yeast GRASP Grh1 Colocalizes with COPII and Is Dispensable for Organizing the Secretory Pathway. *Traffic* 11, 1168–1179. doi:10.1111/j.1600-0854.2010.01089.x

Lhomond, S., Avril, T., Dejeans, N., Voutetakis, K., Doultsinos, D., McMahon, M., et al. (2018). Dual IRE1 RNase Functions Dictate Glioblastoma Development. *EMBO Mol. Med.* 10. doi:10.15252/emmm.201707929

Li, H. Y., Lv, B. B., and Bi, Y. H. (2018). FABP4 Accelerates Glioblastoma Cell Growth and Metastasis through Wnt10b Signalling. *Eur. Rev. Med. Pharmacol. Sci.* 22, 7807–7818. doi:10.26355/eurrev_201811_16405

Li, W., Dong, S., Wei, W., Wang, G., Zhang, A., Pu, P., et al. (2016). The Role of Transcriptional Coactivator TAZ in Gliomas. *Oncotarget* 7, 82686–82699. doi:10.18632/oncotarget.12625

Li, Z., Fu, W.-J., Chen, X.-Q., Wang, S., Deng, R.-S., Tang, X.-P., et al. (2022). Autophagy-based Unconventional Secretion of HMGB1 in Glioblastoma Promotes Chemosensitivity to Temozolomide through Macrophage M1-like Polarization. *J. Exp. Clin. Cancer Res.* 41 (1), 74. doi:10.1186/s13046-022-02291-8

Lima, S., Milstien, S., and Spiegel, S. (2017). Sphingosine and Sphingosine Kinase 1 Involvement in Endocytic Membrane Trafficking. *J. Biol. Chem.* 292, 3074–3088. doi:10.1074/jbc.m116.762377

Lin, W., Lin, Y., Li, J., Harding, H. P., Ron, D., and Jamison, S. (2011). A Deregulated Integrated Stress Response Promotes Interferon-γ-Induced Medulloblastoma. *J. Neurosci. Res.* 89, 1586–1595. doi:10.1002/jnr.22693

Liu, G., Yu, J., Wu, R., Shi, L., Zhang, X., Zhang, W., et al. (2021). GRP78 Determines Glioblastoma Sensitivity to UBA1 Inhibition-Induced UPR Signaling and Cell Death. *Cell Death Dis.* 12, 733. doi:10.1038/s41419-021-04023-w

Liu, X., and Fuentes, E. J. (2019). Emerging Themes in PDZ Domain Signaling. *Int. Rev. Cell Mol. Biol.* 343, 129–218. doi:10.1016/bs.ircmb.2018.05.013

Liu, X., Zhang, Z., Ruan, J., Pan, Y., Magupalli, V. G., Wu, H., et al. (2016). Inflammasome-activated Gasdermin D Causes Pyroptosis by Forming Membrane Pores. *Nature* 535, 153–158. doi:10.1038/nature18629

Lo, W. L., Hsu, T. I., Yang, W. B., Kao, T. J., Wu, M. H., Huang, Y. N., et al. (2020). Betulinic Acid-Mediated Tuning of PERK/CHOP Signaling by Sp1 Inhibition as a Novel Therapeutic Strategy for Glioblastoma. *Cancers (Basel)* 12. doi:10.3390/cancers12040981

Locher, K. P. (2016). Mechanistic Diversity in ATP-Binding Cassette (ABC) Transporters. *Nat. Struct. Mol. Biol.* 23, 487–493. doi:10.1038/nsmb.3216

Lopes, M. H., Hajj, G. N., Muras, A. G., Mancini, G. L., Castro, R. M., Ribeiro, K. C., et al. (2005). Interaction of Cellular Prion and Stress-Inducible Protein 1 Promotes Neuritogenesis and Neuroprotection by Distinct Signaling Pathways. *J. Neurosci.* 25 (49), 11330–11339. doi:10.1523/JNEUROSCI.2313-05.2005

Lopes, M. H., Santos, T. G., Rodrigues, B. R., Queiroz-Hazarbassanov, N., Cunha, I. W., Wasilewska-Sampaio, A. P., et al. (2015). Disruption of Prion Protein-HOP Engagement Impairs Glioblastoma Growth and Cognitive Decline and Improves Overall Survival. *Oncogene* 34, 3305–3314. doi:10.1038/onc.2014.261

Lopez-Castejon, G., and Brough, D. (2011). Understanding the Mechanism of IL-1β Secretion. *Cytokine & Growth Factor Rev.* 22, 189–195. doi:10.1016/j.cytogfr.2011.10.001

Louis, D. N., Perry, A., Wesseling, P., Brat, D. J., Cree, I. A., Figarella-Branger, D., et al. (2021). The 2021 WHO Classification of Tumors of the Central Nervous System: a Summary. *Neuro Oncol.* 23 (8), 1231–1251. doi:10.1093/neuonc/noab106

Lu, J., Xu, Z., Duan, H., Ji, H., Zhen, Z., Li, B., et al. (2020). Tumor-associated Macrophage Interleukin-β Promotes Glycerol-3-phosphate Dehydrogenase Activation, Glycolysis and Tumorigenesis in Glioma Cells. *Cancer Sci.* 111, 1979–1990. doi:10.1111/cas.14408

Ly, K., Luna Saavedra, Y. G., Canuel, M., Routhier, S., Desjardins, R., Hamelin, J., et al. (2014). Annexin A2 Reduces PCSK9 Protein Levels via a Translational Mechanism and Interacts with the M1 and M2 Domains of PCSK9. *J. Biol. Chem.* 289, 17732–17746. doi:10.1074/jbc.m113.541094

Ma, J., Yang, Y.-R., Chen, W., Chen, M.-H., Wang, H., Wang, X.-D., et al. (2016). Fluoxetine Synergizes with Temozolomide to Induce the CHOP-dependent Endoplasmic Reticulum Stress-Related Apoptosis Pathway in Glioma Cells. *Oncol. Rep.* 36, 676–684. doi:10.3892/or.2016.4860

Ma, Q., Yang, Y., Feng, D., Zheng, S., Meng, R., Fa, P., et al. (2015). MAGI3 Negatively Regulates Wnt/β-Catenin Signaling and Suppresses Malignant Phenotypes of Glioma Cells. *Oncotarget* 6, 35851–35865. doi:10.18632/oncotarget.5323

Maceyka, M., Sankala, H., Hait, N. C., Le Stunff, H., Liu, H., Toman, R., et al. (2005). SphK1 and SphK2, Sphingosine Kinase Isoenzymes with Opposing Functions in Sphingolipid Metabolism. *J. Biol. Chem.* 280, 37118–37129. doi:10.1074/jbc.m502207200

MacKenzie, A., Wilson, H. L., Kiss-Toth, E., Dower, S. K., North, R. A., and Surprenant, A. (2001). Rapid Secretion of Interleukin-1β by Microvesicle Shedding. *Immunity* 15, 825–835. doi:10.1016/s1074-7613(01)00229-1

Mambula, S. S., and Calderwood, S. K. (2006). Heat Shock Protein 70 Is Secreted from Tumor Cells by a Nonclassical Pathway Involving Lysosomal Endosomes. *J. Immunol.* 177, 7849–7857. doi:10.4049/jimmunol.177.11.7849

Manea, A. J., and Ray, S. K. (2021). Regulation of Autophagy as a Therapeutic Option in Glioblastoma. *Apoptosis* 26 (11-12), 574–599. doi:10.1007/s10495-021-01691-z

Manjithaya, R., Anjard, C., Loomis, W. F., and Subramani, S. (2010). Unconventional Secretion of Pichia pastoris Acb1 Is Dependent on GRASP

Protein, Peroxisomal Functions, and Autophagosome Formation. *J. Cell Biol.* 188, 537–546. doi:10.1083/jcb.200911149

Mann, M. J., and Hendershot, L. M. (2006). UPR Activation Alters Chemosensitivity of Tumor Cells. *Cancer Biol. Ther.* 5, 736–740. doi:10.4161/cbt.5.7.2969

Markouli, M., Strepkos, D., Papavassiliou, A. G., and Piperi, C. (2020). Targeting of Endoplasmic Reticulum (ER) Stress in Gliomas. *Pharmacol. Res.* 157, 104823. doi:10.1016/j.phrs.2020.104823

Maule, F., Bresolin, S., Rampazzo, E., Boso, D., Puppa, A. D., Esposito, G., et al. (2016). Annexin 2A Sustains Glioblastoma Cell Dissemination and Proliferation. *Oncotarget* 7, 54632–54649. doi:10.18632/oncotarget.10565

Mayer, G., Poirier, S., and Seidah, N. G. (2008). Annexin A2 Is a C-Terminal PCSK9-Binding Protein that Regulates Endogenous Low Density Lipoprotein Receptor Levels. *J. Biol. Chem.* 283, 31791–31801. doi:10.1074/jbc.m805971200

Minchenko, D. O., Khita, O. O., Tsymbal, D. O., Danilovskyi, S. V., Rudnytska, O. V., Halkin, O. V., et al. (2020). Expression of IDE and PITRM1 Genes in ERN1 Knockdown U87 Glioma Cells: Effect of Hypoxia and Glucose Deprivation. *Endocr. Regul.* 54, 183–195. doi:10.2478/enr-2020-0021

Minchenko, D. O., Riabovol, O. O., Tsymbal, D. O., Ratushna, O. O., and Minchenko, O. H. (2016). Inhibition of IRE1 Signaling Affects the Expression of Genes Encoded Glucocorticoid Receptor and Some Related Factors and Their Hypoxic Regulation in U87 Glioma Cells. *Endocr. Regul.* 50, 127–136. doi:10.1515/enr-2016-0014

Mori, S., Wu, C.-Y., Yamaji, S., Saegusa, J., Shi, B., Ma, Z., et al. (2008). Direct Binding of Integrin αvβ3 to FGF1 Plays a Role in FGF1 Signaling. *J. Biol. Chem.* 283, 18066–18075. doi:10.1074/jbc.m801213200

Morré, D. J. (1981). An Alternative Pathway for Secretion of Lipoprotein Particles in Rat Liver. *Eur. J. Cell Biol.* 26, 21–25.

Myers, R. L., Chedid, M., Tronick, S. R., and Chiu, I. M. (1995). Different Fibroblast Growth Factor 1 (FGF-1) Transcripts in Neural Tissues, Glioblastomas and Kidney Carcinoma Cell Lines. *Oncogene* 11, 785–789.

Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., et al. (1995). Characterization of a Cis-Golgi Matrix Protein, GM130. *J. Cell Biol.* 131, 1715–1726. doi:10.1083/jcb.131.6.1715

Ng, F., and Tang, B. L. (2016). Unconventional Protein Secretion in Animal Cells. *Methods Mol. Biol.* 1459, 31–46. doi:10.1007/978-1-4939-3804-9_2

Nickel, W., and Rabouille, C. (2009). Mechanisms of Regulated Unconventional Protein Secretion. *Nat. Rev. Mol. Cell Biol.* 10, 148–155. doi:10.1038/nrm2617

Nickel, W. (2003). The Mystery of Nonclassical Protein Secretion. *Eur. J. Biochem.* 270, 2109–2119. doi:10.1046/j.1432-1033.2003.03577.x

Nüchel, J., Tauber, M., Nolte, J. L., Mörgelin, M., Türk, C., Eckes, B., et al. (2021). An mTORC1-GRASP55 Signaling axis Controls Unconventional Secretion to Reshape the Extracellular Proteome upon Stress. *Mol. Cell* 81, 3275–3293. doi:10.1016/j.molcel.2021.06.017

Ou, A., Yung, W. K. A., and Majd, N. (2020). Molecular Mechanisms of Treatment Resistance in Glioblastoma. *Int. J. Mol. Sci.* 22. doi:10.3390/ijms22010351

Palade, G. (1975). Intracellular Aspects of the Process of Protein Synthesis. *Science* 189, 347–358. doi:10.1126/science.1096303

Parsons, D. W., Jones, S., Zhang, X., Lin, J. C.-H., Leary, R. J., Angenendt, P., et al. (2008). An Integrated Genomic Analysis of Human Glioblastoma Multiforme. *Science* 321, 1807–1812. doi:10.1126/science.1164382

Paugh, B. S., Bryan, L., Paugh, S. W., Wilczynska, K. M., Alvarez, S. M., Singh, S. K., et al. (2009). Interleukin-1 Regulates the Expression of Sphingosine Kinase 1 in Glioblastoma Cells. *J. Biol. Chem.* 284, 3408–3417. doi:10.1074/jbc.m807170200

Peinado, H., Zhang, H., Matei, I. R., Costa-Silva, B., Hoshino, A., Rodrigues, G., et al. (2017). Pre-metastatic Niches: Organ-specific Homes for Metastases. *Nat. Rev. Cancer* 17, 302–317. doi:10.1038/nrc.2017.6

Peñaranda-Fajardo, N. M., Meijer, C., Liang, Y., Dijkstra, B. M., Aguirre-Gamboa, R., den Dunnen, W. F. A., et al. (2019). ER Stress and UPR Activation in Glioblastoma: Identification of a Noncanonical PERK Mechanism Regulating GBM Stem Cells through SOX2 Modulation. *Cell Death Dis.* 10, 690. doi:10.1038/s41419-019-1934-1

Perraud, F., Yoshimura, K., Louis, B., Dalemans, W., Ali-Hadji, D., Schultz, H., et al. (1992). The Promoter of the Human Cystic Fibrosis Transmembrane Conductance Regulator Gene Directing SV40 T Antigen Expression Induces Malignant Proliferation of Ependymal Cells in Transgenic Mice. *Oncogene* 7, 993–997.

Piccini, A., Carta, S., Tassi, S., Lasiglié, D., Fossati, G., and Rubartelli, A. (2008). ATP Is Released by Monocytes Stimulated with Pathogen-Sensing Receptor Ligands and Induces IL-1β and IL-18 Secretion in an Autocrine Way. *Proc. Natl. Acad. Sci. U.S.A.* 105, 8067–8072. doi:10.1073/pnas.0709684105

Pituch, K. C., Miska, J., Krenciute, G., Panek, W. K., Li, G., Rodriguez-Cruz, T., et al. (2018). Adoptive Transfer of IL13Rα2-Specific Chimeric Antigen Receptor T Cells Creates a Pro-inflammatory Environment in Glioblastoma. *Mol. Ther.* 26, 986–995. doi:10.1016/j.ymthe.2018.02.001

Pluquet, O., Dejeans, N., Bouchecareilh, M., Lhomond, S., Pineau, R., Higa, A., et al. (2013). Posttranscriptional Regulation of PER1 Underlies the Oncogenic Function of IREα. *Cancer Res.* 73, 4732–4743. doi:10.1158/0008-5472.can-12-3989

Poore, B., Yuan, M., Arnold, A., Price, A., Alt, J., Rubens, J. A., et al. (2019). Inhibition of mTORC1 in Pediatric Low-Grade Glioma Depletes Glutathione and Therapeutically Synergizes with Carboplatin. *Neuro Oncol.* 21 (2), 252–263. doi:10.1093/neuonc/noy150

Popa, S. J., Stewart, S. E., and Moreau, K. (2018). Unconventional Secretion of Annexins and Galectins. *Seminars Cell & Dev. Biol.* 83, 42–50. doi:10.1016/j.semcdb.2018.02.022

Popov, B., Gadjeva, V., Valkanov, P., Popova, S., and Tolekova, A. (2003). Lipid Peroxidation, Superoxide Dismutase and Catalase Activities in Brain Tumor Tissues. *Archives Physiology Biochem.* 111, 455–459. doi:10.3109/13813450312331342328

Pyrko, P., Schönthal, A. H., Hofman, F. M., Chen, T. C., and Lee, A. S. (2007). The Unfolded Protein Response Regulator GRP78/BiP as a Novel Target for Increasing Chemosensitivity in Malignant Gliomas. *Cancer Res.* 67, 9809–9816. doi:10.1158/0008-5472.can-07-0625

Qu, Y., Franchi, L., Nunez, G., and Dubyak, G. R. (2007). Nonclassical IL-1β Secretion Stimulated by P2X7 Receptors Is Dependent on Inflammasome Activation and Correlated with Exosome Release in Murine Macrophages. *J. Immunol.* 179, 1913–1925. doi:10.4049/jimmunol.179.3.1913

Quail, D. F., and Joyce, J. A. (2017). The Microenvironmental Landscape of Brain Tumors. *Cancer Cell* 31, 326–341. doi:10.1016/j.ccell.2017.02.009

Rabouille, C., Malhotra, V., and Nickel, W. (2012). Diversity in Unconventional Protein Secretion. *J. Cell Sci.* 125 (Pt 22), 5251–5255. doi:10.1242/jcs.103630

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cell Biol.* 27, 230–240. doi:10.1016/j.tcb.2016.11.007

Radulescu, A. E., Mukherjee, S., and Shields, D. (2011). The Golgi Protein P115 Associates with γ-Tubulin and Plays a Role in Golgi Structure and Mitosis Progression. *J. Biol. Chem.* 286, 21915–21926. doi:10.1074/jbc.m110.209460

Rees, D. C., Johnson, E., and Lewinson, O. (2009). ABC Transporters: the Power to Change. *Nat. Rev. Mol. Cell Biol.* 10, 218–227. doi:10.1038/nrm2646

Rehm, A., Stern, P., Ploegh, H. L., and Tortorella, D. (2001). Signal Peptide Cleavage of a Type I Membrane Protein, HCMV US11, Is Dependent on its Membrane Anchor. *EMBO J.* 20, 1573–1582. doi:10.1093/emboj/20.7.1573

Rizzo, F. R., Musella, A., De Vito, F., Fresegna, D., Bullitta, S., Vanni, V., et al. (2018). Tumor Necrosis Factor and Interleukin-1β Modulate Synaptic Plasticity during Neuroinflammation. *Neural Plast.* 2018, 8430123. doi:10.1155/2018/8430123

Romero-Ramirez, L., Cao, H., Nelson, D., Hammond, E., Lee, A.-H., Yoshida, H., et al. (2004). XBP1 Is Essential for Survival under Hypoxic Conditions and Is Required for Tumor Growth. *Cancer Res.* 64, 5943–5947. doi:10.1158/0008-5472.can-04-1606

Ronellenfitsch, M. W., Zeiner, P. S., Mittelbronn, M., Urban, H., Pietsch, T., Reuter, D., et al. (2018). Akt and mTORC1 Signaling as Predictive Biomarkers for the EGFR Antibody Nimotuzumab in Glioblastoma. *Acta Neuropathol. Commun.* 6 (1), 81. doi:10.1186/s40478-018-0583-4

Rosenzweig, R., Nillegoda, N. B., Mayer, M. P., and Bukau, B. (2019). The Hsp70 Chaperone Network. *Nat. Rev. Mol. Cell Biol.* 20, 665–680. doi:10.1038/s41580-019-0133-3

Roth, J. (2002). Protein N-Glycosylation along the Secretory Pathway: Relationship to Organelle Topography and Function, Protein Quality Control, and Cell Interactions. *Chem. Rev.* 102, 285–304. doi:10.1021/cr000423j

Roth, W., Wild-Bode, C., Platten, M., Grimmel, C., Melkonyan, H. S., Dichgans, J., et al. (2000). Secreted Frizzled-Related Proteins Inhibit Motility and Promote Growth of Human Malignant Glioma Cells. *Oncogene* 19 (37), 4210–4220. doi:10.1038/sj.onc.1203783

Rubartelli, A., Cozzolino, F., Talio, M., and Sitia, R. (1990). A Novel Secretory Pathway for Interleukin-1 Beta, a Protein Lacking a Signal Sequence. *EMBO J.* 9, 1503–1510. doi:10.1002/j.1460-2075.1990.tb08268.x

Rubartelli, A., and Sitia, R. (1991). Interleukin Iβ and Thioredoxin Are Secreted through a Novel Pathway of Secretion. *Biochem. Soc. Trans.* 19, 255–259. doi:10.1042/bst0190255

Sabatier, J. M., Vives, E., Mabrouk, K., Benjouad, A., Rochat, H., Duval, A., et al. (1991). Evidence for Neurotoxic Activity of Tat from Human Immunodeficiency Virus Type 1. *J. Virol.* 65, 961–967. doi:10.1128/jvi.65.2.961-967.1991

Sarkar, S., Li, Y., Mirzaei, R., Rawji, K. S., Poon, C. C., Wang, J., et al. (2020). Demeclocycline Reduces the Growth of Human Brain Tumor-Initiating Cells: Direct Activity and through Monocytes. *Front. Immunol.* 11, 272. doi:10.3389/fimmu.2020.00272

Schlottmann, I., Ehrhart-Bornstein, M., Wabitsch, M., Bornstein, S. R., and Lamounier-Zepter, V. (2014). Calcium-dependent Release of Adipocyte Fatty Acid Binding Protein from Human Adipocytes. *Int. J. Obes.* 38, 1221–1227. doi:10.1038/ijo.2013.241

Schotman, H., Karhinen, L., and Rabouille, C. (2008). dGRASP-mediated Noncanonical Integrin Secretion Is Required for Drosophila Epithelial Remodeling. *Dev. Cell* 14, 171–182. doi:10.1016/j.devcel.2007.12.006

Schuerwegh, A. J., Stevens, W. J., Bridts, C. H., and De Clerck, L. S. (2001). Evaluation of Monensin and Brefeldin A for Flow Cytometric Determination of Interleukin-1 Beta, Interleukin-6, and Tumor Necrosis Factor-Alpha in Monocytes. *Cytometry* 46, 172–176. doi:10.1002/cyto.1102

Scriven, P., Coulson, S., Haines, R., Balasubramanian, S., Cross, S., and Wyld, L. (2009). Activation and Clinical Significance of the Unfolded Protein Response in Breast Cancer. *Br. J. Cancer* 101, 1692–1698. doi:10.1038/sj.bjc.6605365

Seidah, N. G., Poirier, S., Denis, M., Parker, R., Miao, B., Mapelli, C., et al. (2012). Annexin A2 Is a Natural Extrahepatic Inhibitor of the PCSK9-Induced LDL Receptor Degradation. *PLoS One* 7, e41865. doi:10.1371/journal.pone.0041865

Shah, S. S., Rodriguez, G. A., Musick, A., Walters, W. M., de Cordoba, N., Barbarite, E., et al. (2019). Targeting Glioblastoma Stem Cells with 2-Deoxy-D-Glucose (2-DG) Potentiates Radiation-Induced Unfolded Protein Response (UPR). *Cancers (Basel)* 11, 159. doi:10.3390/cancers11020159

Shen, H., Giordano, F., Wu, Y., Chan, J., Zhu, C., Milosevic, I., et al. (2014). Coupling between Endocytosis and Sphingosine Kinase 1 Recruitment. *Nat. Cell Biol.* 16, 652–662. doi:10.1038/ncb2987

Shi, Z., Zhang, K., Zhou, H., Jiang, L., Xie, B., Wang, R., et al. (2020). Increased miR-34c Mediates Synaptic Deficits by Targeting Synaptotagmin 1 through ROS-JNK-P53 Pathway in Alzheimer's Disease. *Aging Cell* 19 (3), e13125. doi:10.1111/acel.13125

Shorter, J., Watson, R., Giannakou, M. E., Clarke, M., Warren, G., and Barr, F. A. (1999). GRASP55, a Second Mammalian GRASP Protein Involved in the Stacking of Golgi Cisternae in a Cell-free System. *EMBO J.* 18, 4949–4960. doi:10.1093/emboj/18.18.4949

Shoshan, Y., and Siegal, T. (1996). Control of Vasogenic Edema in a Brain Tumor Model: Comparison between Dexamethasone and Superoxide Dismutase. *Neurosurgery* 39, 1206–1214. doi:10.1097/00006123-199612000-00025

Sicari, D., Maria, A., and Chevet, E. (2019). Control of Protein Homeostasis in the Early Secretory Pathway: Current Status and Challenges. *Cells* 8, 1347. doi:10.3390/cells8111347

Singh, N., Miner, A., Hennis, L., and Mittal, S. (2021). Mechanisms of Temozolomide Resistance in Glioblastoma - a Comprehensive Review. *Cancer Drug Resist* 4, 17–43. doi:10.20517/cdr.2020.79

Sluiman, H. J. (1984). A Pathway of Plasma Membrane Biogenesis Bypassing the Golgi Apparatus during Cell Division in the Green Alga Cylindrocapsa Geminella. *J. Cell Sci.* 72, 89–100. doi:10.1242/jcs.72.1.89

Son, S. M., Cha, M.-Y., Choi, H., Kang, S., Choi, H., Lee, M.-S., et al. (2016). Insulin-degrading Enzyme Secretion from Astrocytes Is Mediated by an Autophagy-Based Unconventional Secretory Pathway in Alzheimer Disease. *Autophagy* 12, 784–800. doi:10.1080/15548627.2016.1159375

Son, S. M., Kang, S., Choi, H., and Mook-Jung, I. (2015). Statins Induce Insulin-Degrading Enzyme Secretion from Astrocytes via an Autophagy-Based Unconventional Secretory Pathway. *Mol. Neurodegener.* 10, 56. doi:10.1186/s13024-015-0054-3

Song, Y., and Masison, D. C. (2005). Independent Regulation of Hsp70 and Hsp90 Chaperones by Hsp70/Hsp90-Organizing Protein Sti1 (Hop1). *J. Biol. Chem.* 280, 34178–34185. doi:10.1074/jbc.m505420200

Soni, H., Bode, J., Nguyen, C. D. L., Puccio, L., Nessling, M., Piro, R. M., et al. (2020). PERK-mediated Expression of Peptidylglycine α-amidating Monooxygenase Supports Angiogenesis in Glioblastoma. *Oncogenesis* 9, 18. doi:10.1038/s41389-020-0201-8

Spill, F., Reynolds, D. S., Kamm, R. D., and Zaman, M. H. (2016). Impact of the Physical Microenvironment on Tumor Progression and Metastasis. *Curr. Opin. Biotechnol.* 40, 41–48. doi:10.1016/j.copbio.2016.02.007

Stefan, S. M. (2019). Multi-target ABC Transporter Modulators: what Next and where to Go? *Future Med. Chem.* 11, 2353–2358. doi:10.4155/fmc-2019-0185

Steringer, J. P., Lange, S., Čujová, S., Šachl, R., Poojari, C., Lolicato, F., et al. (2017). Key Steps in Unconventional Secretion of Fibroblast Growth Factor 2 Reconstituted with Purified Components. *Elife* 6. doi:10.7554/eLife.28985

Steringer, J. P., Müller, H.-M., and Nickel, W. (2015). Unconventional Secretion of Fibroblast Growth Factor 2-A Novel Type of Protein Translocation across Membranes? *J. Mol. Biol.* 427, 1202–1210. doi:10.1016/j.jmb.2014.07.012

Sun, G., Cao, Y., Dai, X., Li, M., and Guo, J. (2019). Hsc70 Interacts with β4GalT5 to Regulate the Growth of Gliomas. *Neuromol Med.* 21, 33–41. doi:10.1007/s12017-018-08520-8

Sun, W., Depping, R., and Jelkmann, W. (2014). Interleukin-1β Promotes Hypoxia-Induced Apoptosis of Glioblastoma Cells by Inhibiting Hypoxia-Inducible Factor-1 Mediated Adrenomedullin Production. *Cell Death Dis.* 5 (1), e1020. doi:10.1038/cddis.2013.562

Suyama, K., Watanabe, M., Sakabe, K., Otomo, A., Okada, Y., Terayama, H., et al. (2014). GRP78 Suppresses Lipid Peroxidation and Promotes Cellular Antioxidant Levels in Glial Cells Following Hydrogen Peroxide Exposure. *PLoS One* 9, e86951. doi:10.1371/journal.pone.0086951

Takahashi, J. A., Fukumoto, M., Igarashi, K., Oda, Y., Kikuchi, H., and Hatanaka, M. (1992). Correlation of Basic Fibroblast Growth Factor Expression Levels with the Degree of Malignancy and Vascularity in Human Gliomas. *J. Neurosurg.* 76, 792–798. doi:10.3171/jns.1992.76.5.0792

Tirasophon, W., Welihinda, A. A., and Kaufman, R. J. (1998). A Stress Response Pathway from the Endoplasmic Reticulum to the Nucleus Requires a Novel Bifunctional Protein Kinase/endoribonuclease (Ire1p) in Mammalian Cells. *Genes Dev.* 12, 1812–1824. doi:10.1101/gad.12.12.1812

Torrado, L. C., Temmerman, K., Müller, H.-M., Mayer, M. P., Seelenmeyer, C., Backhaus, R., et al. (2009). An Intrinsic Quality-Control Mechanism Ensures Unconventional Secretion of Fibroblast Growth Factor 2 in a Folded Conformation. *J. Cell Sci.* 122, 3322–3329. doi:10.1242/jcs.049791

Tundo, G. R., Sbardella, D., Ciaccio, C., Bianculli, A., Orlandi, A., Desimio, M. G., et al. (2013). Insulin-degrading Enzyme (IDE). *J. Biol. Chem.* 288 (4), 2281–2289. doi:10.1074/jbc.m112.393108

Ulloa, F., Gonzàlez-Juncà, A., Meffre, D., Barrecheguren, P. J., Martínez-Mármol, R., Pazos, I., et al. (2015). Blockade of the SNARE Protein Syntaxin 1 Inhibits Glioblastoma Tumor Growth. *PLoS One* 10, e0119707. doi:10.1371/journal.pone.0119707

Van Krieken, R., Tsai, Y. L., Carlos, A. J., Ha, D. P., and Lee, A. S. (2021). ER Residential Chaperone GRP78 Unconventionally Relocalizes to the Cell Surface via Endosomal Transport. *Cell Mol. Life Sci.* 78, 5179–5195. doi:10.1007/s00018-021-03849-z

van Ziel, A. M., Largo-Barrientos, P., Wolzak, K., Verhage, M., and Scheper, W. (2019). Unconventional Secretion Factor GRASP55 Is Increased by Pharmacological Unfolded Protein Response Inducers in Neurons. *Sci. Rep.* 9, 1567. doi:10.1038/s41598-018-38146-6

Villeneuve, J., Bassaganyas, L., Lepreux, S., Chiritoiu, M., Costet, P., Ripoche, J., et al. (2018). Unconventional Secretion of FABP4 by Endosomes and Secretory Lysosomes. *J. Cell Biol.* 217, 649–665. doi:10.1083/jcb.201705047

Vinke, F. P., Grieve, A. G., and Rabouille, C. (2011). The Multiple Facets of the Golgi Reassembly Stacking Proteins. *Biochem. J.* 433, 423–433. doi:10.1042/bj20101540

Virrey, J. J., Dong, D., Stiles, C., Patterson, J. B., Pen, L., Ni, M., et al. (2008). Stress Chaperone GRP78/BiP Confers Chemoresistance to Tumor-Associated Endothelial Cells. *Mol. Cancer Res.* 6, 1268–1275. doi:10.1158/1541-7786.mcr-08-0060

Wang, F., Yang, L., Shi, L., Li, Q., Zhang, G., Wu, J., et al. (2015). Nuclear Translocation of Fibroblast Growth Factor-2 (FGF2) Is Regulated by

Karyopherin-β2 and Ran GTPase in Human Glioblastoma Cells. *Oncotarget* 6, 21468–21478. doi:10.18632/oncotarget.4097

Wang, H., Han, M., Whetsell, W., Jr., Wang, J., Rich, J., Hallahan, D., et al. (2014). Tax-interacting Protein 1 Coordinates the Spatiotemporal Activation of Rho GTPases and Regulates the Infiltrative Growth of Human Glioblastoma. *Oncogene* 33, 1558–1569. doi:10.1038/onc.2013.97

Wang, L., Liu, Z., Balivada, S., Shrestha, T., Bossmann, S., Pyle, M., et al. (2012). Interleukin-1β and Transforming Growth Factor-β Cooperate to Induce Neurosphere Formation and Increase Tumorigenicity of Adherent LN-229 Glioma Cells. *Stem Cell Res. Ther.* 3 (1), 5. doi:10.1186/scrt96

Wang, Z., Min, X., Xiao, S.-H., Johnstone, S., Romanow, W., Meininger, D., et al. (2013). Molecular Basis of Sphingosine Kinase 1 Substrate Recognition and Catalysis. *Structure* 21, 798–809. doi:10.1016/j.str.2013.02.025

Wen, X., Chen, X., and Chen, X. (2020). Increased Expression of GRP78 Correlates with Adverse Outcome in Recurrent Glioblastoma Multiforme Patients. *Turk Neurosurg.* 30, 11–16. doi:10.5137/1019-5149.JTN.21840-17.4

Wesche, J., Małecki, J., Więdłocha, A., Skjerpen, C. S., Claus, P., and Olsnes, S. (2006). FGF-1 and FGF-2 Require the Cytosolic Chaperone Hsp90 for Translocation into the Cytosol and the Cell Nucleus. *J. Biol. Chem.* 281, 11405–11412. doi:10.1074/jbc.m600477200

Wilkens, S. (2015). Structure and Mechanism of ABC Transporters. *F1000Prime Rep.* 7, 14. doi:10.12703/P7-14

Witzgall, R. (2018). Golgi Bypass of Ciliary Proteins. *Seminars Cell & Dev. Biol.* 83, 51–58. doi:10.1016/j.semcdb.2018.03.010

Wu, H., Li, T., and Zhao, J. (2020). GRASP55: A Multifunctional Protein. *Cpps* 21, 544–552. doi:10.2174/1389203721666200218105302

Wu, W., Yu, T., Wu, Y., Tian, W., Zhang, J., and Wang, Y. (2019). The miR155HG/miR-185/ANXA2 Loop Contributes to Glioblastoma Growth and Progression. *J. Exp. Clin. Cancer Res.* 38, 133. doi:10.1186/s13046-019-1132-0

Xu, Y., Cui, L., Dibello, A., Wang, L., Lee, J., Saidi, L., et al. (2018). DNAJC5 Facilitates USP19-dependent Unconventional Secretion of Misfolded Cytosolic Proteins. *Cell Discov.* 4, 11. doi:10.1038/s41421-018-0012-7

Yacoub, A., Hamed, H. A., Allegood, J., Mitchell, C., Spiegel, S., Lesniak, M. S., et al. (2010). PERK-dependent Regulation of Ceramide Synthase 6 and Thioredoxin Play a Key Role in mda-7/IL-24-induced Killing of Primary Human Glioblastoma Multiforme Cells. *Cancer Res.* 70, 1120–1129. doi:10.1158/0008-5472.can-09-4043

Yamaji, S., Saegusa, J., Ieguchi, K., Fujita, M., Mori, S., Takada, Y. K., et al. (2010). A Novel Fibroblast Growth Factor-1 (FGF1) Mutant that Acts as an FGF Antagonist. *PLoS One* 5, e10273. doi:10.1371/journal.pone.0010273

Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., et al. (2007). Transcriptional Induction of Mammalian ER Quality Control Proteins Is Mediated by Single or Combined Action of ATF6α and XBP1. *Dev. Cell* 13, 365–376. doi:10.1016/j.devcel.2007.07.018

Yang, J., and Yang, Q. (2020). Identification of Core Genes and Screening of Potential Targets in Glioblastoma Multiforme by Integrated Bioinformatic Analysis. *Front. Oncol.* 10, 615976. doi:10.3389/fonc.2020.615976

Yang, W., Xiu, Z., He, Y., Huang, W., Li, Y., and Sun, T. (2020). Bip Inhibition in Glioma Stem Cells Promotes Radiation-Induced Immunogenic Cell Death. *Cell Death Dis.* 11, 786. doi:10.1038/s41419-020-03000-z

Yang, Z., and Klionsky, D. J. (2010). Mammalian Autophagy: Core Molecular Machinery and Signaling Regulation. *Curr. Opin. Cell Biol.* 22, 124–131. doi:10.1016/j.ceb.2009.11.014

Yao, Z., Zhang, X., Zhao, F., Wang, S., Chen, A., Huang, B., et al. (2020). Ursodeoxycholic Acid Inhibits Glioblastoma Progression via Endoplasmic Reticulum Stress Related Apoptosis and Synergizes with the Proteasome Inhibitor Bortezomib. *ACS Chem. Neurosci.* 11, 1337–1346. doi:10.1021/acschemneuro.0c00095

Ye, J., and Koumenis, C. (2009). ATF4, an ER Stress and Hypoxia-Inducible Transcription Factor and its Potential Role in Hypoxia Tolerance and Tumorigenesis. *Cmm* 9, 411–416. doi:10.2174/156652409788167096

Ye, Y. (2018). Regulation of Protein Homeostasis by Unconventional Protein Secretion in Mammalian Cells. *Seminars Cell & Dev. Biol.* 83, 29–35. doi:10.1016/j.semcdb.2018.03.006

Yoo, J.-S., Moyer, B. D., Bannykh, S., Yoo, H.-M., Riordan, J. R., and Balch, W. E. (2002). Non-conventional Trafficking of the Cystic Fibrosis Transmembrane Conductance Regulator through the Early Secretory Pathway. *J. Biol. Chem.* 277, 11401–11409. doi:10.1074/jbc.m110263200

Zeghouf, M., Guibert, B., Zeeh, J.-C., and Cherfils, J. (2005). Arf, Sec7 and Brefeldin A: a Model towards the Therapeutic Inhibition of Guanine Nucleotide-Exchange Factors. *Biochem. Soc. Trans.* 33, 1265–1268. doi:10.1042/bst0331265

Zehe, C., Engling, A., Wegehingel, S., Schäfer, T., and Nickel, W. (2006). Cell-surface Heparan Sulfate Proteoglycans Are Essential Components of the Unconventional Export Machinery of FGF-2. *Proc. Natl. Acad. Sci. U.S.A.* 103, 15479–15484. doi:10.1073/pnas.0605997103

Zhang, M., Kenny, S. J., Ge, L., Xu, K., and Schekman, R. (2015). Translocation of Interleukin-1β into a Vesicle Intermediate in Autophagy-Mediated Secretion. *Elife* 4. doi:10.7554/eLife.11205

Zhang, X., Wang, L., Lak, B., Li, J., Jokitalo, E., and Wang, Y. (2018). GRASP55 Senses Glucose Deprivation through O-GlcNAcylation to Promote Autophagosome-Lysosome Fusion. *Dev. Cell* 45, 245–261. doi:10.1016/j.devcel.2018.03.023

Zhang, Y., Yang, X., Zhu, X.-L., Bai, H., Wang, Z.-Z., Zhang, J.-J., et al. (2021). S100A Gene Family: Immune-Related Prognostic Biomarkers and Therapeutic Targets for Low-Grade Glioma. *Aging* 13 (11), 15459–15478. doi:10.18632/aging.203103

Zhao, J., Li, L., and Leissring, M. A. (2009). Insulin-degrading Enzyme Is Exported via an Unconventional Protein Secretion Pathway. *Mol. Neurodegener.* 4, 4. doi:10.1186/1750-1326-4-4

Zhao, Y., Zhang, Q., Xi, J., Xiao, B., Li, Y., and Ma, C. (2015). Neuroprotective Effect of Fasudil on Inflammation through PI3K/Akt and Wnt/β-Catenin Dependent Pathways in a Mice Model of Parkinson's Disease. *Int. J. Clin. Exp. Pathol.* 8, 2354–2364.

Zhong, X., Chen, H.-q., Yang, X.-l., Wang, Q., Chen, W., and Li, C. (2019). CFTR Activation Suppresses Glioblastoma Cell Proliferation, Migration and Invasion. *Biochem. Biophysical Res. Commun.* 508, 1279–1285. doi:10.1016/j.bbrc.2018.12.080

Zhou, W., Ke, S. Q., Huang, Z., Flavahan, W., Fang, X., Paul, J., et al. (2015). Periostin Secreted by Glioblastoma Stem Cells Recruits M2 Tumour-Associated Macrophages and Promotes Malignant Growth. *Nat. Cell Biol.* 17, 170–182. doi:10.1038/ncb3090

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer (LA) declared a shared affiliation, with no collaboration, with the authors (RI, MP, RA, ME, JB, GC, CF, AF, JA, SS, MS, and ML) to the handling editor at the time of the review.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

REVIEW
published: 16 June 2022
doi: 10.3389/fcell.2022.884020

Role of SNAREs in Unconventional Secretion—Focus on the VAMP7-Dependent Secretion

Somya Vats[1] *and Thierry Galli*[1,2]*

[1]Institute of Psychiatry and Neuroscience of Paris (IPNP), INSERM U1266, Membrane Traffic in Healthy and Diseased Brain, Université Paris Cité, Paris, France, [2]GHU PARIS Psychiatrie & Neurosciences, Paris, France

Intracellular membrane protein trafficking is crucial for both normal cellular physiology and cell-cell communication. The conventional secretory route follows transport from the Endoplasmic reticulum (ER) to the plasma membrane via the Golgi apparatus. Alternative modes of secretion which can bypass the need for passage through the Golgi apparatus have been collectively termed as Unconventional protein secretion (UPS). UPS can comprise of cargo without a signal peptide or proteins which escape the Golgi in spite of entering the ER. UPS has been classified further depending on the mode of transport. Type I and Type II unconventional secretion are non-vesicular and non-SNARE protein dependent whereas Type III and Type IV dependent on vesicles and on SNARE proteins. In this review, we focus on the Type III UPS which involves the import of cytoplasmic proteins in membrane carriers of autophagosomal/endosomal origin and release in the extracellular space following SNARE-dependent intracellular membrane fusion. We discuss the role of vesicular SNAREs with a strong focus on VAMP7, a vesicular SNARE involved in exosome, lysosome and autophagy mediated secretion. We further extend our discussion to the role of unconventional secretion in health and disease with emphasis on cancer and neurodegeneration.

Keywords: unconventional protein secretion, VAMP7, SNARE, cancer, neurodegeneration

OPEN ACCESS

Edited by:
Julien Villeneuve, Institute for Functional Genomics (IGF), CNRS, France

Reviewed by:
Sidney Whiteheart, University of Kentucky, United States
Marioara Chiritoiu-Butnaru, Institute of Biochemistry of the Romanian Academy, Romania

***Correspondence:**
Thierry Galli
thierry.galli@inserm.fr

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *25 February 2022*
Accepted: *27 May 2022*
Published: *16 June 2022*

Citation:
Vats S and Galli T (2022) Role of SNAREs in Unconventional Secretion—Focus on the VAMP7-Dependent Secretion. Front. Cell Dev. Biol. 10:884020. doi: 10.3389/fcell.2022.884020

THE SECRETORY PATHWAY

The secretory pathway deals with synthesis and delivery of proteins either membrane associated or not into the extracellular space and as receptors at the cell surface (Popescu, 2012). Secreted proteins which make up the secretome account for 9–15% of the total human proteome and serve major roles in cellular physiology, pathology and intercellular communication. Depending on the mode of secretion, the secretory pathway can be either conventional or unconventional. In this review we will focus on the molecular and cellular mechanisms of secretion in the extracellular space, and refer to other reviews regarding the transport of receptors to the cell surface.

CONVENTIONAL PROTEIN SECRETION

Classical or conventional secretory pathway begins at the Endoplasmic reticulum (ER) where new secreted protein synthesis occurs. A major early event in this route is the insertion of proteins destined to secretion, such as proteins of the extracellular matrix, cytokines, peptidic hormones and neuropeptides, into the lumen of the ER. This translocation is mediated by a short hydrophobic

sequence at the amino-terminus called leader sequence or signal peptide (Viotti, 2016). Hormone and neurotransmitter receptors, adhesion molecules and ionic pumps of the plasma membrane are additionally equipped with one or several transmembrane domains in addition of the leader sequence. These newly synthesized proteins then move to the Golgi apparatus (GA), after passing through and ER-Golgi intermediate compartment (ERGIC). After the GA, proteins destined to secretion are packed in secretory vesicles which subsequently transport them towards the plasma membrane. Finally, these secretory vesicles fuse with the plasma membrane thereby releasing their contents in the extracellular space. Conventional secretion can be constitutive or regulated, referring in general to a regulation by intracellular calcium concentration, sometimes to other second messengers (Benham, 2012). Constitutive secretion such as release of collagen, proteoglycans and interleukins occurs in all cells constantly while regulated secretion such as release of peptidic hormones like insulin or neuropeptides occurs in some specialized animal cells upon signaling cue. In the ER, secreted proteins undergo several important modifications: cleavage of the leader sequence, proteolysis and glycosylations. From the ER, the proteins exit in COP-II vesicles and take the route to the GA where they undergo further additional reactions of glycosylation and deglycosylation, sulfatation or phosphorylation (Ungar, 2009; Huang and Wang, 2017). These modifications occur in a well-ordered sequential manner from the cis-to the medial-to the trans-Golgi network (TGN). Secreted proteins are packaged into secretory vesicles at the exit of the TGN, in mechanisms involving different types of adaptors (Di Martino et al., 2019; Tan and Gleeson, 2019). The resulting secretory granules can further mature with a condensation of their content and the retrieval of some of its components (Hammel et al., 2010). Secretory vesicles are then transported towards the plasma membrane where they finally fuse (Burgess and Kelly, 1987; Benham, 2012; Viotti, 2016). Thul *et al.*, in 2017 published a subcellular map of the human proteome in which they identified 2,918 proteins secreted by the conventional secretion by using bioinformatic tools to score for signal peptide and transmembrane domains (Thul et al., 2017). Uhlen *et al.*, further enriched this knowledge in their comprehensive report on human secretome in which they tried to decipher the destinations of actively secreted human proteins (Uhlén et al., 2019).

UNCONVENTIONAL PROTEIN SECRETION

Work published over the past decade unearthed alternative routes which can bypass the need for passage through the GA and has been collectively termed as Unconventional protein secretion (UPS). Most of the proteins secreted by UPS are leaderless proteins, i.e., they lack targeting signal sequences and their mode of secretion has been classified into four classes: Type I, Type II and Type III and Type IV secretion.

Type I secretion is lipidic pore-mediated translocation of cytoplasmic proteins across the plasma membrane; Type II is ABC transporter-based secretion of acylated proteins, and Type III is packaging of cytoplasmic proteins in vesicles of autophagosomal/endosomal origin which fuse with the plasma membrane and release these proteins in the extracellular space. Type IV UPS involves transmembrane proteins with or without signal sequences which pass the ER and reach the plasma membrane for secretion without going through the Golgi apparatus. Type I and Type II secretion are non-vesicular and non-SNARE protein dependent whereas Type III and Type IV dependent on vesicles and on SNARE proteins (Rabouille, 2017; Dimou and Nickel, 2018). **Figure 1** summarizes the general features of conventional and unconventional secretion. Most of the UPS pathways seem to be triggered by stress conditions such as nutrient deprivation, ER stress, mechanical stress or inflammation and also in the context of cell growth (Wojnacki et al., 2020). This precludes the proper functioning of the ER-Golgi secretion system thereby aggravating the need of an alternate secretory system. The need to bypass the ER-Golgi pathway also arises for proteins such as Fibroblast growth factor 2 (FGF2) which could be rendered biologically inactive upon undergoing glycosylation (Rabouille, 2017; Dimou and Nickel, 2018). Finally, unconventional protein secretion is a way for secretion of proteins such as High mobility group box1 (HMGB1) and acyl-CoA binding protein (ACBP) which have different intracellular and extracellular functions in physiological versus stress conditions (Gardella et al., 2002; Duran et al., 2010). The secretion of extracellular vesicles is one of the most prominent unconventional secretory mechanisms unveiled in the last decades. It was initially discovered in cancer cells as the exfoliation of membrane ecto-enzymes in the form of micro-vesicles referred to as exosomes (Trams et al., 1981). Since then, the formation and release of extracellular vesicles has redefined many rules of secretory mechanism. Indeed, exosomes are formed by invagination of the limiting membrane of late endosomes, defining intraluminal vesicles, in a mechanism which require the ESCRT machinery (Adell et al., 2014). Exosomes are small and rather homogenous whereas other types of larger extracellular vesicles might be more heterogenous in nature as it was recently shown in the case of the release of amphisomes, a mixed secretory organelle with both autophagosomal and late endosomal origin (Jeppesen et al., 2019). The situation is further complicated by the occurrence of microvesicles or ectosomes which originate from the plasma membrane and share biophysical properties with exosomes but are still distinct extracellular vesicles (Mathieu et al., 2021). The recent work from our laboratory and the Demetriades and Debnath laboratories are coherent with the notion that late endosomes and autophagosomes are connected and that secretion might involve a mixed content (Leidal et al., 2020; Wojnacki et al., 2020; Nüchel et al., 2021). During their formation, intraluminal vesicles capture components of the limiting membrane of late endosomes such as tetraspanins (CD81, CD63) and cytosolic proteins. Cytosolic proteins captured into intraluminal vesicles lack a leader sequence like that of secreted peptides in the conventional route and there is not a defined consensus sequence that would target cytosolic proteins to intraluminal vesicles. Thus, whether intraluminal vesicles capture specific proteins or a random pool of the cytosol is debated. On one hand, GFP expressed into the cytosol can be

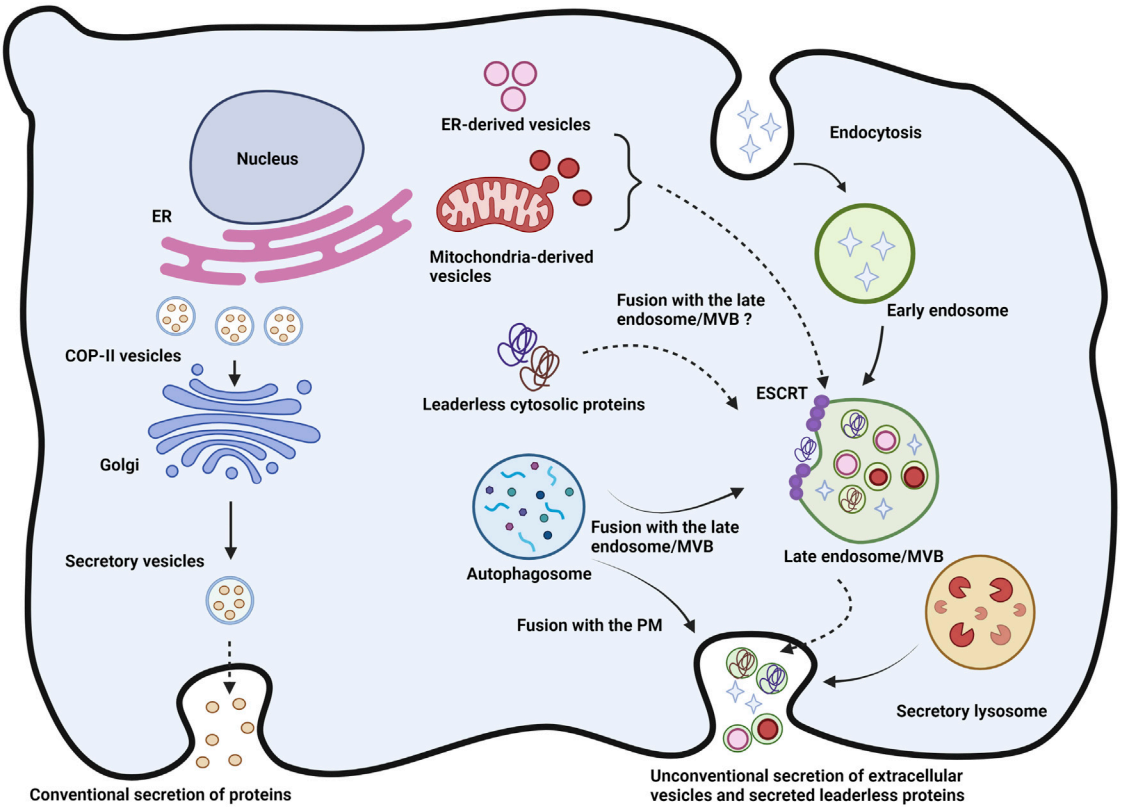


FIGURE 1 | Figure of unconventional vs. conventional secretions: Newly synthesized proteins from the ER and exported in COP-II vesicles towards the Golgi apparatus wherein they are packed in secretory vesicles for export *via* the conventional secretory pathway. Unconventional secretion, in particular the Type III secretion represented here involves packaging of cytosolic proteins as well as the possible targeting of autophagic, ER-derived and mitochondria-derived vesicles to the multivesicular bodies (MVBs) or late endosomes. The resultant intraluminal vesicle can then fuse with the plasma membrane and release exosomes and other mixed content. Autophagosomes and secretory lysosomes can fuse with the plasma membrane directly to release their content in the extracellular space. MVB: Multivesicular body; ILV: Intraluminal vesicle; PM: Plasma membrane. Role of SNAREs and their partners in unconventional secretion.

found in extracellular vesicles proportionally to its expression (Mathieu et al., 2021). On the other hand, certain proteins such cyclin D1 appear to be concentrated in extracellular vesicles in a mechanism relying on Hsc70 (Song et al., 2021). In any case, targeting to intraluminal vesicles and in consequence to extracellular vesicles lacks the identification of specific signals. In addition to cytosol, late endosomes are able to capture elements of the mitochondria *via* mitochondria-derived vesicles (McLelland et al., 2016), of endoplasmic reticulum, the Golgi apparatus and autophagosomes (Jeppesen et al., 2019; Wojnacki et al., 2020; Nüchel et al., 2021). Cytosolic misfolded proteins were shown to be captured by endoplasmic reticulum protein USP19, then transferred to late endosomes for secretion (Lee et al., 2016). It is not yet clear if these membranes are engulfed into nascent intraluminal vesicles or if they fuse with the limiting membrane of late endosome and then are incorporated into intraluminal vesicles. Of note, secretion of extracellular vesicles is necessarily accompanied by the release of the soluble content of late endosomes, and this is not yet well characterized. Secretion of extracellular vesicles might be regulated by calcium in certain cells but not all, and several studies suggest that mTOR inhibitors can stimulate this release (Wojnacki et al., 2020; Nüchel et al., 2021).

SNAREs (Soluble *N*-ethylmaleimide-sensitive factor attachment proteins receptors) are the key components of the intracellular membrane fusion machinery. Classically, SNAREs have been divided into two categories: v-SNAREs and t-SNAREs. As the name suggests, v-SNAREs are present on the transport vesicles whereas t-SNAREs are present on the target membrane (Jahn and Scheller, 2006; Südhof and Rothman, 2009).

Unconventional secretion of extracellular vesicles has been shown to depend on v-SNAREs such as VAMP3, VAMP7 and SEC22B in several studies in different cell types which we discussed below and is depicted in **Figure 2**. The members of the vesicle-associated membrane protein (VAMP) family have varied intracellular location. VAMP2 has been shown to be present at secretory granules and synaptic vesicles, VAMP3 at secretory granules and early endosomes, VAMP4 in the trans-Golgi, VAMP8 is present on recycling endosomes and also shares its location on late endosomes/MVBs along with VAMP7 (Steegmaier et al., 1999; Chen and Scheller, 2001; Pryor et al., 2004; Marshall et al., 2015; Gordon et al., 2017).

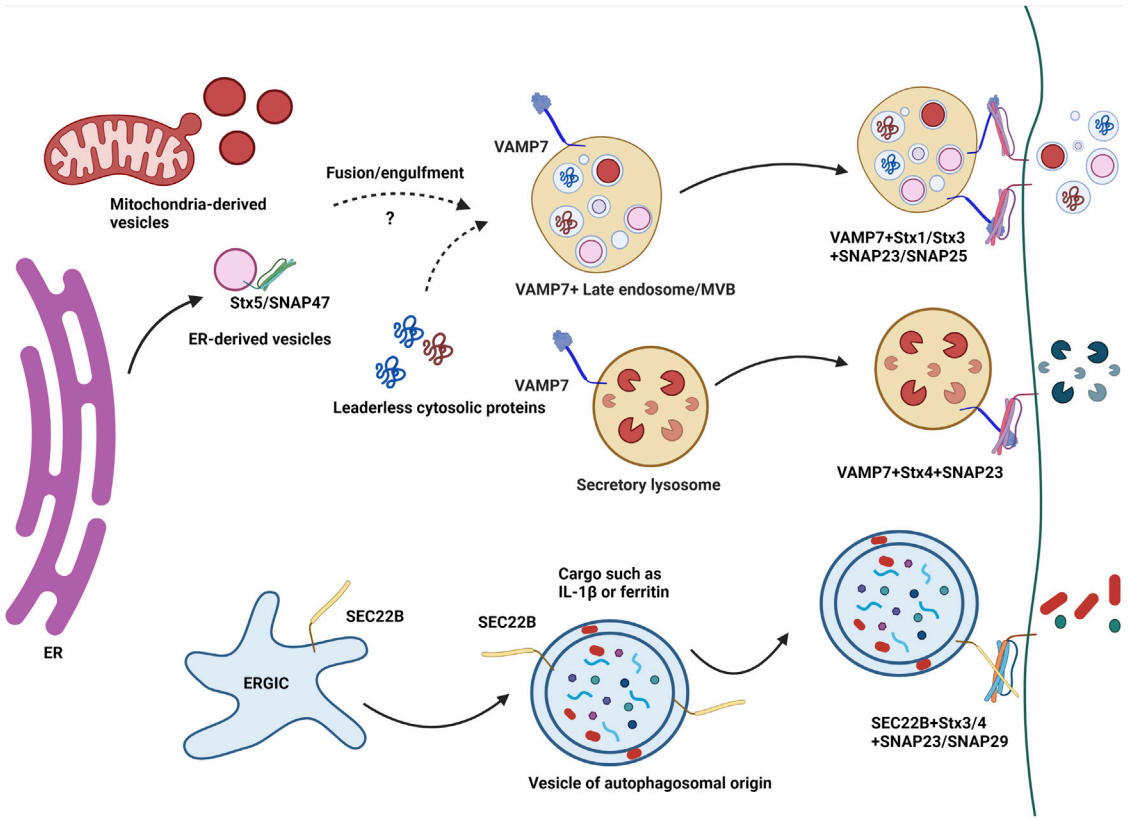


FIGURE 2 | A simplified model of the role of SNAREs in unconventional secretion. Unconventional secretion of extracellular vesicles has been shown to depend on v-SNAREs such as VAMP7 and SEC22B. ER-derived vesicles and mitochondria-derived vesicles may merge with VAMP7$^+$ late endosomes/MVBs in a SNARE dependent manner involving VAMP7, Stx5 and SNAP47. Lysosomal exocytosis i.e. the unconventional secretion of lysosomal contents upon the fusion of lysosomes with the plasma membrane involves VAMP7 along with t-SNARES Syntaxin4 and SNAP23. Vojo Deretic's group has shown the involvement of SEC22B with SNAP23 or SNAP29 and PM SNAREs Syntaxin3/4 in the unconventional secretion of leaderless proteins such as IL-1β and ferritin.

VAMP7, is a SNARE which is insensitive to tetanus and botulinum neurotoxins (hence its other name TI-VAMP for Tetanus neurotoxin Insensitive Vesicle-Associated Membrane Protein) (Galli et al., 1998). VAMP7 is mainly localized to late endosomes and also to the Golgi apparatus and small peripheral vesicles, and particularly colocalizes with CD63 a marker of secretory late endosomes and lysosomes (Advani et al., 1999; Coco et al., 1999; Pols et al., 2013). A particular point of note is that although VAMP7 and VAMP8 share subcellular location on late endosomes and MVBs as mentioned above, according to the human protein atlas and in several studies (Wade et al., 2001; Sato et al., 2011), the expression of VAMP8 is limited more in epithelial and immune cells and is negligible in the brain whereas the expression of VAMP7 has been observed in all cell types (https://www.proteinatlas.org). VAMP7 has the classical SNARE sequence with a SNARE motif and a C-terminal transmembrane domain but also includes an N-terminal extension which is called Longin domain (Filippini et al., 2001). The Longin domain plays an auto-inhibitory role through intramolecular interaction with the SNARE motif (Vivona et al., 2010) thus controlling the fusogenic activity of VAMP7 (Martinez-Arca et al., 2000, 2003). VAMP7 interacts with SNARE partners located at the plasma membrane: Syntaxin 1, Syntaxin 3, SNAP-23 and SNAP-25, autophagosome including Syntaxin 17 and SNAP-29 and ERGIC SNAP-47 (Alberts et al., 2003; Kuster et al., 2015). In particular, VAMP7/Syntaxin 1/SNAP25 and VAMP7/Syntaxin 3/SNAP-23 mediate the fusion of secretory late endosomes with the plasma membrane (Chaineau et al., 2009). Accordingly, Verweij et al. used CD63-pHluorin as an optical reporter to monitor MVB-PM fusion events and observed an increase in release of CD63$^+$ exosomes upon phosphorylation of the t-SNARE SNAP23 by histamine H1 receptor mediated signalling (Verweij et al., 2018). Lysosomal exocytosis is an unconventional secretion of lysosomal contents upon the fusion of lysosomes with the plasma membrane. VAMP7 along with t-SNARES Syntaxin4 and SNAP23 mediates lysosomal secretion in fibroblasts (Martinez et al., 2000; Rao et al., 2004; Proux-Gillardeaux et al., 2007) and lysosomal secretion of ATP in astrocytes (Verderio et al., 2012).

Our latest work showed that, in differentiating neuronal cells, VAMP7-dependent late endosomal secretion is also involved in releasing reticulons and atlastins, components of the endoplasmic reticulum, particularly the short form of reticulon 3 (Wojnacki et al., 2020), all molecules which have been linked to axonal

growth and regeneration, and neurodegeneration (Yan et al., 2006; Behrendt et al., 2019). This important result was provided by detailed proteomic analysis of the cell lysate and secretome of WT, autophagy-null ATG5 KO and VAMP7 KO PC12 cells. We found that WT cells released proteins which were significantly less abundant in the VAMP7 KO secretome significantly increased in ATG5 KO (Reticulon/RTN1, CALCOCO1, Atlastin/ATL1, SQSTM1/p62, MAP1LC3B/LC3b, RTN4, MAP1LC3A/LC3a, GABARAP, GABARAPL2, RTN3, ATL3) therefore correlating with decreased neurite growth in VAMP7 KO and increased neurite growth and ramification in ATG5 KO PC12 cells. We did not find any KFERQ containing proteins, i.e., markers of the chaperone-mediate autophagy (CMA) pathway (Kirchner et al., 2019; Sahu et al., 2011), which would be significantly enriched in ATG5 KO and decreased in VAMP7 KO secretome. In conclusion, we found that VAMP7 KO and ATG5 KO, which have opposite effects on neurite growth, had clear opposite effects in the secretion of RTN3 which is related to ER-phagy. This secretion appeared particularly enhanced when degradation by autophagy of the endoplasmic reticulum is blocked, such as in ATG5 KO neuronal cells and upon treatment with autophagy blocker bafilomycin A1. This led us to define secretory reticulophagy as a new VAMP7-dependent secretory activity (Vats and Galli, 2021). Interestingly, our findings also align with a recent paper demonstrating that components of the LC3 conjugation machinery regulate and specify cargo loading and secretion *via* extracellular vesicles. Indeed, Leidal et al., found that RNA-binding proteins get packed into extracellular vesicles (EVs) by binding to LC3 via LC3 interacting regions. They term this secretion as LC3-dependent EV loading and secretion (LDELS) (Leidal et al., 2020). Autophagy dependent secretion of modified histone H3 which can be enhanced upon rapamycin treatment or by hypoxia has also been reported (Sulkowski et al., 2021). Also of note, is the fact that VAMP7 was the only identified v-SNARE bearing an LC3-interacting region (Gu et al., 2019). In conclusion, VAMP7 is strongly connected to autophagy-related UPS. To gain further insights on how ATG5 and VAMP7 might regulate neurite growth, we also carried out lipidomic analysis of WT, VAMP7 KO and ATG5 KO PC12 cells. In ATG5 KO cells, enhanced levels of several glucosylceramides (GluCers) and reduced sphingomyelins (SMs) is in good agreement with previous report on the inhibitory effects of glucosylceramide synthase inhibitor on neurite outgrowth in PC12 cells (Mutoh et al., 1998) and accumulation of ceramides in Arabidopsis upon ATG5 inactivation (Havé et al., 2019). VAMP7 KO cells exhibited reduced levels of phosphatidylethanolamines (PEs) in good agreement with the finding that the ethanolamine moiety of PE derived from phosphatidylserine is actively re-acylated only in PC12 cells undergoing NGF-induced neuritogenesis (Ikemoto and Okuyama, 2000). This result is particularly interesting because LC3 and other ATG8 molecules bind PE (Kabeya et al., 2004; Thukral et al., 2015). It will be critical to further characterize how UPS regulates lipid homeostasis.

