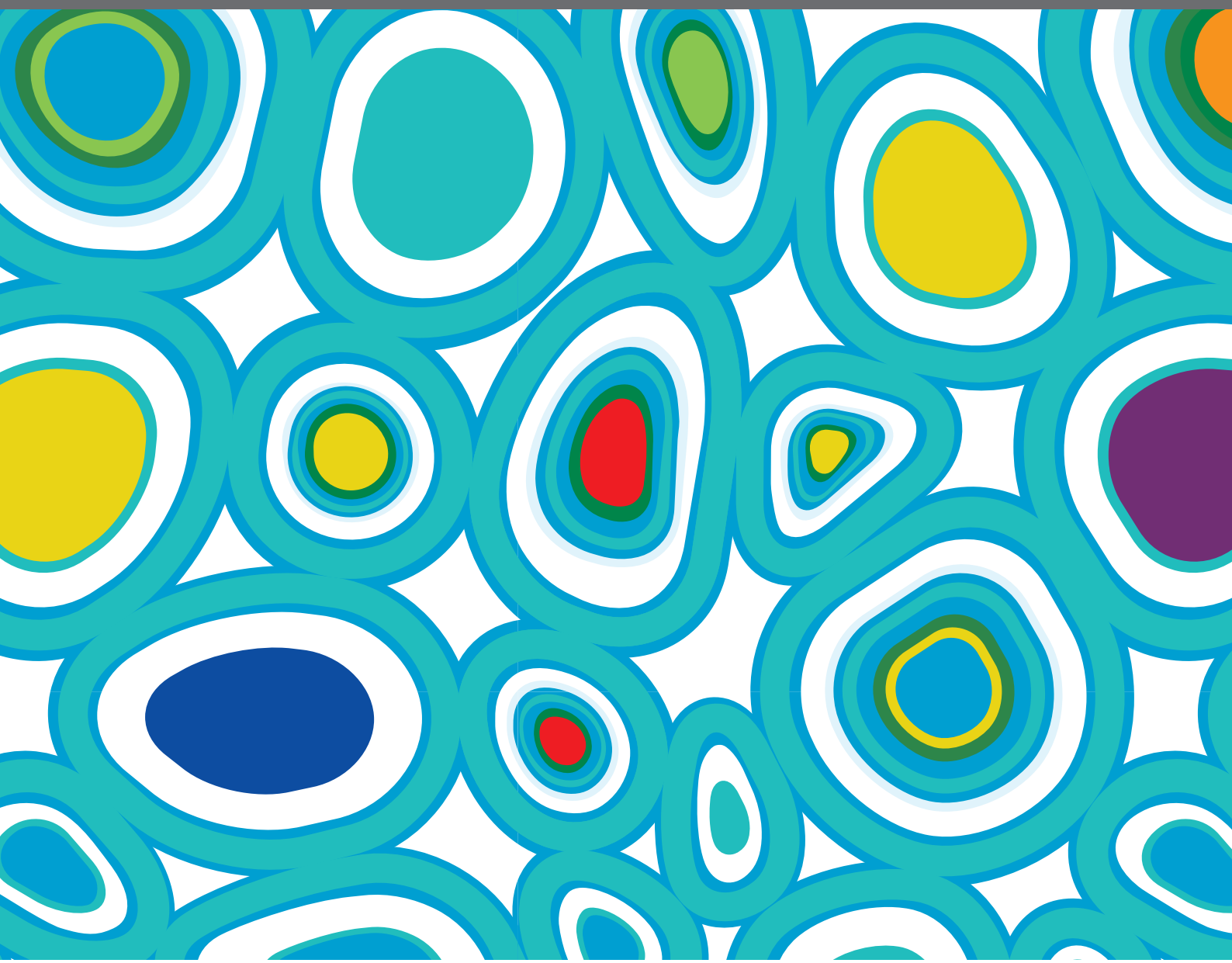
MOLECULAR MECHANISMS AND PHYSIOLOGICAL SIGNIFICANCE OF ORGANELLE INTERACTIONS AND COOPERATION - VOLUME II

EDITED BY: Michael Schrader, Markus Islinger and Joe Costello
PUBLISHED IN: Frontiers in Cell and Developmental Biology



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ISSN 1664-8714
ISBN 978-2-83250-838-1
DOI 10.3389/978-2-83250-838-1

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MOLECULAR MECHANISMS AND PHYSIOLOGICAL SIGNIFICANCE OF ORGANELLE INTERACTIONS AND COOPERATION - VOLUME II

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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.

Citation: Schrader, M., Islinger, M., Costello, J., eds. (2022). Molecular Mechanisms and Physiological Significance of Organelle Interactions and Cooperation - Volume II. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-83250-838-1

Table of Contents

frontiers | Frontiers in Cell and Developmental Biology

TYPE Editorial
PUBLISHED 08 November 2022
DOI 10.3389/fcell.2022.1076670

OPEN ACCESS

EDITED AND REVIEWED BY
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SPECIALTY SECTION
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

RECEIVED 21 October 2022
ACCEPTED 26 October 2022
PUBLISHED 08 November 2022

CITATION
Costello J, Islinger M and Schrader M (2022), Editorial: "Molecular mechanisms and physiological significance of organelle interactions and cooperation—Volume II". *Front. Cell Dev. Biol.* 10:1076670. doi: 10.3389/fcell.2022.1076670

Editorial: "Molecular mechanisms and physiological significance of organelle interactions and cooperation—Volume II"

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KEYWORDS

membrane contact sites, organelles, peroxisomes, mitochondria, lipid droplets, organelle interaction

Editorial on the Research Topic
"Molecular mechanisms and physiological significance of organelle interactions and cooperation—Volume II"

The formation of subcellular compartments is a characteristic feature of eukaryotic life. Membrane-bound organelles such as mitochondria, peroxisomes, or lipid droplets do however not function as isolated entities; they need to cooperate and to communicate with other subcellular compartments in order to support metabolic pathways and functioning of the cell as a unit. In this regard, organelle cooperation/communication events have been associated with apoptosis, antiviral defence, organelle division and biogenesis, ROS metabolism and signalling, and various metabolic pathways such as breakdown of fatty acids. Coordinated interplay between organelles is often mediated by membrane contact sites (MCS), which bring organelles in close apposition (Simmen et al., 2017; Prinz et al., 2020). MCS can contribute to the transfer of lipids, ions and metabolites for cooperative metabolic pathways, facilitate efficient signalling and communication, or contribute to the positioning of organelles within cells. Interacting proteins (or lipids) which act as tethers to bridge the opposing organelle membranes form MCS. The research field of membrane contacts and organelle interaction continues to grow rapidly. New contact sites are still being discovered and the number of tethers and proteins associated with MCS is constantly growing (Eisenberg-Bord et al., 2016; Silva et al., 2020). However, it is still not fully understood, how MCS are regulated (Kors et al, 2022a), what their physiological functions are, and how they impact on human health and disease.

The ever expanding roles of the VAPs, a group of membrane proteins which play a critical role in connecting the ER to other organelles, are reviewed by Kors et al. The authors discuss how the FFAT motif of VAPs allows interaction with over 50 confirmed binding partners and how regulation of these interactions can allow specific control of membrane contact site formation.

Liao et al. summarize the current knowledge on the function and molecular composition of lipid droplet (LD) contacts with mitochondria, lysosomes and the ER. They highlight novel unexpected functions and discuss how LD contact site formation is influenced or regulated by organelle interaction with the cytoskeleton.

Regulation of membrane contact sites by various different mechanisms is an emerging but still relatively understudied area of research. Ravi et al. explore a novel area of membrane contact site regulation, that of the role of lipid modulators in regulating organelle crosstalk by modifying the lipid composition of membranes. Specifically focusing on Phosphotidylinositol-5-phosphate-4-kinases (PI5P4Ks), which regulate phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2) levels, they explain how regulation of lipid phosphorylation on organelle membranes is able to regulate interaction with lipid-interacting proteins.

The number of cellular processes, which involve membrane contact sites, is increasingly expanding. In line with, this Islam et al. present new hypotheses for how membrane contact sites could influence the regulation of low density lipoprotein receptor (LDLR) internalisation and degradation. They highlight several contact site proteins that appear to be involved in this process and suggest that more emphasis should be given to understanding how membrane contact sites influence specific events in endocytosis and trafficking.

Sargsyan et al. present an interesting report on the role of calcium in peroxisomes, a topic which has not been extensively explored previously. They discuss the evidence for the presence of calcium in peroxisomes and potential peroxisomal calcium-handling proteins and also speculate on how membrane contact sites with other organelles may facilitated calcium exchange.

Many peroxisomal functions require factors also relevant to other cellular compartments. Bittner et al. review proteins, which are shared by peroxisomes and other organelles. They discuss the mechanisms to achieve dual targeting, their regulation and functional consequences with a major focus on yeast and fungi. They propose that the regulation of dual targeting will further emerge as a major factor to control organelle interplay and communication.

How lipids are transferred at membrane contact sites is still not fully understood. Using a mutagenesis strategy, Wei et al. identified Vps13 as a protein, which is essential for peroxisome formation in yeast mutants with reduced peroxisome-ER contacts. The findings suggest that in the yeast *Hansenula polymorpha* Vps13 plays a redundant function in lipid transfer from the ER to peroxisomes.

Wu and van der Klei present a first detailed structure-function analysis of *Hansenula polymorpha* ER protein Pex32, a protein of the Pex23 protein family, which only occur in yeast and filamentous fungi. *Hp*Pex32 is required for the formation of peroxisome-ER contact sites. A domain analysis now revealed that the N-terminal transmembrane domain of Pex32 contains ER sorting information, whereas the C-terminal DysF domain is required to concentrate Pex32 at peroxisome-ER contacts and can associate with peroxisomes.

Li et al. discuss the impact of peroxisome-derived H_2O_2 on the regulation of autophagy/pexophagy. They describe our current knowledge on how H_2O_2 might interfere as a signalling molecule in autophagy regulation by oxidizing thiol groups in autophagy-relevant proteins. The authors propose the concept of a peroxisome-autophagy signalling axis, which might adapt the cellular organelle status to distinct metabolic or stress situations.

Van Roermund et al. investigated the metabolic communication between the peroxisomal matrix and the surrounding cytosol. They show that a heterodimer of the yeast peroxisomal ABC transporters Pxa1p and Pxa2p, which transport long chain acyl-CoAs, can as well import cytosolic ATP into peroxisomes. In contrast to the known peroxisomal ATP transporter Ant1p, the ABC transporter heterodimer does not work as an ATP—ADP/AMP antiporter system thereby increasing the flexibility of peroxisomes to increase intraorganellar ATP concentrations. The ATP transporting function is also preserved in the human peroxisomal ABC transporters ABCD1, ABCD2 and ABCD3. Moreover, yeast peroxisomes appear to harbour a third ATP importing system, as the authors further detected that the mitochondrial membrane ATP transporter Aac2p is dually targeted to mitochondria and peroxisomes in *Saccharomyces cerevisiae*. Thus, yeast peroxisomes appear to utilize three complementing ATP importing systems to adapt intraperoxisomal ATP levels to distinct metabolic states of the cell.

The reviews and research articles presented in volume II of this special topic once again demonstrate the impressive breadth of research currently being undertaken to understand the molecular mechanisms and physiological significance of organelle interactions and cooperation.

Author contributions

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

Funding

Supported by BBSRC (BB/R016844/1; BB/T002255/1; BB/W015420/1). UK Research and Innovation Future Leader Fellowship Award (MR/T019409/1 to JC).

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

Eisenberg-Bord, M., Shai, N., Schuldiner, M., and Bohnert, M. (2016). A tether is a tether is a tether: Tethering at membrane contact sites. *Dev. Cell* 39, 395–409. doi:10.1016/j.devcel.2016.10.022

Kors, S., Kurian, S. M., Costello, J. L., and Schrader, M. (2022). Controlling contacts-Molecular mechanisms to regulate organelle membrane tethering. *Bioessays.* 30, 2200151. doi:10.1002/bies.202200151

Prinz, W. A., Toulmay, A., and Balla, T. (2020). The functional universe of membrane contact sites. *Nat. Rev. Mol. Cell Biol.* 21, 7–24. doi:10.1038/s41580-019-0180-9

Silva, B. S., DiGiovanni, L., Kumar, R., Carmichael, R. E., Kim, P. K., and Schrader, M. (2022). Maintaining social contacts: The physiological relevance of organelle interactions. *Biochim. Biophys. Acta - Mol. Cell Res.* 1867 (11), 118800. doi:10.1016/j.bbamcr.2020.118800

Simmen, T., and Tagaya, M. (2017). "Organelle communication at membrane contact sites (MCS): From curiosity to center stage," in *Cell biology and biomedical research BT - organelle contact sites: From molecular mechanism to disease*. Editors M. Tagaya and T. Simmen (Singapore: Springer Singapore), 1–12. doi:10.1007/978-981-10-4567-7_1

frontiers in Cell and Developmental Biology

REVIEW
published: 16 December 2021
doi: 10.3389/fcell.2021.814047

The Peroxisome-Autophagy Redox Connection: A Double-Edged Sword?

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Peroxisomes harbor numerous enzymes that can produce or degrade hydrogen peroxide (H_2O_2). Depending on its local concentration and environment, this oxidant can function as a redox signaling molecule or cause stochastic oxidative damage. Currently, it is well-accepted that dysfunctional peroxisomes are selectively removed by the autophagy-lysosome pathway. This process, known as "pexophagy," may serve a protective role in curbing peroxisome-derived oxidative stress. Peroxisomes also have the intrinsic ability to mediate and modulate H_2O_2-driven processes, including (selective) autophagy. However, the molecular mechanisms underlying these phenomena are multifaceted and have only recently begun to receive the attention they deserve. This review provides a comprehensive overview of what is known about the bidirectional relationship between peroxisomal H_2O_2 metabolism and (selective) autophagy. After introducing the general concepts of (selective) autophagy, we critically examine the emerging roles of H_2O_2 as one of the key modulators of the lysosome-dependent catabolic program. In addition, we explore possible relationships among peroxisome functioning, cellular H_2O_2 levels, and autophagic signaling in health and disease. Finally, we highlight the most important challenges that need to be tackled to understand how alterations in peroxisomal H_2O_2 metabolism contribute to autophagy-related disorders.

Keywords: autophagy, disease, hydrogen peroxide, oxidative damage, peroxisomes, pexophagy, thiol-based redox signaling

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *12 November 2021*
Accepted: *02 December 2021*
Published: *16 December 2021*

Citation:
Li H, Lismont C, Revenco I, Hussein MAF, Costa CF and Fransen M (2021) The Peroxisome-Autophagy Redox Connection: A Double-Edged Sword? Front. Cell Dev. Biol. 9:814047. doi: 10.3389/fcell.2021.814047

INTRODUCTION

Autophagy is a conserved catabolic program for the degradation of cytoplasmic components (e.g., dysfunctional organelles, protein aggregates, and non-specific portions of the cytoplasm) within the lysosome (Kaur and Debnath, 2015). Dysregulation of this process has been linked to pathologies such as neurodegeneration, cancer, and diabetes (Kenific and Debnath, 2015; Fang et al., 2019; Muralidharan et al., 2021). Depending on the delivery route of the cytoplasmic material to the lysosome interior, three primary types of autophagy have been recognized in mammalian cells: microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA) (Yim and Mizushima, 2020).

Proteins degraded by CMA contain a KFERQ-like motif that binds to HSC70, a cytosolic chaperone that delivers its cargo to the lysosomal surface for internalization and rapid degradation (Kaushik and Cuervo, 2018) (all protein acronyms are annotated as in the UniProtKB database and the full names can be retrieved in the Glossary). Approximately 30% of all soluble cytosolic proteins contain such a CMA-targeting motif (Chiang et al., 1989), and-after binding of the substrate-

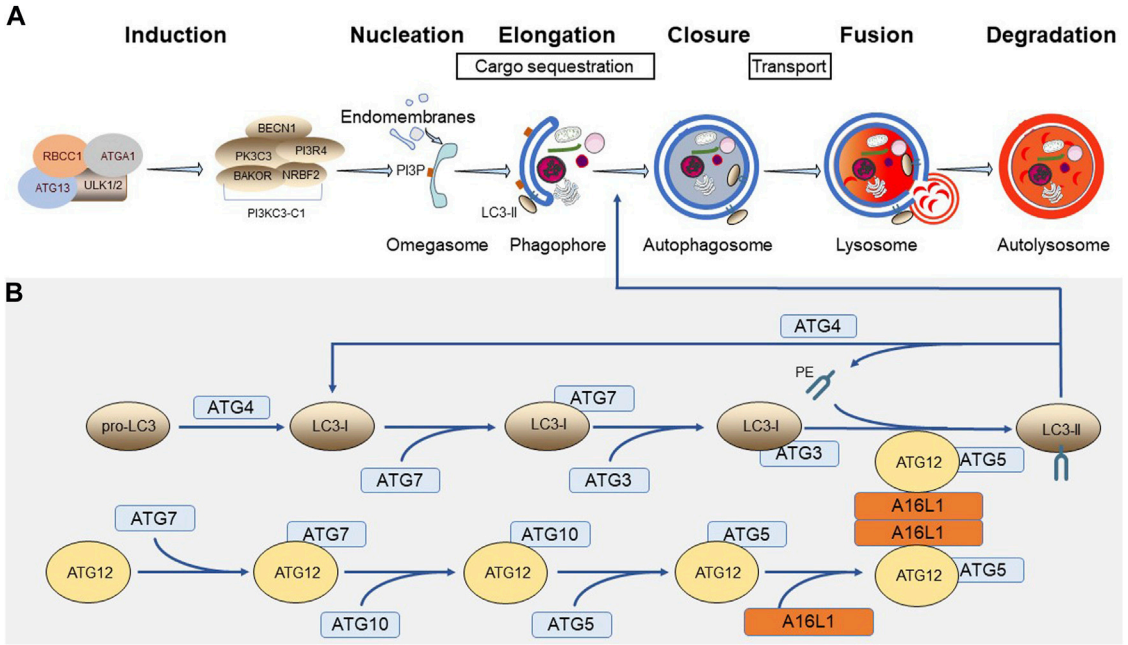

FIGURE 1 | Distinct steps and core components of autophagy. **(A)** Different stages of canonical autophagy. **(B)** Ubiquitin-like conjugation systems involved in phagophore biogenesis.

chaperone complex to the transmembrane protein LAMP2A (Cuervo and Dice, 2000)—the substrate proteins need to unfold before they cross the lysosomal membrane (Rout et al., 2014). These processes occur in cooperation with a set of cochaperones (Rout et al., 2014; Kaushik and Cuervo, 2018).

In microautophagy, the lysosomal or endosomal membrane is deformed to directly engulf cytosolic materials (Yim and Mizushima, 2020). At the morphological level, cargo can be engulfed through lysosomal protrusion, lysosomal invagination, or endosomal invagination (Oku and Sakai, 2018). At the mechanistic level, many aspects remain to be clarified (Mejlvang et al., 2018; Mesquita et al., 2021). However, this degradation route requires neither LAMP2A nor substrate unfolding (Yim and Mizushima, 2020).

Unlike the former two kinds of autophagy, macroautophagy involves the sequestration of cargo at a distinct site from lysosomes. During this process, a double-membrane structure (the phagophore) is formed (Klionsky et al., 2021). This structure wraps around the cytoplasmic target and creates, upon closure, a separate compartment (the autophagosome). This short-lived organelle subsequently fuses with lysosomes to deliver its content for degradation (Kriegenburg et al., 2018). Given that macroautophagy, hereafter referred to as "autophagy," is widely recognized as the major removal pathway for organelles in mammalian cells (Klionsky et al., 2021), we focus here on the double-sided redox connection between peroxisomes and this type of autophagy: on one hand, as a major site of intracellular H_2O_2 metabolism, peroxisomes have the potential to turn on and tune autophagy, and this process may eliminate dysfunctional peroxisomes and protect the cell from oxidative damage; on the other hand, chronic impairment of peroxisome function may lead to an accumulation of H_2O_2 levels that inhibit autophagy, thereby driving a vicious cycle between peroxisome malfunction and cellular redox imbalance.

THE CORE AUTOPHAGY MACHINERY

The canonical autophagy process can be divided into multiple stages, including induction, phagophore nucleation and elongation, cargo sequestration, phagophore closure, autophagosome transport, and cargo degradation via fusion with lysosomes (**Figure 1A**) (Parzych and Klionsky, 2014; Yim and Mizushima, 2020; Melia et al., 2020). Many of these processes are executed by a dedicated cohort of autophagy-related (ATG) proteins (Feng et al., 2014). Here, we mainly focus on the molecular players involved in phagophore biogenesis (**Figure 1B**) (Kawabata and Yoshimori, 2020).

The phagophore (or isolation membrane) is a small cup-shaped pre-autophagosomal structure that can originate from endomembranes (e.g., the ER membrane) containing phosphatidylinositol 3-phosphate (PI3P)-enriched subdomains, often referred to as omegasomes (Roberts and Ktistakis, 2013). PI3P is a phospholipid that is mainly generated by the class III phosphatidylinositol 3-kinase complex I (PI3KC3-C1), which is composed of a catalytic subunit (PK3C3), a PI3-kinase regulatory subunit (PI3R4), an allosteric modulator (BECN1), another regulator (NRBF2), and a phagophore targeting subunit (BAKOR) (Hurley and Young, 2017). Activation of the PI3KC3-C1 complex requires the serine/threonine protein kinase complex ULK. This complex, which integrates upstream nutrient and energy signals to coordinate autophagy

induction, is made up of either ULK1 or ULK2 and 3 non-catalytic subunits (ATG13, RBCC1, and ATGA1) (Hurley and Young, 2017). Upon induction of autophagy, the ULK1 complex becomes active and translocates to the omegasome, where it exerts its function on PI3KC3-C1 (Mercer et al., 2018).

Elongation of the phagophore requires the concerted action of two ubiquitin-like conjugation systems. Here, it is worth noting that the ATG protein family includes two ubiquitin-like protein members, ATG8/MLP3/GBRL (here denoted as LC3) and ATG12 (Mohan and Wollert, 2018). Following translation, the pro-LC3 proteins first need to be cleaved into their LC3-I counterparts to expose a glycine residue at their C-terminus. This event, which is catalyzed by the cysteine protease ATG4, is necessary to become a substrate for ATG7, an E1-like enzyme that can activate both LC3-I and ATG12. ATG7 in turn transfers its activated ubiquitin-like substrates to ATG3 (in case of LC3-I) or ATG10 (in case of ATG12), two E2-like enzymes. Next, ATG12 is transferred onto ATG5 to form a complex with E3-like activity that, upon interaction with the A16L1 dimer (Lystad et al., 2019), is targeted to the autophagosomal membrane, where it stimulates the ATG3-mediated conjugation of LC3-I to phosphatidylethanolamine (PE) (Martens and Fracchiolla, 2020). This lipidated form of LC3, termed LC3-II, is the active form of LC3 and plays an important role in phagophore membrane expansion, cargo selection, and membrane closure (Li and Zhang, 2019). Note that ATG4 can also delipidate LC3-II to release this molecule from the autophagosomal membrane for reuse (Nakatogawa, 2013).

The closed autophagosome needs to be transported to and fused with the lysosome for digestion. These processes require cytoskeletal filaments, motor proteins, RABs, SNAREs, tethering factors, and lysosomal hydrolases. For a detailed overview of these factors, which are not further elaborated on here, the reader is referred to other dedicated reviews (Nakamura and Yoshimori, 2017; Yim and Mizushima, 2020; Zhao et al., 2021).

CARGO RECEPTORS FOR SELECTIVE AUTOPHAGY

Depending on the nature of the substrate, autophagy can be classified into selective or non-selective (bulk) autophagy (Lamark and Johansen, 2021). In bulk autophagy, portions of the cytoplasm are randomly sequestered, degraded, and recycled to compensate for nutrient deficiencies. Selective autophagy, however, rather serves to eliminate functionally redundant or damaged cytoplasmic components, which are selectively sequestered and degraded as a stress response or quality control mechanism. Examples of selective autophagy processes in mammalian cells include aggrephagy (protein aggregates), ER-phagy (ER), ferritinophagy (ferritin), glycophagy (glycogen), lipophagy (lipid droplets), lysophagy (lysosomes), mitophagy (mitochondria), nucleophagy (nuclear fragments), pexophagy (peroxisomes), ribophagy (ribosomes), xenophagy (bacteria and viruses), and zymophagy (zymogen granules) (Gatica et al., 2018; Gubas and Dikic, 2021).

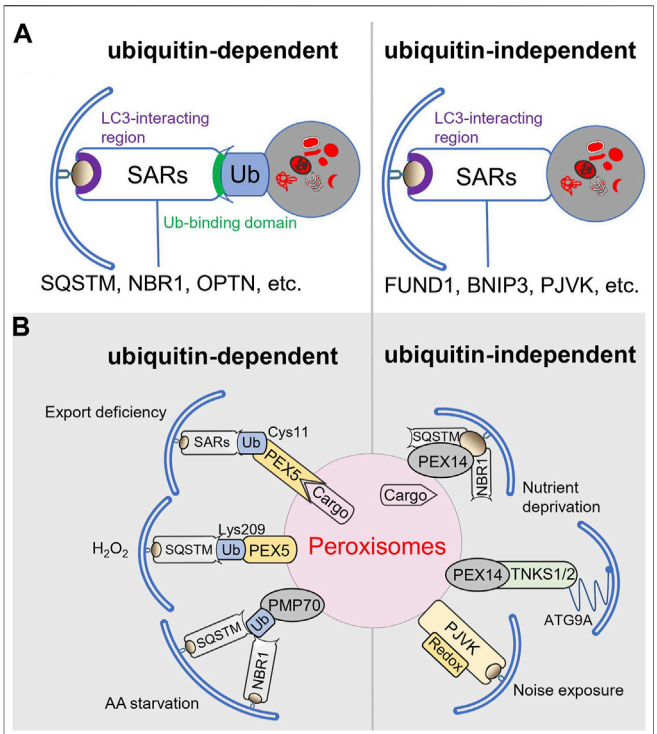

FIGURE 2 | Selective autophagy pathways and pexophagy receptors. **(A)** Types of selective autophagy. **(B)** Cargo receptors involved in selective pexophagy.

Both selective and bulk autophagy utilize the same core autophagy machinery. However, selective autophagy pathways require the additional action of specific autophagy receptors (SARs) including, among others, BNIP3, BNI3L, CACO2, FUND1, NBR1, OPTN, RETR1, and SQSTM (**Figure 2A**) (Gubas and Dikic, 2021). SARs can act either independently or cooperatively to bridge substrates to phagophores (Kirkin et al., 2009; Cemma et al., 2011). Therefore, they possess a cargo-binding domain as well as an LC3-interacting region. Given that autophagic substrates are frequently ubiquitinated, the cargo-binding domain is often a ubiquitin-binding domain (Kirkin and Rogov, 2019). Specific examples of such canonical SARs include SQSTM, NBR1, OPTN, and CACO2 (Kim et al., 2016). Importantly, the specificity, activity, and stability of most SARs are controlled by a diverse range of post-translational modifications (e.g., phosphorylation, ubiquitination, acetylation) and structural changes (e.g., oligomerization) (Gubas and Dikic, 2021), which may vary within different cell types or under specific environmental conditions.

CARGO RECEPTORS FOR PEXOPHAGY

To maintain peroxisome functionality, the superfluous and dysfunctional organelles need to be selectively removed through activation of a specialized form of autophagy, called

pexophagy (Nordgren et al., 2013). Currently, there is good evidence that, in mammalian cells, pexophagy can occur through both ubiquitin-dependent and -independent mechanisms (**Figure 2B**) (Li and Wang, 2021).

Evidence that ubiquitin can indeed function as a self-removal signal for peroxisomes was obtained from an elegant proof-of-concept study showing that heterologous expression of peroxisomal membrane protein (PMPs)-ubiquitin fusion proteins in COS-7 or HeLa cells triggered pexophagy on condition that the ubiquitin moiety was facing the cytosol (Kim et al., 2008). Currently, the autophagy receptors SQSTM and NBR1 are both recognized to participate in this process (Kim et al., 2008; Deosaran et al., 2013; Germain and Kim, 2020). However, given that 1) exogenous expression of NBR1, but not SQSTM, promotes peroxisome clustering and lysosome targeting, 2) SQSTM increases the efficiency of NBR1-mediated pexophagy, and 3) SQSTM is not required for pexophagy upon NBR1 overexpression, their precise roles may differ (Deosaran et al., 2013). In addition, it cannot be ruled out that the relative contribution of NBR1 and SQSTM (or any other SAR) to pexophagy varies depending on the initial stimulus. In this context, it is worthwhile noting that in oxidative stress-induced pexophagy the contribution of SQSTM appears to be more important than that of NBR1 (Zhang et al., 2013; Jo et al., 2020b). Also, for a long time, it was unclear which endogenously ubiquitinated protein was serving as a prime recruitment factor for the SARs involved in pexophagy. Currently, it is common knowledge that PEX5, the cycling import receptor for peroxisomal matrix proteins, plays an active role in this process (Subramani, 2015; Li and Wang, 2021). This association is mainly based on the combined observations that 1) after delivery of its cargo into the peroxisome lumen, the protein is monoubiquitinated on a conserved cysteine residue (Cys11 in human PEX5) in order to be extracted from the peroxisomal membrane by the receptor export machinery (Carvalho et al., 2007; Francisco et al., 2017), 2) peroxisome-associated PEX5 can also be ubiquitinated at Lys209 in response to H_2O_2 treatment (Zhang et al., 2015), and 3) conditions resulting in an accumulation of (mono)ubiquitinated PEX5 on the peroxisomal membrane trigger peroxisome removal (Nordgren et al., 2015; Zhang et al., 2015; Lee et al., 2018). Importantly, these findings do not exclude that other ubiquitinated peroxisome-associated proteins may also be involved. Here, it should be noted that, besides peroxisome-associated PEX5, also PMP70 is ubiquitinated during amino acid starvation (Sargent et al., 2016). In addition, it is currently accepted that, under basal conditions, the ubiquitination state of PMPs is maintained at a low level by the peroxisome-associated pool of USP30, a ubiquitin-specific protease (Marcassa et al., 2018; Riccio et al., 2019).

Pexophagy can also take place in a ubiquitin-independent manner. Indeed, LC3-II can be recruited to the peroxisomal membrane through direct or indirect interactions with PEX14 or PJVK (Hara-Kuge and Fujiki, 2008; Jiang et al., 2015a; Li et al., 2017; Defourny et al., 2019). PEX14 is a peroxisomal membrane protein that normally functions as a docking factor for cargo-loaded PEX5 (Fransen et al., 1998), and PJVK is a redox-sensitive peroxisome-associated protein involved in sound-induced peroxisome proliferation in auditory hair cells (Defourny et al., 2019). Under conditions of starvation, where PEX5 is mostly cargo-unloaded, PEX14 can interact with LC3-II, thereby promoting peroxisome degradation (Hara-Kuge and Fujiki, 2008). In a follow-up study, the same authors reported that, upon starvation, PEX14 can also interact with NBR1 and SQSTM1, thereby (most likely) triggering conformational changes in PEX14 and increasing its affinity for LC3-II (Jiang et al., 2015b). Finally, large-scale protein-protein interaction studies have uncovered that PEX14 can also interact with TNKS1 and TNKS2, which were subsequently demonstrated to localize to peroxisomes and promote pexophagy under amino acid starvation conditions (Li et al., 2017). Given that TNKS1 and TNKS2 can also interact with ATG9A, an autophagosomal protein that promotes phagophore membrane growth (Nishimura and Tooze, 2020), it was suggested that the PEX14-TNKS/2-ATG9A complex may function as a non-canonical pexophagy receptor upon nutrient starvation (Li et al., 2017).

TRIGGERS AND SIGNALING PATHWAYS CONTROLLING (SELECTIVE) AUTOPHAGY

Autophagy serves a critical role in stress response and quality control networks, and imbalances in this process have been recognized as an important contributor to disease states such as neurodegeneration, cardiac ischemia-reperfusion, liver disease, Crohn's disease, and infections (Murrow and Debnath, 2013). Major cellular stresses that can be linked to autophagy include nutrient and growth factor deprivation, hypoxia, ER stress, DNA damage, and oxidative stress. In the following subsections, we briefly outline how each of these factors can promote autophagy. For more detailed information, we refer the reader to other reviews.

Nutrient Deprivation

The basic processes underlying autophagy are controlled by complex signaling pathways. Key regulators include the mechanistic target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AAPK), two kinases whose activities respectively inhibit and stimulate autophagy through a coordinated phosphorylation of ULK1 (Egan et al., 2011; Kim et al., 2011): mTORC1 integrates signals from growth factors, nutrients, oxygen levels, and energy status; and AAPK is a sensor and regulator of cellular energy status (Alers et al., 2012). Both mTORC1 and AAPK are master regulators of cell metabolism, thereby linking autophagy to this process (Murrow and Debnath, 2013; Deleyto-Seldas and Efeyan, 2021). However, their actions are in general antagonistic. For example, nutrient deprivation, a condition well-known to induce autophagy, inhibits and stimulates the activities of mTORC1 and AAPK, respectively (Russell et al., 2014). A starvation-induced activation of AAPK also results in phosphorylation and activation of TSC2, a GTPase activating protein that

functions as a key negative regulator of mTORC1 through the TSC complex-RHEB signaling axis (Inoki et al., 2003).