A persistent question in the field of UPS particularly, the Type III secretion, is how leaderless proteins are packaged into vesicles of autophagosomal/endosomal origins. A recent work by Zhang et al., has described a protein translocation pathway regulated by transmembrane emp24 domain containing protein 10 (TMED10) which can facilitate the transfer of several leaderless UPS cargos into the ERGIC and furthermore into secretory vesicles by the oligomerization of TMED10 (Zhang et al., 2020). TMED10 interacts with the small GTPase Rab21 and this might regulate packaging and release of UPS cargos (Del Olmo et al., 2019). Interestingly enough, VAMP7 interacts with Vps9 and Ankyrin repeat protein (Varp) (Burgo et al., 2009), an exchange factor for Rab21 (Zhang et al., 2006) and effector of Rab32/38 (Wang et al., 2008). Varp interacts with the closed conformation of VAMP7 (Schäfer et al., 2012). Our lab showed that VAMP7 is the starting point of a molecular network that combines proteins belonging to the main classes involved in vesicular trafficking: Varp, kinesin 1 (Kif5A), a molecular motor partner of Varp, GolginA4, a Golgi attachment factor partner of Varp, and the spectraplakin MACF1, an effector of Rab21 (Burgo et al., 2012), which binds both actin and microtubules. We found that this network can send VAMP7 vesicles to the cell periphery along microtubules, thus allowing exocytosis (Burgo et al., 2012; Wang et al., 2018). Varp also interacts with Vps29, a retromer complex subunit involved in Alzheimer disease (Shannon et al., 2014), and this interaction mediates its endosomal membrane targeting. Interestingly, transport of GLUT1 from endosomes to the cell surface requires Varp, VPS29, and VAMP7 and depends on the direct interaction between VPS29 and Varp (Hesketh et al., 2014). Recent work on GRASP55-dependent unconventional secretion also provides strength to the notion that late endosomes and autophagosomes are connected and that secretion might involve a mixed content (Nüchel et al., 2021). GRASP-55 further appeared in the proximome of VAMP7 (Hesketh et al., 2020). Synaptotagmin 7 was found as a VAMP7 partner (Rao et al., 2004) and it is involved in exosome secretion (Hoshino et al., 2013). In conclusion, at least some members of the VAMP7 interactome such as Rab21, GRASP-55 and Synaptotagmin7 appear to be involved in unconventional secretion as discussed above. The detailed molecular mechanisms still require investigation.

Owing to the potential functional redundancy in post-Golgi v-SNAREs, constitutive secretion from the Golgi was shown to be unaffected by depletion of VAMPs 3, 4, 7, 8, and YKT6 individually or in combination in human cells (Gordon et al., 2010). However, in *Drosophila*, depletion of YKT6 caused partial inhibition and the combinatorial depletion of YKT6 and VAMP3, an almost complete block in constitutive conventional secretion (Gordon et al., 2017). Interestingly, YKT6 is also required for the secretion of Wnt proteins in exosomes (Gross et al., 2012) and VAMP3 is involved in the exosome secretion evoked by FGF-2 (Kumar et al., 2020). VAMP3 and SNAP23 were also shown to be involved in the unconventional secretion of tissue transglutaminase in mouse fibroblasts and human endothelial cells (Zemskov et al., 2011). How the functions of YKT6 and VAMP3 in both conventional and unconventional secretions might or not be coordinated will require further investigation. Another SNARE of importance in unconventional secretion is SEC22B. SEC22B, is a longin v-SNARE involved in ERGIC trafficking (Jahn and Scheller, 2006). SEC22B with SNAP23 or

SNAP29 and PM SNAREs Syntaxin3/4 is involved in the unconventional secretion of leaderless proteins such as IL-1β and ferritin as shown in human immune cells (Kimura et al., 2017).

V-SNARE VAMP8 can also interact with PM SNAREs such as SNAP23 and Syntaxin4, but there is limited evidence to suggest its role in UPS. Pilliod et al. (2020), show that in neuroblastoma cell lines, the overexpression of VAMP8 can decrease the cellular load of mutated tau proteins by increasing their secretion. However, as the authors mention, the expression of VAMP8 in the brain is extremely low and hence they suggest that another v-SNARE might be involved in the secretion of tau in neurons (Pilliod et al., 2020). Whether this v-SNARE could be VAMP7, however, remains to be proven experimentally. Recently, an alternative protein quality mechanism to tackle misfolded protein was proposed by Lee et al. This pathway termed as misfolding-associated protein secretion (MAPS) is dependent on the ER associated deubiquitylase USP19 and is involved in the unconventional secretion of cytosolic misfolded proteins. They further show the involvement of late endosome resident SNAREs VAMP7 and VAMP8 in this secretion (Lee et al., 2016). The function of VAMP8 in the fusion of autophagosome with lysosome is very clear. This membrane fusion involves Stx17 and SNAP29 as t-SNARE (Diao et al., 2015; Huang et al., 2021). SNAP29's role in this membrane fusion mechanism is negatively regulated by O-GlcN-acetylation (Guo et al., 2014). Interestingly enough we found an inhibitory effect of the overexpression of SNAP-29 on the exocytic functions of VAMP7 (Kuster et al., 2015). Altogether, this suggests that SNAP-29 expression and regulation might play a central role in the balance between degradative autophagy (which involves VAMP8) and autophagic secretion (which involves VAMP7).

In conclusion, several v-SNAREs (VAMP7, VAMP3, YKT6, SEC22B) might be involved in unconventional secretion of EVs and that might depend on the cell type and signalling mechanisms. Nevertheless, compelling evidence point to VAMP7 and SNAP-23 as central v- and t-SNAREs in this process.

ROLE OF UNCONVENTIONAL SECRETION IN HEALTH AND DISEASE

The characterization of the role of unconventionally secreted proteins in shaping the physiological and pathological cellular microenvironment is still developing. Indeed, tumor microenvironment has emerged as a main feature in cancer initiation and progression (Anderson and Simon, 2020). In addition, non-neuronal cells which contribute to the neuronal microenvironment as much as neurons have been implicated in neurodegeneration (Phatnani and Maniatis, 2015). Secreted small molecules and metabolites, nucleic acids, diffusible proteins and extracellular vesicles, are all components of the secretome, which is a source of biomarkers (Uhlén et al., 2019). The secretome can represent the cellular microenvironment in health and disease, as exemplified in the case of the senescence-associated secretome which appears as an indicator of age and medical risk (Schafer et al., 2020).

Unconventional Secretion in Cancer

A cancerous mass typically consists of heterogenous cancerous cells as well as resident and infiltrating host cells. This entire mixed population of cells can secrete factors either in a conventional or unconventional manner in the extracellular space. Hence, when we talk about cancer secretome, it most likely includes proteins secreted by both cancerous and non-cancerous cells. These tumor cells, host stromal cells, secreted factors and extracellular matrix proteins together make the tumor microenvironment. The fate of cancer progression is largely dependent on interactions between tumor and host cells and the secreted factors secreted by them facilitates this intercellular communication. Hence, cancer secretomes can be of great potential interest as putative therapeutic targets. Apart from mediating interaction with host stromal cells, the secreted factors aid in recruitment of vascular endothelial cells, infiltrating immune cells and cancer associated fibroblasts (Paltridge et al., 2013). Tumor cell secretome is comprised of cytokines, growth factors, enzymes, glycoproteins and extracellular vesicles. Proteomic studies have shown the tumor cell secretome to be markedly different from healthy cell secretome. Thereby, it has been envisioned that cancer secretomes can be a treasure trove of potentially specific cancer biomarkers and can aid in cancer screening and detection (Xue et al., 2008; Mustafa et al., 2017; Madden et al., 2020). Secreted proteins can affect self or adjacent cells or nearby tissues in an autocrine, paracrine or endocrine manner. Tumor cell secretion can induce malignant transformation of normal epithelial cells nearby. Vascular endothelial growth factor (VEGF) secreted by tumor cells plays a role in angiogenesis and enhancing vascularization (Barbera-Guillem et al., 2002). Secreted Epidermal growth factor (EGF) and Transforming growth factor-β (TGF-β) triggers signaling pathways such as PI3K/Akt and Ras/Raf/MAPK which aid in progression of cancer (Larue and Bellacosa, 2005). Matrix metalloproteinases which digest extracellular matrices help with tumor invasiveness and migration while secreted cytokines enhance inflammation by recruiting inflammatory cells (Zucker and Vacirca, 2004; Paltridge et al., 2013). The communication between neurons and tumor cells including glioblastoma cells was proposed to play an important role owing to the release of small molecules like glutamate (Takano et al., 2001; Venkataramani et al., 2019) and serine (Banh et al., 2020).

Exosomes have been shown to be important in cancer and they are also thought to have therapeutic interests, which have already been recently reviewed (Dai et al., 2020). In addition, autophagy is thought to suppress early-stage but to promote late-stage tumor development, a dual effect which might be related to autophagy-dependent paracrine mechanisms thus tumor microenvironment. Because the core of solid tumors is hypoxic therefore under metabolic stress, autophagy is likely to play an important function in tumor initiation and growth (Jin and White, 2008). As pointed in the sections above, VAMP7 acts as a central v-SNARE in regulating unconventional secretion. In specific relation to the above-mentioned molecular and cellular mechanisms of UPS, recent genetic studies particularly using transcriptomics have linked VAMP7 expression to several cancers (Zhu et al., 2020; Wang et al., 2021; Xu et al., 2021; Li et al., 2022). Furthermore, VAMP7 mediates the exosomal release of miR-375 (Kumar et al., 2021), which was involved in

glioblastoma and matrix metalloproteases (Steffen et al., 2008). GRASP55 was shown to mediate the release of matrix metalloproteases by late endosomes and autophagosomes (Nüchel et al., 2021) and matrix metalloproteases play a key role in cancer cell invasion and dissemination (Kessenbrock et al., 2010). Interestingly, matrix metalloproteases have been identified in extracellular vesicles (Shimoda and Khokha, 2017). It will be important to characterize the potential role of VAMP7- and GRASP55-dependent unconventional secretion of miRNA and matrix metalloproteases in tumor development. Additionally, regulated lysosomal exocytosis which involves VAMP7 has also been shown to enhance sarcoma progression by exacerbating the release of lysosomal hydrolases (Machado et al., 2015).

Unconventional Secretion in Neurodegeneration

Extracellular vesicles, particularly small EVs or exosomes have been shown to have an important role in the propagation of neuropathology (Vassileff et al., 2020). Lee et al., reported a misfolding associated protein secretion pathway which uses deubiquitylase USP19 to export misfolded cytosolic proteins (Lee et al., 2016). Defects in protein quality control is a central cause in several neurodegenerative diseases. Parkinson's disease is characterized by protein degradation, endolysosomal and mitochondrial dysfunctions including autophagy impairments. Familial and sporadic forms of Parkinson's disease involve mutations in PARK genes like LRRK2, PRKN, VPS35, SNCA which can affect many cell types but seem to lead to the death of only dopaminergic neurons in the brain (Panicker et al., 2021). Non-cell autonomous mechanisms related to the secretome particularly involving astrocytes (Di Domenico et al., 2019) could be part of the complex physiopathology of Parkinson's disease.

There are several reports of aggregate prone proteins getting secreted in the extracellular space following the Type III UPS. Increasing lysosomal exocytosis can protect human dopaminergic neurons from alpha-synuclein toxicity by releasing it in the extracellular space (Tsunemi et al., 2019). Mutant huntingtin (mHtt), a protein whose aggregation results in Huntington's disease has also been shown to be secreted via late endosomal/lysosomal unconventional secretion (Trajkovic et al., 2017). The phosphorylation of mHtt at S421 also affects the intracellular transport of VAMP7 positive vesicles (Colin et al., 2008). Earlier work from our lab shows the transport of amyloid precursor protein and the endogenous GPI anchored cellular prion protein in a VAMP7 dependent manner (Molino et al., 2015; Wang et al., 2018). A recent report also suggests that mHtt is unconventionally secreted in a GRASP55 dependent manner (Ahat et al., 2021). VAMP7-dependent secretion mediates the release of α-syn aggregates (Xie et al., 2021). Late endosomes are important for the clearance of protein aggregates associated with neurodegenerative disease (Filimonenko et al., 2007) but it is not known if this could be dependent on late endosomal secretion. It will be now important to characterize the potential role of VAMP7- and GRASP55-dependent unconventional secretion of protein aggregates and other elements of late endosomes and autophagosomes. Whether the secretory mechanisms are part of the initiation of neurodegeneration, an early event related to the microenvironment of fragile neurons and/or participating in the propagation of prion-like proteins (Rastogi et al., 2021) remain to be explored in details.

CONCLUSION: A PERSPECTIVAL INTEGRATED VISION OF SECRETION

Compared to synaptic vesicle exocytosis, unconventional secretion of late endosomes is rather slow, likely calcium-independent in most cases, neither quantal nor truly scalable (due to the heterogeneity of late endosomes and ILVs), not sustainable (biogenesis is very complex, no true recycling mechanism unlike synaptic vesicles) and possibly even serendipitous regarding the intraluminal vesicles' capture of cytosol (in the absence of a specific targeting mechanism, there might only be a concentration mechanism for certain cargoes). One might even think that unconventional secretion utilizing late endosomes, for all these shortcomings might represent a primitive form of secretion. From this point of view, it is now rather critical to have an evolutionary perspective on secretory mechanisms: how, when did the different modes of secretion appear during evolution and which are the protein ancestors mediating the basic mechanisms.

In this review, we attempt to focus on the vesicle mediated Type III UPS in which several v-SNAREs are involved. We take an in-depth look at the current body of work which contribute towards delineating the role of VAMP7 in unconventional secretion. Owing to the important role played by unconventional secretion in health and disease, we believe that further explorations into the mechanistic details of the VAMP7 mediated unconventional secretion can provide a clearer view of its impact on cellular physiology and pathology.

AUTHOR CONTRIBUTIONS

SV and TG conceptualized and wrote the manuscript. TG supervised the writing and editing of the manuscript.

FUNDING

Work in our group was funded by grants from the French National Research Agency (MetDePaDi ANR-16-CE16-0012), the Institut National du Cancer (PLBIO 2018-149), the Fondation pour la Recherche Médicale (FRM, Labélisation and postdoctoral fellowship to SV), Fondation de France (00096652), and Fondation Bettencourt-Schueller (Coup d'Elan) to TG.

ACKNOWLEDGMENTS

We thank all members of the laboratory for their assistance and the discussions. We also thank the website Biorender for the aid in figure creation.

REFERENCES

Adell, M. A. Y., Vogel, G. F., Pakdel, M., Müller, M., Lindner, H., Hess, M. W., et al. (2014). Coordinated Binding of Vps4 to ESCRT-III Drives Membrane Neck Constriction during MVB Vesicle Formation. *J. Cell Biol.* 205, 33–49. doi:10.1083/jcb.201310114

Advani, R. J., Yang, B., Prekeris, R., Lee, K. C., Klumperman, J., and Scheller, R. H. (1999). VAMP-7 Mediates Vesicular Transport from Endosomes to Lysosomes. *J. Cell Biol.* 146, 765–776. doi:10.1083/jcb.146.4.765

Ahat, E., Bui, S., Zhang, J., da Veiga Leprevost, F., Sharkey, L., Reid, W., et al. (2021). GRASP55 Regulates Mutant Huntingtin Unconventional Secretion and Aggregation. *BioRxiv*. doi:10.1101/2021.10.05.463252

Alberts, P., Rudge, R., Hinners, I., Muzerelle, A., Martinez-Arca, S., Irinopoulou, T., et al. (2003). Cross Talk between Tetanus Neurotoxin-Insensitive Vesicle-Associated Membrane Protein-Mediated Transport and L1-Mediated Adhesion. *MBoC* 14, 4207–4220. doi:10.1091/mbc.E03-03-0147

Anderson, N. M., and Simon, M. C. (2020). The Tumor Microenvironment. *Curr. Biol.* 30, R921–R925. doi:10.1016/j.cub.2020.06.081

Banh, R. S., Biancur, D. E., Yamamoto, K., Sohn, A. S. W., Walters, B., Kuljanin, M., et al. (2020). Neurons Release Serine to Support mRNA Translation in Pancreatic Cancer. *Cell* 183, 1202–1218. e25. doi:10.1016/j.cell.2020.10.016

Barbera-Guillem, E., Nyhus, J. K., Wolford, C. C., Friece, C. R., and Sampsel, J. W. (2002). Vascular Endothelial Growth Factor Secretion by Tumor-Infiltrating Macrophages Essentially Supports Tumor Angiogenesis, and IgG Immune Complexes Potentiate the Process. *Cancer Res.* 62, 7042–7049.

Behrendt, L., Kurth, I., and Kaether, C. (2019). A Disease Causing ATLASTIN 3 Mutation Affects Multiple Endoplasmic Reticulum-Related Pathways. *Cell. Mol. Life Sci.* 76, 1433–1445. doi:10.1007/s00018-019-03010-x

Benham, A. M. (2012). Protein Secretion and the Endoplasmic Reticulum. *Cold Spring Harb. Perspect. Biol.* 4, a012872. doi:10.1101/cshperspect.a012872

Burgess, T. L., and Kelly, R. B. (1987). Constitutive and Regulated Secretion of Proteins. *Annu. Rev. Cell. Biol.* 3, 243–293. doi:10.1146/annurev.cb.03.110187.001331

Burgo, A., Proux-Gillardeaux, V., Sotirakis, E., Bun, P., Casano, A., Verraes, A., et al. (2012). A Molecular Network for the Transport of the TI-VAMP/VAMP7 Vesicles from Cell Center to Periphery. *Dev. Cell* 23, 166–180. doi:10.1016/j.devcel.2012.04.019

Burgo, A., Sotirakis, E., Simmler, M. C., Verraes, A., Chamot, C., Simpson, J. C., et al. (2009). Role of Varp, a Rab21 Exchange Factor and TI-VAMP/VAMP7 Partner, in Neurite Growth. *EMBO Rep.* 10, 1117–1124. doi:10.1038/embor.2009.186

Chaineau, M., Danglot, L., and Galli, T. (2009). Multiple Roles of the Vesicular-SNARE TI-VAMP in Post-Golgi and Endosomal Trafficking. *FEBS Lett.* 583, 3817–3826. doi:10.1016/j.febslet.2009.10.026

Chen, Y. A., and Scheller, R. H. (2001). SNARE-mediated Membrane Fusion. *Nat. Rev. Mol. Cell Biol.* 2, 98–106. doi:10.1038/35052017

Coco, S., Raposo, G., Martinez, S., Fontaine, J.-J., Takamori, S., Zahraoui, A., et al. (1999). Subcellular Localization of Tetanus Neurotoxin-Insensitive Vesicle-Associated Membrane Protein (VAMP)/VAMP7 in Neuronal Cells: Evidence for a Novel Membrane Compartment. *J. Neurosci.* 19, 9803–9812. doi:10.1523/JNEUROSCI.19-22-09803.1999

Colin, E., Zala, D., Liot, G., Rangone, H., Borrell-Pagès, M., Li, X.-J., et al. (2008). Huntingtin Phosphorylation Acts as a Molecular Switch for Anterograde/retrograde Transport in Neurons. *EMBO J.* 27, 2124–2134. doi:10.1038/emboj.2008.133

Dai, J., Su, Y., Zhong, S., Cong, L., Liu, B., Yang, J., et al. (2020). Exosomes: Key Players in Cancer and Potential Therapeutic Strategy. *Sig Transduct. Target Ther.* 5, 145. doi:10.1038/s41392-020-00261-0

Del Olmo, T., Lacarrière-Keïta, C., Normandin, C., Jean, D., Boisvert, F.-M., and Jean, S. (2019). RAB21 Interacts with TMED10 and Modulates its Localization and Abundance. *Biol. Open* 8. doi:10.1242/bio.045336

Di Domenico, A., Carola, G., Calatayud, C., Pons-Espinal, M., Muñoz, J. P., Richaud-Patin, Y., et al. (2019). Patient-Specific iPSC-Derived Astrocytes Contribute to Non-Cell-Autonomous Neurodegeneration in Parkinson's Disease. *Stem Cell Rep.* 12, 213–229. doi:10.1016/j.stemcr.2018.12.011

Di Martino, R., Sticco, L., and Luini, A. (2019). Regulation of Cargo Export and Sorting at the trans-Golgi Network. *FEBS Lett.* 593, 2306–2318. doi:10.1002/1873-3468.13572

Diao, J., Liu, R., Rong, Y., Zhao, M., Zhang, J., Lai, Y., et al. (2015). ATG14 Promotes Membrane Tethering and Fusion of Autophagosomes to Endolysosomes. *Nature* 520, 563–566. doi:10.1038/nature14147

Dimou, E., and Nickel, W. (2018). Unconventional Mechanisms of Eukaryotic Protein Secretion. *Curr. Biol.* 28, R406–R410. doi:10.1016/j.cub.2017.11.074

Duran, J. M., Anjard, C., Stefan, C., Loomis, W. F., and Malhotra, V. (2010). Unconventional Secretion of Acb1 Is Mediated by Autophagosomes. *J. Cell Biol.* 188, 527–536. doi:10.1083/jcb.200911154

Filimonenko, M., Stuffers, S., Raiborg, C., Yamamoto, A., Malerød, L., Fisher, E. M. C., et al. (2007). Functional Multivesicular Bodies Are Required for Autophagic Clearance of Protein Aggregates Associated with Neurodegenerative Disease. *J. Cell Biol.* 179, 485–500. doi:10.1083/jcb.200702115

Filippini, F., Rossi, V., Galli, T., Budillon, A., D'Urso, M., and D'Esposito, M. (2001). Longins: a New Evolutionary Conserved VAMP Family Sharing a Novel SNARE Domain. *Trends Biochem. Sci.* 26, 407–409. doi:10.1016/s0968-0004(01)01861-8

Galli, T., Zahraoui, A., Vaidyanathan, V. V., Raposo, G., Tian, J. M., Karin, M., et al. (1998). A Novel Tetanus Neurotoxin-Insensitive Vesicle-Associated Membrane Protein in SNARE Complexes of the Apical Plasma Membrane of Epithelial Cells. *MBoC* 9, 1437–1448. doi:10.1091/mbc.9.6.1437

Gardella, S., Andrei, C., Ferrera, D., Lotti, L. V., Torrisi, M. R., Bianchi, M. E., et al. (2002). The Nuclear Protein HMGB1 Is Secreted by Monocytes via a Non-classical, Vesicle-mediated Secretory Pathway. *EMBO Rep.* 3, 995–1001. doi:10.1093/embo-reports/kvf198

Gordon, D. E., Bond, L. M., Sahlender, D. A., and Peden, A. A. (2010). A Targeted siRNA Screen to Identify SNAREs Required for Constitutive Secretion in Mammalian Cells. *Traffic* 11, 1191–1204. doi:10.1111/j.1600-0854.2010.01087.x

Gordon, D. E., Chia, J., Jayawardena, K., Antrobus, R., Bard, F., and Peden, A. A. (2017). VAMP3/Syb and YKT6 Are Required for the Fusion of Constitutive Secretory Carriers with the Plasma Membrane. *PLoS Genet.* 13, e1006698. doi:10.1371/journal.pgen.1006698

Gross, J. C., Chaudhary, V., Bartscherer, K., and Boutros, M. (2012). Active Wnt Proteins Are Secreted on Exosomes. *Nat. Cell Biol.* 14, 1036–1045. doi:10.1038/ncb2574

Gu, Y., Princely Abudu, Y., Kumar, S., Bissa, B., Choi, S. W., Jia, J., et al. (2019). Mammalian Atg8 Proteins Regulate Lysosome and Autolysosome Biogenesis through SNARE S. *EMBO J.* 38, e101994. doi:10.15252/embj.2019101994

Guo, B., Liang, Q., Li, L., Hu, Z., Wu, F., Zhang, P., et al. (2014). O-GlcNAc-modification of SNAP-29 Regulates Autophagosome Maturation. *Nat. Cell Biol.* 16, 1215–1226. doi:10.1038/ncb3066

Hammel, I., Lagunoff, D., and Galli, S. J. (2010). Regulation of Secretory Granule Size by the Precise Generation and Fusion of Unit Granules. *J. Cell Mol. Med.* 14, 1904–1916. doi:10.1111/j.1582-4934.2010.01071.x

Hesketh, G. G., Papazotos, F., Pawling, J., Rajendran, D., Knight, J. D. R., Martinez, S., et al. (2020). The GATOR-Rag GTPase Pathway Inhibits mTORC1 Activation by Lysosome-Derived Amino Acids. *Science* 370, 351–356. doi:10.1126/science.aaz0863

Hesketh, G. G., Pérez-Dorado, I., Jackson, L. P., Wartosch, L., Schäfer, I. B., Gray, S. R., et al. (2014). VARP Is Recruited on to Endosomes by Direct Interaction with Retromer, where Together They Function in Export to the Cell Surface. *Dev. Cell* 29, 591–606. doi:10.1016/j.devcel.2014.04.010

Hoshino, D., Kirkbride, K. C., Costello, K., Clark, E. S., Sinha, S., Grega-Larson, N., et al. (2013). Exosome Secretion Is Enhanced by Invadopodia and Drives Invasive Behavior. *Cell Rep.* 5, 1159–1168. doi:10.1016/j.celrep.2013.10.050

Huang, H., Ouyang, Q., Zhu, M., Yu, H., Mei, K., and Liu, R. (2021). mTOR-mediated Phosphorylation of VAMP8 and SCFD1 Regulates Autophagosome Maturation. *Nat. Commun.* 12, 6622. doi:10.1038/s41467-021-26824-5

Huang, S., and Wang, Y. (2017). Golgi Structure Formation, Function, and Post-translational Modifications in Mammalian Cells. *F1000Res.* 6, 2050. doi:10.12688/f1000research.11900.1

Jahn, R., and Scheller, R. H. (2006). SNAREs - Engines for Membrane Fusion. *Nat. Rev. Mol. Cell Biol.* 7, 631–643. doi:10.1038/nrm2002

Jeppesen, D. K., Fenix, A. M., Franklin, J. L., Higginbotham, J. N., Zhang, Q., Zimmerman, L. J., et al. (2019). Reassessment of Exosome Composition. *Cell* 177, 428–445. e18. doi:10.1016/j.cell.2019.02.029

Jin, S. V., and White, E. (2008). Tumor Suppression by Autophagy through the Management of Metabolic Stress. *Autophagy* 4, 563–566. doi:10.4161/auto.5830

Kessenbrock, K., Plaks, V., and Werb, Z. (2010). Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell* 141, 52–67. doi:10.1016/j.cell.2010.03.015

Kimura, T., Jia, J., Kumar, S., Choi, S. W., Gu, Y., Mudd, M., et al. (2017). Dedicated SNARE S and Specialized TRIM Cargo Receptors Mediate Secretory Autophagy. *EMBO J.* 36, 42–60. doi:10.15252/embj.201695081

Kumar, A., Ren, Y., Sundaram, K., Mu, J., Sriwastva, M. K., Dryden, G. W., et al. (2021). miR-375 Prevents High-Fat Diet-Induced Insulin Resistance and Obesity by Targeting the Aryl Hydrocarbon Receptor and Bacterial Tryptophanase (tnaA) Gene. *Theranostics* 11, 4061–4077. doi:10.7150/thno.52558

Kumar, R., Tang, Q., Müller, S. A., Gao, P., Mahlstedt, D., Zampagni, S., et al. (2020). Fibroblast Growth Factor 2-Mediated Regulation of Neuronal Exosome Release Depends on VAMP3/Cellubrevin in Hippocampal Neurons. *Adv. Sci.* 7, 1902372. doi:10.1002/advs.201902372

Kuster, A., Nola, S., Dingli, F., Vacca, B., Gauchy, C., Beaujouan, J.-C., et al. (2015). The Q-Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor (Q-SNARE) SNAP-47 Regulates Trafficking of Selected Vesicle-Associated Membrane Proteins (VAMPs). *J. Biol. Chem.* 290, 28056–28069. doi:10.1074/jbc.M115.666362

Larue, L., and Bellacosa, A. (2005). Epithelial-mesenchymal Transition in Development and Cancer: Role of Phosphatidylinositol 3′ Kinase/AKT Pathways. *Oncogene* 24, 7443–7454. doi:10.1038/sj.onc.1209091

Lee, J.-G., Takahama, S., Zhang, G., Tomarev, S. I., and Ye, Y. (2016). Unconventional Secretion of Misfolded Proteins Promotes Adaptation to Proteasome Dysfunction in Mammalian Cells. *Nat. Cell Biol.* 18, 765–776. doi:10.1038/ncb3372

Leidal, A. M., Huang, H. H., Marsh, T., Solvik, T., Zhang, D., Ye, J., et al. (2020). The LC3-Conjugation Machinery Specifies the Loading of RNA-Binding Proteins into Extracellular Vesicles. *Nat. Cell Biol.* 22, 187–199. doi:10.1038/s41556-019-0450-y

Li, D., Xu, M., Wang, Z., Huang, P., Huang, C., Chen, Z., et al. (2022). The EMT-Induced lncRNA NR2F1-AS1 Positively Modulates NR2F1 Expression and Drives Gastric Cancer via miR-29a-3p/VAMP7 axis. *Cell Death Dis.* 13, 84. doi:10.1038/s41419-022-04540-2

Machado, E., White-Gilbertson, S., van de Vlekkert, D., Janke, L., Moshiach, S., Campos, Y., et al. (2015). Regulated Lysosomal Exocytosis Mediates Cancer Progression. *Sci. Adv.* 1, e1500603. doi:10.1126/sciadv.1500603

Madden, E. C., Gorman, A. M., Logue, S. E., and Samali, A. (2020). Tumour Cell Secretome in Chemoresistance and Tumour Recurrence. *Trends Cancer* 6, 489–505. doi:10.1016/j.trecan.2020.02.020

Marshall, M. R., Pattu, V., Halimani, M., Maier-Peuschel, M., Müller, M.-L., Becherer, U., et al. (2015). VAMP8-dependent Fusion of Recycling Endosomes with the Plasma Membrane Facilitates T Lymphocyte Cytotoxicity. *J. Cell Biol.* 210, 135–151. doi:10.1083/jcb.201411093

Martinez, I., Chakrabarti, S., Hellevik, T., Morehead, J., Fowler, K., and Andrews, N. W. (2000). Synaptotagmin VII Regulates Ca2+-Dependent Exocytosis of Lysosomes in Fibroblasts. *J. Cell Biol.* 148, 1141–1150. doi:10.1083/jcb.148.6.1141

Martinez-Arca, S., Alberts, P., Zahraoui, A., Louvard, D., and Galli, T. (2000). Role of Tetanus Neurotoxin Insensitive Vesicle-Associated Membrane Protein (TI-VAMP) in Vesicular Transport Mediating Neurite Outgrowth. *J. Cell Biol.* 149, 889–900. doi:10.1083/jcb.149.4.889

Martinez-Arca, S., Rudge, R., Vacca, M., Raposo, G., Camonis, J., Proux-Gillardeaux, V., et al. (2003). A Dual Mechanism Controlling the Localization and Function of Exocytic V-SNAREs. *Proc. Natl. Acad. Sci. U.S.A.* 100, 9011–9016. doi:10.1073/pnas.1431910100

Mathieu, M., Névo, N., Jouve, M., Valenzuela, J. I., Maurin, M., Verweij, F. J., et al. (2021). Specificities of Exosome versus Small Ectosome Secretion Revealed by Live Intracellular Tracking of CD63 and CD9. *Nat. Commun.* 12, 4389. doi:10.1038/s41467-021-24384-2

McLelland, G.-L., Lee, S. A., McBride, H. M., and Fon, E. A. (2016). Syntaxin-17 Delivers PINK1/parkin-dependent Mitochondrial Vesicles to the Endolysosomal System. *J. Cell Biol.* 214, 275–291. doi:10.1083/jcb.201603105

Molino, D., Nola, S., Lam, S. M., Verraes, A., Proux-Gillardeaux, V., Boncompain, G., et al. (2015). Role of Tetanus Neurotoxin Insensitive Vesicle-Associated Membrane Protein in Membrane Domains Transport and Homeostasis. *Cell. Logist.* 5, e1025182. doi:10.1080/21592799.2015.1025182

Mustafa, S., Pan, L., Marzoq, A., Fawaz, M., Sander, L., Rückert, F., et al. (2017). Comparison of the Tumor Cell Secretome and Patient Sera for an Accurate Serum-Based Diagnosis of Pancreatic Ductal Adenocarcinoma. *Oncotarget* 8, 11963–11976. doi:10.18632/oncotarget.14449

Nüchel, J., Tauber, M., Nolte, J. L., Mörgelin, M., Türk, C., Eckes, B., et al. (2021). An mTORC1-GRASP55 Signaling axis Controls Unconventional Secretion to Reshape the Extracellular Proteome upon Stress. *Mol. Cell* 81, 3275–3293. e12. doi:10.1016/j.molcel.2021.06.017

Paltridge, J. L., Belle, L., and Khew-Goodall, Y. (2013). The Secretome in Cancer Progression. *Biochimica Biophysica Acta (BBA) - Proteins Proteomics* 1834, 2233–2241. doi:10.1016/j.bbapap.2013.03.014

Panicker, N., Ge, P., Dawson, V. L., and Dawson, T. M. (2021). The Cell Biology of Parkinson's Disease. *J. Cell Biol.* 220, e202012095. doi:10.1083/jcb.202012095

Phatnani, H., and Maniatis, T. (2015). Astrocytes in Neurodegenerative Disease: Table 1. *Cold Spring Harb. Perspect. Biol.* 7, a020628. doi:10.1101/cshperspect.a020628

Pilliod, J., Desjardins, A., Pernègre, C., Jamann, H., Larochelle, C., Fon, E. A., et al. (2020). Clearance of Intracellular Tau Protein from Neuronal Cells via VAMP8-Induced Secretion. *J. Biol. Chem.* 295, 17827–17841. doi:10.1074/jbc.RA120.013553

Pols, M. S., van Meel, E., Oorschot, V., ten Brink, C., Fukuda, M., Swetha, M. G., et al. (2013). hVps41 and VAMP7 Function in Direct TGN to Late Endosome Transport of Lysosomal Membrane Proteins. *Nat. Commun.* 4, 1361. doi:10.1038/ncomms2360

Popescu, S. C. (2012). A Model for the Biosynthesis and Transport of Plasma Membrane-Associated Signaling Receptors to the Cell Surface. *Front. Plant Sci.* 3, 71. doi:10.3389/fpls.2012.00071

Proux-Gillardeaux, V., Raposo, G., Irinopoulou, T., and Galli, T. (2007). Expression of the Longin Domain of TI-VAMP Impairs Lysosomal Secretion and Epithelial Cell Migration. *Biol. Cell* 99, 261–271. doi:10.1042/BC20060097

Pryor, P. R., Mullock, B. M., Bright, N. A., Lindsay, M. R., Gray, S. R., Richardson, S. C. W., et al. (2004). Combinatorial SNARE Complexes with VAMP7 or VAMP8 Define Different Late Endocytic Fusion Events. *EMBO Rep.* 5, 590–595. doi:10.1038/sj.embor.7400150

Rabouille, C. (2017). Pathways of Unconventional Protein Secretion. *Trends Cell Biol.* 27, 230–240. doi:10.1016/j.tcb.2016.11.007

Rao, S. K., Huynh, C., Proux-Gillardeaux, V., Galli, T., and Andrews, N. W. (2004). Identification of SNAREs Involved in Synaptotagmin VII-Regulated Lysosomal Exocytosis. *J. Biol. Chem.* 279, 20471–20479. doi:10.1074/jbc.M400798200

Rastogi, S., Sharma, V., Bharti, P. S., Rani, K., Modi, G. P., Nikolajeff, F., et al. (2021). The Evolving Landscape of Exosomes in Neurodegenerative Diseases: Exosomes Characteristics and a Promising Role in Early Diagnosis. *Ijms* 22, 440. doi:10.3390/ijms22010440

Sato, M., Yoshimura, S., Hirai, R., Goto, A., Kunii, M., Atik, N., et al. (2011). The Role of VAMP7/TI-VAMP in Cell Polarity and Lysosomal Exocytosis *In Vivo*. *Traffic* 12, 1383–1393. doi:10.1111/j.1600-0854.2011.01247.x

Schäfer, I. B., Hesketh, G. G., Bright, N. A., Gray, S. R., Pryor, P. R., Evans, P. R., et al. (2012). The Binding of Varp to VAMP7 Traps VAMP7 in a Closed, Fusogenically Inactive Conformation. *Nat. Struct. Mol. Biol.* 19, 1300–1309. doi:10.1038/nsmb.2414

Schafer, M. J., Zhang, X., Kumar, A., Atkinson, E. J., Zhu, Y., Jachim, S., et al. (2020). The Senescence-Associated Secretome as an Indicator of Age and Medical Risk. *JCI Insight* 5, e133668. doi:10.1172/jci.insight.133668

Shannon, B., Soto-Ortolaza, A., Rayaprolu, S., Cannon, H. D., Labbé, C., Benitez, B. A., et al. (2014). Genetic Variation of the Retromer Subunits VPS26A/B-VPS29 in Parkinson's Disease. *Neurobiol. Aging* 35–1958. e1–2. doi:10.1016/j.neurobiolaging.2014.03.004

Shimoda, M., and Khokha, R. (2017). Metalloproteinases in Extracellular Vesicles. *Biochimica Biophysica Acta (BBA) - Mol. Cell Res.* 1864, 1989–2000. doi:10.1016/j.bbamcr.2017.05.027

Song, L., Tian, X., and Schekman, R. (2021). Extracellular Vesicles from Neurons Promote Neural Induction of Stem Cells through Cyclin D1. *J. Cell Biol.* 220, e202101075. doi:10.1083/jcb.202101075

Steegmaier, M., Klumperman, J., Foletti, D. L., Yoo, J.-S., and Scheller, R. H. (1999). Vesicle-associated Membrane Protein 4 Is Implicated inTrans-Golgi Network Vesicle Trafficking. *MBoC* 10, 1957–1972. doi:10.1091/mbc.10.6.1957

Steffen, A., Le Dez, G., Poincloux, R., Recchi, C., Nassoy, P., Rottner, K., et al. (2008). MT1-MMP-dependent Invasion Is Regulated by TI-VAMP/VAMP7. *Curr. Biol.* 18, 926–931. doi:10.1016/j.cub.2008.05.044

Sulkowski, P. L., Nicholson, H. E., Li, M. Z., Perrimon, N., Liu, Z., and Kaelin, W. G. (2021). Horizontal Transfer of Histone H3 by Mammalian Cells. *BioRxiv*. doi:10.1101/2021.06.30.450524

Südhof, T. C., and Rothman, J. E. (2009). Membrane Fusion: Grappling with SNARE and SM Proteins. *Science* 323, 474–477. doi:10.1126/science.1161748

Takano, T., Lin, J. H.-C., Arcuino, G., Gao, Q., Yang, J., and Nedergaard, M. (2001). Glutamate Release Promotes Growth of Malignant Gliomas. *Nat. Med.* 7, 1010–1015. doi:10.1038/nm0901-1010

Tan, J. Z. A., and Gleeson, P. A. (2019). Cargo Sorting at the Trans-Golgi Network for Shunting into Specific Transport Routes: Role of Arf Small G Proteins and Adaptor Complexes. *Cells* 8, 531. Cells 8. doi:10.3390/cells8060531

Thul, P. J., Åkesson, L., Wiking, M., Mahdessian, D., Geladaki, A., Ait Blal, H., et al. (2017). A Subcellular Map of the Human Proteome. *Science* 356, eaal3321. doi:10.1126/science.aal3321

Trajkovic, K., Jeong, H., and Krainc, D. (2017). Mutant Huntingtin Is Secreted via a Late Endosomal/lysosomal Unconventional Secretory Pathway. *J. Neurosci.* 37, 9000–9012. doi:10.1523/JNEUROSCI.0118-17.2017

Trams, E. G., Lauter, C. J., Norman Salem, J., and Heine, U. (1981). Exfoliation of Membrane Ecto-Enzymes in the Form of Micro-vesicles. *Biochimica Biophysica Acta (BBA) - Biomembr.* 645, 63–70. doi:10.1016/0005-2736(81)90512-5

Tsunemi, T., Perez-Rosello, T., Ishiguro, Y., Yoroisaka, A., Jeon, S., Hamada, K., et al. (2019). Increased Lysosomal Exocytosis Induced by Lysosomal Ca2+ Channel Agonists Protects Human Dopaminergic Neurons from α-Synuclein Toxicity. *J. Neurosci.* 39, 5760–5772. doi:10.1523/JNEUROSCI.3085-18.2019

Uhlén, M., Karlsson, M. J., Hober, A., Svensson, A.-S., Scheffel, J., Kotol, D., et al. (2019). The Human Secretome. *Sci. Signal.* 12, eaaz0274. doi:10.1126/scisignal.aaz0274

Ungar, D. (2009). Golgi Linked Protein Glycosylation and Associated Diseases. *Seminars Cell & Dev. Biol.* 20, 762–769. doi:10.1016/j.semcdb.2009.03.004

Vassileff, N., Cheng, L., and Hill, A. F. (2020). Extracellular Vesicles - Propagators of Neuropathology and Sources of Potential Biomarkers and Therapeutics for Neurodegenerative Diseases. *J. Cell Sci.* 133, jcs243139. doi:10.1242/jcs.243139

Vats, S., and Galli, T. (2021). Introducing Secretory reticulophagy/ER-Phagy (SERP), a VAMP7-dependent Pathway Involved in Neurite Growth. *Autophagy* 17, 1037–1039. doi:10.1080/15548627.2021.1883886

Venkataramani, V., Tanev, D. I., Strahle, C., Studier-Fischer, A., Fankhauser, L., Kessler, T., et al. (2019). Glutamatergic Synaptic Input to Glioma Cells Drives Brain Tumour Progression. *Nature* 573, 532–538. doi:10.1038/s41586-019-1564-x

Verderio, C., Cagnoli, C., Bergami, M., Francolini, M., Schenk, U., Colombo, A., et al. (2012). TI-VAMP/VAMP7 Is the SNARE of Secretory Lysosomes Contributing to ATP Secretion from Astrocytes. *Biol. Cell* 104, 213–228. doi:10.1111/boc.201100070

Verweij, F. J., Bebelman, M. P., Jimenez, C. R., Garcia-Vallejo, J. J., Janssen, H., Neefjes, J., et al. (2018). Quantifying Exosome Secretion from Single Cells Reveals a Modulatory Role for GPCR Signaling. *J. Cell Biol.* 217, 1129–1142. doi:10.1083/jcb.201703206

Viotti, C. (2016). ER to Golgi-Dependent Protein Secretion: The Conventional Pathway. *Methods Mol. Biol.* 1459, 3–29. doi:10.1007/978-1-4939-3804-9_1

Vivona, S., Liu, C. W., Strop, P., Rossi, V., Filippini, F., and Brunger, A. T. (2010). The Longin SNARE VAMP7/TI-VAMP Adopts a Closed Conformation. *J. Biol. Chem.* 285, 17965–17973. doi:10.1074/jbc.M110.120972

Wade, N., Bryant, N. J., Connolly, L. M., Simpson, R. J., Luzio, J. P., Piper, R. C., et al. (2001). Syntaxin 7 Complexes with Mouse Vps10p Tail Interactor 1b, Syntaxin 6, Vesicle-Associated Membrane Protein (VAMP)8, and VAMP7 in B16 Melanoma Cells. *J. Biol. Chem.* 276, 19820–19827. doi:10.1074/jbc.M010838200

Wang, F., Zhang, H., Zhang, X., Wang, Y., Ren, F., Zhang, X., et al. (2008). Varp Interacts with Rab38 and Functions as its Potential Effector. *Biochem. Biophysical Res. Commun.* 372, 162–167. doi:10.1016/j.bbrc.2008.05.017

Wang, G., and Galli, T. (2018). Reciprocal Link between Cell Biomechanics and Exocytosis. *Traffic* 19, 741–749. doi:10.1111/tra.12584

Wang, G., Nola, S., Bovio, S., Bun, P., Coppey-Moisan, M., Lafont, F., et al. (2018). Biomechanical Control of Lysosomal Secretion Via the VAMP7 Hub: A Tug-Of-War between VARP and LRRK1. *iScience* 4, 127–143. doi:10.1016/j.isci.2018.05.016

Wang, L., Jiang, X., Zhang, X., and Shu, P. (2021). Prognostic Implications of an Autophagy-Based Signature in Colorectal Cancer. *Medicine* 100, e25148. doi:10.1097/MD.0000000000025148

Wojnacki, J., Nola, S., Bun, P., Cholley, B., Filippini, F., Pressé, M. T., et al. (2020). Role of VAMP7-Dependent Secretion of Reticulon 3 in Neurite Growth. *Cell Rep.* 33, 108536. doi:10.1016/j.celrep.2020.108536

Xie, Y. X., Naseri, N. N., Fels, J., Kharel, P., Na, Y., Burré, J., et al. (2021). Lysosomal Exocytosis Releases Pathogenic α-Synuclein Species from Neurons. *BioRxiv*. doi:10.1101/2021.04.10.439302

Xu, T., Yuan, Y., He, C., and Yang, K. (2021). Construction and Evaluation of a Risk Score Model for Autophagy-Related Genes in Esophageal Adenocarcinoma. *Med. Sci. Monit.* 27, e927850. doi:10.12659/MSM.927850

Xue, H., Lu, B., and Lai, M. (2008). The Cancer Secretome: a Reservoir of Biomarkers. *J. Transl. Med.* 6, 52. doi:10.1186/1479-5876-6-52

Yan, R., Shi, Q., Hu, X., and Zhou, X. (2006). Reticulon Proteins: Emerging Players in Neurodegenerative Diseases. *Cell. Mol. Life Sci.* 63, 877–889. doi:10.1007/s00018-005-5338-2

Zemskov, E. A., Mikhailenko, I., Hsia, R.-C., Zaritskaya, L., and Belkin, A. M. (2011). Unconventional Secretion of Tissue Transglutaminase Involves Phospholipid-dependent Delivery into Recycling Endosomes. *PLoS One* 6, e19414. doi:10.1371/journal.pone.0019414

Zhang, M., Liu, L., Lin, X., Wang, Y., Li, Y., Guo, Q., et al. (2020). A Translocation Pathway for Vesicle-Mediated Unconventional Protein Secretion. *Cell* 181, 637–652. e15. doi:10.1016/j.cell.2020.03.031

Zhang, X., He, X., Fu, X.-Y., and Chang, Z. (2006). Varp Is a Rab21 Guanine Nucleotide Exchange Factor and Regulates Endosome Dynamics. *J. Cell Sci.* 119, 1053–1062. doi:10.1242/jcs.02810

Zhu, L., Dong, L., Feng, M., Yang, F., Jiang, W., Huang, Z., et al. (2020). Profiles of Autophagy-Related Genes in Esophageal Adenocarcinoma. *BMC Cancer* 20, 943. doi:10.1186/s12885-020-07416-w

Zucker, S., and Vacirca, J. (2004). Role of Matrix Metalloproteinases (MMPs) in Colorectal Cancer. *Cancer Metastasis Rev.* 23, 101–117. doi:10.1023/a:1025867130437

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

REVIEW
published: 27 June 2022
doi: 10.3389/fcell.2022.910983

Diverse Control Mechanisms of the Interleukin-1 Cytokine Family

Charles L. Evavold[1]* *and Jonathan C. Kagan*[2]*

[1]Ragon Institute of MGH, MIT and Harvard, Cambridge, MA, United States, [2]Division of Gastroenterology, Boston Children's Hospital and Harvard Medical School, Boston, MA, United States

The majority of interleukin-1 (IL-1) family cytokines lack amino terminal secretion signals or transmembrane domains for secretion along the conventional biosynthetic pathway. Yet, these factors must be translocated from the cytoplasm across the plasma membrane into the extracellular space in order to regulate inflammation. Recent work has identified an array of mechanisms by which IL-1 family cytokines can be released into the extracellular space, with supramolecular organizing centers known as inflammasomes serving as dominant drivers of this process. In this review, we discuss current knowledge of the mechanisms of IL-1 family cytokine synthesis, processing, and release from cells. Using this knowledge, we propose a model whereby host metabolic state dictates the route of IL-1β secretion, with implications for microbial infection and sterile inflammation.

Keywords: IL-1, inflammasomes, pyroptosis, hyperactivation, gasdermin D, secretion, cytokines, inflammation

OPEN ACCESS

Edited by:
Julien Villeneuve, Institute for Functional Genomics (IGF) (CNRS), France

Reviewed by:
Jelena Bezbradica, University of Oxford, United Kingdom
Marioara Chiritoiu-Butnaru, Institute of Biochemistry of the Romanian Academy, Romania

***Correspondence:**
Charles L. Evavold
cevavold@mgh.harvard.edu
Jonathan C. Kagan
jonathan.kagan@childrens.harvard.edu

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *01 April 2022*
Accepted: *17 May 2022*
Published: *27 June 2022*

Citation:
Evavold CL and Kagan JC (2022) Diverse Control Mechanisms of the Interleukin-1 Cytokine Family. Front. Cell Dev. Biol. 10:910983. doi: 10.3389/fcell.2022.910983

INTRODUCTION

Production and secretion of interleukin-1 (IL-1) family cytokines is closely linked to inflammation. All IL-1 family cytokines, except IL-1Ra, lack an amino terminal (N-terminal) secretion signal for secretion by the endoplasmic reticulum (ER)-Golgi vesicular pathway (Garlanda et al., 2013). Several family members, such as IL-1α, IL-1β, and IL-36α/β/γ, are considered pro-inflammatory. Other members, such as IL-1Ra and IL-36Ra, serve inhibitory or buffering roles that counteract the pro-inflammatory functions of IL-1α/β and IL-36 cytokine signaling, respectively. Select IL-1 family cytokines can also serve anti-inflammatory functions in the case of IL-37 and IL-38 or context-dependent pro-inflammatory and anti-inflammatory functions in the case of IL-18 and IL-33.

IL-1α and IL-1β (sometimes referred to in aggregate as IL-1) have related functions within the host through action on their shared heterodimeric receptor IL-1R1 and IL-1R accessory protein known as IL-1R3 (Mosley et al., 1987a; Mosley et al., 1987b; Sims et al., 1988; Greenfeder et al., 1995). Through cloning of pro-IL-1β, it was readily appreciated that this inactive precursor molecule did not contain an N terminal signal sequence highlighting a major conundrum on how the bioactive form of this cytokine might exit the cell to act on its cognate receptor (Auron et al., 1984; Rubartelli et al., 1990). The IL-1 receptor complex, when ligated to IL-1α or IL-1β, but not when ligated to the inhibitory protein IL-1Ra, can recruit the signaling adaptor MyD88 (Wesche et al., 1997). MyD88 recruitment and its downstream pro-inflammatory signaling events are similar to the sensing of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) of the Toll-like receptor (TLR) family. As such, many of the pro-inflammatory functions of TLRs are recapitulated by IL-1 family receptors. A major action of IL-1R signaling is the activation of the transcription factor NF-κB leading to production of pro-inflammatory cytokines, upregulation of antigen presentation, and pro-survival signaling in various cell types (O'Neill, 2008). In addition, IL-1R signaling can provide mitogenic signals in the case of T and B lymphocytes, as reviewed elsewhere

(Evavold and Kagan, 2018). Recent work has also highlighted that IL-1 signaling can induce an antiviral state in fibroblasts (Orzalli et al., 2018; Aarreberg et al., 2019).

Signaling through other IL-1 receptors appears to follow analogous processes to IL-1R, whereby the cognate ligand of an IL-1 family cytokine binds a heterodimeric receptor that induces the recruitment and activation of MyD88 (Garlanda et al., 2013; O'Neill, 2008). The anti-inflammatory action of some IL-1 family members may stem from differential usage of MyD88 for pro-inflammatory versus anti-inflammatory responses. For example, IL-33 binding to the specific IL-33 receptor known as IL-1R4 (also known as ST2) can be considered pro-inflammatory on type 2 T helper (Th2) cells and mast cells (Ali et al., 2007; Chackerian et al., 2007). IL-33 bound IL-1R4 can then recruit the accessory protein IL-1R3, as is the case for the IL-1 receptor complex, to recruit and activate MyD88 (Garlanda et al., 2013). Conversely, IL-33 signaling on T regulatory cells (Tregs) can be considered anti-inflammatory through induction of proliferation of this inherently anti-inflammatory cell type and production of the tissue repair factor known as amphiregulin (Arpaia et al., 2015; Kuswanto et al., 2016). Analogous to TLR contextual signaling, TLR4 and TLR5 expressing Tregs also appear to use TLR-MyD88-dependent signaling for anti-inflammatory and tissue repair related responses (Caramalho et al., 2003; Crellin et al., 2005).

Thus, as IL-1 family cytokines can have location and cell-type-dependent responses, leading to either the induction or resolution of inflammation, this family of cytokines is under increased regulation compared to conventionally secreted counterparts. Regulation of the induction, maturation, and secretion of these cytokines is the focus of this review.

OVERVIEW OF IL-1 FAMILY CYTOKINES

As stated above, IL-1 is the prototypical member of the IL-1 family of cytokines. IL-1 acts on many cell types to induce inflammation including, but not limited to, endothelial cells, epithelial cells, myeloid cells, and lymphocytes (Evavold and Kagan, 2018). IL-1 can also trigger the secretion of additional conventional cytokines and chemokines, such as IL-6 and IL-8 respectively, that promote local inflammation through increasing the permeability of endothelial cells for immune cell recruitment and systemic inflammation through induction and maintenance of fever and production of acute phase proteins in the liver (Garlanda et al., 2013).

IL-1α exists as a pro-form cytokine primarily within the nucleus of cells (Werman et al., 2004; Lamacchia et al., 2013). Some cell types, such as epithelial cells, appear to constitutively express IL-1α, though pro-inflammatory signaling can induce the production of new pools of IL-1α. The subcellular localization of this cytokine is attributed to a nuclear localization signal (NLS) within the pro-domain (Werman et al., 2004; Wessendorf et al., 1993). IL-1α is best known for its pro-inflammatory activities resulting from ligation and activation of the IL-1 receptor complex. This necessitates that IL-1α egresses the nucleus and makes it to the extracellular space to act on IL-1 receptor complexes on other cells (**Figure 1**). While pro-form IL-1α can signal through the IL-1 receptor complex (Kim et al., 2013), the potency of IL-1α on its cognate receptor increases after processing by select proteases (**Figure 2**). Examples of such proteases include calpains, which are calcium-dependent cysteine proteases located at the inner leaflet of the plasma membrane (Kobayashi et al., 1990; Afonina et al., 2011). Thus, while IL-1α can be released upon cellular necrosis, the activity of IL-1α is increased following regulated secretion that includes disruption of the nucleus and calcium (Ca) flux (Gross et al., 2012; England et al., 2014). These events occur during certain cell death processes such as induction of pyroptosis through the action of inflammasomes, which will be discussed in the following sections (Keller et al., 2008; Gross et al., 2012). Recent studies indicate that IL-1α can also be released from cells after sublytic inflammasome stimulations and from living cells, such as occurs during phagocyte hyperactivation or early pyroptotic stimulations (Gardner et al., 2015; Evavold et al., 2018; Tapia et al., 2019; Wiggins et al., 2019; Aizawa et al., 2020; Tsuchiya et al., 2021). While the canonical inflammasome component caspase-1 can mediate the calpain-dependent processing and subsequent release of IL-1α (Gross et al., 2012; Tsuchiya et al., 2021), caspase-1 is unable to directly process pro-IL-1α (Howard et al., 1991). In contrast, recent work has identified that inflammatory caspase-5/-11 can directly process IL-1α into a more bioactive molecule (Wiggins et al., 2019). The increased bioactivity of processed IL-1α can also be contextually controlled *in trans*. Under these circumstances, a necrotic cell may release pro-form IL-1α that is then cleaved by proteases from a different cell, such as mast cell chymase, neutrophil elastase, or cytolytic T-lymphocyte (CTL) and natural killer (NK) cell granzyme B (Lüthi et al., 2009; Clancy et al., 2018). Moreover, IL-1α can be activated after cleavage by the coagulation cascade associated protease thrombin (Burzynski et al., 2019) (**Figure 2**).

IL-1β is generally associated with myeloid lineage cells such as macrophages, dendritic cells, and neutrophils (Chan and Schroder, 2020). In their resting (non-inflammatory state), these myeloid cells do not express pro-form IL-1β and typically require a pro-inflammatory signal to initiate transcription and translation, such as after TLR activation upon microbial encounters (**Figure 1**). Conversely, certain cell types, such as keratinocytes, may constitutively express low levels of IL-1β without pro-inflammatory stimuli (Mizutani et al., 1991a). Pro-IL-1β is found within the cytosol (Chan and Schroder, 2020). This pro-form cytokine requires proteolytic processing to become biologically active on the IL-1 receptor complex (Howard et al., 1991; Thornberry et al., 1992) (**Figure 2**). This cytokine also must be released from the cytosol into the extracellular space to reach IL-1 receptor complexes on other cells (**Figure 1**). Thus, in contrast to IL-1α, which must cross the nuclear and plasma membrane to access the extracellular space, cytosolic IL-1β must only traverse the plasma membrane. The lower threshold of crossing the membrane of a single compartment for IL-1β to escape the cell might be explained through the additional regulation at the induction of transcription, compared to a pre-existing pool of nuclear IL-1α in some cell types. Moreover, unlike IL-1α, IL-1β has an absolute

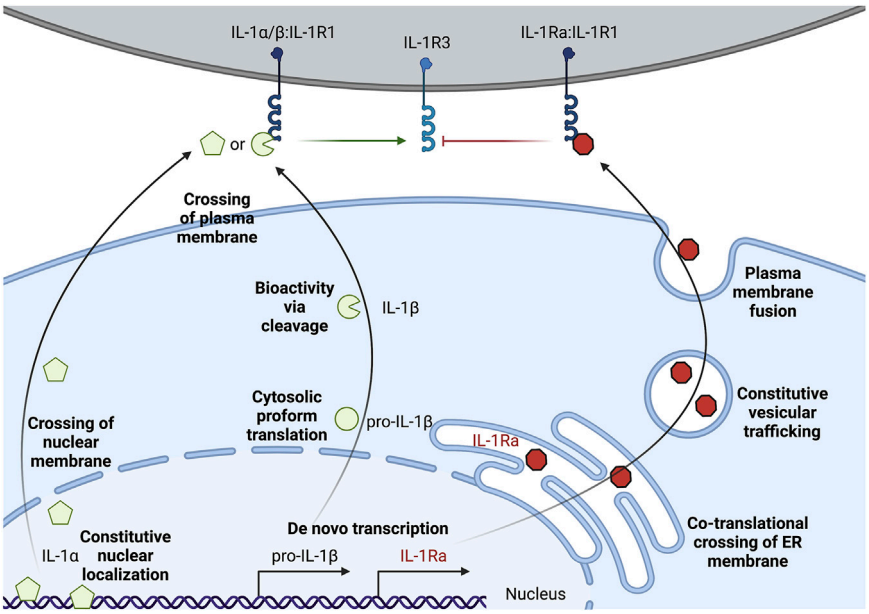


FIGURE 1 | Multi-level regulation of unconventional secretion of IL-1. IL-1α can be constitutively expressed, but accumulates in the nuclear compartment. Secretion requires crossing the topological barriers of the nuclear membrane and the plasma membrane. Proform IL-1α is biologically active on the IL-1 receptor complex, but cleavage by certain proteases such as calpains can increase activity. Pro-IL-1β is often transcriptionally induced upon sensation of lower level threats to the host such as extracellular PAMPs or pro-inflammatory cytokines. Pro-IL-1β is translated and remains in the cytosol, and must cross the topological barrier of the plasma membrane for secretion. Cleavage of pro-IL-1β by proteases such as caspase-1 is required for bioactivity on the IL-1 receptor complex. IL-1Ra is the only IL-1 family member that is conventionally secreted through the biosynthetic pathway. IL-1Ra is transcriptionally induced alongside sensation of inflammatory cues and conventional secretion of this cytokine may buffer the action of pro-inflammatory IL-1α/IL-1β by blocking their interaction with IL-1R1.

requirement for its cleavage to achieve bioactivity. Thus, multiple strategies of IL-1 regulation appear to mediate IL-1α and IL-1β release from cells.

The cleavage and release of IL-1β is often closely linked to the action of inflammasomes (Evavold and Kagan, 2019; Chan and Schroder, 2020). Inflammatory caspase-1 cleaves pro-IL-1β into mature IL-1β (Kostura et al., 1989; Thornberry et al., 1992; Li et al., 1995). Caspase-1 also cleaves the protein gasdermin D (GSDMD) to mediate pyroptotic lysis of cells (He et al., 2015; Kayagaki et al., 2015; Shi et al., 2015). GSDMD cleavage by caspase-1 releases a fragment that oligomerizes into pores in host cell membranes (Aglietti et al., 2016; Liu et al., 2016a; Ding et al., 2016; Sborgi et al., 2016). GSDMD pores can mediate IL-1β release in direct and indirect ways (Evavold et al., 2018; Heilig et al., 2018). Moreover, inflammatory caspases-4, -5, -11 can also mediate the cleavage and secretion of IL-1β, but this process requires the secondary activation of the NLRP3 inflammasome and caspase-1 for direct IL-1β cleavage (Kayagaki et al., 2015; Rühl and Broz, 2015; Shi et al., 2015). The necroptotic pathway can also cause membrane permeability and rupture *via* the pore forming protein MLKL and pro-IL-1β processing *via* the NLRP3 inflammasome (Gutierrez et al., 2017). Recent work has also illustrated that caspase-8 can mediate the cleavage and release of IL-1β in contexts where traditional inflammasome components are lacking or under conditions of TAK1 inhibition (Orning et al., 2018; Sarhan et al., 2018; Muendlein et al., 2020). Finally, pro-IL-1β can be cleaved in the extracellular space *in trans via* mast cell-associated chymase, neutrophil-associated elastase, proteinase-3, matrix metalloprotease 9, and CTL and NK cell-associated granzyme A (Black et al., 1988; Hazuda et al., 1990; Mizutani et al., 1991b; Coeshott et al., 1999) (**Figure 2**).

IL-1Ra is an inhibitory protein to the IL-1 receptor complex (Arend et al., 1989; Arend et al., 1994). IL-1Ra is the only IL-1 family member that contains an N-terminal signal sequence for translation at the endoplasmic reticulum, trafficking through the Golgi, and fusion and release at the plasma membrane (**Figure 1**) (Garlanda et al., 2013). The highly inflammatory nature of IL-1α and IL-1β on cells expressing the IL-1 receptor complex may explain why this inhibitory member of the IL-1 family evolved to be conventionally secreted. IL-1Ra binds to the same IL-1R1 as IL-1α and IL-1β, thus limiting the pro-inflammatory signaling that these cytokines induce (Arend et al., 1989; Arend et al., 1994). IL-1Ra bound IL-1R1 cannot productively signal through IL-1R3. This may serve as a local and systemic buffering system to limit low levels of autoinflammation during constitutive death processes or during resolution of inflammation. Other cytokine systems, such as the conventional cytokine IL-6, also have mechanisms to buffer the signaling propensity of the pro-inflammatory cytokine *via* the production of secreted decoy receptors (Yousif et al., 2021). In addition to IL-1Ra-dependent inhibition of IL-1R1 signaling, IL-1 is also scavenged by a membrane bound and soluble decoy receptor known as IL-1R2 (Colotta et al., 1993; Re et al., 1996; Kuhn et al., 2007; Lorenzen et al., 2012). These two strategies in addition to the cell-intrinsic and *in trans* regulation of IL-1α and IL-1β cytokine processing and release illustrate that these cytokines

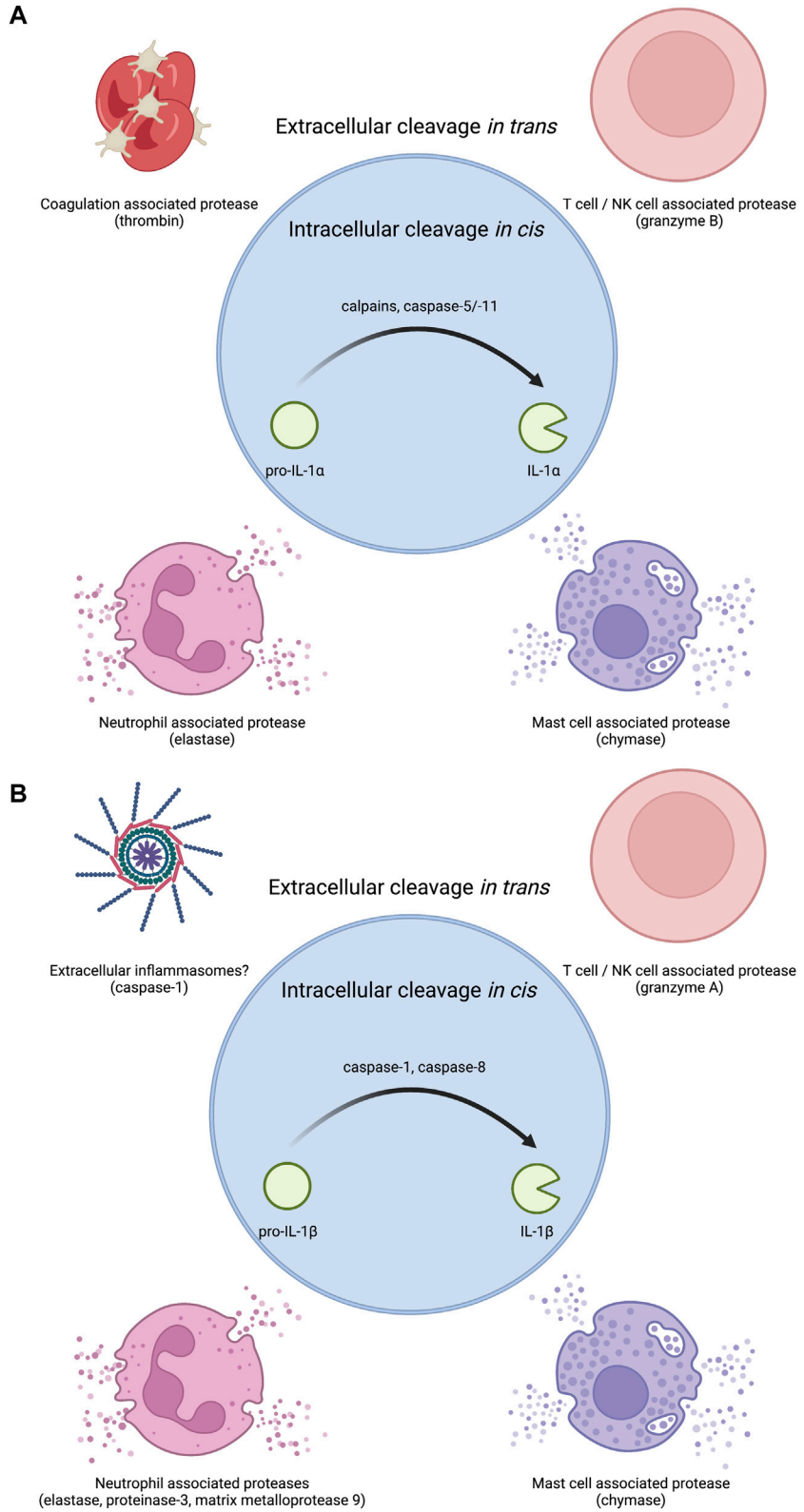


FIGURE 2 | Proteases that regulate IL-1 bioactivity *in cis* or *in trans*. **(A)** The precursor protein for IL-1α is inherently bioactive. Several proteases have been shown to increase this bioactivity. Intracellular proteases that can regulate IL-1α *in cis* include calcium activated proteases such as calpains as well as the non-canonical inflammasome associated caspase-5 and caspase-11 in human and mouse respectively. Extracellular proteases that can regulate IL-1α *in trans* include coagulation

(*Continued*)

FIGURE 2 | associated thrombin, T cell/NK cell associated granzyme B, neutrophil associated elastase, and mast cell associated chymase. **(B)** Pro-IL-1β must be processed into IL-1β to become bioactive. Several proteases have been shown to mediate this conversion to bioactivity. Intracellular proteases that can regulate IL-1β *in cis* include inflammasome associated caspase-1 or diverse complexes that can contain caspase-8. Extracellular proteases that can regulate IL-1β *in trans* include T cell/NK cell associated granzyme A, neutrophil associated proteases elastase, proteinase-3, and matrix metalloprotease 9, and mast cell associated chymase. As inflammasome specks can exist in the extracellular space after pyroptotic lysis, extracellular inflammasomes may also be capable of regulating extracellular pro-IL-1β cleavage likely through caspase-1.

are highly inflammatory. Indeed, several autoinflammatory diseases, such as cryopyrin-associated periodic syndrome (CAPS) and familial Mediterranean fevers (FMF), and autoimmunity diseases, such as rheumatoid arthritis (RA) and multiple sclerosis (MS), are associated with overproduction and secretion of IL-1 (Garlanda et al., 2013). Recombinant IL-1Ra (known as Anakinra) is used as a therapy in some of these indications, and monoclonal antibodies against IL-1β (such as Canakinumab) demonstrate similar reduction in inflammation associated with neutralizing the bioactivity of this cytokine (Dinarello, 2018).

IL-18 is expressed constitutively in certain cell types such as epithelial and myeloid cells (Puren et al., 1999). Similar to IL-1β, IL-18 is an inactive, pro-form cytokine produced in the cytosol of cells (Okamura et al., 1995; Ghayur et al., 1997). IL-18 is cleaved into a bioactive cytokine *via* inflammasome activated caspase-1 (Ghayur et al., 1997; Gu et al., 1997). IL-18 can also be directly cleaved by inflammatory caspase-4 (Kobayashi et al., 2013; Knodler et al., 2014). This contrasts with the indirect role of caspase-4 in activating the NLRP3 inflammasome for IL-1β processing *via* caspase-1 (Kayagaki et al., 2015; Rühl and Broz, 2015). Notably, these molecular themes of IL-1 cleavage apply to humans and mice, but not all mammals. A subset of carnivores (excluding canines) can utilize a hybrid inflammatory caspase to detect bacterial cell wall lipopolysaccharides (LPS), akin to human caspase-4, and also mediate IL-1β cleavage directly (Devant et al., 2021). As such, this hybrid enzyme, known as caspase-1/4, operates as a one-protein signaling pathway that bypasses the need for an inflammasome and directly links LPS detection to IL-1β cleavage in an analogous manner to human caspase-4 direct cleavage of IL-18.

The specific IL-18 receptor is known as IL-1R5 (formerly IL-18 receptor α chain). When IL-1R5 binds cleaved IL-18, IL-1R5 recruits the signaling competent accessory protein known as IL-1R7 (formerly known as IL-18 receptor β chain) (Dinarello, 2018). Downstream signaling occurs through recruitment of MyD88, as is the case for the activated IL-1 receptor complex. IL-18 signaling can be considered pro-inflammatory as it can mediate inflammation *via* immune cell recruitment to tissues and upregulation of antigen presentation (Garlanda et al., 2013). IL-18 also functions to impact adaptive immunity in concert with conventionally secreted pro-inflammatory cytokines from myeloid cells, such as IL-12 and IL-15 (Okamura et al., 1995; Evavold and Kagan, 2018). The original name for IL-18 was IFN-γ inducing factor because IL-18 in combination with IL-12 (or IL-15) can instruct T lymphocytes to differentiate towards the *Th1* helper subset, and thus encourage IFN-γ production *via Th1* and NK cell lymphocytes (Okamura et al., 1995; Ghayur et al., 1997). Consistent with this pro-inflammatory role of IL-18, several autoinflammatory and autoimmune diseases are associated with increased serum concentrations of IL-18 including CAPS, FMF, and MS (Garlanda et al., 2013). Unlike IL-1, IL-18 does not induce fevers when administered exogenously (Gatti et al., 2002). While IL-18 has been purported to have protective roles in colitis, subsequent work suggests that IL-18 mediates inflammation and epithelial barrier dysfunction (Nowarski et al., 2015). Similar to the buffering activity of the membrane-bound decoy receptor IL-1R2 towards the bioactivity of IL-1, the host produces a conventionally secreted protein called IL-18 binding protein (IL-18bp) to scavenge IL-18 and likely dampen the inflammatory activities of IL-18 (Novick et al., 1999). While more studies are needed to delineate the magnitude and kinetics of production of receptor antagonists, binding proteins, and decoy receptors for other IL-1 family members, IL-18bp is well characterized as a buffering system for IL-18 driven inflammation (Novick et al., 1999; Kim et al., 2000; Novick et al., 2001). During homeostasis, serum concentrations of IL-18bp seem to be constitutively higher than serum concentrations of IL-18 by at least an order of magnitude (Novick et al., 2001). As IL-18bp can bind in a 1 to 1 M fashion to IL-18 with tight affinity, this means that at baseline even homeostatic production of IL-18 is buffered or chelated by IL-18bp (Novick et al., 1999; Kim et al., 2000; Novick et al., 2001). During inflammation, IL-18 levels must surmount the levels of IL-18bp to mediate bioactivity on the cognate cytokine receptor. Interestingly, IL-18bp is upregulated by IFN-γ during inflammation (Mühl et al., 2000; Hurgin et al., 2002). As IL-18 can induce the production of IFN-γ as mentioned above (Okamura et al., 1995; Ghayur et al., 1997), this transcriptional feedback loop may initiate resolution of inflammation unless high levels of IL-18 continue to be produced. Other factors that affect the IL-18 to IL-18bp setpoint require further characterization, but this example illustrates that IL-1 family members are under additional extracellular regulation likely to limit inappropriate inflammation at baseline and promote return to homeostasis quickly following resolution of a pathogenic insult.

Similar to IL-1α, IL-33 is expressed as a nuclear pro-form cytokine that has inherent bioactivity when released from cells in an unprocessed form (Carriere et al., 2007; Talabot-Ayer et al., 2009; Bessa et al., 2014). Thus, as is the case in IL-1α regulation, the presence of an NLS and pro-domain act as two barriers to IL-33-mediated inflammation. In contrast to IL-1β and IL-18, caspase-1 processing of IL-33 may abrogate bioactivity (Cayrol and Girard, 2009). Similarly processing by apoptotic executioner caspases such as caspase-3/-7 also leads to diminished bioactivity (Lüthi et al., 2009). It is unknown whether this processing can occur within the nucleus during cell death programs or whether it primarily occurs as the nuclear compartment is damaged and IL-33 egresses through the cytosol on its way to the extracellular

space. Moreover, inflammasomes have been shown to exist in inflamed tissues apart from the initial source pyroptotic cell (Baroja-Mazo et al., 2014; Franklin et al., 2014). While it is unknown how much caspase-1 activity might be retained within these extracellular "ASC specks," the presence of relatively few pyroptotic events may have effects on the bioactivity of IL-33 released from other cells in a local tissue environment. These data suggest that IL-33 can be a contextual signal for caspase-independent necrotic or necroptotic cell death processes (Lüthi et al., 2009; Ohno et al., 2009). IL-33 can be processed *in trans* by proteases, such as mast cell-associated chymase and neutrophil elastase, that increase bioactivity (Bae et al., 2012; Lefrançais et al., 2012; Waern et al., 2013; Roy et al., 2014). IL-33 binds to the specific receptor known as IL-1R4 (formerly ST2) to mediate recruitment of the signaling competent accessory protein IL-1R3 that is also used by the IL-1 receptor complex and IL-18 receptor complex (Ali et al., 2007; Chackerian et al., 2007). The activated IL-33 receptor complex can then recruit MyD88 to activate NF-κB-dependent processes (Garlanda et al., 2013). IL-33 can incur pro-inflammatory functions through activation and proliferation of the *Th2* helper subset of T lymphocytes in contexts such as multicellular parasite infection or allergy (Ali et al., 2007; Bartemes et al., 2012; Garlanda et al., 2013). IL-33 can act as an anti-inflammatory cytokine through proliferation and upregulation of the tissue repair cytokine amphiregulin in T regulatory cells in contexts such as muscle injury (Arpaia et al., 2015; Kuswanto et al., 2016).

The IL-36 subfamily consists of IL-36α, IL-36β, IL-36γ, IL-36Ra, and IL-38 (Dinarello, 2018). These members all bind to the specific IL-36 receptor chain known as IL-1R6 (formerly known as IL-1 receptor-related protein 2) (Towne et al., 2004). The production and response to IL-36 cytokines primarily occurs at barrier sites such as the squamous epithelium of the skin (Boutet et al., 2016). Keratinocytes transcribe and translate IL-36γ after sensation of PAMPs such as poly (I:C) and flagellin (Lian et al., 2012). IL-36γ is released after poly (I:C) treatment of keratinocytes in a caspase-3/-7-dependent manner that also requires upstream caspase-1 activation (Lian et al., 2012). While little is known regarding the processing and secretory mechanism of IL-36 members, the association with inflammasome related caspase-1 and apoptotic caspase-3/-7 may suggest that the gasdermin family of pore forming molecules may play a role in secretion of IL-36 from keratinocytes as is the case for IL-1β and IL-18. While recombinant full-length IL-36 cytokines can elicit bioactivity, N-terminally truncated IL-36 increases bioactivity on the IL-36 receptor complex (Towne et al., 2011). The IL-36 cytokines do not have obvious caspase cleavage motifs, but there may be distinct proteases that cleave IL-36 either in a secreting cell or *in trans* as is the case for other IL-1 family members. This might proceed through either caspase-1-dependent GSDMD pore formation or caspase-3/-7-dependent GSDME pore formation. IL-36 can signal to epithelial cells, such as skin keratinocytes, to produce chemokines that may mediate inflammation through recruitment of immune cells to the site of IL-36 release (Li et al., 2014). Moreover, IL-36 is produced in lesions associated with the autoimmune disorder psoriasis (Johnston et al., 2011; Marrakchi et al., 2011). The inhibitory protein IL-36Ra inhibits IL-36 receptor signaling by blocking binding of the activating ligands IL-36α, IL-36β, and IL-36γ to IL-1R6 in an analogous way to IL-1Ra action on the IL-1 receptor complex (Dinarello, 2018). Deficiency in IL-36Ra is associated with pustular psoriatic lesions in the skin, again highlighting that beyond IL-36 processing and release that additional regulation at the level of receptor binding is required to prevent autoinflammation and autoimmunity for inflammatory IL-1 family members (Blumberg et al., 2007; Marrakchi et al., 2011; Sugiura et al., 2014). IL-38 is a partial antagonist of IL-36-dependent inflammation as IL-38 binds the same IL-36 receptor complex as agonist IL-36 cytokines (van de Veerdonk et al., 2012). IL-38 has an anti-inflammatory role as it can block IL-22 and IL-17A production in response to *Candida albicans* (van de Veerdonk et al., 2012; Han et al., 2019). As IL-38 is elevated in patients with the autoinflammation such as asthma and autoimmune diseases such as SLE and RA, this cytokine may act similarly to IL-1Ra, IL-18bp, and IL-36Ra in buffering the inflammatory actions of agonist IL-36 cytokines (Rudloff et al., 2015; Boutet et al., 2016; Chu et al., 2016). The potential role of processing and mechanisms of IL-38 release await further characterization.

IL-37 is another IL-1 family member that is transcriptionally regulated and sequestered to the nucleus until programmed release into the extracellular space where it can exert anti-inflammatory functions (Sharma et al., 2008; Nold et al., 2010). IL-37 is expressed in epithelial cells, lymphocytes, and myeloid cells (Dinarello, 2018). Mice do not express an orthologue of human IL-37, but ectopic expression in mice and murine cells demonstrates anti-inflammatory properties (Nold et al., 2010). IL-37 binds to IL-1R5 which is the same specific ligand receptor for IL-18 signaling (Kumar et al., 2002; Nold-Petry et al., 2015). In contrast to IL-18/IL-1R5 recruitment of IL-1R7 for IL-18 signaling, IL-37 binding to IL-1R5 recruits the chain IL-1R8 (also known as SIGIRR) (Li et al., 2015; Nold-Petry et al., 2015). IL-37 is released in both a cleaved and unprocessed form after inflammasome activation in human myeloid cells, but processing is not necessary for bioactivity of IL-37 (Kumar et al., 2002; Bulau et al., 2014; Li et al., 2015). Release of IL-37 after inflammasome signaling may serve to mitigate or buffer the inflammatory potential of inflammasome released IL-1 or other sources of inflammation due to the presence of microbial ligands in an infected tissue. One model that has been proposed for how IL-37 could be anti-inflammatory is through sequestering MyD88 to the TIR domain of IL-1R8, thus depriving other TLR and pro-inflammatory IL-1 family receptors of their required signaling adaptor (Gong et al., 2010). This intracellular buffering of pro-inflammatory signaling again highlights the potency of IL-1 family members and the requirement for multiple levels of regulation to their inflammatory actions. As several members of the IL-1 family seem to utilize contextual processing and release *via* the inducible organelles known as inflammasomes, we will provide updates on regulation of inflammasome signaling and membrane permeabilization in subsequent sections.

REGULATION OF INFLAMMASOMES

Inflammasomes are threat-assessing organelles that assemble in response to cytosolic perturbations indicative of pathogen

invasion or sterile damage (Evavold and Kagan, 2019; Chan and Schroder, 2020). Inflammasomes have many layers of regulation that affect the cleavage and secretion of bioactive IL-1 family cytokines. This regulation can take the form of transcriptional control of inflammasome components and substrates, post-translational control of location and conformation of inflammasome components, and control of negative regulators of inflammasome signaling. Inflammasomes consist of a seed protein, oligomerization unit, and enzymatic effector (Evavold and Kagan, 2019). Many intracellular PRRs have been determined to serve as seed proteins for inflammasome activation, including proteins of the NLR family, the protein Pyrin, the protein AIM2, and the recently discovered protein CARD8.

Some inflammasomes require a two-signal integration of threat level for optimal activation (Evavold and Kagan, 2019; Chan and Schroder, 2020). The two-signal requirement of certain inflammasomes serves as a logic gate to prevent the inappropriate release of bioactive IL-1 and inflammatory cell death. This logic gate is best exemplified by the synergistic recognition of microbial ligands by the TLR family and subsequent activation of the NLRP3 inflammasome in myeloid cells, such as macrophages. Unstimulated macrophages do not express appreciable amounts of the inflammasome seed protein NLRP3 or pro-IL-1β. Only upon PRR detection of PAMPs, such as TLR4 sensing bacterial LPS, or through the action of certain pro-inflammatory cytokines, such as TNFR sensing TNFα, can an NF-κB-dependent transcriptional response upregulate NLRP3 and pro-IL-1β. Thus, low-level threats of extracellular microbial ligands, stress ligands, or pro-inflammatory cytokines can poise a sentinel cell to survey for the presence of higher threats such as pathogen invasion of the cytosol or manipulation of host machinery. This transcriptional upregulation of inflammasome components and inflammasome substrates has been termed "priming" or "signal one." Other inflammasomes such as the AIM2 inflammasome and the caspase-11 inflammasome are under the control of a transcriptional signal one, though these receptors typically require the induction of an interferon (IFN) response for their transcriptional upregulation. Beyond the upregulation of transcriptional responses, priming can also post-translationally modify inflammasome proteins or alter lipid organization on membranous organelles to mediate conformational changes or subcellular location of inflammasome proteins.

The second signal in inflammasome activation is the trigger for seed oligomerization. This process is intrinsically controlled by receptor location because all known inflammasome receptors are located within the cytosol (or nucleus) and are thus topologically separated from low-level threats, such as microbial ligands in the extracellular space (Evavold and Kagan, 2019). A higher threat, such as microbial ligands in the sterile cytosol or dysfunction of a cellular process, are thus used as indications of pathogen invasion and result in a commensurate inflammatory response of release of bioactive IL-1 and in some cases lytic cell death.

Inflammasomes consist of several distinct seed proteins that can sense diverse inputs, but triggering of these receptors converge on oligomerization of adaptor ASC (and in some cases NLRC4) to promote the activation of inflammatory caspase-1. ASC is recruited to most inflammasome seeds, such as NLRP3, NLRP6, AIM2, and Pyrin, through PYRIN-PYRIN domain interactions. The NAIP proteins sense proteins structurally related to components of bacterial secretion or motility machinery to recruit the adaptor NLRC4. The oligomerization of the adaptors ASC and NLRC4 in the above inflammasomes serves to recruit pro-caspase-1 and induce activation of caspase-1 through enforced proximity. Oligomers of NLRC4 or ASC recruit pro-caspase-1 through CARD-CARD domain interactions. Increasing the local concentration of caspase-1 within the inflammasome filament allows for pro-caspase-1 and various caspase-1 heterodimers to process other caspase-1 molecules *in trans* at two linker locations (Thornberry et al., 1992; Boucher et al., 2018). These cleavage events cause the formation of distinct species of active caspase-1 heterodimers including an inflammasome localized, highly active species consisting of a p33 and p10 fragment and a solubilized species consisting of a p20 and p10 fragment (Boucher et al., 2018). This sequential cleavage illustrates tight regulation on the duration and magnitude of caspase-1 activity within cells that may be intrinsically related to the size or available oligomerization surfaces of inflammasome assemblies (Boucher et al., 2018; Evavold and Kagan, 2019).

ROLE OF IL-1 CLEAVAGE IN BIOACTIVITY, MEMBRANE LOCALIZATION, AND SECRETION

Caspase-1 activity is intimately related to the cleavage of intracellular substrates such as the select IL-1 family members IL-1β, IL-18, IL-33, and IL-37 (Chan and Schroder, 2020). As stated above, inflammasome associated caspase-1 can cleave IL-1β, IL-18, and IL-37 to increase their binding and bioactivity to their respective cytokine receptors (Thornberry et al., 1992; Ghayur et al., 1997; Gu et al., 1997; Kumar et al., 2002). In the case of IL-33, caspase-1 may process the cytokine into a moiety that is no longer bioactive (Cayrol and Girard, 2009). In the context of IL-1β and IL-18, cleavage of pro-form cytokine can change the overall isoelectric point of the protein (Monteleone et al., 2018). The pro-domain of IL-1β is negatively charged, whereas the polypeptide corresponding to the mature p17 fragment is positively charged. Thus, cleavage of pro-IL-1β into IL-1β releases an overall positively charged mature cytokine that becomes enriched in the inner leaflet of the plasma membrane through charge-charge interactions with negatively charged phospholipid headgroups, such as PI(4,5)P2 (Monteleone et al., 2018). Accumulation of IL-1β at the plasma membrane can facilitate fast release through GSDMD pores or slow release by underdetermined mechanisms. Caspase-1 also facilitates the secretion of bioactive IL-1 family cytokines through regulation of the pore forming protein GSDMD (Kayagaki et al., 2015; Shi et al., 2015; Evavold et al., 2018; Heilig et al., 2018).

GSDMD pores are recognized to be size and charge-dependent conduits for the secretion of IL-1 from hyperactivating and

sublytic inflammasome stimulations (Evavold et al., 2018; Heilig et al., 2018; Xia et al., 2021). The structure of the human GSDMD pore was recently determined through cryo-EM of lipid nanodisk containing oligomerized N-terminal fragments of GSDMD (Xia et al., 2021). Through charge reversal point mutations in the context of GSDMD and the cargo mature IL-1β, it was determined using liposome release assays and sublytic inflammasome stimulations in reconstituted murine macrophages that GSDMD allows the enriched release of mature IL-1β through electrostatic filtering (Xia et al., 2021). This appears to primarily operate through repulsion of negatively charged pro-IL-1β from the pore channel as opposed to selective preference for mature IL-1β.

REGULATION OF GSDMD PORES

All gasdermin family members, except Pejvakin, contain an N-terminal domain that can form a plasma membrane pore (Ding et al., 2016). As such, the gasdermin family has been the subject of recent investigation of unconventional protein secretion, membrane permeability, and cell death. GSDMD exists as a latent protein within the cytosol of resting cells (Kayagaki et al., 2015; Shi et al., 2015). Upon inflammasome activation, GSDMD is cleaved in a flexible linker region that contains a caspase cleavage site (Liu et al., 2020; Wang et al., 2020). Inflammatory caspases (e.g., caspase-1/-4/-5/-11) recognize GSDMD *via* an exosite in the C terminal fragment (Liu et al., 2020; Wang et al., 2020). Caspase-8 can also cleave GSDMD—possibly during death receptor signaling, alternative inflammasome activation, TAK1 inhibition, or during *Yersinia* infection (Gaidt et al., 2016; Orning et al., 2018; Sarhan et al., 2018; Donado et al., 2020). While caspase-8 can also be recruited and activated on ASC assemblies of canonical inflammasomes (Sagulenko et al., 2013; Vajjhala et al., 2015), this may primarily occur in contexts where pyroptosis is delayed or defective, such as genetic deficiencies in caspase-1 and GSDMD (Schneider et al., 2017; Tsuchiya et al., 2019).

The primary role of inflammatory caspases and caspase-8 in activating GSDMD is releasing the pore forming N terminal fragment from the auto-inhibitory C terminal domain (Ding et al., 2016; Liu et al., 2019). However, in certain contexts such as gut inflammation, full length GSDMD may mediate the unconventional secretion of IL-1β (Bulek et al., 2020). This study did not see robust cleavage of GSDMD by immunoblot assay but noted a genetic requirement of GSDMD for IL-1β release. Other studies have found that sublytic stimulations, such as infections with mutant *S. aureus*, may cleave GSDMD for IL-1β secretion below the limit of detection by immunoblot (Evavold et al., 2018; Bjanes et al., 2021). Thus, determining whether full length gasdermins might truly form membrane pores awaits further characterization—though there is evidence that point mutations in GSDMD at the binding interface between the N and C terminus can relieve autoinhibition and cause membrane binding and pore formation by the full-length protein (Liu et al., 2019). Either the removal of an inhibitory post-translational modification or addition of an activating modification may alter GSDMD pore formation through the function of the C-terminal autoinhibitory domain, the accessibility of the caspase cleavage site, or membrane binding and oligomerization potential of the N-terminal domain. Indeed, GSDMD was recently described to be modified by host metabolites at cysteine residues in the N-terminus (Humphries et al., 2020; Bambouskova et al., 2021). These modifications appear to block the cleavage of full length GSDMD by inflammatory caspases thus limiting GSDMD oligomerization and pore formation (Humphries et al., 2020; Bambouskova et al., 2021). Of note, one of these cysteine residues has also been implicated in oligomerization of a GSDMD pore after cleavage (Liu et al., 2016a; Rathkey et al., 2018; Hu et al., 2020; Humphries et al., 2020). Use of non-specific cysteine modifying agents, such as necrosulfanamide and disulfiram, can covalently modify cysteine 192 that may sterically hinder the ability of GSDMD N-terminal fragments to oligomerize (Rathkey et al., 2018; Hu et al., 2020). Moreover, the change of the corresponding cysteine to alanine or more conservatively to serine can impact oligomerization and cell death in 293T cells (Liu et al., 2016a; Hu et al., 2020; Humphries et al., 2020). Recent work from our group has determined that reactive oxygen species (ROS) metabolites can enhance GSDMD pore formation that requires cysteine 192 (Devant et al., 2022). More work is required to delineate the role of post-translational modifications (PTMs) in regulating gasdermin function.

The N-terminal fragment of GSDMD has affinity for negatively charged phospholipids such as phosphatidylserine and PI(4,5)P2 found in the inner leaflet of the plasma membrane (Liu et al., 2016a; Ding et al., 2016). Furthermore, GSDMD can bind to other negatively charged lipids, such as cardiolipin, that is present in bacterial or mitochondrial membranes (Aglietti et al., 2016; Liu et al., 2016a; Ding et al., 2016; Sborgi et al., 2016). GSDMD mediates lysis of bacteria after intracellular expression or treatment of liquid cultures and has recently been shown to target mitochondria in the context of pyroptosis (Liu et al., 2016a; Ding et al., 2016). How GSDMD accesses cardiolipin, which is normally found on the inner membranes of intact mitochondria and is topologically hidden by the bacterial cell wall, has not been determined. Cardiolipin becomes externalized after stress (Iyer et al., 2013; Elliott et al., 2018), so GSDMD may target damaged or stressed mitochondria and bacteria.

At the plasma membrane, GSDMD pores can mediate calcium flux from the hypercalcemic extracellular space into the hypocalcemic cytosol (Martín-Sánchez et al., 2016; Russo et al., 2016; Rühl et al., 2018). As IL-1β and GSDMD both localize to PI(4,5)P2-containing regions of the plasma membrane (Liu et al., 2016a; Monteleone et al., 2018), the formation of a GSDMD pore may allow a transient release of calcium that promotes removal of membrane enriched IL-1β through the action of PLC-γ cleavage of PI(4,5)P2 into DAG. During this transient removal of IL-1β from the membrane, PI(4,5)P2 metabolism by PLC may also mediate conformational changes of the pore to limit the amount of IL-1β that is released (Santa Cruz Garcia et al., 2022). Sustained calcium flux is a trigger for membrane repair processes, such as

lysosomal exocytosis or ESCRTIII-dependent membrane blebbing, to remove compromised sections of the plasma membrane (Cooper and McNeil, 2015; Rühl et al., 2018). Moreover, this calcium flux mediates the rapid conformational opening or closing of GSDMD through a mechanism that may involve metabolism of phospholipids [e.g., PI3K formation of PI(4,5)P2 and PI(3,4,5)P3 or degradation of these species to DAG by calcium-dependent phospholipases such as PLC-γ] (Santa Cruz Garcia et al., 2022). Therefore, ion flux and lipid metabolism may regulate GSDMD pore formation and the rate of secretion of mature IL-1β.

Downstream of GSDMD cleavage, the Ragulator-Rag protein complex, which controls mTOR signaling, is required for GSDMD oligomerization and pore formation (Evavold et al., 2021). The role of Ragulator-Rag in GSDMD regulation was linked to the production of ROS metabolites, which are necessary to promote GSDMD oligomer formation and pyroptosis (Evavold et al., 2021). How ROS metabolites affect GSDMD pore formation is still unknown, but they could directly affect GSDMD cysteine residues either through addition of activating PTMs or removal of inactivating PTMs. In support of this model, our recent work has determined that oxidation state of cysteines in the N terminus of GSDMD are drastically different in RagA-deficient cells that do not form pores compared to wild type macrophages (Devant et al., 2022). Moreover, defects in GSDMD pore formation in RagA-deficient cells could be rescued through diverse ROS inducers (Devant et al., 2022). Beyond these indications that ROS may directly regulate GSDMD oligomerization within living cells, ROS may mediate additional indirect effects on related cellular processes such as autophagy or oxidation of host membranes. Additional studies are required to determine the mechanisms by which ROS can affect GSDMD pores.

GSDMD-INDEPENDENT IL-1 SECRETION

GSDMD mediates membrane permeability and IL-1 secretion during acute inflammasome signaling (Evavold et al., 2018; Heilig et al., 2018). Permeabilization of the plasma membrane by alternate means is often sufficient to encourage secretion of IL-1 that is independent or secondary to GSDMD. Physical disruption of the membrane is sufficient to mediate IL-1 release secondary to cell lysis after treatment with uric acid crystals (Rashidi et al., 2019). In GSDMD- or caspase-1-deficient cells, long term inflammasome stimulation can lead to IL-1 secretion that is dependent on GSDME or subsequent membrane rupture and likely involves a slow induction of apoptotic signaling (Schneider et al., 2017; Heilig et al., 2020; Zhou and Abbott, 2021). GSDME, like GSDMD, can form membrane pores in both stressed mitochondria and the plasma membrane to facilitate either direct release of IL-1 or the initiation of membrane lysis (Rogers et al., 2017; Wang et al., 2017; Rogers et al., 2019). GSDME requires the activation of apoptotic executioner caspases such as caspase-3/-7 for processing into an N-terminal pore forming fragment (Rogers et al., 2017; Wang et al., 2017). Like GSDMD, GSDME may also promote IL-1α maturation *via* calcium flux and calpain activation (Aizawa et al., 2020). Thus, the primary channels in myeloid cells that mediate IL-1 secretion after inflammasome signaling or caspase-8 activation are GSDMD and GSDME.

GSDME can be activated *in trans* by delivery of granules from CTL and NK cells that contain granzyme B protease (Zhang et al., 2020a). It is conceivable that granzyme-mediated GSDME activation could lead to NLRP3 inflammasome activation through potassium efflux and membrane damage. Other gasdermin family members exist that may show cell type or stimulation specific cleavage and pore forming abilities. Granzyme A may mediate cleavage and activation of GSDMB (Zhou et al., 2020). As this pore could also mediate potassium efflux, granzymes may encourage NLRP3 inflammasome processing and release of IL-1 downstream of GSDMB activation. Therefore, different cell types and stimulations may result in the activation of specific gasdermins allowing for release of IL-1 family members in conjunction with or independent of inflammasomes.

In the context of necroptotic signaling, RIPK3 phosphorylation of the pore forming protein MLKL causes membrane damage that can result in cell lysis. This membrane damage can allow for potassium efflux from the cell leading to activation of the NLRP3 inflammasome (Conos et al., 2017; Gutierrez et al., 2017). NLRP3 activation in this context is required for secretion of bioactive IL-1 primarily through control of IL-1β cleavage, whereas MLKL permeabilization and subsequent lysis is sufficient to allow for passive release independent of GSDMD (Gutierrez et al., 2017). As has been shown for GSDMD, MLKL membrane damage is negatively regulated by ESCRTIII-dependent membrane repair processes (Gong et al., 2017).

Buffering cell culture stimulations or organ explants with the amino acid glycine has been shown to inhibit lysis in response to inflammasome activation and ischemia reperfusion injury (Weinberg et al., 1987; Frank et al., 2000; Fink and Cookson, 2006). While originally thought to discourage osmotic pressure on cell membranes, the discovery of GSDMD and the characterization of the permissive transport of ions and water across the membrane suggest that glycine must inhibit lysis independently of osmotic pressure. Glycine is experimentally used to separate GSDMD pore formation from pyroptotic lysis during inflammasome stimulations (Evavold et al., 2018; Heilig et al., 2018). These experiments illustrated that IL-1β was able to directly traverse GSDMD pores on the membrane and did not require membrane rupture for release. A recent study has discovered that the protein NINJ1 mediates membrane rupture downstream of diverse triggers such as GSDMD pore formation, bacterial toxin pore formation, and late apoptotic signaling that might permit GSDME pore formation (Kayagaki et al., 2021). Notably, MLKL activation during necroptotic signaling appears sufficient to mediate membrane lysis independent of NINJ1. NINJ1-deficient cells provide additional evidence that GSDMD can directly convey IL-1β across the plasma membrane of inflammasome-activated macrophages. Recent

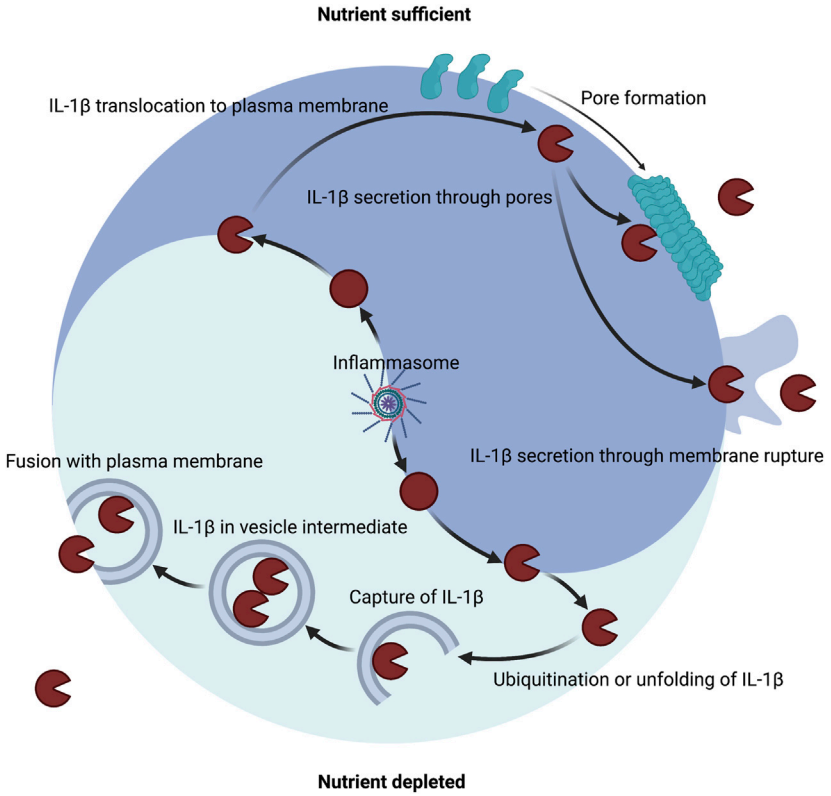


FIGURE 3 | Host metabolic state dictates the route of IL-1β secretion. A major mechanism of IL-1β secretion involves permeabilization of the plasma membrane. Inflammasomes can control the maturation of pro-IL-1β into IL-1β in both nutrient replete or nutrient depleted settings. Inflammasomes can release the pore-forming moiety of GSDMD to encourage membrane permeability for direct secretion and can induce membrane rupture for indirect secretion. Several other membrane permeabilization strategies are sufficient to secrete IL-1β including other gasdermin family members, necroptotic MLKL, bacterial pore forming toxins, and physical disruption. An alternate mechanism of IL-1β secretion may exist that involves capture or translocation into a vesicle intermediate during nutrient depleted or proteotoxic stress settings.

work suggests that glycine may impinge upon NINJ1 oligomerization and membrane rupture, though evidence of whether this is a direct effect on NINJ1 or operates on an unknown activation signal of NINJ1 has not been determined (Borges et al., 2021). Thus, glycine buffering and NINJ1 deficiency can be used to chemically and genetically separate IL-1 secretion from lysis in many contexts (Evavold et al., 2018; Heilig et al., 2018; Bjanes et al., 2021; Borges et al., 2021; Kayagaki et al., 2021).

TRANSIENT MEMBRANE PERMEABILITY AND HYPERACTIVATION

Transient membrane permeability may represent a mechanism of IL-1 secretion (Evavold et al., 2018; Rühl et al., 2018). Recent work has established that cell death and lysis are not necessary consequences of inflammasome activation (Chen et al., 2014; Conos et al., 2016; Gaidt et al., 2016; Wolf et al., 2016; Zanoni et al., 2016). Cells that secrete IL-1 while maintaining energetic viability and resisting membrane rupture are considered hyperactive (Zanoni et al., 2016; Evavold et al., 2018). Certain cell types such as a neutrophils and dendritic cells demonstrate intrinsic resistance to pyroptotic lysis that may represent different membrane reparative capacities, caspase activation dynamics, and expression levels of pore forming proteins such as GSDMD (Chen et al., 2014; Zanoni et al., 2016; Boucher et al., 2018). Some cell types, such as human and porcine blood monocytes, can release IL-1β without cell death as occurs during exogenous treatment of cells with PAMPs such as LPS (Gaidt et al., 2016). However, stimulation of monocytes with combinations of PAMPs can convert a non-lytic release of IL-1β to lytic release in a GSDMD- and ROS-dependent manner (Semino et al., 2018).

An increasing set of stimuli has been reported to induce inflammasome activities and IL-1 release from living (hyperactive) cells (Shimada et al., 2010; Wolf et al., 2016; Zanoni et al., 2016; Evavold et al., 2018). It has been noted that a single inflammasome stimulus can elicit pyroptosis or hyperactivation within the same cell type that presumably

depends on the strength of inflammasome signaling (Xia et al., 2021). Cell types may also display varied expression of NINJ1 that result in different thresholds or propensity for cell lysis. This may explain why some cell types such as skin keratinocytes display membrane ballooning after GSDMD and GSDME activation without appreciable cell lysis (Orzalli et al., 2021).

While more mechanistic studies are necessary to define the molecular events that determine inflammasome-dependent activities in dead (pyroptotic) or live (hyperactive) cells, physiological consequences of these activities have proven notable. In particular, the cell fate of hyperactivation has gained attention for its superior ability to activate adaptive immune responses (Zanoni et al., 2016). By adding the IL-1 family to the repertoire of cytokines secreted by activated DCs, modulating hyperactivation has implications for next generation vaccines. Inflammasomes, specifically within hyperactive DCs are able to speed up the differentiation of antigen specific $CD8^+$ T cells and the production of long-lived memory T resident memory cells, which are associated with protective immunity in cancer (Zhivaki et al., 2020). In the context of *S. aureus* infection, similar links between cell hyperactivation and protective immunity have been observed (Sanchez et al., 2017). The metabolic profile of hyperactive cells is distinct from naïve or traditionally activated cells, as these cells maintain mitochondrial oxidative phosphorylation while simultaneously utilizing glycolytic activities (Wolf et al., 2016; Zanoni et al., 2016; Di Gioia et al., 2020). In contrast, traditionally activated cells undergo a shift from oxidative phosphorylation to glycolysis. These different metabolic activities and maintenance of mitochondrial polarization may regulate IL-1 secretion, membrane reparative capacity, and cell death (Di Gioia et al., 2020). As the host dynamically regulates metabolism under stress conditions or infection (Pernas, 2021), cells may have evolved alternative methods to secrete IL-1 beyond direct membrane pores in order to retain the threat contextualization of secreted pro-inflammatory IL-1 family members.

METABOLIC CONTROL OF IL-1 SECRETION

As stated in the prior sections, under nutrient replete conditions a major mechanism of IL-1 secretion is direct conveyance across the plasma membrane through GSDMD pores and other membrane permeabilization strategies (**Figure 3**). During metabolic dysfunction or starvation, alternative mechanisms may mediate IL-1 secretion (**Figure 3**). IL-1β can be detected in vesicle intermediates during ER stress and starvation (Dupont et al., 2011; Zhang et al., 2015; Kimura et al., 2017; Zhang et al., 2020b). IL-1β can be ubiquitinated, which may encourage degradation through autophagy and the proteasome or impinge upon cleavage by inflammatory caspases (Harris et al., 2011; Ainscough et al., 2014; Eldridge et al., 2017; Vijayaraj et al., 2021). Autophagy is also known to impinge upon inflammasome signaling (Saitoh et al., 2008; Shi et al., 2012; Liu et al., 2016b). Thus, paradoxically autophagic capture of inflammasomes and cleaved substrates such as of IL-1β may serve as a possible mechanism for increased cellular survival by limiting inflammasome signaling but also promote secretion of low quantities of IL-1β. As such, mature IL-1β may also be secreted *via* autophagic means, but the precise trafficking to prevent degradation has not been determined (Dupont et al., 2011; Kimura et al., 2017). During inflammasome activation, GSDMD may still play a role in autophagic release of IL-1 (Karmakar et al., 2020). In neutrophils, GSDMD is genetically required for IL-1β release (Heilig et al., 2018; Monteleone et al., 2018), but this appears to be independent of plasma membrane localization and pore formation (Karmakar et al., 2020). Instead, GSDMD targets intracellular granules that may allow for IL-1β incorporation into secretory granules (Karmakar et al., 2020). This targeting of secretory granules may allow for a feed forward amplification loop whereby release of granule proteases into the cytosol processes additional IL-1β and GSDMD and calcium flux elicited from the hypercalcemic granules or lysosomes lead to lysosomal exocytosis (Karmakar et al., 2020). Furthermore, deficiency of the autophagy component ATG7 diminished IL-1β in neutrophils suggesting that autophagosomes may also contribute to secretion in addition to perforated secretory granules. Disruption of lysosomes by GSDMD may also explain why autophagosomes may not become degradatory in certain contexts of IL-1β capture.

Ragulator-Rag is purported to control GSDMD pore formation *via* control of GSDMD cleavage during caspase-8 activation in the context of TAK1 inhibition and regulate GSDMD oligomerization through metabolic control of ROS production (Evavold et al., 2021; Zheng et al., 2021). Ragulator-Rag can also mediate repair of endo-membrane damage as evident after treatment with lysosomal damaging agents (Jia et al., 2018; Jia et al., 2020). This may invoke direct activation of macroautophagy as well as indirect upregulation of lysosome biogenesis and autophagy genes downstream of mTOR inactivation and subsequent nuclear translocation of de-phosphorylated TFEB (Sardiello et al., 2009; Settembre et al., 2011; Efeyan et al., 2013; Jia et al., 2018; Jia et al., 2020). Recent work has also suggested that mitochondrial dysfunction is sensed by the Ragulator-Rag complex presumably for autophagic capture of damaged or stressed mitochondria (Condon et al., 2021). Ragulator-Rag may be a general regulator of membrane homeostasis by surveying damaged membranous organelles. Thus, Ragulator-Rag may act to prevent GSDMD-mediated membrane damage in many distinct ways ranging from control of cleavage, oligomerization, and removal of damaged organelles (Jia et al., 2018; Condon et al., 2021; Evavold et al., 2021; Zheng et al., 2021).

Autophagic capture and release of mature IL-1β may operate under diverse metabolic perturbations that could occur in response to stress or microbial invasion (Tattoli et al., 2012; Ravindran et al., 2016). Investigation of whether Ragulator-Rag deficiency, starvation, or other mechanisms of mTOR inhibition decrease GSDMD-mediated IL-1 release while encouraging autophagic means of release are needed. Metabolic perturbations have long been known to affect initiation of cell death signaling through apoptotic, pyroptotic, and necroptotic pathways (Zhang et al., 2009; Andersen and Kornbluth, 2013;

Próchnicki and Latz, 2017; Pajuelo et al., 2018). These metabolic perturbations may serve as evolutionary hallmarks of threats to the host such as sterile stressors or pathogenic invasion. Recent studies have identified nuanced metabolic control of terminal stages of death pathways as is evident with control of GSDMD at the stage of cleavage by tricarboxylic acid cycle (TCA) metabolites, oligomerization by ROS metabolites, and pore conformation by phospholipid catabolism (Humphries et al., 2020; Bambouskova et al., 2021; Evavold et al., 2021; Santa Cruz Garcia et al., 2022). As microbes may also have evolved mechanisms to manipulate these endogenous metabolic checkpoints, alternative mechanisms of IL-1 release are crucial to convey threat levels to other cells. IL-1 family cytokines can poise or prime cells for cell-intrinsic immunity or detection of higher-level threats (Garlanda et al., 2013; Evavold and Kagan, 2019). IL-1 family cytokines can also encourage local inflammation and recruitment of additional innate and adaptive leukocytes (Garlanda et al., 2013). In addition, IL-1 family cytokines can reprogram organismal metabolism through fever (Garlanda et al., 2013). The intersection between host defense and metabolism is a burgeoning area of investigation. Studies on IL-1 family cytokines as both initiators and responders to host metabolic state are sure to follow.

CONCLUDING REMARKS AND OUTSTANDING QUESTIONS

Of the IL-1 family members, IL-1α and IL-1β have been the most characterized in terms of bioactivity, activation, and secretion. Based on current evidence for the multi-step regulation of these prototypical nuclear and cytosolic IL-1 family members, we speculate that similar mechanisms may exist for the activation and secretion of other leaderless IL-1 family members. Specifically, we predict that the crossing of topological barriers, such as the nuclear and/or plasma membranes, represents a point of regulation for other IL-1 family members. Whereas nuclear IL-1 family members may be constitutively expressed yet confined by an added physical barrier, cytosolic IL-1 family members may be primarily regulated by context-dependent transcription and refined proteolytic cleavage by the secreting cell or other cell types. Further studies are required to determine the signals that instruct the nuclear release of IL-1α, IL-33, and IL-37, and in the case of newly synthesized membrane-bound IL-1α, more work is required to delineate the mechanisms that instruct the trafficking to and crossing of the plasma membrane. Perhaps due to inflammatory nature of secreted IL-1 family cytokines, compensatory mechanisms regulate IL-1 proteins post-secretion. For example, decoy receptors, binding proteins, and inactive IL-1 family structural analogues (termed receptor antagonists) further buffer the bioactivity of the IL-1 family in the extracellular space. These buffering systems likely exist to limit inflammation in the context of homeostatic death processes and may be upregulated during the resolution phase of inflammation. Moreover, metabolic control of IL-1 family cytokines likely constitutes another pathway of regulation. While intact metabolism may primarily affect IL-1β secretion *via* encouraging membrane permeability, alternate routes of secretion may occur in nutrient deplete contexts or proteotoxic stress. For instance, translocation or capture of IL-1β into vesicle intermediates may rely on the metabolic status of the cell. Analogous metabolic mechanisms may also exist in the regulation of other IL-1 family members. In terms of membrane permeabilization strategies employed by the host to secrete IL-1, GSDMD and GSDME are the best characterized. Recent studies have begun to discover host or pathogen driven activation programs for other gasdermin family members. Additional studies have implicated distinct mechanisms of membrane permeabilization or rupture mediated through MLKL, NINJ1, and bacterial pore forming toxins as well as physical disruption as being sufficient for mediating release of IL-1. Cell type specific or pathogen specific programs may therefore exist that mediate the secretion of particular IL-1 family cytokines in response to unique membrane permeabilization strategies.

AUTHOR CONTRIBUTIONS

CE and JK wrote and edited this manuscript. CE drafted figures.

FUNDING

This study was supported by NIH grants AI133524, AI093589, AI116550 and P30DK34854 to JK. CE is supported by the Ragon Early Independence Fellowship.

ACKNOWLEDGMENTS

We thank Isabella Fraschilla for thoughtful reading and revision of the manuscript. We apologize to members of the community whose studies were not cited herein due to space constraints. Figures were generated using Biorender.

REFERENCES

Aarreberg, L. D., Esser-Nobis, K., Driscoll, C., Shuvarikov, A., Roby, J. A., and Gale, M., Jr. (2019). Interleukin-1β Induces mtDNA Release to Activate Innate Immune Signaling via cGAS-STING. *Mol. Cell* 74, 801–815. doi:10.1016/j.molcel.2019.02.038

Afonina, I. S., Tynan, G. A., Logue, S. E., Cullen, S. P., Bots, M., Lüthi, A. U., et al. (2011). Granzyme B-dependent Proteolysis Acts as a Switch to Enhance the Proinflammatory Activity of IL-1α. *Mol. Cell* 44, 265–278. doi:10.1016/j.molcel.2011.07.037

Aglietti, R. A., Estevez, A., Gupta, A., Ramirez, M. G., Liu, P. S., Kayagaki, N., et al. (2016). GsdmD P30 Elicited by Caspase-11 during Pyroptosis Forms Pores in Membranes. *Proc. Natl. Acad. Sci. U.S.A.* 113, 7858–7863. doi:10.1073/pnas.1607769113

Ainscough, J. S., Frank Gerberick, G., Zahedi-Nejad, M., Lopez-Castejon, G., Brough, D., Kimber, I., et al. (2014). Dendritic Cell IL-1α and IL-1β Are

Polyubiquitinated and Degraded by the Proteasome. *J. Biol. Chem.* 289, 35582–35592. doi:10.1074/jbc.m114.595686

Aizawa, E., Karasawa, T., Watanabe, S., Komada, T., Kimura, H., Kamata, R., et al. (2020). GSDME-dependent Incomplete Pyroptosis Permits Selective IL-1α Release under Caspase-1 Inhibition. *iScience* 23, 101070. doi:10.1016/j.isci.2020.101070

Ali, S., Huber, M., Kollewe, C., Bischoff, S. C., Falk, W., and Martin, M. U. (2007). IL-1 Receptor Accessory Protein Is Essential for IL-33-induced Activation of T Lymphocytes and Mast Cells. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18660–18665. doi:10.1073/pnas.0705939104

Andersen, J. L., and Kornbluth, S. (2013). The Tangled Circuitry of Metabolism and Apoptosis. *Mol. Cell* 49, 399–410. doi:10.1016/j.molcel.2012.12.026

Arend, W. P., Joslin, F. G., Thompson, R. C., and Hannum, C. H. (1989). An IL-1 Inhibitor from Human Monocytes. Production and Characterization of Biologic Properties. *J. Immunol.* 143, 1851–1858.

Arend, W. P., Malyak, M., Smith, M. F., Jr., Whisenand, T. D., Slack, J. L., Sims, J. E., et al. (1994). Binding of IL-1 Alpha, IL-1 Beta, and IL-1 Receptor Antagonist by Soluble IL-1 Receptors and Levels of Soluble IL-1 Receptors in Synovial Fluids. *J. Immunol.* 153, 4766–4774.

Arpaia, N., Green, J. A., Moltedo, B., Arvey, A., Hemmers, S., Yuan, S., et al. (2015). A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell* 162, 1078–1089. doi:10.1016/j.cell.2015.08.021

Auron, P. E., Webb, A. C., Rosenwasser, L. J., Mucci, S. F., Rich, A., Wolff, S. M., et al. (1984). Nucleotide Sequence of Human Monocyte Interleukin 1 Precursor cDNA. *Proc. Natl. Acad. Sci. U.S.A.* 81, 7907–7911. doi:10.1073/pnas.81.24.7907

Bae, S., Kang, T., Hong, J., Lee, S., Choi, J., Jhun, H., et al. (2012). Contradictory Functions (Activation/termination) of Neutrophil Proteinase 3 Enzyme (PR3) in Interleukin-33 Biological Activity. *J. Biol. Chem.* 287, 8205–8213. doi:10.1074/jbc.m111.295055

Bambouskova, M., Potuckova, L., Paulenda, T., Kerndl, M., Mogilenko, D. A., Lizotte, K., et al. (2021). Itaconate Confers Tolerance to Late NLRP3 Inflammasome Activation. *Cell Rep.* 34, 108756. doi:10.1016/j.celrep.2021.108756

Baroja-Mazo, A., Martín-Sánchez, F., Gomez, A. I., Martínez, C. M., Amores-Iniesta, J., Compan, V., et al. (2014). The NLRP3 Inflammasome Is Released as a Particulate Danger Signal that Amplifies the Inflammatory Response. *Nat. Immunol.* 15, 738–748. doi:10.1038/ni.2919

Bartemes, K. R., Iijima, K., Kobayashi, T., Kephart, G. M., McKenzie, A. N., and Kita, H. (2012). IL-33-Responsive Lineage−CD25+CD44hi Lymphoid Cells Mediate Innate Type 2 Immunity and Allergic Inflammation in the Lungs. *J. I.* 188, 1503–1513. doi:10.4049/jimmunol.1102832

Bessa, J., Meyer, C. A., de Vera Mudry, M. C., Schlicht, S., Smith, S. H., Iglesias, A., et al. (2014). Altered Subcellular Localization of IL-33 Leads to Non-resolving Lethal Inflammation. *J. Autoimmun.* 55, 33–41. doi:10.1016/j.jaut.2014.02.012

Bjanes, E., Sillas, R. G., Matsuda, R., Demarco, B., Fettrelet, T., DeLaney, A. A., et al. (2021). Genetic Targeting of Card19 Is Linked to Disrupted NINJ1 Expression, Impaired Cell Lysis, and Increased Susceptibility to Yersinia Infection. *PLoS Pathog.* 17, e1009967. doi:10.1371/journal.ppat.1009967

Black, R. A., Kronheim, S. R., Cantrell, M., Deeley, M. C., March, C. J., Prickett, K. S., et al. (1988). Generation of Biologically Active Interleukin-1 Beta by Proteolytic Cleavage of the Inactive Precursor. *J. Biol. Chem.* 263, 9437–9442. doi:10.1016/s0021-9258(19)76559-4

Blumberg, H., Dinh, H., Trueblood, E. S., Pretorius, J., Kugler, D., Weng, N., et al. (2007). Opposing Activities of Two Novel Members of the IL-1 Ligand Family Regulate Skin Inflammation. *J. Exp. Med.* 204, 2603–2614. doi:10.1084/jem.20070157

Borges, J. P., Sætra, R. S. R., Volchuk, A., Bugge, M., Kilburn, B., Goldenberg, N. M., et al. (2021). *Glycine Targets NINJ1-Mediated Plasma Membrane Rupture to Provide Cytoprotection*. Laurel Hollow, New York: Cold Spring Harbor Laboratory.

Boucher, D., Monteleone, M., Coll, R. C., Chen, K. W., Ross, C. M., Teo, J. L., et al. (2018). Caspase-1 Self-Cleavage Is an Intrinsic Mechanism to Terminate Inflammasome Activity. *J. Exp. Med.* 215, 827–840. doi:10.1084/jem.20172222

Boutet, M.-A., Bart, G., Penhoat, M., Amiaud, J., Brulin, B., Charrier, C., et al. (2016). Distinct Expression of Interleukin (IL)-36α, β and γ, Their Antagonist IL-36Ra and IL-38 in Psoriasis, Rheumatoid Arthritis and Crohn's Disease. *Clin. Exp. Immunol.* 184, 159–173. doi:10.1111/cei.12761

Bulau, A.-M., Nold, M. F., Li, S., Nold-Petry, C. A., Fink, M., Mansell, A., et al. (2014). Role of Caspase-1 in Nuclear Translocation of IL-37, Release of the Cytokine, and IL-37 Inhibition of Innate Immune Responses. *Proc. Natl. Acad. Sci. U.S.A.* 111, 2650–2655. doi:10.1073/pnas.1324140111

Bulek, K., Zhao, J., Liao, Y., Rana, N., Corridoni, D., Antanaviciute, A., et al. (2020). Epithelial-derived Gasdermin D Mediates Nonlytic IL-1β Release during Experimental Colitis. *J. Clin. Invest.* 130, 4218–4234. doi:10.1172/JCI138103

Burzynski, L. C., Humphry, M., Pyrillou, K., Wiggins, K. A., Chan, J. N. E., Figg, N., et al. (2019). The Coagulation and Immune Systems Are Directly Linked through the Activation of Interleukin-1α by Thrombin. *Immunity* 50, 1033–1042. doi:10.1016/j.immuni.2019.03.003

Caramalho, I., Lopes-Carvalho, T., Ostler, D., Zelenay, S., Haury, M., and Demengeot, J. (2003). Regulatory T Cells Selectively Express Toll-like Receptors and Are Activated by Lipopolysaccharide. *J. Exp. Med.* 197, 403–411. doi:10.1084/jem.20021633

Carriere, V., Roussel, L., Ortega, N., Lacorre, D.-A., Americh, L., Aguilar, L., et al. (2007). IL-33, the IL-1-like Cytokine Ligand for ST2 Receptor, Is a Chromatin-Associated Nuclear Factor *In Vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 282–287. doi:10.1073/pnas.0606854104

Cayrol, C., and Girard, J.-P. (2009). The IL-1-like Cytokine IL-33 Is Inactivated after Maturation by Caspase-1. *Proc. Natl. Acad. Sci. U.S.A.* 106, 9021–9026. doi:10.1073/pnas.0812690106

Chackerian, A. A., Oldham, E. R., Murphy, E. E., Schmitz, J., Pflanz, S., and Kastelein, R. A. (2007). IL-1 Receptor Accessory Protein and ST2 Comprise the IL-33 Receptor Complex. *J. Immunol.* 179, 2551–2555. doi:10.4049/jimmunol.179.4.2551

Chan, A. H., and Schroder, K. (2020). Inflammasome Signaling and Regulation of Interleukin-1 Family Cytokines. *J. Exp. Med.* 217, 20190314. jem. doi:10.1084/jem.20190314

Chen, K. W., Gross, C. J., Sotomayor, F. V., Stacey, K. J., Tschopp, J., Sweet, M. J., et al. (2014). The Neutrophil NLRC4 Inflammasome Selectively Promotes IL-1β Maturation without Pyroptosis during Acute Salmonella Challenge. *Cell Rep.* 8, 570–582. doi:10.1016/j.celrep.2014.06.028

Chu, M., Chu, I., Yung, E., Lam, C., Leung, T., Wong, G., et al. (2016). Aberrant Expression of Novel Cytokine IL-38 and Regulatory T Lymphocytes in Childhood Asthma. *Molecules* 21, 933. doi:10.3390/molecules21070933

Clancy, D. M., Sullivan, G. P., Moran, H. B. T., Henry, C. M., Reeves, E. P., McElvaney, N. G., et al. (2018). Extracellular Neutrophil Proteases Are Efficient Regulators of IL-1, IL-33, and IL-36 Cytokine Activity but Poor Effectors of Microbial Killing. *Cell Rep.* 22, 2937–2950. doi:10.1016/j.celrep.2018.02.062

Coeshott, C., Ohnemus, C., Pilyavskaya, A., Ross, S., Wieczorek, M., Kroona, H., et al. (1999). Converting Enzyme-independent Release of Tumor Necrosis Factor α and IL-1β from a Stimulated Human Monocytic Cell Line in the Presence of Activated Neutrophils or Purified Proteinase 3. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6261–6266. doi:10.1073/pnas.96.11.6261

Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., et al. (1993). Interleukin-1 Type II Receptor: a Decoy Target for IL-1 that Is Regulated by IL-4. *Science* 261, 472–475. doi:10.1126/science.8332913

Condon, K. J., Orozco, J. M., Adelmann, C. H., Spinelli, J. B., van der Helm, P. W., Roberts, J. M., et al. (2021). Genome-wide CRISPR Screens Reveal Multitiered Mechanisms through Which mTORC1 Senses Mitochondrial Dysfunction. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2022120118. doi:10.1073/pnas.2022120118

Conos, S. A., Chen, K. W., De Nardo, D., Hara, H., Whitehead, L., Núñez, G., et al. (2017). Active MLKL Triggers the NLRP3 Inflammasome in a Cell-Intrinsic Manner. *Proc. Natl. Acad. Sci. U. S. A.* 114, E961–E969. doi:10.1073/pnas.1613305114

Conos, S. A., Lawlor, K. E., Vaux, D. L., Vince, J. E., and Lindqvist, L. M. (2016). Cell Death Is Not Essential for Caspase-1-Mediated Interleukin-1β Activation and Secretion. *Cell Death Differ.* 23, 1827–1838. doi:10.1038/cdd.2016.69

Cooper, S. T., and McNeil, P. L. (2015). Membrane Repair: Mechanisms and Pathophysiology. *Physiol. Rev.* 95, 1205–1240. doi:10.1152/physrev.00037.2014

Crellin, N. K., Garcia, R. V., Hadisfar, O., Allan, S. E., Steiner, T. S., and Levings, M. K. (2005). Human CD4+T Cells Express TLR5 and its Ligand Flagellin Enhances the Suppressive Capacity and Expression of FOXP3 in CD4+CD25+T Regulatory Cells. *J. Immunol.* 175, 8051–8059. doi:10.4049/jimmunol.175.12.8051

Devant, P., Boršić, E., Ngwa, E. M., Thiagarajah, J. R., Hafner-Bratkovič, I., Evavold, C. L., et al. (2022). *The Pore-Forming Protein Gasdermin D Is a Cellular Redox Sensor*. Laurel Hollow, New York: Cold Spring Harbor Laboratory.