Hypoxia

Hypoxia, a condition in which oxygen availability is limited, is a significant contributor to cell damage in many acute (e.g., ischemic stroke) and chronic (e.g., pulmonary hypertension) disease processes (Lee et al., 2019). Hypoxia can induce autophagy through activation of multiple oxygen-sensitive signaling pathways (Fang et al., 2015). One such pathway involves hypoxia-inducible factors (HIFs). Under normoxic conditions, transcription factors belonging to the HIF protein family are rapidly degraded by the ubiquitin-proteasome system. However, under hypoxic conditions, these proteins are stabilized and translocated to the nucleus to initiate the transcription of genes involved in cellular adaptation and survival, including a set of genes essential for autophagy (e.g., ATG5, ATG7, ATG9A, BECN1, BNIP3, and BNI3L) (Daskalaki et al., 2018). In addition, HIF1 can act as regulator of autophagy by altering the expression levels of genes involved in glucose metabolism (Kierans and Taylor, 2020). Finally, autophagy can also be induced in a HIF-independent manner through hypoxic stress-induced activation of 1) the MK08 signaling pathway (Frazier et al., 2007), 2) the AAPK/TSC2 pathway (Papandreaou et al., 2008), or 3) the unfolded protein response in the ER (Rouschop et al., 2010; Yang et al., 2019). Note that, under normoxic conditions, ER stress can lead to either autophagy stimulation or inhibition (Rashid et al., 2015).

DNA Integrity

A third factor that can induce autophagy is diminished DNA integrity caused by, for example, UV-sunlight or metabolically-derived reactive oxygen species (ROS) (Juretschke and Beli, 2021). Such insults trigger a set of DNA damage response signaling pathways that lead to activation of PARP1, FOXO3, ATM, and P53: 1) PARP1 is a predominantly nuclear enzyme that converts NAD^+ into poly(ADP-ribose), and hyperactivation of this enzyme causes NAD^+ and ATP depletion, a condition promoting AAPK-mediated autophagy activation (Czarny et al., 2015); 2) FOXO3 is a transcription factor known to control the expression levels of multiple autophagy-related genes, including MLP3B and BNIP3, and binding of FOXO3 to the protein kinase ATM triggers autophosphorylation and activation of the latter protein (Rodriguez-Rocha et al., 2011); 3) activation of ATM triggers the initiation of a phosphorylation cascade that regulates the activity of various downstream targets, including AAPK and P53 (Rodriguez-Rocha et al., 2011); and 4) phosphorylation of P53, a multifunctional transcription factor, results in a transcriptional upregulation of TSC2 and PTEN, a negative regulator of PI3K signaling (Rodriguez-Rocha et al., 2011). In the end, all these events contribute to suppression of mTORC1 activity.

Oxidative Stress

Nutrient and growth factor deprivation, hypoxia, ER stress, and DNA damage can all be linked to perturbations in the cellular redox balance, another autophagy-modulating factor (Li et al., 2015; Sedlackova and Korolchuk, 2020). Indeed, physiologically relevant oxidants such as H_2O_2 can oxidatively modify proteins that are directly or indirectly involved in autophagy regulation and execution, thereby potentially effecting their localization, binding affinities, and/or activities (Sedlackova and Korolchuk, 2020). For example, exposure of cells to H_2O_2 can 1) upregulate the transcriptional expression of BECN, BNIP3, BNI3L, MLP3C, and SQSTM through activation of HIF1, P53, FOXO3, NFKB, and NF2L2 (Li et al., 2015), 2) activate PI3K signaling through inactivation of PTEN (Koundouros and Poulogiannis, 2018), 3) suppress mTORC1 activity through activation of ATM and AAPK (Wible and Bratton, 2018), and 4) oxidize and inhibit ATG4 (Wible and Bratton, 2018). For the underlying molecular mechanisms and physiological consequences, we refer the reader to *Redox Regulation of Autophagy*. Importantly, although it is generally thought that oxidative stress always induces autophagy as part of a cellular safeguard mechanism to limit oxidative injury, also other critical factors (e.g., the amount of ROS, nutrient availability, etc.) determine whether autophagy is effectively induced or suppressed under conditions of oxidative stress (Kma and Baruah, 2021).

TRIGGERS AND SIGNALING PATHWAYS CONTROLLING PEXOPHAGY

Over the past decade, multiple studies have shown that pexophagy can be triggered by various stress stimuli including amino acid depletion (Li et al., 2017; Dutta et al., 2021), oxidative stress (Zhang et al., 2013; Jo et al., 2015; Zhang et al., 2015; Tripathi et al., 2016; Lee et al., 2018; Defourny et al., 2019; Jo et al., 2020b; Daussy et al., 2020; Dutta et al., 2021), hypoxia (Walter et al., 2014; Mu et al., 2020), viral infection (Daussy et al., 2020), and dysfunctional peroxisome biogenesis (Yamashita et al., 2014; Nordgren et al., 2015; Dahabieh et al., 2021; Wei et al., 2021). In the following subsections, these triggers will be discussed in more detail (**Figure 3**).

Amino Acid Starvation

Free amino acids play a vital role in cellular metabolism. Examples include but are not limited to protein synthesis and energy metabolism. Amino acids activate mTORC1 signaling and amino acid starvation suppresses this pathway, thereby inducing autophagy and pexophagy (Germain and Kim, 2020; Deretic and Kroemer, 2021). On one hand, starvation-induced pexophagy can be linked to PEX2, a peroxisomal membrane-associated E3 ubiquitin ligase that is rapidly degraded by the proteasome under basal conditions (Sargent et al., 2016). Upon inhibition of mTORC1 (e.g., during amino acid starvation or upon rapamycin treatment), PEX2 is stabilized and promotes PMP ubiquitination, thereby triggering pexophagy (Sargent et al., 2016). On the other hand, starvation-induced pexophagy can be further enhanced by a PEX14-dependent recruitment of TNKS1/2 onto the peroxisomal membrane (Li et al., 2017). The *in vivo* observation that hepatic peroxisome content is dramatically decreased in rats fed with a low protein diet (Van Zutphen et al., 2014) is in line with the idea that there is indeed a

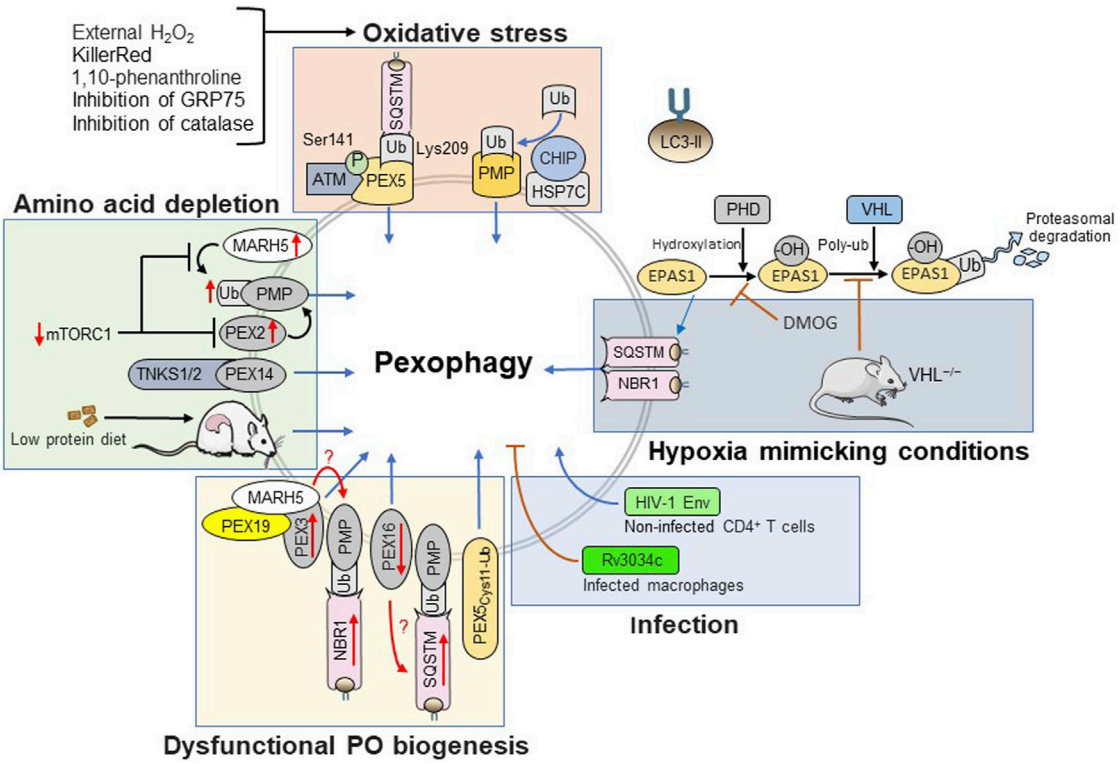

FIGURE 3 | Triggers and signaling pathways controlling pexophagy.

causal link between amino acid starvation and pexophagy. However, direct effectors contributing to pexophagy upon mTORC1 inhibition remain to be identified. Nevertheless, in this regard, it must be noted that a very recent study demonstrated that 1) MARH5, an E3 ubiquitin ligase promoting mitophagy, can also be recruited to the peroxisomal membrane through interaction with the PMP import receptor PEX19 and its membrane docking protein PEX3, 2) recruitment of this E3 ligase to the peroxisomal membrane results in the ubiquitination of PMP70, and 3) MARH5 is playing an important role in mTORC1 inhibition-mediated pexophagy (Zheng et al., 2022). In addition, it is known that prolonged nutrient deprivation causes a significant decrease and increase in the intracellular GSH (Desideri et al., 2012) and H_2O_2 levels (Scherz-Shouval et al., 2007b; Chen et al., 2009), respectively.

Oxidative Stress

Over the last decade, multiple studies have suggested a strong link between oxidative stress and pexophagy, both at the cellular and organismal level. Examples include treatment of Chang liver cells with the chemical 1,10-phenanthroline (Jo et al., 2015), addition of H_2O_2 to HepG2 or HEK-293 cells (Zhang et al., 2013), treatment of mouse embryonic fibroblasts with 3-methyladenine (Ivashchenko et al., 2011), loss of GRP75 in neuroblastoma cells (Jo et al., 2020b), peroxisomal KillerRed-mediated ROS production in NIH/3T3 cells (Chen et al., 2020), suppression of catalase expression or activity in serum-starved HepG2 or RPE1 cells (Lee et al., 2018), and prolonged fasting of catalase-deficient mice (Dutta et al., 2021). Note that catalase is a major peroxisomal matrix protein that catalyzes the degradation of H_2O_2. Although the underlying molecular details have not yet been fully elucidated, different types of oxidative stressors can induce pexophagy through distinct mechanisms. For example, while it has been claimed that external H_2O_2 triggers pexophagy through activation of ATM (Zhang et al., 2015; Tripathi et al., 2016), this kinase appears to be dispensable for the removal of peroxisomes that are oxidatively damaged by activation of peroxisomal KillerRed (Chen et al., 2020). Here, it is important to point out that 1) activation of ATM not only suppresses mTORC1 activity (Wible and Bratton, 2018), but also triggers phosphorylation of PEX5 at Ser 141, an event that subsequently results in its ubiquitination at Lys 209 and the recruitment of SQSTM to the peroxisomal membrane (Zhang et al., 2015; Tripathi et al., 2016), and 2) intraperoxisomal ROS production by KillerRed triggers a HSP7C-mediated recruitment of CHIP, a ubiquitin E3 ligase, onto oxidatively stressed peroxisomes, thereby promoting their selective ubiquitination and autophagic degradation (Chen et al., 2020). Finally, it is worth noting that pexophagy in response to oxidative stress is no mammalian-specific phenomenon. For example, it has been reported that exposure of plant leaves to cadmium induces peroxisomal glycolate oxidase activity (McCarthy et al., 2001), which in

turn leads to increased intraperoxisomal H_2O_2 levels and pexophagy induction (Calero and Muñoz et al., 2019).

Hypoxia

Given that peroxisomal respiration can be responsible for up to 20% of the oxygen consumption in tissues such a liver (De Duve and Baudhuin, 1966), it may not come as a surprise that limited oxygen availability (e.g., as a consequence of inadequate vascular networks in solid tumors) and hypoxia mimicking conditions (e.g., upon treatment of cells with HIF prolyl hydroxylase inhibitors such as DMOG) have been found to trigger pexophagy in an EPAS1-dependent manner, at least in certain cell types (Walter et al., 2014; Schönenberger et al., 2015; Mu et al., 2020). EPAS1 is a hypoxia-inducible transcription factor that, under normoxic conditions, is rapidly targeted for proteasomal degradation through hydroxylation by oxygen-sensing prolyl hydroxylases and subsequent recruitment of the von Hippel-Lindau (VHL) ubiquitination complex (Påhlman and Mohlin, 2018). *In vivo* evidence suggested that hypoxia-induced pexophagy involves both NBR1 and SQSTM (Walter et al., 2014). However, although there is evidence that hypoxia can induce ROS formation (Paddenberg et al., 2003; Rathore et al., 2008), the precise mechanisms underlying EPAS1-mediated pexophagy are unclear. Intriguingly, another study reported that peroxisomes are intact and even essential for growth of K562 and HEK-293 cells under hypoxia (Jain et al., 2020). Importantly, these researchers also demonstrated that this phenotype, which was attributed to the organelle's role in maintenance of membrane fluidity, strongly depended on the medium lipid content and cell seeding conditions (Jain et al., 2020). As such, these seemingly conflicting data may be explained by differences in experimental conditions.

Viral and Bacterial Infections

Two recent studies have shown that pexophagy can also be modulated by pathogenesis-related proteins. One study demonstrated that the human immunodeficiency virus type 1 (HIV1) envelope glycoprotein (Env) has the potential to induce pexophagy in non-infected bystander $CD4^+$ T cells (Daussy et al., 2020). The authors also demonstrated that HIV1 Env can provoke excessive ROS production, a condition that eventually leads to apoptosis thereby very likely contributing to the acquired immunodeficiency syndrome in HIV1-infected patients (Daussy et al., 2020). The other study showed that, upon infection of macrophages, *Mycobacterium tuberculosis* Rv3034c, a putative acetyltransferase, can 1) suppress pexophagy through phosphorylation of mTORC1, an event associated with the down-regulation of pexophagy-associated proteins (e.g., ATG5, NBR1, and SQSTM), and 2) activate peroxisome proliferator activated receptor-γ, a transcription factor that initiates the transcription of peroxisome biogenesis (e.g., PEX3, PEX5, and PEX19) and proliferation (e.g., PEX11B, FIS1, and DNM1L) factors (Ganguli et al., 2020). These changes are likely to favor redox homeostasis, thereby allowing the parasite to avoid ROS-mediated killing (Ganguli et al., 2020). Once again, the precise underlying molecular mechanisms remain unclear.

Dysfunctional Peroxisome Biogenesis

Another emerging pexophagy trigger is dysregulated PMP or matrix protein import. For example, the peroxisomal membrane proteins PEX3 and PEX16 are essential for PMP assembly, and both overexpression of PEX3 (Yamashita et al., 2014) or silencing of PEX16 (Wei et al., 2021) have been shown to induce pexophagy in an NBR1-and SQSTM-dependent manner, respectively. In addition, conditions leading to an accumulation of (mono) ubiquitinated PEX5 on the peroxisomal membrane (Nordgren et al., 2015; Park et al., 2021b; Dahabieh et al., 2021) can also trigger pexophagy. Note that the latter observation strongly indicates that peroxisome-associated monoubiquitinated PEX5 acts as a key surveillance factor for selective elimination of peroxisomes with a defective PEX5 export machinery (Nordgren et al., 2015; Law et al., 2017; Nazarko, 2017).

REDOX REGULATION OF AUTOPHAGY

Currently, it is widely accepted that autophagy represents a prime mechanism of protection against oxidative damage (Ornatowski et al., 2020; Yun et al., 2020). In addition, autophagic activity is governed by complex redox-mediated signaling pathways that, depending on the context, exert positive or negative regulatory activities at the transcriptional and/or protein level (Scherz-Shouval et al., 2007a; Park et al., 2021a; Redza-Dutordoir and Averill-Bates, 2021; Zhou et al., 2021). In the following subsections, we first briefly explain the mechanisms behind H_2O_2 signaling. Next, we elaborate further on how autophagic activity can be directly (e.g., through oxidative modification of autophagy-related proteins) or indirectly (e.g., through oxidative modification of transcription factors or signaling proteins) modulated by H_2O_2, the major ROS in redox regulation of biological activities (Lismont et al., 2019b; Sies and Jones, 2020).

The Concept of H_2O_2 Signaling

A main mechanism by which H_2O_2 achieves specificity as signaling molecule is through direct oxidation of thiolate groups (RS^-) in target proteins (Sies and Jones, 2020). These groups can react with H_2O_2 to form sulfenic acid (RSOH), an intermediate in inter- or intramolecular disulfide bond formation that–in the presence of high H_2O_2 concentrations–can be further oxidized to sulfinic (RSO_2H) or sulfonic (RSO_3H) acid. The latter modification is irreversible and causes permanent oxidative damage. Disulfide bond formation can act as a molecular switch to regulate the activity, localization, and stability of redox-sensitive proteins. Importantly, protein thiols with a low reactivity towards H_2O_2 can also form disulfide bonds through a redox relay mechanism whereby thiol peroxidases shuttle oxidative equivalents from H_2O_2 to other target proteins (Stöcker et al., 2018).

H_2O_2 as a Modulator of ATG Activity

Accumulating evidence points to H_2O_2 as a potent modulator of ATG activity (Lizama-Manibusan and McLaughlin, 2013). For example, it has been demonstrated that the human cysteine proteases ATG4A and ATG4B are direct targets for oxidation

by H_2O_2, thereby rendering them enzymatically inactive through formation of inter- or intramolecular disulfide bridges (Scherz-Shouval et al., 2007b; Zheng et al., 2020). Given the dual function of ATG4 as pro-LC3 cleavage and LC3-II delipidating enzyme (**Figure 1**), these activities need to be tightly controlled to ensure LC3-I lipidation and autophagy progression when cells are exposed to oxidative insults. To cope with this dual role, it has been proposed that 1) the oxidative modification of ATG4 is mainly taking place at autophagosomes in close vicinity to H_2O_2-generating platforms such as mitochondria (Scherz-Shouval et al., 2007b), and 2) the ATG4-dependent cleavage of pro-LC3 into their LC3-I counterparts is more efficient than LC3-II deconjugation (and thus less impacted by partial inhibition of ATG4 activity) (Wible and Bratton, 2018). Nevertheless, although disulfide-bonded ATG4 can be efficiently reduced by the thioredoxin system, it can be expected that harsh or long-term exposure to oxidative stress will eventually fully inhibit ATG4 activity, thereby rather blocking than inducing LC3 lipid conjugation and autophagy (Wible and Bratton, 2018). Besides ATG4, also ATG3, ATG7, and ATG10 have been demonstrated to be redox-sensitive (Filomeni et al., 2010; Frudd et al., 2018). Under basal conditions, ATG3 and ATG7 form inactive thioester-bonded complexes with LC3; upon stimulation of autophagy, ATG3 and ATG7 become active and dissociate from LC3, thereby freeing their catalytic thiols; and under oxidative stress conditions, the non-LC3-shielded thiols in ATG3 and ATG7 form intermolecular disulfide linkages, thereby preventing LC3 lipidation, autophagosome maturation, and autophagy (Frudd et al., 2018). In analogy, it can be expected that the catalytic cysteine of ATG10, another E2-like enzyme, displays a redox-sensitive behavior (Filomeni et al., 2010). However, despite the observation that ATG10 is sensitive to oxidation by H_2O_2, this has apparently no impact on the conjugation of ATG5 to ATG12, at least not under the conditions tested (Frudd et al., 2018).

H_2O_2 as a Modulator of SARs Activity

Another important redox-regulated protein in autophagy is the autophagy receptor SQSTM. This protein can undergo self-polymerization through intermolecular disulfide bond formation, thereby facilitating cargo selection and degradation through high-avidity binding to LC3-II on nascent autophagic membranes (Cha-Molstad et al., 2018). The formation of such disulfide-linked conjugates is promoted by oxidative stress conditions, thereby activating prosurvival autophagy (Carroll et al., 2018). Whether or not the other SARs have the capacity to form similar disulfide-linked complexes, remains to be established.

H_2O_2 as a Modulator of Transcriptional Autophagy Regulation

H_2O_2-induced posttranslational modifications can also modulate the stability, subcellular localization, and/or activity of many transcription factors (Marinho et al., 2014; Li et al., 2015). Here, we briefly summarize the main impact of H_2O_2 on HIF1A, P53, NF2L2, and FOXO, all of which have been implicated in autophagy regulation. For more details regarding the complex molecular mechanisms involved in the adaptive responses, we refer the reader to the references cited. HIF1A is the main driver of transcriptional responses to hypoxia, and H_2O_2-induced activation of HIF1A promotes the transcription of BNIP3 and BNI3L, thereby promoting selective mitophagy (Fan et al., 2019; Asgari et al., 2021). The tumor suppressor protein P53 can, depending on its intracellular location (e.g., cytoplasm versus nuclear) and the cellular environment (e.g., normal physiological conditions versus nutrient starvation or hypoxia), modulate autophagy at multiple levels and through diverse mechanisms (Hu et al., 2019). For example, nuclear-localized P53 can directly upregulate the expression levels of TSC2 and the β-scaffolding subunit of AAPK, thereby enhancing autophagy through inhibition of mTORC1 signaling; and cytoplasmic P53 can inhibit autophagosome formation through binding to RBCC1, an A16L1 interactor and component of the ULK complex (Hu et al., 2019). NF2L2 is a transcription factor that controls the expression of genes containing an antioxidant response element in their promoter, such as SQSTM (Puissant et al., 2012). H_2O_2 can enhance the expression, stability, and nuclear localization of NF2L2 through sequestration of oxidized KEAP1, a thiol-rich protein that promotes the continuous ubiquitin-mediated degradation of NF2L2 under basal conditions (Li et al., 2015; Yin et al., 2015). Finally, H_2O_2 can also activate FOXO transcription factors, which stimulate the transcription of LC3, BNIP3, and ATG12 (Sengupta et al., 2009; Li et al., 2015; Deng et al., 2021a).

H_2O_2 as a Modulator of Autophagy Signaling Pathways

Finally, H_2O_2 can also indirectly promote or inhibit autophagy via modulation of the AAPK, PI3K, and mitogen-activated protein kinase (MK) signaling pathways. Importantly, the outcome is context specific. For example, 1) exposure of HEK-293 cells to H_2O_2 results in S-glutathionylation of Cys299 and Cys304 (likely Cys297 and Cys302 in UniProt ID P54646) in AAPK2, a catalytic subunit of AAPK, and 2) these oxidative modifications stimulate AAPK activity through release of the autoinhibitory domain from its catalytic core, even under non-ATP depleting conditions (Zmijewski et al., 2010). On the other hand, oxidation of Cys130 and Cys174 has been reported to interfere with AAPK activity under energy starvation conditions, at least in mouse cardiomyocytes (Shao et al., 2014). For PI3K, it was shown that the α- and β-catalytic subunits respectively inhibit and promote autophagy in response to moderate and high levels of ROS (Kma and Baruah, 2021). The α-subunit inhibits autophagy through activation of AKT, a serine/threonine kinase that activates mTORC1 activity and arrests autophagic gene expression; and the β-subunit promotes autophagy through stimulating the activities of PI3KC3-C1 and FOXO (Kma and Baruah, 2021). High levels of ROS can also potentiate the PI3K/AKT pathway through inactivation of PTEN, a phosphatase that counteracts PI3K signaling through dephosphorylation of phosphatidylinositol (3,4,5)-trisphosphate to

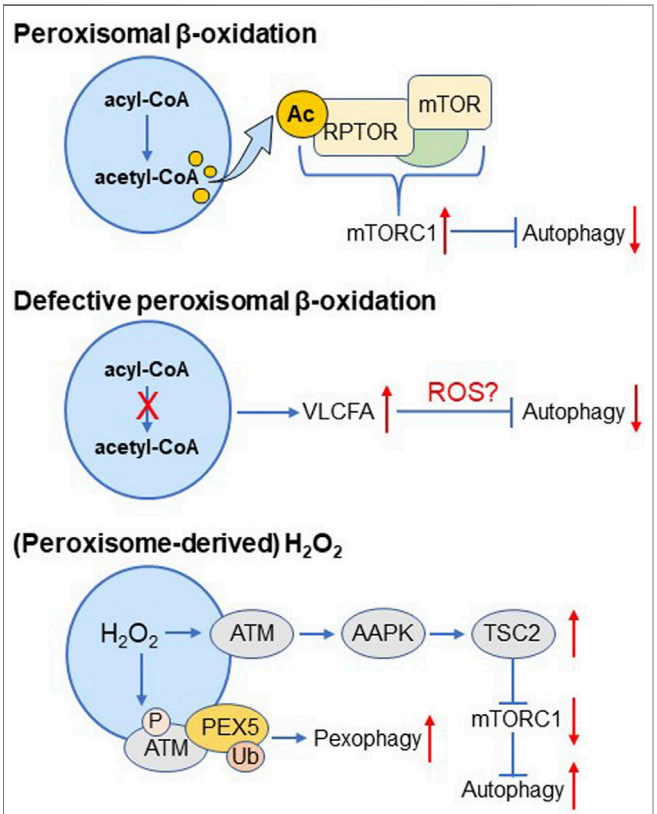

FIGURE 4 | The peroxisome-autophagy signaling axes.

phosphatidylinositol (4,5)-bisphosphate (Koundouros and Poulogiannis, 2018).

MKs are a group of ROS-regulated serine-threonine protein kinases that play a role in diverse cellular processes (Son et al., 2011), including autophagy (Cagnol and Chambard, 2010; Zhou et al., 2015; He et al., 2018). This class of kinases can be grouped in three subclasses: the extracellular signal-regulated kinases, the c-jun N-terminal kinases, and the p38 kinases (Son et al., 2011; Sui et al., 2014). In general, these MKs can be activated by various oxidative stressors, including H_2O_2 (Son et al., 2011), and this subsequently triggers the initiation of complex signaling cascades that eventually modulate, among other processes, autophagic activity. For example, ROS-induced activation of the extracellular signal-regulated kinase pathway can induce adaptive and protective autophagy-associated responses in urinary protein-irritated renal tubular epithelial cells (Deng et al., 2021b); c-jun N-terminal kinase activation can enhance autophagy through 1) upregulation of LC3 (Sun et al., 2011) and DRAM1, a damage-regulated autophagy modulator (Lorin et al., 2010), and 2) the liberation of BECN1 from BCL2/B2CL1 (Zhou et al., 2011); and ROS-induced activation of p38 can induce the expression of various autophagy-related genes (McClung et al., 2010).

THE PEROXISOME-AUTOPHAGY SIGNALING AXES

Peroxisomes act as master regulators of cellular lipid and H_2O_2 metabolism (Van Veldhoven, 2010; Lismont et al., 2015), and emerging evidence hints changes in peroxisomal lipid or H_2O_2 metabolism have the potential to modulate autophagic activity (**Figure 4**). Specifically, peroxisomal β-oxidation-derived acetyl-CoA can downregulate autophagy by enhancing acetylation of the mTORC1 subunit RPTOR, a process driving mTORC1 activation (He et al., 2020); defects in peroxisomal β-oxidation can suppress autophagy through redox imbalances associated with an accumulation of very-long-chain fatty acids (VLCFAs) (Fourcade et al., 2015; Launay et al., 2015); and (peroxisome-derived) H_2O_2 can activate the peroxisomal pool of ATM (Tripathi et al., 2016), an event that enhances 1) autophagic flux through AAPK-TSC2-mediated suppression of mTORC1 activity, and 2) pexophagy through phosphorylation and subsequent ubiquitination of the peroxisome-associated pool of PEX5 (Zhang et al., 2013; Tripathi and Walker, 2016). At first sight, these findings appear somewhat paradoxical. However, this may highlight the complexity of the peroxisome-autophagy signaling axis and point to the importance of other factors. For example, it is well known that disturbances in peroxisomal fitness are intrinsically linked to mitochondrial redox imbalances (Fransen et al., 2017). In addition, we recently found that peroxisomes with a dysfunctional H_2O_2 metabolism are not necessarily predisposed to pexophagy, even though peroxisome-derived H_2O_2 has the potential to oxidize redox-sensitive cysteine residues in PEX5, PTEN, NFKB1, TF65, and FOXO3 (Lismont et al., 2019a), all proteins whose activities can be linked to pexophagy or autophagy regulation (Subramani, 2015; Füllgrabe et al., 2016; Koundouros and Poulogiannis, 2018). Importantly, given that H_2O_2 shows a Janus-faced effect on autophagy (see *Redox Regulation of Autophagy*), it remains to be investigated whether the observed oxidative modifications lead to autophagy stimulation or inhibition. In addition, it still must be clarified if and to which extent other redox-sensitive autophagy-related proteins can act as a target of peroxisome-derived H_2O_2.

THE OXIDATIVE STRESS-PEXOPHAGY SIGNALING AXES

Oxidative stress is generally considered as one of the key mediators of cellular aging, a process that coincides with a decline in autophagic activity (Leidal et al., 2018) and a build-up of peroxisomes with a disturbed H_2O_2 metabolism (Legakis et al., 2002; Houri et al., 2020). Given that 1) in senescent cells, peroxisomes accumulate excessive amounts of PEX5 on their membranes (Legakis et al., 2002), 2) extraction of PEX5 from the peroxisomal membrane requires monoubiquitination of the protein at Cys11 (Carvalho et al., 2007), 3) Cys11 of human PEX5 functions as a redox switch that modulates the protein's activity in response to intracellular oxidative stress (Apanasets et al., 2014), and 4) excessive peroxisomal H_2O_2 production decreases the intracellular levels of the (peroxisome-associated)

$PEX5_{Cys11\text{-}ubiquitin}$ thioester conjugate (Lismont et al., 2019a), these findings point to an oxidative stress-induced decrease in PEX5-mediated pexophagy. On the other hand, dysfunctional autophagy/pexophagy will also lead to accumulation of SQSTM, a SAR linking autophagy and NF2L2 signaling through KEAP1 sequestration (Jiang et al., 2015a; Bartolini et al., 2018). This in turn enhances the expression of peroxisome proliferator-activated receptor-γ coactivator-1α, a protein whose expression is antioxidant response element-regulated (Gureev et al., 2019) and causes peroxisomal remodeling and biogenesis (Bagattin et al., 2010). As such, oxidative insults and defects in autophagy can lead to an accumulation of oxidatively burned-out peroxisomes, thereby fueling a vicious circle of oxidative injury (Vasko and Goligorsky, 2013).

PEXOPHAGY AND DISEASE

Pexophagy acts as a global regulator of peroxisome homeostasis and quality control and, as such, it does not come as a surprise that perturbations in this process have been linked to multiple disease conditions. Examples include the peroxisome biogenesis disorders (Nazarko, 2017), cancer (Walter et al., 2014; Dahabieh et al., 2018), lipopolysaccharide-induced acute kidney injury (Vasko, 2016), malnutrition-associated liver steatosis (van Zutphen et al., 2014), diabetes (Chu et al., 2020), noise-induced hearing loss (Defourny et al., 2019), HIV1 infections (Daussy et al., 2020), and neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Jo et al., 2020a). In the following paragraphs, we outline these examples in more detail.

In case of peroxisome biogenesis disorders, it was suggested that 1) in patients with mutations in genes coding for proteins constituting the ubiquitin-PEX5 export machinery, the disease phenotype is rather caused by excessive removal of peroxisomes than by defects in the peroxisomal matrix protein import machinery (Nazarko, 2017), and 2) low doses of autophagy inhibitors improve peroxisomal matrix protein import and peroxisome function without compromising cell viability (Law et al., 2017). Unfortunately, these findings could not be confirmed by others (Klouwer et al., 2021).

Both excessive and defective pexophagy have been linked to cancer (Dahabieh et al., 2018). For example, loss of peroxisomes due to enhanced pexophagy leads to metabolic alterations that have been suggested to promote a malignant phenotype in human clear cell renal cell carcinomas (Walter et al., 2014); and high expression levels of PEX6, PEX26 or MTOR, three negative regulators of pexophagy, have been associated with decreased patient survival in diffuse large B-cell lymphoma, lung cancer and melanoma cohorts. In the latter case, interference with the function of these proteins increased pexophagy and thwarted drug resistance in human melanoma and lymphoma cells (Dahabieh et al., 2021).

Pexophagy was also found to protect auditory hair cells against noise-induced oxidative damage (Defourny et al., 2019) and to attenuate lipopolysaccharide-induced acute kidney injury (Vasko et al., 2013; Vasko, 2016). In these conditions, the removal of dysfunctional peroxisomes may serve a quality control function to prevent ROS accumulation. On the other hand, enhanced peroxisome turnover because of HIV1 Env expression (Daussy et al., 2020) or mutations in GRP75 (Jo et al., 2020b) sensitize, respectively bystander $CD4^+$ T lymphocytes and neuronal cells to oxidative injury, thereby potentially contributing to viral spreading and the progression of Parkinson's disease. Finally, severe malnutrition-induced pexophagy contributes to hepatic mitochondrial dysfunction (Van Zutphen et al., 2014), and short-term inhibition of pexophagy benefits the health of pancreatic β-cells through elevation of ether phospholipid biosynthesis and by counteracting depletion of n-3 polyunsaturated fatty acids after fat-feeding (Chu et al., 2020).