Devant, P., Cao, A., and Kagan, J. C. (2021). Evolution-inspired Redesign of the LPS Receptor Caspase-4 into an Interleukin-1beta Converting Enzyme. *Sci. Immunol.* 6. doi:10.1126/sciimmunol.abh3567

Di Gioia, M., Spreafico, R., Springstead, J. R., Mendelson, M. M., Joehanes, R., Levy, D., et al. (2020). Endogenous Oxidized Phospholipids Reprogram Cellular Metabolism and Boost Hyperinflammation. *Nat. Immunol.* 21, 42–53. doi:10.1038/s41590-019-0539-2

Dinarello, C. A. (2018). Overview of the IL-1 Family in Innate Inflammation and Acquired Immunity. *Immunol. Rev.* 281, 8–27. doi:10.1111/imr.12621

Ding, J., Wang, K., Liu, W., She, Y., Sun, Q., Shi, J., et al. (2016). Pore-forming Activity and Structural Autoinhibition of the Gasdermin Family. *Nature* 535, 111–116. doi:10.1038/nature18590

Donado, C. A., Cao, A. B., Simmons, D. P., Croker, B. A., Brennan, P. J., and Brenner, M. B. (2020). A Two-Cell Model for IL-1β Release Mediated by Death-Receptor Signaling. *Cell Rep.* 31, 107466. doi:10.1016/j.celrep.2020.03.030

Dupont, N., Jiang, S., Pilli, M., Ornatowski, W., Bhattacharya, D., and Deretic, V. (2011). Autophagy-based Unconventional Secretory Pathway for Extracellular Delivery of IL-1β. *EMBO J.* 30, 4701–4711. doi:10.1038/emboj.2011.398

Efeyan, A., Zoncu, R., Chang, S., Gumper, I., Snitkin, H., Wolfson, R. L., et al. (2013). Regulation of mTORC1 by the Rag GTPases Is Necessary for Neonatal Autophagy and Survival. *Nature* 493, 679–683. doi:10.1038/nature11745

Eldridge, M. J. G., Sanchez-Garrido, J., Hoben, G. F., Goddard, P. J., and Shenoy, A. R. (2017). The Atypical Ubiquitin E2 Conjugase UBE2L3 Is an Indirect Caspase-1 Target and Controls IL-1β Secretion by Inflammasomes. *Cell Rep.* 18, 1285–1297. doi:10.1016/j.celrep.2017.01.015

Elliott, E. I., Miller, A. N., Banoth, B., Iyer, S. S., Stotland, A., Weiss, J. P., et al. (2018). Cutting Edge: Mitochondrial Assembly of the NLRP3 Inflammasome Complex Is Initiated at Priming. *J. I.* 200, 3047–3052. doi:10.4049/jimmunol.1701723

England, H., Summersgill, H. R., Edye, M. E., Rothwell, N. J., and Brough, D. (2014). Release of Interleukin-1α or Interleukin-1β Depends on Mechanism of Cell Death. *J. Biol. Chem.* 289, 15942–15950. doi:10.1074/jbc.m114.557561

Evavold, C. L., Hafner-Bratkovič, I., Devant, P., D'Andrea, J. M., Ngwa, E. M., Boršić, E., et al. (2021). Control of Gasdermin D Oligomerization and Pyroptosis by the Ragulator-Rag-mTORC1 Pathway. *Cell* 184, 4495–4511. doi:10.1016/j.cell.2021.06.028

Evavold, C. L., and Kagan, J. C. (2018). How Inflammasomes Inform Adaptive Immunity. *J. Mol. Biol.* 430, 217–237. doi:10.1016/j.jmb.2017.09.019

Evavold, C. L., and Kagan, J. C. (2019). Inflammasomes: Threat-Assessment Organelles of the Innate Immune System. *Immunity* 51, 609–624. doi:10.1016/j.immuni.2019.08.005

Evavold, C. L., Ruan, J., Tan, Y., Xia, S., Wu, H., and Kagan, J. C. (2018). The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages. *Immunity* 48, 35–44. doi:10.1016/j.immuni.2017.11.013

Fink, S. L., and Cookson, B. T. (2006). Caspase-1-dependent Pore Formation during Pyroptosis Leads to Osmotic Lysis of Infected Host Macrophages. *Cell Microbiol.* 8, 1812–1825. doi:10.1111/j.1462-5822.2006.00751.x

Frank, A., Rauen, U., and Groot, H. d. (2000). Protection by glycine against Hypoxic Injury of Rat Hepatocytes: Inhibition of Ion Fluxes through Nonspecific Leaks. *J. Hepatology* 32, 58–66. doi:10.1016/s0168-8278(00)80190-7

Franklin, B. S., Bossaller, L., De Nardo, D., Ratter, J. M., Stutz, A., Engels, G., et al. (2014). The Adaptor ASC Has Extracellular and 'prionoid' Activities that Propagate Inflammation. *Nat. Immunol.* 15, 727–737. doi:10.1038/ni.2913

Gaidt, M. M., Ebert, T. S., Chauhan, D., Schmidt, T., Schmid-Burgk, J. L., Rapino, F., et al. (2016). Human Monocytes Engage an Alternative Inflammasome Pathway. *Immunity* 44, 833–846. doi:10.1016/j.immuni.2016.01.012

Gardner, S. E., Humphry, M., Bennett, M. R., and Clarke, M. C. H. (2015). Senescent Vascular Smooth Muscle Cells Drive Inflammation through an Interleukin-1α-dependent Senescence-Associated Secretory Phenotype. *Atvb* 35, 1963–1974. doi:10.1161/atvbaha.115.305896

Garlanda, C., Dinarello, C. A., and Mantovani, A. (2013). The Interleukin-1 Family: Back to the Future. *Immunity* 39, 1003–1018. doi:10.1016/j.immuni.2013.11.010

Gatti, S., Beck, J., Fantuzzi, G., Bartfai, T., and Dinarello, C. A. (2002). Effect of Interleukin-18 on Mouse Core Body Temperature. *Am. J. Physiology-Regulatory, Integr. Comp. Physiology* 282, R702–R709. doi:10.1152/ajpregu.00393.2001

Ghayur, T., Banerjee, S., Hugunin, M., Butler, D., Herzog, L., Carter, A., et al. (1997). Caspase-1 Processes IFN-γ-Inducing Factor and Regulates LPS-Induced IFN- γ Production. *Nature* 386, 619–623. doi:10.1038/386619a0

Gong, J., Wei, T., Stark, R. W., Jamitzky, F., Heckl, W. M., Anders, H. J., et al. (2010). Inhibition of Toll-like Receptors TLR4 and 7 Signaling Pathways by SIGIRR: a Computational Approach. *J. Struct. Biol.* 169, 323–330. doi:10.1016/j.jsb.2009.12.007

Gong, Y.-N., Guy, C., Olauson, H., Becker, J. U., Yang, M., Fitzgerald, P., et al. (2017). ESCRT-III Acts Downstream of MLKL to Regulate Necroptotic Cell Death and its Consequences. *Cell* 169, 286–300. e16. doi:10.1016/j.cell.2017.03.020

Greenfeder, S. A., Nunes, P., Kwee, L., Labow, M., Chizzonite, R. A., and Ju, G. (1995). Molecular Cloning and Characterization of a Second Subunit of the Interleukin 1 Receptor Complex. *J. Biol. Chem.* 270, 13757–13765. doi:10.1074/jbc.270.23.13757

Gross, O., Yazdi, A. S., Thomas, C. J., Masin, M., Heinz, L. X., Guarda, G., et al. (2012). Inflammasome Activators Induce Interleukin-1α Secretion via Distinct Pathways with Differential Requirement for the Protease Function of Caspase-1. *Immunity* 36, 388–400. doi:10.1016/j.immuni.2012.01.018

Gu, Y., Kuida, K., Tsutsui, H., Ku, G., Hsiao, K., Fleming, M. A., et al. (1997). Activation of Interferon-γ Inducing Factor Mediated by Interleukin-1β Converting Enzyme. *Science* 275, 206–209. doi:10.1126/science.275.5297.206

Gutierrez, K. D., Davis, M. A., Daniels, B. P., Olsen, T. M., Ralli-Jain, P., Tait, S. W. G., et al. (2017). MLKL Activation Triggers NLRP3-Mediated Processing and Release of IL-1β Independently of Gasdermin-D. *J. I.* 198, 2156–2164. doi:10.4049/jimmunol.1601757

Han, Y., Mora, J., Huard, A., da Silva, P., Wiechmann, S., Putyrski, M., et al. (2019). IL-38 Ameliorates Skin Inflammation and Limits IL-17 Production from γδ T Cells. *Cell Rep.* 27, 835–846. doi:10.1016/j.celrep.2019.03.082

Harris, J., Hartman, M., Roche, C., Zeng, S. G., O'Shea, A., Sharp, F. A., et al. (2011). Autophagy Controls IL-1β Secretion by Targeting Pro-IL-1β for Degradation. *J. Biol. Chem.* 286, 9587–9597. doi:10.1074/jbc.m110.202911

Hazuda, D. J., Strickler, J., Kueppers, F., Simon, P. L., and Young, P. R. (1990). Processing of Precursor Interleukin 1 Beta and Inflammatory Disease. *J. Biol. Chem.* 265, 6318–6322. doi:10.1016/s0021-9258(19)39328-7

He, W.-t., Wan, H., Hu, L., Chen, P., Wang, X., Huang, Z., et al. (2015). Gasdermin D Is an Executor of Pyroptosis and Required for Interleukin-1β Secretion. *Cell Res.* 25, 1285–1298. doi:10.1038/cr.2015.139

Heilig, R., Dick, M. S., Sborgi, L., Meunier, E., Hiller, S., and Broz, P. (2018). The Gasdermin-D Pore Acts as a Conduit for IL-1β Secretion in Mice. *Eur. J. Immunol.* 48, 584–592. doi:10.1002/eji.201747404

Heilig, R., Dilucca, M., Boucher, D., Chen, K. W., Hancz, D., Demarco, B., et al. (2020). Caspase-1 Cleaves Bid to Release Mitochondrial SMAC and Drive Secondary Necrosis in the Absence of GSDMD. *Life Sci. Alliance* 3, e202000735. doi:10.26508/lsa.202000735

Howard, A. D., Kostura, M. J., Thornberry, N., Ding, G. J., Limjuco, G., Weidner, J., et al. (1991). IL-1-converting Enzyme Requires Aspartic Acid Residues for Processing of the IL-1 Beta Precursor at Two Distinct Sites and Does Not Cleave 31-kDa IL-1 Alpha. *J. Immunol.* 147, 2964–2969.

Hu, J. J., Liu, X., Xia, S., Zhang, Z., Zhang, Y., Zhao, J., et al. (2020). FDA-approved Disulfiram Inhibits Pyroptosis by Blocking Gasdermin D Pore Formation. *Nat. Immunol.* 21, 736–745. doi:10.1038/s41590-020-0669-6

Humphries, F., Shmuel-Galia, L., Ketelut-Carneiro, N., Li, S., Wang, B., Nemmara, V. V., et al. (2020). Succination Inactivates Gasdermin D and Blocks Pyroptosis. *Science* 369, 1633–1637. doi:10.1126/science.abb9818

Hurgin, V., Novick, D., and Rubinstein, M. (2002). The Promoter of IL-18 Binding Protein: Activation by an IFN-γ-Induced Complex of IFN Regulatory Factor 1 and CCAAT/enhancer Binding Protein β. *Proc. Natl. Acad. Sci. U.S.A.* 99, 16957–16962. doi:10.1073/pnas.262663399

Iyer, S. S., He, Q., Janczy, J. R., Elliott, E. I., Zhong, Z., Olivier, A. K., et al. (2013). Mitochondrial Cardiolipin Is Required for Nlrp3 Inflammasome Activation. *Immunity* 39, 311–323. doi:10.1016/j.immuni.2013.08.001

Jia, J., Abudu, Y. P., Claude-Taupin, A., Gu, Y., Kumar, S., Choi, S. W., et al. (2018). Galectins Control mTOR in Response to Endomembrane Damage. *Mol. Cell* 70, 120–135. doi:10.1016/j.molcel.2018.03.009

Jia, J., Claude-Taupin, A., Gu, Y., Choi, S. W., Peters, R., Bissa, B., et al. (2020). Galectin-3 Coordinates a Cellular System for Lysosomal Repair and Removal. *Dev. Cell* 52, 69–87. doi:10.1016/j.devcel.2019.10.025

Johnston, A., Xing, X., Guzman, A. M., Riblett, M., Loyd, C. M., Ward, N. L., et al. (2011). IL-1F5, -F6, -F8, and -F9: a Novel IL-1 Family Signaling System that Is Active in Psoriasis and Promotes Keratinocyte Antimicrobial Peptide Expression. *J. I.* 186, 2613–2622. doi:10.4049/jimmunol.1003162

Karmakar, M., Minns, M., Greenberg, E. N., Diaz-Aponte, J., Pestonjamasp, K., Johnson, J. L., et al. (2020). N-GSDMD Trafficking to Neutrophil Organelles Facilitates IL-1β Release Independently of Plasma Membrane Pores and Pyroptosis. *Nat. Commun.* 11, 2212. doi:10.1038/s41467-020-16043-9

Kayagaki, N., Kornfeld, O. S., Lee, B. L., Stowe, I. B., O'Rourke, K., Li, Q., et al. (2021). NINJ1 Mediates Plasma Membrane Rupture during Lytic Cell Death. *Nature* 591, 131–136. doi:10.1038/s41586-021-03218-7

Kayagaki, N., Stowe, I. B., Lee, B. L., O'Rourke, K., Anderson, K., Warming, S., et al. (2015). Caspase-11 Cleaves Gasdermin D for Non-canonical Inflammasome Signalling. *Nature* 526, 666–671. doi:10.1038/nature15541

Keller, M., Rüegg, A., Werner, S., and Beer, H.-D. (2008). Active Caspase-1 Is a Regulator of Unconventional Protein Secretion. *Cell* 132, 818–831. doi:10.1016/j.cell.2007.12.040

Kim, B., Lee, Y., Kim, E., Kwak, A., Ryoo, S., Bae, S. H., et al. (2013). The Interleukin-1α Precursor Is Biologically Active and Is Likely a Key Alarmin in the IL-1 Family of Cytokines. *Front. Immunol.* 4, 391. doi:10.3389/fimmu.2013.00391

Kim, S.-H., Eisenstein, M., Reznikov, L., Fantuzzi, G., Novick, D., Rubinstein, M., et al. (2000). Structural Requirements of Six Naturally Occurring Isoforms of the IL-18 Binding Protein to Inhibit IL-18. *Proc. Natl. Acad. Sci. U.S.A.* 97, 1190–1195. doi:10.1073/pnas.97.3.1190

Kimura, T., Jia, J., Kumar, S., Choi, S. W., Gu, Y., Mudd, M., et al. (2017). Dedicated SNARE S and Specialized TRIM Cargo Receptors Mediate Secretory Autophagy. *EMBO J.* 36, 42–60. doi:10.15252/embj.201695081

Knodler, L. A., Crowley, S. M., Sham, H. P., Yang, H., Wrande, M., Ma, C., et al. (2014). Noncanonical Inflammasome Activation of Caspase-4/caspase-11 Mediates Epithelial Defenses against Enteric Bacterial Pathogens. *Cell Host Microbe* 16, 249–256. doi:10.1016/j.chom.2014.07.002

Kobayashi, T., Ogawa, M., Sanada, T., Mimuro, H., Kim, M., Ashida, H., et al. (2013). The Shigella OspC3 Effector Inhibits Caspase-4, Antagonizes Inflammatory Cell Death, and Promotes Epithelial Infection. *Cell Host Microbe* 13, 570–583. doi:10.1016/j.chom.2013.04.012

Kobayashi, Y., Yamamoto, K., Saido, T., Kawasaki, H., Oppenheim, J. J., and Matsushima, K. (1990). Identification of Calcium-Activated Neutral Protease as a Processing Enzyme of Human Interleukin 1 Alpha. *Proc. Natl. Acad. Sci. U.S.A.* 87, 5548–5552. doi:10.1073/pnas.87.14.5548

Kostura, M. J., Tocci, M. J., Limjuco, G., Chin, J., Cameron, P., Hillman, A. G., et al. (1989). Identification of a Monocyte Specific Pre-interleukin 1 Beta Convertase Activity. *Proc. Natl. Acad. Sci. U.S.A.* 86, 5227–5231. doi:10.1073/pnas.86.14.5227

Kuhn, P.-H., Marjaux, E., Imhof, A., De Strooper, B., Haass, C., and Lichtenthaler, S. F. (2007). Regulated Intramembrane Proteolysis of the Interleukin-1 Receptor II by α-, β-, and γ-Secretase. *J. Biol. Chem.* 282, 11982–11995. doi:10.1074/jbc.m700356200

Kumar, S., Hanning, C. R., Brigham-Burke, M. R., Rieman, D. J., Lehr, R., Khandekar, S., et al. (2002). INTERLEUKIN-1F7B (IL-1H4/IL-1F7) IS PROCESSED BY CASPASE-1 AND MATURE IL-1F7B BINDS TO THE IL-18 RECEPTOR BUT DOES Not INDUCE IFN-γ PRODUCTION. *Cytokine* 18, 61–71. doi:10.1006/cyto.2002.0873

Kuswanto, W., Burzyn, D., Panduro, M., Wang, K. K., Jang, Y. C., Wagers, A. J., et al. (2016). Poor Repair of Skeletal Muscle in Aging Mice Reflects a Defect in Local, Interleukin-33-dependent Accumulation of Regulatory T Cells. *Immunity* 44, 355–367. doi:10.1016/j.immuni.2016.01.009

Lamacchia, C., Rodriguez, E., Palmer, G., and Gabay, C. (2013). Endogenous IL-1α Is a Chromatin-Associated Protein in Mouse Macrophages. *Cytokine* 63, 135–144. doi:10.1016/j.cyto.2013.04.010

Lefrançais, E., Roga, S., Gautier, V., Gonzalez-de-Peredo, A., Monsarrat, B., Girard, J.-P., et al. (2012). IL-33 Is Processed into Mature Bioactive Forms by Neutrophil Elastase and Cathepsin G. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1673–1678. doi:10.1073/pnas.1115884109

Li, N., Yamasaki, K., Saito, R., Fukushi-Takahashi, S., Shimada-Omori, R., Asano, M., et al. (2014). Alarmin Function of Cathelicidin Antimicrobial Peptide LL37 through IL-36γ Induction in Human Epidermal Keratinocytes. *J. I.* 193, 5140–5148. doi:10.4049/jimmunol.1302574

Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., et al. (1995). Mice Deficient in IL-1β-converting Enzyme Are Defective in Production of Mature IL-1β and Resistant to Endotoxic Shock. *Cell* 80, 401–411. doi:10.1016/0092-8674(95)90490-5

Li, S., Neff, C. P., Barber, K., Hong, J., Luo, Y., Azam, T., et al. (2015). Extracellular Forms of IL-37 Inhibit Innate Inflammation *In Vitro* and *In Vivo* but Require the IL-1 Family Decoy Receptor IL-1R8. *Proc. Natl. Acad. Sci. U.S.A.* 112, 2497–2502. doi:10.1073/pnas.1424626112

Lian, L.-H., Milora, K. A., Manupipatpong, K. K., and Jensen, L. E. (2012). The Double-Stranded RNA Analogue Polyinosinic-Polycytidylic Acid Induces Keratinocyte Pyroptosis and Release of IL-36γ. *J. Investigative Dermatology* 132, 1346–1353. doi:10.1038/jid.2011.482

Liu, T., Tang, Q., Liu, K., Xie, W., Liu, X., Wang, H., et al. (2016). TRIM11 Suppresses AIM2 Inflammasome by Degrading AIM2 via P62-dependent Selective Autophagy. *Cell Rep.* 16, 1988–2002. doi:10.1016/j.celrep.2016.07.019

Liu, X., Zhang, Z., Ruan, J., Pan, Y., Magupalli, V. G., Wu, H., et al. (2016). Inflammasome-activated Gasdermin D Causes Pyroptosis by Forming Membrane Pores. *Nature* 535, 153–158. doi:10.1038/nature18629

Liu, Z., Wang, C., Yang, J., Chen, Y., Zhou, B., Abbott, D. W., et al. (2020). Caspase-1 Engages Full-Length Gasdermin D through Two Distinct Interfaces that Mediate Caspase Recruitment and Substrate Cleavage. *Immunity* 53, 106–114.e5. doi:10.1016/j.immuni.2020.06.007

Liu, Z., Wang, C., Yang, J., Zhou, B., Yang, R., Ramachandran, R., et al. (2019). Crystal Structures of the Full-Length Murine and Human Gasdermin D Reveal Mechanisms of Autoinhibition, Lipid Binding, and Oligomerization. *Immunity* 51, 43–49. doi:10.1016/j.immuni.2019.04.017

Lorenzen, I., Lokau, J., Düsterhöft, S., Trad, A., Garbers, C., Scheller, J., et al. (2012). The Membrane-Proximal Domain of A Disintegrin and Metalloprotease 17 (ADAM17) Is Responsible for Recognition of the Interleukin-6 Receptor and Interleukin-1 Receptor II. *FEBS Lett.* 586, 1093–1100. doi:10.1016/j.febslet.2012.03.012

Lüthi, A. U., Cullen, S. P., McNeela, E. A., Duriez, P. J., Afonina, I. S., Sheridan, C., et al. (2009). Suppression of Interleukin-33 Bioactivity through Proteolysis by Apoptotic Caspases. *Immunity* 31, 84–98. doi:10.1016/j.immuni.2009.05.007

Marrakchi, S., Guigue, P., Renshaw, B. R., Puel, A., Pei, X.-Y., Fraitag, S., et al. (2011). Interleukin-36-receptor Antagonist Deficiency and Generalized Pustular Psoriasis. *N. Engl. J. Med.* 365, 620–628. doi:10.1056/nejmoa1013068

Martín-Sánchez, F., Diamond, C., Zeitler, M., Gomez, A. I., Baroja-Mazo, A., Bagnall, J., et al. (2016). Inflammasome-dependent IL-1β Release Depends upon Membrane Permeabilisation. *Cell Death Differ.* 23, 1219–1231. doi:10.1038/cdd.2015.176

Mizutani, H., Black, R., and Kupper, T. S. (1991). Human Keratinocytes Produce but Do Not Process Pro-interleukin-1 (IL-1) Beta. Different Strategies of IL-1 Production and Processing in Monocytes and Keratinocytes. *J. Clin. Invest.* 87, 1066–1071. doi:10.1172/jci115067

Mizutani, H., Schechter, N., Lazarus, G., Black, R. A., and Kupper, T. S. (1991). Rapid and Specific Conversion of Precursor Interleukin 1 Beta (IL-1 Beta) to an Active IL-1 Species by Human Mast Cell Chymase. *J. Exp. Med.* 174, 821–825. doi:10.1084/jem.174.4.821

Monteleone, M., Stanley, A. C., Chen, K. W., Brown, D. L., Bezbradica, J. S., von Pein, J. B., et al. (2018). Interleukin-1β Maturation Triggers its Relocation to the Plasma Membrane for Gasdermin-D-dependent and -Independent Secretion. *Cell Rep.* 24, 1425–1433. doi:10.1016/j.celrep.2018.07.027

Mosley, B., Dower, S. K., Gillis, S., and Cosman, D. (1987). Determination of the Minimum Polypeptide Lengths of the Functionally Active Sites of Human Interleukins 1 Alpha and 1 Beta. *Proc. Natl. Acad. Sci. U.S.A.* 84, 4572–4576. doi:10.1073/pnas.84.13.4572

Mosley, B., Urdal, D. L., Prickett, K. S., Larsen, A., Cosman, D., Conlon, P. J., et al. (1987). The Interleukin-1 Receptor Binds the Human Interleukin-1 Alpha Precursor but Not the Interleukin-1 Beta Precursor. *J. Biol. Chem.* 262, 2941–2944. doi:10.1016/s0021-9258(18)61450-4

Muendlein, H. I., Jetton, D., Connolly, W. M., Eidell, K. P., Magri, Z., Smirnova, I., et al. (2020). cFLIP L Protects Macrophages from LPS-Induced Pyroptosis via

Inhibition of Complex II Formation. *Science* 367, 1379–1384. doi:10.1126/science.aay3878

Mühl, H., Kämpfer, H., Bosmann, M., Frank, S., Radeke, H., and Pfeilschifter, J. (2000). Interferon-γ Mediates Gene Expression of IL-18 Binding Protein in Nonleukocytic Cells. *Biochem. Biophysical Res. Commun.* 267, 960–963. doi:10.1006/bbrc.1999.2064

Nold, M. F., Nold-Petry, C. A., Zepp, J. A., Palmer, B. E., Bufler, P., and Dinarello, C. A. (2010). IL-37 Is a Fundamental Inhibitor of Innate Immunity. *Nat. Immunol.* 11, 1014–1022. doi:10.1038/ni.1944

Nold-Petry, C. A., Lo, C. Y., Rudloff, I., Elgass, K. D., Li, S., Gantier, M. P., et al. (2015). IL-37 Requires the Receptors IL-18Rα and IL-1R8 (SIGIRR) to Carry Out its Multifaceted Anti-inflammatory Program upon Innate Signal Transduction. *Nat. Immunol.* 16, 354–365. doi:10.1038/ni.3103

Novick, D., Kim, S.-H., Fantuzzi, G., Reznikov, L. L., Dinarello, C. A., and Rubinstein, M. (1999). Interleukin-18 Binding Protein. *Immunity* 10, 127–136. doi:10.1016/s1074-7613(00)80013-8

Novick, D., Schwartsburd, B., Pinkus, R., Suissa, D., Belzer, I., Sthoeger, Z., et al. (2001). A Novel IL-18BP ELISA Shows Elevated Serum IL-18BP in Sepsis and Extensive Decrease of Free IL-18. *Cytokine* 14, 334–342. doi:10.1006/cyto.2001.0914

Nowarski, R., Jackson, R., Gagliani, N., de Zoete, M. R., Palm, N. W., Bailis, W., et al. (2015). Epithelial IL-18 Equilibrium Controls Barrier Function in Colitis. *Cell* 163, 1444–1456. doi:10.1016/j.cell.2015.10.072

O'Neill, L. A. J. (2008). The Interleukin-1 receptor/Toll-like Receptor Superfamily: 10 Years of Progress. *Immunol. Rev.* 226, 10–18. doi:10.1111/j.1600-065x.2008.00701.x

Ohno, T., Oboki, K., Kajiwara, N., Morii, E., Aozasa, K., Flavell, R. A., et al. (2009). Caspase-1, Caspase-8, and Calpain Are Dispensable for IL-33 Release by Macrophages. *J. Immunol.* 183, 7890–7897. doi:10.4049/jimmunol.0802449

Okamura, H., Tsutsui, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., et al. (1995). Cloning of a New Cytokine that Induces IFN-γ Production by T Cells. *Nature* 378, 88–91. doi:10.1038/378088a0

Orning, P., Weng, D., Starheim, K., Ratner, D., Best, Z., Lee, B., et al. (2018). Pathogen Blockade of TAK1 Triggers Caspase-8-dependent Cleavage of Gasdermin D and Cell Death. *Science* 362, 1064–1069. doi:10.1126/science.aau2818

Orzalli, M. H., Prochera, A., Payne, L., Smith, A., Garlick, J. A., and Kagan, J. C. (2021). Virus-mediated Inactivation of Anti-apoptotic Bcl-2 Family Members Promotes Gasdermin-E-dependent Pyroptosis in Barrier Epithelial Cells. *Immunity* 54, 1447–1462. e5. doi:10.1016/j.immuni.2021.04.012

Orzalli, M. H., Smith, A., Jurado, K. A., Iwasaki, A., Garlick, J. A., and Kagan, J. C. (2018). An Antiviral Branch of the IL-1 Signaling Pathway Restricts Immune-Evasive Virus Replication. *Mol. Cell* 71, 825–840. doi:10.1016/j.molcel.2018.07.009

Pajuelo, D., Gonzalez-Juarbe, N., Tak, U., Sun, J., Orihuela, C. J., and Niederweis, M. (2018). NAD+ Depletion Triggers Macrophage Necroptosis, a Cell Death Pathway Exploited by *Mycobacterium tuberculosis*. *Cell Rep.* 24, 429–440. doi:10.1016/j.celrep.2018.06.042

Pernas, L. (2021). Cellular Metabolism in the Defense against Microbes. *J. Cell Sci.* 134, jcs252023. doi:10.1242/jcs.252023

Próchnicki, T., and Latz, E. (2017). Inflammasomes on the Crossroads of Innate Immune Recognition and Metabolic Control. *Cell Metab.* 26, 71–93. doi:10.1016/j.cmet.2017.06.018

Puren, A. J., Fantuzzi, G., and Dinarello, C. A. (1999). Gene Expression, Synthesis, and Secretion of Interleukin 18 and Interleukin 1β Are Differentially Regulated in Human Blood Mononuclear Cells and Mouse Spleen Cells. *Proc. Natl. Acad. Sci. U.S.A.* 96, 2256–2261. doi:10.1073/pnas.96.5.2256

Rashidi, M., Simpson, D. S., Hempel, A., Frank, D., Petrie, E., Vince, A., et al. (2019). The Pyroptotic Cell Death Effector Gasdermin D Is Activated by Gout-Associated Uric Acid Crystals but Is Dispensable for Cell Death and IL-1β Release. *J. I.* 203, 736–748. doi:10.4049/jimmunol.1900228

Rathkey, J. K., Zhao, J., Liu, Z., Chen, Y., Yang, J., Kondolf, H. C., et al. (2018). Chemical Disruption of the Pyroptotic Pore-Forming Protein Gasdermin D Inhibits Inflammatory Cell Death and Sepsis. *Sci. Immunol.* 3. doi:10.1126/sciimmunol.aat2738

Ravindran, R., Loebbermann, J., Nakaya, H. I., Khan, N., Ma, H., Gama, L., et al. (2016). The Amino Acid Sensor GCN2 Controls Gut Inflammation by Inhibiting Inflammasome Activation. *Nature* 531, 523–527. doi:10.1038/nature17186

Re, F., Sironi, M., Muzio, M., Matteucci, C., Introna, M., Orlando, S., et al. (1996). Inhibition of Interleukin-1 Responsiveness by Type II Receptor Gene Transfer: a Surface "receptor" with Anti-interleukin-1 Function. *J. Exp. Med.* 183, 1841–1850. doi:10.1084/jem.183.4.1841

Rogers, C., Erkes, D. A., Nardone, A., Aplin, A. E., Fernandes-Alnemri, T., and Alnemri, E. S. (2019). Gasdermin Pores Permeabilize Mitochondria to Augment Caspase-3 Activation during Apoptosis and Inflammasome Activation. *Nat. Commun.* 10, 1689. doi:10.1038/s41467-019-09397-2

Rogers, C., Fernandes-Alnemri, T., Mayes, L., Alnemri, D., Cingolani, G., and Alnemri, E. S. (2017). Cleavage of DFNA5 by Caspase-3 during Apoptosis Mediates Progression to Secondary Necrotic/pyroptotic Cell Death. *Nat. Commun.* 8, 14128. doi:10.1038/ncomms14128

Roy, A., Ganesh, G., Sippola, H., Bolin, S., Sawesi, O., Dagälv, A., et al. (2014). Mast Cell Chymase Degrades the Alarmins Heat Shock Protein 70, Biglycan, HMGB1, and Interleukin-33 (IL-33) and Limits Danger-Induced Inflammation. *J. Biol. Chem.* 289, 237–250. doi:10.1074/jbc.m112.435156

Rubartelli, A., Cozzolino, F., Talio, M., and Sitia, R. (1990). A Novel Secretory Pathway for Interleukin-1 Beta, a Protein Lacking a Signal Sequence. *EMBO J.* 9, 1503–1510. doi:10.1002/j.1460-2075.1990.tb08268.x

Rudloff, I., Godsell, J., Nold-Petry, C. A., Harris, J., Hoi, A., Morand, E. F., et al. (2015). Brief Report: Interleukin-38 Exerts Antiinflammatory Functions and Is Associated with Disease Activity in Systemic Lupus Erythematosus. *Arthritis & Rheumatology* 67, 3219–3225. doi:10.1002/art.39328

Rühl, S., and Broz, P. (2015). Caspase-11 Activates a Canonical NLRP3 Inflammasome by Promoting K+efflux. *Eur. J. Immunol.* 45, 2927–2936. doi:10.1002/eji.201545772

Rühl, S., Shkarina, K., Demarco, B., Heilig, R., Santos, J. C., and Broz, P. (2018). ESCRT-dependent Membrane Repair Negatively Regulates Pyroptosis Downstream of GSDMD Activation. *Science* 362, 956–960. doi:10.1126/science.aar7607

Russo, H. M., Rathkey, J., Boyd-Tressler, A., Katsnelson, M. A., Abbott, D. W., and Dubyak, G. R. (2016). Active Caspase-1 Induces Plasma Membrane Pores that Precede Pyroptotic Lysis and Are Blocked by Lanthanides. *J. I.* 197, 1353–1367. doi:10.4049/jimmunol.1600699

Sagulenko, V., Thygesen, S. J., Sester, D. P., Idris, A., Cridland, J. A., Vajjhala, P. R., et al. (2013). AIM2 and NLRP3 Inflammasomes Activate Both Apoptotic and Pyroptotic Death Pathways via ASC. *Cell Death Differ.* 20, 1149–1160. doi:10.1038/cdd.2013.37

Saitoh, T., Fujita, N., Jang, M. H., Uematsu, S., Yang, B.-G., Satoh, T., et al. (2008). Loss of the Autophagy Protein Atg16L1 Enhances Endotoxin-Induced IL-1β Production. *Nature* 456, 264–268. doi:10.1038/nature07383

Sanchez, M., Kolar, S. L., Müller, S., Reyes, C. N., Wolf, A. J., Ogawa, C., et al. (2017). O-acetylation of Peptidoglycan Limits Helper T Cell Priming and Permits *Staphylococcus aureus* Reinfection. *Cell Host Microbe* 22, 543–551. doi:10.1016/j.chom.2017.08.008

Santa Cruz Garcia, A. B., Schnur, K. P., Malik, A. B., and Mo, G. C. H. (2022). Gasdermin D Pores Are Dynamically Regulated by Local Phosphoinositide Circuitry. *Nat. Commun.* 13, 52. doi:10.1038/s41467-021-27692-9

Sardiello, M., Palmieri, M., di Ronza, A., Medina, D. L., Valenza, M., Gennarino, V. A., et al. (2009). A Gene Network Regulating Lysosomal Biogenesis and Function. *Science* 325, 473–477. doi:10.1126/science.1174447

Sarhan, J., Liu, B. C., Muendlein, H. I., Li, P., Nilson, R., Tang, A. Y., et al. (2018). Caspase-8 Induces Cleavage of Gasdermin D to Elicit Pyroptosis during Yersinia Infection. *Proc. Natl. Acad. Sci. U. S. A.* 115, E10888–E10897. doi:10.1073/pnas.1809548115

Sborgi, L., Rühl, S., Mulvihill, E., Pipercevic, J., Heilig, R., Stahlberg, H., et al. (2016). GSDMD Membrane Pore Formation Constitutes the Mechanism of Pyroptotic Cell Death. *EMBO J.* 35, 1766–1778. doi:10.15252/embj.201694696

Schneider, K. S., Gross, C. J., Dreier, R. F., Saller, B. S., Mishra, R., Gorka, O., et al. (2017). The Inflammasome Drives GSDMD-independent Secondary Pyroptosis and IL-1 Release in the Absence of Caspase-1 Protease Activity. *Cell Rep.* 21, 3846–3859. doi:10.1016/j.celrep.2017.12.018

Semino, C., Carta, S., Gattorno, M., Sitia, R., and Rubartelli, A. (2018). Progressive Waves of IL-1β Release by Primary Human Monocytes via Sequential Activation of Vesicular and Gasdermin D-Mediated Secretory Pathways. *Cell Death Dis.* 9, 1088. doi:10.1038/s41419-018-1121-9

Settembre, C., Di Malta, C., Polito, V. A., Arencibia, M. G., Vetrini, F., Erdin, S., et al. (2011). TFEB Links Autophagy to Lysosomal Biogenesis. *Science* 332, 1429–1433. doi:10.1126/science.1204592

Sharma, S., Kulk, N., Nold, M. F., Gräf, R., Kim, S.-H., Reinhardt, D., et al. (2008). The IL-1 Family Member 7b Translocates to the Nucleus and Down-Regulates Proinflammatory Cytokines. *J. Immunol.* 180, 5477–5482. doi:10.4049/jimmunol.180.8.5477

Shi, C.-S., Shenderov, K., Huang, N.-N., Kabat, J., Abu-Asab, M., Fitzgerald, K. A., et al. (2012). Activation of Autophagy by Inflammatory Signals Limits IL-1β Production by Targeting Ubiquitinated Inflammasomes for Destruction. *Nat. Immunol.* 13, 255–263. doi:10.1038/ni.2215

Shi, J., Zhao, Y., Wang, K., Shi, X., Wang, Y., Huang, H., et al. (2015). Cleavage of GSDMD by Inflammatory Caspases Determines Pyroptotic Cell Death. *Nature* 526, 660–665. doi:10.1038/nature15514

Shimada, T., Park, B. G., Wolf, A. J., Brikos, C., Goodridge, H. S., Becker, C. A., et al. (2010). *Staphylococcus aureus* Evades Lysozyme-Based Peptidoglycan Digestion that Links Phagocytosis, Inflammasome Activation, and IL-1β Secretion. *Cell Host Microbe* 7, 38–49. doi:10.1016/j.chom.2009.12.008

Sims, J. E., March, C. J., Cosman, D., Widmer, M. B., MacDonald, H. R., McMahan, C. J., et al. (1988). cDNA Expression Cloning of the IL-1 Receptor, a Member of the Immunoglobulin Superfamily. *Science* 241, 585–589. doi:10.1126/science.2969618

Sugiura, K., Shoda, Y., and Akiyama, M. (2014). Generalized pustular psoriasis triggered by amoxicillin in monozygotic twins with compound heterozygous IL36RN mutations: comment on the article by Navarini et al. *J. Investigative Dermatology* 134, 578–579. doi:10.1038/jid.2013.354

Talabot-Ayer, D., Lamacchia, C., Gabay, C., and Palmer, G. (2009). Interleukin-33 Is Biologically Active Independently of Caspase-1 Cleavage. *J. Biol. Chem.* 284, 19420–19426. doi:10.1074/jbc.m901744200

Tapia, V. S., Daniels, M. J. D., Palazón-Riquelme, P., Dewhurst, M., Luheshi, N. M., Rivers-Auty, J., et al. (2019). The Three Cytokines IL-1β, IL-18, and IL-1α Share Related but Distinct Secretory Routes. *J. Biol. Chem.* 294, 8325–8335. doi:10.1074/jbc.ra119.008009

Tattoli, I., Sorbara, M. T., Vuckovic, D., Ling, A., Soares, F., Carneiro, L. A. M., et al. (2012). Amino Acid Starvation Induced by Invasive Bacterial Pathogens Triggers an Innate Host Defense Program. *Cell Host Microbe* 11, 563–575. doi:10.1016/j.chom.2012.04.012

Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., et al. (1992). A Novel Heterodimeric Cysteine Protease Is Required for Interleukin-1βprocessing in Monocytes. *Nature* 356, 768–774. doi:10.1038/356768a0

Towne, J. E., Garka, K. E., Renshaw, B. R., Virca, G. D., and Sims, J. E. (2004). Interleukin (IL)-1F6, IL-1F8, and IL-1F9 Signal through IL-1Rrp2 and IL-1RAcP to Activate the Pathway Leading to NF-Kb and MAPKs. *J. Biol. Chem.* 279, 13677–13688. doi:10.1074/jbc.m400117200

Towne, J. E., Renshaw, B. R., Douangpanya, J., Lipsky, B. P., Shen, M., Gabel, C. A., et al. (2011). Interleukin-36 (IL-36) Ligands Require Processing for Full Agonist (IL-36α, IL-36β, and IL-36γ) or Antagonist (IL-36Ra) Activity. *J. Biol. Chem.* 286, 42594–42602. doi:10.1074/jbc.m111.267922

Tsuchiya, K., Hosojima, S., Hara, H., Kushiyama, H., Mahib, M. R., Kinoshita, T., et al. (2021). Gasdermin D Mediates the Maturation and Release of IL-1α Downstream of Inflammasomes. *Cell Rep.* 34, 108887. doi:10.1016/j.celrep.2021.108887

Tsuchiya, K., Nakajima, S., Hosojima, S., Thi Nguyen, D., Hattori, T., Manh Le, T., et al. (2019). Caspase-1 Initiates Apoptosis in the Absence of Gasdermin D. *Nat. Commun.* 10, 2091. doi:10.1038/s41467-019-09753-2

Vajjhala, P. R., Lu, A., Brown, D. L., Pang, S. W., Sagulenko, V., Sester, D. P., et al. (2015). The Inflammasome Adaptor ASC Induces Procaspase-8 Death Effector Domain Filaments. *J. Biol. Chem.* 290, 29217–29230. doi:10.1074/jbc.m115.687731

van de Veerdonk, F. L., Stoeckman, A. K., Wu, G., Boeckermann, A. N., Azam, T., Netea, M. G., et al. (2012). IL-38 Binds to the IL-36 Receptor and Has Biological Effects on Immune Cells Similar to IL-36 Receptor Antagonist. *Proc. Natl. Acad. Sci. U.S.A.* 109, 3001–3005. doi:10.1073/pnas.1121534109

Vijayaraj, S. L., Feltham, R., Rashidi, M., Frank, D., Liu, Z., Simpson, D. S., et al. (2021). The Ubiquitylation of IL-1β Limits its Cleavage by Caspase-1 and Targets it for Proteasomal Degradation. *Nat. Commun.* 12, 2713. doi:10.1038/s41467-021-22979-3

Waern, I., Lundequist, A., Pejler, G., and Wernersson, S. (2013). Mast Cell Chymase Modulates IL-33 Levels and Controls Allergic Sensitization in Dust-Mite Induced Airway Inflammation. *Mucosal Immunol.* 6, 911–920. doi:10.1038/mi.2012.129

Wang, K., Sun, Q., Zhong, X., Zeng, M., Zeng, H., Shi, X., et al. (2020). Structural Mechanism for GSDMD Targeting by Autoprocessed Caspases in Pyroptosis. *Cell* 180, 941–955. doi:10.1016/j.cell.2020.02.002

Wang, Y., Gao, W., Shi, X., Ding, J., Liu, W., He, H., et al. (2017). Chemotherapy Drugs Induce Pyroptosis through Caspase-3 Cleavage of a Gasdermin. *Nature* 547, 99–103. doi:10.1038/nature22393

Weinberg, J. M., Davis, J. A., Abarzua, M., and Rajan, T. (1987). Cytoprotective Effects of glycine and Glutathione against Hypoxic Injury to Renal Tubules. *J. Clin. Invest.* 80, 1446–1454. doi:10.1172/jci113224

Werman, A., Werman-Venkert, R., White, R., Lee, J.-K., Werman, B., Krelin, Y., et al. (2004). The Precursor Form of IL-1α Is an Intracrine Proinflammatory Activator of Transcription. *Proc. Natl. Acad. Sci. U.S.A.* 101, 2434–2439. doi:10.1073/pnas.0308705101

Wesche, H., Henzel, W. J., Shillinglaw, W., Li, S., and Cao, Z. (1997). MyD88: An Adapter that Recruits IRAK to the IL-1 Receptor Complex. *Immunity* 7, 837–847. doi:10.1016/s1074-7613(00)80402-1

Wessendorf, J. H., Garfinkel, S., Zhan, X., Brown, S., and Maciag, T. (1993). Identification of a Nuclear Localization Sequence within the Structure of the Human Interleukin-1 Alpha Precursor. *J. Biol. Chem.* 268, 22100–22104. doi:10.1016/s0021-9258(20)80653-x

Wiggins, K. A., Parry, A. J., Cassidy, L. D., Humphry, M., Webster, S. J., Goodall, J. C., et al. (2019). IL-1α Cleavage by Inflammatory Caspases of the Noncanonical Inflammasome Controls the Senescence-associated Secretory Phenotype. *Aging Cell* 18, e12946. doi:10.1111/acel.12946

Wolf, A. J., Reyes, C. N., Liang, W., Becker, C., Shimada, K., Wheeler, M. L., et al. (2016). Hexokinase Is an Innate Immune Receptor for the Detection of Bacterial Peptidoglycan. *Cell* 166, 624–636. doi:10.1016/j.cell.2016.05.076

Xia, S., Zhang, Z., Magupalli, V. G., Pablo, J. L., Dong, Y., Vora, S. M., et al. (2021). Gasdermin D Pore Structure Reveals Preferential Release of Mature Interleukin-1. *Nature* 593, 607–611. doi:10.1038/s41586-021-03478-3

Yousif, A. S., Ronsard, L., Shah, P., Omatsu, T., Sangesland, M., Bracamonte Moreno, T., et al. (2021). The Persistence of Interleukin-6 Is Regulated by a Blood Buffer System Derived from Dendritic Cells. *Immunity* 54, 235–246. e5. doi:10.1016/j.immuni.2020.12.001

Zanoni, I., Tan, Y., Di Gioia, M., Broggi, A., Ruan, J., Shi, J., et al. (2016). An Endogenous Caspase-11 Ligand Elicits Interleukin-1 Release from Living Dendritic Cells. *Science* 352, 1232–1236. doi:10.1126/science.aaf3036

Zhang, D.-W., Shao, J., Lin, J., Zhang, N., Lu, B.-J., Lin, S.-C., et al. (2009). RIP3, an Energy Metabolism Regulator that Switches TNF-Induced Cell Death from Apoptosis to Necrosis. *Science* 325, 332–336. doi:10.1126/science.1172308

Zhang, M., Kenny, S. J., Ge, L., Xu, K., and Schekman, R. (2015). Translocation of Interleukin-1β into a Vesicle Intermediate in Autophagy-Mediated Secretion. *Elife* 4. doi:10.7554/eLife.11205

Zhang, M., Liu, L., Lin, X., Wang, Y., Li, Y., Guo, Q., et al. (2020). A Translocation Pathway for Vesicle-Mediated Unconventional Protein Secretion. *Cell* 181, 637–652. doi:10.1016/j.cell.2020.03.031

Zhang, Z., Zhang, Y., Xia, S., Kong, Q., Li, S., Liu, X., et al. (2020). Gasdermin E Suppresses Tumour Growth by Activating Anti-tumour Immunity. *Nature* 579, 415–420. doi:10.1038/s41586-020-2071-9

Zheng, Z., Deng, W., Bai, Y., Miao, R., Mei, S., Zhang, Z., et al. (2021). The Lysosomal Rag-Ragulator Complex Licenses RIPK1 and Caspase-8-Mediated Pyroptosis by Yersinia. *Science* 372. doi:10.1126/science.abg0269

Zhivaki, D., Borriello, F., Chow, O. A., Doran, B., Fleming, I., Theisen, D. J., et al. (2020). Inflammasomes within Hyperactive Murine Dendritic Cells Stimulate Long-Lived T Cell-Mediated Anti-tumor Immunity. *Cell Rep.* 33, 108381. doi:10.1016/j.celrep.2020.108381

Zhou, B., and Abbott, D. W. (2021). Gasdermin E Permits Interleukin-1 Beta Release in Distinct Sublytic and Pyroptotic Phases. *Cell Rep.* 35, 108998. doi:10.1016/j.celrep.2021.108998

Zhou, Z., He, H., Wang, K., Shi, X., Wang, Y., Su, Y., et al. (2020). Granzyme A from Cytotoxic Lymphocytes Cleaves GSDMB to Trigger Pyroptosis in Target Cells. *Science* 368. doi:10.1126/science.aaz7548

Conflict of Interest: JK consults for IFM Therapeutics and consults and holds equity in Corner Therapeutics, Larkspur Biosciences and Neumora Therapeutics. None of these relationships influenced the manuscript.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Cell and Developmental Biology

REVIEW
published: 28 June 2022
doi: 10.3389/fcell.2022.926421

Unconventional Secretion, Gate to Homeoprotein Intercellular Transfer

Alain Joliot[1]* *and Alain Prochiantz*[2]

[1]INSERM U932, Institut Curie Centre de Recherche, PSL Research University, Paris, France, [2]Centre for Interdisciplinary Research in Biology (CIRB), Collège de France, CNRS UMR 7241, INSERM U1050, PSL Research University, Labex MemoLife, Paris, France

Unconventional secretion allows for the secretion of fully mature and biologically active proteins mostly present in the cytoplasm or nucleus. Besides extra vesicle-driven secretion, non-extravesicular pathways also exist that specifically rely on the ability of the secreted proteins to translocate directly across the plasma membrane. This is the case for several homeoproteins, a family of over 300 transcription factors characterized by the structure of their DNA-binding homeodomain. The latter highly conserved homeodomain is necessary and sufficient for secretion, a process that requires PI(4,5)P2 binding, as is the case for FGF2 and HIV Tat unconventional secretion. An important feature of homeoproteins is their ability to cross membranes in both directions and thus to transfer between cells. This confers to homeoproteins their paracrine activity, an essential facet of their physiological functions.

Keywords: PIP 2, internalization, intercelluar communication, paracrine action, homeoprotein, unconventional secretion

OPEN ACCESS

Edited by:
Min Zhang,
Tsinghua University, China

Reviewed by:
Shenjie Wu,
University of California, Berkeley, United States
Kerstin Schipper,
Heinrich Heine University of Düsseldorf, Germany

***Correspondence:**
Alain Joliot
alain.joliot@curie.fr

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *22 April 2022*
Accepted: *07 June 2022*
Published: *28 June 2022*

Citation:
Joliot A and Prochiantz A (2022) Unconventional Secretion, Gate to Homeoprotein Intercellular Transfer. Front. Cell Dev. Biol. 10:926421. doi: 10.3389/fcell.2022.926421

INTRODUCTION

Unconventional protein secretion gathers multiple and heterogenous pathways defined by absence of the hallmarks that characterize the conventional pathway, such as the presence of a signal sequence at the N-terminus of the secreted polypeptide and the use of an invariant endoplasmic reticulum to Golgi journey blocked by Brefeldin A (Viotti, 2016). Beside relying on alternative routes to cross the plasma membrane, unconventional secretion is uncoupled from translation and therefore, can concern fully mature proteins endowed with genuine intracellular functions. Extracellular vesicle-driven secretion, described in this issue, proved to be predominant for unconventional secretion pathways but it necessarily requires the entrapment of the secreted proteins within vesicles. In parallel, a limited set of proteins devoid of signal sequence were shown to accumulate freely in the culture medium. Among them, the growth factor FGF2 is one of the first reported example, and the mechanism of its secretion was accurately dissected (Steringer and Nickel, 2018). Homeoprotein secretion was first described 20 years ago (Joliot et al., 1998). This observation was unexpected as this protein family was originally identified as a class of transcriptional regulators. Importantly, homeoproteins are not only secreted but also internalized by cells, the combination of these two processes allowing their transfer between cells. Such transfer confers to homeoproteins a paracrine mode of action, now recognized as an essential component of their developmental and physiological functions.

THE HOMEOPROTEIN FAMILY

Homeoproteins were discovered in a genetic screen focused on development in *Drosophila melanogaster* (Shearn et al., 1971). Some of the genes identified, named homeotic due to their

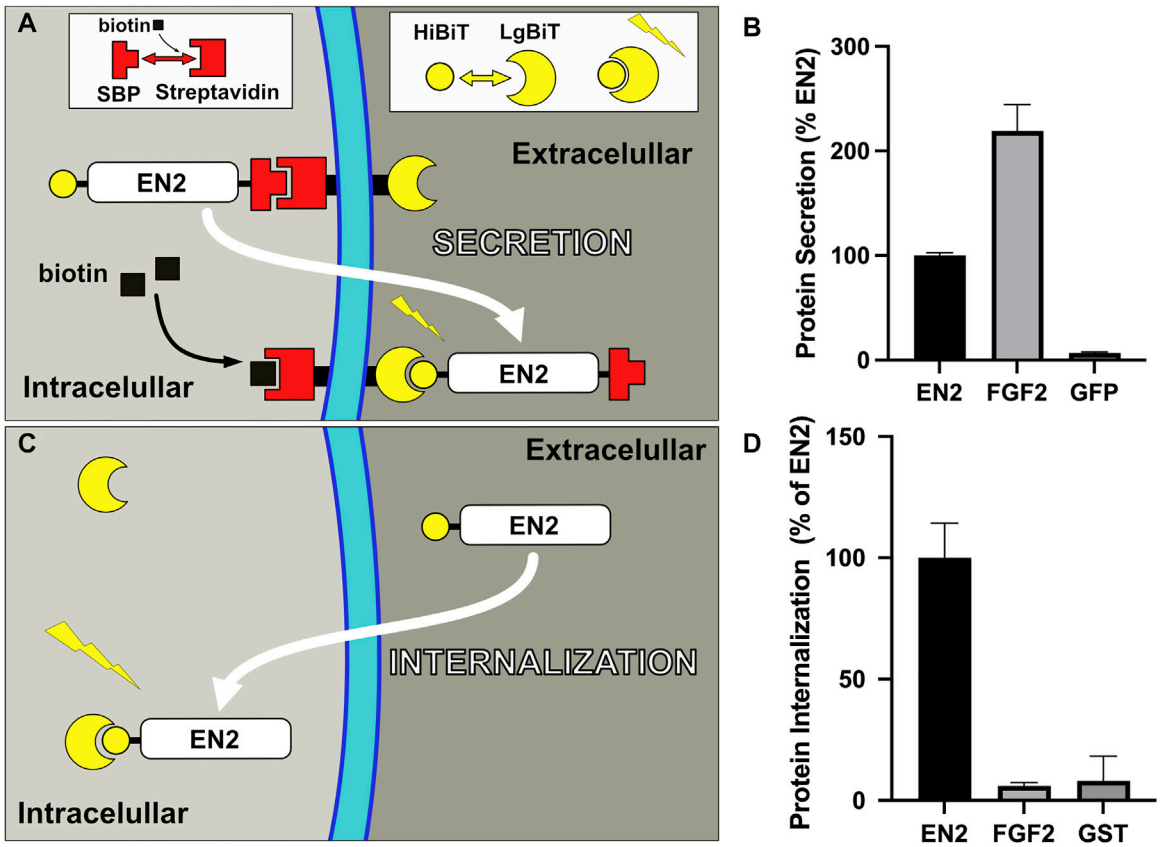


FIGURE 1 | Quantitative translocation assays. **(A)** Secretion assay (TransRush): Thanks to the addition of a SBP tag, EN2 is hooked at the inner side of the plasma membrane using the Rush system and can be released upon biotin addition. The presence of a second tag (HiBiT) allows monitoring secretion of EN2 upon release through complementation with the complementary Nanoluc fragment (LgBiT) present at the cell surface. **(B)** Quantification of the secretion of the indicated proteins with the TransRush assay. Luciferase activity is quantified 1 h after biotin addition. **(C)** Internalization assay: Cytosolic delivery of a HiBiT-tagged recombinant EN2 protein loaded in the medium is monitored through complementation with the complementary Nanoluc fragment (LgBiT) present in the cytosol of the recipient cell. **(D)** Cytosolic delivery of the indicated proteins is quantified 30 min after addition in the medium (**B**,**D** from ref 22).

ability to control the spatial identity of metameric structures, shared a common DNA-binding motif called the homeodomain (Gehring et al., 1994). Homeodomain-containing proteins, or homeoproteins, constitute one of the largest family of transcription factors highly conserved during evolution. More than 300 members are found in the human, where they exert multiple functions throughout life, as *bona fide* transcriptional regulators (Holland et al., 2007).

Originally, it is in the course of experiments aiming at perturbing the transcriptional activity of homeoproteins by mechanical loading of a purified homeodomain fragment in fragilized neuronal cells, we made the unexpected observation of homeodomain spontaneous uptake (Joliot A. et al., 1991). Later on, we demonstrated that full-length homeoproteins are efficiently internalized and also secreted, despite the absence of a classical secretion signal sequence (Joliot et al., 1998). Once in the extracellular medium, homeoproteins are detected in the soluble fraction following 100,000×*g* centrifugation (Joliot et al., 1998) and are able to interact with cell surface carbohydrates (Layalle et al., 2011), ruling out their incorporation into extracellular vesicles. This behavior is similar to that described for FGF2 and the HIV Tat protein, distinct to homeoproteins although similarly highly basic, suggesting the possibility of similarities in secretion mechanisms. It was then demonstrated that these unusual trafficking properties confer to homeoproteins new functions that superimpose on their transcriptional activity. They will be specifically discussed in the last part of this review.

HOMEOPROTEIN SECRETION

Homeoproteins predominantly localize to the nucleus, as expected for transcription factors. By subcellular fractionation, they are also detected in the membrane fraction and selectively distribute into raft domains that are characteristic of the plasma membrane (Joliot et al., 1997). Since it is estimated that no more than 10% of the intracellular pool of homeoproteins is secreted (Maizel et al., 1999), a sensitive assay is required to monitor their secretion. In a recent study, we have implemented a new strategy called TransRush, combining the Ru system to control protein trafficking (Boncompain et al., 2012) and nanoluciferase bi-

molecular complementation (Dixon et al., 2016) to monitor secretion (**Figure1A**). Thanks to the addition of two tags, the protein is hooked at the inner side of the plasma membrane using the RUSH system, and its accumulation in the extracellular space monitored through bi-molecular complementation with a luciferase fragment attached at the outer side of the plasma membrane. Secretion of the hooked protein is quantified following its release by biotin addition compared to control conditions and normalized by the cell content (**Figure 1B**). Both FGF2 and chick Engrailed2 homeoprotein (EN2) secretions could be accurately quantified, revealing a higher secretion efficacy for the former (Amblard et al., 2020a).

The mechanism of homeoprotein secretion was precisely dissected with EN2, used as a paradigm for this protein family. It shows striking similarities with that of FGF2 (Temmerman et al., 2008) and HIV Tat (Rayne et al., 2010) proteins and in particular, a mandatory requirement for Phosphatidylinositol(4,5)bisphosphate [PI(4,5)P2] (Amblard et al., 2020a). PI(4,5)P2 are minor components of the cell lipidome but specifically localize in the inner leaflet of the plasma membrane (Borges-Araújo and Fernandes, 2020). The efficacy of EN2 secretion strictly correlates with the levels of PI(4,5)P2, modulated by enzymatic or pharmacologic treatments. PI(4,5)P2 are known to be essential for the recruitment of various proteins at the cytosolic face of the plasma membrane, such as proteins involved in actin remodeling and in signal transduction (McLaughlin et al., 2002). They act in a similar way with FGF2, Tat and EN2, allowing for their recruitment at their site of secretion. Indeed, EN2 directly interacts with PI(4,5)P2 in artificial bilayers (Amblard et al., 2020a). The nature of the lipid polar head is an important determinant of EN2 interaction as PI4P and PS, show decreasing affinity for EN2. Comparing the respective affinities of the three proteins for PI(4,5)P2 is uneasy due to the diversity of techniques used but when analyzed with a same setting, FGF2 and EN2 display similar affinities (Amblard et al., 2020a). In a live cell context, EN2 interaction with PI(4,5)P2 is supported by the delocalization of the PI(4,5)P2-sensor PHPLC ∂ upon induction of EN2 secretion and by the release of EN2 from membranes treated with neomycin, a classical PI(4,5)P2 competitor.

Contrasting with FGF2, EN2 significantly interacts with PI4P, with a fourfold lower affinity for this lipid compared to PI(4,5)P2, but PI4P could not substitute for PI(4,5)P2 in EN2 secretion (Amblard et al., 2020a). This might reflect the fact that EN2 interaction with PI(4,5)P2 also depends on the acyl part of the molecule as it is not observed with the polar head alone. The contribution of the hydrophobic part of the bilayer is further supported by the direct interaction of EN2, but not FGF2, with a cholesterol-enriched PC bilayer, in agreement with its preferential association with cholesterol-enriched membranes (Joliot et al., 1997). However, cholesterol incorporation in PI(4,5)P2-containing membranes increases the affinity for both proteins (Temmerman et al., 2008; Amblard et al., 2020a), and plasma membrane depletion of cholesterol by methyl-ß-cyclodextrin impairs their secretion (Amblard et al., 2020a). Such interplay between PI(4,5)P2 and cholesterol are also observed for other proteins, through the induction of lipid phase demixing (Wang et al., 2016), or through stabilization of fluid PI(4,5)P2 domain by reducing electrostatic repulsion (Jiang et al., 2014).

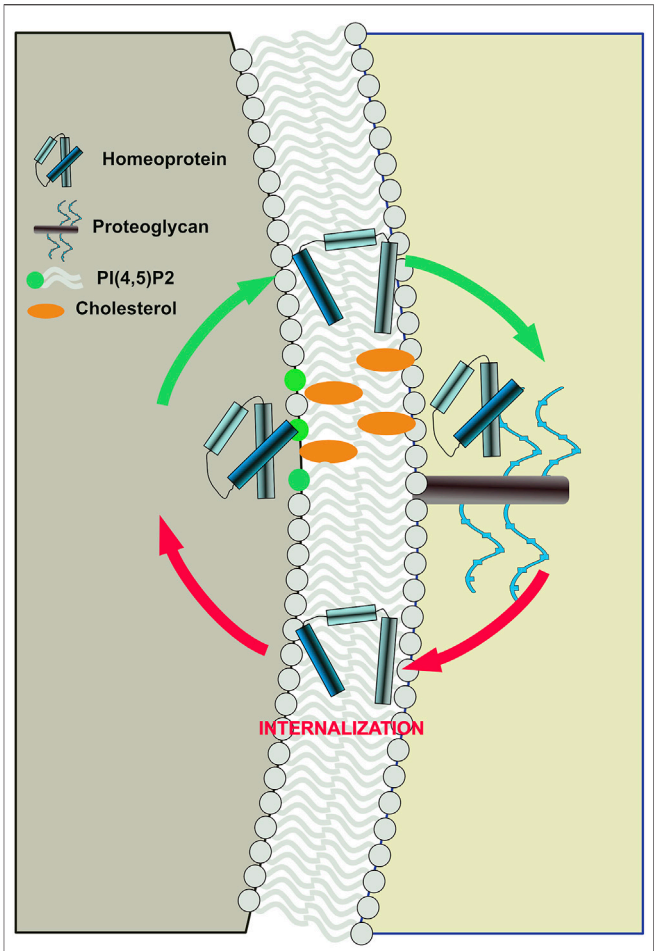


FIGURE 2 | Proposed model of bidirectional translocation of EN2 homeoprotein across the plasma membrane. Interaction with PI(4,5)P2 and cholesterol at the inner side and with glycosaminoglycan at the outer side, act as a conformational switches for EN2 allowing its exchange between the polar and apolar environments.

The distinctive feature of PI(4,5)P2 interaction with FGF2, Tat and EN2 is to promote their translocation across the plasma membrane, well beyond its mere recruitment role. Upon interaction with PI(4,5)P2, FGF2 (Steringer et al., 2012) and Tat (Zeitler et al., 2015) assemble into oligomers that create pore-forming structures, ultimately leading to secretion. Although not formally ruled-out, formation of pores or oligomers was not observed with EN2. On the other hand, the conformation of the homeodomain motif analyzed by NMR is significantly modified in presence of membrane mimetics and is characterized by the partial insertion of the monomer within the acyl chains (Carlier et al., 2015). Differences in the translocation mechanism of the three proteins would not be so surprising in the light of the additional translocation properties of homeoproteins, leading to their internalization.

One could note that PI(4,5)P2 or cholesterol depletion does not fully inhibit homeoprotein secretion and furthermore, that the TransRush assay used to identify the role of PI(4,5)P2 in the secretion of EN2 specifically targets plasma membrane translocation events as it only quantify the secretion of the protein hooked at the inner side of the plasma membrane. Alternative secretion pathways involving other cell compartments could not be excluded.

HOMEOPROTEIN SECRETION AND INTERNALIZATION, TWO FACES OF A SAME PROCESS

As mentioned earlier, internalization is the first unusual trafficking property identified in several homeoproteins which also relies on unconventional mechanisms (Sagan et al., 2013). Its persistence at low temperatures that precludes endocytosis events (Joliot AH. et al., 1991) and the non-vesicular distribution of the internalized protein (Joliot A. et al., 1991), both agree with a translocation-driven process, in a way opposite to secretion. Contrasting with FGF2, extracellular delivery of recombinant EN2 quickly leads to its cytosolic accumulation that could also be quantified using the split-Nanoluciferase assay (**Figures 1C,D**).

Despite plasma membrane asymmetry, homeoprotein internalization surprisingly displays the same requirement for PI(4,5)P2 and cholesterol (Amblard et al., 2020a). Because of the strategic localization of the lipid polar heads at the interface between the polar and apolar environments constituted by the cytosol and the acyl chains respectively, PI(4,5)P2 interaction might act as a conformational switch for homeoproteins to exchange between the two environments in either direction (**Figure 2**). This implies that on the external face of the plasma membrane, devoid of PI(4,5)P2, other components would regulate these exchanges. Cell surface glycosaminoglycans (GAGs) are attractive candidates because they are critical for homeoprotein internalization (Beurdeley et al., 2012). Interestingly, homeoprotein interaction with GAGs appear to differ between various homeoproteins. Such specificity is illustrated by the binding of OTX2 to highly sulfated chondroitin (CS-E) at the surface of their target cells (Beurdeley et al., 2012), whereas EN2 preferentially interact with heparan sulfate (**Figure 2**) (Cardon et al., 2021). Interestingly, the presence of cell surface heparan sulfates at the outer leaflet of the plasma membrane is also mandatory for the completion of FGF2 secretion (Zehe et al., 2006).

HOMEOPROTEIN SECRETION AND SEQUENCE REQUIREMENT

EN2 homeoprotein was chosen to unravel the mechanism of homeoprotein secretion but most if not all homeoproteins are also able of intercellular transfer (Lee et al., 2019). Indeed, the homeodomain motif that defines the homeoprotein family is on its own sufficient to recapitulate the whole secretion/internalization process (Tassetto et al., 2005). Interestingly, the homeodomain is one of the most frequent motif retrieved in an unbiased PI(4,5)P2 interaction screen of a human protein fragment library (Bidlingmaier et al., 2011). The homeodomain belongs to the helix-loop-helix class of DNA-binding motifs and among the three alpha helices that compose its structure in solution, the third one is enriched in basic and aromatic residues and is critical for translocation (Derossi et al., 1994). This 16 amino-acid long motif, also known as penetratin, is a founder member of the cell-penetrating peptide family, used as vectors to deliver cytosolic cargoes into the cell (Kurrikoff et al., 2016). A second motif adjacent to the third helix is specifically required for secretion (Dupont et al., 2007). Since this motif also promotes nuclear export, its function is more likely linked to the trafficking of the protein towards the plasma membrane rather than to the translocation process *per se*.

Contrasting with FGF2 and Tat proteins, the residues within the homeodomain which are required for PI(4,5)P2 interaction remain uncharacterized but specific mutations in EN2 lying close to the homeodomain were shown to lower simultaneously the affinity for PI(4,5)P2 and the efficacy of transfer (secretion and internalization), possibly by impacting on homeodomain conformation or accessibility. The first one contains a substitution of two tryptophan residues with lysins in a motif known to mediate protein-protein interaction (Maizel et al., 1999). In the second one, a cysteine residue that promote EN2 homodimerization is substituted for a serine (Amblard et al., 2020b).

REGULATION OF HOMEOPROTEIN SECRETION

Unconventional and conventional secretion pathways differ not only by their mechanism but also how they might be regulated. Proteins that use unconventional pathways can localize in different parts of the cell and thus, before secretion, must reach the plasma membrane. Homeoproteins mainly reside in the nucleus, but they can be actively exported toward the cytosol thanks to the presence of a nuclear export signal (Maizel et al., 1999). Strikingly, mutations (Tassetto et al., 2005) or post-translational modifications (Maizel et al., 2002) that lower homeoprotein nuclear targeting also impair its secretion, suggesting that homeoprotein secretion requires its passage through the nucleus.

Homeoprotein intercellular transfer implies that, once secreted, they are internalized by adjacent cells, but the latter internalization can antagonize secretion when occurring in the secreting cell. Accordingly, we recently demonstrated that these two processes, secretion and internalization, are inversely regulated by the cell redox state (Amblard et al., 2020b), further supporting the view that they are two opposite faces of a same process. Near-physiological modulation of H_2O_2 levels through ectopic expression of H2O2-producing or -degrading enzymes reveals that high and low H_2O_2 levels favors secretion and internalization, respectively. As most of the motifs shown to regulate the transfer of EN2 reside outside the homeodomain, it is

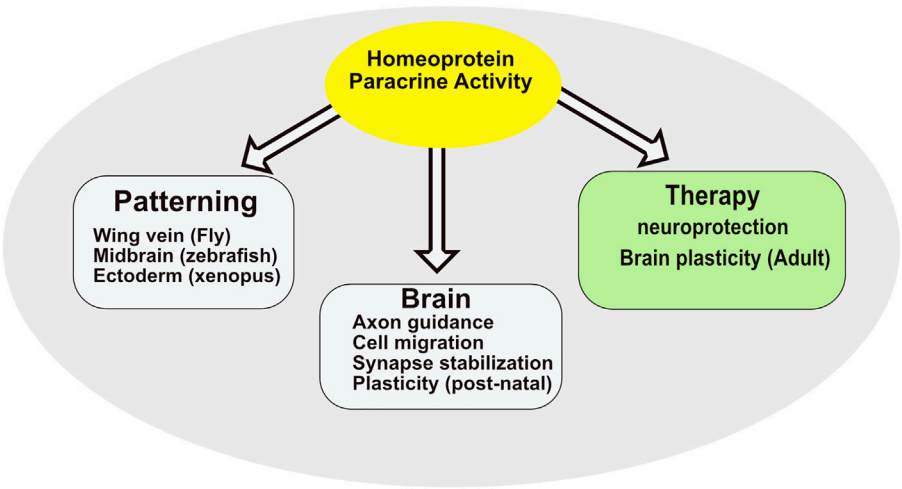


FIGURE 3 | Summary of homeoprotein paracrine activity.

likely that the regulation of secretion would differ depending on the nature of the homeoprotein.

At the plasma membrane, PI(4,5)P2 and cholesterol levels are determinant to modulate the recruitment and subsequent secretion of homeoproteins, without excluding the implication of other partners as reported for FGF2 (Zacherl et al., 2015). The concentration and distribution of these two lipids can be regulated at multiple levels. Even within a single cell, plasma membrane PI(4,5)P2 levels can vary significantly, due for instance to the asymmetric distribution of the enzymes of that control their metabolism (Myeong et al., 2021). Whether secretion is polarized within a single cell is an open question. In particular, the possible involvement of cytonemes (Ramírez-Weber and Kornberg, 1999), key players in paracrine signaling by connecting producing and recipient cells, is an attractive hypothesis.

Although key players have been identified, a full understanding of the translocation mechanism of homeoprotein is still lacking. Interestingly, the minimal internalization sequence penetratin is able to induce lipid hexagonal phase when incubated with cellular lipid extracts (Berlose et al., 1996) and to induce lipid curvature in artificial vesicles (Lamazière et al., 2008), suggesting that induction of lipid bilayer remodeling might be part of the process.

PHYSIOLOGY OF HOMEOPROTEIN SECRETION: GATE TO THEIR TRANSFER

Visualization of homeoprotein secretion *in vivo* is hampered by the low endogenous levels of homeoproteins combined to their predominant nuclear localization but was reported in a few situations (Wizenmann et al., 2009; Kim et al., 2014). This contrasts with the multiple physiological functions requiring homeoprotein secretion (**Figure 3**). Until now, all these functions were linked to the transfer of the homeoprotein into recipient cells rather than to its extracellular presentation, for example by cytonemes, to classical receptors, yet to be identified. Even at the functional level, internalization and secretion are inseparable. The fact that both rely on similar mechanisms would explain how they have been co-opted simultaneously during evolution.

Transfer has been studied for handful of homeoproteins, with a focus on the physiological functions associated with this novel signaling pathway. Here we will restrict the discussion to animal cells, even though intercellular transfer was also reported in plants. This choice is dictated by the fact that, in plants, HP Knotted-1 transfer primarily involves plasmodesmata described as intercellular bridges (Ruiz-Medrano et al., 2004). Consequently, it may not correspond to a true secretion-internalization sequence although it displays unexpected similarities with the situation in animals (Tassetto et al., 2005).

EARLY AND LATE DEVELOPMENTAL FUNCTIONS OF HOMEOPROTEIN TRANSFER

Early in development, it was demonstrated that, in the *Drosophila* wing disk, transfer of Engrailed from the Patched domains towards anterior cells not expressing Engrailed is necessary for the formation of the anterior cross vein (Layalle et al., 2011). This was established by the extracellular expression of single chain antibodies (EN1/2-scFv) with Engrailed neutralizing activity. Interestingly, this induction requires a physiological synergic interaction with decapentaplegic (DPP) a morphogen of the TGFß family. As will be described below, such a signaling interaction between a HP and a classical signaling pathway was also reported in the chick tectum for axon guidance by EN2 and in the neural tube for the regulation of oligodendrocyte precursor (OPC) migration by PAX6 (Wizenmann et al., 2009; Di Lullo et al., 2011). Early morphogenetic activity of HP transfer is

also involved in the patterning of the chick tectum by EN2 and the regulation of Cajal-Retzius cell migration by PAX6 in the mouse neuroepithelium (Rampon et al., 2015; Amblard et al., 2020b; Kaddour et al., 2020).

As already alluded to, still during development, but at slightly later stages, PAX6 transfer interacts with netrin to regulate OPC migration in the chick neural tube (Di Lullo et al., 2011). Still during late embryonic development, EN2 (possibly EN1), secreted by the chick optic tectum, where EN1 and EN2 show graded (anterior low, posterior high) expression, guides the migration of retinal ganglion cell (RGC) axons and participates in the distribution of nasal and temporal axons onto posterior and anterior tectum domains, respectively(Wizenmann et al., 2009). The latter paracrine EN2 activity was demonstrated *in vivo* thanks to the extracellular expression of neutralizing EN1/2-scFvs. *In vitro* studies allowed for the demonstration that the latter paracrine activity of EN2 requires its ability to regulate local protein translation, within growth cones, through the activation of eIF4I translation initiation factor. Further *in vitro* experiments led to conclude that the ability of EN2 to provoke the collapse of anterior RGC axon growth cones (Brunet et al., 2005), a guidance mechanism, requires a physiological interaction with EphrinA5 and Adenosine signaling at the growth cone level (Stettler et al., 2012). Similar results were reported for VAX1, the secretion of which at the optic chiasma is necessary for proper decussation (Kim et al., 2014). VAX1 activity follows its recognition of target optic chiasma cells through its binding to specific glycosaminoglycans.

POST-NATAL PHYSIO-PATHOLOGICAL FUNCTIONS OF HP HOMEOPROTEIN TRANSFER

At post-natal stages the cerebral cortex adapts to the environment through morphological and physiological changes that take place during transient periods of plasticity, called critical periods (CPs) (Hensch, 2005). Such periods correspond to the maturation of a specific class of GABAergic interneurons expression parvalbumin (PV cells). These inhibitory interneurons form synapses with the cell body of excitatory pyramidal cells in layers III/IV of the cerebral cortex and their maturation during CP shifts the Excitatory/Inhibitory (E/I) balance toward inhibition (Hensch et al., 1998). A classic case of CP is the maturation of the visual cortex and is illustrated by the loss of visual acuity of an eye sutured during CP. This amblyopic phenotype can be reversed if the eye is reopened before the end of CP, but not after CP closure, unless adult plasticity is activated, in particular by blocking OTX2 import into PV cells (Beurdeley et al., 2012; Bernard et al., 2016).

It was shown that the opening of plasticity is triggered by the internalization of OTX2, a homeoprotein synthesized by the choroid-plexus and secreted into the cerebrospinal fluid (Sugiyama et al., 2008). The capture of OTX2 specifically by PV cells is permitted by the assembly of matrixial perineural nets (PNNs) that enwrap these cells and the specific binding of OTX2 to chondroitin sulfate (CSE/CSD) glycosaminoglycans (GAGs) present in the matrix. In the visual system, PNN assembly is induced by eye opening and photoreceptor activation (Sugiyama et al., 2008; Beurdeley et al., 2012; Miyata et al., 2012). The binding of OTX2 to CSE/CSDE GAGs is permitted by a small sequence, the RK peptide. Glycosaminoglycan-binding sequences similar to this RK peptide are present in a large number of other homeoproteins, including VAX1, EN1 and EN2 where they also trigger specific GAG recognition (Kim et al., 2014; Prochiantz and Di Nardo, 2015). Functional studies are only provided for these few proteins, but this observation suggests the existence of a sugar code for the specific recognition of target cells in homeoprotein transduction.

OTX2 is transported from the choroid plexus to PV cells throughout the cerebral cortex. Accordingly, its ability to regulate plasticity during development and in the adult is probably not limited to the visual system and was experimentally extended to the auditory cortex and the medial prefrontal cortex (Lee et al., 2017). A striking observation is that *Otx2* heterozygote mice are hypoanxious and that this trait is maintained in the adult, unless OTX2 is virally overexpressed in the choroid plexus (Vincent et al., 2021). Conversely, a normally anxious wild-type adult mouse can be made hypoanxious through the induction of an OTX2-scFv and the ensuing neutralization of OTX2 in the cerebrospinal fluid (Vincent et al., 2021).

OTHER EXAMPLE OF THERAPEUTIC STRATEGIES BASED ON HOMEOPROTEIN TRANSDUCTION

The possibility to cure experimental amblyopia or to regulate anxiety-like behaviors by modifying, permanently or transiently, OTX2 import by PV cells suggests that this novel signaling pathway might open new avenues in the study, possibly cure, of disease affecting the nervous system. Still for OTX2, its transfer within the retina from producing cells, probably bipolar neurons, to RGCs was demonstrated (Sugiyama et al., 2008) and from the retinal pigmented epithelium (RPE) to photoreceptors strongly suggested (Pensieri et al., 2021). The neutralization of extracellular OTX2 by an OTX2-scFv secreted by retinal parvalbumin producing cells leads to a decrease of visual acuity associated with the alteration of inner retinal functions and *Otx2* knock out, specifically in the RPE, leads to photoreceptor cell death (Torero Ibad et al., 2020; Pensieri et al., 2021). This putative trophic OTX2 activity was verified in the glaucoma model of induced excitotoxicity in the retina rapidly followed by RGC degeneration (Torero Ibad et al., 2011). OTX2 injection in the optic cup followed by its capture by RGCs completely protects against excitotoxicity and preserves visual acuity. In parallel it was shown that OTX2 promotes the survival of adult purified rodent RGCs *in vitro* and induces the regeneration of their axons from cultured retinal explants. This ability to promote axon regeneration was confirmed *in vivo* in the optic nerve crush paradigm (Ibad et al., 2022).

The potential therapeutic activity of EN1/2 proteins in the mouse was evaluated for mesencephalic dopaminergic (mDA) neurons of the Substantia Nigra pars compacta (SNpc) and for

α-Motoneurons (αMNs) from the spinal cord ventral horns. EN1/2 expressed in the mDA neurons from the SNpc and Ventral Tegmental Area (VTA) mDA neurons exerts pro-survival activity in these cells as demonstrated by their progressive retrograde degeneration in the *En1* heterozygote mouse (*En1*-Het), associated with Parkinson-Disease (PD)-like motor and non-motor phenotypes (Sonnier et al., 2007; Nordströma et al., 2015). The bulk injection of EN1 or EN2 at the SNpc level, followed by its neuronal capture preserves mDA neurons from death in mouse and macaque PD models (Alvarez-Fischer et al., 2011; Rekaik et al., 2015; Blaudin de Thé et al., 2018; Thomasson, 2019). In the mouse, it was observed that EN1/2 acts at different levels, including an increase in the translation of mitochondrial complex-I, NdufS1 and NdufS3 proteins, a direct repression of genetic mobile elements of the LINE-1 family and the restoration of a healthy pattern for several heterochromatin marks, including MeCP2, Nucleolin, H3K27me3 and H3K9me3 (Alvarez-Fischer et al., 2011; Rekaik et al., 2015; Blaudin de Thé et al., 2018). This epigenetic activity probably explains why a single injection has long-lasting curative effects in mouse and macaque PD models.

α-Motoneurons in the ventral spinal cord do not express EN1/2 but are in post-synaptic contact with *En1*-expressing V1 interneurons and exhibit slow retrograde degeneration in the *En1*-Het mouse (Abonce et al., 2020). This degeneration is also observed *in vivo* following the viral expression of an EN1/2 scFv, demonstrating the EN1 secreted by V1 interneurons exerts a trophic activity on αMNs (Abonce et al., 2020). This trophic activity was confirmed by αMN protection by a single intrathecal injection of EN1 at the lumbar 5 (L5) level. The latter injection of EN1 is followed by its specific addressing to αMNs thanks to the EN1 GAG-binding domain. As shown for the mDA neurons, EN1 protecting activity is long-lasting with a duration of 2 months at least following a single injection, suggesting epigenetic mechanisms not yet studied in detail.

CONCLUSION

In the light of their distinct cell trafficking requirement, how and when intracrine and paracrine activities of homeoproteins have been acquired along evolution is an intriguing question. Since transcriptional activity and intercellular transfer are both intimately linked to the presence of the homeodomain, this duality of activities might have been intrinsic to the advent of homeoproteins. Although displaying some similarities with prokaryote transcription factors of the helix-turn-helix class, genuine homeoproteins are first detected in unicellular eukaryotes (Derelle et al., 2007) and one of the most ancestral functions attributed to these proteins is linked to sexual mating (Sun et al., 2019) and consequently to intercellular communication. Thanks to their unique and unconventional secretion and internalization properties, homeoproteins might have constituted a primitive from of signaling, which does not require the presence of specific protein receptors. While being primitive, this mode of signaling has been conserved judging by the ability of most homeoproteins to transfer between cells, may be thanks to their ability to synergize with more stringent signaling pathways based on ligand/receptor interactions.

AUTHOR CONTRIBUTIONS

AJ and AP wrote sections of the manuscript.

FUNDING

This work was supported by the Centre National de la Recherche Scientifique (CNRS), Institut National de la Sante et de la Recherche Medicale, College de France and the Agence Nationale de la Recherche (ANR-17-CE11-0050, CROSS and ANR-20CE44-0018, Glycotarget).

REFERENCES

Abonce, S. E. V., Lebœuf, M., Moya, K. L., and Prochiantz, A. (2020). Homeoprotein ENGRAILED-1 Promotes Motoneuron Survival and Motor Functions. *bioRxiv*. doi:10.1101/734020

Alvarez-Fischer, D., Fuchs, J., Castagner, F., Stettler, O., Massiani-Beaudoin, O., Moya, K. L., et al. (2011). Engrailed Protects Mouse Midbrain Dopaminergic Neurons against Mitochondrial Complex I Insults. *Nat. Neurosci.* 14, 1260–1266. doi:10.1038/nn.2916

Amblard, I., Dupont, E., Alves, I., Miralvès, J., Queguiner, I., and Joliot, A. (2020a). Bidirectional Transfer of Engrailed Homeoprotein across the Plasma Membrane Requires PIP2. *J. Cell Sci.* 133. doi:10.1242/jcs.244327

Amblard, I., Thauvin, M., Rampon, C., Queguiner, I., Pak, V. V., Belousov, V., et al. (2020b). H2O2 and Engrailed 2 Paracrine Activity Synergize to Shape the Zebrafish Optic Tectum. *Commun. Biol.* 3, 536. doi:10.1038/s42003-020-01268-7

Berlose, J.-P., Convert, O., Derossi, D., Brunissen, A., and Chassaing, G. (1996). Conformational and Associative Behaviours of the Third Helix of Antennapedia Homeodomain in Membrane-Mimetic Environments. *Eur. J. Biochem.* 242, 372–386. doi:10.1111/j.1432-1033.1996.0372r.x

Bernard, C., Vincent, C., Testa, D., Bertini, E., Ribot, J., Di Nardo, A. A., et al. (2016). A Mouse Model for Conditional Secretion of Specific Single-Chain Antibodies Provides Genetic Evidence for Regulation of Cortical Plasticity by a Non-cell Autonomous Homeoprotein Transcription Factor. *PLoS Genet.* 12, e1006035. doi:10.1371/journal.pgen.1006035

Beurdeley, M., Spatazza, J., Lee, H. H. C., Sugiyama, S., Bernard, C., Di Nardo, A. A., et al. (2012). Otx2 Binding to Perineuronal Nets Persistently Regulates Plasticity in the Mature Visual Cortex. *J. Neurosci.* 32, 9429–9437. doi:10.1523/JNEUROSCI.0394-12.2012

Bidlingmaier, S., Wang, Y., Liu, Y., Zhang, N., and Liu, B. (2011). Comprehensive Analysis of Yeast Surface Displayed cDNA Library Selection Outputs by Exon Microarray to Identify Novel Protein-Ligand Interactions. *Mol. Cell Proteomics* 10. M110 005116. doi:10.1074/mcp.M110.005116

Blaudin de Thé, F. X., Rekaik, H., Peze-Heidsieck, E., Massiani-Beaudoin, O., Joshi, R. L., Fuchs, J., et al. (2018). Engrailed Homeoprotein Blocks Degeneration in Adult Dopaminergic Neurons through LINE-1 Repression. *EMBO J.* 37, e97374. doi:10.15252/embj.201797374

Boncompain, G., Divoux, S., Gareil, N., de Forges, H., Lescure, A., Latreche, L., et al. (2012). Synchronization of Secretory Protein Traffic in Populations of Cells. *Nat. Methods* 9, 493–498. doi:10.1038/nmeth.1928

Borges-Araújo, L., and Fernandes, F. (2020). Structure and Lateral Organization of Phosphatidylinositol 4,5-bisphosphate. *Molecules* 25, 3885. doi:10.3390/molecules25173885

Brunet, I., Weinl, C., Piper, M., Trembleau, A., Volovitch, M., Harris, W., et al. (2005). The Transcription Factor Engrailed-2 Guides Retinal Axons. *Nature* 438, 94–98. doi:10.1038/nature04110

Cardon, S., Bolbach, G., Hervis, Y. P., Lopin-Bon, C., Jacquinet, J.-C., Illien, F., et al. (2021). A Cationic Motif in Engrailed-2 Homeoprotein Controls its Internalization via Selective Cell-Surface Glycosaminoglycans Interactions. doi:10.1101/2021.07.29.454375

Carlier, M.-F., Pernier, J., Pernier, J., Montaville, P., Shekhar, S., and Kühn, S. (2015). Cytoskeleton Dynamics and Motility groupControl of Polarized Assembly of Actin Filaments in Cell Motility. *Cell. Mol. Life Sci.* 72, 3051–3067. doi:10.1007/s00018-015-1914-2

Derelle, R., Lopez, P., Guyader, H. L., and Manuel, M. (2007). Homeodomain Proteins Belong to the Ancestral Molecular Toolkit of Eukaryotes. *Evol. Dev.* 9, 212–219. doi:10.1111/j.1525-142X.2007.00153.x

Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994). The Third Helix of the Antennapedia Homeodomain Translocates through Biological Membranes. *J. Biol. Chem.* 269, 10444–10450. doi:10.1016/s0021-9258(17)34080-2

Di Lullo, E., Haton, C., Le Poupon, C., Volovitch, M., Joliot, A., Thomas, J.-L., et al. (2011). Paracrine Pax6 Activity Regulates Oligodendrocyte Precursor Cell Migration in the Chick Embryonic Neural Tube. *Development* 138, 4991–5001. doi:10.1242/dev.066282

Dixon, A. S., Schwinn, M. K., Hall, M. P., Zimmerman, K., Otto, P., Lubben, T. H., et al. (2016). NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem. Biol.* 11, 400–408. doi:10.1021/acschembio.5b00753

Dupont, E., Prochiantz, A., and Joliot, A. (2007). Identification of a Signal Peptide for Unconventional Secretion. *J. Biol. Chem.* 282, 8994–9000. doi:10.1074/jbc.M609246200

Gehring, W. J., Affolter, M., and Bürglin, T. (1994). Homeodomain Proteins. *Annu. Rev. Biochem.* 63, 487–526. doi:10.1146/annurev.bi.63.070194.002415

Hensch, T. K. (2005). Critical Period Plasticity in Local Cortical Circuits. *Nat. Rev. Neurosci.* 6, 877–888. doi:10.1038/nrn1787

Hensch, T. K., Fagiolini, M., Mataga, N., Stryker, M. P., Baekkeskov, S., and Kash, S. F. (1998). Local GABA Circuit Control of Experience-dependent Plasticity in Developing Visual Cortex. *Science* 282, 1504–1508. doi:10.1126/science.282.5393.1504

Holland, P. W., Booth, H. A. F., and Bruford, E. A. (2007). Classification and Nomenclature of All Human Homeobox Genes. *BMC Biol.* 5, 47. doi:10.1186/1741-7007-5-47

Ibad, R., Moya, K., Quenech'du, N., and Prochiantz, A. (2022). OTX2 Stimulates Adult Retinal Ganglion Cell Regeneration. *Neural Regen. Res.* 17, 690–696. doi:10.4103/1673-5374.320989

Jiang, Z., Redfern, R. E., Isler, Y., Ross, A. H., and Gericke, A. (2014). Cholesterol Stabilizes Fluid Phosphoinositide Domains. *Chem. Phys. Lipids* 182, 52–61. doi:10.1016/j.chemphyslip.2014.02.003

Joliot, A. H., Triller, A., Volovitch, M., Pernelle, C., and Prochiantz, A. (1991b). alpha-2,8-Polysialic Acid Is the Neuronal Surface Receptor of Antennapedia Homeobox Peptide. *New Biol.* 3, 1121–1134.

Joliot, A., Maizel, A., Rosenberg, D., Trembleau, A., Dupas, S., Volovitch, M., et al. (1998). Identification of a Signal Sequence Necessary for the Unconventional Secretion of Engrailed Homeoprotein. *Curr. Biol.* 8, 856–863. doi:10.1016/s0960-9822(07)00346-6

Joliot, A., Pernelle, C., Deagostini-Bazin, H., and Prochiantz, A. (1991a). Antennapedia Homeobox Peptide Regulates Neural Morphogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 88, 1864–1868. doi:10.1073/pnas.88.5.1864

Joliot, A., Trembleau, A., Raposo, G., Calvet, S., Volovitch, M., and Prochiantz, A. (1997). Association of Engrailed Homeoproteins with Vesicles Presenting Caveolae-like Properties. *Development* 124, 1865–1875. doi:10.1242/dev.124.10.1865

Kaddour, H., Coppola, E., Di Nardo, A. A., Le Poupon, C., Mailly, P., Wizenmann, A., et al. (2020). Extracellular Pax6 Regulates Tangential Cajal-Retzius Cell Migration in the Developing Mouse Neocortex. *Cereb. Cortex* 30, 465–475. doi:10.1093/cercor/bhz098

Kim, N., Min, K. W., Kang, K. H., Lee, E. J., Kim, H.-T., Moon, K., et al. (2014). Regulation of Retinal Axon Growth by Secreted Vax1 Homeodomain Protein. *Elife* 3, e02671. doi:10.7554/eLife.02671

Kurrikoff, K., Gestin, M., and Langel, Ü. (2016). Recentin Vivoadvances in Cell-Penetrating Peptide-Assisted Drug Delivery. *Expert Opin. Drug Deliv.* 13, 373–387. doi:10.1517/17425247.2016.1125879

Lamazière, A., Wolf, C., Lambert, O., Chassaing, G., Trugnan, G., and Ayala-Sanmartin, J. (2008). The Homeodomain Derived Peptide Penetratin Induces Curvature of Fluid Membrane Domains. *PLoS ONE* 3, e1938. doi:10.1371/journal.pone.0001938

Layalle, S., Volovitch, M., Mugat, B., Bonneaud, N., Parmentier, M.-L., Prochiantz, A., et al. (2011). Engrailed Homeoprotein Acts as a Signaling Molecule in the Developing Fly. *Development* 138, 2315–2323. doi:10.1242/dev.057059

Lee, E. J., Kim, N., Park, J. W., Kang, K. H., Kim, W.-I., Sim, N. S., et al. (2019). Global Analysis of Intercellular Homeodomain Protein Transfer. *Cell Rep.* 28, 712–722. e3. doi:10.1016/j.celrep.2019.06.056

Lee, H. H. C., Bernard, C., Ye, Z., Acampora, D., Simeone, A., Prochiantz, A., et al. (2017). Genetic Otx2 Mis-Localization Delays Critical Period Plasticity across Brain Regions. *Mol. Psychiatry* 22, 680–688. doi:10.1038/mp.2017.1

Maizel, A., Bensaude, O., Prochiantz, A., and Joliot, A. (1999). A Short Region of its Homeodomain Is Necessary for Engrailed Nuclear Export and Secretion. *Development* 126, 3183–3190. doi:10.1242/dev.126.14.3183

Maizel, A., Tassetto, M., Filhol, O., Cochet, C., Prochiantz, A., and Joliot, A. (2002). Engrailed Homeoprotein Secretion Is a Regulated Process. *Development* 129, 3545–3553. doi:10.1242/dev.129.15.3545

McLaughlin, S., Wang, J., Gambhir, A., and Murray, D. (2002). PIP2 and Proteins: Interactions, Organization, and Information Flow. *Annu. Rev. Biophys. Biomol. Struct.* 31, 151–175. doi:10.1146/annurev.biophys.31.082901.134259

Miyata, S., Komatsu, Y., Yoshimura, Y., Taya, C., and Kitagawa, H. (2012). Persistent Cortical Plasticity by Upregulation of Chondroitin 6-sulfation. *Nat. Neurosci.* 15, 414–422. S1-2. doi:10.1038/nn.3023

Myeong, J., Park, C.-G., Suh, B.-C., and Hille, B. (2021). Compartmentalization of Phosphatidylinositol 4,5-bisphosphate Metabolism into Plasma Membrane Liquid-Ordered/raft Domains. *Proc. Natl. Acad. Sci. U.S.A.* 118. doi:10.1073/pnas.2025343118

Nordström, U., Beauvais, G., Ghosh, A., Pulikkaparambil Sasidharan, B. C., Lundblad, M., Fuchs, J., et al. (2015). Progressive Nigrostriatal Terminal Dysfunction and Degeneration in the Engrailed1 Heterozygous Mouse Model of Parkinson's Disease. *Neurobiol. Dis.* 73, 70–82. doi:10.1016/j.nbd.2014.09.012

Pensieri, P., Mantilleri, A., Plassard, D., Furukawa, T., Moya, K. L., Prochiantz, A., et al. (2021). Photoreceptor cKO of OTX2 Enhances OTX2 Intercellular Transfer in the Retina and Causes Photophobia. *eNeuro* 8. ENEURO.0229-21.2021. doi:10.1523/ENEURO.0229-21.2021

Prochiantz, A., and Di Nardo, A. A. (2015). Homeoprotein Signaling in the Developing and Adult Nervous System. *Neuron* 85, 911–925. doi:10.1016/j.neuron.2015.01.019

Ramírez-Weber, F. A., and Kornberg, T. B. (1999). Cytonemes: Cellular Processes that Project to the Principal Signaling Center in. *Drosoph. imaginal discs* 97, 599–607.

Rampon, C., Gauron, C., Lin, T., Meda, F., Dupont, E., Cosson, A., et al. (2015). Control of Brain Patterning by Engrailed Paracrine Transfer: a New Function of the Pbx Interaction Domain. *Development* 142, 1840–1849. doi:10.1242/dev.114181

Rayne, F., Debaisieux, S., Yezid, H., Lin, Y.-L., Mettling, C., Konate, K., et al. (2010). Phosphatidylinositol-(4,5)-bisphosphate Enables Efficient Secretion of HIV-1 Tat by Infected T-Cells. *EMBO J.* 29, 1348–1362. doi:10.1038/emboj.2010.32

Rekaik, H., Blaudin de Thé, F.-X., Fuchs, J., Massiani-Beaudoin, O., Prochiantz, A., and Joshi, R. L. (2015). Engrailed Homeoprotein Protects Mesencephalic Dopaminergic Neurons from Oxidative Stress. *Cell Rep.* 13, 242–250. doi:10.1016/j.celrep.2015.08.076

Ruiz-Medrano, R., Xoconostle-Cazares, B., and Kragler, F. (2004). The Plasmodesmatal Transport Pathway for Homeotic Proteins, Silencing Signals and Viruses. *Curr. Opin. Plant Biol.* 7, 641–650. doi:10.1016/j.pbi.2004.09.012

Sagan, S., Burlina, F., Alves, I. D., Bechara, C., Dupont, E., and Joliot, A. (2013). Homeoproteins and Homeoprotein-Derived Peptides: Going in and Out. *Cpd* 19, 2851–2862. doi:10.2174/1381612811319160002

Shearn, A., Rice, T., Garen, A., and Gehring, W. (1971). Imaginal Disc Abnormalities in Lethal Mutants of Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 68, 2594–2598. doi:10.1073/pnas.68.10.2594

Sonnier, L., Le Pen, G., Hartmann, A., Bizot, J.-C., Trovero, F., Krebs, M.-O., et al. (2007). Progressive Loss of Dopaminergic Neurons in the Ventral Midbrain of Adult Mice Heterozygote for Engrailed1. *J. Neurosci.* 27, 1063–1071. doi:10.1523/JNEUROSCI.4583-06.2007

Steringer, J. P., Bleicken, S., Andreas, H., Zacherl, S., Laussmann, M., Temmerman, K., et al. (2012). Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2)-dependent Oligomerization of Fibroblast Growth Factor 2 (FGF2) Triggers the Formation of a Lipidic Membrane Pore Implicated in Unconventional Secretion. *J. Biol. Chem.* 287, 27659–27669. doi:10.1074/jbc.M112.381939

Steringer, J. P., and Nickel, W. (2018). A Direct Gateway into the Extracellular Space: Unconventional Secretion of FGF2 through Self-Sustained Plasma Membrane Pores. *Seminars Cell & Dev. Biol.* 83, 3–7. doi:10.1016/j.semcdb.2018.02.010

Stettler, O., Joshi, R. L., Wizenmann, A., Reingruber, J., Holcman, D., Bouillot, C., et al. (2012). Engrailed Homeoprotein Recruits the Adenosine A1 Receptor to Potentiate Ephrin A5 Function in Retinal Growth Cones. *Development* 139, 215–224. doi:10.1242/dev.063875

Sugiyama, S., Di Nardo, A. A., Aizawa, S., Matsuo, I., Volovitch, M., Prochiantz, A., et al. (2008). Experience-Dependent Transfer of Otx2 Homeoprotein into the Visual Cortex Activates Postnatal Plasticity. *Cell* 134, 508–520. doi:10.1016/j.cell.2008.05.054

Sun, S., Coelho, M. A., Heitman, J., and Nowrousian, M. (2019). Convergent Evolution of Linked Mating-type Loci in Basidiomycete Fungi. *PLoS Genet.* 15, e1008365. doi:10.1371/journal.pgen.1008365

Tassetto, M., Maizel, A., Osorio, J., and Joliot, A. (2005). Plant and Animal Homeodomains Use Convergent Mechanisms for Intercellular Transfer. *EMBO Rep.* 6, 885–890. doi:10.1038/sj.embor.7400487

Temmerman, K., Ebert, A. D., Müller, H.-M., Sinning, I., Tews, I., and Nickel, W. (2008). A Direct Role for Phosphatidylinositol-4,5-Bisphosphate in Unconventional Secretion of Fibroblast Growth Factor 2. *Traffic* 9, 1204–1217. doi:10.1111/j.1600-0854.2008.00749.x

Thomasson, N., Pioli, E., Friedel, C., Monseur, A., Lavaur, J., Moya, K. L., et al. (2019). Engrailed-1 Induces Long-lasting Behavior Benefit in an Experimental Parkinson Primate Model. *Mov. Disord.* 34, 1082–1084. doi:10.1002/mds.27714

Torero Ibad, R., Mazhar, B., Vincent, C., Bernard, C., Dégardin, J., Simonutti, M., et al. (2020). OTX2 Non-cell Autonomous Activity Regulates Inner Retinal Function. *eNeuro* 7. 0012, 19ENEURO.0012-19.2020. doi:10.1523/ENEURO.0012-19.2020

Torero Ibad, R., Rheey, J., Mrejen, S., Forster, V., Picaud, S., Prochiantz, A., et al. (2011). Otx2 Promotes the Survival of Damaged Adult Retinal Ganglion Cells and Protects against Excitotoxic Loss of Visual Acuity *In Vivo*. *J. Neurosci.* 31, 5495–5503. doi:10.1523/JNEUROSCI.0187-11.2011

Vincent, C., Gilabert-Juan, J., Gibel-Russo, R., Alvarez-Fischer, D., Krebs, M.-O., Le Pen, G., et al. (2021). Non-cell-autonomous OTX2 Transcription Factor Regulates Anxiety-Related Behavior in the Mouse. *Mol. Psychiatry* 26, 6469–6480. doi:10.1038/s41380-021-01132-y

Viotti, C. (2016). "ER to Golgi-dependent Protein Secretion: The Conventional Pathway," in *Unconventional Protein Secretion, Methods in Molecular Biology*. Editors A. Pompa and F. De Marchis (New York, NY: Springer New York), 3–29. doi:10.1007/978-1-4939-3804-9_1

Wang, Y.-H., Bucki, R., and Janmey, P. A. (2016). Cholesterol-Dependent Phase-Demixing in Lipid Bilayers as a Switch for the Activity of the Phosphoinositide-Binding Cytoskeletal Protein Gelsolin. *Biochemistry* 55, 3361–3369. doi:10.1021/acs.biochem.5b01363

Wizenmann, A., Brunet, I., Lam, J. S. Y., Sonnier, L., Beurdeley, M., Zarbalis, K., et al. (2009). Extracellular Engrailed Participates in the Topographic Guidance of Retinal Axons *In Vivo*. *Neuron* 64, 355–366. doi:10.1016/j.neuron.2009.09.018

Zacherl, S., La Venuta, G., Müller, H.-M., Wegehingel, S., Dimou, E., Sehr, P., et al. (2015). A Direct Role for ATP1A1 in Unconventional Secretion of Fibroblast Growth Factor 2. *J. Biol. Chem.* 290, 3654–3665. doi:10.1074/jbc.M114.590067

Zehe, C., Engling, A., Wegehingel, S., Schäfer, T., and Nickel, W. (2006). Cell-surface Heparan Sulfate Proteoglycans Are Essential Components of the Unconventional Export Machinery of FGF-2. *Proc. Natl. Acad. Sci. U.S.A.* 103, 15479–15484. doi:10.1073/pnas.0605997103

Zeitler, M., Steringer, J. P., Müller, H.-M., Mayer, M. P., and Nickel, W. (2015). HIV-tat Protein Forms Phosphoinositide-dependent Membrane Pores Implicated in Unconventional Protein Secretion. *J. Biol. Chem.* 290, 21976–21984. doi:10.1074/jbc.M115.667097

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

REVIEW
published: 12 July 2022
doi: 10.3389/fcell.2022.915117

Tunneling Nanotubes Facilitate Intercellular Protein Transfer and Cell Networks Function

*Laura Turos-Korgul†, Marta Dorota Kolba†, Piotr Chroscicki, Aleksandra Zieminska and Katarzyna Piwocka**

Laboratory of Cytometry, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

The past decade witnessed a huge interest in the communication machinery called tunneling nanotubes (TNTs) which is a novel, contact-dependent type of intercellular protein transfer (IPT). As the IPT phenomenon plays a particular role in the cross-talk between cells, including cancer cells as well as in the immune and nervous systems, it therefore participates in remodeling of the cellular networks. The following review focuses on the placing the role of tunneling nanotube-mediated protein transfer between distant cells. Firstly, we describe different screening methods used to study IPT including tunneling nanotubes. Further, we present various examples of TNT-mediated protein transfer in the immune system, cancer microenvironment and in the nervous system, with particular attention to the methods used to verify the transfer of individual proteins.

Keywords: intercellular protein transfer, proteome, cellular network, tunneling nanotubes (TNT), SILAC mass spectrometry, codeIT, cancer microenvironment

OPEN ACCESS

Edited by:
Julien Villeneuve, Institute for Functional Genomics (IGF) (CNRS), France

Reviewed by:
Shunsuke Kimura, Keio University, Japan
Wei-Hua Yan, Wenzhou Medical University, China

****Correspondence:***
Katarzyna Piwocka
k.piwocka@nencki.edu.pl

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *07 April 2022*
Accepted: *20 June 2022*
Published: *12 July 2022*

Citation:
Turos-Korgul L, Kolba MD, Chroscicki P, Zieminska A and Piwocka K (2022) Tunneling Nanotubes Facilitate Intercellular Protein Transfer and Cell Networks Function. Front. Cell Dev. Biol. 10:915117. doi: 10.3389/fcell.2022.915117

INTRODUCTION

The intercellular communication has crucial impact on proper functioning of tissues and organisms. Until recently, major part of the research on intercellular communication delved into mechanisms independent on direct contact between cells, namely communication through secreted factors, e.g., cytokines, chemokines or a plethora of extracellular vesicles. The direct contact-dependent mechanisms remained less studied, with the sole exception of gap junctions which enable the transfer of small molecules. However, in recent years, more data point to the fact that also larger molecules and organelles can be transported between cells in a contact-dependent manner. One of the new possible routes enabling this kind of transport, are mediated by tunneling nanotubes, shortly TNTs. TNTs are membranous channels, connecting two or more distant cells which are able to transport different types of cargo, including vesicles (Zhu et al., 2015), individual proteins (Pasquier et al., 2012; Desir et al., 2019; Kretschmer et al., 2019) and mitochondria (Spees et al., 2006; Pasquier et al., 2013; Jackson et al., 2016; Vignais et al., 2017; Saha et al., 2022). These structures are actin-rich, have diameters ranging from 50 to 200 nm and 30 μm mean length. It is also commonly accepted that TNTs are straight conduits hovering above the substratum (Önfelt et al., 2004; Korenkova et al., 2020; Dagar et al., 2021). There is a growing interest to investigate the precise mechanisms governing these types of interactions. In this review, we discuss several methods used to study intercellular protein transport in general. Furthermore, we review the role of tunneling nanotubes in this process and various approaches used to confirm the transport of particular molecules between cells through TNTs.

THE PHENOMENON OF INTERCELLULAR PROTEIN TRANSFER—THE NOVEL PERSPECTIVE OF CELLULAR NETWORK

Generally, the processes of intercellular transport of large molecules are the most vastly reported in immune cells. These studies in the major part relate to the intercellular transfer of membrane-associated proteins, including MHC proteins, co-stimulatory proteins, NK-cell receptors for MHC class I protein, polio-virus receptor (CD155), membrane-associated antigens or antigen-specific BCR (Davis, 2007; Ahmed and Xiang, 2011). The list of possible mechanisms of this transport consists internalization and recycling pathway, dissociation-associated pathway, exosome uptake, exocytosis or more specialized secretory pathways, the enzymatic cleavage of cell-surface proteins, trogocytosis, local membrane fusion and membrane nanotube formation (Davis, 2007; Ahmed and Xiang, 2011). An increasing body of evidence both *in vitro* and *in vivo* indicates that intercellular protein transfer (IPT) is a common phenomenon, at least in the immune system (Davis, 2007; Ahmed and Xiang, 2011).

To verify whether IPT is a general phenomenon, Niu and colleagues created a mathematical formula describing IPT and experimentally verified their model predictions (Niu et al., 2009). In a 2-day long confluent co-culture of donor and acceptor cells they found, that the transfer of three different membrane proteins was bidirectional and direct contact-dependent. The transfer efficiency varied as a function of their lateral membrane mobility, related to the molecular mass, membrane fluidity and the ratio between donor and acceptor cells. Cell-cell adhesion enhanced the membrane protein transfer, therefore authors supposed that the underlying mechanism was based on transient local plasma membrane fusions. However, they did not exclude the possibility of the involvement of tunneling nanotubes in this process. In general, authors demonstrated that IPT is not restricted to a few types of proteins and occurs between multiple cell types. It must be taken into account that the possible universality of IPT challenges the classic theories of cell autonomy.

"No Cell Is an Island" Perspective

Rechavi et al. (2009) proposed that DNA content of a cell can be considered as the "hardware," whereas the transcriptome and proteome—as the "personality" of a cell (Rechavi et al., 2009). In this regard, the "personality" of each cell is constantly shared with other interacting cells. Therefore, cells cannot be more considered as unchangeable units of life but rather—units of life which continuously "become" what they are supposed to be in a specific situation. Although this perspective makes experimental biology more complex, it is probably closer to the physiology of living tissues and allows a holistic view on the functionality of a tissue. So far, only the neuronal system was studied with this perspective. It is commonly accepted that, although the brain consists of separate cells, the memory as well as other functions are based on intercellular contacts between neurons through synapses (Rechavi et al., 2009). IPT, as mentioned above, has several proven functional implications on biological processes, such as immune responses (Davis, 2007; Ahmed and Xiang, 2011) and presumably enables a fast modification of behaviour of large groups of cells without the need to change their gene expression profile (Niu et al., 2009). In general, research on IPT, including TNT-mediated transfer, shifts the focus on systemic level of tissue functionality. However, to achieve a more global characterization of all the proteins that transfer, there is a need for the development of high throughput technologies facilitating the identification of these groups of proteins.

METHODOLOGY FOR INTERCELLULAR PROTEIN TRANSFER RESEARCH

Trans-SILAC

The trans-SILAC technique is a method to identify the full repertoire of transferred proteins and therefore demark the non-cell-autonomous proteome (Rechavi et al., 2009). This technique derives from SILAC (stable isotope labelling by amino acids in cell culture), combines it with FACS (fluorescence-activated cell sorting) and provides bioinformatic tools to facilitate the identification of rare heavy proteins in a large pool of light proteins within the proteome of acceptor cells. In short, this method relies on > 98% enrichment of donor cells' proteome in heavy isotopologues of amino acids of choice and subsequent co-culture with acceptor cells, previously cultured in the presence of light amino acids. Further on, acceptor cells get sorted by FACS on the basis of a fluorescent cytoplasmic marker (if present) or on the basis of plasma membrane markers. To additionally narrow the scope of search, a chosen subpopulation of acceptor cells might be sorted e.g. cells which received a chosen fluorescent cargo. Finally, "heavy" proteins are identified in acceptor cells by bioinformatic tools—these are the proteins which were synthesised by donor cells and transferred to acceptor cells in the scope of IPT (**Figure 1**). For the first time, the trans-SILAC technique was successfully used to identify sets of proteins transferred from B cells to NK cells in a direct contact-dependent and actin-dependent manner (Rechavi et al., 2009). Identified proteins grouped into functional protein networks, e.g., related to "cancer, immunological disease, and hematological disease," identified by the network explorer feature of the Ingenuity Pathways Analysis (IPA) platform. Importantly, authors highlighted that this is an exemplary presentation of the utility of the trans-SILAC method which can be used for versatile purposes: it is available for the study of various cell types, the conditions and the time of co-culture can be specifically chosen. Moreover, one can track the direct contact-dependent or -independent IPT, as well as IPT correlated with the transfer of a given cargo or a protein of interest. No specialized instruments are required, the software created for the analysis of the LC-MS/MS data has been reposited with the original paper and is available on-line. Another use of trans-SILAC technique reported in the literature enabled the discovery that senescent cells communicate through direct contact-dependent IPT with NK cells (Biran et al., 2015). As previous reports focused on the secretory phenotype of senescent

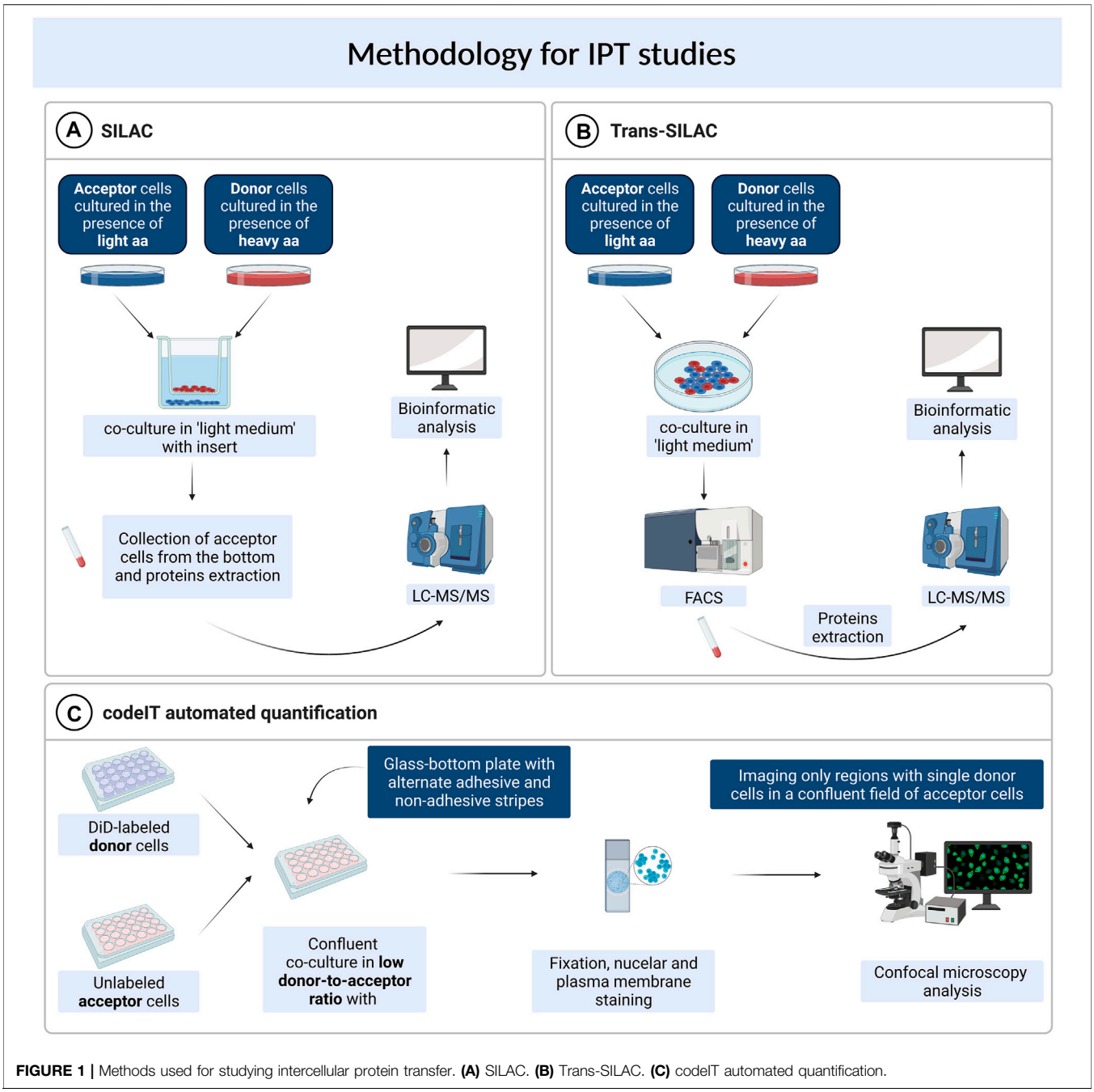


FIGURE 1 | Methods used for studying intercellular protein transfer. **(A)** SILAC. **(B)** Trans-SILAC. **(C)** codeIT automated quantification.

cells, the direct-contact dependent communication was a novelty in this regard. Authors found that among 47 proteins transferred from donor to NK cells, 90% transferred exclusively from cells with induced senescence, with no mass restriction for the transfer. They specifically enriched three biological processes, namely glycolysis, regulation of the actin cytoskeleton, and antigen processing and presentation (Gene Ontology analysis). What is more, the IPT led to increased NK cell activation and cytotoxicity towards senescent cells. In spite of the fact that the majority of IPT correlated with the protein abundance in donor cells, 12.5% of the identified proteins transferred independently of protein abundance in donor cells. It indicates that IPT is not a passive process but can be actively regulated.

SILAC

Parallely, a similar concept was used to identify IPT that was independent on direct contact, with the use of the sole SILAC method (Li et al., 2010). In this study, murine lung cells previously grown in "heavy" media and irradiated, were co-cultured in a transwell system with murine bone marrow cells. After 48 h, acceptor cells were collected from the bottom chamber of the transwell and analysed by LC-MS/MS for the presence of

"heavy" proteins (**Figure 1**). A set of seven proteins was transferred, including the retinoblastoma-binding protein 7 (RBBP7), however, the irradiation step was critical for IPT to occur. Authors found that irradiation injury of lung cells led to secretion of proteins that have not been previously regarded as secreted. These findings further highlight the biological relevance of IPT.

Contact-Dependent Intercellular Transfer Automated Quantification

Another high throughput technique available for the study of intercellular transfer, including TNT-mediated transfer, is not restricted to proteins and can be used to identify and quantify regulators and cargo of contact-dependent intercellular transfer (termed: codeIT) (Frei et al., 2015). This method is a microscopy-based screening (**Figure 1**), and can be assigned also to individual donor cells, quantified by confocal microscopy and image analysis in 2D or 3D, therefore preserving spatial information. Importantly, codeIT is suited for any fluorescently labelled molecule or structure, including pathogens. It was used for the identification of regulators of transfer of endocytic vesicles labelled with lipophylic VybrantDye DiD. For the identification of regulators of the codeIT process, one can use siRNA-based screen. To exclude the possibility of intercellular transfer through the shared medium, the low donor-to-acceptor ratio should be used (in the range 1:100–1:400). To increase transfer with such a limited number of donors, the confluent co-culture is recommended. In this set-up (extracellular vesicles transfer), DiD is a specific dye and marker for codeIT. The study revealed that majority of donor cells transferred cellular vesicles in a direct contact-dependent manner. DiD transfer intensity followed a normal distribution. Additionally, the dotted pattern of the transfer as well as a correlation between the intensity of signal and the volume (sum of voxels) excluded the suspicion of dye diffusion and supported the transfer of DiD-labelled "packages". Observed transfer was dependent on F-actin and serum components. A screen of 36 gene candidates revealed several regulators of codeIT, including Myo10, Cdc42 and several Rab proteins.

THERE IS A NEED FOR HIT VALIDATION

It is important to note that high throughput techniques described above require further steps to validate the transfer of the identified proteins with supplementary methods. For example, the putative regulators of codeIT, identified by siRNA-based silencing, were further overexpressed as EGFP-tagged proteins, including truncated proteins and point-mutants, and searched for their influence on contact-dependent transfer of the studied vesicles with flow cytometry methods. Therefore the described assay offers a possibility of differentiating the role of candidate protein separately in donor and acceptor cells. In regard to SILAC-based methods, the proteins identified as transferred can be further validated by Western blotting, flow cytometry or other approaches. For example, the transfer of proteins from murine lung cells identified by SILAC, was validated by studying the expression levels of the proteins of interest in acceptor cells by Western blotting (Li et al., 2010). However, this method of hit validation is limited to proteins transferred in significant amounts. Additionally, to exclude the possibility of increased transcription of the relevant genes, a Real-Time PCR was performed. Finally, to ensure that proteins are transferred into the cytosol of the cell, a trypsin digestion to destroy the proteins adsorbed to outer membrane followed by Western blotting was performed. This further supported the IPT hits identified by SILAC. The protein transfer can be also validated by flow cytometry, which has been used to verify the transfer of 17 selected proteins between B cells and NK cells, (Rechavi et al., 2009). Cytosolic proteins were expressed as EGFP-tagged proteins in donor cells, whereas membrane-associated proteins were identified by specific fluorochrome-conjugated monoclonal antibodies. Altogether, it is important to point out that the specific detection/visualization is necessary to confirm direct transfer of specific proteins from donor to acceptor cell, identified first by high throughput techniques.

CAN TUNNELING NANOTUBES MEDIATE INTERCELLULAR PROTEIN TRANSFER?

Tunneling Nanotubes as a Mechanism of Intercellular Protein Transfer

Tunneling nanotubes (TNTs) are membrane-bound intercellular conduits enabling the transport of various cellular components directly from cell to cell (Rustom et al., 2004). TNTs were shown to enable the transfer of endocytic vesicles, lysosomes and mitochondria as well as membrane-bound proteins (Mittal et al., 2019). They can also be highjacked for direct intercellular viral spread or can transfer misfolded proteins, leading to the propagation of prion diseases and neurodegenerative diseases (Mittal et al., 2019). The involvement of TNTs in the intercellular transfer of membrane-bound proteins has been reported so far—examples will be cited in the following parts of this review. Although, the TNT-mediated transfer of cytosolic proteins, is much less studied, TNTs impede the transfer of small cytosolic molecules such as calcein or GFP (Rustom et al., 2004). Nevertheless, the TNTs formation and protein transfer mechanisms are still not fully known, including the observation that both, open- and closed-ended TNTs exist. Their specific and tight regulation allowing for intercellular transfer is still a matter of debate.

Possible Mechanisms and Regulators of Tunneling Nanotubes Formation and Function

The exact mechanism of tunneling nanotubes formation still remains not fully known and is likely to depend on the cell type and microenvironment. Resent research suggests two main mechanisms by which cells can form TNTs: filopodial interplay and cell dislodgement. In the first case, when filopodia protrudes from one cell and elongates until it encounters the other one, the

conversion towards TNT occurs (Rustom et al., 2004; Bukoreshtliev et al., 2009). When cells stay in contact for a given period of time, then move apart from each other while remaining connected through a thin membranous structure, TNT is formed by a cell dislodgement mechanism (Önfelt et al., 2004; Sowinski et al., 2008, 2011). It is worth noting that these two mechanisms can occur simultaneously, or change dependently on the conditions (Rustom et al., 2004; Wang et al., 2012).

Biran et al. (2015) showed that at least some part of IPT is independent on protein abundance in donor cells and therefore IPT can be a regulated process (Biran et al., 2015). Moreover, the screen of regulators of codeIT, including TNT-mediated transfer, revealed several proteins involved in the regulation of this process (Frei et al., 2015). Myo10 was identified as one of the regulators. As it localises mainly to the tips of cell protrusions, it suggests that those structures are involved. The truncation mutant of Myo10, lacking the motor domain, inhibited transfer only when expressed in donor cells. Cdc42 is another codeIT/TNTs regulator, probably located upstream, as it was not transferred between cells, in contrast to all other regulatory molecules. The Cdc42 knockdown inhibited protein transfer, whereas overexpression of two different mutants of Cdc42 revealed that a cycling between active and inactive forms of Cdc42 is indispensable for this process. Finally, several Rab proteins described to be localised to early, recycling, and tubular endosomes, were found to regulate intercellular protein transfer. The activity of Rab11a, a regulator of recycling endosomes, was important for the process both in donors and acceptors. Its role might be the delivery of cell-cell adhesion molecules to the plasma membrane to enable formation of a tight contact between cells. Moreover, authors proposed several possible roles of Rab35, a known marker of tubular endosomes: determination of sites of F-actin polymerisation, initiation of formation of nanotube precursors or the cadherin-mediated anchoring of nanotubes to target cells. Finally, knockdown of the early endosomal marker Rab5a or EEA1, a known Rab5a-effector, reduced or enhanced transfer, respectively. The list of other identified regulators includes Rab7a, Rab8a and Myo5c (Frei et al., 2015). Altogether those data indicate that mechanisms regulating formation of membrane protrusions are involved in TNTs formation, however this may not be directly associated with the transport of proteins, as only open TNTs are able to finalize the transfer process.

Additional experiments with the transfer of EGFP-tagged regulators showed that all codeIT regulators, except for Cdc42, transferred themselves. This indicates that DiD-labelled vesicles originate from intercellular membranes and the endocytic pathway (Frei et al., 2015). These discoveries led to the model of intercellular protein transfer as a process dependent on F-actin-rich protrusions, positive for Myo10 and regulated by Cdc42, whereas the membrane material is delivered by the endosomal pathway (Frei et al., 2015). The above-described discoveries suggest that TNTs might offer the possible mechanism for contact-dependent intercellular transfer between non-immune cells. Moreover, cytosolic proteins might be transferred enclosed within endosomes, which were identified as the compartment transferred between cells. We also implemented this assumption and used trans-SILAC technique to identify proteins transferred between stromal and leukemic cells within DiD-labelled cellular vesicles. Based on our results, we propose that the intercellular transfer of proteins within these vesicles is an active and tightly regulated process (Kolba et al., 2019).

TNT-MEDIATED TRANSFER OF PROTEINS IN CANCER

An ever-increasing body of literature shows that different types of proteins can be transferred via tunneling nanotubes in the cancer microenvironment (**Figure 2**). This phenomenon concerns both, membrane and cytosolic proteins, which belong to different functional groups, partially described below. Several methods are reported to address this issue, mainly live cell imaging, time-lapse video microscopy and immunofluorescence.

Stress Adaptation

Kretschmer et al. showed that androgen receptor blockade and metabolic stress result in induction of TNTs formation between stressed and unstressed prostate cancer cells (PCa) as well as between prostate cancer cells and osteoblasts. They identified three stress adaptor proteins: clusterin (CLU), YB-1 and Hsp27 which localize within TNTs formed between prostate cancer cells (PCa) (Kretschmer et al., 2019). Proximity Ligation Assay further supported these observations. Importantly, authors did not observe stress granules within TNTs, indicating that such structures are not transferred between cells, at least in this model. Using live imaging and fluorescently labelled proteins they confirmed that clusterin is transported bi-directionally *via* TNTs. Moreover, silencing CLU and YB-1 in PCa cells significantly decreased TNTs formation under stress conditions. What is more, it was pointed out that there is a possible regulation that relied upon PI3K pathway in TNTs formation after androgen receptor blockade. Disruption of TNTs formation reduced prostate cancer cells' survival when treated with androgen deprivation. This indicates a possible role of TNT-mediated transport in the adaptation of prostate cancer cells to stress and cell survival.

Transfer of Pro-Oncogenic Proteins

It was reported that a mutated form of KRAS protein, involved in development of cancer and chemoresistance (Misale et al., 2012), can be transferred *via* TNTs between colorectal cancer cells (CRC) (Desir et al., 2019). Moreover, CRC harbouring mutant *KRAS* variant (*KRAS G13D*) formed more TNTs than CRC cells with wild-type *KRAS*. GFP-tagged mutant *KRAS* transferred via TNTs from CRC LOVO cells (expressing mutant *KRAS*) to HCT-8 cells (wild-type *KRAS*) was confirmed by fluorescence microscopy and fluorescence recovery after bleaching (FRAP) experiments. Furthermore, co-culture of HCT-8 cells transfected with mutant KRAS with non-transfected HCT-8 cells not only promoted TNTs formation but also proved that mutant *KRAS* can be transported through them, which was presented using fluorescence time-lapse microscopy. Additionally, the transfer of

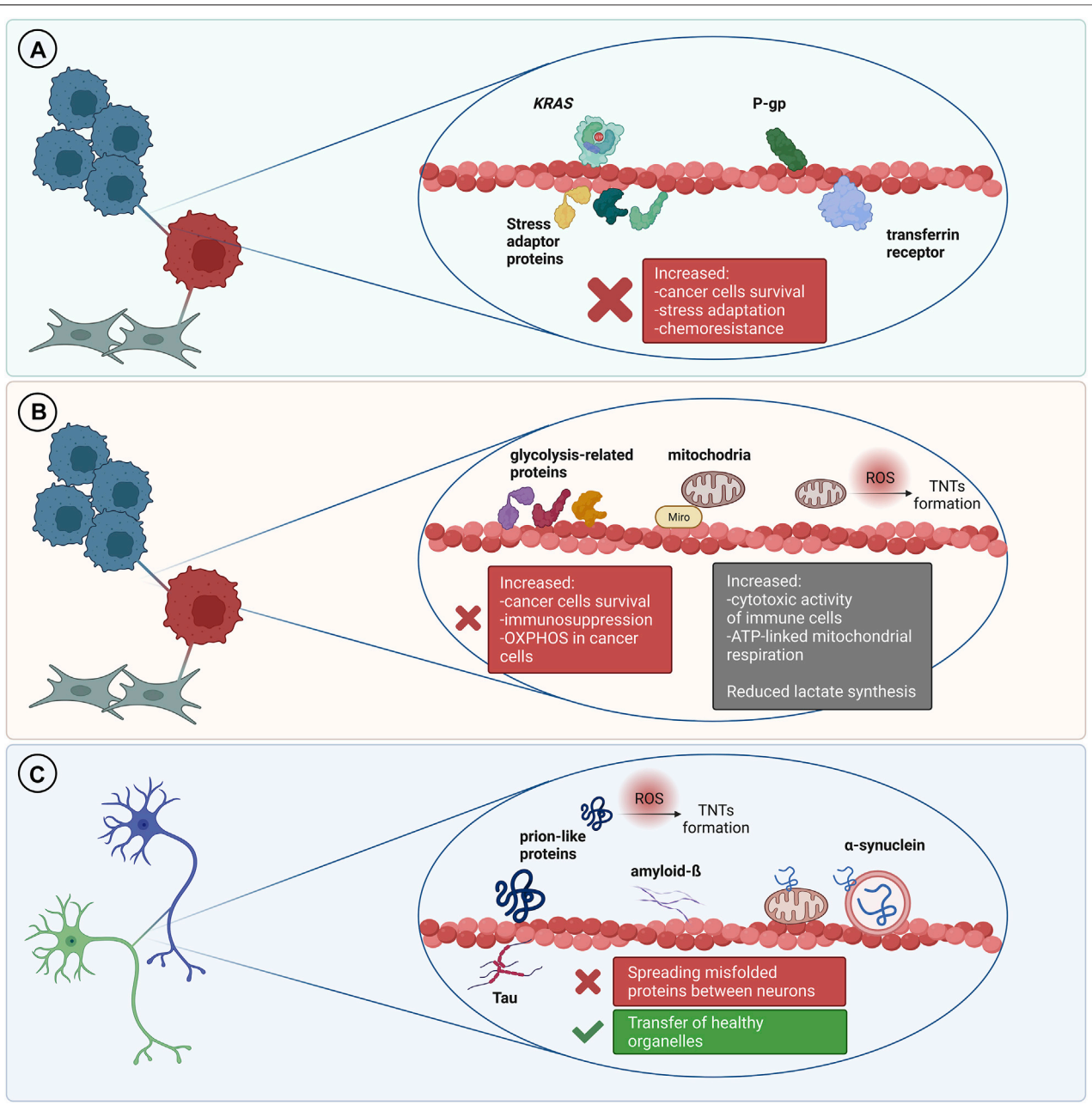


FIGURE 2 | TNT-mediated transfer of various proteins and its outcomes. **(A)** In the cancer microenvironment it is usually associated with cancer progression and increased chemoresistance. **(B)** Transfer of metabolic-related proteins and mitochondria may result in increased immunosuppression but it can also be a cause of increased cytotoxic activity of immune cells. Transfer of mitochondria to cancer cells is often associated with increased chemoresistance and cancer progression. **(C)** TNT can mediate transfer of misfolded proteins between cells in the nervous system which leads to progression of neurodegenerative disorders. On the other hand, TNT can be a route for the transmission of healthy organelles as a rescue mechanism.