CONCLUSION AND PERSPECTIVES

Pexophagy is a complex cellular process that is tightly regulated at multiple levels and by distinct stimuli. The data presented in this review support the view that changes in the intracellular redox state have the potential to balance this process through activity modulation of autophagy-related proteins, transcription factors, kinases, phosphatases, and PEX5. An increasing number of studies started to examine the relationship between peroxisomal H_2O_2 emission and pexophagy, with a focus on the role of peroxisome-associated ubiquitin-PEX5. Major hurdles that have slowed down these studies include the lack of 1) easily accessible and reliable tools to monitor pexophagy in a dynamic manner, 2) compounds that rapidly and selectively trigger peroxisome degradation, and 3) study models that allow the modulation of peroxisomal H_2O_2 production in a time- and dose-dependent manner. Here, it is important to highlight that traditional platforms for studying pexophagy mainly focus on immunoblot and (immuno)cytochemistry analyses of key autophagy and peroxisome markers, which only provide a snapshot of a dynamic situation. In addition, unlike what is sometimes thought, amino acid starvation-induced pexophagy is a non-selective process, as also other kinds of cargo (e.g., portions of the cytosol, endoplasmic reticulum, and mitochondria) are sequestered during this type of "metabolic" autophagy (Deretic and Kroemer, 2021; and references therein). Furthermore, although there is mounting evidence that disturbances in peroxisomal H_2O_2 metabolism can trigger pexophagy, some frequently cited key experiments have been carried out by treating cells with external H_2O_2 (Zhang et al., 2013), a condition incomparable with intraperoxisomal H_2O_2 production (Lismont et al., 2021). As such, the recent development of 1) a genetically modified human cell line in which the intraperoxisomal production of H_2O_2 can be selectively modulated in a dose- and time-dependent manner (Lismont et al., 2019a), 2) a peroxisome-targeted variant of mKeima, a pH-sensitive red fluorescent protein suitable for imaging pexophagy in cellulo (Marcassa et al., 2018; Jo et al., 2020b), and 3) new fluorescent probes for *in vitro* and *in vivo* quantification of H_2O_2 (Ye et al., 2020), offers new opportunities to dynamically monitor (e.g., by flow cytometry) and study pexophagy flux in living cells in response to controlled fluctuations in peroxisomal H_2O_2 levels.

Despite the tremendous progress made in recent years, additional work is needed to better understand the peroxisome-autophagy redox connection and to sort out the exact nature of the mechanisms underlying the seemingly contradictory observations regarding the role of amino acid starvation, oxidative stress, and hypoxia in pexophagy regulation. Questions that deserve further research include but are not limited to: Which proteins with an established role in autophagy regulation are direct or indirect targets of peroxisome-derived H_2O_2? How do the corresponding oxidative modifications affect the activities of these proteins? Are the cellular responses induced dose-, time-, and cell type-specific? Do the *in vitro* studies recapitulate the *in vivo* situation? Obtaining answers to these questions will not only help us to unravel the molecular mechanisms underlying the Janus-role of pexophagy in health and disease, but also aid researchers to screen for pharmacological pexophagy regulators that can be used in a clinical setting to compensate for genetic and age-related changes in peroxisome homeostasis.

AUTHOR CONTRIBUTIONS

HL and MF conceptualized and wrote the manuscript. HL generated the figures. HL, CL, IR, MAH, CC, and MF made a substantial intellectual contribution to the work, contributed to the editing, and approved the submitted version of the manuscript.

FUNDING

This work was supported by the KU Leuven (grant number: C14/18/088), the Research Foundation–Flanders (grant numbers G095315N and G091819N), and the European Union's Horizon 2020 Research and Innovation Programme (Marie Skłodowska-Curie grant agreement No. 812968 (PERICO)). HL was a recipient of a doctoral fellowship from the China Scholarship Council (201906790005), CL was supported by postdoctoral fellowships from the KU Leuven (PDM/18/188) and the Research Foundation–Flanders (1213620N), and MAH was supported by a scholarship from the Ministry of Higher Education of the Arab Republic of Egypt.

ACKNOWLEDGMENTS

The authors acknowledge Servier Medical Art (https://smart.servier.com) for artistic templates.

REFERENCES

Alers, S., Löffler, A. S., Wesselborg, S., and Stork, B. (2012). Role of AMPK-mTOR-Ulk1/2 in the Regulation of Autophagy: Cross Talk, Shortcuts, and Feedbacks. *Mol. Cel. Biol.* 32, 2–11. doi:10.1128/MCB.06159-11

Apanasets, O., Grou, C. P., Van Veldhoven, P. P., Brees, C., Wang, B., Nordgren, M., et al. (2014). PEX5, the Shuttling Import Receptor for Peroxisomal Matrix Proteins, Is a Redox-Sensitive Protein. *Traffic* 15, 94–103. doi:10.1111/tra.12129

Asgari, R., Yarani, R., Mohammadi, P., and Emami Aleagha, M. S. (2021). HIF-1α in the Crosstalk between Reactive Oxygen Species and Autophagy Process: a Review in Multiple Sclerosis. *Cell. Mol. Neurobiol.* in press. doi:10.1007/s10571-021-01111-5

Bagattin, A., Hugendubler, L., and Mueller, E. (2010). Transcriptional Coactivator PGC-1alpha Promotes Peroxisomal Remodeling and Biogenesis. *Proc. Natl. Acad. Sci. USA* 107, 20376–20381. doi:10.1073/pnas.1009176107

Bartolini, D., Dallaglio, K., Torquato, P., Piroddi, M., and Galli, F. (2018). Nrf2-p62 Autophagy Pathway and its Response to Oxidative Stress in Hepatocellular Carcinoma. *Transl. Res.* 193, 54–71. doi:10.1016/j.trsl.2017.11.007

Cagnol, S., and Chambard, J. C. (2010). ERK and Cell Death: Mechanisms of ERK-Induced Cell Death – Apoptosis, Autophagy and Senescence. *FEBS J.* 277, 2–21. doi:10.1111/j.1742-4658.2009.07366.x

Calero-Muñoz, N., Exposito-Rodriguez, M., Collado-Arenal, A. M., Rodríguez-Serrano, M., Laureano-Marín, A. M., Santamaría, M. E., et al. (2019). Cadmium Induces Reactive Oxygen Species-dependent Pexophagy in *Arabidopsis* Leaves. *Plant Cel Environ* 42, 2696–2714. doi:10.1111/pce.13597

Carroll, B., Otten, E. G., Manni, D., Stefanatos, R., Menzies, F. M., Smith, G. R., et al. (2018). Oxidation of SQSTM1/p62 Mediates the Link between Redox State and Protein Homeostasis. *Nat. Commun.* 9, 256. doi:10.1038/s41467-017-02746-z

Carvalho, A. F., Pinto, M. P., Grou, C. P., Alencastre, I. S., Fransen, M., Sá-Miranda, C., et al. (2007). Ubiquitination of Mammalian Pex5p, the Peroxisomal Import Receptor. *J. Biol. Chem.* 282, 31267–31272. doi:10.1074/jbc.M706325200

Cemma, M., Kim, P. K., and Brumell, J. H. (2011). The Ubiquitin-Binding Adaptor Proteins p62/SQSTM1 and NDP52 Are Recruited Independently to Bacteria-Associated Microdomains to Target Salmonella to the Autophagy Pathway. *Autophagy* 7, 341–345. doi:10.4161/auto.7.3.14046

Cha-Molstad, H., Lee, S. H., Kim, J. G., Sung, K. W., Hwang, J., Shim, S. M., et al. (2018). Regulation of Autophagic Proteolysis by the N-Recognin SQSTM1/p62 of the N-End Rule Pathway. *Autophagy* 14, 359–361. doi:10.1080/15548627.2017.1415190

Chen, B. H., Chang, Y. J., Lin, S., and Yang, W. Y. (2020). Hsc70/Stub1 Promotes the Removal of Individual Oxidatively Stressed Peroxisomes. *Nat. Commun.* 11, 5267. doi:10.1038/s41467-020-18942-3

Chen, Y., Azad, M. B., and Gibson, S. B. (2009). Superoxide Is the Major Reactive Oxygen Species Regulating Autophagy. *Cell Death Differ.* 16, 1040–1052. doi:10.1038/cdd.2009.49

Chiang, H. L., Terlecky, S. R., Plant, C. P., and Dice, J. F. (1989). A Role for a 70-kilodalton Heat Shock Protein in Lysosomal Degradation of Intracellular Proteins. *Science* 246, 382–385. doi:10.1126/science.2799391

Chu, K. Y., Mellet, N., Thai, L. M., Meikle, P. J., and Biden, T. J. (2020). Short-term Inhibition of Autophagy Benefits Pancreatic β-cells by Augmenting Ether Lipids and Peroxisomal Function, and by Countering Depletion of N-3 Polyunsaturated Fatty Acids after Fat-Feeding. *Mol. Metab.* 40, 101023. doi:10.1016/j.molmet.2020.101023

Cuervo, A. M., and Dice, J. F. (2000). Unique Properties of Lamp2a Compared to Other Lamp2 Isoforms. *J. Cel Sci.* 113, 4441–4450. doi:10.1242/jcs.113.24.4441

Czarny, P., Pawlowska, E., Bialkowska-Warzecha, J., Kaarniranta, K., and Blasiak, J. (2015). Autophagy in DNA Damage Response. *Int. J. Mol. Sci.* 16, 2641–2662. doi:10.3390/ijms16022641

Dahabieh, M. S., Di Pietro, E., Jangal, M., Goncalves, C., Witcher, M., Braverman, N. E., et al. (2018). Peroxisomes and Cancer: The Role of a Metabolic Specialist in a Disease of Aberrant Metabolism. *Biochim. Biophys. Acta Rev. Cancer* 1870, 103–121. doi:10.1016/j.bbcan.2018.07.004

Dahabieh, M. S., Huang, F., Goncalves, C., González, R. E. F., Prabhu, S., Bolt, A., et al. (2021). Silencing PEX26 as an Unconventional Mode to Kill Drug-Resistant Cancer Cells and Forestall Drug Resistance. *Autophagy*. in press. doi:10.1080/15548627.2021.1936932

Daskalaki, I., Gkikas, I., and Tavernarakis, N. (2018). Hypoxia and Selective Autophagy in Cancer Development and Therapy. *Front. Cel Dev. Biol.* 6, 104. doi:10.3389/fcell.2018.00104

Daussy, C. F., Galais, M., Pradel, B., Robert-Hebmann, V., Sagnier, S., Pattingre, S., et al. (2020). HIV-1 Env Induces Pexophagy and an Oxidative Stress Leading to Uninfected CD4$^+$ T Cell Death. *Autophagy* 17, 2465–2474. doi:10.1080/15548627.2020.1831814

De Duve, C., and Baudhuin, P. (1966). Peroxisomes (Microbodies and Related Particles). *Physiol. Rev.* 46, 323–357. doi:10.1152/physrev.1966.46.2.323

Defourny, J., Aghaie, A., Perfettini, I., Avan, P., Delmaghan, i. S., and Petit, C. (2019). Pejvakin-mediated Pexophagy Protects Auditory Hair Cells against Noise-Induced Damage. *Proc. Natl. Acad. Sci. USA* 116, 8010–8017. doi:10.1073/pnas.1821844116

Deleyto-Seldas, N., and Efeyan, A. (2021). The mTOR-Autophagy axis and the Control of Metabolism. *Front. Cel Dev. Biol.* 9, 655731. doi:10.3389/fcell.2021.655731

Deng, A., Ma, L., Zhou, X., Wang, X., Wang, S., Chen, X., et al. (2021a). FoxO3 Transcription Factor Promotes Autophagy after Oxidative Stress Injury in HT22 Cells. *Physiol. Pharmacol.* 99, 627–634. doi:10.1139/cjpp-2020-0448

Deng, J. K., Zhang, X., Wu, H. L., Gan, Y., Ye, L., Zheng, H., et al. (2021b). ROS-ERK Pathway as Dual Mediators of Cellular Injury and Autophagy-Associated Adaptive Response in Urinary Protein-Irritated Renal Tubular Epithelial Cells. *J. Diabetes Res.* 2021, 6614848. doi:10.1155/2021/6614848

Deosaran, E., Larsen, K. B., Hua, R., Sargent, G., Wang, Y., Kim, S., et al. (2013). NBR1 Acts as an Autophagy Receptor for Peroxisomes. *J. Cel Sci.* 126, 939–952. doi:10.1242/jcs.114819

Deretic, V., and Kroemer, G. (2021). Autophagy in Metabolism and Quality Control: Opposing, Complementary or Interlinked Functions? *Autophagy*. in press. doi:10.1080/15548627.2021.1933742

Desideri, E., Filomeni, G., and Ciriolo, M. R. (2012). Glutathione Participates in the Modulation of Starvation-Induced Autophagy in Carcinoma Cells. *Autophagy* 8, 1769–1781. doi:10.4161/auto.22037

Dutta, R. K., Maharjan, Y., Lee, J. N., Park, C., Ho, Y. S., and Park, R. (2021). Catalase Deficiency Induces Reactive Oxygen Species Mediated Pexophagy and Cell Death in the Liver during Prolonged Fasting. *Biofactors* 47, 112–125. doi:10.1002/biof.1708

Egan, D., Kim, J., Shaw, R. J., and Guan, K. L. (2011). The Autophagy Initiating Kinase ULK1 Is Regulated via Opposing Phosphorylation by AMPK and mTOR. *Autophagy* 7, 643–644. doi:10.4161/auto.7.6.15123

Fan, P., Xie, X. H., Chen, C. H., Peng, X., Zhang, P., Yang, C., et al. (2019). Molecular Regulation Mechanisms and Interactions between Reactive Oxygen Species and Mitophagy. *DNA Cel Biol.* 38, 10–22. doi:10.1089/dna.2018.4348

Fang, E. F., Hou, Y., Palikaras, K., Adriaanse, B. A., Kerr, J. S., Yang, B., et al. (2019). Mitophagy Inhibits Amyloid-β and Tau Pathology and Reverses Cognitive Deficits in Models of Alzheimer's Disease. *Nat. Neurosci.* 22, 401–412. doi:10.1038/s41593-018-0332-9

Fang, Y., Tan, J., and Zhang, Q. (2015). Signaling Pathways and Mechanisms of Hypoxia-Induced Autophagy in the Animal Cells. *Cell Biol. Int.* 39, 891–898. doi:10.1002/cbin.10463

Feng, Y., He, D., Yao, Z., and Klionsky, D. J. (2014). The Machinery of Macroautophagy. *Cell Res.* 24, 24–41. doi:10.1038/cr.2013.168

Filomeni, G., Desideri, E., Cardaci, S., Rotilio, G., and Ciriolo, M. R. (2010). Under the ROS: Thiol Network Is the Principal Suspect for Autophagy Commitment. *Autophagy* 6, 999–1005. doi:10.4161/auto.6.7.12754

Fourcade, S., Ferrer, I., and Pujol, A. (2015). Oxidative Stress, Mitochondrial and Proteostasis Malfunction in Adrenoleukodystrophy: A Paradigm for Axonal Degeneration. *Free Radic. Biol. Med.* 88, 18–29. doi:10.1016/j.freeradbiomed.2015.05.041

Francisco, T., Rodrigues, T. A., Dias, A. F., Barros-Barbosa, A., Bicho, D., and Azevedo, J. E. (2017). Protein Transport into Peroxisomes: Knowns and Unknowns. *Bioessays* 39, 1700047. doi:10.1002/bies.201700047

Fransen, M., Lismont, C., and Walton, P. (2017). The Peroxisome-Mitochondria Connection: How and Why? *Int. J. Mol. Sci.* 18, 1126. doi:10.3390/ijms18061126

Fransen, M., Terlecky, S. R., and Subramani, S. (1998). Identification of a Human PTS1 Receptor Docking Protein Directly Required for Peroxisomal Protein Import. *Proc. Natl. Acad. Sci. USA* 95, 8087–8092. doi:10.1073/pnas.95.14.8087

Frazier, D. P., Wilson, A., Dougherty, C. J., Li, H., Bishopric, N. H., and Webster, K. A. (2007). PKC-alpha and TAK-1 Are Intermediates in the Activation of C-Jun NH2-terminal Kinase by Hypoxia-Reoxygenation. *Am. J. Physiol. Heart Circ. Physiol.* 292, H1675–H1684. doi:10.1152/ajpheart.01132.2006

Frudd, K., Burgoyne, T., and Burgoyne, J. R. (2018). Oxidation of Atg3 and Atg7 Mediates Inhibition of Autophagy. *Nat. Commun.* 9, 95. doi:10.1038/s41467-017-02352-z

Füllgrabe, J., Ghislat, G., Cho, D. H., and Rubinsztein, D. C. (2016). Transcriptional Regulation of Mammalian Autophagy at a Glance. *J. Cel Sci.* 129, 3059–3066. doi:10.1242/jcs.188920

Ganguli, G., Pattanaik, K. P., Jagadeb, M., and Sonawane, A. (2020). *Mycobacterium tuberculosis* Rv3034c Regulates mTORC1 and PPAR-Gamma Dependant Pexophagy Mechanism to Control Redox Levels in Macrophages. *Cell. Microbiol.* 22, e13214. doi:10.1111/cmi.13214

Gatica, D., Lahiri, V., and Klionsky, D. J. (2018). Cargo Recognition and Degradation by Selective Autophagy. *Nat. Cel Biol.* 20, 233–242. doi:10.1038/s41556-018-0037-z

Germain, K., and Kim, P. K. (2020). Pexophagy: a Model for Selective Autophagy. *Int. J. Mol. Sci.* 21, 578. doi:10.3390/ijms21020578

Gubas, A., and Dikic, I. (2021). A Guide to the Regulation of Selective Autophagy Receptors. *FEBS J.* in press. doi:10.1111/febs.15824

Gureev, A. P., Shaforostova, E. A., and Popov, V. N. (2019). Regulation of Mitochondrial Biogenesis as a Way for Active Longevity: Interaction between the Nrf2 and PGC-1α Signaling Pathways. *Front. Genet.* 10, 435. doi:10.3389/fgene.2019.00435

Hara-Kuge, S., and Fujiki, Y. (2008). The Peroxin Pex14p Is Involved in LC3-dependent Degradation of Mammalian Peroxisomes. *Exp. Cel Res.* 314, 3531–3541. doi:10.1016/j.yexcr.2008.09.015

He, A., Chen, X., Tan, M., Chen, Y., Lu, D., Zhang, X., et al. (2020). Acetyl-CoA Derived from Hepatic Peroxisomal β-Oxidation Inhibits Autophagy and Promotes Steatosis via mTORC1 Activation. *Mol. Cel.* 79, 30–42. e4. doi:10.1016/j.molcel.2020.05.007

He, Y., She, H., Zhang, T., Xu, H., Cheng, L., Yepes, M., et al. (2018). p38 MAPK Inhibits Autophagy and Promotes Microglial Inflammatory Responses by Phosphorylating ULK1. *J. Cel Biol.* 217, 315–328. doi:10.1083/jcb.201701049

Houri, K., Mori, T., Onodera, Y., Tsujimoto, T., Takehara, T., Nakao, S., et al. (2020). miR-142 Induces Accumulation of Reactive Oxygen Species (ROS) by Inhibiting Pexophagy in Aged Bone Marrow Mesenchymal Stem Cells. *Sci. Rep.* 10, 3735. doi:10.1038/s41598-020-60346-2

Hu, W., Chen, S., Thorne, R. F., and Wu, M. (2019). TP53, TP53 Target Genes (DRAM, TIGAR), and Autophagy. *Adv. Exp. Med. Biol.* 1206, 127–149. doi:10.1007/978-981-15-0602-4_6

Hurley, J. H., and Young, L. N. (2017). Mechanisms of Autophagy Initiation. *Annu. Rev. Biochem.* 86, 225–244. doi:10.1146/annurev-biochem-061516-044820

Inoki, K., Zhu, T., and Guan, K. L. (2003). TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival. *Cell* 115, 577–590. doi:10.1016/S0092-8674(03)00929-2

Ivashchenko, O., Van Veldhoven, P. P., Brees, C., Ho, Y. S., Terlecky, S. R., and Fransen, M. (2011). Intraperoxisomal Redox Balance in Mammalian Cells: Oxidative Stress and Interorganellar Cross-Talk. *Mol. Biol. Cel.* 22, 1440–1451. doi:10.1091/mbc.e10-11-0919

Jain, I. H., Calvo, S. E., Markhard, A. L., Skinner, O. S., To, T. L., Ast, T., et al. (2020). Genetic Screen for Cell Fitness in High or Low Oxygen Highlights Mitochondrial and Lipid Metabolism. *Cell* 181, 716–727. doi:10.1016/j.cell.2020.03.029

Jiang, L., Hara-Kuge, S., Yamashita, S. I., and Fujiki, Y. (2015a). Peroxin Pex14p Is the Key Component for Coordinated Autophagic Degradation of Mammalian Peroxisomes by Direct Binding to LC3-II. *Genes Cells* 20, 36–49. doi:10.1111/gtc.12198

Jiang, T., Harder, B., Rojo de la Vega, M., Wong, P. K., Chapman, E., and Zhang, D. D. (2015b). p62 Links Autophagy and Nrf2 Signaling. *Free Radic. Biol. Med.* 88, 199–204. doi:10.1016/j.freeradbiomed.2015.06.014

Jo, D. S., Bae, D. J., Park, S. J., Seo, H. M., Kim, H. B., Oh, J. S., et al. (2015). Pexophagy Is Induced by Increasing Peroxisomal Reactive Oxygen Species in 1,10-Phenanthroline-Treated Cells. *Biochem. Biophys. Res. Commun.* 467, 354–360. doi:10.1016/j.bbrc.2015.09.153

Jo, D. S., Park, N. Y., and Cho, D. H. (2020a). Peroxisome Quality Control and Dysregulated Lipid Metabolism in Neurodegenerative Diseases. *Exp. Mol. Med.* 52, 1486–1495. doi:10.1038/s12276-020-00503-9

Jo, D. S., Park, S. J., Kim, A. K., Park, N. Y., Kim, J. B., Bae, J. E., et al. (2020b). Loss of HSPA9 Induces Peroxisomal Degradation by Increasing Pexophagy. *Autophagy* 16, 1989–2003. doi:10.1080/15548627.2020.1712812

Juretschke, T., and Beli, P. (2021). Causes and Consequences of DNA Damage-Induced Autophagy. *Matrix Biol.* 100-101, 39–53. doi:10.1016/j.matbio.2021.02.004

Kaur, J., and Debnath, J. (2015). Autophagy at the Crossroads of Catabolism and Anabolism. *Nat. Rev. Mol. Cel Biol.* 16, 461–475. doi:10.1038/nrm4024

Kaushik, S., and Cuervo, A. M. (2018). The Coming of Age of Chaperone-Mediated Autophagy. *Nat. Rev. Mol. Cel Biol.* 19, 365–381. doi:10.1038/s41580-018-0001-6

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

Kenific, C. M., and Debnath, J. (2015). Cellular and Metabolic Functions for Autophagy in Cancer Cells. *Trends Cel Biol.* 25, 37–45. doi:10.1016/j.tcb.2014.09.001

Kierans, S. J., and Taylor, C. T. (2021). Regulation of Glycolysis by the Hypoxia-Inducible Factor (HIF): Implications for Cellular Physiology. *J. Physiol.* 599, 23–37. doi:10.1113/JP280572

Kim, B. W., Kwon, D. H., and Song, H. K. (2016). Structure Biology of Selective Autophagy Receptors. *BMB Rep.* 49, 73–80. doi:10.5483/BMBRep.2016.49.2.265

Kim, J., Kundu, M., Viollet, B., and Guan, K. L. (2011). AMPK and mTOR Regulate Autophagy through Direct Phosphorylation of Ulk1. *Nat. Cel Biol.* 13, 132–141. doi:10.1038/ncb2152

Kim, P. K., Hailey, D. W., Mullen, R. T., and Lippincott-Schwartz, J. (2008). Ubiquitin Signals Autophagic Degradation of Cytosolic Proteins and Peroxisomes. *Proc. Natl. Acad. Sci. USA* 105, 20567–20574. doi:10.1073/pnas.0810611105

Kirkin, V., and Rogov, V. V. (2019). A Diversity of Selective Autophagy Receptors Determines the Specificity of the Autophagy Pathway. *Mol. Cel.* 76, 268–285. doi:10.1016/j.molcel.2019.09.005

Kirkin, V., Lamark, T., Sou, Y. S., Bjørkøy, G., Nunn, J. L., Bruun, J. A., et al. (2009). A Role for NBR1 in Autophagosomal Degradation of Ubiquitinated Substrates. *Mol. Cel.* 33, 505–516. doi:10.1016/j.molcel.2009.01.020

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). *Autophagy* 17, 1–382. doi:10.1080/15548627.2020.179728010.1080/15548627.2015.1100356

Klouwer, F. C. C., Falkenberg, K. D., Ofman, R., Koster, J., van Gent, D., Ferdinandusse, S., et al. (2021). Autophagy Inhibitors Do Not Restore Peroxisomal Functions in Cells with the Most Common Peroxisome Biogenesis Defect. *Front. Cel Dev. Biol.* 9, 661298. doi:10.3389/fcell.2021.661298

Kma, L., and Baruah, T. J. (2021). The Interplay of ROS and the PI3K/Akt Pathway in Autophagy Regulation. *Biotechnol. Appl. Biochem.* 2021., 2021 in press. doi:10.1002/bab.2104

Koundouros, N., and Poulogiannis, G. (2018). Phosphoinositide 3-kinase/Akt Signaling and Redox Metabolism in Cancer. *Front. Oncol.* 8, 160. doi:10.3389/fonc.2018.00160

Kriegenburg, F., Ungermann, C., and Reggiori, F. (2018). Coordination of Autophagosome-Lysosome Fusion by Atg8 Family Members. *Curr. Biol.* 28, R512–R518. doi:10.1016/j.cub.2018.02.034

Lamark, T., and Johansen, T. (2021). Mechanisms of Selective Autophagy. *Annu. Rev. Cel Dev. Biol.* 37, 143–169. doi:10.1146/annurev-cellbio-120219-035530

Launay, N., Aguado, C., Fourcade, S., Ruiz, M., Grau, L., Riera, J., et al. (2015). Autophagy Induction Halts Axonal Degeneration in a Mouse Model of X-Adrenoleukodystrophy. *Acta Neuropathol.* 129, 399–415. doi:10.1007/s00401-014-1378-8

Law, K. B., Bronte-Tinkew, D., Di Pietro, E., Snowden, A., Jones, R. O., Moser, A., et al. (2017). The Peroxisomal AAA ATPase Complex Prevents Pexophagy and Development of Peroxisome Biogenesis Disorders. *Autophagy* 13, 868–884. doi:10.1080/15548627.2017.1291470

Lee, J. N., Dutta, R. K., Maharjan, Y., Liu, Z. Q., Lim, J. Y., Kim, S. J., et al. (2018). Catalase Inhibition Induces Pexophagy through ROS Accumulation. *Biochem. Biophys. Res. Commun.* 501, 696–702. doi:10.1016/j.bbrc.2018.05.050

Lee, J. W., Ko, J., Ju, C., and Eltzschig, H. K. (2019). Hypoxia Signaling in Human Diseases and Therapeutic Targets. *Exp. Mol. Med.* 51, 1–13. doi:10.1038/s12276-019-0235-1

Legakis, J. E., Koepke, J. I., Jedeszko, C., Barlaskar, F., Terlecky, L. J., Edwards, H. J., et al. (2002). Peroxisome Senescence in Human Fibroblasts. *Mol. Biol. Cel.* 13, 243–255. doi:10.1091/mbc.e02-06-0322

Leidal, A. M., Levine, B., and Debnath, J. (2018). Autophagy and the Cell Biology of Age-Related Disease. *Nat. Cel Biol.* 20, 1338–1348. doi:10.1038/s41556-018-0235-8

Li, J., and Wang, W. (2021). Mechanisms and Functions of Pexophagy in Mammalian Cells. *Cells* 10, 1094. doi:10.3390/cells10051094

Li, L., Tan, J., Miao, Y., Lei, P., and Zhang, Q. (2015). ROS and Autophagy: Interactions and Molecular Regulatory Mechanisms. *Cel. Mol. Neurobiol.* 35, 615–621. doi:10.1007/s10571-015-0166-x

Li, W., and Zhang, L. (2019). Regulation of ATG and Autophagy Initiation. *Adv. Exp. Med. Biol.* 1206, 41–65. doi:10.1007/978-981-15-0602-4_2

Li, X., Han, H., Zhou, M. T., Yang, B., Ta, A. P., Li, N., et al. (2017). Proteomic Analysis of the Human Tankyrase Protein Interaction Network Reveals its Role in Pexophagy. *Cell Rep.* 20, 737–749. doi:10.1016/j.celrep.2017.06.077

Lismont, C., Nordgren, M., Brees, C., Knoops, B., Van Veldhoven, P. P., and Fransen, M. (2019a). Peroxisomes as Modulators of Cellular Protein Thiol Oxidation: a New Model System. *Antioxid. Redox Signal.* 30, 22–39. doi:10.1089/ars.2017.6997

Lismont, C., Nordgren, M., Van Veldhoven, P. P., and Fransen, M. (2015). Redox Interplay between Mitochondria and Peroxisomes. *Front. Cel Dev. Biol.* 3, 35. doi:10.3389/fcell.2015.00035

Lismont, C., Revenco, I., and Fransen, M. (2019b). Peroxisomal Hydrogen Peroxide Metabolism and Signaling in Health and Disease. *Int. J. Mol. Sci.* 20, 3673. doi:10.3390/ijms20153673

Lismont, C., Revenco, R., Li, H., Costa, C. F., Lenaerts, L., Hussein, M. A. F., et al. (2021). Peroxisome-derived Hydrogen Peroxide Can Modulate the Sulfenylation Profiles of Key Redox Signaling Proteins. Preprint. *BioRxiv*. doi:10.1101/2021.10.08.463647

Lizama-Manibusan, B., and McLaughlin, B. (2013). Redox Modification of Proteins as Essential Mediators of CNS Autophagy and Mitophagy. *FEBS Lett.* 587, 2291–2298. doi:10.1016/j.febslet.2013.06.007

Lorin, S., Pierron, G., Ryan, K. M., Codogno, P., and Djavaheri-Mergny, M. (2010). Evidence for the Interplay between JNK and P53-DRAM Signalling Pathways in the Regulation of Autophagy. *Autophagy* 6, 153–154. doi:10.4161/auto.6.1.10537

Lystad, A. H., Carlsson, S. R., and Simonsen, A. (2019). Toward the Function of Mammalian ATG12-ATG5-Atg16l1 Complex in Autophagy and Related Processes. *Autophagy* 15, 1485–1486. doi:10.1080/15548627.2019.1618100

Marcassa, E., Kallinos, A., Jardine, J., Rusilowicz-Jones, E. V., Martinez, A., Kuehl, S., et al. (2018). Dual Role of USP30 in Controlling Basal Pexophagy and Mitophagy. *EMBO Rep.* 19, e45595. doi:10.15252/embr.201745595

Marinho, H. S., Real, C., Cyrne, L., Soares, H., and Antunes, F. (2014). Hydrogen Peroxide Sensing, Signaling and Regulation of Transcription Factors. *Redox Biol.* 2, 535–562. doi:10.1016/j.redox.2014.02.006

Martens, S., and Fracchiolla, D. (2020). Activation and Targeting of ATG8 Protein Lipidation. *Cel Discov.* 6, 23. doi:10.1038/s41421-020-0155-1

McCarthy, I., Romero-Puertas, M. C., Palma, J. M., Sandalio, L. M., Corpas, F. J., Gómez, M., et al. (2001). Cadmium Induces Senescence Symptoms in Leaf Peroxisomes of Pea Plants. *Plant Cel Environ* 24, 1065–1073. doi:10.1046/j.1365-3040.2001.0075010.1046/j.1365-3040.2001.00750.x

McClung, J. M., Judge, A. R., Powers, S. K., and Yan, Z. (2010). p38 MAPK Links Oxidative Stress to Autophagy-Related Gene Expression in Cachectic Muscle Wasting. *Am. J. Physiol. Cel Physiol.* 298, C542–C549. doi:10.1152/ajpcell.00192.2009

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. Cel Biol.* 217, 3640–3655. doi:10.1002/bies.20180000810.1083/jcb.201711002

Melia, T. J., Lystad, A. H., and Simonsen, A. (2020). Autophagosome Biogenesis: from Membrane Growth to Closure. *J. Cel Biol.* 219, e202002085. doi:10.1083/jcb.202002085

Mercer, T. J., Gubas, A., and Tooze, S. A. (2018). A Molecular Perspective of Mammalian Autophagosome Biogenesis. *J. Biol. Chem.* 293, 5386–5395. doi:10.1074/jbc.R117.810366

Mesquita, A., Glenn, J., and Jenny, A. (2021). Differential Activation of eMI by Distinct Forms of Cellular Stress. *Autophagy* 17, 1828–1840. doi:10.1080/15548627.2020.1783833

Mohan, J., and Wollert, T. (2018). Human Ubiquitin-like Proteins as central Coordinators in Autophagy. *Interf. Focus* 8, 20180025. doi:10.1098/rsfs.2018.0025

Mu, Y., Maharjan, Y., Dutta, R. K., Kim, H., Wei, X., Kim, J. H., et al. (2020). Dimethyloxaloylglycine Induces Pexophagy in a HIF-2alpha Dependent Manner Involving Autophagy Receptor P62. *Biochem. Biophys. Res. Commun.* 525, 46–52. doi:10.1016/j.bbrc.2020.02.051

Muralidharan, C., Conteh, A. M., Marasco, M. R., Crowder, J. J., Kuipers, J., de Boer, P., et al. (2021). Pancreatic Beta Cell Autophagy Is Impaired in Type 1 Diabetes. *Diabetologia* 64, 865–877. doi:10.1007/s00125-021-05387-6

Murrow, L., and Debnath, J. (2013). Autophagy as a Stress-Response and Quality-Control Mechanism: Implications for Cell Injury and Human Disease. *Annu. Rev. Pathol.* 8, 105–137. doi:10.1146/annurev-pathol-020712-163918

Nakamura, S., and Yoshimori, T. (2017). New Insights into Autophagosome-Lysosome Fusion. *J. Cel Sci.* 130, 1209–1216. doi:10.1242/jcs.196352

Nakatogawa, H. (2013). Two Ubiquitin-like Conjugation Systems that Mediate Membrane Formation during Autophagy. *Essays Biochem.* 55, 39–50. doi:10.1042/bse0550039

Nazarko, T. Y. (2017). Pexophagy Is Responsible for 65% of Cases of Peroxisome Biogenesis Disorders. *Autophagy* 13, 991–994. doi:10.1080/15548627.2017.1291480

Nishimura, T., and Tooze, S. A. (2020). Emerging Roles of ATG Proteins and Membrane Lipids in Autophagosome Formation. *Cel Discov.* 6, 32. doi:10.1038/s41421-020-0161-3

Nordgren, M., Francisco, T., Lismont, C., Hennebel, L., Brees, C., Wang, B., et al. (2015). Export-deficient Monoubiquitinated PEX5 Triggers Peroxisome Removal in SV40 Large T Antigen-Transformed Mouse Embryonic Fibroblasts. *Autophagy* 11, 1326–1340. doi:10.1080/15548627.2015.1061846

Nordgren, M., Wang, B., Apanasets, O., and Fransen, M. (2013). Peroxisome Degradation in Mammals: Mechanisms of Action, Recent Advances, and Perspectives. *Front. Physiol.* 4, 145. doi:10.3389/fphys.2013.00145

Oku, M., and Sakai, Y. (2018). Three Distinct Types of Microautophagy Based on Membrane Dynamics and Molecular Machineries. *Bioessays* 40, e1800008. doi:10.1002/bies.201800008

Ornatowski, W., Lu, Q., Yegambaram, M., Garcia, A. E., Zemskov, E. A., Maltepe, E., et al. (2020). Complex Interplay between Autophagy and Oxidative Stress in the Development of Pulmonary Disease. *Redox Biol.* 36, 101679. doi:10.1016/j.redox.2020.101679

Paddenberg, R., Ishaq, B., Goldenberg, A., Faulhammer, P., Rose, F., Weissmann, N., et al. (2003). Essential Role of Complex II of the Respiratory Chain in Hypoxia-Induced ROS Generation in the Pulmonary Vasculature. *Am. J. Physiol. Lung Cel. Mol. Physiol.* 284, L710–L719. doi:10.1152/ajplung.00149.2002

Påhlman, S., and Mohlin, S. (2018). Hypoxia and Hypoxia-Inducible Factors in Neuroblastoma. *Cell Tissue Res.* 372, 269–275. doi:10.1007/s00441-017-2701-1

Papandreou, I., Lim, A. L., Laderoute, K., and Denko, N. C. (2008). Hypoxia Signals Autophagy in Tumor Cells via AMPK Activity, Independent of HIF-1, BNIP3, and BNIP3L. *Cel Death Differ.* 15, 1572–1581. doi:10.1038/cdd.2008.84

Park, H., Kim, J., Shin, C., and Lee, S. (2021a). Intersection between Redox Homeostasis and Autophagy: Valuable Insights into Neurodegeneration. *Antioxidants* 10, 694. doi:10.3390/antiox10050694

Park, N. Y., Jo, D. S., Park, S. J., Lee, H., Bae, J. E., Hong, Y., et al. (2021b). Depletion of HNRNPA1 Induces Peroxisomal Autophagy by Regulating PEX1 Expression. *Biochem. Biophys. Res. Commun.* 545, 69–74. doi:10.1016/j.bbrc.2021.01.083

Parzych, K. R., and Klionsky, D. J. (2014). An Overview of Autophagy: Morphology, Mechanism, and Regulation. *Antioxid. Redox Signal.* 20, 460–473. doi:10.1089/ars.2013.5371

Puissant, A., Fenouille, N., and Auberger, P. (2012). When Autophagy Meets Cancer through p62/SQSTM1. *Am. J. Cancer Res.* 2, 397–413.