GFP-tagged mutant *KRAS* to wild-type *KRAS* CRC cells was also confirmed by flow cytometry. Moreover, co-culture of wild-type CRC cells with cells harbouring a mutant version of *KRAS,* increased phosphorylation of ERK when compared to wild-type CRC cells cultured alone. This observation indicates a possible role of transferred *KRAS* mutant in inducing ERK activation. This allows to conclude that mutant *KRAS* increases the cross-talk between CRC cells and can be transferred horizontally via tunneling nanotubes.

TNT-mediated transfer of another member of Ras superfamily—H-Ras was shown by Rainy and co-workers. This small GTPase localizes to the inner plasma membrane

and can be transferred via TNTs from B cells to T cells (Rainy et al., 2013). This phenomenon was diminished after inhibition of actin polymerization and separation of co-cultured cells with insert which indicated the contact-dependent character of such transport. Researchers applied optical tweezers and 4D spinning disk confocal microscopy to observe TNTs formation between B lymphoblastoid cells and Jurkat cells. GFP-labelled H-Ras protein was transferred through TNTs and GFP-labelled membrane patches segregated from the TNTs were present on the acceptor cells. Authors applied FRAP technique to measure the diffusion of GFP-H-RasG12V both in the TNT and in the membrane patches present on acceptor cells. While photobleaching of GFP-positive regions of TNTs resulted in quick fluorescence recovery, photobleaching of GFP-rich membrane patches present on acceptor cells did not result in fluorescence recovery which confirmed TNT-mediated transport of GFP membrane patches to Jurkat cells. Moreover, labelling of Jurkat cells with an anti-CD86 antibody that specifically recognizes the extracellular domain of CD86—a transmembrane B-cell marker, confirmed that transferred membrane patches maintain their in-out orientation in acceptor T cells. What is more, using specific mutants of H-Ras with prominent cytosolic localization, authors showed that plasma membrane localization of H-Ras is essential for its transfer through tunneling nanotubes.

Transfer of Cancer Stem Cell Markers

CD133 protein, widely used as a marker for cancer stem cell isolation, was detected within tunnelling nanotubes formed between human primary $CD34^+$ hematopoietic progenitors and KG1a—acute myeloid leukemia cells (Reichert et al., 2016). Authors applied time-lapse video microscopy to observe the movement of CD133-GFP puncta along TNTs. They did not report retrograde transport to the donor cells, however, local accumulation of CD133-GFP protein was presented. Reichert et al. suggested that these aggregates could not cross junctional complexes between KG1a and $CD34^+$ HPSC cells. To determine if CD133 transfer occurs at the plasma membrane or *via* cytoplasmic structures, they stained live GFP-CD133 KG1a cells with fluorochrome-coupled anti-CD133 antibody. These experiments showed that CD133 transport occurs mainly *via* cell surface (Reichert et al., 2016).

Proteins Involved in Drug Resistance

Additionally, the well-known drug transporter, P-gp, was reported to be transferred within TNTs between cancer cells. This phenomenon was shown in cultured breast cancer MCF-7 cells by Pasquier and co-workers (Pasquier et al., 2012), where TNTs, together with microparticles, mediated transfer of P-gp between MCF-7 cells. This led to extragenetic emergence of multidrug resistance in a drug-sensitive population of breast cancer cells. First, using flow cytometry, they confirmed that in the co-culture of sensitive and drug-resistant MCF-7 cells, the level of P-gp protein increases with culturing time. Moreover, multidrug resistance activity of co-cultured cells increased, which was shown in the drug efflux assay with calcein AM. Both events were contact-dependent. However, the data was acquired from a mixed population of sensitive and resistant cells, not from single subpopulations. In these co-cultures TNTs were presented never earlier than after 3 days of incubation. Authors labelled sensitive MCF-7 cells with Cell Tracker Violet (to stain cytoplasm) and stained co-culture with WGA (to stain cell membranes) with additional staining with anti-P-gp antibody. Analyses of fixed specimens revealed co-localization of these three dyes and the presence of P-gp enriched "bridges" between MCF-7 resistant and sensitive cells.

Transfer of Proteins Involved in Tunneling Nanotubes Formation

As already mentioned, proteins involved in tunneling nanotubes formation can be transferred between cells *via* TNTs (D'Aloia et al., 2021). Burtey et al. applied high resolution 4-dimensional confocal microscopy to demonstrate that transferrin receptor (Tf-R) is transferred between HeLa cells. Moreover, Rab8 small GTPase was co-transferred with Tf-R *via* TNTs to acceptor cells (Burtey et al., 2015), and transferrin receptor was detected in vesicular structures visible along tunneling nanotubes. After inhibition of clathrin-mediated endocytosis, transfer of Tf-R decreased, which indicates that endocytosis of the transferrin receptor is required for its TNT-mediated transfer. Similar experiments with GFP-tagged DN mutant of Rab8 co-expressed with Tf-RmCherry demonstrated that Rab8 is also crucial for transfer of Tf-R. Also in the culture of 5637 cells (bladder cancer cells), live imaging microscopy data showed that fluorescently tagged RalA GTPase, which is well-known for its role in TNTs development (Hase et al., 2009), is transported between these cells through TNTs. Another protein significant in TNTs development—LST1 (Schiller et al., 2012) was also effectively transported in this way. It is worth mentioning that both proteins interact with RalGPS2, which was not detected within TNTs but plays a major role in the molecular machinery underlying TNTs formation between bladder cancer cells presented by authors. Interesting observations from Schiller and colleagues (Schiller et al., 2013) showed that transmembrane, but not soluble, HLA-EGFP protein can be transferred between HeLa cells, and inhibition of actin polymerization diminished transfer rate and overexpression of LST1, considered as TNTs formation regulator. All of the data described above is summarized in **Table 1**.

TRANSFER OF METABOLIC-RELATED PROTEINS

An increasing number of evidence demonstrates that IPT is also frequently associated with metabolic adaptation of the recipient cells. Such phenomenon was recently investigated in the context of cancer progression, immune response, drug resistance and tissue rejuvenation (Hekmatshoar et al., 2018; Mittal et al., 2019) (**Figure 2**). In tumors, metabolism of malignant cells is characterized by the Warburg effect—an increased glucose uptake and lactate fermentation, even in the presence of oxygen and fully functional mitochondria. This leads to

TABLE 1 | Transfer of proteins through TNTs in cancer.

Cell Type	Transferred proteins	Protein localization	Methods	References
Prostate cancer cells (PC3, LNCaP)	stress adaptor proteins: CLU, YB-1, Hsp27	CLU- cytosol, HSP27 plasma membrane and cytosol, YB-1 plasma membrane, cytosol, ER, vesicles	Immunofluorescence	Kretschmer et al. (2019)
colorectoral cancer cells (HCT-8, LOVO)	mutant KRAS G12D	Plasma membrane	Fluorescence microscopy, FRAP, time-lapse microscopy, flow cytometry	Desir et al. (2019)
B721.221 (B cells), Jurkat (T cells)	H-Ras	Plasma membrane	Confocal microscopy, FRAP	Rainy et al. (2013)
MCF-7 (breast cancer cells)	P-gp	Plasma membrane	Live cell microscopy, immunofluorescence, flow cytometry	Pasquier et al. (2012)
HeLa	transferrin receptor	Plasma membrane	High resolution 4D confocal microscopy	Burtey et al. (2015)
5637 (bladder cancer cells)	RalA, LST1	Plasma membrane	Live cell imaging	D'Aloia et al. (2021)
HeLa	MHC I	Plasma membrane	Confocal microscopy	Schiller et al. (2013)

FRAP, fluorescence recovery after bleaching.

mitochondria reprograming to supply anabolic pathways to and support rapid proliferation (DeBerardinis and Chandel, 2016). Alterations in mtDNA, including mutations, depletions or reduced copy numbers, are common hallmarks of cancer, including response to chemotherapy, therefore, restoration of the mitochondrial function is important for development of the resistance and cancer progression (Guerra et al., 2017).

Soon after TNTs were discovered, mitochondria were identified among TNT cargos (Spees et al., 2006; Önfelt et al., 2006).

Most of the 1,100 different proteins that build human mitochondria (Rath et al., 2021) is synthesized in the cytosol as protein precursors (Neupert and Herrmann, 2007), which are unstable and can compromise the cellular protein homeostasis (Liu et al., 2019; Nowicka et al., 2021). Thus, the intercellular transfer of the whole organelles, potentially provides the immediate effect without disturbing cytosolic proteostasis of the recipient cells. Even though, there are other pathways that allow mitochondrial uptake by the cell, including extracellular vesicles, free-mitochondria uptake, cell fusion and gap-junctions, TNTs have recently gained most of the attention due to its targeted and inducible characteristics (reviewed in Yan et al., 2021; Zampieri et al., 2021). TNTs formation was shown to be enhanced by starvation, reactive oxygen species or chemotherapeutic drugs (Zhu et al., 2005; Desir et al., 2019). At the molecular level, Lu and colleagues found that number of TNTs positively correlated with metabolism-related Akt-mTOR signaling in malignant urothelial T24 cells (Lu et al., 2017). Another study showed that multiple myeloma cells utilize plasma membrane NADPH oxidase 2 (NOX2) to generate ROS and stimulate mesenchymal stem cells (MSCs) for TNTs formation and mitochondria transfer (Marlein et al., 2017). What is more, the process of mitochondria motility through TNTs is based on cytoskeletal filaments and is regulated by a calcium-sensitive adaptor protein—Miro1 (mitochondrial Rho GTPase 1, synonym: Rhot1), which ties mitochondrion with motor complex (Fransson et al., 2006; Ahmad et al., 2014). Remarkably, phosphorylation of Miro1 protein causes dissipation of the damaged mitochondrion from the motor protein complex and induce autophagy (Narendra et al., 2008; Safiulina et al., 2019). Despite the molecular mechanism of TNT function remains elusive, it seems that TNT-mediated communication between cells is precisely regulated and delivers mitochondria of a good quality.

Importance in Cancer

The first evidence of functional horizontal mitochondrial transfer was presented on human MSCs and skin fibroblasts, which rescued mitochondria function in mtDNA-depleted lung carcinoma cell line—A549 rho^0 cells. After direct co-culture, A549 cells re-established the level of intracellular ATP and oxygen consumption with simultaneous decrease of extracellular lactate and ROS production. This indicated a recovered respiratory function and oxidative metabolism. The examination of mitochondrial and DNA polymorphisms in the rescued clones confirmed the successful horizontal mitochondria transfer between cells, as well as excluded the role of cell fusion in this process (Spees et al., 2006). Even though the direct evidence for TNT activity in this work was not provided, the co-localization of mitochondria with TNT was documented by microscopic imaging in numerous following studies. Till now, mitochondria transfer to cancer cells *via* TNTs was confirmed in different *in vitro* and *ex vivo* experimental set-ups including lung (Ahmad et al., 2014), breast (Pasquier et al., 2013; Tan et al., 2015), ovarian (Pasquier et al., 2013), bladder (Lu et al., 2017), brain (Pinto et al., 2021) and blood cancers (Moschoi et al., 2016; Marlein et al., 2017; Wang et al., 2018; Burt et al., 2019; Kolba et al., 2019). Reviewed in Vignais et al. (2017), Hekmatshoar et al. (2018), and Yan et al. (2021).

Effect on Immunometabolism

In many cases, modification of cell bioenergetics by increased OXPHOS at the expense of glycolysis was observed. Recently, Saha and colleagues discovered also that cancer cells utilize TNTs to hijack mitochondria from immune cells to suppress their cytotoxic activity. By combining the transient (MitoTracker,

dye-based) and stable (genetic-based) fluorescent labelling of mitochondria with microscopic and flow cytometry detection techniques, authors documented that mitochondria are transferred unidirectionally from T cells ($CD3^+$/$CD3^+$ $CD8^+$/ NKT) to breast cancer cells *via* TNTs. The possibility of indirect mitochondria transfer *via* extracellular vesicles was excluded, as it did not occur in the transwell co-culture set-up. Mitochondria hijacking resulted in increased basal and spare mitochondrial respiration as well as enhanced proliferation of cancer cells. On the other hand, organelle outflow from T cells resulted in a reduced aerobic respiration and decreased number of cells (Saha et al., 2022). In the immune cells, the cytotoxic activity and metabolism are correlated (Mathis and Shoelson, 2011; Klein Geltink et al., 2018). Thus, TNT-mediated transfer provides both metabolic and immunosuppressive benefits for cancer growth (Saha et al., 2022). The effect of TNT-mediated communication on immunometabolism was also observed in the context of non-malignant cells. Specifically, macrophages were shown to obtain mitochondria from MSCs *via* TNTs in *in vitro* and *in vivo* models of *Escherichia coli pneumonia*. As expected, mitochondria transfer led to significant and strong increase in basal and ATP-linked mitochondrial respiration in macrophages, as measured by enhanced oxygen consumption rate and reduced lactate synthesis. Simultaneously, co-culture with MSCs enhanced their bacteria phagocytic capacity. Both respiratory and phagocytic effect was partially inhibited by pre-treatment of MSCs with cytochalasin B—an actin polymerization inhibitor, which inhibits TNT-mediated communication (Jackson et al., 2016). Incomplete inhibitory effect can be explained by the fact that transfer of functional mitochondria between those cells can also be governed by extracellular vesicles that lead to the same phenotypic changes (Morrison et al., 2017).

Immunometabolic effect of mitochondria transfer was also observed in active Th17 cells, which generate most of their ATP in glycolysis (Kono et al., 2018). Notably, Luz-Crawford and colleagues have found that Th17 cells among other primary T cell subpopulations, are most efficient in taking up mitochondria during direct co-culture with bone marrow MSCs. Mitochondria acceptors showed elevated aerobic respiration and reduced production of pro-inflammatory IL-17. Moreover, authors discovered that a higher percentage of Th17 effector memory cells that received mitochondria acquire a regulatory T cell phenotype, compared to their counterparts which did not received mitochondria. This indicates that Th17 pro-inflamatory activity is negatively regulated by MSC cells through TNT-mediated IPT. Furthermore, co-culture of Th17 cells with MSCs derived from patients with rheumatoid arthritis, in contrast to healthy MSCs, showed decreased mitochondria transfer, meaning that altered Th17 regulation through mitochondria uptake can be involved in pathogenesis of rheumatoid arthritis (Luz-Crawford et al., 2019). Cdc42 protein is also recognized as a central regulator of Th17/Treg balance and determines the pathogenic phenotype of Th17 cells, characterized by upregulated glycolysis. Intriguingly, T-cells obtained from mice with Cdc42-deficiency manifested increased susceptibility to intestinal damage and pathogenic inflammation (Kalim et al., 2018), therefore supporting the hypothesis postulated by Luz-Crawford et al.

TNT-MEDIATED SELF-INFECTION OF NEURONS IN NEURODEGENERATIVE DISORDERS

It is well established that spreading of amyloidogenic proteins occurs through secretory mechanisms, including exosomes, thus contributing to exacerbation of neurodegenerative diseases (Lee et al., 2011). However, growing evidence indicates that TNT-mediated protein transfer also plays a role in propagating neurodegenerative pathologies (**Figure 2**.). It was demonstrated that a variety of misfolded proteins, including tau (Abounit et al., 2016; Tardivel et al., 2016; Chastagner et al., 2020), α-synuclein (Dilsizoglu Senol et al., 2021), prions (Gousset et al., 2009; Zhu et al., 2015) and mutant huntingtin (Costanzo et al., 2013; Tang, 2018), can be transferred through TNTs in neuronal cells. Importantly, not only neurons' infection, but also their exposure to amyloidogenic proteins supports intercellular transfer by increasing the number of TNT connections between cells (Costanzo et al., 2013; Zhu et al., 2015; Abounit et al., 2016; Tardivel et al., 2016). Neurodegenerative diseases and accumulation of cytotoxic protein assemblies are associated with oxidative stress. Therefore, it has been proposed that the prion-like proteins might contribute to generation of reactive oxygen species, which in turn stimulate TNTs formation as a stress response mechanism (Abounit et al., 2016; Victoria and Zurzolo, 2017). Propagation of aggregates can differ depending on their origin. However, both endogenously formed (Chastagner et al., 2020) and internalized (Abounit et al., 2016) tau aggregates were found within TNTs formed between neurons. Misfolded proteins can be transferred through TNTs, either inside the vesicles or as protein aggregates associated with organelles or proteins. In CAD (Cath.-a-differentiated) cells, the prions responsible for transmissible spongiform encephalopathies and Parkinson's Disease-causing α-synuclein aggregates can be found within TNTs in endolysosomal vesicles and lysosomes, respectively. Both PrPSc and α-synuclein were found to colocalize with endosomal and lysosomal markers, confirming the transport (Zhu et al., 2015; Dilsizoglu Senol et al., 2021). Moreover, α-synuclein fibrils can damage lysosome structure and promote peripheral redistribution of α-synuclein-bearing lysosomes in neuronal cells, leading to enhanced α-synuclein transfer *via* TNTs to neighbouring cells (Dilsizoglu Senol et al., 2021). Studying TNTs is currently mostly based on imaging methods, such as fluorescence microscopy (FM). However, due to low-resolution of FM it is often challenging to obtain desirable data. Employing more advanced imaging tools, such as super-resolution (SR) microscopy or combining different approaches, seems to overcome this issue. For instance, structured illumination microscopy (SIM) demonstrated α-synuclein localization both inside lysosomes and at their membrane, whilst correlative light-electron microscopy enabled identifying α-synuclein positive

TABLE 2 | Transfer of proteins through TNTs in neurodegenerative disorders.

Cell type	Transferred proteins	Methods	Additional informations	References
CAD; HeLa	α-synuclein	Confocal microscopy, Co-localization studies, SR SIM, live spinning-disk microscopy CLEM	α-syn aggregates transfer inside lysosomes; transfer of healthy lysosomes to damaged cells	Dilsizoglu Senol et al. (2021)
SH-SY5Y; human post-mortem brain pericytes	α-synuclein	Confocal microscopy, SEM, electrophysiology		Dieriks et al. (2017)
1321N1; differentiated microglia-like THP1; SH-SY5Y	α-synuclein	Confocal microscopy, STED	α-syn aggregates associated with mitochondrial outer membrane	Valdinocci et al. (2021)
Human ESC-derived astrocytes	α-synuclein	Confocal microscopy, TEM	Transfer of healthy mitochondria to damaged cells	Rostami et al. (2017)
Mouse primary microglia; Human monocyte-derived microglia; Mouse organotypic slice culture (OSCs); Human post-mortem brain sections	α-synuclein	Confocal microscopy, *in vivo* 2-photon microscopy, flow cytometry	α-syn aggregates redistribution and degradation; transfer of healthy mitochondria to α-syn-overloaded microglia	Scheiblich et al. (2021)
Human iPSC-derived astrocytes and microglia	α-synuclein amyloid- ß	Confocal microscopy	Microglia degrade aggregates more efficiently	Rostami et al. (2021)
CAD; HeLa	tau	Epifluorescence microscopy		Abounit et al. (2016)
CAD; rat primary neurons	tau	Confocal microscopy, spinning-disk confocal microscopy, co-localization, TEM	Soluble and fibrillar tau co-localizes with actin	Tardivel et al. (2016)
CAD;SH-SY5Y; mouse primary neurons	tau	Confocal microscopy, flow cytometry, IncuCyte	Endogenously formed tau aggregates transfer	Chastagner et al. (2020)
CAD;Mouse primary cerebellar granule neurons	mHtt	Wide-field fluorescence microscopy, flow cytometry		Costanzo et al. (2013)
Mouse striatal neuronal cells (cell line and primary cells)	mHtt	Flow cytometry, confocal microscopy, SEM, TEM	"Rhes tunnels"; Rhes protein-associated transfer	Sharma and Subramaniam (2019)
CAD; Mouse primary cerebellar granule neurons; Mouse primary bone-marrow-derived dendritic cells; Mouse primary embryonic hippocampal neurons	PrPSc	Confocal microscopy, spinning-disk confocal microscopy		Gousset et al. (2009)
CAD	PrPSc	Co-localization Confocal microscopy, flow cytometry		Zhu et al. (2015)

SR SIM, super resolution structures illumination microscopy; CLEM, correlative light-electron microscopy; SEM, scanning electron microscopy; STED, stimulated emission depletion microscopy; TEM, transmission electron microscopy, IncuCyte—real-time live-cell imaging and analysis system.

lysosomes by FM and studying their corresponding size and morphology by electron microscopy (EM) (Dilsizoglu Senol et al., 2021). Mitochondria-associated TNT-mediated α-synuclein transfer was also reported. SR microscopy data obtained *via* stimulated emission depletion microscopy (STED) presented α-synuclein bound to the mitochondrial outer membrane, both in cytoplasm and within TNTs of connected cells (Valdinocci et al., 2021). Mutant huntingtin and other poly-Q expanded proteins can be selectively transported by Rhes protein primarily in lysosomal vesicles within TNT-like Rhes-induced protrusions in murine striatal neuronal cells (Sharma and Subramaniam, 2019). Finally, using a combination of live imaging, light- and cryo-electron microscopy approaches allowed studying TNTs' complex structure at nanometre resolution in murine CAD and human neuroblastoma SH-SY5Y model cell lines. It was reported that single TNTs observed by FM consist of a bundle of individual TNTs (iTNTs) held together and stabilized by N-Cadherin. Each iTNT is filled with a parallel actin bundle, which enables cargo transport, presumably involving myosin motor proteins. Additionally, correlative focused-ion beam SEM (FIB-SEM) demonstrated that TNTs can be open on both ends (Sartori-Rupp et al., 2019). Electrophysiology assay can be used to assess electrical properties and type of TNTs, whether TNTs allow transport of cargo or not (Dieriks et al., 2017). However, to determine the efficacy of TNT-mediated cargo transfer, the flow cytometry methodology is often used in studying neurodegenerative disorders (Costanzo et al., 2013; Sharma

and Subramaniam, 2019; Chastagner et al., 2020). To confirm that protein transfer occurs through contact-dependent mechanism, acceptor cells can be incubated in the supernatant of donor cells or both cell types can be physically separated, i.e., by filters, and then analyzed by flow cytometry (Costanzo et al., 2013; Sharma and Subramaniam, 2019). Noteworthy, non-neuronal cells might also contribute to spreading of misfolded proteins in the brain. Human pericytes (Dieriks et al., 2017) and astrocytes (Rostami et al., 2017) frequently form TNTs and transfer α-synuclein aggregates. TNTs can be formed in homotypic and heterotypic co-cultures (Chastagner et al., 2020; Dilsizoglu Senol et al., 2021). Chastagner et al. (2020) identified tau fibrils TNT-mediated transfer from neurons to astrocytes and Gousset et al. (2009) demonstrated that dendritic cells are capable of transporting prions to CNS (central nervous system) *via* TNTs, thus emphasizing the variety of cells contributing to misfolded proteins spreading via direct cell-cell connections. Importantly, a recent study presented evidence for a TNT-mediated green fluorescent protein transport from astrocytes to neurons *in vivo*, indicating a possible similar mechanism for prion-like proteins propagation in the brain (Chen and Cao, 2021). Interestingly, emerging evidence demonstrates aggregates degradation supported by TNT-mediated protein distribution. Microglia overloaded with α-synuclein can transfer cytotoxic proteins to healthy microglia, thus supporting aggregates degradation, inflammation attenuation and overall decrease of cytotoxicity, improving microglial survival *in vitro* and *in vivo* (Scheiblich et al., 2021). Similarly, TNT-mediated crosstalk between astrocytes and microglia increases α-synuclein and amyloid-ß aggregates degradation. Microglia, however, are more efficient in aggregates clearance than astrocytes (Rostami et al., 2021). Altogether it seems that in such context, the microenvironment can utilize the TNT network and microglia connections to protect neurons from aggregate-mediated cytotoxicity. Some studies reported that acceptor cells which received a pathological protein send out healthy lysosomes (Dilsizoglu Senol et al., 2021) or mitochondria (Rostami et al., 2017; Scheiblich et al., 2021) in return, possibly as a TNT-mediated rescue mechanism for infected cells. Although there are emerging reports on the presence of TNTs in CNS (Alarcon-Martinez et al., 2020) and TNT-mediated intercellular protein transfer in the brain (Chen and Cao, 2021), it remains challenging to study TNTs in neurodegenerative diseases *in vivo*. All of the data described in this chapter is summarized in **Table 2**.

CONCLUDING REMARKS

Accumulating evidence has indicated the important role of tunneling nanotubes in the intercellular protein transfer between distant cells, as well as other molecules or organelles involved in many pathological conditions (mainly cancer and neurodegenerative disorders). In this review we focused on the one hand, on different examples of intercellular and TNT-mediated intercellular protein transfer between distant cells of different origin, and on the other hand, on methods used for studying IPT, mainly with regards to transfer *via* TNTs. Despite the advanced approaches used to investigate the intercellular protein transfer, like trans-SILAC or codeIT, and great development of other techniques used for hit validation, particularly microscopy, further efforts on this topic are required. Considering particular fragility of TNTs structure, it seems that in many cases the greatest remaining challenge is to transfer the results obtained using *in vitro* cellular models into *in vivo* conditions.

AUTHOR CONTRIBUTIONS

LT-K contributed to the writing of the text, prepared figures, **Table 1** and bibliography, MDK and PC contributed to the writing of the text, AZ contributed to the writing of the text and prepared **Table 2**. KP discussed and revised the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

This work was supported by National Science Centre grants: OPUS 2018/29/B/NZ3/01778 (KP) and PRELUDIUM BIS 2019/35/O/NZ3/01936 (KP).

ACKNOWLEDGMENTS

Figures were created with BioRender.com online software.

REFERENCES

Abounit, S., Wu, J. W., Duff, K., Victoria, G. S., and Zurzolo, C. (2016). Tunneling Nanotubes: A Possible Highway in the Spreading of Tau and Other Prion-like Proteins in Neurodegenerative Diseases. *Prion* 10, 344–351. doi:10.1080/19336896.2016.1223003

Ahmad, T., Mukherjee, S., Pattnaik, B., Kumar, M., Singh, S., Kumar, M., et al. (2014). Miro1 Regulates Intercellular Mitochondrial Transport & Enhances Mesenchymal Stem Cell Rescue Efficacy. *EMBO J.* 33, 994–1010. doi:10.1002/embj.201386030

Ahmed, K. A., and Xiang, J. (2011). Mechanisms of Cellular Communication through Intercellular Protein Transfer. *J. Cell. Mol. Med.* 15, 1458–1473. doi:10.1111/j.1582-4934.2010.01008.x

Alarcon-Martinez, L., Villafranca-Baughman, D., Quintero, H., Kacerovsky, J. B., Dotigny, F., Murai, K. K., et al. (2020). Interpericyte Tunnelling Nanotubes Regulate Neurovascular Coupling. *Nature* 585, 91–95. doi:10.1038/s41586-020-2589-x

Biran, A., Perelmutter, M., Gal, H., Burton, D. G. A., Ovadya, Y., Vadai, E., et al. (2015). Senescent Cells Communicate via Intercellular Protein Transfer. *Genes Dev.* 29, 791–802. doi:10.1101/gad.259341.115

Bukoreshtliev, N. V., Wang, X., Hodneland, E., Gurke, S., Barroso, J. F. V., and Gerdes, H.-H. (2009). Selective Block of Tunneling Nanotube (TNT) Formation Inhibits Intercellular Organelle Transfer between PC12 Cells. *FEBS Lett.* 583, 1481–1488. doi:10.1016/j.febslet.2009.03.065

Burt, R., Dey, A., Aref, S., Aguiar, M., Akarca, A., Bailey, K., et al. (2019). Activated Stromal Cells Transfer Mitochondria to Rescue Acute Lymphoblastic Leukemia Cells from Oxidative Stress. *Blood* 134, 1415–1429. doi:10.1182/blood.2019001398

Burtey, A., Wagner, M., Hodneland, E., Skaftnesmo, K. O., Schoelermann, J., Mondragon, I. R., et al. (2015). Intercellular Transfer of Transferrin Receptor by a Contact-, Rab8-dependent Mechanism Involving Tunneling Nanotubes. *FASEB J.* 29, 4695–4712. doi:10.1096/fj.14-268615

Chastagner, P., Loria, F., Vargas, J. Y., Tois, J., I Diamond, M., Okafo, G., et al. (2020). Fate and Propagation of Endogenously Formed Tau Aggregates in Neuronal Cells. *EMBO Mol. Med.* 12, e12025. doi:10.15252/emmm.202012025

Chen, J., and Cao, J. (2021). Astrocyte-to-neuron Transportation of Enhanced Green Fluorescent Protein in Cerebral Cortex Requires F-Actin Dependent Tunneling Nanotubes. *Sci. Rep.* 11, 16798. doi:10.1038/s41598-021-96332-5

Costanzo, M., Abounit, S., Marzo, L., Danckaert, A., Chamoun, Z., Roux, P., et al. (2013). Transfer of Polyglutamine Aggregates in Neuronal Cells Occurs in Tunneling Nanotubes. *J. Cell Sci.* 126, 3678–3685. doi:10.1242/jcs.126086

Dagar, S., Pathak, D., Oza, H. V., and Mylavarapu, S. V. S. (2021). Tunneling Nanotubes and Related Structures: Molecular Mechanisms of Formation and Function. *Biochem. J.* 478, 3977–3998. doi:10.1042/BCJ20210077

D'Aloia, A., Arrigoni, E., Costa, B., Berruti, G., Martegani, E., Sacco, E., et al. (2021). RalGPS2 Interacts with Akt and PDK1 Promoting Tunneling Nanotubes Formation in Bladder Cancer and Kidney Cells Microenvironment. *Cancers* 13, 6330. doi:10.3390/cancers13246330

Davis, D. M. (2007). Intercellular Transfer of Cell-Surface Proteins Is Common and Can Affect Many Stages of an Immune Response. *Nat. Rev. Immunol.* 7, 238–243. doi:10.1038/nri2020

DeBerardinis, R. J., and Chandel, N. S. (2016). Fundamentals of Cancer Metabolism. *Sci. Adv.* 2, e1600200. doi:10.1126/sciadv.1600200

Desir, Wong, Wong, Chen, Turbyville, Clark, Chen, fnm, Shetty, fnm, Clark, fnm, et al. (2019). Intercellular Transfer of Oncogenic KRAS via Tunneling Nanotubes Introduces Intracellular Mutational Heterogeneity in Colon Cancer Cells. *Cancers* 11, 892. doi:10.3390/cancers11070892

Dieriks, B. V., Park, T. I.-H., Fourie, C., Faull, R. L. M., Dragunow, M., and Curtis, M. A. (2017). α-Synuclein Transfer through Tunneling Nanotubes Occurs in SH-Sy5y Cells and Primary Brain Pericytes from Parkinson's Disease Patients. *Sci. Rep.* 7, 42984. doi:10.1038/srep42984

Dilsizoglu Senol, A., Samarani, M., Syan, S., Guardia, C. M., Nonaka, T., Liv, N., et al. (2021). α-Synuclein Fibrils Subvert Lysosome Structure and Function for the Propagation of Protein Misfolding between Cells through Tunneling Nanotubes. *PLoS Biol.* 19, e3001287. doi:10.1371/journal.pbio.3001287

Fransson, Å., Ruusala, A., and Aspenström, P. (2006). The Atypical Rho GTPases Miro-1 and Miro-2 Have Essential Roles in Mitochondrial Trafficking. *Biochem. Biophysical Res. Commun.* 344, 500–510. doi:10.1016/j.bbrc.2006.03.163

Frei, D. M., Hodneland, E., Rios-Mondragon, I., Burtey, A., Neumann, B., Bulkescher, J., et al. (2015). Novel Microscopy-Based Screening Method Reveals Regulators of Contact-dependent Intercellular Transfer. *Sci. Rep.* 5, 12879. doi:10.1038/srep12879

Gousset, K., Schiff, E., Langevin, C., Marijanovic, Z., Caputo, A., Browman, D. T., et al. (2009). Prions Hijack Tunnelling Nanotubes for Intercellular Spread. *Nat. Cell Biol.* 11, 328–336. doi:10.1038/ncb1841

Guerra, F., Arbini, A. A., and Moro, L. (2017). Mitochondria and Cancer Chemoresistance. *Biochimica Biophysica Acta (BBA) - Bioenergetics* 1858, 686–699. doi:10.1016/j.bbabio.2017.01.012

Hase, K., Kimura, S., Takatsu, H., Ohmae, M., Kawano, S., Kitamura, H., et al. (2009). M-Sec Promotes Membrane Nanotube Formation by Interacting with Ral and the Exocyst Complex. *Nat. Cell Biol.* 11, 1427–1432. doi:10.1038/ncb1990

Hekmatshoar, Y., Nakhle, J., Galloni, M., and Vignais, M.-L. (2018). The Role of Metabolism and Tunneling Nanotube-Mediated Intercellular Mitochondria Exchange in Cancer Drug Resistance. *Biochem. J.* 475, 2305–2328. doi:10.1042/BCJ20170712

Jackson, M. V., Morrison, T. J., Doherty, D. F., McAuley, D. F., Matthay, M. A., Kissenpfennig, A., et al. (2016). Mitochondrial Transfer via Tunneling Nanotubes Is an Important Mechanism by Which Mesenchymal Stem Cells Enhance Macrophage Phagocytosis in the *In Vitro* and *In Vivo* Models of ARDS. *Stem Cells* 34, 2210–2223. doi:10.1002/stem.2372

Kalim, K. W., Yang, J.-Q., Li, Y., Meng, Y., Zheng, Y., and Guo, F. (2018). Reciprocal Regulation of Glycolysis-Driven Th17 Pathogenicity and Regulatory T Cell Stability by Cdc42. *J. I.* 200, 2313–2326. doi:10.4049/jimmunol.1601765

Klein Geltink, R. I., Kyle, R. L., and Pearce, E. L. (2018). Unraveling the Complex Interplay Between T Cell Metabolism and Function. *Annu. Rev. Immunol.* 36, 461–488. doi:10.1146/annurev-immunol-042617-053019

Kolba, M. D., Dudka, W., Zaręba-Kozioł, M., Kominek, A., Ronchi, P., Turos, L., et al. (2019). Tunneling Nanotube-Mediated Intercellular Vesicle and Protein Transfer in the Stroma-Provided Imatinib Resistance in Chronic Myeloid Leukemia Cells. *Cell Death Dis.* 10, 817. doi:10.1038/s41419-019-2045-8

Kono, M., Yoshida, N., Maeda, K., Skinner, N. E., Pan, W., Kyttaris, V. C., et al. (2018). Pyruvate Dehydrogenase Phosphatase Catalytic Subunit 2 Limits Th17 Differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 115, 9288–9293. doi:10.1073/pnas.1805717115

Korenkova, O., Pepe, A., and Zurzolo, C. (2020). Fine Intercellular Connections in Development: TNTs, Cytonemes, or Intercellular Bridges? *CST* 4, 30–43. doi:10.15698/cst2020.02.212

Kretschmer, A., Zhang, F., Somasekharan, S. P., Tse, C., Leachman, L., Gleave, A., et al. (2019). Stress-induced Tunneling Nanotubes Support Treatment Adaptation in Prostate Cancer. *Sci. Rep.* 9, 7826. doi:10.1038/s41598-019-44346-5

Lee, S.-J., Lim, H.-S., Masliah, E., and Lee, H.-J. (2011). Protein Aggregate Spreading in Neurodegenerative Diseases: Problems and Perspectives. *Neurosci. Res.* 70, 339–348. doi:10.1016/j.neures.2011.05.008

Li, M., Aliotta, J. M., Asara, J. M., Wu, Q., Dooner, M. S., Tucker, L. D., et al. (2010). Intercellular Transfer of Proteins as Identified by Stable Isotope Labeling of Amino Acids in Cell Culture. *J. Biol. Chem.* 285, 6285–6297. doi:10.1074/jbc.M109.057943

Liu, Y., Wang, X., Coyne, L. P., Yang, Y., Qi, Y., Middleton, F. A., et al. (2019). Mitochondrial Carrier Protein Overloading and Misfolding Induce Aggresomes and Proteostatic Adaptations in the Cytosol. *Mol. Biol. Cell.* 30, 1272–1284. doi:10.1091/mbc.E19-01-0046

Lu, J., Zheng, X., Li, F., Yu, Y., Chen, Z., Liu, Z., et al. (2017). Tunneling Nanotubes Promote Intercellular Mitochondria Transfer Followed by Increased Invasiveness in Bladder Cancer Cells. *Oncotarget* 8, 15539–15552. doi:10.18632/oncotarget.14695

Luz-Crawford, P., Hernandez, J., Djouad, F., Luque-Campos, N., Caicedo, A., Carrère-Kremer, S., et al. (2019). Mesenchymal Stem Cell Repression of Th17 Cells Is Triggered by Mitochondrial Transfer. *Stem Cell Res. Ther.* 10, 232. doi:10.1186/s13287-019-1307-9

Marlein, C. R., Zaitseva, L., Piddock, R. E., Robinson, S. D., Edwards, D. R., Shafat, M. S., et al. (2017). NADPH Oxidase-2 Derived Superoxide Drives Mitochondrial Transfer from Bone Marrow Stromal Cells to Leukemic Blasts. *Blood* 130, 1649–1660. doi:10.1182/blood-2017-03-772939

Mathis, D., and Shoelson, S. E. (2011). Immunometabolism: an Emerging Frontier. *Nat. Rev. Immunol.* 11, 81–83. doi:10.1038/nri2922

Misale, S., Yaeger, R., Hobor, S., Scala, E., Janakiraman, M., Liska, D., et al. (2012). Emergence of KRAS Mutations and Acquired Resistance to Anti-EGFR Therapy in Colorectal Cancer. *Nature* 486, 532–536. doi:10.1038/nature11156

Mittal, R., Karhu, E., Wang, J. S., Delgado, S., Zukerman, R., Mittal, J., et al. (2019). Cell Communication by Tunneling Nanotubes: Implications in Disease and Therapeutic Applications. *J. Cell Physiol.* 234, 1130–1146. doi:10.1002/jcp.27072

Morrison, T. J., Jackson, M. V., Cunningham, E. K., Kissenpfennig, A., McAuley, D. F., O'Kane, C. M., et al. (2017). Mesenchymal Stromal Cells Modulate Macrophages in Clinically Relevant Lung Injury Models by Extracellular Vesicle Mitochondrial Transfer. *Am. J. Respir. Crit. Care Med.* 196, 1275–1286. doi:10.1164/rccm.201701-0170OC

Moschoi, R., Imbert, V., Nebout, M., Chiche, J., Mary, D., Prebet, T., et al. (2016). Protective Mitochondrial Transfer from Bone Marrow Stromal Cells to Acute Myeloid Leukemic Cells during Chemotherapy. *Blood* 128, 253–264. doi:10.1182/blood-2015-07-655860

Narendra, D., Tanaka, A., Suen, D.-F., and Youle, R. J. (2008). Parkin Is Recruited Selectively to Impaired Mitochondria and Promotes Their Autophagy. *J. Cell Biol.* 183, 795–803. doi:10.1083/jcb.200809125

Neupert, W., and Herrmann, J. M. (2007). Translocation of Proteins into Mitochondria. *Annu. Rev. Biochem.* 76, 723–749. doi:10.1146/annurev.biochem.76.052705.163409

Niu, X., Gupta, K., Yang, J. T., Shamblott, M. J., and Levchenko, A. (2009). Physical Transfer of Membrane and Cytoplasmic Components as a General Mechanism of Cell-Cell Communication. *J. Cell Sci.* 122, 600–610. doi:10.1242/jcs.031427

Nowicka, U., Chroscicki, P., Stroobants, K., Sladowska, M., Turek, M., Uszczynska-Ratajczak, B., et al. (2021). Cytosolic Aggregation of Mitochondrial Proteins Disrupts Cellular Homeostasis by Stimulating the Aggregation of Other Proteins. *eLife* 10, e65484. doi:10.7554/eLife.65484

Önfelt, B., Nedvetzki, S., Benninger, R. K. P., Purbhoo, M. A., Sowinski, S., Hume, A. N., et al. (2006). Structurally Distinct Membrane Nanotubes between Human Macrophages Support Long-Distance Vesicular Traffic or Surfing of Bacteria. *J. Immunol.* 177, 8476–8483. doi:10.4049/jimmunol.177.12.8476

Önfelt, B., Nedvetzki, S., Yanagi, K., and Davis, D. M. (2004). Cutting Edge: Membrane Nanotubes Connect Immune Cells. *J. Immunol.* 173, 1511–1513. doi:10.4049/jimmunol.173.3.1511

Pasquier, J., Galas, L., Boulangé-Lecomte, C., Rioult, D., Bultelle, F., Magal, P., et al. (2012). Different Modalities of Intercellular Membrane Exchanges Mediate Cell-To-Cell P-Glycoprotein Transfers in MCF-7 Breast Cancer Cells. *J. Biol. Chem.* 287, 7374–7387. doi:10.1074/jbc.M111.312157

Pasquier, J., Guerrouahen, B. S., Al Thawadi, H., Ghiabi, P., Maleki, M., Abu-Kaoud, N., et al. (2013). Preferential Transfer of Mitochondria from Endothelial to Cancer Cells through Tunneling Nanotubes Modulates Chemoresistance. *J. Transl. Med.* 11, 94. doi:10.1186/1479-5876-11-94

Pinto, G., Saenz-de-Santa-Maria, I., Chastagner, P., Perthame, E., Delmas, C., Toulas, C., et al. (2021). Patient-derived Glioblastoma Stem Cells Transfer Mitochondria through Tunneling Nanotubes in Tumor Organoids. *Biochem. J.* 478, 21–39. doi:10.1042/BCJ20200710

Rainy, N., Chetrit, D., Rouger, V., Vernitsky, H., Rechavi, O., Marguet, D., et al. (2013). H-Ras Transfers from B to T Cells via Tunneling Nanotubes. *Cell Death Dis.* 4, e726. doi:10.1038/cddis.2013.245

Rath, S., Sharma, R., Gupta, R., Ast, T., Chan, C., Durham, T. J., et al. (2021). MitoCarta3.0: an Updated Mitochondrial Proteome Now with Sub-organelle Localization and Pathway Annotations. *Nucleic Acids Res.* 49, D1541–D1547. doi:10.1093/nar/gkaa1011

Rechavi, O., Goldstein, I., and Kloog, Y. (2009). Intercellular Exchange of Proteins: The Immune Cell Habit of Sharing. *FEBS Lett.* 583, 1792–1799. doi:10.1016/j.febslet.2009.03.014

Reichert, D., Scheinpflug, J., Karbanová, J., Freund, D., Bornhäuser, M., and Corbeil, D. (2016). Tunneling Nanotubes Mediate the Transfer of Stem Cell Marker CD133 between Hematopoietic Progenitor Cells. *Exp. Hematol.* 44, 1092–1112. e2. doi:10.1016/j.exphem.2016.07.006

Rostami, J., Holmqvist, S., Lindström, V., Sigvardson, J., Westermark, G. T., Ingelsson, M., et al. (2017). Human Astrocytes Transfer Aggregated Alpha-Synuclein via Tunneling Nanotubes. *J. Neurosci.* 37, 11835–11853. doi:10.1523/JNEUROSCI.0983-17.2017

Rostami, J., Mothes, T., Kolahdouzan, M., Eriksson, O., Moslem, M., Bergström, J., et al. (2021). Crosstalk between Astrocytes and Microglia Results in Increased Degradation of α-synuclein and Amyloid-β Aggregates. *J. Neuroinflammation* 18, 124. doi:10.1186/s12974-021-02158-3

Rustom, A., Saffrich, R., Markovic, I., Walther, P., and Gerdes, H.-H. (2004). Nanotubular Highways for Intercellular Organelle Transport. *Science* 303, 1007–1010. doi:10.1126/science.1093133

Safiulina, D., Kuum, M., Choubey, V., Gogichaishvili, N., Liiv, J., Hickey, M. A., et al. (2019). Miro Proteins Prime Mitochondria for Parkin Translocation and Mitophagy. *EMBO J.* 38, e99384. doi:10.15252/embj.201899384

Saha, T., Dash, C., Jayabalan, R., Khiste, S., Kulkarni, A., Kurmi, K., et al. (2022). Intercellular Nanotubes Mediate Mitochondrial Trafficking between Cancer and Immune Cells. *Nat. Nanotechnol.* 17, 98–106. doi:10.1038/s41565-021-01000-4

Sartori-Rupp, A., Cordero Cervantes, D., Pepe, A., Gousset, K., Delage, E., Corroyer-Dulmont, S., et al. (2019). Correlative Cryo-Electron Microscopy Reveals the Structure of TNTs in Neuronal Cells. *Nat. Commun.* 10, 342. doi:10.1038/s41467-018-08178-7

Scheiblich, H., Dansokho, C., Mercan, D., Schmidt, S. V., Bousset, L., Wischhof, L., et al. (2021). Microglia Jointly Degrade Fibrillar Alpha-Synuclein Cargo by Distribution through Tunneling Nanotubes. *Cell* 184, 5089–5106. e21. doi:10.1016/j.cell.2021.09.007

Schiller, C., Diakopoulos, K. N., Rohwedder, I., Kremmer, E., von Toerne, C., Ueffing, M., et al. (2012). LST1 Promotes the Assembly of a Molecular Machinery Responsible for Tunneling Nanotube Formation. *J. Cell Sci.* 126, 767–777. doi:10.1242/jcs.114033

Schiller, C., Huber, J. E., Diakopoulos, K. N., and Weiss, E. H. (2013). Tunneling Nanotubes Enable Intercellular Transfer of MHC Class I Molecules. *Hum. Immunol.* 74, 412–416. doi:10.1016/j.humimm.2012.11.026

Sharma, M., and Subramaniam, S. (2019). Rhes Travels from Cell to Cell and Transports Huntington Disease Protein via TNT-like Protrusion. *J. Cell Biol.* 218, 1972–1993. doi:10.1083/jcb.201807068

Sowinski, S., Alakoskela, J.-M., Jolly, C., and Davis, D. M. (2011). Optimized Methods for Imaging Membrane Nanotubes between T Cells and Trafficking of HIV-1. *Methods* 53, 27–33. doi:10.1016/j.ymeth.2010.04.002

Sowinski, S., Jolly, C., Berninghausen, O., Purbhoo, M. A., Chauveau, A., Köhler, K., et al. (2008). Membrane Nanotubes Physically Connect T Cells over Long Distances Presenting a Novel Route for HIV-1 Transmission. *Nat. Cell Biol.* 10, 211–219. doi:10.1038/ncb1682

Spees, J. L., Olson, S. D., Whitney, M. J., and Prockop, D. J. (2006). Mitochondrial Transfer between Cells Can Rescue Aerobic Respiration. *Proc. Natl. Acad. Sci. U.S.A.* 103, 1283–1288. doi:10.1073/pnas.0510511103

Tan, A. S., Baty, J. W., Dong, L.-F., Bezawork-Geleta, A., Endaya, B., Goodwin, J., et al. (2015). Mitochondrial Genome Acquisition Restores Respiratory Function and Tumorigenic Potential of Cancer Cells without Mitochondrial DNA. *Cell Metab.* 21, 81–94. doi:10.1016/j.cmet.2014.12.003

Tang, B. L. (2018). Unconventional Secretion and Intercellular Transfer of Mutant Huntingtin. *Cells* 7, 59. doi:10.3390/cells7060059

Tardivel, M., Bégard, S., Bousset, L., Dujardin, S., Coens, A., Melki, R., et al. (2016). Tunneling Nanotube (TNT)-mediated Neuron-To Neuron Transfer of Pathological Tau Protein Assemblies. *acta neuropathol. Commun.* 4, 117. doi:10.1186/s40478-016-0386-4

Valdinocci, D., Kovarova, J., Neuzil, J., and Pountney, D. L. (2021). Alpha-Synuclein Aggregates Associated with Mitochondria in Tunnelling Nanotubes. *Neurotox. Res.* 39, 429–443. doi:10.1007/s12640-020-00285-y

Victoria, G. S., and Zurzolo, C. (2017). The Spread of Prion-like Proteins by Lysosomes and Tunneling Nanotubes: Implications for Neurodegenerative Diseases. *J. Cell Biol.* 216, 2633–2644. doi:10.1083/jcb.201701047

Vignais, M.-L., Caicedo, A., Brondello, J.-M., and Jorgensen, C. (20172017). Cell Connections by Tunneling Nanotubes: Effects of Mitochondrial Trafficking on Target Cell Metabolism, Homeostasis, and Response to Therapy. *Stem Cells Int.* 2017, 1–14. doi:10.1155/2017/6917941

Wang, J., Liu, X., Qiu, Y., Shi, Y., Cai, J., Wang, B., et al. (2018). Cell Adhesion-Mediated Mitochondria Transfer Contributes to Mesenchymal Stem Cell-Induced Chemoresistance on T Cell Acute Lymphoblastic Leukemia Cells. *J. Hematol. Oncol.* 11, 11. doi:10.1186/s13045-018-0554-z

Wang, Z.-G., Liu, S.-L., Tian, Z.-Q., Zhang, Z.-L., Tang, H.-W., and Pang, D.-W. (2012). Myosin-Driven Intercellular Transportation of Wheat Germ Agglutinin Mediated by Membrane Nanotubes between Human Lung Cancer Cells. *ACS Nano* 6, 10033–10041. doi:10.1021/nn303729r

Yan, W., Diao, S., and Fan, Z. (2021). The Role and Mechanism of Mitochondrial Functions and Energy Metabolism in the Function Regulation of the Mesenchymal Stem Cells. *Stem Cell Res. Ther.* 12, 140. doi:10.1186/s13287-021-02194-z

Zampieri, L. X., Silva-Almeida, C., Rondeau, J. D., and Sonveaux, P. (2021). Mitochondrial Transfer in Cancer: A Comprehensive Review. *Int. J. Mol. Sci.* 22, 3245. doi:10.3390/ijms22063245

Zhu, D., Tan, K. S., Zhang, X., Sun, A. Y., Sun, G. Y., and Lee, J. C.-M. (2005). Hydrogen Peroxide Alters Membrane and Cytoskeleton Properties and Increases Intercellular Connections in Astrocytes. *J. Cell Sci.* 118, 3695–3703. doi:10.1242/jcs.02507

Zhu, S., Victoria, G. S., Marzo, L., Ghosh, R., and Zurzolo, C. (2015). Prion Aggregates Transfer through Tunneling Nanotubes in Endocytic Vesicles. *Prion* 9, 125–135. doi:10.1080/19336896.2015.1025189

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Cell and Developmental Biology

TYPE Original Research
PUBLISHED 07 September 2022
DOI 10.3389/fcell.2022.878620

OPEN ACCESS

EDITED BY
Sarah Elizabeth Stewart,
La Trobe University, Australia

REVIEWED BY
Suresh Mathivanan,
La Trobe University, Australia
Yoshinori Takahashi,
Penn State Milton S. Hershey Medical Center, United States

*CORRESPONDENCE
Ralf Jacob,
jacob@staff.uni-marburg.de

SPECIALTY SECTION
This article was submitted to Membrane Traffic,
a section of the journal
Frontiers in Cell and Developmental Biology

RECEIVED 18 February 2022
ACCEPTED 20 July 2022
PUBLISHED 07 September 2022

CITATION
Bänfer S, Kutscher S, Fleck F, Dienst M, Preußer C, Pogge von Strandmann E and Jacob R (2022), Late domain dependent E-cadherin recruitment into extracellular vesicles.
Front. Cell Dev. Biol. 10:878620.
doi: 10.3389/fcell.2022.878620

Late domain dependent E-cadherin recruitment into extracellular vesicles

Sebastian Bänfer[1], Sophie Kutscher[1], Fenja Fleck[1], Martina Dienst[1], Christian Preußer[2], Elke Pogge von Strandmann[2] and Ralf Jacob[1]*

[1]Department of Cell Biology and Cell Pathology, Philipps University Marburg, Marburg, Germany, [2]Center for Tumor Biology and Immunology (ZTI), Institute for Tumor Immunology, Philipps University Marburg, Marburg, Germany

E-cadherin, a transmembrane protein involved in epithelial cell-cell adhesion and signaling, is found in exosomal fractions isolated from human body fluids. A cellular mechanism for recruitment of E-cadherin into extracellular vesicles (EVs) has not yet been defined. Here, we show that E-cadherin is incorporated into the membrane of EVs with the extracellular domain exposed at the vesicle surface. This recruitment depends on the endosomal sorting complex required for transport I (ESCRT-I) component Tsg101 and a highly conserved tetrapeptide P(S/T)AP late domain motif in the cytoplasmic tail of E-cadherin that mediates interaction with Tsg101. Mutation of this motif results in a loss of interaction and a dramatic decrease in exosomal E-cadherin secretion. We conclude, that the process of late domain mediated exosomal recruitment is exerted by this endogenous non-ESCRT transmembrane protein.

KEYWORDS

E-cadherin, exosomes, late domain, ESCRT (endosomal sorting complex required for transport), multivesicular bodies (MVB), extracellular vesicles

Introduction

EVs are extracellular vesicles with diameters ranging from 30–150 nm (van Niel et al., 2018). They are generated within the endosomal system as intraluminal vesicles (ILVs) and secreted during the fusion of multivesicular endosomes (MVEs) with the plasma membrane into the outer milieu. Almost every cell type can secrete EVs under physiological or pathological conditions. Their cargo composition is manifold and cell-type specific. Accordingly, cellular machinery to recruit cargo into ILVs is diverse. A prominent example is the endosomal sorting complex required for transport (ESCRT), which acts stepwise in the formation of MVEs and ILVs. Alternative ILV-sorting mechanisms employ the generation of ceramide by neutral type II sphingomyelinase, which hydrolyses sphingomyelin to ceramide (Trajkovic et al., 2008). The members CD9, CD63, CD81 and CD82 of the tetraspanin family have also been shown to be involved in ESCRT-independent cargo-sorting to EVs by the formation of dynamic membrane microdomains (Theos

et al., 2006; van Niel et al., 2011; Charrin et al., 2014; Gauthier et al., 2017). Moreover, clustering of the cytosolic adaptor syntenin and the auxiliary component ALIX with the transmembrane proteoglycan syndecan supports their EV recruitment (Baietti et al., 2012). The observation that syntenin also controls Arf6-mediated syndecan-recycling through endosomal compartments emphasizes interconnectivity of vesicular pathways for endocytic recycling and exosomal recruitment. This is further evidenced by the aggregation-dependent rerouting of the transferrin receptor from membrane-recycling to sorting into EVs (Vidal et al., 1997).

E-cadherin is a membrane-anchored glycoprotein that couples calcium-dependent cell-cell adhesion to the cytoskeleton and intracellular signaling pathways in epithelial cells. Upon destabilization of intercellular adhesion by depletion of extracellular Ca^{2+} ions E-cadherin is endocytosed into endosomal vesicles (Kartenbeck et al., 1991). Some of these actively internalized E-cadherin polypeptides are then recycled back to the basolateral plasma membrane (Le et al., 1999). Lock and Stow showed that newly synthesized as well as endocytosed E-cadherin traverses Rab11-positive recycling endosomes before entering the plasma membrane (Lock and Stow, 2005). Their observations indicate a constant uptake of small quantities of E-cadherin in epithelial monolayers with a markedly increase in E-cadherin-endocytosis following destabilization of cell-cell contacts. The endocytic uptake of E-cadherin itself depends on the formation of clathrin coats and is regulated by AP2 and clathrin recruitment as well as the concerted action of the formin Diaphanous and Myosin-II (Levayer et al., 2011). Although the main cellular functions of E-cadherin are exerted at the plasma membrane of epithelial cells, there is accumulating evidence for extracellular E-cadherin in human body fluids. E-cadherin can be shed from the plasma membrane by proteolytic cleavage as soluble E-cadherin (Grabowska and Day, 2012) or recruited to the exosomal membrane (Zhang et al., 2020). Tang et al. (2018) have recently shown that EVs exposing E-cadherin can induce angiogenesis *in vitro* and *in vivo* by a crosstalk between the nuclear factor-κB (NFκB) and β-catenin signaling cascades. The newly formed vasculature then leads to ovarian cancer progression and metastasis. However, cellular components involved in E-cadherin recruitment into EVs have remained elusive.

We now report data showing that in epithelial MDCK cells a small proportion of E-cadherin is recruited into ILVs, which are secreted at the plasma membrane as EVs. Recruitment involves interaction of a highly conserved P(S/T)AP late domain motif in the cytoplasmic tail of E-cadherin with the ESCRT I component Tsg101. Mutagenesis of this motif or Tsg101-knockdown reduce the recruitment efficiency of E-cadherin into EVs. In addition to the previously published observation of soluble cargo recruitment (Bänfer et al., 2018), this study suggests that P(S/T)AP late domains are also involved in the recruitment of endogenous membrane-anchored polypeptides into EVs.

Materials and methods

DNA constructs

Canine E-cadherin inserted into the peGFP-N1 vector was kindly provided by W. James Nelson (Adams et al., 1998). Cadherin domains 2 to 5 (amino acids 272–671) were deleted by two-step mutagenesis PCR using the primer pair 5′- CAC CCAGGCAGTCTTCCAAGGATATCTCAAGCTCACAGA TAACC -3′ and 5′- GGTTATCTGTGAGCTTGAGATATCCTT GGAAGACTGCCTGGGTG -3′ to generate plasmid pE-cadherinΔE2-5GFP$_{PTAP}$. Mutation of the E-cadherin PTAP motif into ASAA was induced by overlap extension PCR with inside primers 5′- CG GAC ACT GAC gCT Agc GCT gCT CCT TAT GAC-3′ and 5′- GTC ATA AGG AGc AGC gcT AGc GTC AGT GTC CG-3’ (mutated nucleotides are depicted in small letters). Successful generation of plasmid constructs was validated by sequence analysis.

Antibodies and nanobodies

The following monoclonal and polyclonal antibodies or nanobodies were used in this study: anti-E-cadherin (C-terminus: BD Transduction Laboratories, 61018; N-terminus: Genetex/Biozol GTX134997), anti-Tsg101 (Abcam, 4A10), anti-GFP (Takara, 632592), anti-GFP-nanobodies (Chromotek), anti-actin (BD Transduction Laboratories, 612656), anti-Hrs (Enzo, A-5; GeneTex, GTX89364), anti-GAPDH (Abcam, 6C5), anti-giantin (Covance, PRB-114C), anti-PDI (BD Transduction Laboratories, 610946), anti-TOM20 (Santa Cruz, Sc11415).

Alignment and sequence logos

E-cadherin sequences were aligned with ClustalOmega (McWilliam et al., 2013). Sequence logos aligned to the decapeptide (765)DTPTAPPYD(773) were generated by WebLogo (Crooks et al., 2004).

Cell culture and transfection

MDCK (Madin-Darby Canine Kidney) type II cells were cultured in MEM high glucose supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FCS at 37°C in humidified atmosphere containing 5% CO_2. Transfections were performed with Lipofectamine 2000

(Invitrogen). For generation of stable cell lines, MDCK cells were split in high ratios 2 days after transfection and selected in MEM medium containing 0.4 mg/ml Zeocin or an equivalent antibiotic for selection. We transferred single clones to 24 well plates with Trypsin/EDTA-soaked Whatman slices. Subsequently, the clones were analyzed for expression of the exogenous proteins by immunoblot and fluorescence microscopy. Only those clones were selected that exhibited a transfection efficiency of at least 90% transfected cells.

RNA interference

Cells were transfected as previously described (Bänfer et al., 2018) at day 1 after seeding with the following siRNA duplexes (Invitrogen): Tsg101: 5′-GGU UAC CCG UUU AGA UCA A [dT][dT]-3′, 5′-UUG AUC UAA ACG GGU AAC C [dT][dT]-3'; Hrs: 5′-UUC UUC UCC CAG UAG UUC C [dT][dT]-3′, 5′-GGA ACU ACU GGG AGA AGA A [dT][dT]-3′ and 5′-GGA ACG AGC CCA AGU ACA A [dT][dT]-3′, 5′-UUG UAC UUG GGC UCG UUC C [dT][dT]-3'.

Preparation of extracellular vesicless

The cells were washed three times with PBS and incubated overnight with MEM and 10% fetal calf serum (FCS). To avoid contamination of the exosomal fraction by bovine serum EVs, cell culture media were subjected to centrifugation at 100,000 g for 2 h prior to overnight incubation. Medium was collected and submitted to a series of centrifugation steps as previously described (Bänfer et al., 2018). Cell culture supernatants were centrifuged at 100,000 g for 1 h. The resulting pellet was washed in PBS++ (PBS supplemented with 1 mM CaCl2 and 1 mM MgCl2), repelleted again at 100,000 g for 1 h and then resuspended in either PBS++ or in SDS-PAGE sample buffer for further use. All steps were performed at 4°C.

Proteinase K protection assay

Exosomal pellets were resuspended in PBS++, pooled, and subsequently split into three identical aliquots. Proteinase digestion was then performed with 0.5 mg/ml to 1 mg/ml proteinase K (Fermentas) in presence or absence of 1% Triton X-100 for 30 min at 37°C. As control, one of the aliquots was incubated without proteinase K.

Co-immunoprecipitation

MDCK cells stably expressing eGFP or eGFP fusion proteins were washed with PBS^{++}, followed by mechanical detachment of the cells in PBS^{++}. The cells were pelleted by centrifugation at 500 *g* for 3 min at 4°C and rinsed twice with PBS^{++}. Cell lysis was achieved by application of lysis buffer (10 mM HEPES, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 0.5% SDS and proteinase inhibitor cocktail, pH 7.5). Cell lysates (17,000 *g*, 15 min, 4°C) were then incubated with GFP-nanobody agarose (GFP-Trap, Chromotek) or control beads without GFP-nanobodies for 1.5 h at 4°C. Finally, beads were rinsed four times with Co-IP washing buffer (10 mM HEPES, 150 mM NaCl, 0.5 mM EDTA) and boiled in SDS-PAGE loading buffer for Western blot analysis.