Rashid, H. O., Yadav, R. K., Kim, H. R., and Chae, H. J. (2015). ER Stress: Autophagy Induction, Inhibition and Selection. *Autophagy* 11, 1956–1977. doi:10.1080/15548627.2015.1091141

Rathore, R., Zheng, Y. M., Niu, C. F., Liu, Q. H., Korde, A., Ho, Y. S., et al. (2008). Hypoxia Activates NADPH Oxidase to Increase [ROS]i and [Ca^{2+}]i through the Mitochondrial ROS-PKCepsilon Signaling axis in Pulmonary Artery Smooth Muscle Cells. *Free Radic. Biol. Med.* 45, 1223–1231. doi:10.1016/j.freeradbiomed.2008.06.012

Redza-Dutordoir, M., and Averill-Bates, D. A. (2021). Interactions between Reactive Oxygen Species and Autophagy. *Biochim. Biophys. Acta Mol. Cel. Res.* 1868, 119041. doi:10.1016/j.bbamcr.2021.119041

Riccio, V., Demers, N., Hua, R., Vissa, M., Cheng, D. T., Strilchuk, A. W., et al. (2019). Deubiquitinating Enzyme USP30 Maintains Basal Peroxisome Abundance by Regulating Pexophagy. *J. Cel Biol.* 218, 798–807. doi:10.1083/jcb.201804172

Roberts, R., and Ktistakis, N. T. (2013). Omegasomes: PI3P Platforms that Manufacture Autophagosomes. *Essays Biochem.* 55, 17–27. doi:10.1042/bse0550017

Rodriguez-Rocha, H., Garcia-Garcia, A., Panayiotidis, M. I., and Franco, R. (2011). DNA Damage and Autophagy. *Mutat. Res.* 711, 158–166. doi:10.1016/j.mrfmmm.2011.03.007

Rouschop, K. M. A., van den Beucken, T., Dubois, L., Niessen, H., Bussink, J., Savelkouls, K., et al. (2010). The Unfolded Protein Response Protects Human Tumor Cells during Hypoxia through Regulation of the Autophagy Genes MAP1LC3B and ATG5. *J. Clin. Invest.* 120, 127–141. doi:10.1172/JCI40027

Rout, A. K., Strub, M. P., Piszczek, G., and Tjandra, N. (2014). Structure of Transmembrane Domain of Lysosome-Associated Membrane Protein Type 2a (LAMP-2A) Reveals Key Features for Substrate Specificity in Chaperone-Mediated Autophagy. *J. Biol. Chem.* 289, 35111–35123. doi:10.1074/jbc.M114.609446

Russell, R. C., Yuan, H. X., and Guan, K. L. (2014). Autophagy Regulation by Nutrient Signaling. *Cel Res.* 24, 42–57. doi:10.1038/cr.2013.166

Sargent, G., van Zutphen, T., Shatseva, T., Zhang, L., Giovanni, V. S., Bandsma, R., et al. (2016). PEX2 Is the E3 Ubiquitin Ligase Required for Pexophagy during Starvation. *J. Cel Biol.* 214, 677–690. doi:10.1083/jcb.201511034

Scherz-Shouval, R., Shvets, E., and Elazar, Z. (2007a). Oxidation as a post-translational Modification that Regulates Autophagy. *Autophagy* 3, 371–373. doi:10.4161/auto.4214

Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L., and Elazar, Z. (2007b). Reactive Oxygen Species Are Essential for Autophagy and Specifically Regulate the Activity of Atg4. *EMBO J.* 26, 1749–1760. doi:10.1038/sj.emboj.7601623

Schönenberger, M. J., Krek, W., and Kovacs, W. J. (2015). EPAS1/HIF-2α Is a Driver of Mammalian Pexophagy. *Autophagy* 11, 967–969. doi:10.1080/15548627.2015.1045180

Sedlackova, L., and Korolchuk, V. I. (2020). The Crosstalk of NAD, ROS and Autophagy in Cellular Health and Ageing. *Biogerontology* 21, 381–397. doi:10.1007/s10522-020-09864-0

Sengupta, A., Molkentin, J. D., and Yutzey, K. E. (2009). FoxO Transcription Factors Promote Autophagy in Cardiomyocytes. *J. Biol. Chem.* 284, 28319–28331. doi:10.1074/jbc.M109.024406

Shao, D., Oka, S., Liu, T., Zhai, P., Ago, T., Sciarretta, S., et al. (2014). A Redox-dependent Mechanism for Regulation of AMPK Activation by Thioredoxin1 during Energy Starvation. *Cell Metab* 19, 232–245. doi:10.1016/j.cmet.2013.12.013

Sies, H., and Jones, D. P. (2020). Reactive Oxygen Species (ROS) as Pleiotropic Physiological Signalling Agents. *Nat. Rev. Mol. Cel Biol.* 21, 363–383. doi:10.1038/s41580-020-0230-3

Son, Y., Cheong, Y. K., Kim, N. H., Chung, H. T., Kang, D. G., and Pae, H. O. (2011). Mitogen-activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways? *J. Signal. Transduct.* 2011, 792639. doi:10.1155/2011/792639

Stöcker, S., Van Laer, K., Mijuskovic, A., and Dick, T. P. (2018). The Conundrum of Hydrogen Peroxide Signaling and the Emerging Role of Peroxiredoxins as Redox Relay Hubs. *Antioxid. Redox Signal.* 28, 558–573. doi:10.1089/ars.2017.7162

Subramani, S. (2015). A Mammalian Pexophagy Target. *Nat. Cel Biol.* 17, 1371–1373. doi:10.1038/ncb3253

Sui, X., Kong, N., Ye, L., Han, W., Zhou, J., Zhang, Q., et al. (2014). p38 and JNK MAPK Pathways Control the Balance of Apoptosis and Autophagy in Response to Chemotherapeutic Agents. *Cancer Lett.* 344, 174–179. doi:10.1016/j.canlet.2013.11.019

Sun, T., Li, D., Wang, L., Xia, L., Ma, J., Guan, Z., et al. (2011). c-Jun NH2-terminal Kinase Activation Is Essential for Up-Regulation of LC3 during Ceramide-Induced Autophagy in Human Nasopharyngeal Carcinoma Cells. *J. Transl. Med.* 9, 161. doi:10.1186/1479-5876-9-161

Tripathi, D. N., and Walker, C. L. (2016). The Peroxisome as a Cell Signaling Organelle. *Curr. Opin. Cel Biol.* 39, 109–112. doi:10.1016/j.ceb.2016.02.017

Tripathi, D. N., Zhang, J., Jing, J., Dere, R., and Walker, C. L. (2016). A New Role for ATM in Selective Autophagy of Peroxisomes (Pexophagy). *Autophagy* 12, 711–712. doi:10.1080/15548627.2015.1123375

Van Veldhoven, P. P. (2010). Biochemistry and Genetics of Inherited Disorders of Peroxisomal Fatty Acid Metabolism. *J. Lipid Res.* 51, 2863–2895. doi:10.1194/jlr.R005959

van Zutphen, T., Ciapaite, J., Bloks, J. W., Ackereley, C., Gerding, A., Jurdzinski, A., et al. (2014). Malnutrition-associated Liver Steatosis and ATP Depletion Is Caused by Peroxisomal and Mitochondrial Dysfunction. *J. Hepatol.* 65, 1198–1208. doi:10.1016/j.jhep.2016.05.046

Vasko, R., and Goligorsky, M. S. (2013). Dysfunctional Lysosomal Autophagy Leads to Peroxisomal Oxidative Burnout and Damage during Endotoxin-Induced Stress. *Autophagy* 9, 442–444. doi:10.4161/auto.23344

Vasko, R. (2016). Peroxisomes and Kidney Injury. *Antioxid. Redox Signal.* 25, 217–231. doi:10.1089/ars.2016.6666

Vasko, R., Ratliff, B. B., Bohr, S., Nadel, E., Chen, J., Xavier, S., et al. (2013). Endothelial Peroxisomal Dysfunction and Impaired Pexophagy Promotes Oxidative Damage in Lipopolysaccharide-Induced Acute Kidney Injury. *Antioxid. Redox Signal.* 19, 211–230. doi:10.1089/ars.2012.4768

Walter, K. M., Schönenberger, M. J., Trötzmüller, M., Horn, M., Elsässer, H. P., Moser, A. B., et al. (2014). Hif-2α Promotes Degradation of Mammalian Peroxisomes by Selective Autophagy. *Cel Metab* 20, 882–897. doi:10.1016/j.cmet.2014.09.017

Wei, X., Maharjan, Y., Dorotea, D., Dutta, R. K., Kim, D., Kim, H., et al. (2021). Knockdown of PEX16 Induces Autophagic Degradation of Peroxisomes. *Int. J. Mol. Sci.* 22, 7989. doi:10.3390/ijms22157989

Wible, D. J., and Bratton, S. B. (2018). Reciprocity in ROS and Autophagic Signaling. *Curr. Opin. Toxicol.* 7, 28–36. doi:10.1016/j.cotox.2017.10.006

Yamashita, S. I., Abe, K., Tatemichi, Y., and Fujiki, Y. (2014). The Membrane Peroxin PEX3 Induces Peroxisome-Ubiquitination-Linked Pexophagy. *Autophagy* 10, 1549–1564. doi:10.4161/auto.29329

Yang, Y., Ma, F., Liu, Z., Su, Q., Liu, Y., Liu, Z., et al. (2019). The ER-Localized Ca^{2+}-Binding Protein Calreticulin Couples ER Stress to Autophagy by Associating with Microtubule-Associated Protein 1A/1B Light Chain 3. *J. Biol. Chem.* 294, 772–782. doi:10.1074/jbc.RA118.005166

Ye, S., Hu, J. J., Zhao, Q. A., and Yang, D. (2020). Fluorescent Probes for *In Vitro* and *In Vivo* Quantification of Hydrogen Peroxide. *Chem. Sci.* 11, 11989–11997. doi:10.1039/d0sc04888g

Yim, W. W. Y., and Mizushima, N. (2020). Lysosome Biology in Autophagy. *Cel Discov* 6, 6. doi:10.1038/s41421-020-0141-7

Yin, J., Duan, J., Cui, Z., Ren, W., Li, T., and Yin, Y. (2015). Hydrogen Peroxide-Induced Oxidative Stress Activates NF-kB and Nrf2/Keap1 Signals and Triggers Autophagy in Piglets. *RSC Adv.* 5, 15479. doi:10.1039/C4RA13557A

Yun, H. R., Jo, Y. H., Kim, J., Shin, Y., Kim, S. S., and Choi, T. G. (2020). Roles of Autophagy in Oxidative Stress. *Int. J. Mol. Sci.* 21, 3289. doi:10.3390/ijms21093289

Zhang, J., Kim, J., Alexander, A., Cai, S., Tripathi, D. N., Dere, R., et al. (2013). A Tuberous Sclerosis Complex Signalling Node at the Peroxisome Regulates mTORC1 and Autophagy in Response to ROS. *Nat. Cel Biol.* 15, 1186–1196. doi:10.1038/ncb2822

Zhang, J., Tripathi, D. N., Jing, J., Alexander, A., Kim, J., Powell, R. T., et al. (2015). ATM Functions at the Peroxisome to Induce Pexophagy in Response to ROS. *Nat. Cel. Biol.* 17, 1259–1269. doi:10.1038/ncb3230

Zhao, Y. G., Codogno, P., and Zhang, H. (2021). Machinery, Regulation and Pathophysiological Implications of Autophagosome Maturation. *Nat. Rev. Mol. Cel Biol.* 22, 733–750. doi:10.1038/s41580-021-00392-4

Zheng, J., Chen, X., Liu, Q., Zhong, G., and Zhuang, M. (2022). Ubiquitin Ligase MARCH5 Localizes to Peroxisomes to Regulate Pexophagy. *J. Cel Biol.* 221, e202103156. doi:10.1083/jcb.202103156

Zheng, X., Yang, Z., Gu, Q., Xia, F., Fu, Y., Liu, P., et al. (2020). The Protease Activity of Human ATG4B Is Regulated by Reversible Oxidative Modification. *Autophagy* 16, 1838–1850. doi:10.1080/15548627.2019.1709763

Zhou, F., Yang, Y., and Xing, D. (2011). Bcl-2 and Bcl-xL Play Important Roles in the Crosstalk between Autophagy and Apoptosis. *FEBS J.* 278, 403–413. doi:10.1111/j.1742-4658.2010.07965.x

Zhou, J., Li, X. Y., Liu, Y. J., Feng, J., Wu, Y., Shen, H. M., et al. (2021). Full-coverage Regulations of Autophagy by ROS: from Induction to Maturation. *Autophagy*. in press. doi:10.1080/15548627.2021.1984656

Zhou, Y. Y., Li, Y., Jiang, W. Q., and Zhou, L. F. (2015). MAPK/JNK Signalling: a Potential Autophagy Regulation Pathway. *Biosci. Rep.* 35, e00199. doi:10.1042/BSR20140141

Zmijewski, J. W., Banerjee, S., Bae, H., Friggeri, A., Lazarowski, E. R., and Abraham, E. (2010). Exposure to Hydrogen Peroxide Induces Oxidation and Activation of AMP-Activated Protein Kinase. *J. Biol. Chem.* 285, 33154–33164. doi:10.1074/jbc.M110.143685

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 handling editor declared a past co-authorship with one of the authors, MF.

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GLOSSARY

AAPK AMP-activated protein kinase

ATG autophagy-related protein

ATM ataxia telangiectasia mutated

BAKOR beclin 1-associated autophagy-related key regulator

BCL B-cell CLL/lymphoma

B2Cl Bcl-2-like protein

BECN beclin

BNIP ATM, ataxia telangiectasia mutated; BCL2/adenovirus E1B 19 kDa protein-interacting protein

BNI3L BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like

CACO calcium-binding and coiled-coil domain-containing protein

CHIP C-terminus of Hsc70-interacting protein

CMA chaperone-mediated autophagy

DMOG dimethyloxalylglycine

DNM1L dynamin-1-like protein

DRAM DNA damage-regulated autophagy modulator protein

Env envelope glycoprotein

EPAS endothelial PAS domain-containing protein

ER endoplasmic reticulum;

FIS fission protein

FOXO forkhead box protein

FUND FUN14 domain-containing protein

GRP stress-70 protein

HIF hypoxia-inducible factor

HIV1 human immunodeficiency virus type 1

HSP7C constitutive heat shock protein 70

HSC70 heat shock cognate 70 kDa protein

HSPA heat shock protein family A

KEAP Kelch-like ECH-associated protein

LAMP lysosome-associated membrane glycoprotein

LC3 microtubule associated protein 1 light chain 3

MARH5 membrane-associated RING-CH protein V

MK mitogen-activated protein kinase

MLP3 microtubule-associated proteins 1A/1B light chain

NBR next to BRCA1 gene protein

mTORC mechanistic target of rapamycin complex

NF2L2 nuclear factor erythroid 2-related factor

NFKB nuclear factor NF-kappa-B

NRBF nuclear receptor-binding factor

OPTN optineurin

P53 cellular tumor antigen p53

PARP poly (ADP-ribose) polymerase

PE phosphatidylethanolamine

PEX peroxin

PI3KC phosphatidyl 3-kinase complex

PI3P phosphatidylinositol 3-phosphate

PI3R4 phosphoinositide 3-kinase regulatory subunit

PJVK pejvakin

PK3C phosphatidylinositol 3-kinase catalytic subunit

PMP peroxisomal membrane protein

PTEN Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase

RAB RAS-associated binding

RBCC RB1-inducible coiled-coil protein

RETR reticulophagy regulator

RHEB RAS homologue enriched in brain

ROS reactive oxygen species

RPTOR regulatory-associated protein of mTOR. SAR, specific autophagy receptor

SNARE soluble N-ethylmaleimide-sensitive fusion protein attachment receptor

SQSTM sequestosome

TF transcription factor

TNKS poly (ADP-ribose) polymerase tankyrase

TSC tuberin

ULK UNC-51 like autophagy activating kinase

USP ubiquitin carboxyl-terminal hydrolase

VHL Von Hippel-Lindau

VLCFA very-long-chain fatty acid

frontiers in Cell and Developmental Biology

PERSPECTIVE
published: 07 January 2022
doi: 10.3389/fcell.2021.791758

Crucial Players for Inter-Organelle Communication: PI5P4Ks and Their Lipid Product PI-4,5-P_2 Come to the Surface

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While organelles are individual compartments with specialized functions, it is becoming clear that organellar communication is essential for maintaining cellular homeostasis. This cooperation is carried out by various interactions taking place on the membranes of organelles. The membranes themselves contain a multitude of proteins and lipids that mediate these connections and one such class of molecules facilitating these relations are the phospholipids. There are several phospholipids, but the focus of this perspective is on a minor group called the phosphoinositides and specifically, phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2). This phosphoinositide, on intracellular membranes, is largely generated by the non-canonical Type II PIPKs, namely, Phosphotidylinositol-5-phosphate-4-kinases (PI5P4Ks). These evolutionarily conserved enzymes are emerging as key stress response players in cells. Further, PI5P4Ks have been shown to modulate pathways by regulating organelle crosstalk, revealing roles in preserving metabolic homeostasis. Here we will attempt to summarize the functions of the PI5P4Ks and their product PI-4,5-P_2 in facilitating inter-organelle communication and how they impact cellular health as well as their relevance to human diseases.

Keywords: phosphoinositides, phosphotidylinositol-5-phosphate-4-kinases, PI-4, 5-P2, organelle, metabolism, peroxisomes, lipids

OPEN ACCESS

Edited by:
Joe Costello,
University of Exeter, United Kingdom

Reviewed by:
Masashi Maekawa,
Keio University, Japan
Raghu Padinjat,
National Centre for Biological Sciences, India

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

Received: *08 October 2021*
Accepted: *24 November 2021*
Published: *07 January 2022*

Citation:
Ravi A, Palamiuc L and Emerling BM (2022) Crucial Players for Inter-Organelle Communication: PI5P4Ks and Their Lipid Product PI-4,5-P_2 Come to the Surface. Front. Cell Dev. Biol. 9:791758. doi: 10.3389/fcell.2021.791758

INTRODUCTION

Lipids are essential components of cellular membranes. The type and the amount of lipids vary within the membranes at the organism, cell, as well as the organellar level. The lipid composition, quantitative and qualitative, of the membranes defines not only their physical characteristics but also, their biological properties. The majority of the membrane lipids are grouped into glycerophospholipids (GPLs), sphingolipids, and sterols (van Meer et al., 2008). GPLs are characterized by a glycerol backbone which forms the link between the head group that consists of a phosphate and an alcohol and the tail which consists of varying lengths of fatty acid chains (Harayama and Riezman, 2018). One such group of GPLs are the phosphoinositols (PIs).

PIs derive their name from the inositol head group, with the most predominant fatty acyl chains seen in the PIs in tissues being the stearoyl and arachidonoyl-acyl chains (Harayama and Riezman, 2018). PIs give rise to seven different species of phosphoinositides by the phosphorylation and dephosphorylation at the 3,4 and 5 position of the inositol moieties by the precise actions of specific kinases and phosphatases (Balla, 2013; Burke, 2018; Palamiuc et al., 2020). While found in minute amounts as compared to the other lipids on membranes, PIs play essential roles in regulating various

TABLE 1 | PI-4,5-P_2 at the organelles: Binding partners and functions. PI-4,5-P2 interacting partners and organellar regulation. The table highlights some of the major interacting partners of PI-4,5-P_2 on the plasma membrane, nucleus and other organelles and the functions they regulate. While the PM and nucleus functions are attributed to the type I kinases (PI4P5Ks), Golgi and ER have been shown to have roles regulated by PI-4,5-P_2 generates by both family of kinases. Whereas autophagosome, lysosome and peroxisome interactions are carried out predominantly by PI-4,5-P_2 generated by PI5P4Ks (Type II kinases).

Organelle	Binding partner(s)	Function	References
Plasma membrane	PLCδ	Hydrolysis to IP3 and DAG – second messengers	Falkenburger et al. (2013)
	CAPS, Synaptogamin1 and Syntaxin	Exocytosis	Martin (2015)
	AP2	Endocytosis	Jost et al. (1998)
	F-actin regulatory protein	Migration	Sun et al. (1999)
	?	Cell adhesion, spreading and migration	Yoneda et al. (2020)
	E-Syts on the ER	Ca^{2+} signaling	Giordano et al. (2013)
Nucleus	Pol I and Pol II	Transcription	Sobol et al. (2018) Sobol et al. (2013) Castano et al. (2019)
Endoplasmic reticulum	?	ER-Golgi transport	Itoh et al. (1998)
Golgi	Dynamin2, PAP1, PLD1	Transport carrier formation from TGN	Jones et al. (1998) Andreev et al. (1999) Freyberg et al. (2001)
	βIII-spectrin	Golgi-ER transport	Godi et al. (1998)
	ARNO family	Golgi structure	Monier et al. (1998)
Autophagosome	?	Inhibition of autophagy initiation	Vicinanza et al. (2015)
Lysosome	ESCRTIII (?)	Lysosome-autophagosome fusion and possibly cholesterol trafficking into the lysosome	Lundquist et al. (2018)
Peroxisome	Syt-7 on the lysosome	Trafficking of cholesterol from lysosome	Chu et al. (2015)
	E-Syts on the ER	Trafficking to cholesterol to the ER	Xiao et al. (2019)
	ESCRTIII (?)	Trafficking of VLCFA from LDs	Ravi et al. (2021)

cellular functions such as cytoskeletal remodeling, vesicular budding, membrane dynamics to regulating ion channels and signaling pathways. This is carried out by recruitment and interaction with, as well as activation of proteins, in a spatial and temporal manner (Falkenburger et al., 2010). Every species of phosphoinositide has its own cohort of interacting partners. This, along with the fact that membranes differ in their lipid composition, allows for distinct functional entities. In this review, we will mainly focus on one particular PI species, phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2).

PI-4,5-P_2, along with phosphotidylinositol-4-phosphate (PI-4-P), accounts for the bulk of all PIs (Di Paolo and De Camilli, 2006) and are generated by two distinct families of PI kinases. Initially these enzymes were identified as the Type I and Type II PI4P-kinases (Phosphatidylinositol-4-phosphate kinases) based on their biochemical properties and immunochemical cross-reactivities (Bazenet et al., 1990; Ling et al., 1989). It was later discovered that the Type II kinases actually produced PI-4,5-P_2 by phosphorylating the 4-position of phosphatidylinositol 5-phosphate (PI-5-P), a previously unknown PI at time and interestingly the last PI to be discovered in higher organisms (Rameh et al., 1997), whereas the Type I kinases phosphorylated the 5-position of phosphatidylinositol 4-phosphate (PI-4-P) to produce the same PI-4,5-P_2 species. Thus, giving rise to the current nomenclature of the PI families as the Type I kinases or PI4P5Ks (Phosphatidylinositol 4-phosphate 5-kinases) and the Type II or PI5P4Ks (Phosphatidylinositol 5-phosphate 4-kinases). Moreover, in mammalian cells, both kinase families have three distinct isoforms each, namely, α, β, and γ (Bulley et al., 2015; Clarke and Irvine, 2012; Clarke and Irvine, 2013). Apart from having very diverse immunological and catalytic functions, these kinases also generate PI-4,5-P_2 on different cellular membranes (**Table 1**) (Balla, 2013).

PI-4,5-P_2 Functions on Cellular Membranes and Correlations for Organellar Cooperation

With PI-4-P being 10 times more abundant than PI-5-P on the plasma membrane (PM), PI-4,5-P_2 generated here is predominantly by the action of Type I PIPKs or PI4P5Ks and constitute only 1–3% of the total lipid content on the membrane (King et al., 1987; King et al., 1989; McLaughlin et al., 2002). As a substrate, PI-4,5-P_2 can be hydrolyzed to IP_3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol), by the activity of phospholipase C (PLC), which in turn serve as second messengers in various intracellular signaling cascades (Falkenburger et al., 2013). PI-4,5-P_2 is also a precursor for PI-4-P and PI-3,4,5-P_3 both of which play a role themselves in signaling and membrane dynamics (Auger et al., 1989; Varnai et al., 2006).

Additionally, PI-4,5-P_2 plays a major role in membrane remodeling and trafficking. As will be discussed in the later sections, this function is essential not only at the level of the PM but is also a feature in key inter-organellar events mediated by these lipids to regulate cellular and metabolic homeostasis. At the PM, fusion and fission cycles can lead to exocytosis and endocytosis, both of which are invariably dependent on PI-

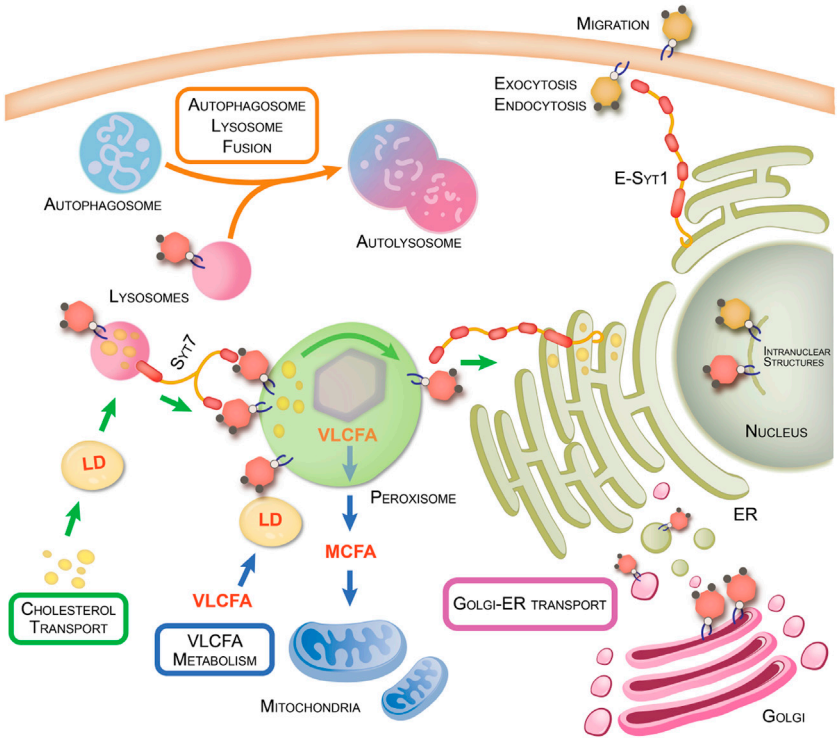

FIGURE 1 | PI-4,5-P_2 regulation of organellar interactions and cellular metabolism. Organelle-organelle interactions within the cell are key to regulating various cellular processes and transport of nutrients and materials to maintain metabolic homeostasis. Disruption of these interactions can lead to various diseased states such as cancer and neurodegenerative disorders. Here we summarize the various functions regulated by PI4P5K-generated PI-4,5-P_2 (yellow hexagons) and PI5P4K-generated PI-4,5-P_2 (red hexagons). This figure also highlights organellar interactions regulated by PI5P4Ks and their products as well the proteins involved and the metabolic pathways impacted by such interactions. Refer also to **Table 1** for details of PI-4,5-P_2 interactors and functions on the plasma membrane.

4,5-P_2. These are mediated by the ability of PI-4,5-P_2 to bind various proteins via structured basic regions such as pleckstrin homology (PH) and C2-domains (Balla, 2013). PI-4,5-P_2 is also a requisite for Ca^{2+} dependent PM-Endoplasmic Reticulum (ER) interaction by binding C2-domain containing E-Syts (Extended-synaptogamins) that are embedded in the ER membrane (Giordano et al., 2013). Interestingly, for a lipid that is quantitatively low on the PM, PI-4,5-P_2 has shown to be a central mediator of a multitude of cellular functions and not surprisingly, loss of or mutation in kinases and phosphatases that regulate PI-4,5-P_2 levels can lead to various diseased states (Pendaries et al., 2003; McCrea and De Camilli, 2009). Even though the bulk of the PI-4,5-P_2 in the cell is generated by the Type I kinases, here we are focusing on the small but significant pool of PI-4,5-P_2 that is created by the action of the Type II kinases, which to date is under appreciated yet is emerging to be fundamental for many essential cellular and metabolic events. The role of Type I kinases and their product is discussed in detail in another review (Katan and Cockcroft, 2020). **Figure 1** and **Table 1** also highlight some of the interactions mediated by PI-4,5-P_2 generated via the activity of the Type I kinases.

With the Type I or PI4P5Ks being considered the main pathway for PI-4,5-P_2 synthesis and PI-4-P being more abundant compared to the minor phosphoinositide, PI-5-P, the Type II or PI5P4Ks were relegated to the role of merely regulating the level of this lipid in the cells. However, recent studies have eloquently shown that PI-4,5-P_2 generated by PI5P4Ks are not just by-products but have the capacity to modulate cellular metabolism by regulating organellar functions (Hu et al., 2018; Xiao et al., 2019; Ravi et al., 2021). Of the three isoforms described, PI5P4Kα is the most active and PI5P4Kγ the least active (Clarke and Irvine, 2013; Giudici et al., 2016). As a substrate, PI-5-P is also the most elusive of the phosphoinositides, with its separation and identification in the cell made difficult due to technical limitations. Since its discovery almost 25 years ago (Rameh et al., 1997), new roles and localization in the cellular compartments is being constantly uncovered. Studies have shown that the levels of PI-5-P change in response to various stimuli such as insulin, oxidative stress, bacterial infection etc. and in turn regulate numerous cellular functions such as cell signaling, vesicular transport and even play a role in the nucleus (Hasegawa et al., 2017; Ghosh et al., 2019).

One of the first biochemical studies of cellular compartments showed enrichment of PI-5-P at both the Golgi and the PM (Sarkes and Rameh, 2010). The product of its phosphorylation, PI-4,5-P_2 has been detected on the stacked cisternae of the Golgi and its role has been studied in connection with maintaining the structural and functional integrity of the Golgi. PI-4,5-P_2-mediated interactions with various proteins at the Golgi is

essential for its structural organization, formation of carriers from the trans-Golgi network (TGN) as well as Golgi-ER transport (De Matteis et al., 2002; De Matteis and D'Angelo, 2007). Moreover, PI5P4Ks are functionally involved in various signaling events at the Golgi (Mackey et al., 2014). In fact, PI5P4Kγ has been localized to the ER suggesting PI-4,5-P_2 synthesis at this organelle by these kinases (Itoh et al., 1998) and with PI-4,5-P_2 playing a role in ER-Golgi transport, it remains to be seen whether and what percent of the lipid is generated by PI5P4Ks (De Matteis et al., 2002). However, this is contradictory to the studies demonstrating that the gamma isoform has very little inherent kinase function *in vitro* (Clarke et al., 2008). The mechanism by which PI5P4Kγ functions, and the role it plays in regulating cellular processes, remains to be explored further.

Interestingly, PI-5-P has also been found in the nucleus where it not only serves as a substrate for PI5P4Ks at that location but has been implicated in various nuclear outputs (Poli et al., 2019). PI5P4Kβ is the only isoform that has a unique nuclear localization signal that allows it to be targeted to the nucleus. However, the PI5P4Ks are dimeric enzymes and therefore the beta isoform has been shown to dimerize with the alpha, directing it into the nucleus as well (Bultsma et al., 2010). While PI-4,5-P_2 has been shown to have nuclear functions such as the involvement with Pol I and Pol II mediated transcription as well as associating with splicing compartments, these functions have mostly been attributed to the canonical Type I PI4P5Ks. Thus far, the main function of the Type II PI5P4Ks at the nucleus seems to be quelling the starvation-induced increase in levels of PI-5-P. Details of the role of PI-4,5-P_2 at the organelles mentioned thus far are discussed in Tan X et al. (2015) Also, see **Table 1** for various interacting proteins of PI-4,5-P_2 and the roles played in inter-organellar communication.

Vicinanza et al., show that PI-5-P is essential for autophagosome formation and overexpression studies showed that PI5P4Kγ localizes to the autophagosomes more frequently than both the PI5P4Kα and β isoforms. Knockdown of the kinases leads to the increase in the autophagosome formation, indicating that PI-4,5-P_2 generation attenuates autophagosome biogenesis (Vicinanza et al., 2015). On the contrary another study showed that ATG16L1 interacts with PI-4,5-P_2 on the PM to activate this process (Ravikumar et al., 2010). PI5P4Ks have also been shown to localize to the lysosome (Lundquist et al., 2018). Further, the loss of PI5P4Kα and PI5P4Kβ is sufficient to prevent the fusion of autophagosomes with lysosomes, which inhibits the process of autophagy and leads to accumulation of autophagosomes. This function of the PI5P4Ks requires a concomitant loss of p53 or a similar cellular stress (Lundquist et al., 2018). Together, these data indicate an important role for PI-4,5-P_2 in the process of autolysosome formation. Moreover, a recent study showed that the endosomal sorting complex required for transport (ESCRT) is essential for lysosomal membrane repair (Gupta et al., 2021). These complexes are involved in membrane remodeling, which follows fusion events such as those between organelles. Interestingly, ESCRT complex proteins interact specifically with PI-4,5-P_2 (McCullough et al., 2015), which could explain the importance of the lipid on the lysosomal membrane and its role in autophagy. For an in-depth discussion of PI-4,5-P_2, and other phosphoinositide in autophagy, refer to Palamiuc et al. (2020).

Studies have also shown the localization of PI5P4Kα and the role of the PI5P4Ks in general at the peroxisomes. Peroxisomes, though highly essential for regulating various metabolic functions, are not well studied in mammalian cells. In the recent years, peroxisomes, their actions, as well as their role in regulating lipid metabolism have come to the forefront, bringing with them the PI5P4Ks and their product, PI-4,5-P_2 into the spotlight.

PI5P4Ks as Key Regulators of Peroxisomal Functions by Sustaining PI-4,5-P_2 Homeostasis

Peroxisomes are single-membraned organelles with multifaceted functions, ranging from ether lipid biosynthesis to fatty acid (FA) oxidation and reactive oxygen species (ROS) metabolism (He et al., 2021). They respond to metabolic and environmental cues, putting them at the center of various signaling nodes in the cell. Also, because of their roles, peroxisomes interact with and regulate other organellar functions such as the lysosomes, lipid droplets (LDs) and the mitochondria (He et al., 2021). Multiple studies have placed PI-4,5-P_2 at the peroxisomes (Jeynov et al., 2006; Chu et al., 2015; Ravi et al., 2021). Furthermore, recent work has showed that this pool of PI-4,5-P_2 is generated by PI5P4Kα (Hu et al., 2018). We, in a recent publication, were the first to physically localize PI5P4Kα to the peroxisome in the mouse prostate tissue (Ravi et al., 2021). Further, using imaging techniques, we showed the localization of PI-4,5-P_2 to the peroxisomes, confirming the lipid blot data from Chu et al. We also categorically demonstrated that the knockout of the two most active isoforms of the Type II kinases, namely, PI5P4Kα and PI5P4Kβ were sufficient to deplete the peroxisomal pool of PI-4,5-P_2 and this can be rescued by adding back the wild-type (WT) but not by the kinase-dead PI5P4Kα (Ravi et al., 2021).

PI-4,5-P_2, as mentioned earlier, can bind to a wide range of proteins with PH- or C2- domains to mediate diverse cellular functions. Similarly, as shown by Chu et al., peroxisomal PI-4,5-P_2 interacts with the C2 domain containing Synaptogamin VII (Syt7) on the lysosomal membrane to regulate cholesterol trafficking from the lysosome to the peroxisome. Their work revealed a previously unappreciated role for peroxisomes in cholesterol transport. While NPC1 (Niemann-Pick disease, type C1) on lysosomes and ABCD1 (ATP Binding Cassette Subfamily D Member 1) on peroxisomes are indispensable for the formation of lysosome-peroxisome membrane contact (LPMC), Syt7 allows for the stabilization of these sites by binding to PI-4,5-P_2 on the peroxisome. Knockdown on Syt7 leads to accumulation of cholesterol in the lysosomes of the cells, uncovering a crucial role for the LPMC in cholesterol transport (Chu et al., 2015). In their following paper, they further demonstrated that knockdown of PI5P4Kα but not PI5P4Kβ or PI5P4Kγ leads to accumulation cholesterol in the lysosome, similar to the loss of Syt7 (Hu et al., 2018). Notably, we showed that the β isoform of the kinases is also able to rescue, albeit to a lesser extent than the α isoform, PI-4,5-P_2 on peroxisomal

membranes (Ravi et al., 2021). This correlates with the fact that PI5P4Kα is the most catalytically active of the isoforms and also may explain why Hu et al., did not observe a marked increase in cholesterol accumulation in the lysosome upon PI5P4Kβ or PI5P4Kγ knockdown. They also go on to show that while the loss of PI5P4Kα is sufficient to regulate peroxisomal PI-4,5-P_2 it does not seem to affect lysosomal PI-4,5-P_2 (Hu et al., 2018). This supports the role of the PI5P4Ks in autophagosome-lysosome fusion, which requires the loss of both the α and the β isoforms (Lundquist et al., 2018). Since both lysosomes and peroxisomes are sites of localization for the PI5P4Ks, it was also elegantly demonstrated *in vitro* that, peroxisomes and not lysosomes isolated from PI5P4Kα knockdown affect LPMC formation, conclusively showing the role of PI-4,5-P_2 on the peroxisomes in this process (Chu et al., 2015). Further, similar to the PM-ER interaction, the PI-4,5-P_2 on the peroxisomes also binds E-Syts on the ER, to traffic cholesterol. This lysosome-peroxisome-ER transport explains a previously not understood mechanism about the how exogenous cholesterol is trafficked from lysosomes to the ER (Xiao et al., 2019).

Other than cholesterol metabolism, peroxisomes are also sites of breakdown of very long chain fatty acids (VLCFAs) to medium chain fatty acids (MCFAs), known as peroxisomal β-oxidation (Poirier et al., 2006). Our study has shown a role for the PI5P4Ks in peroxisomal β-oxidation as seen by the change in peroxisomal gene signature upon loss of α and β isoforms (Ravi et al., 2021). For this process to occur, the peroxisomes need to take up VLCFAs, which are stored in the form of LDs. Concisely, peroxisome-LD interactions, which as with the LPMC formation, require ABCD1 on peroxisomal membranes and M1 Spastin on LDs, following which the FAs are trafficked across the membranes of the organelles (Chang et al., 2019). While loss of the PI5P4Ks does not affect the peroxisome's interaction with LDs, they are no longer able to take up the FAs. This phenotype can be rescued by adding back the WT but not the kinase-dead PI5P4Kα, indicating that PI-4,5-P_2 is essential for the trafficking event (Ravi et al., 2021). Chang et al., showed that upon tethering LDs to the peroxisomes, M1 Spastin is also responsible for recruiting ESCRT-III proteins to the surface, which is essential for FA uptake (Chang et al., 2019). Interestingly, as previously mentioned, ESCRT-III complex proteins preferentially associate with PI-4,5-P_2. This could provide a possible explanation for the involvement of PI-4,5-P_2 in regulating FA trafficking. The chain shortened FAs from peroxisomal β-oxidation are then utilized by mitochondria to break them down into carbon dioxide and water, in a process that generates ATP (Fransen et al., 2017). Studies have shown that peroxisomal storage and biogenesis disorders lead to mitochondrial dysfunction (Lismont et al., 2015; Tanaka et al., 2019). Similarly, knockdown of the PI5P4Ks leads to major structural and functional defects in the mitochondria downstream of peroxisome dysregulation (Ravi et al., 2021). These two organelles not only play a key role in lipid metabolism, but in redox mechanisms as well, tying them together in maintaining cellular homeostasis and PI-4,5-P_2 generated by the PI5P4Ks is surfacing to be crucial in maintaining this balance. See **Figure 1** and **Table 1**.

Health and Disease: PI5P4Ks Emerge as Exciting Targets

It is not surprising, considering the wide-ranging role PI-4,5-P_2 plays, that PI5P4Ks will have such a drastic effect on cellular signaling and metabolism. Depending on the disease state, the activity of PI5P4Ks can be leveraged to bring about a change in the system. While targeting a kinase might seem like a daunting task, PI5P4Ks are a more feasible target. This is predominantly because their substrate, PI-5-P, mainly accumulates under conditions of stress. Our previous work demonstrated that PI5P4Ks are essential for tumor formation upon loss of p53, suggesting the PI5P4Ks are attractive targets for p53 mutant cancers (Emerling et al., 2013) and become relevant in regulating autophagosome-lysosome fusion under these conditions (Lundquist et al., 2018). Further, we have shown elevated expression of the PI5P4Ks in breast tumors compared to normal breast tissue (Emerling et al., 2013) and other studies have shown the impact of the kinases in breast cancer (Luoh et al., 2004; Keune et al., 2013). Similarly, PI5P4Ks have been demonstrated to be upregulated in several cancer subtypes, including glioblastomas, AML, and sarcomas (Fiume et al., 2015; Jude et al., 2015; Zhang et al., 2018; Lima et al., 2019; Shin et al., 2019; Ravi et al., 2021). Moreover, we recently illustrated the requirement of the PI5P4Ks for not only the establishment of sarcoma tumors but also in maintaining them, possibly through their role in regulating peroxisome-mitochondrial interplay (Ravi et al., 2021). While in diseases such as cancer, it might be necessary to inhibit the PI5P4Ks, other diseases/conditions might benefit from enhancing their activity. Accordingly, inhibiting autophagy is an appealing strategy in cancer therapeutics, whereas the reverse is true from an ageing standpoint. Studies have shown that PI-4,5-P_2 levels are often lowered in neurodegenerative disorders, such as Alzheimer's disease (Arancio, 2008). Lysosomal and peroxisomal storage disorders, encompass a large group of metabolic diseases, which are associated with the inability of the cells to properly process their cargo, such as cholesterol. These could serve as prime targets where we could possibly exploit the enzymatic function of the PI5P4Ks to our advantage. All things considered, in the recent past, PI5P4Ks and their product PI-4,5-P_2 have risen from insignificance to being without a doubt one of the key metabolic sensors and regulators within the cell as well as pivotal players for inter-organelle communication necessary for cell survival. With drugs being developed against these kinases (Davis et al., 2013; Clarke et al., 2015; Al-Ramahi et al., 2017; Kitagawa et al., 2017; Manz et al., 2020; Sivakumaren et al., 2020; Chen et al., 2021), targeting them in the near future in various diseases is looking brighter.

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

AR and BE wrote the manuscript. LP and AR designed the artwork for the figure and table.

FUNDING

This work is supported by grants from the NCI and ACS (R01 CA237536 and RSG-20-064-01-TBE) to BE.

REFERENCES

Al-Ramahi, I., Giridharan, S. S. P., Chen, Y. C., Patnaik, S., Safren, N., Hasegawa, J., et al. (2017). Inhibition of PIP4Kγ Ameliorates the Pathological Effects of Mutant Huntingtin Protein. *Elife* 6, e29123. doi:10.7554/eLife.29123

Andreev, J., Simon, J.-P., Sabatini, D. D., Kam, J., Plowman, G., Randazzo, P. A., et al. (1999). Identification of a New Pyk2 Target Protein with Arf-GAP Activity. *Mol. Cel Biol* 19, 2338–2350. doi:10.1128/mcb.19.3.2338

Arancio, O. (2008). PIP2: a New Key Player in Alzheimer's Disease. *Cellscience* 5, 44–47.

Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P., and Cantley, L. C. (1989). PDGF-dependent Tyrosine Phosphorylation Stimulates Production of Novel Polyphosphoinositides in Intact Cells. *Cell* 57, 167–175. doi:10.1016/0092-8674(89)90182-7

Balla, T. (2013). Phosphoinositides: Tiny Lipids with Giant Impact on Cell Regulation. *Physiol. Rev.* 93, 1019–1137. doi:10.1152/physrev.00028.2012

Bazenet, C. E., Ruano, A. R., Brockman, J. L., and Anderson, R. A. (1990). The Human Erythrocyte Contains Two Forms of Phosphatidylinositol-4-Phosphate 5-kinase Which Are Differentially Active toward Membranes. *J. Biol. Chem.* 265, 18012–18022. doi:10.1016/s0021-9258(18)38264-4

Bulley, S. J., Clarke, J. H., Droubi, A., Giudici, M.-L., and Irvine, R. F. (2015). Exploring Phosphatidylinositol 5-phosphate 4-kinase Function. *Adv. Biol. Regul.* 57, 193–202. doi:10.1016/j.jbior.2014.09.007

Bultsma, Y., Keune, W.-J., and Divecha, N. (2010). PIP4Kβ Interacts with and Modulates Nuclear Localization of the High-Activity PtdIns5P-4-Kinase Isoform PIP4Kα. *Biochem. J.* 430, 223–235. doi:10.1042/bj20100341

Burke, J. E. (2018). Structural Basis for Regulation of Phosphoinositide Kinases and Their Involvement in Human Disease. *Mol. Cel* 71, 653–673. doi:10.1016/j.molcel.2018.08.005

Castano, E., Yildirim, S., Faberova, V., Krausova, A., Ulicna, L., Paprckova, D., et al. (2019). Nuclear Phosphoinositides-Versatile Regulators of Genome Functions. *Cells* 8, 649. doi:10.3390/cells8070649

Chang, C.-L., Weigel, A. V., Ioannou, M. S., Pasolli, H. A., Xu, C. S., Peale, D. R., et al. (2019). Spastin Tethers Lipid Droplets to Peroxisomes and Directs Fatty Acid Trafficking through ESCRT-III. *J. Cel Biol* 218, 2583–2599. doi:10.1083/jcb.201902061

Chen, S., Chandra Tjin, C., Gao, X., Xue, Y., Jiao, H., Zhang, R., et al. (2021). Pharmacological Inhibition of PI5P4Kalpha/beta Disrupts Cell Energy Metabolism and Selectively Kills P53-Null Tumor Cells. *Proc. Natl. Acad. Sci. U S A.* 118, e2002486118. doi:10.1073/pnas.2002486118

Chu, B.-B., Liao, Y.-C., Qi, W., Xie, C., Du, X., Wang, J., et al. (2015). Cholesterol Transport through Lysosome-Peroxisome Membrane Contacts. *Cell* 161, 291–306. doi:10.1016/j.cell.2015.02.019

Clarke, J. H., Emson, P. C., and Irvine, R. F. (2008). Localization of Phosphatidylinositol Phosphate Kinase IIγ in Kidney to a Membrane Trafficking Compartment within Specialized Cells of the Nephron. *Am. J. Physiology-Renal Physiol.* 295, F1422–F1430. doi:10.1152/ajprenal.90310.2008

Clarke, J. H., Giudici, M.-L., Burke, J. E., Williams, R. L., Maloney, D. J., Marugan, J., et al. (2015). The Function of Phosphatidylinositol 5-phosphate 4-kinase γ (PI5P4Kγ) Explored Using a Specific Inhibitor that Targets the PI5P-Binding Site. *Biochem. J.* 466, 359–367. doi:10.1042/bj20141333

Clarke, J. H., and Irvine, R. F. (2013). Evolutionarily Conserved Structural Changes in Phosphatidylinositol 5-phosphate 4-kinase (PI5P4K) Isoforms Are Responsible for Differences in Enzyme Activity and Localization. *Biochem. J.* 454, 49–57. doi:10.1042/bj20130488

Clarke, J. H., and Irvine, R. F. (2012). "Phosphatidylinositol 5-phosphate 4-kinase," in *Encyclopedia of Signaling Molecules*. Editor S. Choi (New York, NY: Springer New York), 1369–1377.

Davis, M. I., Sasaki, A. T., Shen, M., Emerling, B. M., Thorne, N., Michael, S., et al. (2013). A Homogeneous, High-Throughput Assay for Phosphatidylinositol 5-phosphate 4-kinase with a Novel, Rapid Substrate Preparation. *PLoS One* 8, e54127. doi:10.1371/journal.pone.0054127

De Matteis, M. A., and D'Angelo, G. (2007). The Role of the Phosphoinositides at the Golgi Complex. *Biochem. Soc. Symp.* 74, 107–116. doi:10.1042/bss2007c10

De Matteis, M. A., Godi, A., and Corda, D. (2002). Phosphoinositides and the Golgi Complex. *Curr. Opin. Cel Biol.* 14, 434–447. doi:10.1016/s0955-0674(02)00357-5

Di Paolo, G., and De Camilli, P. (2006). Phosphoinositides in Cell Regulation and Membrane Dynamics. *Nature* 443, 651–657. doi:10.1038/nature05185

Emerling, B. M., Hurov, J. B., Poulogiannis, G., Tsukazawa, K. S., Choo-Wing, R., Wulf, G. M., et al. (2013). Depletion of a Putatively Druggable Class of Phosphatidylinositol Kinases Inhibits Growth of P53-Null Tumors. *Cell* 155, 844–857. doi:10.1016/j.cell.2013.09.057

Falkenburger, B. H., Dickson, E. J., and Hille, B. (2013). Quantitative Properties and Receptor reserve of the DAG and PKC branch of Gq-Coupled Receptor Signaling. *J. Gen. Physiol.* 141, 537–555. doi:10.1085/jgp.201210887

Falkenburger, B. H., Jensen, J. B., Dickson, E. J., Suh, B.-C., and Hille, B. (2010). SYMPOSIUM REVIEW: Phosphoinositides: Lipid Regulators of Membrane Proteins. *J. Physiol.* 588, 3179–3185. doi:10.1113/jphysiol.2010.192153

Fiume, R., Stijf-Bultsma, Y., Shah, Z. H., Keune, W. J., Jones, D. R., Jude, J. G., et al. (2015). PIP4K and the Role of Nuclear Phosphoinositides in Tumour Suppression. *Biochim. Biophys. Acta (Bba) - Mol. Cel Biol. Lipids* 1851, 898–910. doi:10.1016/j.bbalip.2015.02.014

Fransen, M., Lismont, C., and Walton, P. (2017). The Peroxisome-Mitochondria Connection: How and Why. *Int. J. Mol. Sci.* 18, 1126. doi:10.3390/ijms18061126

Freyberg, Z., Sweeney, D., Siddhanta, A., Bourgoin, S., Frohman, M., and Shields, D. (2001). Intracellular Localization of Phospholipase D1 in Mammalian Cells. *MBoC* 12, 943–955. doi:10.1091/mbc.12.4.943

Ghosh, A., Sharma, S., Shinde, D., Ramya, V., and Raghu, P. (2019). A Novel Mass Assay to Measure Phosphatidylinositol-5-Phosphate from Cells and Tissues. *Biosci. Rep.* 39, BSR20192502. doi:10.1042/BSR20192502

Giordano, F., Saheki, Y., Idevall-Hagren, O., Colombo, S. F., Pirruccello, M., Milosevic, I., et al. (2013). PI(4,5)P2-Dependent and Ca2+-Regulated ER-PM Interactions Mediated by the Extended Synaptotagmins. *Cell* 153, 1494–1509. doi:10.1016/j.cell.2013.05.026

Giudici, M.-L., Clarke, J. H., and Irvine, R. F. (2016). Phosphatidylinositol 5-phosphate 4-kinase γ (PI5P4Kγ), a Lipid Signalling enigma. *Adv. Biol. Regul.* 61, 47–50. doi:10.1016/j.jbior.2015.11.007

Godi, A., Santone, I., Pertile, P., Devarajan, P., Stabach, P. R., Morrow, J. S., et al. (1998). ADP Ribosylation Factor Regulates Spectrin Binding to the Golgi Complex. *Proc. Natl. Acad. Sci.* 95, 8607–8612. doi:10.1073/pnas.95.15.8607

Gupta, S., Yano, J., Mercier, V., Htwe, H. H., Shin, H. R., Rademaker, G., et al. (2021). Lysosomal Retargeting of Myoferlin Mitigates Membrane Stress to Enable Pancreatic Cancer Growth. *Nat. Cel Biol* 23, 232–242. doi:10.1038/s41556-021-00644-7

Harayama, T., and Riezman, H. (2018). Understanding the Diversity of Membrane Lipid Composition. *Nat. Rev. Mol. Cel Biol* 19, 281–296. doi:10.1038/nrm.2017.138

Hasegawa, J., Strunk, B. S., and Weisman, L. S. (2017). PI5P and PI(3,5)P2: Minor, but Essential Phosphoinositides. *Cell Struct. Funct.* 42, 49–60. doi:10.1247/csf.17003

He, A., Dean, J. M., and Lodhi, I. J. (2021). Peroxisomes as Cellular Adaptors to Metabolic and Environmental Stress. *Trends Cel Biol.* 31, 656–670. doi:10.1016/j.tcb.2021.02.005

Hu, A., Zhao, X.-T., Tu, H., Xiao, T., Fu, T., Wang, Y., et al. (2018). PIP4K2A Regulates Intracellular Cholesterol Transport through Modulating PI(4,5)P2 Homeostasis. *J. Lipid Res.* 59, 507–514. doi:10.1194/jlr.m082149

Itoh, T., Ijuin, T., and Takenawa, T. (1998). A Novel Phosphatidylinositol-5-Phosphate 4-Kinase (Phosphatidylinositol-Phosphate Kinase IIγ) Is

Phosphorylated in the Endoplasmic Reticulum in Response to Mitogenic Signals. *J. Biol. Chem.* 273, 20292–20299. doi:10.1074/jbc.273.32.20292

Jeynov, B., Lay, D., Schmidt, F., Tahirovic, S., and Just, W. W. (2006). Phosphoinositide Synthesis and Degradation in Isolated Rat Liver Peroxisomes. *FEBS Lett.* 580, 5917–5924. doi:10.1016/j.febslet.2006.09.058

Jones, S. M., Howell, K. E., Henley, J. R., Cao, H., and McNiven, M. A. (1998). Role of Dynamin in the Formation of Transport Vesicles from the Trans-golgi Network. *Science* 279, 573–577. doi:10.1126/science.279.5350.573

Jost, M., Simpson, F., Kavran, J. M., Lemmon, M. A., and Schmid, S. L. (1998). Phosphatidylinositol-4,5-bisphosphate Is Required for Endocytic Coated Vesicle Formation. *Curr. Biol.* 8, 1399–1404. doi:10.1016/s0960-9822(98)00022-0

Jude, J. G., Spencer, G. J., Huang, X., Somerville, T. D. D., Jones, D. R., Divecha, N., et al. (2015). A Targeted Knockdown Screen of Genes Coding for Phosphoinositide Modulators Identifies PIP4K2A as Required for Acute Myeloid Leukemia Cell Proliferation and Survival. *Oncogene* 34, 1253–1262. doi:10.1038/onc.2014.77

Katan, M., and Cockcroft, S. (2020). Phosphatidylinositol(4,5)bisphosphate: Diverse Functions at the Plasma Membrane. *Essays Biochem.* 64, 513–531. doi:10.1042/ebc20200041

Keune, W.-J., Sims, A. H., Jones, D. R., Bultsma, Y., Lynch, J. T., Jirström, K., et al. (2013). Low PIP4K2B Expression in Human Breast Tumors Correlates with Reduced Patient Survival: A Role for PIP4K2B in the Regulation of E-Cadherin Expression. *Cancer Res.* 73, 6913–6925. doi:10.1158/0008-5472.can-13-0424

King, C. E., Hawkins, P. T., Stephens, L. R., and Michell, R. H. (1989). Determination of the Steady-State Turnover Rates of the Metabolically Active Pools of Phosphatidylinositol 4-phosphate and Phosphatidylinositol 4,5-bisphosphate in Human Erythrocytes. *Biochem. J.* 259, 893–896. doi:10.1042/bj2590893

King, C. E., Stephens, L. R., Hawkins, P. T., Guy, G. R., and Michell, R. H. (1987). Multiple Metabolic Pools of Phosphoinositides and Phosphatidate in Human Erythrocytes Incubated in a Medium that Permits Rapid Transmembrane Exchange of Phosphate. *Biochem. J.* 244, 209–217. doi:10.1042/bj2440209

Kitagawa, M., Liao, P.-J., Lee, K. H., Wong, J., Shang, S. C., Minami, N., et al. (2017). Dual Blockade of the Lipid Kinase PIP4Ks and Mitotic Pathways Leads to Cancer-Selective Lethality. *Nat. Commun.* 8, 2200. doi:10.1038/s41467-017-02287-5

Lima, K., Coelho-Silva, J. L., Kinker, G. S., Pereira-Martins, D. A., Traina, F., Fernandes, P. A. C. M., et al. (2019). PIP4K2A and PIP4K2C Transcript Levels Are Associated with Cytogenetic Risk and Survival Outcomes in Acute Myeloid Leukemia. *Cancer Genet.* 233-234, 56–66. doi:10.1016/j.cancergen.2019.04.002

Ling, L. E., Schulz, J. T., and Cantley, L. C. (1989). Characterization and Purification of Membrane-Associated Phosphatidylinositol-4-Phosphate Kinase from Human Red Blood Cells. *J. Biol. Chem.* 264, 5080–5088. doi:10.1016/s0021-9258(18)83702-4

Lismont, C., Nordgren, M., Van Veldhoven, P. P., and Fransen, M. (2015). Redox Interplay between Mitochondria and Peroxisomes. *Front. Cel Dev. Biol.* 3, 35. doi:10.3389/fcell.2015.00035

Lundquist, M. R., Goncalves, M. D., Loughran, R. M., Possik, E., Vijayaraghavan, T., Yang, A., et al. (2018). Phosphatidylinositol-5-Phosphate 4-Kinases Regulate Cellular Lipid Metabolism by Facilitating Autophagy. *Mol. Cel* 70, 531–544. doi:10.1016/j.molcel.2018.03.037

Luoh, S.-W., Venkatesan, N., and Tripathi, R. (2004). Overexpression of the Amplified Pip4k2β Gene from 17q11-12 in Breast Cancer Cells Confers Proliferation Advantage. *Oncogene* 23, 1354–1363. doi:10.1038/sj.onc.1207251

Mackey, A. M., Sarkes, D. A., Bettencourt, I., Asara, J. M., and Rameh, L. E. (2014). PIP4kγ Is a Substrate for mTORC1 that Maintains Basal mTORC1 Signaling during Starvation. *Sci. Signal.* 7, ra104. doi:10.1126/scisignal.2005191

Manz, T. D., Sivakumaren, S. C., Yasgar, A., Hall, M. D., Davis, M. I., Seo, H.-S., et al. (2020). Structure-Activity Relationship Study of Covalent Pan-Phosphatidylinositol 5-Phosphate 4-Kinase Inhibitors. *ACS Med. Chem. Lett.* 11, 346–352. doi:10.1021/acsmedchemlett.9b00402

Martin, T. F. J. (2015). PI(4,5)P_2-binding Effector Proteins for Vesicle Exocytosis. *Biochim. Biophys. Acta* 1851, 785–793. doi:10.1016/j.bbalip.2014.09.017

McCrea, H. J., and De Camilli, P. (2009). Mutations in Phosphoinositide Metabolizing Enzymes and Human Disease. *Physiology* 24, 8–16. doi:10.1152/physiol.00035.2008

McCullough, J., Clippinger, A. K., Talledge, N., Skowyra, M. L., Saunders, M. G., Naismith, T. V., et al. (2015). Structure and Membrane Remodeling Activity of ESCRT-III Helical Polymers. *Science* 350, 1548–1551. doi:10.1126/science.aad8305

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

Monier, S., Chardin, P., Robineau, S., and Goud, B. (1998). Overexpression of the ARF1 Exchange Factor ARNO Inhibits the Early Secretory Pathway and Causes the Disassembly of the Golgi Complex. *J. Cel Sci* 111 (Pt 22) (Pt 22), 3427–3436. doi:10.1242/jcs.111.22.3427

Palamiuc, L., Ravi, A., and Emerling, B. M. (2020). Phosphoinositides in Autophagy: Current Roles and Future Insights. *FEBS J.* 287, 222–238. doi:10.1111/febs.15127

Pendaries, C., Tronchère, H., Plantavid, M., and Payrastre, B. (2003). Phosphoinositide Signaling Disorders in Human Diseases. *FEBS Lett.* 546, 25–31. doi:10.1016/s0014-5793(03)00437-x

Poirier, Y., Antonenkov, V. D., Glumoff, T., and Hiltunen, J. K. (2006). Peroxisomal β-oxidation-A Metabolic Pathway with Multiple Functions. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1763, 1413–1426. doi:10.1016/j.bbamcr.2006.08.034

Poli, A., Zaurito, A. E., Abdul-Hamid, S., Fiume, R., Faenza, I., and Divecha, N. (2019). Phosphatidylinositol 5 Phosphate (PI5P): From behind the Scenes to the Front (Nuclear) Stage. *Int. J. Mol. Sci.* 20, 2080. doi:10.3390/ijms20092080

Rameh, L. E., Tolias, K. F., Duckworth, B. C., and Cantley, L. C. (1997). A New Pathway for Synthesis of Phosphatidylinositol-4,5-Bisphosphate. *Nature* 390, 192–196. doi:10.1038/36621

Ravi, A., Palamiuc, L., Loughran, R. M., Triscott, J., Arora, G. K., Kumar, A., et al. (2021). PI5P4Ks Drive Metabolic Homeostasis through Peroxisome-Mitochondria Interplay. *Develop. Cel* 56, 1661–1676. doi:10.1016/j.devcel.2021.04.019

Ravikumar, B., Moreau, K., Jahreiss, L., Puri, C., and Rubinsztein, D. C. (2010). Plasma Membrane Contributes to the Formation of Pre-autophagosomal Structures. *Nat. Cel Biol* 12, 747–757. doi:10.1038/ncb2078

Sarkes, D., and Rameh, L. E. (2010). A Novel HPLC-Based Approach Makes Possible the Spatial Characterization of Cellular PtdIns5P and Other Phosphoinositides. *Biochem. J.* 428, 375–384. doi:10.1042/bj20100129

Shin, Y. J., Sa, J. K., Lee, Y., Kim, D., Chang, N., Cho, H. J., et al. (2019). PIP4K2A as a Negative Regulator of PI3K in PTEN-Deficient Glioblastoma. *J. Exp. Med.* 216, 1120–1134. doi:10.1084/jem.20172170

Sivakumaren, S. C., Shim, H., Zhang, T., Ferguson, F. M., Lundquist, M. R., Browne, C. M., et al. (2020). Targeting the PI5P4K Lipid Kinase Family in Cancer Using Covalent Inhibitors. *Cel Chem. Biol.* 27, 525–537. doi:10.1016/j.chembiol.2020.02.003

Sobol, M., Krausová, A., Yildirim, S., Kalasová, I., Fáberová, V., Vrkoslav, V., et al. (2018). Nuclear Phosphatidylinositol 4,5-bisphosphate Islets Contribute to Efficient RNA Polymerase II-dependent Transcription. *J. Cel Sci* 131, jcs211094. doi:10.1242/jcs.211094

Sobol, M., Yildirim, S., Philimonenko, V. V., Marášek, P., Castaño, E., and Hozák, P. (2013). UBF Complexes with Phosphatidylinositol 4,5-bisphosphate in Nucleolar Organizer Regions Regardless of Ongoing RNA Polymerase I Activity. *Nucleus* 4, 478–486. doi:10.4161/nucl.27154

Sun, H. Q., Yamamoto, M., Mejillano, M., and Yin, H. L. (1999). Gelsolin, a Multifunctional Actin Regulatory Protein. *J. Biol. Chem.* 274, 33179–33182. doi:10.1074/jbc.274.47.33179

Tan, X., Thapa, N., Choi, S., and Anderson, R. A. (2015). Emerging Roles of PtdIns(4,5)P2 - beyond the Plasma Membrane. *J. Cel Sci* 128, 4047–4056. doi:10.1242/jcs.175208

Tanaka, H., Okazaki, T., Aoyama, S., Yokota, M., Koike, M., Okada, Y., et al. (2019). Peroxisomes Control Mitochondrial Dynamics and the Mitochondrion-dependent Apoptosis Pathway. *J. Cel Sci* 132, jcs224766. doi:10.1242/jcs.224766

van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008). Membrane Lipids: where They Are and How They Behave. *Nat. Rev. Mol. Cel Biol* 9, 112–124. doi:10.1038/nrm2330

Varnai, P., Thyagarajan, B., Rohacs, T., and Balla, T. (2006). Rapidly Inducible Changes in Phosphatidylinositol 4,5-bisphosphate Levels Influence Multiple Regulatory Functions of the Lipid in Intact Living Cells. *J. Cel Biol* 175, 377–382. doi:10.1083/jcb.200607116

Vicinanza, M., Korolchuk, V. I., Ashkenazi, A., Puri, C., Menzies, F. M., Clarke, J. H., et al. (2015). PI(5)P Regulates Autophagosome Biogenesis. *Mol. Cel* 57, 219–234. doi:10.1016/j.molcel.2014.12.007

Xiao, J., Luo, J., Hu, A., Xiao, T., Li, M., Kong, Z., et al. (2019). Cholesterol Transport through the Peroxisome-ER Membrane Contacts Tethered by PI(4,5)P2 and Extended Synaptotagmins. *Sci. China Life Sci.* 62, 1117–1135. doi:10.1007/s11427-019-9569-9

Yoneda, A., Kanemaru, K., Matsubara, A., Takai, E., Shimozawa, M., Satow, R., et al. (2020). Phosphatidylinositol 4,5-bisphosphate Is Localized in the Plasma Membrane Outer Leaflet and Regulates Cell Adhesion and Motility. *Biochem. Biophysical Res. Commun.* 527, 1050–1056. doi:10.1016/j.bbrc.2020.05.040

Zhang, S., Li, Z., Yan, X., Bao, L., Deng, Y., Zeng, F., et al. (2018). Regulatory Network and Prognostic Effect Investigation of PIP4K2A in Leukemia and Solid Cancers. *Front. Genet.* 9, 721. doi:10.3389/fgene.2018.00721

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frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
published: 19 January 2022
doi: 10.3389/fcell.2021.788921

Peroxisomal ATP Uptake Is Provided by Two Adenine Nucleotide Transporters and the ABCD Transporters

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Peroxisomes are essential organelles involved in various metabolic processes, including fatty acid β-oxidation. Their metabolic functions require a controlled exchange of metabolites and co-factors, including ATP, across the peroxisomal membrane. We investigated which proteins are involved in the peroxisomal uptake of ATP in the yeast *Saccharomyces cerevisiae*. Using wild-type and targeted deletion strains, we measured ATP-dependent peroxisomal octanoate β-oxidation, intra-peroxisomal ATP levels employing peroxisome-targeted ATP-sensing reporter proteins, and ATP uptake in proteoliposomes prepared from purified peroxisomes. We show that intra-peroxisomal ATP levels are maintained by different peroxisomal membrane proteins each with different modes of action: 1) the previously reported Ant1p protein, which catalyzes the exchange of ATP for AMP or ADP, 2) the ABC transporter protein complex Pxa1p/Pxa2p, which mediates both uni-directional acyl-CoA and ATP uptake, and 3) the mitochondrial Aac2p protein, which catalyzes ATP/ADP exchange and has a dual localization in both mitochondria and peroxisomes. Our results provide compelling evidence for a complementary system for the uptake of ATP in peroxisomes.

Keywords: peroxisome, ABCD, beta oxidation, SLC25A, ATP uptake

OPEN ACCESS

Edited by:
Michael Schrader,
University of Exeter, United Kingdom

Reviewed by:
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Medical University of Vienna, Austria
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *03 October 2021*
Accepted: *27 December 2021*
Published: *19 January 2022*

Citation:
van Roermund CWT, IJlst L, Linka N, Wanders RJ and Waterham HR (2022) Peroxisomal ATP Uptake Is Provided by Two Adenine Nucleotide Transporters and the ABCD Transporters. Front. Cell Dev. Biol. 9:788921. doi: 10.3389/fcell.2021.788921

INTRODUCTION

Peroxisomes are single-membrane bounded organelles found in cells of all eukaryotic species. They can be involved in a large variety of metabolic pathways which may differ per species but always includes the degradation of fatty acids through β-oxidation. In mammals, including humans, peroxisomes also play an important role in ether phospholipid biosynthesis, fatty acid alpha-oxidation, bile acid synthesis, glyoxylate detoxification and H_2O_2 degradation (Wanders and Waterham, 2006; Van Veldhoven, 2010). Genetic defects in the biogenesis and/or functioning of peroxisomes affect these metabolic pathways and usually have severe clinical consequences, as is demonstrated in the Zellweger spectrum disorders (Waterham et al., 2016) and the various single peroxisomal enzyme deficiencies (Wanders, 2018).

In most metabolic pathways, peroxisomes only catalyze a specific subset of enzyme reactions with other reactions catalyzed in the cytosol, mitochondria and/or endoplasmic reticulum (Wanders, 2014). The involvement of different cellular compartments implies that the various metabolites involved, i.e.

substrates and products, and co-factors, i.e. NAD, ATP, CoA, need to be transported across the peroxisomal membrane. In the past decades, the enzymology and biochemical functions of peroxisomes have largely been resolved. However, the mechanisms involved in peroxisomal metabolite transport have remained largely unknown. Yet, the importance of this transport is underlined by the existence of two inherited human diseases that are caused by defects in the peroxisomal half ABC transporter proteins ABCD1 and ABCD3 (Wanders, 2018), which function in the peroxisomal import of the CoA esters of very-long-chain fatty acids and branched-chain fatty acids, respectively.

Current consensus holds that peroxisomes are equipped with two fundamentally different mechanisms for metabolite transport across their membrane, which includes 1) diffusion of small Mw metabolites (<400 Da) via channel-forming membrane proteins, and 2) carrier-mediated transport of higher Mw metabolites, such as acyl-CoAs and ATP. Genetic complementation approaches, sequence similarity searches, and proteomic analyses of highly purified peroxisomes of mouse (Mi et al., 2007; Wiese et al., 2007), human (Gronemeyer et al., 2013), plants (Plett et al., 2020), and the yeast *Saccharomyces cerevisiae* (Yi et al., 2002; Chen and Williams, 2018) have led to the identification of several integral peroxisomal membrane proteins which, based on (partial) sequence similarity shared with known transport proteins, may function as peroxisomal metabolite transport proteins.

In mammals, including humans, three half ABC transporter proteins have been identified in the peroxisomal membrane: ABCD1 (also known as adrenoleukodystrophy protein, ALDP) (Aubourg et al., 1993; Mosser et al., 1993), ABCD2 (also known as adrenoleukodystrophy-related protein, ALDRP) (Lombard-Platet et al., 1996; Holzinger et al., 1997) and ABCD3 (also known as 70-kDa peroxisomal membrane protein, PMP70) (Kamijo et al., 1993). These proteins were shown to function as homodimers and import long, very-long-chain and branched-chain acyl-CoA esters into peroxisomes (van Roermund et al., 2012; Okamoto et al., 2018). So far, only three additional mammalian peroxisomal membrane proteins with a presumed function in metabolite transport have been identified. The first one is SLC25A17, also known as PMP34, which, based on sequence similarity, is a member of the mitochondrial carrier family (MCF). Reconstitution experiments in proteoliposomes followed by substrate exchange studies revealed that, *in vitro*, this protein is able to transport CoA, FAD, FMN, and AMP, and to a lesser extent NAD^+, PAP (adenosine 3′,5′-diphosphate) and ADP (Agrimi et al., 2012).

The second protein is PXMP2, which was shown to have channel-forming properties (Rokka et al., 2009). The third protein is PXMP4, which shares some similarity with bacterial permeases, but has not been functionally studied (Reguenga et al., 1999; Visser et al., 2007).

Peroxisomes in *S. cerevisiae* contain two half ABC transporters, Pxa1p and Pxa2p, which are involved in the import of long-chain acyl-CoA esters (e.g., C18:1) (Hettema et al., 1996; Shani et al., 1996; Swartzman et al., 1996; Roermund et al., 2008). In contrast to their human orthologues, Pxa1p and Pxa2p were shown to function as heterodimers. Two additional peroxisomal membrane proteins with a presumed function in metabolite transport have been identified in *S. cerevisiae*.

Ant1p is an MCF member with strong similarity to human PMP34 but, in contrast to PMP34, demonstrated to catalyze the exchange of cytosolic ATP for peroxisomal AMP or ADP. AMP is generated upon the intra-peroxisomal ATP-dependent activation of fatty acids by the acyl-CoA synthetase Faa2p (Palmieri et al., 2001; van Roermund et al., 2001) while ADP is generated by the peroxisomal nudix family members NPY1 and PCD1 (Plett et al., 2020).

The second protein, Pex11p, is known to be involved in peroxisomal fission, but also was reported to have transport or channel-forming properties (van Roermund et al., 2000; Mindthoff et al., 2016).

We use *S. cerevisiae* as model system (van Roermund et al., 2003) to unravel the mechanism of metabolite transport across the peroxisomal membrane. In contrast to human cells, in which both mitochondria and peroxisomes perform β-oxidation, fatty acid degradation in yeast cells takes place exclusively in peroxisomes and thus requires the import of fatty acids, and the co-factors ATP and CoA. Medium-chain fatty acids with carbon lengths of 8–12 enter yeast peroxisomes in their free acid form and are activated into CoA esters inside peroxisomes via the peroxisomal acyl-CoA synthetase Faa2p (Hettema et al., 1996; Roermund et al., 2008). This activation is ATP- and CoA-dependent. Long-chain fatty acids, however, are first activated outside peroxisomes and then imported as acyl-CoA ester by the ABC transporter protein complex Pxa1p/Pxa2p (Hettema et al., 1996; Roermund et al., 2008), followed by release of coenzyme A at the luminal side of peroxisomes and re-esterification by a peroxisomal synthetase (van Roermund et al., 2012; Carrier et al., 2019; van Roermund CWT et al., 2020).

Although in yeast, intra-peroxisomal ATP is essential for the peroxisomal β-oxidation of fatty acids following their import via the free fatty-acid route as well as the ABC transporter protein-mediated pathway, relatively little is known about the peroxisomal uptake of ATP except for the above mentioned involvement of Ant1p (Palmieri et al., 2001; van Roermund et al., 2001). Our observation that a knock-out of Ant1p in *S. cerevisiae* does not completely abolishes peroxisomal β-oxidation, however, implied the existence of additional ways to import ATP into peroxisomes. In this study we show that the uptake of ATP into peroxisomes is indeed mediated by different peroxisomal membrane proteins. In addition to Ant1p, these include the ABC transporter protein complex Pxa1p/Pxa2p, which thus catalyzes peroxisomal ATP uptake as well as acyl-CoA import, and the MCF carrier Aac2p, a predominantly mitochondrial protein, which we found partially localized to peroxisomes and which catalyzes the exchange of cytosolic ATP for peroxisomal ADP.

RESULTS

Ant1p and the ABC Transporter Protein Complex Pxa1p/Pxa2p Transport ATP Across the Peroxisomal Membrane

We previously showed that in the yeast *S. cerevisiae*, medium chain fatty acids such as octanoate (C8:0) are imported into peroxisomes as free fatty acids. To become substrate for β-oxidation they subsequently are activated into their corresponding fatty acyl-

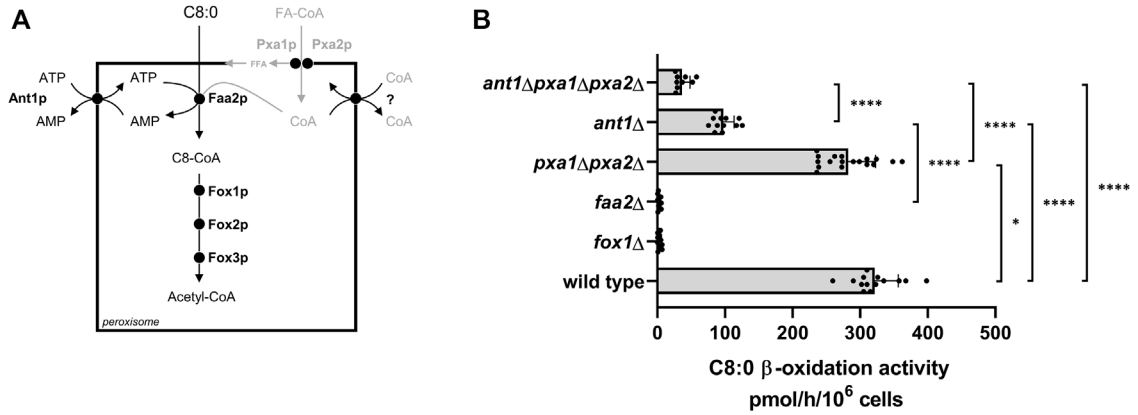

FIGURE 1 | C8:0 β-oxidation activity in wild-type and mutant yeast strains. **(A)** In yeast, β-oxidation of fatty acids occurs exclusively in peroxisomes requiring the import of fatty acids, ATP and CoA. Medium-chain fatty acids (C8–C12) enter yeast peroxisomes as free fatty acids and are subsequently activated to their corresponding CoA ester by the peroxisomal enzyme acyl-CoA synthetase Faa2p. This activation step is ATP and CoA dependent. For completeness, the acyl-CoA uptake route, not involved in the uptake of medium-chain fatty acids, and a yet to identify CoA transporter are depicted in gray. **(B)** Yeast cells were cultured overnight in oleate medium and β-oxidation rates were measured using [1–^{14}C] labelled octanoate (C8:0) as substrate. Data are means ± SD of values from 5–18 independent experiments. Only most relevant statistic relations are indicated in **Figure 1B**, all are given in **Supplementary Table S1**. ****, ***, **, and * indicate significance with a *p*-value of $p < 0.0001$, $p < 0.001$, $p < 0.01$, and $p < 0.05$ respectively.

CoA ester by the intra-peroxisomal ATP-dependent acyl-CoA synthetase Faa2 (**Figure 1A**) (Hettema et al., 1996; Roermund et al., 2008). In accordance with this, the β-oxidation of C8:0 in mutant cells in which the *FAA2* gene is deleted (*faa2Δ*) is fully deficient, similar as in *fox1Δ* cells in which the *FOX1* gene encoding acyl-CoA oxidase, the first enzyme of the β-oxidation pathway, is deleted (**Figure 1B**). Earlier work also showed that the peroxisomal membrane protein Ant1p functions as an antiporter of ATP against AMP or ADP and thus most probably is responsible for the peroxisomal uptake of ATP required for the intra-peroxisomal activation of fatty acids (Palmieri et al., 2001; van Roermund et al., 2001). Indeed, deletion of *ANT1* resulted in a significant decrease in the C8:0 β-oxidation activity (**Figure 1B**). However, the C8:0 β-oxidation activity in the *ant1*Δ cells was still ~30% of the activity measured in wild-type cells, which implied the involvement of additional ATP uptake system(s) in the peroxisomal membrane. To identify these, we measured C8:0 β-oxidation activities in *ant1*Δ cells in which, in addition, genes encoding other known peroxisomal membrane proteins were deleted. Surprisingly, we observed that C8:0 β-oxidation activity was further reduced to ~10% when we also deleted both *PXA1* and *PXA2* in the *ant1*Δ cells (**Figure 1B**). In *pxa1*Δ *pxa2*Δ double mutant cells, the C8:0 β-oxidation was only slightly decreased compared to wild-type cells. These findings pointed to a novel, unanticipated role for the ABC transporter protein complex Pxa1p/Pxa2p in peroxisomal ATP uptake in addition to its established role in the peroxisomal import of fatty acyl-CoAs.

ATP Uptake in Proteoliposomes Prepared From Peroxisomes of Different Mutant Strains

To provide additional evidence for a role of Pxa1p/Pxa2p in peroxisomal ATP uptake, we next studied the uptake of radio-labeled ATP in proteoliposomes prepared from peroxisomal membranes isolated from wild-type cells, *ant1*Δ, *pxa1*Δ *pxa2*Δ, and *ant1*Δ *pxa1*Δ *pxa2*Δ mutant cells.

In the absence of internal adenine nucleotides as counter-exchange substrate, we observed low and similar levels of ATP uptake in proteoliposomes prepared from *ant1*Δ and wild-type cells, while no ATP uptake was observed in proteoliposomes prepared from *pxa1*Δ *pxa2*Δ and *ant1*Δ *pxa1*Δ *pxa2*Δ mutant cells (**Figure 2A**). When we preloaded the proteoliposomes with AMP, ATP uptake was reduced to ~30% in proteoliposomes prepared from *ant1*Δ mutant cells, similar in proteoliposomes prepared from *pxa1*Δ *pxa2*Δ mutant cells, and virtually absent in proteoliposomes prepared from *ant1*Δ *pxa1*Δ *pxa2*Δ mutant cells when compared to ATP uptake in proteoliposomes prepared from wild-type cells (**Figure 2B**). Combined, these findings 1) show that Ant1p is responsible for most of the peroxisomal ATP uptake, 2) confirm that Ant1p functions as peroxisomal antiporter of ATP against AMP (Palmieri et al., 2001; van Roermund et al., 2001); 3) are in agreement with the C8:0 β-oxidation activities measured in the corresponding mutant cells; and 4) support a role for Pxa1p/Pxa2p in unidirectional peroxisomal uptake of ATP.

When we preloaded the proteoliposomes with ADP, ATP uptake was similar in proteoliposomes prepared from *pxa1*Δ *pxa2*Δ mutant cells and wild-type cells, and reduced to ~45% in proteoliposomes prepared from *ant1Δ* mutant cells. This confirms that Ant1p can also function as an antiporter of ATP against ADP as previously shown (Palmieri et al., 2006). Interestingly, *ant1*Δ *pxa1*Δ *pxa2*Δ mutant cells still showed ~20% ATP uptake when compared to wild-type cells (**Figure 2C**), which indicated the involvement of at least one additional peroxisomal ATP transporter that can mediate the exchange of ATP for ADP.

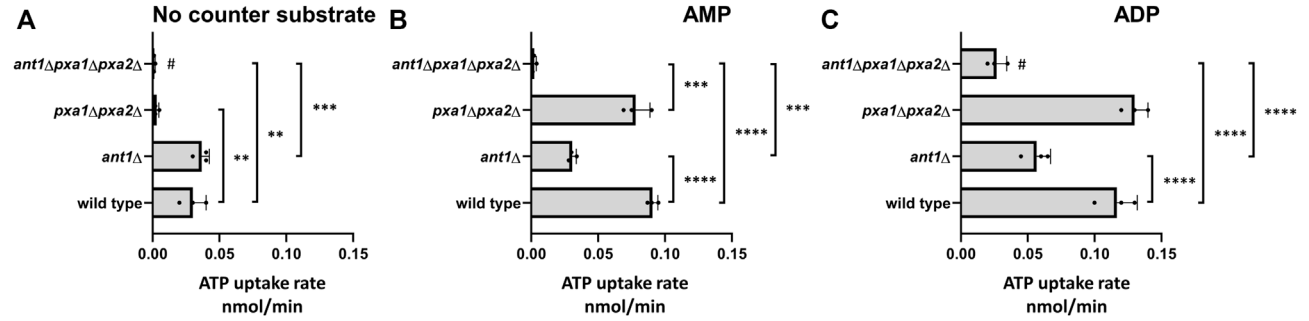

FIGURE 2 | ATP uptake into liposomes reconstituted with peroxisomal membranes purified from different yeast strains. Isolated peroxisomal membranes were reconstituted in proteoliposomes with no counter substrate **(A)**, or pre-loaded with AMP **(B)** or ADP **(C)**. Uptake of [α-^{32}P]ATP was measured as described in Methods. Data are means ± SD of values from three independent experiments. Only most relevant statistic relations are indicated in **Figure 2**, all are given in **Supplementary Table S1**. ****, ***, **, and * indicate significance with a *p*-value of $p < 0.0001$, $p < 0.001$, $p < 0.01$, and $p < 0.05$ respectively and # indicates significant difference between ADP or no counter substrate.

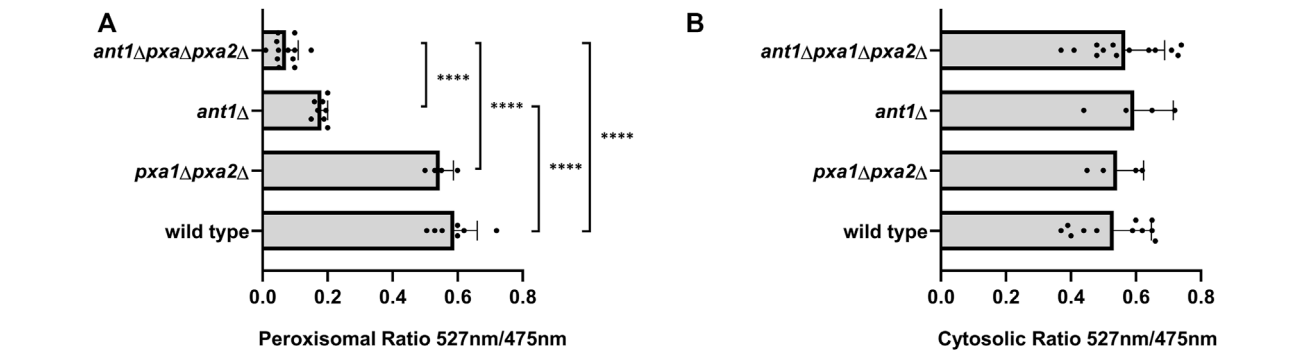

FIGURE 3 | Comparison of ATP levels determined with FRET-based ATeam reporter proteins in wild-type and mutant yeast strains. Cells were transformed with the different ATeam reporter proteins, grown overnight in oleate medium and transferred to 25 mM MES buffer (pH 6.0) supplemented with 20 g/L glucose prior to measurements. **(A)** Peroxisomal ATP levels determined with reporter proteins ATeam-per and ATeam-per (mut) **(B)** ATP levels determined with reporter proteins ATeam-cyt and ATeam-cyt (mut). FRET was determined by measuring 527/475 nm. Ratios were calculated after subtraction of the background signal from wild-type cells transformed with an empty vector. The semi-relative ATP levels were then calculated by subtracting the 572/475 ratio of the ATeam(mut) reporter from the 572/475 of ATeam reporter. Data are means ± SD of values from 4–13 independent experiments. Only most relevant statistic relations are indicated in **Figure 3**, all are given in **Supplementary Table S1**. ****, ***, **, and * indicate significance with a *p*-value of $p < 0.0001$, $p < 0.001$, $p < 0.01$, and $p < 0.05$ respectively.

Direct Measurement of Intra-Peroxisomal ATP Levels

To provide *in vivo* evidence for the role of Ant1p and Pxa1p/Pxa2p in peroxisomal ATP uptake, we expressed modified versions of the FRET-based ATeam reporter protein (Imamura et al., 2009; Bermejo et al., 2010) in the different yeast strains to measure the relative ATP levels in the peroxisomes (ATeam-per), i.e. extended with a carboxy-terminal peroxisomal targeting signal) and the cytosol (ATeam-cyt). To correct for ATP-independent background fluorescence, we used mutated versions of the same reporter proteins that have no affinity for ATP (ATeam-per (mut) and ATeam-cyt (mut)). The relative ATP levels in cytosol and peroxisomes of *pxa1*Δ *pxa2*Δ mutant cells were similar as observed in cytosol and peroxisomes of wild-type cells (**Figure 3**). However, the relative ATP levels in peroxisomes of *ant1*Δ and *ant1*Δ *pxa1*Δ *pxa2*Δ mutant cells were significantly reduced (**Figure 3A**) while the relative cytosolic ATP levels in these strains were similar when compared to wild-type cells (**Figure 3B**). The observation that the relative peroxisomal ATP levels in the *ant1*Δ *pxa1*Δ *pxa2*Δ mutant cells were significantly lower than in the *ant1*Δ cells confirmed that Pxa1p/Pxa2p also mediates ATP uptake, in addition to Ant1p.

Identification of an Additional Peroxisomal ATP Transporter

The ~10% residual C8:0 β-oxidation activity measured in the *ant1*Δ *pxa1*Δ *pxa2*Δ mutant cells (**Figure 1B**) as well as the ~20% residual *in vitro* ATP/ADP exchange observed in proteoliposomes prepared from peroxisomes of the *ant1*Δ *pxa1*Δ *pxa2*Δ mutant cells (**Figure 2C**), indicated the involvement of at least one additional peroxisomal ATP transporter. We hypothesized that in addition to Ant1p, which is a member of the MCF family and exclusively localized to peroxisomes, there might be one or more other members of this carrier family that have a dual localization in both the

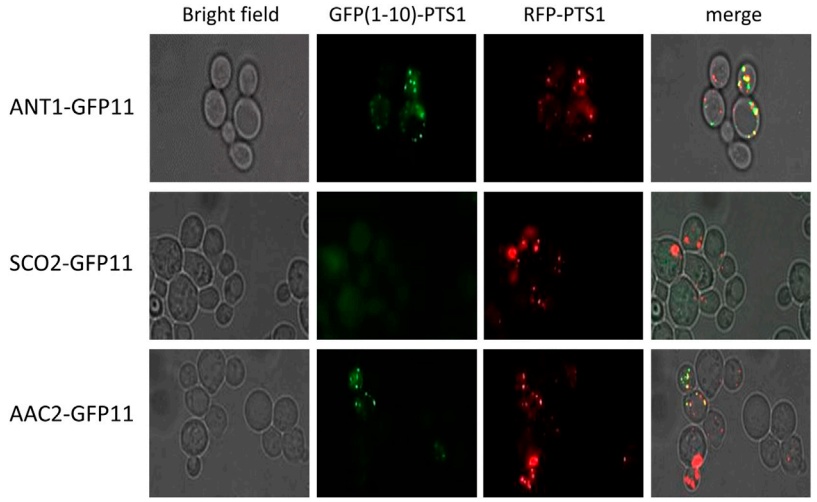

FIGURE 4 | Peroxisomal localisation of Aac2p using self-assembling GFP assay. Wild-type cells were transformed with GFP(1–10)-PTS1, GFP(11)-tagged Ant1p, Sco2p, or Aac2p, as well as RFP-PTS1 for confirmation of peroxisomal localisation. All cells were cultured overnight in ethanol medium. Images show bright field to visualize the localization of the cells (left); fluorescence of self-assembling GFP (GFP(1–10)-PTS1; left centre), RFP-PTS1 (right centre) and the overlay of bright field, self-assembling GFP and RFP-PTS1 (Merge; right).

mitochondrial and the peroxisomal membrane. In order to study this, we developed a sensitive cell-based assay employing self-assembling GFP (Cabantous and Waldo, 2006) that allows to determine a possible peroxisomal localization of MCF proteins (see **Supplementary Figure S2**). To this end, we tagged the C-terminus of selected MCF proteins with the GFP(11) peptide sequence and co-expressed these with a peroxisome-targeted GFP(1–10)-PTS1 in wild-type yeast cells. We tested five mitochondrial MCF proteins that are known to function as adenine nucleotide carriers: Aac1p, Aac2p, Aac3p, Yea6p, and Leu5p. As positive control we used Ant1p, and as negative control we used the mitochondrial MCF protein Sco2p, which is known to mediate copper transport to cytochrome c oxidase and thus is assumed not to show co-localization in peroxisomes.

In contrast to GFP(11)-tagged Ant1p, we did not observe GFP fluorescence for GFP(11)-tagged Aac1p, Aac3p, Yea6p, Leu5p and Sco2p when co-expressed with peroxisome-targeted GFP(1–10)-PTS1. GFP(11)-tagged Aac2p, however, displayed a clear punctated GFP fluorescence pattern similar as observed for GFP(11)-tagged Ant1p, which indicated a peroxisomal co-localization. To confirm the peroxisomal co-localization of Aac2p, we co-expressed GFP(11)-tagged Aac2p with GFP(1–10)-PTS1 in wild-type cells expressing a peroxisomal RFP-PTS1 reporter protein. This revealed co-localization of the punctated green GFP fluorescence with the peroxisomal red RFP-PTS1 fluorescence similar as observed for Ant1p (**Figure 4**). These findings imply that Aac2p has a dual subcellular localization in both mitochondria and peroxisomes and thus could be responsible for the residual peroxisomal ATP in peroxisomes observed in the *ant1*Δ *pxa1*Δ *pxa2*Δ mutant cells. Interestingly, Aac2p was shown previously to function as an ATP/ADP carrier (Palmieri et al., 2006; Bamber et al., 2007; Klingenberg, 2008; Duncan et al., 2018), which fits very well with the residual *in vitro* ATP/ADP exchange we measured in proteoliposomes prepared from peroxisomes of the *ant1*Δ *pxa1*Δ *pxa2*Δ mutant cells (**Figure 2C**).

Increased Mitochondrial Targeting of Aac2p Diminishes Residual Peroxisomal ATP Levels

In order to demonstrate that peroxisome-localized Aac2p is responsible for the residual C8:0 β-oxidation activity in the *ant1*Δ *pxa1*Δ *pxa2*Δ mutant cells, we attempted to delete the *AAC2* gene in these cells, but this did not result in a viable strain. As an alternative approach, we introduced a strong mitochondrial targeting signal (MTS) to the N terminus of Aac2p (van Roermund et al., 2016) in order to increase mitochondrial and decrease peroxisomal targeting of Aac2p. Using our self-assembling GFP assay, we observed that the addition of the strong MTS indeed reduced the peroxisomal localization of GFP(11)-tagged Aac2p to 2–3% of cells compared to 15% of GFP(11)-tagged Aac2p without the MTS (**Figure 5**).

Next, we introduced the MTS sequence via homologous genomic recombination to the N terminus of Aac2p in wild-type, the *ant1Δ* and *ant1Δ pxa1Δ pxa2Δ* mutant cells. In all three strains, the expression of MTSAAC2 resulted in lower C8:0 β-oxidation activities when compared to the activities in the same strains that do not express MTSAAC2 (**Figure 6A**).

These findings strongly suggest that peroxisome-localized Aac2p indeed is responsible for the residual C8:0 β-oxidation activity observed in *ant1Δ pxa1Δ pxa2Δ* mutant cells.

We also observed a decrease in the peroxisomal ATP levels in wild-type cells expressing MTSAAC2, but this did not reach significance ($p = 0.089$) (**Figure 6B**). Thus, the combined activity of Ant1p and Pxa1p/Pxa2p appears sufficient to maintain the ATP levels in wild-type cells expressing MTSAAC2. Expression

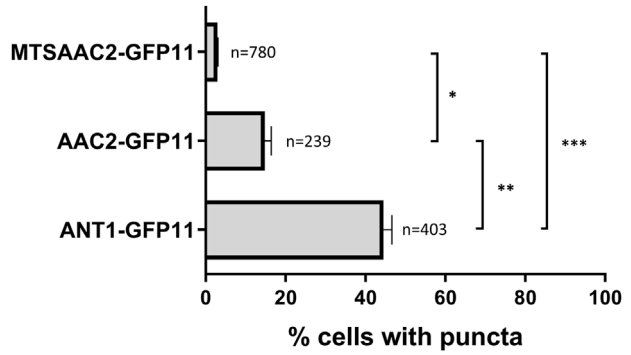

FIGURE 5 | Quantification of cells with Ant1p, Aac2p or MTSAac2p located in the peroxisomal membrane. Wild-type cells co-expressing the indicated GFP(11)-tagged MCF protein and the peroxisomal GFP(1–10)-PTS1 protein were analyzed for the number of cells displaying punctated GFP fluorescence, resulting from the physical interaction between GFP(11)-tagged MCF proteins and the peroxisomal GFP(1–10)-PTS1 protein. Expressed as percentage of number of analyzed cells indicated next to the bars. A one-way ANOVA test with Tukey's multiple comparisons test was performed. ***, **, and * indicate significance with a p-value of $p < 0.001$, $p < 0.01$, and $p < 0.05$ respectively.

of MTSAAC2 in *ant1Δ* cells resulted in a significant decrease in ATP levels. We did not study the effect of MTSAAC2 on the peroxisomal ATP levels in *ant1Δ pxa1Δ pxa2Δ* mutant cells because the residual ATP levels in this mutant strain were already near the detection limit of the ATeam reporter proteins.

Human ABCD1, ABCD2, and ABCD3 Can Also Transport ATP

After having established that Pxa1p/Pxa2p can mediate ATP uptake into peroxisomes, we studied whether the human orthologues HsABCD1, HsABCD2, and HsABCD3 can also mediate peroxisomal ATP uptake in addition to transport of fatty acyl-CoAs. We previously showed that yeast-codon optimized HsABCD1, HsABCD2 and HsABCD3 can be functionally expressed as homodimers in *S. cerevisiae*, and display different substrate specificities (van Roermund et al., 2014).

Expression of HsABCD1 in *ant1Δ pxa1Δ pxa2Δ* mutant cells cultured on oleate medium resulted in a more than 3-fold increase in C8:0 β-oxidation activity (**Figure 7A**) and co-expression with the peroxisomal ATeam reporter proteins showed a marked increase in the relative peroxisomal ATP levels (**Figure 7D**). Thus, similar as its yeast orthologues, HsABCD1 can also mediate ATP uptake. Expression of ABCD2 or ABCD3 in *ant1Δ pxa1Δ pxa2Δ* mutant cells did not result in a significant increase in C8:0 β-oxidation activity (**Figure 7A**).