Immunostaining, immunofluorescence microscopy and image processing

Immunofluorescence analysis was performed essentially as previously described (Bänfer et al., 2018). The cells were grown on cover slips and fixed with 4% paraformaldehyde for 20 min. Afterwards, cells were permeabilized with 0.1% Triton-X-100 for 20 min and blocked in 5% BSA/PBS^{++} for 1 h. Primary antibodies were added in blocking reagent for 2 h or overnight. Secondary antibodies labelled with the indicated Alexa Fluor dyes were applied in PBS^{++} for 1 h. Nuclei were stained with Hoechst 33342. Following incubation, cells were washed with PBS^{++} and mounted with Mowiol. Confocal images were acquired on a Leica STELLARIS microscope equipped with a ×93 glycerol planapochromat objective (Leica Microsystems). Processing of images was done with Leica LAS X and Volocity 5 (PerkinElmer). We calculated co-localization between markers as Manders' coefficient using the Volocity software package. Structures with coefficients <0.5 were classified as "not-colocalized".

Nano-flow cytometry

For nanoFCM, a Nano Analyzer (NanoFCM Co. Ltd., Nottingham, United Kingdom) equipped with a 488 nm laser, was calibrated using 200 nm polystyrene beads (NanoFCM Co.) with a defined concentration of 2.08 × 108 particles/ml, which were also used as a reference for particle concentration. In addition, monodisperse silica beads (NanoFCM Co. Ltd.) of four different sizes served as size reference standards to calibrate the size of EVs. Freshly filtered (0.1 µm) 1 × PBS was analyzed as background signal and subtracted from the other measurements. Each dot plot was derived from data collected approximately 4,000 events with a sample pressure of 1.0 kPa. For immunofluorescence staining, the following antibodies were used (BioLegend): FITC-conjugated mouse anti-human/canine CD9 antibody (clone HI9a), and anti-human Ecad, with secondary PE-conjugated donkey anti-rabbit IgG antibody (clone Poly4064); as isotype controls, FITC-conjugated mouse IgG1, κ (clone MOCP-21), and PE-

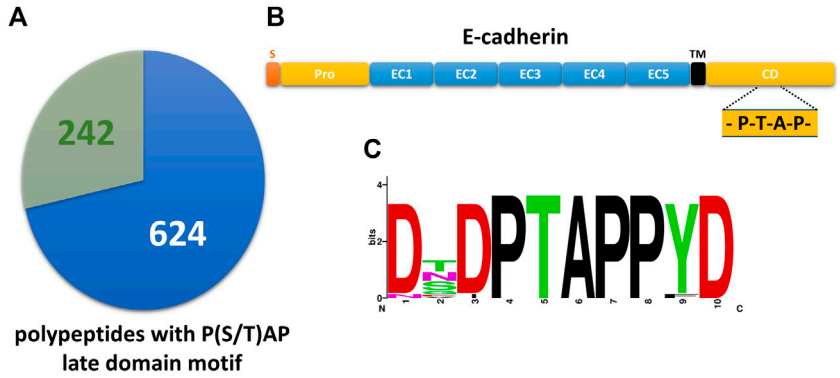


FIGURE 1
The cytoplasmic tail of E-cadherin comprises a highly conserved PTAP-late domain motif. **(A)** Pie chart showing 242 primary polypeptide sequences of the UniProtKB/Swiss-Prot database that contain a P(S/T)AP late domain motif and are listed in ExoCarta (see also Supplementary Table). 624 late domain containing sequences from the UniProtKB/Swiss-Prot database are not listed in ExoCarta. **(B)** Domain structure of E-cadherin. S, signaling sequence; pro, pro-domain; EC, E-cadherin domain; TM, transmembrane domain; CD, cytoplasmic domain. The location of the PTAP late domain motif is indicated. **(C)** Alignment of E-cadherin late domain-like motifs found in 32 vertebrates was used to generate a sequence logo of the decapeptide (765)DTPTAPPYD(773).

conjugated donkey anti-rabbit IgG antibody (clone Poly4064); 1 ng/µl of each antibody in 50 µL 1 × PBS. After removing antibody aggregates by centrifugation at 12,000× g for 10 min, the supernatant was added to 5 × 108 purified EVs, followed by incubation for 90 min at 25°C under constant shaking. Stained EV were diluted 1:100 in 1xPBS for NanoFCM analysis.

Results

E-cadherin is recruited into extracellular vesicless and interacts with Tsg101

A PROSITE database search of the UniProtKB/Swiss-Prot database revealed 866 candidate polypeptides that contain at least one P(S/T)AP late domain motif (Figure 1A, Supplementary Table S1). 242 of these protein candidates are listed in the ExoCarta database as EV cargo proteins including 16 transmembrane proteins. Among them 5 candidates belong to the cadherin family. Primary sequences of E-cadherin orthologs revealed a conserved PS/TAP motif in the cytosolic domains of e.g., human (. . . $P_{767}T_{768}A_{769}P_{770}$ . . .) and dog (. . . $P_{825}T_{826}A_{827}P_{828}$ . . .) E-cadherin (Figures 1B,C). To assess if E-cadherin is recruited into ILVs of MVEs that can be released as EVs, epithelial MDCK cells were immunostained for the ESCRT-0 protein Hrs, which labels MVEs (Bache et al., 2003). About 6% of these Hrs-positive structures were co-stained with endogenously expressed E-cadherin, which is significantly above the co-staining efficiency of E-cadherin with mitochondrial TOM20 as negative control (Figure 2). These observations document that a small fraction of E-cadherin is closely related to MVEs, which are the source compartment for exosomal release.

We then isolated EVs from MDCK cell medium by differential centrifugation as previously published (Bänfer et al., 2018) and monitored the presence of E-cadherin in the EV fraction by immunoblot. Antibodies directed against Tsg101 were used as positive control for the validation of successful EV isolation. Figure 3A indicates that E-cadherin as well as Tsg101 are enriched in isolated EVs. Antibodies directed against endoplasmic reticulum protein disulfide isomerase (PDI), the Golgi component giantin and mitochondrial TOM20 were used as negative controls to verify the purity of isolated eEVs. To clarify the orientation of E-cadherin with its single transmembrane domain on the EV membrane, purified EVs were treated with the unspecific protease proteinase K in the presence or absence of Triton X-100 (Figure 3B). Antibodies directed against E-cadherin did not detect full length E-cadherin after proteinase K-treatment. This indicates, that the N-terminal extracellular domain of E-cadherin was accessible for the protease and therefore degraded. Others have shown that a significant fraction of E-cadherin can be proteolytically shed into the extracellular space through cleavage by secretases and caspases into 80 kDa soluble E-cadherin (sE-cadherin) and the remaining membrane-attached cytoplasmic tail of about 22 kDa (David and Rajasekaran, 2012). We also detected this smaller band with an antibody directed against the cytoplasmic domain of E-cadherin and this band was insensitive to proteinase K digestion. It was solely degraded if EV-membrane lipids were solubilized by Triton X-100. It is important to note that Tsg101 and the designed eGFP variant containing the late domain motif at the C-terminus (eGFP-PSAP), which reside in

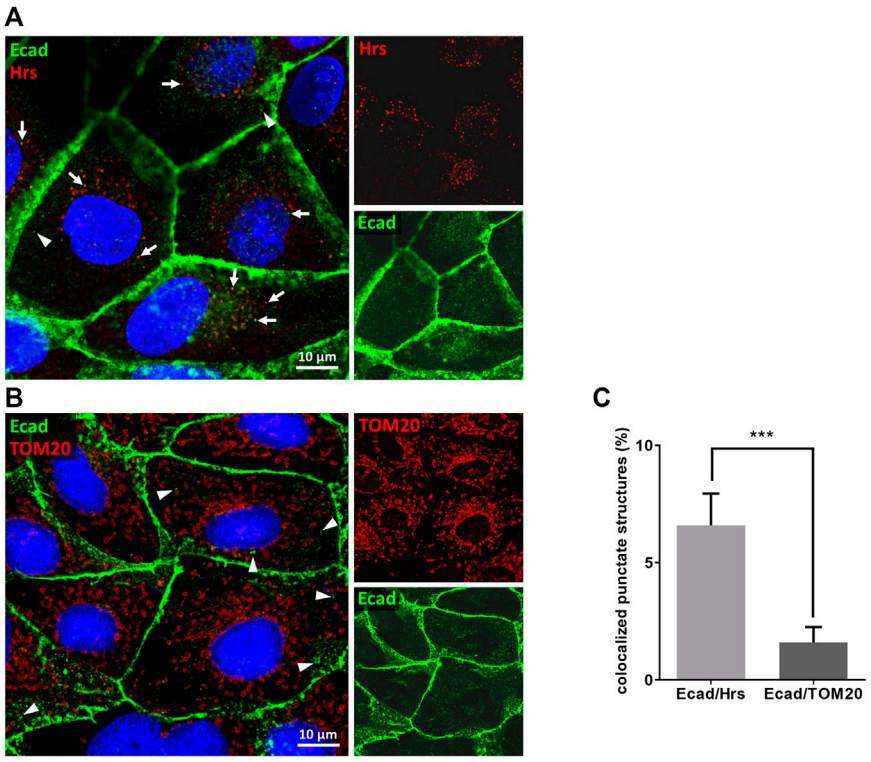


FIGURE 2
E-cadherin colocalizes with Hrs in MDCK cells. Confocal codistribution analysis of immunostained E-cadherin with the MVE-protein Hrs **(A)** or mitochondrial outer membrane protein TOM20 **(B)**. MDCK cells were cultivated for 2 days, fixed and stained by immunofluorescence with mAb anti-E-cadherin/Alexa Fluor 555 and pAb anti-Hrs/Alexa Fluor 647 or pAb anti-TOM20/Alexa Fluor 647. Nuclei are depicted in blue. Co-stained vesicular structures are indicated by arrows. Arrowheads point at punctate E-cadherin-positive structures that are not co-stained with Hrs or TOM20. **(C)** Manders' correlation coefficient was used for quantification of experiments. Means ± s.e.m., 15–20 cells per experiment, $n = 3$ independent experiments. Nuclei were excluded from quantification. Statistical analysis in this figure: Student's unpaired *t*-test, ***$p < 0.001$.

the lumen of EVs (Bänfer et al., 2018), showed a similar sensitivity pattern in this assay. Thus, E-cadherin is oriented with the large extracellular domain facing the outer surface of EVs while the cytoplasmic tail points into the EV lumen (Figure 3C). In essence, the cytoplasmic tail of E-cadherin, which harbors a PS/TAP motif, soluble eGFP-PSAP and the ESCRT-component Tsg101 are found in the EV lumen.

Next, we sought to study putative interaction of E-cadherin with Tsg101. Therefore, we incubated $MDCK_{Ecad\text{-}GFP}$ and $MDCK_{Tsg101\text{-}GFP}$ cells stably expressing E-cadherin-GFP or Tsg101-GFP (Bänfer et al., 2018). GFP-Trap beads were used for precipitation of the GFP fusion proteins from cell lysates. Indeed, Tsg101 was pulled down by E-cadherin-GFP (Figure 4A). Moreover, Tsg101-GFP precipitated E-cadherin. This is a first hint for interaction between E-cadherin and Tsg101 in MDCK cells. However, we cannot conclude from these experiments, whether the interaction is direct or indirect.

In order to find out if Tsg101 plays a functional role in recruitment of E-cadherin into EVs, which could be in analogy to the mechanism published for soluble galectin-3 (Bänfer et al., 2018), we performed experiments were siRNA was used to specifically deplete the cellular content of Tsg101. Figures 4B,C show specific depletion to about 25% of residual Tsg101. Under these conditions EV recruitment of E-cadherin significantly declined by about 50% (Figures 4B,D). The EV-pool of actin, which has also been listed as EV cargo molecule (Xu et al., 2016), was not dramatically affected by Tsg101-depletion indicating that the cells still secrete representative EV quantities. These data suggest that expression of Tsg101 is linked to efficient incorporation of E-cadherin into secreted EVs, most likely by recognition of a cytoplasmic binding motif and the initiation of E-cadherin transport into budding ILVs.

EV recruitment of E-cadherin with a mutated late domain motif

We thus addressed the question if the cytoplasmic PTAP late domain motif of E-cadherin mediates ILV-recruitment of

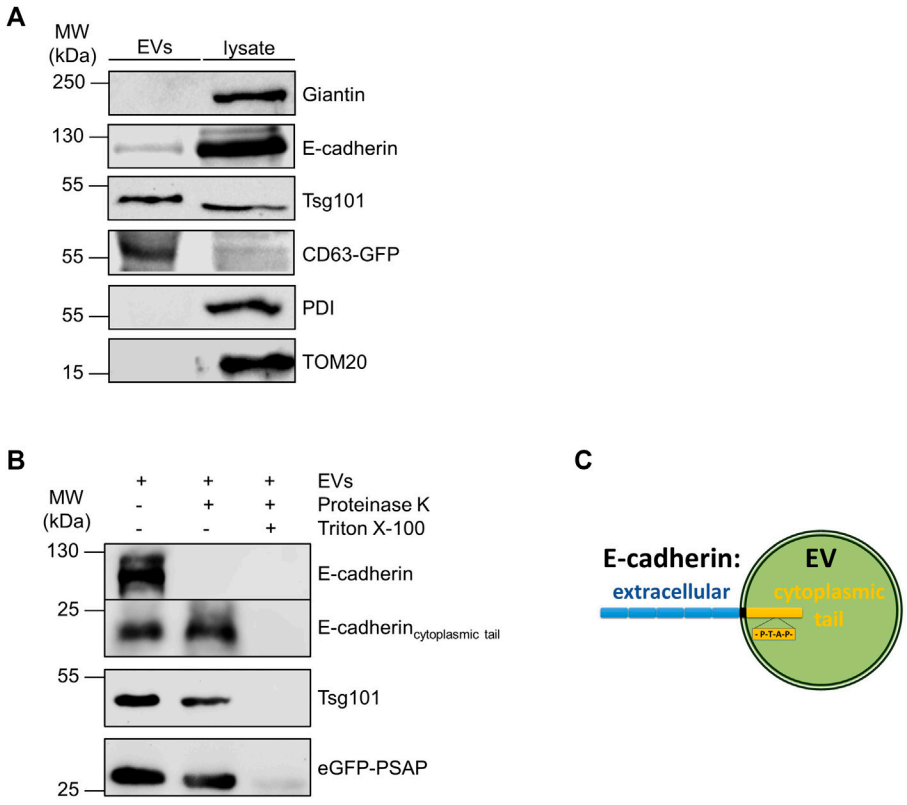


FIGURE 3
Identification of E-cadherin on isolated EVs. **(A)** Western blotting analysis of EV fractions and cell lysates. Representative results, *n* = 3 independent experiments. EVs and the corresponding cell lysates were analyzed by immunoblot with antibodies directed against E-cadherin and Tsg101. Antibodies directed against giantin (Golgi), PDI (ER) and TOM20 (mitochondria) were used as negative controls to validate the purity of EV-isolation. EVs isolated from $MDCK_{CD63\text{-}GFP}$ cells were immunoblotted with anti-GFP antibodies and used as positive controls. **(B)** Proteinase protection assay. Antibodies directed against the cytoplasmic tail of E-cadherin were used for E-cadherin staining. Antibodies directed against Tsg101 and GFP were used as indicated. Representative results, *n* = 3 independent experiments. **(C)** Schematic drawing of the orientation of E-cadherin in the exosomal membrane. Schematic drawing of the orientation of E-cadherin in the membrane of an extracellular vesicle (EV). The extracellular part of the protein is shown in blue, and the part directed to the interior of the vesicle is shown in yellow.

E-cadherin and interaction between E-cadherin and Tsg101. To increase the transfection efficiency and to facilitate the generation of stable cell clones, we first generated a GFP-tagged variant of E-cadherin with a deletion of cadherin domains two to five (E-cadherinΔE2-$5GFP_{PTAP}$) (Figure 5A). In a second step, the cytoplasmic PTAP motif of this variant was mutated to ASAA (E-cadherinΔE2-$5GFP_{ASAA}$), mimicking a mutation that is known to abrogate the release of Marburg virus-like particles (Dolnik et al., 2010). The two constructs were transiently transfected into MDCK cells. 48 h after transfection the cells were fixed and immunostained for Hrs and Tsg101 as MVE-markers (Figure 5B). Quantification using Manders' correlation coefficients revealed that 9.61 ± 1.7% of cytoplasmic E-cadherinΔE2-$5GFP_{PTAP}$ structures localized to Tsg101-positive vesicles representing the MVB formation site (Figures 5B,C). In contrast, co-staining of E-cadherinΔE2-$5GFP_{ASAA}$ resulted in a significantly reduced overlap of 2.30 ± 0.7%, thus supporting the idea of a relevant role of the cytoplasmic PTAP late domain in ILV-sorting of this transmembrane polypeptide. Consequences of PTAP late domain mutation on EV recruitment of E-cadherin were studied in MDCK cells stably expressing E-cadherinΔE2-5GFP ($MDCK_{E\text{-}cadherin\Delta E2\text{-}5GFP}$) or E-cadherinΔE2-$5GFP_{ASAA}$ ($MDCK_{E\text{-}cadherin\Delta E2\text{-}5GFPASAA)}$. Here indeed, we found that E-cadherinΔE2-5GFP was enriched in EVs, whereas the quantities of E-cadherinΔE2-$5GFP_{ASAA}$ were drastically reduced in isolated EVs (Figure 5D). Evidence for a central role of the cytosolic E-cadherin PTAP motif in Tsg101-interaction was provided by diminished

Tsg101 precipitation of E-cadherinΔE2-5GFP$_{ASAA}$ (Figure 5E). In conclusion, the PTAP motif in the cytoplasmic tail is essential for efficient Tsg101 interaction and EV-mediated release of E-cadherin.

Finally, we monitored the spectrum of EV populations released by MDCK cells using nano-flow cytometry (nFCM). Therefore, EVs were collected from the supernatants of MDCK or MDCK$_{Gal3\text{-}GFP}$ cells and exposed polypeptides were fluorescently stained with antibodies directed against CD9, CD63 or E-cadherin to discriminate between distinct EV cargoes by flow cytometry (Figure 6). EV analysis at single particle level then revealed a strong correlation between the presence of E-cadherin and galectin-3 on extracellular vesicles. Galectin-3 is recruited by PSAP-mediated sorting into EVs (Bänfer et al., 2018) and shows the highest overlap with E-cadherin on these vesicles. Nearly all galectin-3-positive vesicles contain E-cadherin (20.1 % versus 1.3%). However, not all E-cadherin-positive vesicles are also loaded with galectin-3, which can be explained by a cargo-specific EV loading efficiency and modulation of this process by additional cellular factors. Less stringent correlation was detected between vesicles positive for the tetraspanins CD9-or CD-63- and E-cadherin. Altogether, these observations indicate that significant quantities of E-cadherin and galectin-3 are sorted into identical extracellular vesicles.

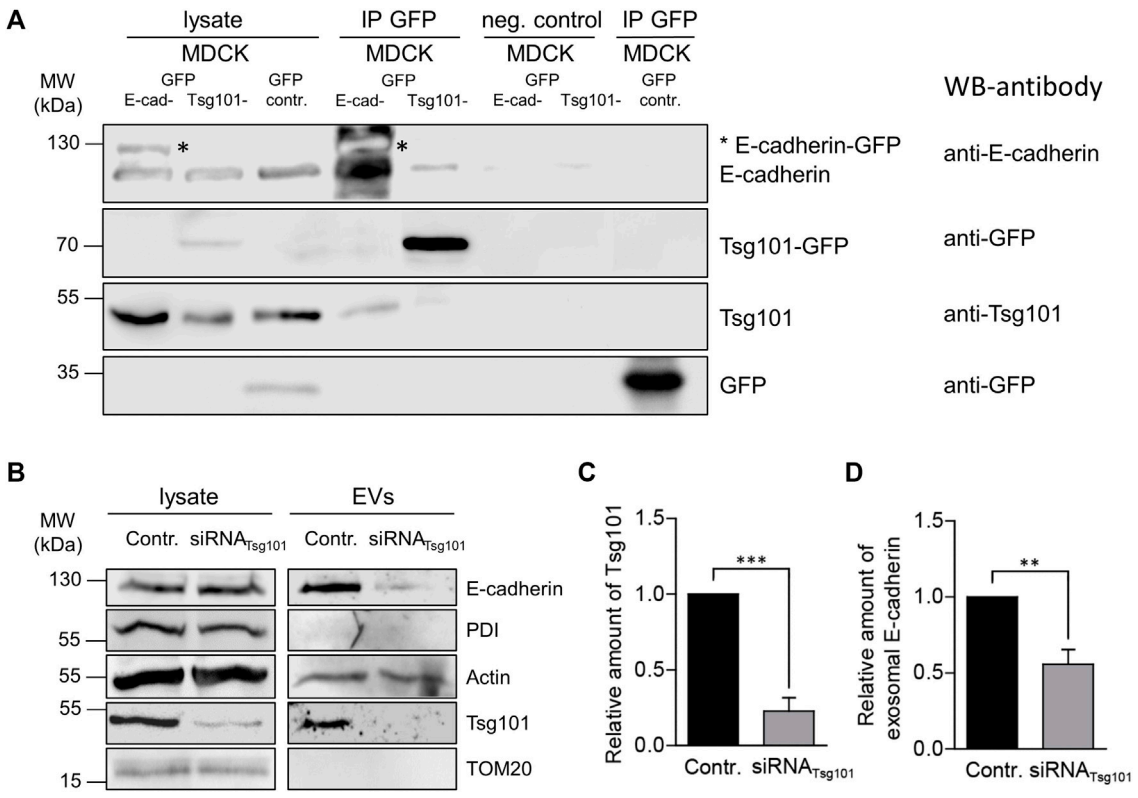


FIGURE 4
Tsg-101 dependent EV recruitment of E-cadherin. **(A)** Co-immunoprecipitation of E-cadherin-GFP or Tsg101-GFP from MDCK cell lysates. E-cadherin-GFP or Tsg101-GFP fusion proteins and their binding partners were immunoprecipitated with GFP-nanobody beads and detected by immunoblot using antibodies directed against E-cadherin, GFP and Tsg101. Non-specific precipitation was monitored by using control beads without nanobodies (neg. control) and GFP from MDCK$_{GFP}$ cells (GFP-contr.). Antibodies used for western blots (WB) are indicated. Representative results, $n = 3$ independent experiments. **(B)** Tsg101 knockdown in MDCK cells and the corresponding EVs isolated from cell culture media. EVs and the corresponding cell lysates were analyzed by immunoblot using antibodies directed against E-cadherin and Tsg101. Antibodies directed against PDI were used as negative controls to validate the purity of EV-isolation. Actin was monitored as EV cargo molecule. **(C)** Efficient knockdown through Tsg101 siRNA administration was verified by quantification of Tsg101 in cell lysates from 3 independent experiments. Tsg101 quantities were normalized to GAPDH. Means ± s.e.m. **(D)** Quantification of the immunoblot analysis of the exosomal fraction after Tsg101 knockdown in MDCK cells as in **(C)**. E-cadherin was significantly reduced in Tsg101 siRNA-treated cells. Normalized to the E-cadherin quantities in the respective cell lysates. Means ± s.e.m., $n = 3$ independent experiments. Statistical analysis in this figure: Student's unpaired *t*-test, $^{***}p < 0.001$; $^{**}p < 0.005$; $^{*}p < 0.01$.

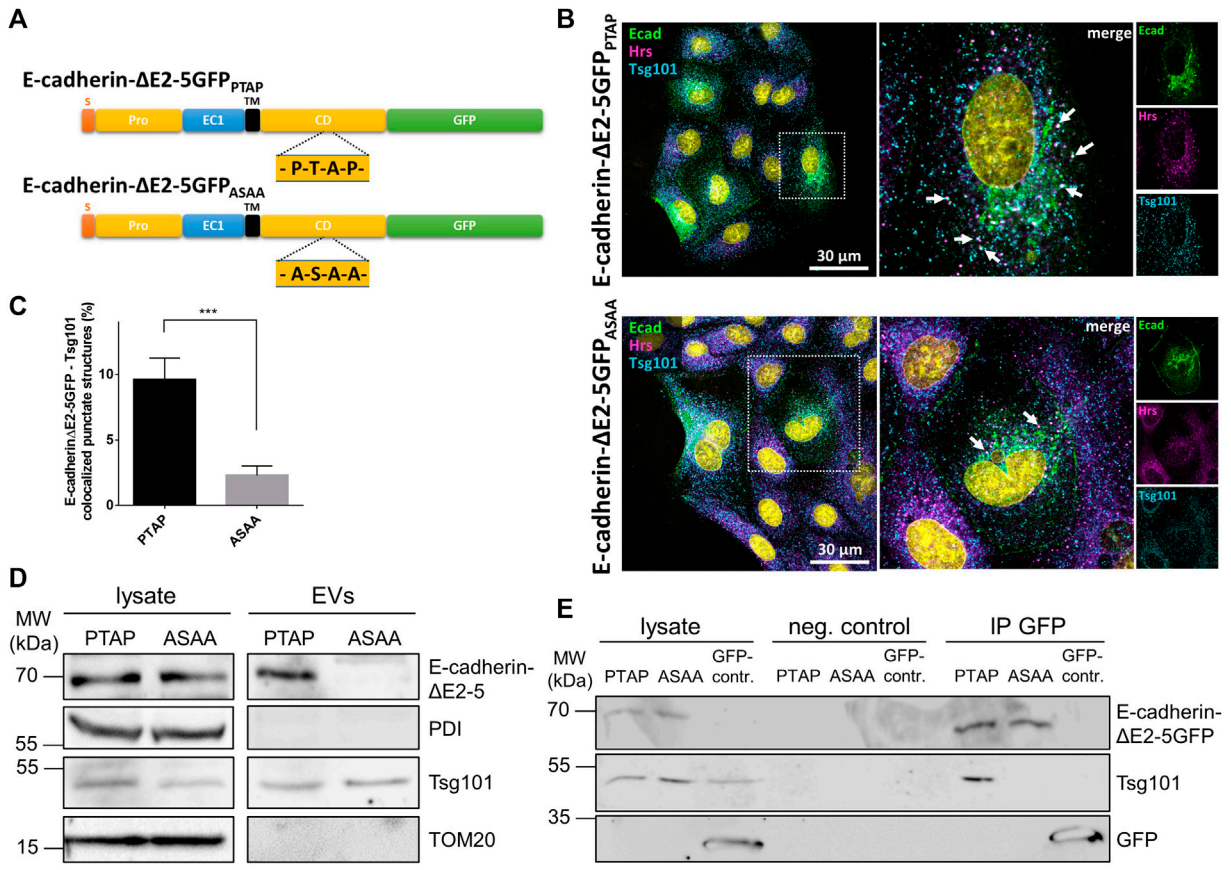


FIGURE 5
Subcellular localization and EV recruitment of E-cadherin variants. **(A)** Schematic drawing of the two E-cadherin deletion constructs. S, signaling sequence; pro, pro-domain; EC, E-cadherin domain; TM, transmembrane domain; CD, cytoplasmic domain. The location of the PTAP late domain motif and the mutagenized ASAA stretch are indicated. **(B)** Confocal codistribution analysis of MDCK cells transiently transfected with E-cadherinΔE2-5GFP or E-cadherinΔE2-5GFP$_{ASAA}$ with the MVE-proteins Hrs and Tsg101. The cells were cultivated for 2 days post transfection, fixed and stained by immunofluorescence with mAb anti-Tsg101/Alexa Fluor 555 and pAb anti-Hrs/Alexa Fluor 647. Enlarged views of areas encircled by dotted lines are depicted on the right. Nuclei are depicted in cyan. Vesicular structures co-stained for E-cadherin, Hrs, and Tsg101 are indicated by arrows. **(C)** Colocalization of the two E-cadherinΔE2-5GFP variants and Tsg101 was estimated from at least 15 cells per experiment *via* the Manders' colocalization coefficient. Data are represented as means ± s.e.m., significance was tested with Student's *t*-test (***$p < 0.001$), $n = 3$ independent experiments. **(D)** EVs isolated from MDCK$_{E\text{-}cadherinΔE2\text{-}5GFPPTAP}$ (PTAP) and MDCK$_{E\text{-}cadherinΔE2\text{-}5GFPASAA}$ (ASAA) culture media and the corresponding cell lysates were analyzed by immunoblot using antibodies directed against E-cadherin and Tsg101. Antibodies directed against PDI and TOM20 were used as negative controls to validate the purity of EV-isolation. **(E)** Co-immunoprecipitation of E-cadherin variants with Tsg101 from MDCK$_{E\text{-}cadherinΔE2\text{-}5GFPPTAP}$ (PTAP) and MDCK$_{E\text{-}cadherinΔE2\text{-}5GFPASAA}$ (ASAA) cell lysates. E-cadherinΔE2-5GFP or E-cadherinΔE2-5GFP$_{ASAA}$ and their binding partners were immunoprecipitated with GFP-nanobody beads and detected by immunoblot using antibodies directed against GFP or Tsg101. Non-specific precipitation was monitored by using control beads without nanobodies (neg. control) and GFP from MDCK$_{GFP}$ cells (GFP-contr.).

Discussion

EVs provide an additional exchange platform for intercellular and interorgan communication. We had previously shown that galectin-3, a soluble lectin, can be loaded by an ESCRT-I-mediated mechanism based on a P(S/T)AP late domain into ILVs of MVEs. This study suggests a similar recruitment of E-cadherin, a type I transmembrane protein, into ILV-membranes.

Generally, proteins are targeted to MVEs after they are endocytosed from the plasma membrane (Lakkaraju and Rodriguez-Boulan, 2008). Hrs interacts with Tsg101 and has been described as an adaptor for ubiquitin-independent endosomal sorting of interleukin-2 receptor beta from early to LAMP1-positive late endosomes resulting in degradation of the receptor (Yamashita et al., 2008). Similarly, the G protein-coupled protease-activated receptor-1 and the purinergic receptor P2Y1 both contain a YPX_3L motif, to which the ESCRT-associated protein ALG-2 interacting protein X (ALIX) binds (Dores et al., 2012). Moreover, recruitment of activated epidermal growth factor receptor (EGFR) into ILVs requires action of the ESCRT machinery (Katzmann et al., 2001). Here, ubiquitination serves as a sorting signal for selective entry

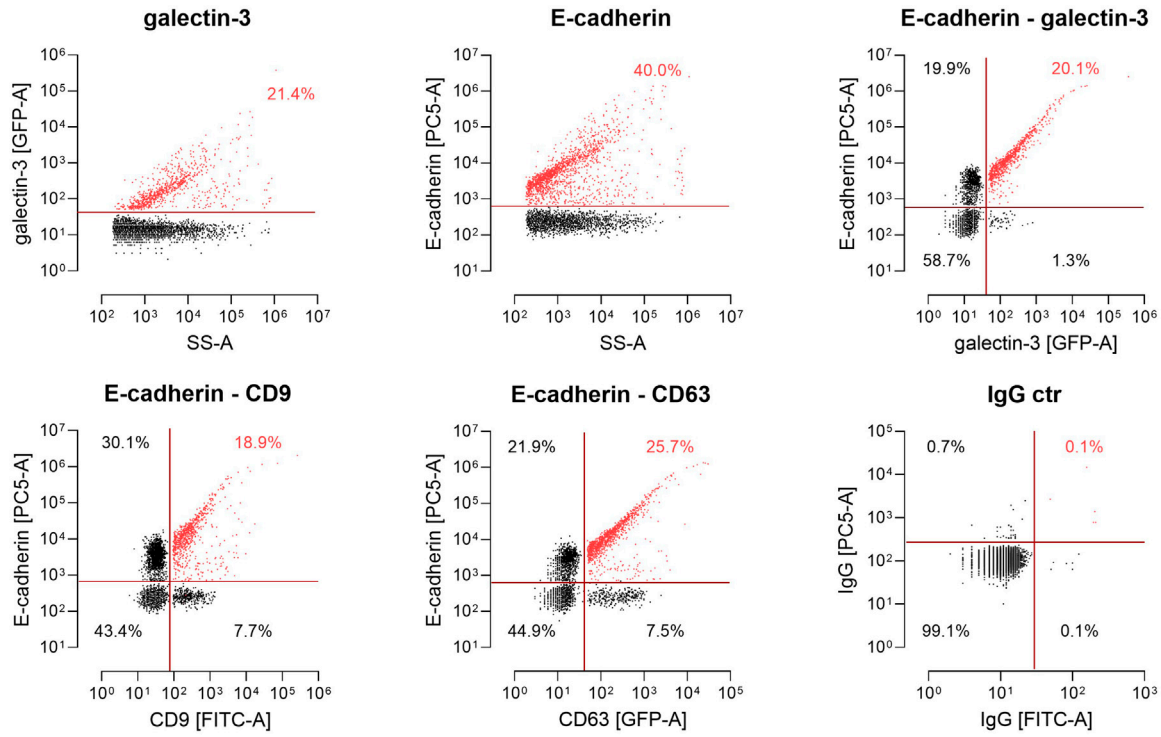


FIGURE 6
Single-particle phenotyping of EVs derived from $MDCK_{Gal3\text{-}GFP}$ cells. Representative plots of galectin-3-GFP and E-cadherin (Ecad) expression on single EVs using the GFP signal and PE-conjugated antibodies by nano-flow cytometry (nFCM).Bivariate dot-plots of indicated fluorescence versus side scatter (SS-A). In addition, EVs harboring a CD63-GFP or fluorescently labeled with FITC-conjugated antibodies specific to CD9 were stained with PE-conjugated antibodies specific to Ecad. For E-cadherin-detection on vesicle surfaces, the Genetex/Biozol antibody GTX134997 was used. Double positives for CD9/Ecad and CD63/Ecad are depicted. Fluorescently labeled IgG isotypes were used as a control. Numbers indicate events detected in the corresponding gate in percent of total events.

of endocytosed cargo into ILVs, which are going to be degraded following delivery to the lysosomal lumen. Sequestering of cargos destined for degradation or exoscytosis expands the fate of MVEs beyond lysosomal fusion and ILV degradation. Thus, EGFR can be sorted by Rab31 into CD63-positive MVEs to prevent its lysosomal degradation (Wei et al., 2021). In this case flotillins are engaged to drive EGFR-containing ILV formation, which also depends on cholesterol and ceramide within lipid raft microdomains. This clearly shows how tightly balanced and intertwined the trafficking scenarios in the endosomal membrane system are. The question how ILVs containing E-cadherin are sorted away from the degradation pathway into MVEs for EV-release has not been solved yet and remains to be clarified.

As mentioned above specific lipid species are sorted into MVEs. The unique, poorly degradable phospholipid lysobisphosphatidic acid (LBPA) accumulates on MVE membranes and interacts with ALIX (Kobayashi et al., 1999; Matsuo et al., 2004). ALIX may thus support LBPA-enrichment at sites of ILV formation. Evidence for the involvement of ceramids in the biogenesis of EVs comes from experiments using inhibitors of neutral sphingomyelinases (Trajkovic et al., 2008; Menck et al., 2017). Ceramide is capable to self-associate through hydrogen bonding and can induce the coalescence of small microdomains into larger domains, which promotes microdomain-induced budding (Gulbins and Kolesnick, 2003). In polarized cells ceramides predominantly mediate the release of EVs from the basolateral membrane domain (Matsui et al., 2021). This study also describes apically secreted EVs that are formed in the presence of ALIX but independently of other ESCRT components. Together with our observation that the formation of galectin-3- as well as E-cadherin-positive EVs correlates with the presence of Tsg101, these data suggest that at least three distinct molecular mechanisms for the recruitment of EV cargo exist in epithelial cells, with one of them using the canonical ESCRT pathway. Consequently, this would lead to EV subpopulations composed of individual protein pools, which are formed in separate MVEs. Our nFCM-data point into the same direction. This idea of discrete EV biogenesis in individual endosomal compartments is confirmed by the finding that MVEs positive for the EV components

CD9 or CD63 are stained separately in the cytoplasm of MDCK cells (Matsui et al., 2021). Release of specific EV subpopulations would then expand the extracellular vesicle bouquet and thereby enhance the spectrum of vesicle-mediated cell-cell communication in a living organism.

Two fates for E-cadherin on the EV membrane are plausible, it can remain intact or be cleaved of as soluble E-cadherin (Tang et al., 2018). Here, we found intact E-cadherin on isolated EVs, which would be more advantageous for long distance communication. EVs are extremely stable in human body fluids (Kalra et al., 2013) and thus provide a membrane environment that helps to increase the half-life of E-cadherin. Questions remain on the functional role of E-cadherin exposed on the EV membrane. Tang et al. (2018) reported that E-cadherin-positive EVs secreted from ovarian cells can promote angiogenesis. The cadherin heterogeneously interacts with VE-cadherin on the surface of endothelial cells. VE-mediated signaling then leads to increased nuclear accumulation of β-catenin and activation of the NFκB signaling cascade to induce angiogenesis. On the other hand, Zhang et al. (2020) claimed that EVs carrying E-cadherin promote the migration and invasion of adenocarcinomic human alveolar basal epithelial cells. They isolated EVs from the bronchoalveolar lavage fluid from patients with lung cancer. Release of E-cadherin positive EVs increases the E-cadherin concentration within the tumor microenvironment, thus facilitating lung cancer metastasis. Both analysis of E-cadherin-positive EVs and their functional effects are related to cancer progression. Moreover, in bone marrow dendritic cells E-cadherin is required to mediate the release of β-catenin into EVs (Chairoungdua et al., 2010). EV discharge of β-catenin might suppress tumor metastasis through down-regulation of the Wnt signaling pathway. This is an interesting observation, since interaction with E-cadherin is part of a process to recruit β-catenin into EVs. Considering these heterogenous examples of E-cadherin function on EVs, it remains to define in future studies regulatory elements that modulate formation and release of E-cadherin-positive EVs also under non-pathologic conditions.

Data availability statement

All datasets generated for this study are included in the article and/or the Supplementary Material.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SB, SK, FF, MD, CP, EP, and RJ. The first draft of the manuscript was written by RJ and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany (GRK 2213). Parts of the study were funded by the Deutsche Forschungsgemeinschaft grant number GRK2573.

Acknowledgments

We are grateful to W. Ackermann for technical assistance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.878620/full#supplementary-material

References

Adams, C. L., Chen, Y. T., Smith, S. J., and Nelson, W. J. (1998). Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. *J. Cell. Biol.* 142, 1105–1119. doi:10.1083/jcb.142.4.1105

Bache, K. G., Brech, A., Mehlum, A., and Stenmark, H. (2003). Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. *J. Cell. Biol.* 162, 435–442. doi:10.1083/jcb.200302131

Baietti, M. F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., et al. (2012). Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat. Cell. Biol.* 14, 677–685. doi:10.1038/ncb2502

Bänfer, S., Schneider, D., Dewes, J., Strauss, M. T., Freibert, S. A., Heimerl, T., et al. (2018). Molecular mechanism to recruit galectin-3 into multivesicular bodies for polarized exosomal secretion. *Proc. Natl. Acad. Sci. U. S. A.* 115, E4396–E4405. doi:10.1073/pnas.1718921115

Chairoungdua, A., Smith, D. L., Pochard, P., Hull, M., and Caplan, M. J. (2010). Exosome release of beta-catenin: A novel mechanism that antagonizes Wnt signaling. *J. Cell. Biol.* 190, 1079–1091. doi:10.1083/jcb.201002049

Charrin, S., Jouannet, S., Boucheix, C., and Rubinstein, E. (2014). Tetraspanins at a glance. *J. Cell. Sci.* 127, 3641–3648. doi:10.1242/jcs.154906

Crooks, G. E., Hon, G., Chandonia, J. M., and Brenner, S. E. (2004). WebLogo: A sequence logo generator. *Genome Res.* 14, 1188–1190. doi:10.1101/gr.849004

David, J. M., and Rajasekaran, A. K. (2012). Dishonorable discharge: The oncogenic roles of cleaved E-cadherin fragments. *Cancer Res.* 72, 2917–2923. doi:10.1158/0008-5472.CAN-11-3498

Dolnik, O., Kolesnikova, L., Stevermann, L., and Becker, S. (2010). Tsg101 is recruited by a late domain of the nucleocapsid protein to support budding of Marburg virus-like particles. *J. Virol.* 84, 7847–7856. doi:10.1128/JVI.00476-10

Dores, M. R., Chen, B., Lin, H., Soh, U. J., Paing, M. M., Montagne, W. A., et al. (2012). ALIX binds a YPX(3)L motif of the GPCR PAR1 and mediates ubiquitin-independent ESCRT-III/MVB sorting. *J. Cell. Biol.* 197, 407–419. doi:10.1083/jcb.201110031

Gauthier, S. A., Perez-Gonzalez, R., Sharma, A., Huang, F. K., Alldred, M. J., Pawlik, M., et al. (2017). Enhanced exosome secretion in Down syndrome brain - a protective mechanism to alleviate neuronal endosomal abnormalities. *Acta Neuropathol. Commun.* 5, 65. doi:10.1186/s40478-017-0466-0

Grabowska, M. M., and Day, M. L. (2012). Soluble E-cadherin: More than a symptom of disease. *Front. Biosci.* 17, 1948–1964. doi:10.2741/4031

Gulbins, E., and Kolesnick, R. (2003). Raft ceramide in molecular medicine. *Oncogene* 22, 7070–7077. doi:10.1038/sj.onc.1207146

Kalra, H., Adda, C. G., Liem, M., Ang, C. S., Mechler, A., Simpson, R. J., et al. (2013). Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. *Proteomics* 13, 3354–3364. doi:10.1002/pmic.201300282

Kartenbeck, J., Schmelz, M., Franke, W. W., and Geiger, B. (1991). Endocytosis of junctional cadherins in bovine kidney epithelial (MDBK) cells cultured in low Ca2+ ion medium. *J. Cell. Biol.* 113, 881–892. doi:10.1083/jcb.113.4.881

Katzmann, D. J., Babst, M., and Emr, S. D. (2001). Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell.* 106, 145–155. doi:10.1016/s0092-8674(01)00434-2

Kobayashi, T., Beuchat, M. H., Lindsay, M., Frias, S., Palmiter, R. D., Sakuraba, H., et al. (1999). Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport. *Nat. Cell. Biol.* 1, 113–118. doi:10.1038/10084

Lakkaraju, A., and Rodriguez-Boulan, E. (2008). Itinerant exosomes: Emerging roles in cell and tissue polarity. *Trends Cell. Biol.* 18, 199–209. doi:10.1016/j.tcb.2008.03.002

Le, T. L., Yap, A. S., and Stow, J. L. (1999). Recycling of E-cadherin: A potential mechanism for regulating cadherin dynamics. *J. Cell. Biol.* 146, 219–232. doi:10.1083/jcb.146.999.219

Levayer, R., Pelissier-Monier, A., and Lecuit, T. (2011). Spatial regulation of Dia and Myosin-II by RhoGEF2 controls initiation of E-cadherin endocytosis during epithelial morphogenesis. *Nat. Cell. Biol.* 13, 529–540. doi:10.1038/ncb2224

Lock, J. G., and Stow, J. L. (2005). Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin. *Mol. Biol. Cell.* 16, 1744–1755. doi:10.1091/mbc.e04-10-0867

Matsui, T., Osaki, F., Hiragi, S., Sakamaki, Y., and Fukuda, M. (2021). ALIX and ceramide differentially control polarized small extracellular vesicle release from epithelial cells. *EMBO Rep.* 22, e51475. doi:10.15252/embr.202051475

Matsuo, H., Chevallier, J., Mayran, N., Le Blanc, I., Ferguson, C., Faure, J., et al. (2004). Role of LBPA and Alix in multivesicular liposome formation and endosome organization. *Science* 303, 531–534. doi:10.1126/science.1092425

McWilliam, H., Li, W., Uludag, M., Squizzato, S., Park, Y. M., Buso, N., et al. (2013). Analysis tool web services from the EMBL-EBI. *Nucleic Acids Res.* 41, W597–W600. doi:10.1093/nar/gkt376

Menck, K., Sonmezer, C., Worst, T. S., Schulz, M., Dihazi, G. H., Streit, F., et al. (2017). Neutral sphingomyelinases control extracellular vesicles budding from the plasma membrane. *J. Extracell. Vesicles* 6, 1378056. doi:10.1080/20013078.2017.1378056

Tang, M. K. S., Yue, P. Y. K., Ip, P. P., Huang, R. L., Lai, H. C., Cheung, A. N. Y., et al. (2018). Soluble E-cadherin promotes tumor angiogenesis and localizes to exosome surface. *Nat. Commun.* 9, 2270. doi:10.1038/s41467-018-04695-7

Theos, A. C., Truschel, S. T., Tenza, D., Hurbain, I., Harper, D. C., Berson, J. F., et al. (2006). A lumenal domain-dependent pathway for sorting to intralumenal vesicles of multivesicular endosomes involved in organelle morphogenesis. *Dev. Cell.* 10, 343–354. doi:10.1016/j.devcel.2006.01.012

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., et al. (2008). Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 319, 1244–1247. doi:10.1126/science.1153124

van Niel, G., Charrin, S., Simoes, S., Romao, M., Rochin, L., Saftig, P., et al. (2011). The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. *Dev. Cell.* 21, 708–721. doi:10.1016/j.devcel.2011.08.019

van Niel, G., D'Angelo, G., and Raposo, G. (2018). Shedding light on the cell biology of extracellular vesicles. *Nat. Rev. Mol. Cell. Biol.* 19, 213–228. doi:10.1038/nrm.2017.125

Vidal, M., Mangeat, P., and Hoekstra, D. (1997). Aggregation reroutes molecules from a recycling to a vesicle-mediated secretion pathway during reticulocyte maturation. *J. Cell. Sci.* 110 (16), 1867–1877. doi:10.1242/jcs.110.16.1867

Wei, D., Zhan, W., Gao, Y., Huang, L., Gong, R., Wang, W., et al. (2021). RAB31 marks and controls an ESCRT-independent exosome pathway. *Cell. Res.* 31, 157–177. doi:10.1038/s41422-020-00409-1

Xu, R., Greening, D. W., Zhu, H. J., Takahashi, N., and Simpson, R. J. (2016). Extracellular vesicle isolation and characterization: Toward clinical application. *J. Clin. Invest.* 126, 1152–1162. doi:10.1172/JCI81129

Yamashita, Y., Kojima, K., Tsukahara, T., Agawa, H., Yamada, K., Amano, Y., et al. (2008). Ubiquitin-independent binding of Hrs mediates endosomal sorting of the interleukin-2 receptor beta-chain. *J. Cell. Sci.* 121, 1727–1738. doi:10.1242/jcs.024455

Zhang, Y., Liu, Z., Li, S., Wang, M., Dai, D., Jing, H., et al. (2020). Upregulation of E-cadherin in bronchoalveolar lavage fluid-derived exosomes in patients with lung cancer. *Thorac. Cancer* 11, 41–47. doi:10.1111/1759-7714.13220

frontiers | Frontiers in Cell and Developmental Biology

TYPE Original Research
PUBLISHED 05 October 2022
DOI 10.3389/fcell.2022.912118

OPEN ACCESS

EDITED BY
Julien Villeneuve,
Institute for Functional Genomics (IGF) (CNRS), France

REVIEWED BY
Geoffrey Canet,
Centre hospitalier de l'Université Laval, Canada
Marie-Laure Parmentier,
Centre National de la Recherche Scientifique (CNRS), France
Thomas Curel,
Centre National de la Recherche Scientifique (CNRS), France, in collaboration with reviewer M-LP

*CORRESPONDENCE
Nicole Leclerc,
Nicole.leclerc@umontreal.ca

SPECIALTY SECTION
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

RECEIVED 04 April 2022
ACCEPTED 20 September 2022
PUBLISHED 05 October 2022

CITATION
Pilliod J, Gélinas-Faucher M and Leclerc N (2022), Unconventional secretion of tau by VAMP8 impacts its intra- and extracellular cleavage. *Front. Cell Dev. Biol.* 10:912118. doi: 10.3389/fcell.2022.912118

Unconventional secretion of tau by VAMP8 impacts its intra- and extracellular cleavage

Julie Pilliod[1], Maude Gélinas-Faucher[1] and Nicole Leclerc[1,2]*

[1]Research Center of the University of Montreal Hospital (CRCHUM), Montréal, QC, Canada, [2]Département de Neurosciences, Faculté de Médecine, Université de Montréal, Montréal, QC, Canada

In Alzheimer's disease, Tau, a microtubule-associated protein, becomes hyperphosphorylated, detaches from microtubules, and accumulates in the somato-dendritic compartment where it forms insoluble aggregates. Tau also accumulates in the CSF of patients indicating that it is released by neurons. Consistent with this, several laboratories including ours have shown that Tau is secreted by neurons through unconventional secretory pathways. Recently, we reported that VAMP8, an R-SNARE found on late endosomes, increased Tau secretion and that secreted Tau was cleaved at the C-terminal. In the present study, we examined whether the increase of Tau secretion by VAMP8 affected its intra- and extracellular cleavage. Upon VAMP8 overexpression, an increase of Tau cleaved by caspase-3 in the cell lysate and medium was observed. This was correlated to an increase of active caspase-3 in the cell lysate and medium. Using a Tau mutant not cleavable by caspase-3, we demonstrated that Tau cleavage by caspase-3 was not necessary for its secretion upon VAMP8 overexpression. By adding recombinant Tau to the culture medium, we demonstrated that extracellular Tau cleavage by caspase-3 could occur because of the release of active caspase-3, which was the highest when VAMP8 was overexpressed. When cleavage of Tau by caspase-3 was prevented by using a non-cleavable mutant, secreted Tau was still cleaved at the C-terminal, the asparagine N410 contributing to it. Lastly, we demonstrated that N-terminal of Tau regulated the secretion pattern of a Tau fragment containing the microtubule-binding domain and the C-terminal of Tau upon VAMP8 overexpression. Collectively, the above observations indicate that VAMP8 overexpression affects the intra- and extracellular cleavage pattern of Tau.

KEYWORDS

tau protein, VAMP8, secretion, caspase-3, tau cleavage

Introduction

Tau is a neuronal MAP enriched in the axon that becomes hyperphosphorylated, accumulates in the somato-dendritic compartment and self-aggregates into insoluble filaments called paired helical filaments (PHFs) forming the neurofibrillary tangles (NFTs) in Alzheimer's disease (AD) (Ludin and Matus, 1993; Mandell & Banker, 1996; Lee et al., 2001; Cairns et al., 2007; Iqbal et al., 2016). Tau pathology is correlated to cognitive deficits in patients

which, was confirmed by histopathological examination of post-mortem brain and Tau PET imaging (Tomlinson et al., 1970; Alafuzoff et al., 1987; Braak & Braak, 1991; Arriagada et al., 1992; Bierer et al., 1995; Ossenkoppele et al., 2016; Pontecorvo et al., 2019). The contribution of Tau dysfunction to neurodegeneration is further supported by the enrichment of Tau genetic variants in patients suffering from frontotemporal lobar degeneration (FTLD-Tau) (Cairns et al., 2007). No mutations in Tau gene were found in AD patients but Tau gene polymorphisms may be risk factors for sporadic AD (Schraen-Maschke et al., 2004). In a recent study, a duplication of the Tau gene was correlated to an early-onset dementia with an AD clinical phenotype (Le Guennec et al., 2017). Although all the above observations indicate that Tau pathology is involved in the pathogenesis of AD, its precise role in the process of neurodegeneration remains elusive.

Besides its intracellular accumulation, Tau also accumulates extracellularly in AD as revealed by its increase in the CSF during the progression of the disease. This increase was believed to correlate with neuronal cell death (Hampel et al., 2010). Several recent studies have demonstrated that Tau can be released by neurons through an active process of secretion (Pernegre et al., 2019). The presence of Tau in the interstitial fluid in the absence of neurodegeneration was detected by microdialysis in Tau transgenic mouse brain (Yamada et al., 2011). The release of Tau by neurons was shown to be increased by neuronal activity both *in vitro* and *in vivo* (Pooler et al., 2013; Yamada et al., 2014; Ismael et al., 2021). AD is linked to autophagic and lysosomal dysfunction, which was shown to increase the release of Tau by primary cortical neurons (Mohamed et al., 2014).

The secretory pathways of Tau are still largely unknown. So far, Tau was shown to be only secreted by unconventional pathways. Tau can be released either by its translocation across the plasma membrane or by membranous organelles that can fuse with the plasma membrane (Pernegre et al., 2019). Membranous organelles such as late endosomes, autophagosomes and lysosomes were shown to be involved in Tau release (Pernegre et al., 2019). In a previous study, we reported that Rab7A associated with late endosomes participates in Tau secretion (Rodriguez et al., 2017). More recently, we demonstrated that VAMP8, a R-SNARE associated with late endosomes, increases Tau secretion upon its overexpression in neurons and the neuronal cell line N2a (Antonin et al., 2000; Pryor et al., 2004; Itakura et al., 2012; Pilliod et al., 2020). Other groups have also demonstrated that the endosomal system contributes to Tau secretion. In a recent study, it was reported that Bin1(bridging integrator 1), a protein involved in endocytosis and subcellular trafficking can bind to Tau and regulate its secretion (Prokic et al., 2014; Glennon et al., 2020). Its loss resulted in a significant decrease of Tau secretion by neurons. Interestingly, polymorphisms associated with Bin1 is the second largest genetic risk for sporadic AD (Lambert et al., 2013; Vardarajan et al., 2015). Syntaxins 6 and 8, two SNAREs that play an important role in the membranes trafficking, can interact with Tau C-terminal and increase its secretion (Lee et al., 2021). Syntaxin 6 is found at the trans-Golgi network and early endosomes whereas syntaxin 8 is localized on recycling and late endosomes (Jung et al., 2012).

The above observations revealed that Tau can be secreted by several pathways. In both the CSF and culture medium of neuronal cells, full length-Tau (FL-Tau) and N- and C-terminal truncated forms are detected (Mohamed et al., 2013; Pernegre et al., 2019). It remains unclear whether these forms of Tau are released by distinct secretory pathways. The amount of FL-Tau released by primary neuronal cultures varies from one study to another. In some studies, it was the main form whereas in other studies it was a minor pool of secreted Tau (less than 1%) (Plouffe et al., 2012; Pooler et al., 2013; Mohamed et al., 2014; Bright et al., 2015; Kanmert et al., 2015; Mohamed et al., 2017; Rodriguez et al., 2017).

The above observations revealed that different cleaved forms of Tau are released by neurons. We previously reported that VAMP8 increases Tau secretion, and that secreted Tau was cleaved at the C-terminal (Pilliod et al., 2020). In the present study, we examined whether the increase of Tau secretion by VAMP8 affected its intra- and extracellular cleavage. Upon VAMP8 overexpression, an increase of Tau cleaved by caspase-3 in the cell lysate and in the medium was observed which, was correlated to an increase of active caspase-3 in the cell lysate and the medium. However, our results revealed that Tau cleavage by caspase-3 was not necessary for its secretion upon VAMP8 overexpression. We also demonstrated that the asparagine N410 affected the cleavage of secreted Tau at the C-terminal when VAMP8 was overexpressed. Lastly, we demonstrated that N-terminal of Tau regulated the secretion pattern of a Tau fragment containing the microtubule-binding domain and the C-terminal of Tau upon VAMP8 overexpression. All above observations indicate that VAMP8 influences the intra- and extracellular cleavage pattern of Tau.

Materials and methods

Cell culture

Neuro-2A cells were purchased from ATCC (#CCL-131TM, Manassas, VA, United States) and were cultured in MEM with Earles's Salt, non-essential amino acids supplemented with L-glutamine, Na-pyruvate and Na-bicarbonate (#320-026-CL, Wisent Life Sciences, Saint-Bruno, QC, CANADA) and with 10% foetal bovine serum premium (Wisent, Saint Bruno, QC, CANADA) at 37°C in a humidified 5% CO2 incubator.

Chemicals, antibodies, and plasmids

The protease inhibitor cocktail, cOmplete™ ULTRA tablets from Roche Diagnostics was used (#5892988001, Roche Diagnostics, Indianapolis, IN, United States). For immunoblotting, the following antibodies were used : total Tau

(1:50000 #A0024, Dako, Santa Clara, CA, United States); GFP (1:1000 #3H9, Chromotek Inc., Hauppauge, NY, United States); ɣ-actin (1:10000 #Sc-65635, Santa Cruz, Dallas, TX, United States); Tau-46 (1:500 #ab203179, Abcam, Cambridge, MA, United States); Tau-C3 (1:1000 #AHB0061, Invitrogen, Carlsbad, CA, United States); Caspase3 (1:1000 #9662, CellSignaling); Cleaved Caspase3 (1:1000 #9661, CellSignaling); α-Synuclein (1:1000 #610787, BD Biosciences, Franklin Lakes, NJ, United States). All the secondary antibodies were coupled with HRP from Jackson ImmunoResearch (West Grove, PA, United States). The Flag-Tau, Flag-empty, GFP-VAMP8 and GFP-empty plasmids used for co-transfection of Neuro-2A cells were described previously (Pilliod et al., 2020). Tau-D421A, Tau-Δ421-441, Flag-TauNT and Flag-TauMBD-CT were generated by mutagenesis from Flag-Tau and Tau-N410A+D421A from pEGFP-C1-4R-Tau by removing the GFP tag (Civic Biosciences limitée, Beloeil, QC, Canada). Myc- α-synuclein was obtained from Dr. EA Fon. Recombinant Tau protein (0N4R) was obtained from Biotechne (SP-499, Bio-Techne, Minneapolis, MN, United States).

Plasmid transfection

Neuro-2A cells were plated into 35 mm plates and transfected the next day with plasmids using Genejuice (#70967, Millipore-Sigma, ON, Canada) and 48 h post-transfection cells were lysed for immunoblotting.

Extracellular cleavage of tau assay

To determine whether Tau can be cleaved extracellularly by caspase-3, the medium either of untransfected or transfected cells was collected 48 h post transfection and transferred to a petri dish without cells. 1.5 µg of recombinant Tau with or without protease inhibitors was added to the medium and the dishes were placed in the incubator for 12 h. The protease inhibitors were prepared according to the manufacturer instructions.

Western blot

The culture medium of N2a cells was collected 48 h after transfection and centrifuged at 3,000 rpm for 10min at 23°C to remove cell debris. For cell lysates, cells were washed twice with PBS and once with PBS containing 0.5 M NaCl and lysed in fresh lysis buffer containing Tris 50mM, NaCl 300mM, Triton 100 × 0.5%, a protease inhibitor cocktail (cOmplete™ ULTRA tablets), and a phosphatase inhibitor cocktail (PhosSTOP, Roche Diagnostics), and then incubated on ice for 20 min. Proteins were quantified using Bio-Rad DC Protein assay (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). The medium and the lysates were mixed with Laemmli buffer 1X and boiled for 5 min at 95°C. Equal amount of the culture medium and cell lysates were loaded in each lane and electrophoresed on polyacrylamide gel. Immunoblotting was performed as previously described (Plouffe et al., 2012). All the secondary antibodies purchased from Jackson ImmunoResearch were coupled with HRP. The quantification of the immunoreactive bands from western blot image acquisition was performed using a ChemiDoc MP system (Bio-Rad Laboratories) and densitometry analysis was done with Image Lab software (version 5.0, Bio-Rad Laboratories).

Calculation of normalized tau secretion

In all the graphs presenting the quantification of Tau secretion by western blotting, normalized Tau secretion was calculated by dividing the signal of total Tau in the medium (ExTau) by the signal of total Tau in the cell lysate (InTau). The signal of InTau was normalized to that of actin in the cell lysate.

LDH assay

Lactase dehydrogenase activity (LDH) in media was determined using a LDH Activity Assay Kit (Cayman Chemical Company, Ann Arbor, MI, United States) according to manufacturer instructions. The LDH was measured using a BIO-TEK SYNERGY4 plate reader at Abs 490 nm (Winooski, VT, United States). The mean of the enzyme activity was used for comparison between experimental conditions.

Statistical analysis

The statistical analysis was performed using Prism 8.0c software (GraphPad Software Inc., San Diego, CA, United States). Normality was assumed for the statistical analysis. Findings were considered significant as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, or $^{****}p < 0.0001$. When we compared the means of 3 or more experimental groups (Figures 1, 3), statistical significance was evaluated with an ordinary one-way ANOVA test. The experimental groups were compared to the control group. When the means of two groups were compared, a paired *t*-test was used (Figures 2, 4–6).

Results

Intracellular and extracellular tau is cleaved by caspase-3 upon VAMP8 overexpression

We previously showed that VAMP8 overexpression increased Tau secretion in the neuroblastoma cell line N2a, which was correlated to a decrease of intracellular Tau (Pilliod et al., 2020).

10.3389/fcell.2022.912118

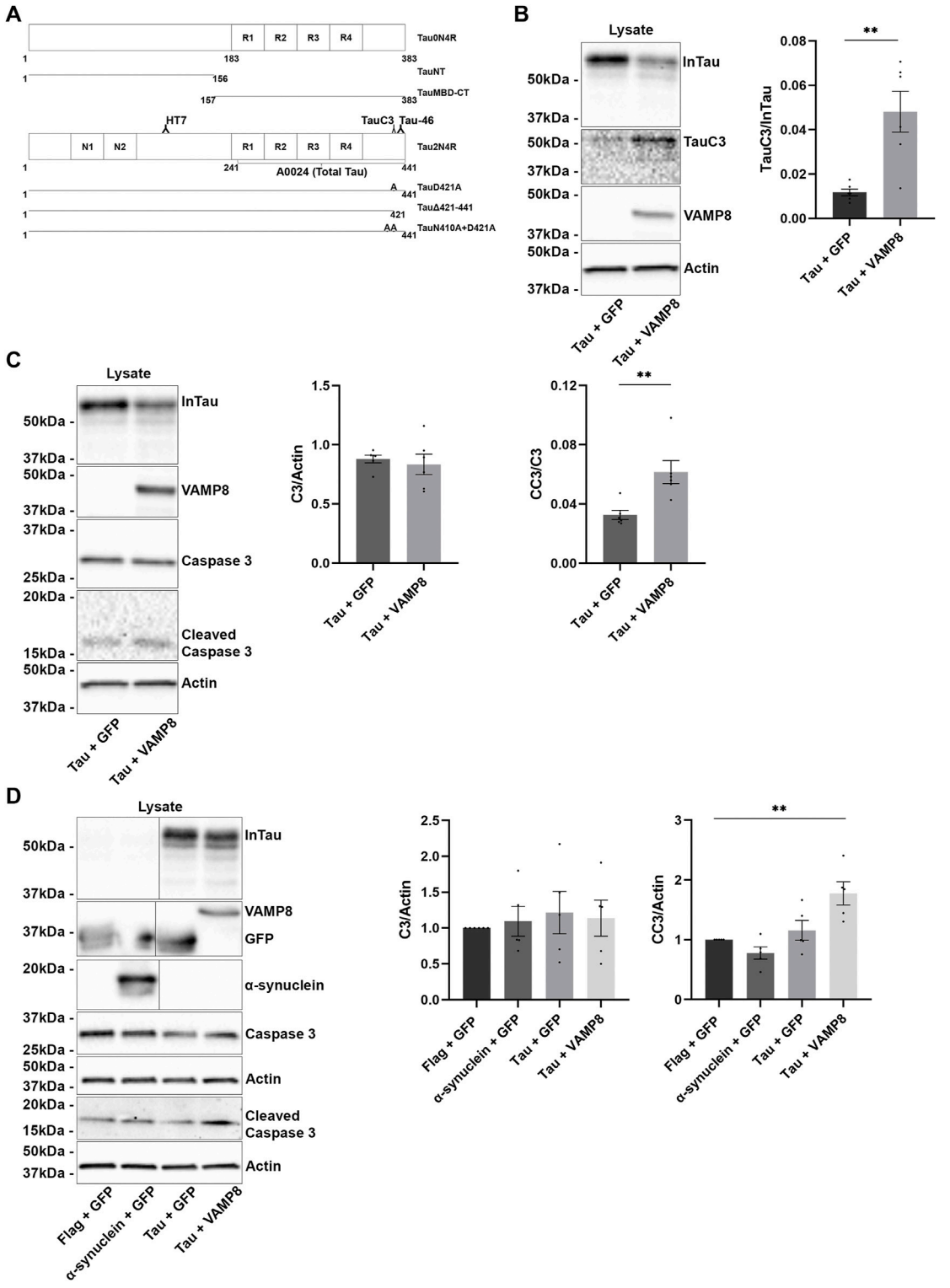


FIGURE 1
Overexpression of VAMP8 increases the cleavage of Tau by Caspase 3 and increases the amount of active caspase-3 in the cell lysate. For all the figures, GFP alone corresponds to GFP-empty vector. **(A)** Schematic representation of Tau constructs and Tau antibodies. All the experiments were carried out with 0N4R Tau isoform. The constructs TauNT and TauMBD-CT were produced from 0N4R. For the antibodies, the isoform 2N4R was used for consistency with the literature. For B and C, N2a cells were transfected either with Flag-Tau and GFP-empty or Flag-Tau and GFP-VAMP8 plasmids. **(B)** Representative Western blot with the anti-Tau antibody A0024 recognizing total intracellular Tau (InTau) and the antibody

(*Continued*)

FIGURE 1
TauC3 directed against Tau cleaved by caspase-3 of the cell lysate revealing that the overexpression of VAMP8 decreased InTau but increased InTau cleaved by caspase-3 (TauC3). For the densitometry analysis of the TauC3/total Tau ratio, InTau (total Tau) was normalized with the actin signal, the loading reference. **(C)** Representative Western blot with the anti-Tau antibody A0024, the anti-caspase-3 antibody (C3) and the anti-cleaved caspase-3 antibody (CC3) of the cell lysate showing that the C3 signal is similar for cells overexpressing either Tau alone or Tau and VAMP8 while the co-expression of Tau and VAMP8 increased intracellular CC3. CC3/C3 ratio was analyzed by densitometry. $n = 6$. Data represent scatter plot and mean±SEM. $^{**}p < 0.01$. **(D)** N2a cells were transfected either with Flag-empty and GFP-empty (Flag + GFP), α-synuclein and GFP-empty, Flag-Tau and GFP-empty, Flag-Tau and GFP-VAMP8 plasmids. Representative Western blot of the cell lysate revealed with the antibodies recognizing Tau (A0024), VAMP8, GFP, α-synuclein, caspase-3, cleaved caspase-3 and actin. No difference of total caspase-3 levels was noted between the experimental conditions. In the case of cleaved by caspase-3, the condition Tau and VAMP8 presented the highest levels. For the densitometry analysis of the signal of caspase-3 and cleaved caspase-3 was normalized with the actin signal, the loading reference. Black frames were used to mark the splice sites of immunoblot images. $n = 5$. Data represent scatter plot and mean±SEM. $^{**}p < 0.01$.