C8:0 β-oxidation activity was not increased when HsABCD2 or HsABCD3 were expressed in *ant1Δ pxa1Δ pxa2Δ* mutant cells cultured on oleate medium (**Figure 7A**). Since the CoA ester of oleate, oleoyl-CoA, is also a good substrate for HsABCD2 and HsABCD3, but less for HsABCD1, we hypothesized that the uptake of oleoyl-CoA competes for the uptake of ATP by the ABCD proteins. To study this, we cultured the *ant1Δ pxa1Δ pxa2Δ* cells expressing HsABCD1, HsABCD2, or HsABCD3 on ethanol instead of oleate and repeated the C8:0 β-oxidation activity measurements. Under these conditions we indeed measured not only a significant increase in C8:0 β-oxidation activity in the cells expressing HsABCD1 but also in the cells expressing HsABCD2 or HsABCD3 when compared to the same cells expressing neither of these proteins (**Figure 7B**).

The substrate preference of ABCD1 for very-long-chain acyl-CoAs also allowed us to study whether acyl-CoA and ATP compete for the same binding site of ABCD1. To this end, we measured C8:0 β-oxidation activity in oleate-grown cells in the presence (**Figure 7C**) and absence (**Figure 7A**) of docosanoic acid (C22:0), which is a good substrate for ABCD1 after intracellular conversion into its CoA-ester (van Roermund et al., 2014). Both the C8:0 β-oxidation activity (**Figure 7C**) and the peroxisomal ATP levels (**Figure 7D**) were lower in the presence of C22:0,

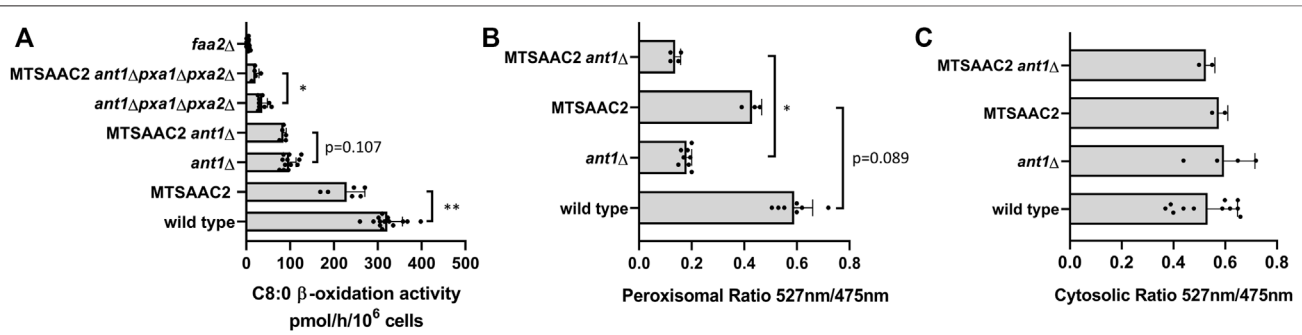

FIGURE 6 | C8:0 β-oxidation activity and ATP levels in wild-type and mutant yeast strains expressing MTS-Aac2p. **(A)** Untransformed strains and the same strains expressing MTSAAC2 were cultured overnight in oleate medium and β-oxidation rates were measured using [1–^{14}C] labelled octanoate (C8:0) as substrate. Data are means ± SD of values from 5–14 independent experiments. **(B)** Untransformed wild type and *ant1Δ* strains and the same strains expressing MTSAAC2 were transformed with ATeam-per or ATeam-per (mut) to determine relative peroxisomal ATP levels as described in legend of **Figure 3**. Data are means ± SD of values from 3–8 independent experiments. **(C)** Untransformed wild type and *ant1Δ* strains and the same strains expressing MTSAAC2p were transformed with ATeam-cyt and ATeam-cyt (mut) to determine relative cytosolic ATP levels as described in legend of **Figure 3**. Data are means ± SD of values from 2–11 independent experiments. Only most relevant statistic relations are indicated in **Figure 6**, all are given in **Supplementary Table S1**. ** and * indicate significance with a p-value of $p < 0.01$ and $p < 0.05$ respectively.

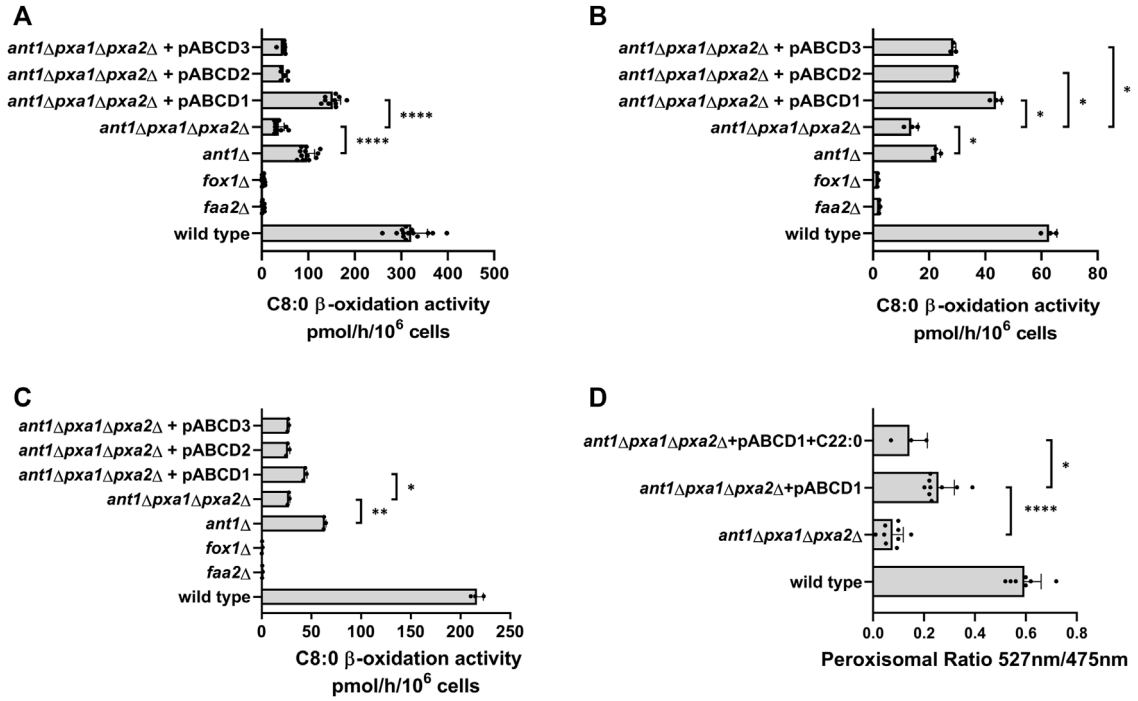

FIGURE 7 | C8:0 β-oxidation activity in wild-type and mutant yeast strains expressing human ABCD proteins. Wild-type and mutant strains, including *ant1Δpxa1Δpxa2Δ* strains expressing ABCD1, ABCD2, or ABCD3 were cultured overnight in oleate medium **(A)**, ethanol medium **(B)** or oleate medium supplemented with 100 μM C22:0 **(C)**. Fatty acid ß-oxidation activity was measured with [1–^{14}C] labelled octanoate (C8:0) **(A–C)**. **(D)** Wild-type and mutant strains, including *ant1Δpxa1Δpxa2Δ* strains expressing ABCD1 were transformed with ATeam-per and ATeam-per (mut) to allow quantification of peroxisomal ATP levels as described in legend of **Figure 3**. Cells were grown overnight in oleate medium or oleate medium supplemented with 100 μM C22:0. Data are means ± SD of values from 3–12 independent experiments. Only most relevant statistic relations are indicated in **Figure 7**, all are given in **Supplementary Table S1**. ****, ***, **, and * indicate significance with a *p*-value of respectively $p < 0.0001$, $p < 0.001$, $p < 0.01$, and $p < 0.05$.

which strongly suggests that the fatty acyl-CoAs and ATP compete for the same binding site in HsABCD1.

Taken together, these results show that all three human ABCD transporters can mediate peroxisomal ATP uptake in addition to transport of fatty acyl-CoAs.

DISCUSSION

Peroxisomes are generally considered to be selectively permeable organelles, which allow molecules with an Mw < 400 Da to cross the peroxisomal membrane passively via one or more (putative) channel-forming proteins, including Pxmp2 (Rokka et al., 2009) and Pex11beta (van Roermund et al., 2000; Mindthoff et al., 2016). Transport across the peroxisomal membrane of more bulky molecules, such as fatty acyl-CoAs and ATP, however, requires dedicated transport proteins. Among others, intra-peroxisomal ATP is essential for the peroxisomal β-oxidation of fatty acids. Previous work has shown that the transport of fatty acyl-CoAs is mediated by different dimeric half-ABC transporters of the ABCD family, which includes the heterodimer Pxa1p/Pxa2p in peroxisomes of *S. cerevisiae* and homodimers of ABCD1, ABCD2, and ABCD3 in human peroxisomes. In this paper we have studied which proteins are involved in the peroxisomal uptake of ATP. ATP has to be transported from the cytosol into the peroxisome since peroxisomes do not possess an ATP-synthesizing or regenerating system. To this end, we generated and used a series of *S. cerevisiae* mutant strains in which we deleted single or combinations of genes encoding putative peroxisomal ATP transporters. As readouts for peroxisomal ATP uptake we used three different and independent assays, including 1) measurement of *in vivo* C8:0 β-oxidation activity, which in yeast is strictly dependent on intra-peroxisomal ATP; 2) *in vitro* ATP uptake/exchange by proteoliposomes prepared from peroxisomal membranes isolated from the different mutant strains; and 3) *in vivo* measurement of relative ATP levels using FRET-based ATeam reporter proteins targeted to the peroxisomes or the cytosol.

We demonstrated that peroxisomes in *S. cerevisiae* contain at least three different transport systems that can mediate ATP uptake, each with a different mode of action. As anticipated, we found the previously reported peroxisomal membrane protein Ant1p to be responsible for most of the peroxisomal ATP uptake as can be concluded from the observation that *in vivo*, a deletion of *ANT1* resulted in a ~70% reduction of the peroxisomal ATP-dependent C8:0 β-oxidation activity and in a marked reduction of the intra-peroxisomal ATP levels. As reported previously and confirmed in our *in vitro* proteoliposome uptake studies, Ant1p functions as an antiporter of ATP against AMP and ADP

(Palmieri et al., 2001; van Roermund et al., 2001). Unexpectedly, we found that the remaining peroxisomal ATP uptake capacity is mediated by the ABC transporter protein complex Pxa1p/Pxa2p, which most probably functions as an ATP uniporter, and Aac2p, a predominantly mitochondrial ATP/ADP antiporter (Bamber et al., 2007; Klingenberg, 2008; Duncan et al., 2018; van Roermund CWT et al., 2020), which we here showed to be partly localized in peroxisomes.

Our finding that heterodimeric Pxa1p/Pxa2p, as well as the human orthologues HsABCD1, HsABCD2, and HsABCD3 as homodimers, can also mediate peroxisomal uptake of non-hydrolyzed ATP in addition to fatty acyl-CoAs was unexpected and is very intriguing. Indeed, ABC transporter proteins typically use ATP hydrolysis to catalyze the transport of substrates across membranes, although few ABC transporter proteins have been reported to function as ATP channels (Abraham et al., 1993; Reisin et al., 1994; Roman et al., 1997; Jansen et al., 2014). Our *in vivo* substrate competition experiments in which we added C22:0 during the C8:0 β-oxidation measurement suggested that ATP and acyl-CoAs compete for the same binding site of HsABCD1, which thus is different from the binding site at which ATP is hydrolyzed to drive the transport of fatty acyl-CoAs across the peroxisomal membrane. This competition is also suggested by the significant increase in C8:0 β-oxidation activity when the *ant1*Δ *pxa1*Δ *pxa2*Δ cells expressing HsABCD1, HsABCD2, or HsABCD3 are cultured on ethanol instead of oleate. We recently reported that Pxa1p/Pxa2p is also involved in peroxisomal uptake of CoA (Roermund et al., 2021). This may suggest that the increased C8:0 β-oxidation observed in the *ant1*Δ *pxa1*Δ *pxa2*Δ cells expressing HsABCD1 and cultured on ethanol could also be due to an ABCD1-mediated increase of intra-peroxisomal CoA levels. However, this explanation seems unlikely because if intra-peroxisomal CoA would be limiting for C8:0 β-oxidation, one would expect that addition of C22:0, which is a good substrate for ABCD1, would increase intra-peroxisomal CoA levels resulting in increased C8:0 βoxidation, while we observed a decrease of the latter. Unfortunately, we and others have not succeeded in reconstituting purified ABCD proteins in liposomes to study these and other aspects in more detail *in vitro*.

Aac2p is a well-established ATP/ADP antiporter localized in the inner mitochondrial membrane (Bamber et al., 2007; Klingenberg, 2008; Duncan et al., 2018; van Roermund CWT et al., 2020). Our finding that Aac2p is partly localized in peroxisomes and thus constitutes a third protein mediating peroxisomal ATP uptake is equally intriguing as the finding of the involvement of Pxa1p/Pxa2p. It also raises the question on how Aac2p is targeted to peroxisomes in addition to its primary targeting to mitochondria, given that the mechanisms of membrane protein import into mitochondria and peroxisomes must be very different, although this is still largely unknown for peroxisomal membrane proteins. It should be noted, however, that a dual localization in mitochondria and peroxisomes is not unique and has been reported for several proteins, including DLP1, FIS1, MIRO, and VDAC (Costello et al., 2018). In the case of Aac2p, the introduction of a stronger mitochondrial targeting signal (MTS) to its N terminus reduced the peroxisomal localization and, consequently, reduced the peroxisomal ATP uptake mediated by peroxisomal Aac2p.

The different substrate affinities and modes of action of Ant1p, Pxa1p/Pxa2p, and Aac2p not only ensure that peroxisomes can maintain their ATP levels to support the intra-peroxisomal ATP-dependent enzyme reactions, but also take care of the export of AMP and ADP generated after intra-peroxisomal hydrolysis of ATP. Indeed, peroxisomes in yeast harbor several enzymes the activity of which depends on ATP hydrolysis leading to the generation of AMP or ADP. These include several acyl-CoA synthetases (Hettema et al., 1996; van Roermund et al., 2012), PCD1 (Cartwright et al., 2000), NPY1 (Xu et al., 2000), VPS34 kinase (Stjepanovic et al., 2017) and LonP proteases (Bartoszewska et al., 2012; Pomatto et al., 2017).

Taken together, our results provide compelling evidence for the presence of multiple systems for the uptake and exchange of ATP in peroxisomes in yeast. Our finding that also the human peroxisomal ABC transporters can mediate peroxisomal ATP uptake strongly suggests that our findings in yeast are transferable to humans.

METHODS

Yeast Strains

We used *S. cerevisiae* BJ1991 (*MATα, pep4-3, prbl-1122, ura3-52, leu2, trp1*) as wild-type strain and for the generation of targeted deletion mutant strains. Gene deletions in BJ1991 were created by replacement of specific genes by the yeast *LEU2* gene, the Kanamycin (*KAN*) or the Bleomycin (*BLE*) resistance gene using homologous recombination. For this study we generated and used the following deletion mutant strains: *ant1Δ* (*ant1*:*KAN*), *pxa1Δ pxa2Δ* (*pxa1*:*LEU2*, *pxa2*:*KAN*), and *ant1Δ pxa1Δ pxa2Δ* (*ant1*:*KAN*, *pxa1*:*LEU2*, *pxa2*:*BLE*), and used two previously described mutant strains *faa2Δ* (*faa2*:*LEU2*) and *fox1Δ* (*fox1*:*KAN*).

Culture Conditions

We cultured yeast cells at 28°C under continuous shaking at 225 rpm. For standard growth, cells were cultured in glucose medium containing 6.7 g/L yeast nitrogen base without amino acids (Difco) and 5 g/L D-glucose. Amino acids were supplemented to the medium when required; 30 mg/L leucine, 20 mg/L uracil, or 20 mg/L tryptophan. To induce peroxisome proliferation, yeast cells were cultured for at least 24 h in glucose medium and then transferred to and cultured overnight in YPO medium (3 g/L yeast extract, 5 g/L peptone, 25 mM potassium phosphate buffer (pH = 6), 1.07 g/L oleate, 2.16 g/L Tween-80) with supplemented amino acids when required.

For fluorescent microscopy of self-assembling GFP, yeast cells were cultured in ethanol medium containing 1 g/L yeast extract, 25 mM potassium phosphate buffer (pH = 6), 6.7 g/L yeast nitrogen base and 2% ethanol and supplemented amino acids when required.

Octanoate (C8:0) β-Oxidation Measurements

We measured β-oxidation activity in intact yeast cells as follows. Cells were cultured overnight in YPO media, harvested by centrifugation, washed and resuspended in 9 g/L NaCl at a cell density of $OD_{600\ nm}$ = 1 (~1.48×10^7 cells/mL). Incubations were performed in 20 mL vials with a rubber septum, containing two tubes, one with the cells in incubation mixture and the other with 500 µL NaOH (2 M). To start the measurements, 20 µL of cell suspension was added to the reaction mixture composed of 20 µL MES buffer (0.5 M; pH = 6), 140 µL NaCl (9 g/L), and 20 µL of 100 µM [1–^{14}C] octanoate (200,000 dpm) as substrate. The reaction was allowed to proceed for 1 h at 28°C after which the reaction was terminated by the addition of 50 µL of perchloric acid (2.6 M). Radiolabelled [^{14}C]-CO_2, released during the β-oxidation of octanoate was trapped overnight in the tube with 500 µL of 2 M NaOH. Acid soluble products (ASP) were collected after extraction with chloroform/methanol/heptane (van Roermund CWT et al., 2020). Both CO_2 and ASP were quantified in a liquid scintillation counter and the β-oxidation rate was determined as the sum of CO_2 and ASP production. The octanoate β-oxidation rate in wild-type cells was 3.2 ± 0.4 nmol/h/10^7 cells.

ATP Uptake Measurements in Proteoliposomes

We isolated peroxisomes in duplicate from wild-type and the *ant1Δ*, *pxa1Δpxa2Δ* and *ant1Δpxa1Δpxa2Δ* mutant strains cultured overnight in oleate medium using cell fractionation and Nycodenz gradient centrifugation as described previously (van Roermund et al., 2001). Gradient fractions were analysed for peroxisomal 3-hydroxyacyl-CoA dehydrogenase (3-HAD) and mitochondrial fumarase activity (van Roermund et al., 2001). Purified peroxisomes from fractions 2–4 of the gradients (**Supplementary Figure S1**) and equivalent to 375 units of peroxisomal 3HAD activity were harvested and dissolved in 150 µL of 50 mM Hepes (pH = 7.4) and 5 mM $MgCl_2$. Of these, peroxisomes equivalent to 50 units of 3HAD activity were added to 1 mL 30 g/L L-α-glycerophosphorylcholine only or supplemented with 10 mM ADP or 10 mM AMP after which the mixtures were frozen in liquid nitrogen. The samples were then thawed at room temperature, resulting in the formation of proteoliposomes, and subjected to size-exclusion chromatography using Sephadex G-25 (Medium) columns (GE Healthcare Life Science) to remove external ADP or AMP. The eluate was used to start the uptake experiment by adding 0.2 mM [α-^{32}P]-ATP (6,000 Ci/mmol). The uptake reaction was terminated via passing the proteoliposomes over Dowex AG1-X8 anion-exchange columns using 150 mM sodium acetate (pH = 7.4) as elution buffer. The incorporated [α-^{32}P]-ATP was quantified by liquid scintillation counting. Time-dependent uptake data were fitted using nonlinear regression analysis based on one-phase exponential association using GraphPad Prism 5.0 software (GraphPad, www.graphpad.com). The initial velocity of uptakes were calculated using the equation slope = (Plateau–Y0)*k, with Y0 set to 0.

Measurement of ATP Levels Using FRET-Based ATeam Reporter Proteins

We measured the relative *in vivo* ATP levels in peroxisomes and the cytosol of wild-type cells and different mutant strains through expression of modified versions of the previously described ATeam sensors (Imamura et al., 2009). As source for the generation of the ATeam reporter proteins used in this study, we ordered the pDR-GW AT1.03 and pDR-GW AT1.03 R122K/R126K plasmids (Bermejo et al., 2010) from Addgene (deposited by Wolf Frommer). The pDR-GW AT1.03 plasmid (http://www.addgene.org/28003) codes for a cytosolic ATeam reporter protein and the pDR-GW AT1.03 R122K/R126K plasmid (http://www.addgene.org/28005) codes for a mutated version of the same ATeam reporter protein that no longer binds ATP. To allow constitutive, carbon source-independent ATeam gene expression in yeast, we first replaced the CTA1 promoter of pIJL30 with the TEF1 promoter generating the yeast expression vector pMK05 (TEF1pr, ARS1/CEN4, Trp1, ampR). The *XbaI-HindIII* fragments from pDR-GW AT1.03 and pDR-GW AT1.03R122K/R126K were then subcloned downstream of the TEF1 promoter into the *XbaI-HindIII* sites of pMK05. The resulting plasmids were designated pATeam-cyt (expressing cytosolic AT1.03) and pATeam-cyt (mut) (expressing the mutated cytosolic AT1.03 R122K/R126K), respectively.

To target the ATeam reporter proteins to peroxisomes, we replaced by site directed mutagenesis the stop codon of the AT1.03 ORFs in pATeam-cyt and pATeam-cyt (mut) by a flexible loop and the coding sequence for the twelve C-terminal amino acids of Fox2p, including the strong peroxisomal targeting sequence PTS1. This resulted in pATeam-per (expressing peroxisomal AT1.03) and pATeam-per (mut) (expressing the mutated peroxisomal AT1.03 R122K/R126K), respectively.

Yeast cells were transformed with the different pATeam plasmids, plated and individual colonies were cultured overnight in YPO medium. The cells were harvested by centrifugation at 230 *g* for 5 min at 4°C, washed once with and then suspended in cold MES-glucose buffer (pH 6.0) composed of 25 mM 2-(N-morpholino) ethanesulfonic acid and 20 g/L D-glucose. We resuspended the cells in MES + glucose to assure that the cytosolic ATP levels (i.e. the substrate for import) during the measurement were comparable in all strains (as confirmed in **Figures 3B**, **6C**) and thus would not provide an additional variable in the measurement of peroxisomal ATP import. Cells were kept on ice until analysis was conducted (5 h max). Prior to the measurements, the suspended cells were diluted with MES-glucose buffer until $OD_{600\ nm}$ = 3, and 200 µL was transferred in duplicate into a 96-wells microplate (Greiner, black round bottom). FRET analysis was then performed in on a Tecan Infinite M200 pro plate reader using an excitation of 435/9 nm and detecting emission at 475/20 nm and 527/20 nm, respectively. Fluorescence intensities at each wavelength were measured 10 times. To assure accurate measurements, prior to the start of each cycle the microplate was shaken in orbitals with an amplitude of 2 mm, a Z position height of 20,000 µm, settle

time of 200 ms, and 0 s lag time. Ratios were calculated after subtraction of the background signal in wild-type cells transformed with an empty plasmid (no ATeam expression). The relative ATP levels were obtained by subtracting the 572/475 ratio of the ATeam-cyt/per (mut) reporter protein from the 572/475 ratio of the corresponding ATeam-cyt/per reporter protein.

Construction of ABCD Expression Plasmids

We designed and ordered a yeast-codon optimized open reading frame (ORF) coding for ABCD1 and flanked by *SacI* and *KpnI*, and cloned this into the yeast expression vectors pIJL30 and pEL30. Construction of ABCD2 and ABCD3 expression plasmids have been described previously (van Roermund et al., 2014).

Subcellular Localisation of MCF Proteins Using a Self-Assembling GFP Assay

We adapted the self-assembling GFP assay (Cabantous and Waldo, 2006) to study the subcellular localization of MCF proteins. To this end, we designed and ordered from Genscript a yeast-codon optimized open reading frame (ORF) coding for GFP(1–10) in pUC57 and re-cloned this into the yeast expression vector pIJL30 (CTA1pr, ARS1/CEN4, Trp1, ampR) allowing cytosolic expression of GFP(1–10). To generate a peroxisome-localized GFP(1–10), we used the pUC57-GFP(1–10) as template and added via PCR amplification the coding sequences of the twelve C-terminal amino acids of FOX2, which include a strong peroxisomal targeting signal PTS1, spaced with a flexible linker. The resulting GFP(1–10)-PTS1 ORF was also subcloned into the pIJL30 expression vector.

To generate a basic cloning vector that allows expression of MCF proteins with a C-terminal extension coding for the GFP(11) domain, we introduced a linker encoding a flexible loop and yeast-codon optimized GFP(11) into the *BamH1* and *HindIII* sites of yeast expression vector pEL30 (van Roermund et al., 2012) (CTA1pr, ARS1/CEN4, URA3, ampR). The resulting plasmid pEL30-GFP(11) allows upstream cloning of ORFs into *SacI*, *KpnI*, *SmaI* and *BamHI* sites in frame with GFP(11).

ORFs encoding the yeast MCF proteins Ant1p, Aac1p, Aac2p, Aac3p, Yea6p, Leu5p, and Sco2p were PCR amplified from genomic DNA of *S. cerevisiae* using ORF-specific PCR primers with small extensions to introduce the appropriate restriction sites and, after restriction, sub-cloned in frame with GFP(11) into pEL30-GFP(11). All PCR-amplified sequences were verified by Sanger sequencing.

Wild-type cells were co-transformed with pEL30-GFP(11) containing one of the MCF proteins and either pIJL30-GFP(1–10)-PTS1 or pIJL30-GFP(1–10). After transformation the cells were cultured in 2% ethanol medium for 24 h, harvested, re-suspended in sterile water and examined on a ZEISS Axio Observer A1 fluorescence microscope using a 450 nm excitation and a 515–565 nm emission filter. The Leica Application Suite was used to capture the images.

Enhancing Mitochondrial Targeting of Aac2p

We introduced the mitochondrial targeting signal (MTS) from the mitochondrial succinate/fumarate carrier of *Arabidopsis thaliana* to the N-terminus of Aac2p (van Roermund et al., 2016), to increase mitochondrial and decrease peroxisomal targeting of Aac2p. To this end, we amplified the ORF of *AAC2* by PCR using an *AAC2*-specific forward primer with a 5′ extension comprising the coding sequence for the MTS. The MTS-*AAC2* ORF was cloned in frame with GFP(11) into pEL30-GFP(11) and verified by Sanger sequencing. Wild-type cells were co-transformed with the resulting pEL30-MTS-Aac2p-GFP(11) vector and the pIJL30-GFP(1–10)-PTS1 vector and used in the self-assembling GFP assay described above to compare the subcellular localization of MTS-Aac2p with Aac2p.

In order to introduce the MTS by homologous recombination at the N terminus of genomically encoded Aac2p, we generated by PCR amplification a DNA fragment comprising a 5′ *AAC2* non-coding sequence followed by the *NAT1* resistance gene under control of the *TEF1* promoter, the sequence for the MTS under control of the *NOP1* promoter, and a 5′ *AAC2* coding sequence. The fragment was transformed into wild-type, *ant1Δ* and *ant1/pxa1/pxa2Δ* mutant strains. After transformation, cells were washed and incubated for 5 h in 5 g/L glucose supplemented with amino acids, so that the *NAT1* resistance gene could be expressed. Cells were then plated on YPD plates supplemented with 100 μg/mL NTC to select for cells expressing the *NAT1* gene. Correct integration of the DNA fragment at the *AAC2* locus was verified by Sanger sequencing. Normal growth was observed in all knock-in strains on either YPD or 5 g/L glucose medium supplemented with amino acids. The different knock-in strains were used to determine the relative peroxisomal and cytosolic ATP levels using the ATeam reporter constructs as described above.

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

CR and LI designed research and analysed data. CR and NL performed experiments. CR, LI, RW, NL, and HW wrote and revised the manuscript.

FUNDING

This work was in part funded by the German Research Foundation (DFG) via grants LI 1781/1-3 (to NL).

ACKNOWLEDGMENTS

We thank Suzan Knottnerus, Andrew McDonald, Juan Carlos Munoz, Demi Rijlaarsdam and Mark de Keizer for their input in parts of this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.788921/full#supplementary-material

REFERENCES

Abraham, E. H., Prat, A. G., Gerweck, L., Seneveratne, T., Arceci, R. J., Kramer, R., et al. (1993). The Multidrug Resistance (Mdr1) Gene Product Functions as an ATP Channel. *Proc. Natl. Acad. Sci.* 90, 312–316. doi:10.1073/pnas.90.1.312

Agrimi, G., Russo, A., Scarcia, P., and Palmieri, F. (2012). The Human Gene SLC25A17 Encodes a Peroxisomal Transporter of Coenzyme A, FAD and NAD+. *Biochem. J.* 443, 241–247. doi:10.1042/bj20111420

Aubourg, P., Mosser, J., Douar, A. M., Sarde, C. O., Lopez, J., and Mandel, J. L. (1993). Adrenoleukodystrophy Gene: Unexpected Homology to a Protein Involved in Peroxisome Biogenesis. *Biochimie* 75, 293–302. doi:10.1016/0300-9084(93)90089-b

Bamber, L., Harding, M., Monne, M., Slotboom, D.-J., and Kunji, E. R. S. (2007). The Yeast Mitochondrial ADP/ATP Carrier Functions as a Monomer in Mitochondrial Membranes. *Proc. Natl. Acad. Sci.* 104, 10830–10834. doi:10.1073/pnas.0703969104

Bartoszewska, M., Williams, C., Kikhney, A., Opaliński, Ł., van Roermund, C. W. T., de Boer, R., et al. (2012). Peroxisomal Proteostasis Involves a Lon Family Protein that Functions as Protease and Chaperone. *J. Biol. Chem.* 287, 27380–27395. doi:10.1074/jbc.m112.381566

Bermejo, C., Haerizadeh, F., Takanaga, H., Chermak, D., and Frommer, W. B. (2010). Dynamic Analysis of Cytosolic Glucose and ATP Levels in Yeast Using Optical Sensors. *Biochem. J.* 432, 399–406. doi:10.1042/bj20100946

Cabantous, S., and Waldo, G. S. (2006). *In Vivo* and *In Vitro* Protein Solubility Assays Using Split GFP. *Nat. Methods* 3, 845–854. doi:10.1038/nmeth932

Carrier, D. J., van Roermund, C. W. T., Schaedler, T. A., Rong, H. L., IJlst, L., Wanders, R. J. A., et al. (2019). Mutagenesis Separates ATPase and Thioesterase Activities of the Peroxisomal ABC Transporter, Comatose. *Sci. Rep.* 9, 10502. doi:10.1038/s41598-019-46685-9

Cartwright, J. L., Gasmi, L., Spiller, D. G., and McLennan, A. G. (2000). The *Saccharomyces cerevisiae* PCD1 Gene Encodes a Peroxisomal Nudix Hydrolase Active toward Coenzyme A and its Derivatives. *J. Biol. Chem.* 275, 32925–32930. doi:10.1074/jbc.m005015200

Chen, X., and Williams, C. (2018). Fungal Peroxisomes Proteomics. *Sub-cellular Biochem.* 89, 67–83. doi:10.1007/978-981-13-2233-4_3

Costello, J. L., Passmore, J. B., Islinger, M., and Schrader, M. (2018). Multi-localized Proteins: The Peroxisome-Mitochondria Connection. *Sub-cellular Biochem.* 89, 383–415. doi:10.1007/978-981-13-2233-4_17

Duncan, A. L., Ruprecht, J. J., Kunji, E. R. S., and Robinson, A. J. (2018). Cardiolipin Dynamics and Binding to Conserved Residues in the Mitochondrial ADP/ATP Carrier. *Biochim. Biophys. Acta (Bba) - Biomembranes* 1860, 1035–1045. doi:10.1016/j.bbamem.2018.01.017

Gronemeyer, T., Wiese, S., Ofman, R., Bunse, C., Pawlas, M., Hayen, H., et al. (2013). The Proteome of Human Liver Peroxisomes: Identification of Five New Peroxisomal Constituents by a Label-free Quantitative Proteomics Survey. *PloS one* 8, e57395. doi:10.1371/journal.pone.0057395

Hettema, E. H., van Roermund, C. W., Distel, B., van den Berg, M., Vilela, C., Rodrigues-Pousada, C., et al. (1996). The ABC Transporter Proteins Pat1 and Pat2 Are Required for Import of Long-Chain Fatty Acids into Peroxisomes of *Saccharomyces cerevisiae*. *EMBO J.* 15, 3813–3822. doi:10.1002/j.1460-2075.1996.tb00755.x

Holzinger, A., Kammerer, S., Berger, J., and Roscher, A. A. (1997). cDNA Cloning and mRNA Expression of the Human Adrenoleukodystrophy Related Protein (ALDRP), a Peroxisomal ABC Transporter. *Biochem. biophysical Res. Commun.* 239, 261–264. doi:10.1006/bbrc.1997.7391

Imamura, H., Huynh Nhat, K. P., Togawa, H., Saito, K., Iino, R., Kato-Yamada, Y., et al. (2009). Visualization of ATP Levels inside Single Living Cells with Fluorescence Resonance Energy Transfer-Based Genetically Encoded Indicators. *Proc. Natl. Acad. Sci.* 106, 15651–15656. doi:10.1073/pnas.0904764106

Jansen, R. S., Duijst, S., Mahakena, S., Sommer, D., Szeri, F., Váradi, A., et al. (2014). ABCC6-mediated ATP Secretion by the Liver Is the Main Source of the Mineralization Inhibitor Inorganic Pyrophosphate in the Systemic Circulation-Brief Report. *Arteriosclerosis, Thrombosis, and Vascular Biology* 34, 1985–1989. doi:10.1161/atvbaha.114.304017

Kamijo, K., Osumi, T., and Hashimoto, T. (1993). PMP70, the 70-kDa Peroxisomal Membrane Protein: a Member of the ATP-Binding Cassette Transporters. *Nihon Rinsho* 51, 2343–2352.

Klingenberg, M. (2008). The ADP and ATP Transport in Mitochondria and its Carrier. *Biochim. Biophys. Acta (Bba) - Biomembranes* 1778, 1978–2021. doi:10.1016/j.bbamem.2008.04.011

Lombard-Platet, G., Savary, S., Sarde, C. O., Mandel, J. L., and Chimini, G. (1996). A Close Relative of the Adrenoleukodystrophy (ALD) Gene Codes for a Peroxisomal Protein with a Specific Expression Pattern. *Proc. Natl. Acad. Sci.* 93, 1265–1269. doi:10.1073/pnas.93.3.1265

Mi, J., Kirchner, E., and Cristobal, S. (2007). Quantitative Proteomic Comparison of Mouse Peroxisomes from Liver and Kidney. *Proteomics* 7, 1916–1928. doi:10.1002/pmic.200600638

Mindthoff, S., Grunau, S., Steinfort, L. L., Girzalsky, W., Hiltunen, J. K., Erdmann, R., et al. (2016). Peroxisomal Pex11 Is a Pore-Forming Protein Homologous to TRPM Channels. *Biochim. Biophys. Acta (Bba) - Mol. Cell Res.* 1863, 271–283. doi:10.1016/j.bbamcr.2015.11.013

Mosser, J., Douar, A.-M., Sarde, C.-O., Kioschis, P., Feil, R., Moser, H., et al. (1993). Putative X-Linked Adrenoleukodystrophy Gene Shares Unexpected Homology with ABC Transporters. *Nature* 361, 726–730. doi:10.1038/361726a0

Okamoto, T., Kawaguchi, K., Watanabe, S., Agustina, R., Ikejima, T., Ikeda, K., et al. (2018). Characterization of Human ATP-Binding Cassette Protein Subfamily D Reconstituted into Proteoliposomes. *Biochem. biophysical Res. Commun.* 496, 1122–1127. doi:10.1016/j.bbrc.2018.01.153

Palmieri, F., Agrimi, G., Blanco, E., Castegna, A., Di Noia, M. A., Iacobazzi, V., et al. (2006). Identification of Mitochondrial Carriers in *Saccharomyces cerevisiae* by Transport Assay of Reconstituted Recombinant Proteins. *Biochim. Biophys. Acta (Bba) - Bioenerg.* 1757, 1249–1262. doi:10.1016/j.bbabio.2006.05.023

Palmieri, L., Rottensteiner, H., Girzalsky, W., Scarcia, P., Palmieri, F., and Erdmann, R. (2001). Identification and Functional Reconstitution of the Yeast Peroxisomal Adenine Nucleotide Transporter. *Embo J.* 20, 5049–5059. doi:10.1093/emboj/20.18.5049

Plett, A., Charton, L., and Linka, N. (2020). Peroxisomal Cofactor Transport. *Biomolecules* 10, 1174. doi:10.3390/biom10081174

Pomatto, L. C. D., Raynes, R., and Davies, K. J. A. (2017). The Peroxisomal Lon Protease LonP2 in Aging and Disease: Functions and Comparisons with Mitochondrial Lon Protease LonP1. *Biol. Rev.* 92, 739–753. doi:10.1111/brv.12253

Reguenga, C., Oliveira, M. E. M., Gouveia, A. M. M., Eckerskorn, C., Sá-Miranda, C., and Azevedo, J. E. (1999). Identification of a 24 kDa Intrinsic Membrane Protein from Mammalian Peroxisomes. *Biochim. Biophys. Acta (Bba) - Gene Struct. Expr.* 1445, 337–341. doi:10.1016/s0167-4781(99)00061-5

Reisin, I. L., Prat, A. G., Abraham, E. H., Amara, J. F., Gregory, R. J., Ausiello, D. A., et al. (1994). The Cystic Fibrosis Transmembrane Conductance Regulator Is a Dual ATP and Chloride Channel. *J. Biol. Chem.* 269, 20584–20591. doi:10.1016/s0021-9258(17)32033-1

Roermund, C. W. T., IJlst, L., Baker, A., Wanders, R. J. A., Theodoulou, F. L., and Waterham, H. R. (2021). The *Saccharomyces cerevisiae* ABC Subfamily D Transporter Pxa1/Pxa2p Co-imports CoASH into the Peroxisome. *FEBS Lett.* 595, 763–772. doi:10.1002/1873-3468.13974

Roermund, C. W. T., Visser, W. F., IJlst, L., Cruchten, A., Boek, M., Kulik, W., et al. (2008). The Human Peroxisomal ABC Half Transporter ALDP Functions as a

Homodimer and Accepts Acyl-CoA Esters. *FASEB j.* 22, 4201–4208. doi:10.1096/fj.08-110866

Rokka, A., Antonenkov, V. D., Soininen, R., Immonen, H. L., Pirilä, P. L., Bergmann, U., et al. (2009). Pxmp2 Is a Channel-Forming Protein in Mammalian Peroxisomal Membrane. *PloS one* 4, e5090. doi:10.1371/journal.pone.0005090

Roman, R. M., Wang, Y., Lidofsky, S. D., Feranchak, A. P., Lomri, N., Scharschmidt, B. F., et al. (1997). Hepatocellular ATP-Binding Cassette Protein Expression Enhances ATP Release and Autocrine Regulation of Cell Volume. *J. Biol. Chem.* 272, 21970–21976. doi:10.1074/jbc.272.35.21970

Shani, N., Sapag, A., Watkins, P. A., and Valle, D. (1996). An *S. cerevisiae* Peroxisomal Transporter, Orthologous to the Human Adrenoleukodystrophy Protein, Appears to Be a Heterodimer of Two Half ABC Transporters: Pxa1p and Pxa2p. *Ann. NY Acad. Sci.* 804, 770–772. doi:10.1111/j.1749-6632.1996.tb18697.x

Stjepanovic, G., Baskaran, S., Lin, M. G., and Hurley, J. H. (2017). Vps34 Kinase Domain Dynamics Regulate the Autophagic PI 3-Kinase Complex. *Mol. Cel.* 67, 528–534. e523. doi:10.1016/j.molcel.2017.07.003

Swartzman, E. E., Viswanathan, M. N., and Thorner, J. (1996). The PAL1 Gene Product Is a Peroxisomal ATP-Binding Cassette Transporter in the Yeast *Saccharomyces cerevisiae*. *J. Cel. Biol.* 132, 549–563. doi:10.1083/jcb.132.4.549

van Roermund, C. W. T., IJlst, L., Baker, A., Wanders, R. J. A., Theodoulou, F. L., and Waterham, H. R. (2020). The *Saccharomyces cerevisiae* ABC Subfamily D Transporter Pxa1/Pxa2p Co-imports CoASH into the Peroxisome. *FEBS Lett.* 595 (6), 763–772. doi:10.1002/1873-3468.13974

van Roermund, C. W. T., Drissen, R., van den Berg, M., IJlst, L., Hettema, E. H., Tabak, H. F., et al. (2001). Identification of a Peroxisomal ATP Carrier Required for Medium-Chain Fatty Acid β-Oxidation and Normal Peroxisome Proliferation in *Saccharomyces cerevisiae*. *Mol. Cell Biol* 21, 4321–4329. doi:10.1128/mcb.21.13.4321-4329.2001

van Roermund, C. W. T., IJlst, L., Majczak, W., Waterham, H. R., Folkerts, H., Wanders, R. J. A., et al. (2012). Peroxisomal Fatty Acid Uptake Mechanism in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 287, 20144–20153. doi:10.1074/jbc.m111.332833

van Roermund, C. W. T., IJlst, L., Wagemans, T., Wanders, R. J. A., and Waterham, H. R. (2014). A Role for the Human Peroxisomal Half-Transporter ABCD3 in the Oxidation of Dicarboxylic Acids. *Biochim. Biophys. Acta (Bba) - Mol. Cell Biol. Lipids* 1841, 563–568. doi:10.1016/j.bbalip.2013.12.001

van Roermund, C. W. T., Schroers, M. G., Wiese, J., Facchinelli, F., Kurz, S., Wilkinson, S., et al. (2016). The Peroxisomal NAD Carrier from Arabidopsis Imports NAD in Exchange with AMP. *Plant Physiol.* 171, 2127–2139. doi:10.1104/pp.16.00540

van Roermund, C. W. T., Tabak, H. F., van Den Berg, M., Wanders, R. J. A., and Hettema, E. H. (2000). Pex11p Plays a Primary Role in Medium-Chain Fatty Acid Oxidation, a Process that Affects Peroxisome Number and Size in *Saccharomyces cerevisiae*. *J. Cel. Biol.* 150, 489–498. doi:10.1083/jcb.150.3.489

van Roermund, C. W. T., Waterham, H. R., IJlst, L., and Wanders, R. J. A. (2003). Fatty Acid Metabolism in *Saccharomyces cerevisiae*. *Cell Mol. Life Sci. (Cmls)* 60, 1838–1851. doi:10.1007/s00018-003-3076-x

Van Veldhoven, P. P. (2010). Biochemistry and Genetics of Inherited Disorders of Peroxisomal Fatty Acid Metabolism. *J. lipid Res.* 51, 2863–2895. doi:10.1194/jlr.r005959

Visser, W. F., van Roermund, C. W. T., IJlst, L., Waterham, H. R., and Wanders, R. J. A. (2007). Metabolite Transport across the Peroxisomal Membrane. *Biochem. J.* 401, 365–375. doi:10.1042/bj20061352

Wanders, R. J. A. (2014). Metabolic Functions of Peroxisomes in Health and Disease. *Biochimie* 98, 36–44. doi:10.1016/j.biochi.2013.08.022

Wanders, R. J. A. (2018). Peroxisomal Disorders: Improved Laboratory Diagnosis, New Defects and the Complicated Route to Treatment. *Mol. Cell. probes* 40, 60–69. doi:10.1016/j.mcp.2018.02.001

Wanders, R. J. A., and Waterham, H. R. (2006). Biochemistry of Mammalian Peroxisomes Revisited. *Annu. Rev. Biochem.* 75, 295–332. doi:10.1146/annurev.biochem.74.082803.133329

Waterham, H. R., Ferdinandusse, S., and Wanders, R. J. A. (2016). Human Disorders of Peroxisome Metabolism and Biogenesis. *Biochim. Biophys. Acta (Bba) - Mol. Cell Res.* 1863, 922–933. doi:10.1016/j.bbamcr.2015.11.015

Wiese, S., Gronemeyer, T., Ofman, R., Kunze, M., Grou, C. P., Almeida, J. A., et al. (2007). Proteomics Characterization of Mouse Kidney Peroxisomes by Tandem Mass Spectrometry and Protein Correlation Profiling. *Mol. Cell Proteomics* 6, 2045–2057. doi:10.1074/mcp.m700169-mcp200

Xu, W., Dunn, C. A., and Bessman, M. J. (2000). Cloning and Characterization of the NADH Pyrophosphatases from *Caenorhabditis elegans* and *Saccharomyces cerevisiae*, Members of a Nudix Hydrolase Subfamily. *Biochem. Biophysical Res. Commun.* 273, 753–758. doi:10.1006/bbrc.2000.2999

Yi, E. C., Marelli, M., Lee, H., Purvine, S. O., Aebersold, R., Aitchison, J. D., et al. (2002). Approaching Complete Peroxisome Characterization by Gas-phase Fractionation. *Electrophoresis* 23, 3205–3216. doi:10.1002/1522-2683(200209)23:18<3205:aid-elps3205>3.0.co;2-y

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frontiers in Cell and Developmental Biology

MINI REVIEW
published: 24 January 2022
doi: 10.3389/fcell.2022.826379

Low-Density Lipoprotein Internalization, Degradation and Receptor Recycling Along Membrane Contact Sites

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Low-density lipoprotein (LDL) internalization, degradation, and receptor recycling is a fundamental process underlying hypercholesterolemia, a high blood cholesterol concentration, affecting more than 40% of the western population. Membrane contact sites influence endosomal dynamics, plasma membrane lipid composition, and cellular cholesterol distribution. However, if we focus on LDL-related trafficking events we mostly discuss them in an isolated fashion, without cellular context. It is our goal to change this perspective and to highlight that all steps from LDL internalization to receptor recycling are likely associated with dynamic membrane contact sites in which endosomes engage with the endoplasmic reticulum and other organelles.

Keywords: low-density lipoprotein receptor (LDLR), low-density lipoprotein (LDL), hypercholesterolemia, membrane contact site, endosomal recycling, endosomal degradation

OPEN ACCESS

Edited by:
Joe Costello,
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Reviewed by:
Doris Höglinger,
Heidelberg University, Germany
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University College London,
United Kingdom

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

Received: *30 November 2021*
Accepted: *04 January 2022*
Published: *24 January 2022*

Citation:
Islam MM, Hlushchenko I and Pfisterer SG (2022) Low-Density Lipoprotein Internalization, Degradation and Receptor Recycling Along Membrane Contact Sites. Front. Cell Dev. Biol. 10:826379. doi: 10.3389/fcell.2022.826379

INTRODUCTION

Lipoproteins are transport shuttles in the circulation, delivering cholesterol to different destinations. The balance of lipoprotein production and clearance determines a person's blood cholesterol level. Accumulation of cholesterol-enriched low-density lipoprotein (LDL) is a hallmark of hypercholesterolemia, the main risk factor for cardiovascular disease (CVD), one of the most common causes of death worldwide (Mach et al., 2019; Borén et al., 2020).

Here we focus on how cells take up LDL in a regulated process mediated by the LDL receptor (LDLR). Patients with a homozygous mutation in *LDLR* can display more than five-fold higher LDL levels and experience severe cardiovascular complications before adolescence. Also, heterozygous carriers of *LDLR* mutations are at severe CVD risk, experiencing drastically elevated LDL concentrations (Cuchel et al., 2014). Interestingly, genetic defects in LDLR and proteins associated with LDLR trafficking predispose to a greater CVD risk, even when compared to individuals with similar blood cholesterol levels (Trinder et al., 2020). Probably, this is due to life-long exposure to elevated LDL levels or longer residence time of LDL particles in the bloodstream. This highlights the relevance of cellular LDL internalization in the development of hypercholesterolemia and CVD, and the importance of elucidating additional aspects of this pathway.

LDL binds to LDLR on the cell surface and is internalized via clathrin-mediated endocytosis (Brown and Goldstein, 1979). In the acidic environment of the early endosome, LDL dissociates from LDLR. Whilst a majority of LDLR is recycled back to the plasma membrane, LDL remains in the maturing endosomal system, resulting in degradation in late endosomes and lysosomes (LELs) (**Figure 1A**) (Brown and Goldstein, 1986). LDLR can join the path to degradation when it does not dissociate from LDL (Davis et al., 1987) or when it is specifically targeted by proprotein convertase

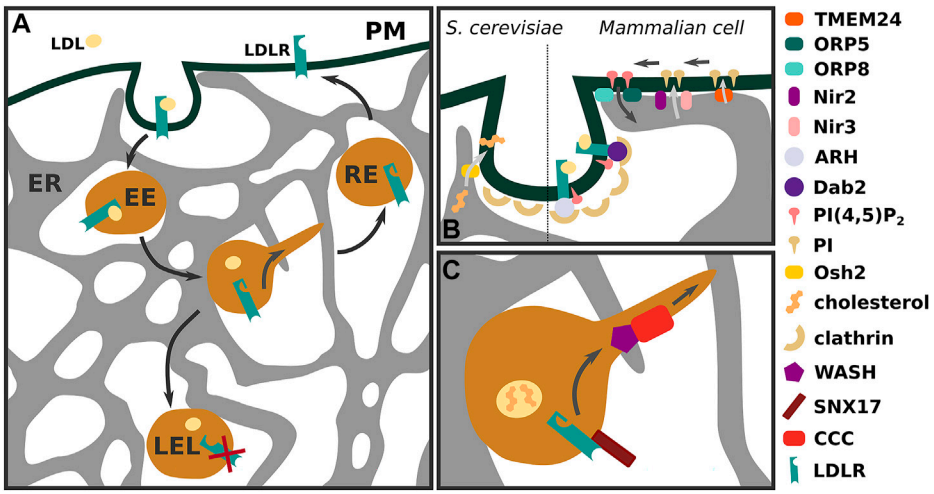

FIGURE 1 | LDLR trafficking in an interconnected membrane system **(A)** Low-density lipoprotein (LDL) receptor (LDLR) bound to its cargo is internalised via clathrin-mediated endocytosis. Within early endosomes (EE) LDLR separates from LDL and is sorted to recycling endosomes (RE) for transport to the plasma membrane. Receptors that fail to separate from LDL or are marked for degradation by extracellular or intracellular factors remain in the maturing endosomal system, resulting in their degradation in late endosomes and lysosomes (LELs). **(B)** Membrane contact sites between endoplasmic reticulum (ER) and plasma membrane are lipid exchange routes in both *S. cerevisiae* and mammalian cells, affecting sterol and PI(4,5)P_2 abundance. In *S. cerevisiae* sterol transfer to the budding vesicle is important for fission. In mammalian cells PI(4,5)P_2 is shuffled between PM and ER via ORP5 and ORP8 proteins and PM PI(4,5)P_2 pools can be replenished by PI transfer via Nir2, Nir3 and TMEM24. **(C)** Sorting of LDLR is enhanced by sorting nexin 17 (SNX17) and requires the WASH and COMMD/CCDC22/CCDC93 (CCC) complex for efficient recycling.

subtilisin/kexin type 9 (PCSK9) (Zhang et al., 2007) or inducible degrader of LDLR (IDOL) (Zelcer et al., 2009).

Efficient cholesterol export from LELs and transport to the endoplasmic reticulum (ER) plays an important role in regulating cholesterol synthesis and LDLR expression. Low cholesterol concentrations in the ER result in nuclear translocation of the transcription factor sterol regulatory element-binding protein 2 (SREBP-2) and activation of cholesterol synthesis and LDLR expression. On the other hand, an elevated cholesterol concentration in the ER results in SREBP-2 retention in the ER, reduced LDLR expression and lower LDL uptake. Consequently, defective cholesterol egress from LELs and reduced transport to the ER leads to higher LDL internalization rates (Ikonen 2008; Luo et al., 2020).

The majority of endosomes are in close contact with the ER and membrane contacts between both organelles influence endosomal function as well as fission of recycling vesicles from early endosomes (Eden et al., 2016; Rowland et al., 2014) (**Figure 1A**). Furthermore, membrane contact sites between the ER and endosomes, Golgi or the plasma membrane facilitate intracellular cholesterol transport, controlling cellular cholesterol balance and influencing transcriptional regulation of LDLR (Wilhelm et al., 2017; Mesmin et al., 2013b; Sandhu et al., 2018). Here we want to highlight those contact sites that appear relevant for LDL internalization and degradation, or enable an efficient retrieval of LDLR to the plasma membrane.

LDL-LDLR Internalization in Light of Membrane Contact Sites

For efficient internalization of the LDL-LDLR complex from the plasma membrane, LDLR is clustered into coated pits (Anderson et al., 1977; Anderson et al., 1982). The initiation, growth, and maturation of coated pits and vesicles is a tightly regulated process dependent on the plasma membrane levels of phosphatidylinositol-(4,5)-bisphosphate (PI(4,5)P_2) (Antonescu et al., 2011). Internalization of the LDL-LDLR complex starts with the binding of the adaptor proteins ARH (autosomal recessive hypercholesterolemia) or Dab2 (disabled-2) to the LDLR cytoplasmic tail (Sirinian et al., 2005; Maurer and Cooper, 2006). Adaptor protein interaction with LDLR and PI(4,5)P_2 is crucial for the formation of clathrin-coated pits, and recruitment of accessory proteins such as AP2 and clathrin stimulate clathrin-coated vesicle generation (**Figure 1B**) (Mettlen et al., 2010).

There are two main routes how membrane contact sites could contribute to the regulation of LDLR internalization. 1) Via influencing the lipid and PI(4,5)P_2 composition of the plasma membrane. 2) Through direct connection with the nascent endosome, regulating the vesicle formation process.

Several mechanisms have been proposed on how membrane contact sites can influence plasma membrane PI(4,5)P_2 levels. Oxysterol-binding protein (OSBP)-related proteins (ORP) 5 and 8 localize to plasma membrane-ER (PM-ER) contact sites dependent on PI(4,5)P_2 and appear to transport phosphatidylserine to the plasma membrane in exchange for PI(4,5)P_2, with ORP5/ORP8 depletion resulting in PM accumulation of PI(4,5)P_2 (Ghai et al., 2017) (**Figure 1B**). In an alternative mechanism, ORP5/ORP8 plasma membrane localization is influenced by both PI4P and PI(4,5)P_2 and in this case plasma membrane PI(4,5)P_2 levels are modulated by PI4P transport at PM-ER contacts (Sohn et al., 2018). Overall, PI(4,5)P_2 formation is limited by the amount of available PI4P and PI precursors, of which PI is synthesized at the ER (Kim et al., 2015; Chang and Liou, 2015; Chang et al., 2013). Nir3 localizes to PM-ER contact sites and maintains a basal PI pool at the plasma

membrane from which PI(4,5)P_2 can be generated via PI 4-kinase and PI4P 5-kinase (Chang and Liou, 2015). Activation of signaling receptors can lead to a rapid local PI(4,5)P_2 depletion through stimulation of phospholipase C (PLC). This evokes Nir2 translocation to PM-ER contact sites and rapid transfer of PI from the ER to the PM in exchange for phosphatidic acid resulting in PI(4,5)P_2 reformation (**Figure 1B**) (Chang and Liou, 2015; Kim et al., 2015). Furthermore, transmembrane protein 24 (TMEM24) can mediate plasma membrane PI replenishment at PM-ER contact sites, resulting in PI4,5P_2 reformation during glucose-stimulated signaling (Lees et al., 2017) (**Figure 1B**).

Therefore, even though we lack direct support for this hypothesis, it appears likely that membrane contact sites influence clathrin-mediated endocytosis in a localized fashion through the modulation of PI(4,5)P_2 abundance, acting together with lipid kinases and phosphatases (Posor et al., 2015).

Interestingly, in *S. cerevisiae* membrane contact sites between the ER and the forming endosome have been observed. This involves the yeast ORP protein Osh2 and results in actin recruitment and vesicle fission (**Figure 1B**) (Encinar del Dedo et al., 2017). Furthermore, Osh2 is involved in mediating sterol transport at these contact sites, which appears to be important for endocytosis when plasma sterol availability is limited (Encinar del Dedo et al., 2021). Also in plants PM-ER contact sites can influence endocytosis. Plant VAP (Vesicle-Associated Membrane Protein-Associated Protein) proteins (VAP27-1 and VAP27-3) mediate contact formation through interaction with PIPs and clathrin at endocytic membranes, facilitating endocytosis (Stefano et al., 2018). This highlights multiple options of how membrane contact sites could influence clathrin-mediated endocytosis and internalization of the LDL-LDLR complex.

Soon after the clathrin-coated vesicle detaches from the plasma membrane, the coat proteins disassemble, PI(4,5)P_2 is hydrolyzed and the vesicles merge into the early endosomal system (Kaksonen and Roux, 2018). Around 80% of early endosomes are in contact with the ER (Friedman et al., 2013), indicating that this is also the case for those containing the LDL-LDLR complex. At this stage, separate trafficking routes emerge, LDLR can be sorted into recycling endosomes, whilst LDL and some LDLRs are staying on a path to degradation in late endosomes and lysosomes (Wijers et al., 2015).

LDLR Recycling in an Interconnected Endosome-ER Meshwork

LDLR recycling is activated once the ligand and receptor dissociate in the early endosomal system. At this stage, a conformational change of the LDLR impedes its degradation and makes it available for recycling (Davis et al., 1987; Surdo et al., 2011). Possibly, sorting nexins (SNXs) play an important role in redirecting LDLR towards the plasma membrane. SNX17 binds to the LDLR cytoplasmic tail and SNX17 overexpression increases the LDL internalization rate (Stockinger, 2002; Burden et al., 2004), suggesting a role in LDLR recycling. However, we lack loss-of-function information to say that LDLR sorting depends on SNX17. Therefore, it is possible that other proteins can initiate LDLR recycling as well.

We know that efficient LDLR recycling requires an intact Wiskott–Aldrich syndrome protein and SCAR homolog (WASH) and COMMD/CCDC22/CCDC93 (CCC) complex (**Figure 1C**) (Bartuzi et al. 2016; Wijers et al., 2019; Rimbert et al., 2020). The WASH components WASH1 and FAM21 co-precipitate with LDLR and WASH1 deficient cells show increased LDLR degradation, reduced surface expression, and LDL uptake. Defects in LDLR recycling in WASH1 deficient cells can be rescued by re-expressing a wild type but not a WASH1 mutant, which fails to initiate F-actin polymerization via Arp2/3 activation (Bartuzi et al., 2016). WASH-mediated actin polymerization plays a pivotal role in endosome fission from the sorting endosome (Derivery et al., 2009). 80% of endosomal tubules undergo fission at an intersection point with the ER. Interestingly, FAM21 localizes to the neck of endosomal tubules and nearly all of these sites overlap with the ER (Rowland et al., 2014). Moreover, WASH activity is regulated by endosomal PI(4)P levels which in turn are influenced by OSBP (Oxysterol Binding Protein) and VAP acting at endosome-ER contact sites (Dong et al., 2016). Tight control of endosomal PI(4)P levels appears important in endosomal fission as OSBP inactivation leads to PI(4)P accumulation and exaggerated actin polymerization (Dong et al., 2016). On the other hand, PI(4)P is coupled to phosphatidylserine delivery to the endosome from the ER *via* ORP10. A defect in this process also impairs effective retrograde trafficking of endosomal cargo and endosomal fission (Kawasaki et al., 2021).

Even though endosomal fission has mostly been studied in connection with endosomal sorting towards the Golgi (Rowland et al., 2014; Dong et al., 2016; Hoyer et al., 2018), it is fair to speculate that ER/endosome interconnections are important for LDLR recycling towards the plasma membrane as well.

LDL Degradation: Holding on to the ER Whilst Reaching out to Other Organelles

LDL embarks on a path to degradation in late endosomes/lysosomes upon dissociating from LDLR. LDLR can join this path if targeted by PCSK9, IDOL, or upon failure to dissociate from LDL. On this path endosome association with the ER increases to nearly 100% (Friedman et al., 2013). This close association appears to play a key role in exporting LDL-derived cholesterol from endosomes to the ER (**Figure 2**). Whilst there are multiple pathways for cholesterol export from endosomes (Kanerva et al., 2013; Takahashi et al., 2021) it has been shown that 30% of endosomal cholesterol is transported directly to the ER (Neufeld et al., 1996). Lysosomal acid lipase liberates LDL-derived cholesterol in the endosomal lumen (Chang et al., 2006). Then cholesterol gets inserted into the LEL limiting membrane through the concerted action of Nieman Pick Type C 2 (NPC2) and NPC1 proteins (Infante et al., 2008).

The first evidence for the involvement of membrane contact sites in redistributing LDL-derived cholesterol came from studies involving oxysterol binding protein (OSBP) related protein

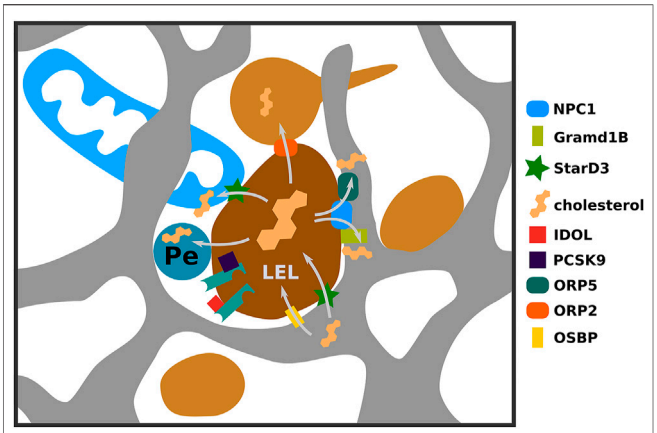

FIGURE 2 | Multiple routes of endosomal cholesterol transport via membrane contact sites. LDL-derived cholesterol is liberated in late endosomes and lysosomes (LEL) and is made available to other cellular compartments via different mechanisms. Here we highlight several contact sites involved in this process. NPC1 via interaction with ORP5 or GramD1B can stimulate contacts between LELs and ER mediating cholesterol efflux to the ER. If NPC1 mediated export to the ER is impaired, cholesterol can be transferred to mitochondria via contacts established via StarD3. Also LELs can engage in contacts with peroxisomes and recycling endosomes for cholesterol export and cholesterol can be transported in reverse direction from ER to LEL.

(ORP) 5 (ORP5) (Du et al., 2011). ORP5, an ER-resident protein, interacts with NPC1 in the LEL limiting membrane, establishing a connection between both organelles. Upon LDL load, depletion of ORP5 leads to cholesterol accumulation in LEL membranes (Du et al., 2011). Recent findings further strengthen that protein-protein interactions with NPC1 facilitate LEL-ER contact site formation and cholesterol transport towards the ER (Höglinger et al., 2019). NPC1 depletion reduces ER-lysosome contacts, whilst NPC1 overexpression increases them. Moreover, NPC1 interacts with Gramd1B/AsterB, a novel contact site protein previously implied in PM to ER cholesterol transport (Sandhu et al., 2018; Höglinger et al., 2019). Similar to NPC1, Gramd1B influences LEL-ER contact site formation, and Gramd1B depletion results in endosomal cholesterol accumulation. Interestingly, LEL-ER contact site restoration stimulates cholesterol export even without NPC1 (Höglinger et al., 2019) suggesting that either close proximity itself can lead to cholesterol transport, or that other proteins mediate transport. One such protein could be ORP1L, which localizes to LELs and influences LEL-ER contact site formation (Rocha et al., 2009). Deficiency of ORP1L leads to cholesterol accumulation in LELs and reduced transport towards the ER (Zhao and Ridgway, 2017). Whilst this can indicate that ORP1L affects transport of LDL derived cholesterol along LEL-ER contact sites, this could also happen via more indirect means of transport.

Besides LEL-ER contact sites, LELs engage in membrane contacts with multiple organelles to ensure efficient cholesterol delivery within cells. Contacts between LELs and recycling endosomes facilitate cholesterol transport towards the plasma membrane (Takahashi et al., 2021) and also LEL-Peroxisome contacts can stimulate LEL cholesterol export (**Figure 2**) (Chu et al., 2015). Moreover, defective ER-Lysosome contacts are compensated by increased LEL-mitochondria contacts, resulting in increased cholesterol transport towards mitochondria, a process which is dependent on the StarD3 protein (Charman et al., 2010; Höglinger et al., 2019). This is a striking effect, especially as StarD3 itself is involved in mediating ER-LEL contacts in cholesterol-depleted conditions to deliver newly synthesized cholesterol towards endosomes (Wilhelm et al., 2017). Reverse cholesterol transport from the ER to endosomes is important for efficient multivesicular body formation as blocking this step results in defective degradation of signaling receptors when access to LDL-cholesterol is limited (Eden et al., 2016). Furthermore, reverse cholesterol transport at LEL-ER contact sites, mediated by OSBP influences mTOR recruitment and activation at LELs, contributing to the regulation of autophagy (Lim et al., 2019).

There are many open questions regarding LDL-cholesterol redistribution via LEL-ER contact sites. These involve the spatio-temporal involvement of proteins in multiple contact sites with different organelles (Gramd1B (Sandhu et al., 2018; Höglinger et al., 2019; Naito et al., 2019; Ferrari et al., 2020; Ercan et al., 2021), ORP5 (Sohn et al., 2018; Du et al., 2011, 2019), ORP1L (Rocha et al., 2009; Boutry and Kim 2021), OSBP (Mesmin et al., 2013a; Lim et al., 2019) and StarD3 (Wilhelm et al., 2017; Höglinger et al., 2019)), and influence of the cellular cholesterol distribution on the formation of LEL-ER contact sites by different players (Rocha et al., 2009; Höglinger et al., 2019; Lim et al., 2019).

CONCLUSION

Multiple different membrane contact sites can converge with the LDL internalization and degradation path. Whilst most reliable data exists for the involvement of contact sites in transporting cholesterol between LELs and the ER, we can only extrapolate that membrane contact sites also influence LDL internalization and LDLR recycling events. We believe that more emphasis should be directed to elucidate how membrane contact sites influence clathrin-mediated endocytosis and LDLR trafficking.

AUTHOR CONTRIBUTIONS

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

FUNDING

This study was supported by the Academy of Finland grants 328861, 325040, Finnish Foundation for Cardiovascular Research, and a Business Finland Research-to-Business 1821/31/2021 grant to SP.

REFERENCES

Anderson, R. G., Brown, M. S., Beisiegel, U., and Goldstein, J. L. (1982). Surface Distribution and Recycling of the Low Density Lipoprotein Receptor as Visualized with Antireceptor Antibodies. *J. Cell Biol.* 93 (3), 523–531. doi:10.1083/jcb.93.3.523

Anderson, R. G. W., Brown, M. S., and Goldstein, J. L. (1977). Role of the Coated Endocytic Vesicle in the Uptake of Receptor-Bound Low Density Lipoprotein in Human Fibroblasts. *Cell* 10 (3), 351–364. doi:10.1016/0092-8674(77)90022-8

Antonescu, C. N., Aguet, F., Danuser, G., and Schmid, S. L. (2011). Phosphatidylinositol-(4,5)-Bisphosphate Regulates Clathrin-Coated Pit Initiation, Stabilization, and Size. *MBoC* 22 (14), 2588–2600. doi:10.1091/mbc.E11-04-0362

Bartuzi, P., Billadeau