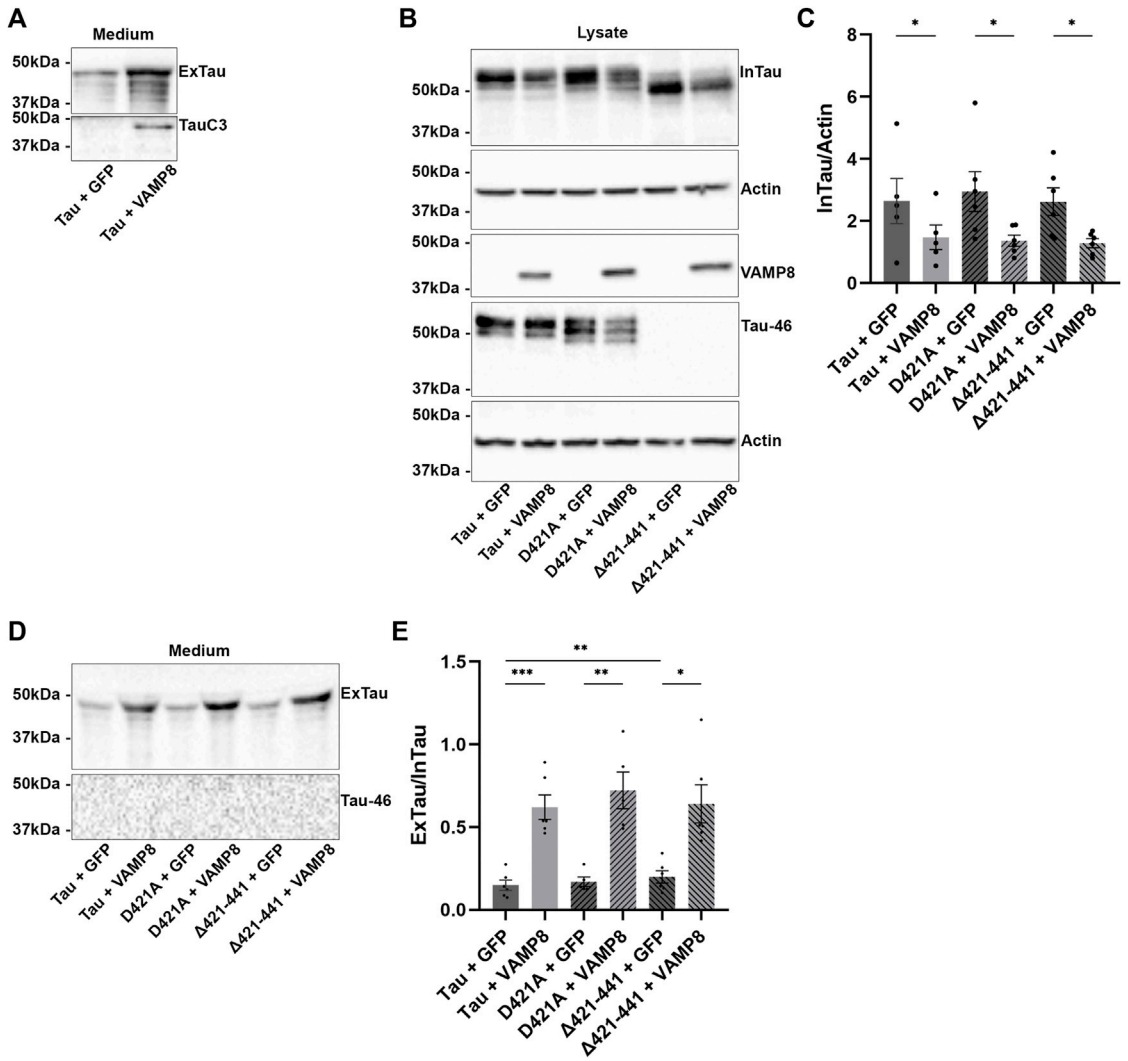


FIGURE 2
Secretion of Tau upon VAMP8 overexpression does not depend on the cleavage of Tau by caspase-3. N2a cells were transfected either with Flag-Tau and GFP-empty, Flag-Tau and GFP-VAMP8, TauD421A and GFP-empty, TauD421A and GFP-VAMP8, TauΔ421-441 and GFP-empty or TauΔ421-441 and GFP-VAMP8 plasmids for 48 h. **(A)** Representative Western blot with TauC3 antibody of the medium showing the detection of Tau cleaved by caspase-3 only detectable with VAMP8 overexpression. **(B)** Representative Western blot with A0024 of the cell lysate showing that the overexpression of VAMP8 decreased intracellular Tau (InTau) of all Tau mutants. **(C)** Densitometry analysis of A0024 signal of InTau. $n = 6$. Data represent scatter plot and mean±SEM. $^{*}p < 0.05$. **(D)** Representative Western blot with A0024 of the medium showing that the overexpression of VAMP8 increased extracellular Tau (ExTau) of all Tau mutants. **(E)** Densitometry analysis of A0024 signal of the ExTau/InTau ratio. $n = 6$. Data represent scatter plot and mean±SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

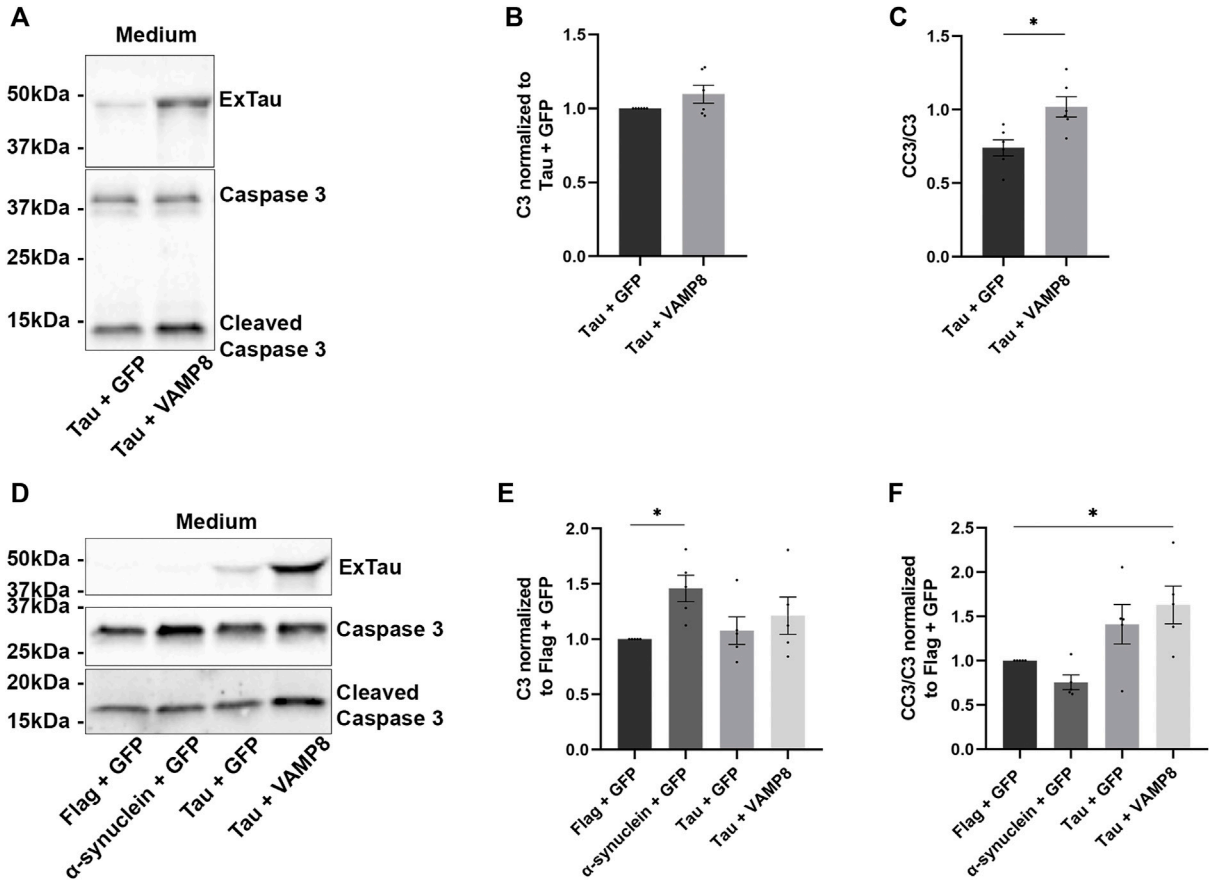


FIGURE 3
Increased secretion of active caspase-3 upon the overexpression of Tau and VAMP8. **(A)** N2a cells were transfected either with Flag-Tau and GFP-empty or Flag-Tau and GFP-VAMP8 plasmids for 48 h. Representative Western blot with A0024 for staining of extracellular Tau (ExTau), the antibody C3 for total caspase-3 and the antibody CC3 for cleaved caspase-3 of the medium revealing that the secretion of total caspase 3 was similar with Tau alone or Tau and VAMP8 overexpression and that cleaved caspase-3 was increased for Tau and VAMP8. **(B,C)** Densitometry analysis of C3 and CC3/C3 ratio in the medium. The signal of C3 and CC3 was normalized with that of Tau and GFP. $n = 6$. Data represent scatter plot and mean ± SEM. *$p < 0.05$. **(D)** N2a cells were co-transfected either with Flag-empty and GFP-empty, α-synuclein and GFP-empty, Flag-Tau and GFP-empty or Flag-Tau and GFP-VAMP8 plasmids. Representative Western blot of the medium to confirm the secretion of Tau, caspase-3 and cleaved caspase-3. **(E,F)** Densitometry analysis of C3 and CC3 signals normalized to that of Flag-empty + GFP-empty. The secretion of CC3 in the medium of Flag-tau and GFP-VAMP8 was significantly higher than that detected in Flag-empty + GFP-empty. N = 5. Data represent scatter plot and mean±SEM. *$p < 0.05$.

Secreted Tau induced by VAMP8 overexpression was cleaved at the C-terminal as revealed by the lack of staining with the anti-Tau antibody, Tau46 that does not recognize Tau cleaved between the 404–441 amino acids located at its C-terminal (Figure 1A). Full-length intracellular Tau was recognized by the antibody Tau46 but no signal was detected in the medium revealing that secreted Tau was cleaved at the C-terminal (Pilliod et al., 2020). In the present study, we examined the intracellular and extracellular forms of cleaved Tau upon VAMP8 overexpression. N2a cells were co-transfected either with Flag-Tau and GFP-empty or Flag-Tau and GFP-VAMP8 plasmids as previously described (Pilliod et al., 2020). Interestingly, an increase of Tau cleaved by caspase-3 was observed in the cell lysate of cells overexpressing VAMP8 indicating that caspase-3 was activated in these cells (Figure 1B). To demonstrate that it was the case, we examined the protein levels of total caspase-3 and cleaved caspase-3, its active form, in the cell lysate of cells overexpressing either Tau alone or Tau and VAMP8. The amount of total caspase-3 was similar in the cell lysate of cells overexpressing either Tau alone or Tau and VAMP8, but a significant increase of cleaved caspase-3 was observed in cells overexpressing Tau and VAMP8 compared to cells overexpressing Tau alone (Figure 1C). This increase of active caspase-3 in the lysate of cells overexpressing Tau and VAMP8 was consistent with the increase of Tau cleaved by caspase-3 in these cells. We then examined whether the increase of

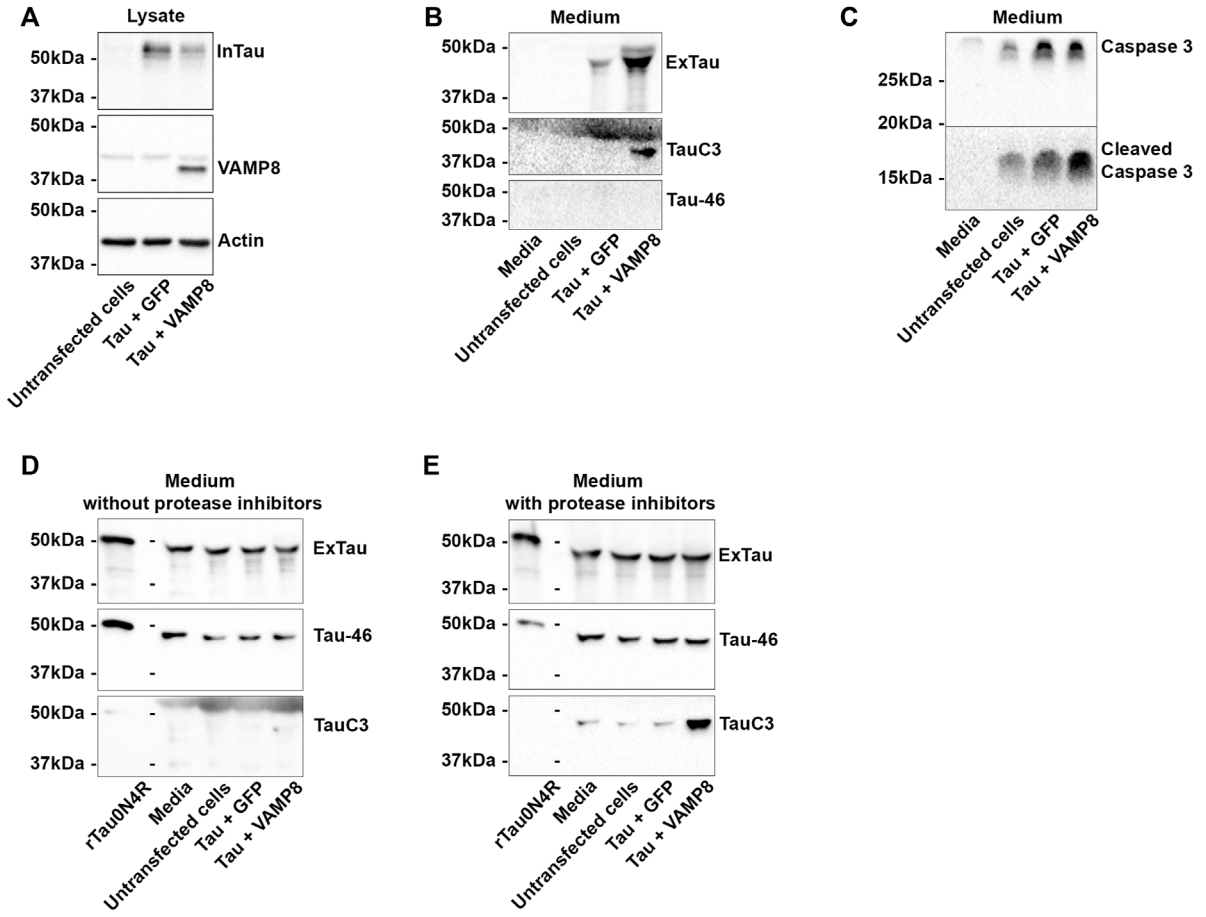


FIGURE 4
Extracellular cleavage of Tau by caspase-3. N2a cells were transfected either with Flag-Tau and GFP-empty or Flag-Tau and GFP-VAMP8 plasmids. Recombinant 0N4R Tau protein (rTau) was added in media after 48 h post transfection. The medium without cells and the medium of untransfected cells were used as controls. **(A)** Representative Western blot of the cell lysate confirming the expression of Tau and VAMP8. **(B)** Representative Western blot of the medium showing the secretion of Tau and Tau cleaved by caspase-3 (TauC3). The lack of signal with the anti-Tau antibody Tau-46 indicated that Tau was cleaved at the C-terminal. **(C)** Representative Western blot with anti-caspase-3 and the anti-cleaved caspase-3 antibodies to confirm their presence in the medium before the addition of rTau. **(D)** Representative Western blot with the anti-Tau antibodies A0024, TauC3 and Tau-46 after addition of rTau showing that the presence of Tau full-length as revealed by Tau46 antibody. A weak band was detected with TauC3 indicating cleavage of rTau by caspase-3. **(E)** Representative Western blot with the anti-Tau antibodies A0024, TauC3 and Tau-46 after addition of rTau and protease inhibitors showing the important increase of TauC3 signal. N = 3.

active caspase-3 was specific to Tau and VAMP8 overexpression. To do so, cells were co-transfected either with GFP-empty and Flag-empty (the empty plasmids of VAMP8 and Tau, respectively), GFP-empty and α-synuclein (a secreted protein linked to Parkinson's disease) or Tau and VAMP8 (Figure 1D). No difference between these different conditions was noted in the amount of total caspase-3. In the case of active caspase-3, only the condition Tau and VAMP8 was statistically different from the control condition, GFP-empty and Flag-empty.

We then examined whether secreted Tau was also cleaved by caspase-3 upon VAMP8 overexpression. As noted for intracellular Tau, extracellular Tau was cleaved by caspase-3 (Figure 2A). No Tau cleaved by caspase-3 was detected in the medium of cells overexpressing Tau alone. The LDH was measured to monitor cell death. No difference was noted between the different conditions indicating that extracellular Tau was not released by cell death (Supplementary Figure S1).

Tau cleavage by caspase-3 is not necessary for its secretion upon VAMP8 overexpression

The above results prompted us to examine whether cleavage of Tau by caspase-3 was necessary for Tau secretion upon VAMP8 overexpression. To test this, a Tau mutant either mimicking Tau cleavage by caspase-3 (Δ421-441) or not cleavable by caspase-3 (D421A) was produced and co-expressed

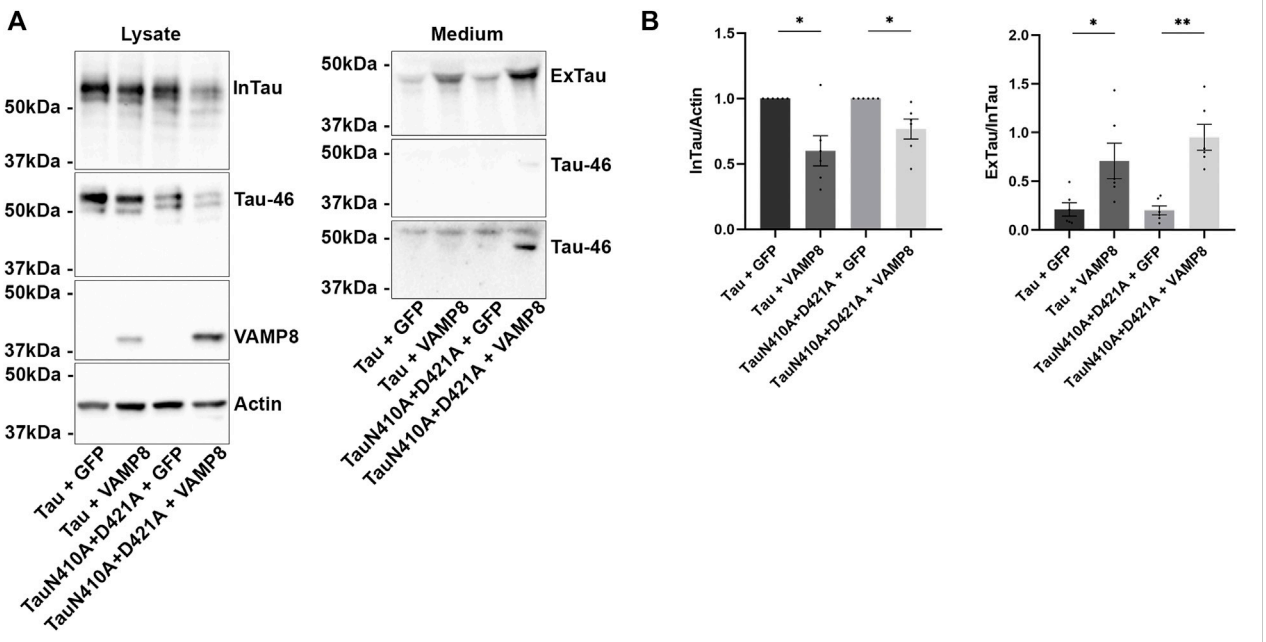


FIGURE 5
The asparagine N410 affects the cleavage of secreted Tau upon VAMP8 overexpression. N2a cells were transfected either with Flag-Tau and GFP-empty, Flag-Tau and GFP-VAMP8, TauN410A+D421A and GFP-empty or TauN410A+D421A and GFP-VAMP8 plasmids for 48 h. **(A)** Representative Western blot with A0024 of the cell lysate and the medium revealing that the overexpression of VAMP8 decreased intracellular Tau (InTau) and increased extracellular Tau (ExTau) for both Tau mutant and wild-type. A signal was detected with the anti-Tau antibody Tau46 in both the cell lysate and medium indicating the presence of uncleaved Tau at the C-terminal. A short and a long exposure times are presented to show that the Tau46 antibody signal was not detected when wild-type Tau was expressed. **(B)** Densitometry analysis of A0024 signal of InTau. Actin was used as a loading reference. Densitometry analysis of A0024 signal of ExTau. Normalized Tau secretion corresponds to the ratio ExTau/InTau. InTau was normalized with the actin signal. N = 6. Data represent scatter plot and mean±SEM. *$p < 0.05$. **$p < 0.01$.

with VAMP8 in N2a cells. The expression of these Tau mutants was confirmed by WB (Figure 2B). The antibody Tau46 did not reveal the mutant Δ421-441 as expected (Figure 2B). A decrease of intracellular D421A and Δ421-441was noted when VAMP8 was overexpressed as observed for wild-type Tau (Figures 2B,C). Consistent with this, an increase of extracellular D421A and Δ421-441 similar to that of wild-type Tau was found upon VAMP8 overexpression (Figure 2D). As noted in our previous study, Tau mutant mimicking its cleavage by caspase-3 was more secreted than wild-type Tau although this effect was less important than that previously observed in Hela cells (Figures 2D,E) (Plouffe et al., 2012). Interestingly, the mutant non-cleavable by caspase-3, D421A, was cleaved at the C-terminal by another protease as revealed by the lack of staining with the Tau46 antibody (Figure 2D). These results demonstrated that cleavage by caspase-3 was not necessary for Tau secretion by VAMP8.

Extracellular cleavage of tau by caspase-3 upon VAMP8 overexpression

The fact that cleavage of Tau by caspase-3 was not necessary for its secretion by VAMP8 prompted us to examine the possibility that Tau could be extracellularly cleaved by caspase-3. Indeed, caspase-3 was previously shown to be secreted by cells (Garcia-Faroldi et al., 2013; Zorn et al., 2013). We examined the amount of total caspase-3 and cleaved caspase-3 in the medium. The amount of total caspase-3 in the medium was similar for cells either overexpressing Tau alone or Tau and VAMP8 (Figures 3A,B). In the case of active caspase-3, the cells overexpressing Tau and VAMP8 presented higher levels than the cells overexpressing Tau alone (Figure 3C). We then verified whether the increase of active caspase-3 in the medium was specific to Tau and VAMP8. To do so, we compared the amount of total caspase-3 and active caspase-3 in the medium of cells co-overexpressing the control plasmids (GFP-empty and Flag-empty), GFP-empty and α-synuclein, Flag-Tau and GFP-empty and Flag-Tau and GFP-VAMP8. The amount of total caspase-3 was similar to that of the control plasmids for all the experimental conditions except for the condition α-synuclein and GFP-empty presenting a higher amount than control plasmids (Figures 3D,E). In the case of active caspase-3, only the condition Tau and VAMP8 was statistically different from the control plasmids (Figures 3D,F). The fact that caspase-3 and its active form could be found in the medium of N2a cells indicated that Tau cleavage could occur in the medium.

To further demonstrate that Tau cleavage by caspase-3 could occur in the medium, recombinant human Tau (0N4R) (rTau)

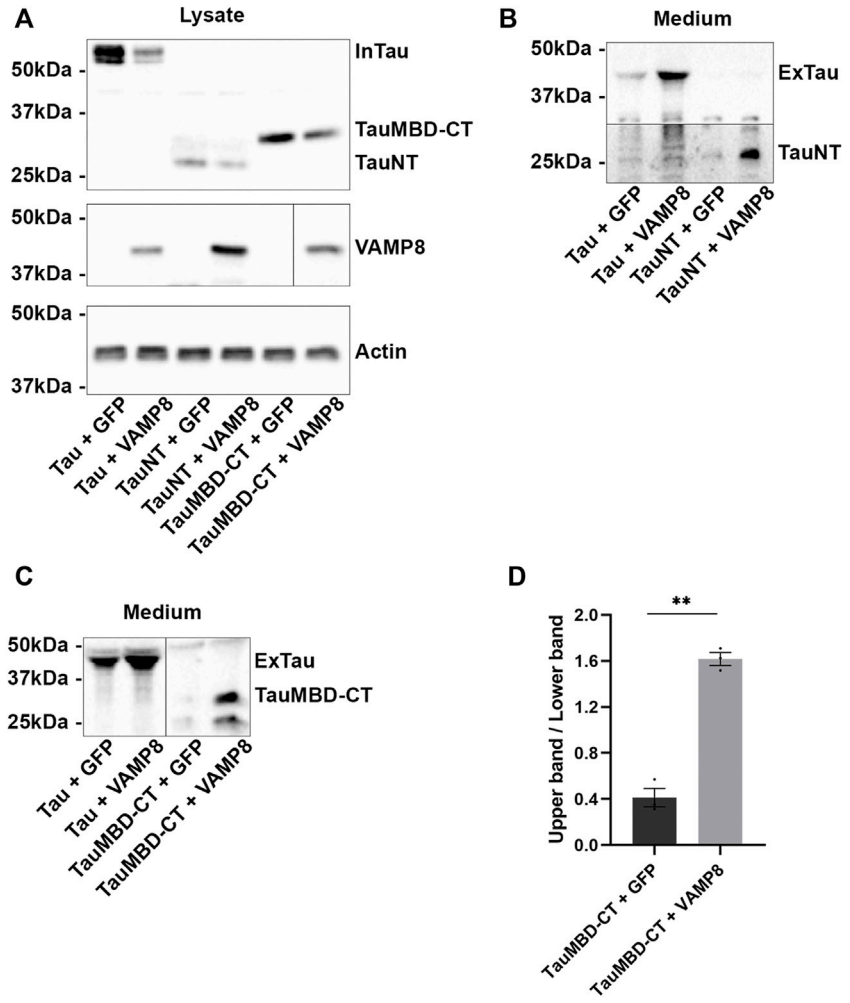


FIGURE 6
Secretion of Tau mediated by VAMP8 is independent of C-terminal cleavage. N2a cells were transfected either with Flag-Tau and GFP-empty, Flag-Tau and GFP-VAMP8, Flag-TauNT and GFP-empty, Flag-TauNT and GFP-VAMP8, Flag-TauMBD-CT and GFP-empty or Flag-TauMBD-CT and GFP-VAMP8 plasmids for 48 h. **(A)** Representative Western blot with the anti-FLAG antibody for intracellular signal of Tau, TauNT and TauMBD-CT. **(B)** Representative Western blot with the anti-Tau antibody HT7 to reveal extracellular TauNT. **(C)** Representative Western blot with the anti-Tau antibody A0024 to reveal extracellular TauMBD-CT. **(D)** Densitometry analysis of the upper band/lower band ratio for TauMBD-CT. Black frames were used to mark the splice sites of immunoblot images. $n = 3$. Data represent scatter plot and mean±SEM. $^{**}p < 0.01$.

was added either to the medium without cells, medium of untransfected cells and medium of cells transfected either with Tau alone or Tau and VAMP8. Two sets of experiments were carried out. In the first set, the medium was collected after 48 h of transfection and transferred to a petri dish without cells. Transfection of Tau and VAMP8 and Tau secretion were confirmed by WB (Figures 4A,B). The presence of caspase-3 and cleaved caspase-3 in the medium was also confirmed by WB (Figure 4C). For all the experiments, rTau (1.5 µg) was added for 12 h in the medium. Most of the signal detected by the anti-Tau antibody A0024 in the medium corresponded to rTau. Indeed, the signal was stronger when rTau was added compared to that obtained for medium only containing secreted Tau when the membranes were revealed side by side (Supplementary Figure S2). Furthermore, the fact that the intensity of Tau signal was similar in medium containing or not secreted Tau also indicated that most of the anti-Tau antibody signal corresponded to rTau. Lastly, the signal of the anti-Tau antibody Tau46 in the medium, which did not reveal secreted Tau, confirmed that the main signal was generated by rTau. FL-Tau revealed by the anti-Tau antibodies, Tau46 and A0024, and fragments of rTau mainly detected with the antibody A0024 were observed (Figure 4D). This indicated that some degradation and/or cleavage had occurred in the medium. A weak staining with the anti-Tau antibody TauC3 recognizing Tau cleaved by caspase-3 was noted in the medium collected from cells overexpressing Tau and

VAMP8, which contained the highest levels of active caspase-3 as shown in Figure 3C (Figure 4D). The number of Tau bands was higher in the medium obtained either from untransfected cells, cells overexpressing Tau or cells overexpressing Tau and VAMP8 than in the medium not incubated with cells. This indicated that proteases were released by cells with and without transfection. In the second set of experiments, rTau and protease inhibitors were added to the medium. The protease inhibitors were directed against metalloproteases and serine and cystein proteases (Roche Diagnostics). Interestingly, a lower number of Tau-positive bands was detected in the medium indicating that Tau was less cleaved by these proteases (Figure 4E). Furthermore, this was correlated to an increase of TauC3 staining in the medium collected from the cells overexpressing Tau and VAMP8. This indicated that Tau was cleaved by caspase-3 in the medium as well as by metalloproteases and/or cysteine and serine proteases.

Asparagine 410 affects the cleavage of secreted tau upon VAMP8 overexpression

Tau was still cleaved in the medium when the site of caspase-3 was mutated to prevent its cleavage as revealed by the lack of staining with the antibody Tau46 for this mutant (Figure 2D). Our previous study revealed that secreted Tau could be cleaved at a site in close vicinity to the phosphorylation site S409 (Plouffe et al., 2012). Tau is known to be cleaved by asparagine endopeptidase (AEP) at the C-terminal (Zhang et al., 2014). Based on this, the asparagine 410 (N410), which could be a potential cleavage site by AEP, was mutated to alanine to prevent its cleavage in the mutant non-cleavable by caspase-3 (N410A+D421A). Interestingly, a recent study reported that N410 can be glycosylated and can modulate Tau pathology (Losev et al., 2021). The secretion of N410A+D421A mutant was tested in N2a cells upon VAMP8 expression. Its secretion was similar to that of wild-type Tau indicating that the cleavage and glycosylation of N410 was not necessary for Tau secretion (Figures 5A,B). In contrast to Tau mutant only resistant to cleavage by caspase-3 (D421A), a weak band reactive to the antibody Tau46 was detected in the medium of N410A+D421A mutant indicating that a portion of secreted Tau was not cleaved at the C-terminal upon VAMP8 overexpression. The above results revealed that the N410 could alter the cleavage of Tau found in the medium upon VAMP8 overexpression.

N-terminal deletion of tau modifies its pattern of secretion upon VAMP8 overexpression

The above results revealed that secreted Tau could be cleaved at the C-terminal by different proteases, but these cleavage events did not have significant impact on Tau secretion upon VAMP8 overexpression. We then asked whether the N-terminal deletion could exert regulatory effects on Tau secretion by VAMP8. To investigate this point, we produced two Tau mutants, one containing Tau N-terminal (TauNT) and one containing Tau microtubule-binding domain and its C-terminal (TauMBD-CT) (Figure 1A). The secretion of TauNT was increased upon VAMP8 overexpression as noted for FL-Tau (Figures 6A,B). Its pattern was similar to that of FL-Tau meaning one main band was detected in the culture medium. Interestingly, the secretion pattern of TauMBD-CT was different upon VAMP8 overexpression compared to that of its overexpression alone (Figure 6C). When it was overexpressed in the absence of VAMP8, two bands were detected in the medium, a very weak upper band and a stronger lower band as revealed with the anti-Tau antibody A0024. Upon the overexpression of VAMP8, this pattern was inverted (Figures 6C,D). Collectively, the above observations indicate that the N-terminal has an effect of the pattern of Tau secretion upon VAMP8 overexpression since its deletion resulted in a different pattern of TauMBD-CT secretion.

Discussion

In the present study, we examined whether the increase of Tau secretion by VAMP8 affected its intra- and extracellular cleavage. Upon VAMP8 overexpression, an increase of Tau cleaved by caspase-3 in the cell lysate was observed. This increase was correlated to an increase of active caspase-3. Using a Tau mutant not cleavable by caspase-3, we demonstrated that Tau cleavage by caspase-3 was not necessary for its secretion upon VAMP8 overexpression. We also demonstrated that Tau cleavage by caspase-3 could occur extracellularly because of the secretion of active caspase-3 by cells overexpressing Tau and VAMP8. Our results also revealed that N410 affected the cleavage of Tau released upon VAMP8 overexpression. Lastly, we observed that the N-terminal of Tau regulated the secretion of a Tau fragment containing the microtubule-binding domain and C-terminal upon VAMP8 overexpression.

Our results demonstrated that the cells overexpressing VAMP8 presented the highest levels of active caspase-3 in the cell lysate. A previous study reported that the protein levels of VAMP8 was regulated by caspases in dendritic cells (Ho et al., 2009). The inhibition of caspases increased its protein levels. Based on this, the increase of active caspase-3 observed in our experimental conditions could be a protective reaction to prevent an excessive overexpression of VAMP8. This increase of active caspase-3 was correlated to an enhanced cleavage of Tau in the cell lysate. In most studies, Tau cleaved by caspase-3 was found to be detrimental to neurons and to contribute to Tau pathology (Means et al., 2016; Cieri et al., 2018). It was also associated with the progression of AD (Basurto-Islas et al., 2008; Jarero-Basulto

et al., 2013; Zhou et al., 2018). However, recent studies indicate that Tau cleavage by caspase-3 could be neuroprotective. A study reported that at the early stages of AD, caspase-3 was activated without leading to neuronal cell death (de Calignon et al., 2010). More recently, a study demonstrated that in mice, blocking Tau cleavage by caspase-3 resulted in memory deficits (Biundo et al., 2017). In *Drosophila*, caspase 3 cleavage of hyperphosphorylated Tau prevented its toxicity and allowed recovery of motor deficits (Chi et al., 2020). From these results, it appears that the increased cleavage of Tau by caspase-3 upon VAMP8 overexpression could be neuroprotective. In such a case, VAMP8 would decrease toxicity of intracellular Tau by this cleavage.

We found that VAMP8 increased the secretion of active caspase-3. It was previously demonstrated that removing active caspase-3 through lysosomal degradation was linked to an increase of survival of neuronal cells induced by NGF (Mnich et al., 2014). VAMP8-induced secretion of active caspase-3 was also shown to be protective in the pancreatic acinar cells. The acute inhibition of VAMP8-mediated secretion resulted in the intracellular accumulation of trypsin causing acinar cell damages during pancreatitis (Messenger et al., 2017). Based on these observations, one can postulate that VAMP8 could be part of an unconventional secretory pathway that is beneficial to cells by eliminating proteins that can become toxic and compromise cell survival. Active caspase-3 released upon VAMP8 overexpression seemed to be functional as indicated by the cleavage of rTau. In a previous study, extracellular caspase-3 released by mouse mast cells through secretory lysosomes was reported to be able to cleave interleukin-33 (Zorn et al., 2013). It was proposed that extracellular active caspase-3 could be involved in the processing of cytokines and thereby contributed to the inflammatory response (Garcia-Faroldi et al., 2013). Surprisingly, active caspase-3 was found in the medium of all the conditions that we tested. Furthermore, as revealed by adding protease inhibitors against metalloproteases and serine and cystein proteases in the medium, the number of Tau fragments was reduced indicating that these proteases were present in the medium. This should be considered when examining protease cleavage of extracellular proteins. The present results on Tau cleavage occurring extracellularly does not fit with a previous study reporting no cleavage of Tau in the culture medium of primary neuronal cultures (Kanmert et al., 2015). The authors did not observe any change in the pattern of secreted Tau forms when protease inhibitors were added to the culture medium. The release of proteases could depend on the type of cells. N2a, neuroblastoma cells, might release more proteases than primary neurons. In AD, endosomes were reported to accumulate in the early stages of the disease. Our results with VAMP8 indicate that in such a condition, the release of proteases could be increased, which could contribute to the presence of Tau fragments in the CSF. Indeed, the presence of caspase activity was reported in CSF of patients suffering from dementia and traumatic brain injury (Harter et al., 2001; Albrecht et al., 2009) (Perez-Barcena et al., 2022).

Upon VAMP8 overexpression, FL-Tau and N- and C-terminal fragments of Tau could be released. This corroborates previous studies that reported the release of C-terminal truncated form of Tau by primary neuronal cultures and neurons produced iPSC (Bright et al., 2015; Kanmert et al., 2015). The released of fragments containing the microtubule-binding domain and the C-terminal is more controversial. The lack of detection of such fragments in previous studies could be explained by the fact that the anti-Tau antibody Tau46 was used either for detection by ELISA or for immunoprecipitation in these studies (Bright et al., 2015; Kanmert et al., 2015). In our experiments, this antibody could not detect secreted Tau because of its cleavage at the C-terminal. The mechanisms underlying the pattern of secreted tau fragments remain poorly characterized. In the case of VAMP8-induced secretion, a positive weak signal with the antibody Tau46 in the medium was only detected when N410 was mutated in alanine indicating that this site affected the cleavage of tau at the C-terminal. N410 could be cleaved by an asparagine endopeptidase (Zhang et al., 2014). However, this cleavage remains to be demonstrated. In a recent study, it was reported that N410 can be glycosylated and that preventing it worsened tau pathology (Losev et al., 2021). Our study confirmed the role of this site in tau processing. Our results also demonstrated that the N-terminal had regulatory effects on VAMP8-induced tau secretion given that the secretion pattern of TauMBD-CT, a Tau fragment containing the microtubule binding domain and the C-terminal was different when VAMP8 was overexpressed. In a previous study, it was reported that the N-terminal was necessary for Tau secretion in lamprey (Kim et al., 2010). In this previous study, it was also noted that exon 2 present in the N-terminal exerted inhibitory effects on Tau secretion. Such effects were not confirmed in cultured cells where Tau isoforms containing exon 2 were secreted (Karch et al., 2012). Collectively, the above observations indicate that both the N- and C-terminal can regulate Tau secretion.

Several unconventional secretory pathways are involved in Tau secretion (Mohamed et al., 2013; Pernegre et al., 2019). Our previous studies demonstrated that late endosomes are involved in Tau secretion. We reported that Rab7A, GTPase associated with late endosomes, and VAMP8, a R-SNARE attached to late endosomes, were involved in Tau secretion (Rodriguez et al., 2017; Pilliod et al., 2020). The contribution of VAMP8 to Tau secretion was observed in N2a and neurons. By TIRF microscopy, we observed a depletion of Tau in the cytoplasm upon the fusion of VAMP8-positive vesicles with the plasma membrane (Pilliod et al., 2020). Other proteins involved in the endocytic pathways such as Bin1 and the two SNAREs, syntaxins 6 and 8 also contribute to Tau secretion (Glennon et al., 2020; Lee et al., 2021). It seems possible that

each of these pathways could permit the release of a specific set of Tau forms. VAMP8 would induce the release of Tau forms cleaved at the C-terminal. FL-Tau and N- and C-terminal truncated forms were detected in the CSF and culture medium of non-neuronal and neuronal cells (Plouffe et al., 2012; Mohamed et al., 2013; Pooler et al., 2013; Mohamed et al., 2014; Bright et al., 2015; Kanmert et al., 2015; Mohamed et al., 2017; Rodriguez et al., 2017; Pernegre et al., 2019). Exosomes were shown to contain both FL-Tau and C- and N-terminal truncated Tau (Saman et al., 2012; Simon et al., 2012; Dujardin et al., 2014; Guix et al., 2018). In the case of phosphorylation, most studies reported that extracellular membrane-free Tau was less phosphorylated than intracellular Tau (Mohamed et al., 2013; Pernegre et al., 2019). Some discrepancies exist in the literature concerning the phosphorylation levels of exosomal Tau. Indeed, high and low levels were reported (Saman et al., 2012; Wang et al., 2017). Tau oligomers were found to be secreted by translocation across the plasma membrane as well as by exosomes (Saman et al., 2012; Asai et al., 2015; Wang et al., 2017; Merezhko et al., 2018). No Tau aggregates were found to be released by an active process of secretion in the medium where Tau was membrane-free (Kanmert et al., 2015). All together, the above observations indicate that experimental conditions and/or cell types can influence the Tau forms that are released.

Tau secretion could be a mechanism for clearance of Tau, meaning that it is beneficial to neurons by removing toxic forms of Tau. This is supported by recent studies where Tau secretion was shown to reverse of Tau-induced cellular alterations. When Tau secretion was decreased because of Bin1 loss, it resulted in an accumulation of Tau and synaptic dysfunction (Glennon et al., 2020). We showed that the increase of Tau secretion by VAMP8 could reverse the microtubule stability induced by the overexpression of Tau in N2a cells (Pilliod et al., 2020). The different unconventional pathways involved in Tau secretion permit the release of diverse Tau species. It remains to be determined which of these pathways allows the release of toxic forms to prevent their accumulation in neurons and which forms of Tau are toxic in the extracellular space. This information is determinant to elaborate a therapeutic strategy to prevent both the intracellular and extracellular accumulation of toxic Tau species. Tau secretion could be used to increase the accessibility of intracellular Tau species involved in the neurodegenerative process that takes place in AD and FTLD. These species could then be neutralized by a therapeutic agent. Indeed, several undergoing clinical trials target extracellular Tau using an anti-Tau antibody to sequester its toxic species (Jadhav et al., 2019). A therapy combining the increase of Tau secretion with the capture of extracellular toxic Tau species by an antibody could be an efficient approach to prevent the intracellular accumulation of pathological Tau and its propagation in the brain.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

Author contributions

JP was involved conceptually and technically in all the experiments included in the manuscript. MG-F performed the experiments of tau mutant not cleavable by asparagine endopeptidase. JP, MG-F, and NL were involved in the analysis and interpretation of the data. JP and NL contributed to the preparation and writing of the manuscript. All the authors read and approved the final manuscript.

Funding

This work was supported by the Canadian Institute of Health Research (CIHR) grant (PJT-155993).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.912118/full#supplementary-material

SUPPLEMENTARY FIGURE S1
LDH data for the experiments on Tau secretion. LDH was measured in the medium for monitoring cell death. The LDH differences (%) between the control condition and the experimental conditions was calculated.

SUPPLEMENTARY FIGURE S2
Comparaison of the signal of secreted Tau and recombinant Tau in the medium. The signal of Tau in the medium revealed by the anti-Tau antibody A0024 was less intense than the signal detected after rTau was added to the medium. The two membranes were revealed side by side.

References

Alafuzoff, I., Iqbal, K., Friden, H., Adolfsson, R., and Winblad, B. (1987). Histopathological criteria for progressive dementia disorders: Clinical-pathological correlation and classification by multivariate data analysis. *Acta Neuropathol.* 74, 209–225. doi:10.1007/BF00688184

Albrecht, P., Schmitz, M., Otto, M., Hemmer, B., Zerr, I., Hartung, H. P., et al. (2009). Free caspase activity in CSF of patients with dementia. *J. Neurol.* 256, 1561–1562. doi:10.1007/s00415-009-5145-z

Antonin, W., Holroyd, C., Tikkanen, R., Honing, S., and Jahn, R. (2000). The R-SNARE endobrevin/VAMP-8 mediates homotypic fusion of early endosomes and late endosomes. *Mol. Biol. Cell* 11, 3289–3298. doi:10.1091/mbc.11.10.3289

Arriagada, P., Growdon, J., Hedley-Whyte, E., and Hyman, B. (1992). Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology* 42, 631–639. doi:10.1212/wnl.42.3.631

Asai, H., Ikezu, S., Tsunoda, S., Medalla, M., Luebke, J., Haydar, T., et al. (2015). Depletion of microglia and inhibition of exosome synthesis halt tau propagation. *Nat. Neurosci.* 18, 1584–1593. doi:10.1038/nn.4132

Basurto-Islas, G., Luna-Munoz, J., Guillozet-Bongaarts, A. L., Binder, L. I., Mena, R., and Garcia-Sierra, F. (2008). Accumulation of aspartic acid421- and glutamic acid391-cleaved tau in neurofibrillary tangles correlates with progression in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 67, 470–483. doi:10.1097/NEN.0b013e31817275c7

Bierer, L., Hof, P., Purohit, D., Carlin, L., Schmeidler, J., Davis, K., et al. (1995). Neocortical neurofibrillary tangles correlate with dementia severity in Alzheimer's disease. *Arch. Neurol.* 52, 81–88. doi:10.1001/archneur.1995.00540250089017

Biundo, F., d'Abramo, C., Tambini, M. D., Zhang, H., Del Prete, D., Vitale, F., et al. (2017). Abolishing Tau cleavage by caspases at Aspartate(421) causes memory/synaptic plasticity deficits and pre-pathological Tau alterations. *Transl. Psychiatry* 7, e1198. doi:10.1038/tp.2017.165

Braak, H., and Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol.* 82, 239–259. doi:10.1007/BF00308809

Bright, J., Hussain, S., Dang, V., Wright, S., Cooper, B., Byun, T., et al. (2015). Human secreted tau increases amyloid-beta production. *Neurobiol. Aging* 36, 693–709. doi:10.1016/j.neurobiolaging.2014.09.007

Cairns, N. J., Bigio, E. H., Mackenzie, I. R., Neumann, M., Lee, V. M., Hatanpaa, K. J., et al. (2007). Neuropathologic diagnostic and nosologic criteria for frontotemporal lobar degeneration: Consensus of the consortium for frontotemporal lobar degeneration. *Acta Neuropathol.* 114, 5–22. doi:10.1007/s00401-007-0237-2

Chi, H., Sun, L., Shiu, R. H., Han, R., Hsieh, C. P., Wei, T. M., et al. (2020). Cleavage of human tau at Asp421 inhibits hyperphosphorylated tau induced pathology in a Drosophila model. *Sci. Rep.* 10, 13482. doi:10.1038/s41598-020-70423-1

Cieri, D., Vicario, M., Vallese, F., D'Orsi, B., Berto, P., Grinzato, A., et al. (2018). Tau localises within mitochondrial sub-compartments and its caspase cleavage affects ER-mitochondria interactions and cellular Ca(2+) handling. *Biochim. Biophys. Acta. Mol. Basis Dis.* 1864, 3247–3256. doi:10.1016/j.bbadis.2018.07.011

de Calignon, A., Fox, L. M., Pitstick, R., Carlson, G. A., Bacskai, B. J., Spires-Jones, T. L., et al. (2010). Caspase activation precedes and leads to tangles. *Nature* 464, 1201–1204. doi:10.1038/nature08890

Dujardin, S., Begard, S., Caillierez, R., Lachaud, C., Delattre, L., Carrier, S., et al. (2014). Ectosomes: A new mechanism for non-exosomal secretion of tau protein. *PLoS One* 9, e100760. doi:10.1371/journal.pone.0100760

Garcia-Faroldi, G., Melo, F. R., Ronnberg, E., Grujic, M., and Pejler, G. (2013). Active caspase-3 is stored within secretory compartments of viable mast cells. *J. Immunol.* 191, 1445–1452. doi:10.4049/jimmunol.1300216

Glennon, E. B., Lau, D. H., Gabriele, R. M. C., Taylor, M. F., Troakes, C., Opie-Martin, S., et al. (2020). Bridging Integrator-1 protein loss in Alzheimer's disease promotes synaptic tau accumulation and disrupts tau release. *Brain Commun.* 2, fcaa011. doi:10.1093/braincomms/fcaa011

Guix, F. X., Corbett, G. T., Cha, D. J., Mustapic, M., Liu, W., Mengel, D., et al. (2018). Detection of aggregation-competent tau in neuron-derived extracellular vesicles. *Int. J. Mol. Sci.* 19, E663. doi:10.3390/ijms19030663

Hampel, H., Blennow, K., Shaw, L. M., Hoessler, Y. C., Zetterberg, H., and Trojanowski, J. Q. (2010). Total and phosphorylated tau protein as biological markers of Alzheimer's disease. *Exp. Gerontol.* 45, 30–40. doi:10.1016/j.exger.2009.10.010

Harter, L., Keel, M., Hentze, H., Leist, M., and Ertel, W. (2001). Caspase-3 activity is present in cerebrospinal fluid from patients with traumatic brain injury. *J. Neuroimmunol.* 121, 76–78. doi:10.1016/s0165-5728(01)00409-x

Ho, Y. H., Cai, D. T., Huang, D., Wang, C. C., and Wong, S. H. (2009). Caspases regulate VAMP-8 expression and phagocytosis in dendritic cells. *Biochem. Biophys. Res. Commun.* 387, 371–375. doi:10.1016/j.bbrc.2009.07.028

Iqbal, K., Liu, F., and Gong, C. X. (2016). Tau and neurodegenerative disease: The story so far. *Nat. Rev. Neurol.* 12, 15–27. doi:10.1038/nrneurol.2015.225

Ismael, S., Sindi, G., Colvin, R. A., and Lee, D. (2021). Activity-dependent release of phosphorylated human tau from Drosophila neurons in primary culture. *J. Biol. Chem.* 297, 101108. doi:10.1016/j.jbc.2021.101108

Itakura, E., Kishi-Itakura, C., and Mizushima, N. (2012). The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. *Cell* 151, 1256–1269. doi:10.1016/j.cell.2012.11.001

Jadhav, S., Avila, J., Scholl, M., Kovacs, G. G., Kovari, E., Skrabana, R., et al. (2019). A walk through tau therapeutic strategies. *Acta Neuropathol. Commun.* 7, 22. doi:10.1186/s40478-019-0664-z

Jarero-Basulto, J. J., Luna-Munoz, J., Mena, R., Kristofikova, Z., Ripova, D., Perry, G., et al. (2013). Proteolytic cleavage of polymeric tau protein by caspase-3: Implications for alzheimer disease. *J. Neuropathol. Exp. Neurol.* 72, 1145–1161. doi:10.1097/NEN.0000000000000013

Jung, J. J., Inamdar, S. M., Tiwari, A., and Choudhury, A. (2012). Regulation of intracellular membrane trafficking and cell dynamics by syntaxin-6. *Biosci. Rep.* 32, 383–391. doi:10.1042/BSR20120006

Kanmert, D., Cantlon, A., Muratore, C. R., Jin, M., O'Malley, T. T., Lee, G., et al. (2015). C-terminally truncated forms of tau, but not full-length tau or its C-terminal fragments, are released from neurons independently of cell death. *J. Neurosci.* 35, 10851–10865. doi:10.1523/JNEUROSCI.0387-15.2015

Karch, C. M., Jeng, A. T., and Goate, A. M. (2012). Extracellular tau levels are influenced by variability in tau that is associated with tauopathies. *J. Biol. Chem.* 287, 42751–42762. doi:10.1074/jbc.M112.380642

Kim, W., Lee, S., and Hall, G. F. (2010). Secretion of human tau fragments resembling CSF-tau in Alzheimer's disease is modulated by the presence of the exon 2 insert. *FEBS Lett.* 584, 3085–3088. doi:10.1016/j.febslet.2010.05.042

Lambert, J. C., Ibrahim-Verbaas, C. A., Harold, D., Naj, A. C., Sims, R., Bellenguez, C., et al. (2013). Meta-analysis of 74, 046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nat. Genet.* 45, 1452–1458. doi:10.1038/ng.2802

Le Guennec, K., Quenez, O., Nicolas, G., Wallon, D., Rousseau, S., Richard, A. C., et al. (2017). 17q21.31 duplication causes prominent tau-related dementia with increased MAPT expression. *Mol. Psychiatry* 22, 1119–1125. doi:10.1038/mp.2016.226

Lee, V., Goedert, M., and Trojanowski, J. (2001). Neurodegenerative tauopathies. *Annu. Rev. Neurosci.* 24, 1121–1159. doi:10.1146/annurev.neuro.24.1.1121

Lee, W. S., Tan, D. C., Deng, Y., van Hummel, A., Ippati, S., Stevens, C., et al. (2021). Syntaxins 6 and 8 facilitate tau into secretory pathways. *Biochem. J.* 478, 1471–1484. doi:10.1042/BCJ20200664

Losev, Y., Frenkel-Pinter, M., Abu-Hussien, M., Viswanathan, G. K., Elyashiv-Revivo, D., Geries, R., et al. (2021). Differential effects of putative N-glycosylation sites in human Tau on Alzheimer's disease-related neurodegeneration. *Cell. Mol. Life Sci.* 78, 2231–2245. doi:10.1007/s00018-020-03643-3

Ludin, B., and Matus, A. (1993). The neuronal cytoskeleton and its role in axonal and dendritic plasticity. *Hippocampus* 3, 61–71. doi:10.1002/hipo.1993.4500030710

Mandell, J., and Banker, G. (1996). Microtubule-associated proteins, phosphorylation gradients, and the establishment of neuronal polarity. *Perspect. Dev. Neurobiol.* 4, 125–135.

Means, J. C., Gerdes, B. C., Kaja, S., Sumien, N., Payne, A. J., Stark, D. A., et al. (2016). Caspase-3-Dependent proteolytic cleavage of tau causes neurofibrillary tangles and results in cognitive impairment during normal aging. *Neurochem. Res.* 41, 2278–2288. doi:10.1007/s11064-016-1942-9

Merezhko, M., Brunello, C. A., Yan, X., Vihinen, H., Jokitalo, E., Uronen, R. L., et al. (2018). Secretion of tau via an unconventional non-vesicular mechanism. *Cell Rep.* 25, 2027–2035. e2024. doi:10.1016/j.celrep.2018.10.078

Messenger, S. W., Jones, E. K., Holthaus, C. L., Thomas, D. D. H., Cooley, M. M., Byrne, J. A., et al. (2017). Acute acinar pancreatitis blocks vesicle-associated membrane protein 8 (VAMP8)-dependent secretion, resulting in intracellular trypsin accumulation. *J. Biol. Chem.* 292, 7828–7839. doi:10.1074/jbc.M117.781815

Mnich, K., Carleton, L. A., Kavanagh, E. T., Doyle, K. M., Samali, A., and Gorman, A. M. (2014). Nerve growth factor-mediated inhibition of apoptosis post-caspase activation is due to removal of active caspase-3 in a lysosome-dependent manner. *Cell Death Dis.* 5, e1202. doi:10.1038/cddis.2014.173

Mohamed, N.-V., Desjardins, A., and Leclerc, N. (2017). Tau secretion is correlated to an increase of Golgi dynamics. *PloS ONE* 12, e0178288. doi:10.1371/journal.pone.0178288

Mohamed, N. V., Herrou, T., Plouffe, V., Piperno, N., and Leclerc, N. (2013). Spreading of tau pathology in Alzheimer's disease by cell-to-cell transmission. *Eur. J. Neurosci.* 37, 1939–1948. doi:10.1111/ejn.12229

Mohamed, N. V., Plouffe, V., Remillard-Labrosse, G., Planel, E., and Leclerc, N. (2014). Starvation and inhibition of lysosomal function increased tau secretion by primary cortical neurons. *Sci. Rep.* 4, 5715. doi:10.1038/srep05715

Ossenkoppele, R., Schonhaut, D. R., Scholl, M., Lockhart, S. N., Ayakta, N., Baker, S. L., et al. (2016). Tau PET patterns mirror clinical and neuroanatomical variability in Alzheimer's disease. *Brain* 139, 1551–1567. doi:10.1093/brain/aww027

Perez-Barcena, J., Rodriguez Pilar, J., Salazar, O., Crespi, C., Frontera, G., Novo, M. A., et al. (2022). Serum caspase-1 as an independent prognostic factor in traumatic brain injured patients. *Neurocrit. Care* 36, 527–535. doi:10.1007/s12028-021-01340-y

Pernegre, C., Duquette, A., and Leclerc, N. (2019). Tau secretion: Good and bad for neurons. *Front. Neurosci.* 13, 649. doi:10.3389/fnins.2019.00649

Pilliod, J., Desjardins, A., Pernegre, C., Jamann, H., Larochelle, C., Fon, E. A., et al. (2020). Clearance of intracellular tau protein from neuronal cells via VAMP8-induced secretion. *J. Biol. Chem.* 295, 17827–17841. doi:10.1074/jbc.RA120.013553

Plouffe, V., Mohamed, N. V., Rivest-McGraw, J., Bertrand, J., Lauzon, M., and Leclerc, N. (2012). Hyperphosphorylation and cleavage at D421 enhance tau secretion. *PLoS One* 7, e36873. doi:10.1371/journal.pone.0036873

Pontecorvo, M. J., Devous, M. D., Kennedy, I., Navitsky, M., Lu, M., Galante, N., et al. (2019). A multicentre longitudinal study of flortaucipir (18F) in normal ageing, mild cognitive impairment and Alzheimer's disease dementia. *Brain* 142, 1723–1735. doi:10.1093/brain/awz090

Pooler, A. M., Phillips, E. C., Lau, D. H., Noble, W., and Hanger, D. P. (2013). Physiological release of endogenous tau is stimulated by neuronal activity. *EMBO Rep.* 14, 389–394. doi:10.1038/embor.2013.15

Prokic, I., Cowling, B. S., and Laporte, J. (2014). Amphiphysin 2 (BIN1) in physiology and diseases. *J. Mol. Med.* 92, 453–463. doi:10.1007/s00109-014-1138-1

Pryor, P. R., Mullock, B. M., Bright, N. A., Lindsay, M. R., Gray, S. R., Richardson, S. C., et al. (2004). Combinatorial SNARE complexes with VAMP7 or VAMP8 define different late endocytic fusion events. *EMBO Rep.* 5, 590–595. doi:10.1038/sj.embor.7400150

Rodriguez, L., Mohamed, N.-V., Desjardins, A., Lippe, R., Fon, E. A., and Leclerc, N. (2017). Rab7A regulates tau secretion. *J. Neurochem.* 141, 592–605. doi:10.1111/jnc.13994

Saman, S., Kim, W., Raya, M., Visnick, Y., Miro, S., Jackson, B., et al. (2012). Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease. *J. Biol. Chem.* 287, 3842–3849. doi:10.1074/jbc.M111.277061

Schraen-Maschke, S., Dhaenens, C. M., Delacourte, A., and Sablonniere, B. (2004). Microtubule-associated protein tau gene: A risk factor in human neurodegenerative diseases. *Neurobiol. Dis.* 15, 449–460. doi:10.1016/j.nbd.2003.12.009

Simon, D., Garcia-Garcia, E., Gomez-Ramos, A., Falcon-Perez, J. M., Diaz-Hernandez, M., Hernandez, F., et al. (2012). Tau overexpression results in its secretion via membrane vesicles. *Neurodegener. Dis.* 10, 73–75. doi:10.1159/000334915

Tomlinson, B. E., Blessed, G., and Roth, M. (1970). Observations on the brains of demented old people. *J. Neurol. Sci.* 11, 205–242. doi:10.1016/0022-510x(70)90063-8

Vardarajan, B. N., Ghani, M., Kahn, A., Sheikh, S., Sato, C., Barral, S., et al. (2015). Rare coding mutations identified by sequencing of Alzheimer disease genome-wide association studies loci. *Ann. Neurol.* 78, 487–498. doi:10.1002/ana.24466

Wang, Y., Balaji, V., Kaniyappan, S., Kruger, L., Irsen, S., Tepper, K., et al. (2017). The release and trans-synaptic transmission of Tau via exosomes. *Mol. Neurodegener.* 12, 5. doi:10.1186/s13024-016-0143-y

Yamada, K., Cirrito, J. R., Stewart, F. R., Jiang, H., Finn, M. B., Holmes, B. B., et al. (2011). *In vivo* microdialysis reveals age-dependent decrease of brain interstitial fluid tau levels in P301S human tau transgenic mice. *J. Neurosci.* 31, 13110–13117. doi:10.1523/JNEUROSCI.2569-11.2011

Yamada, K., Holth, J. K., Liao, F., Stewart, F. R., Mahan, T. E., Jiang, H., et al. (2014). Neuronal activity regulates extracellular tau *in vivo*. *J. Exp. Med.* 211, 387–393. doi:10.1084/jem.20131685

Zhang, Z., Song, M., Liu, X., Kang, S. S., Kwon, I. S., Duong, D. M., et al. (2014). Cleavage of tau by asparagine endopeptidase mediates the neurofibrillary pathology in Alzheimer's disease. *Nat. Med.* 20, 1254–1262. doi:10.1038/nm.3700

Zhou, Y., Shi, J., Chu, D., Hu, W., Guan, Z., Gong, C. X., et al. (2018). Relevance of phosphorylation and truncation of tau to the etiopathogenesis of alzheimer's disease. *Front. Aging Neurosci.* 10, 27. doi:10.3389/fnagi.2018.00027

Zorn, C. N., Pardo, J., Martin, P., Kuhny, M., Simon, M. M., and Huber, M. (2013). Secretory lysosomes of mouse mast cells store and exocytose active caspase-3 in a strictly granzyme B dependent manner. *Eur. J. Immunol.* 43, 3209–3218. doi:10.1002/eji.201343941

Frontiers in
Cell and Developmental Biology

Explores the fundamental biological processes of life, covering intracellular and extracellular dynamics.

The world's most cited developmental biology journal, advancing our understanding of the fundamental processes of life. It explores a wide spectrum of cell and developmental biology, covering intracellular and extracellular dynamics.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact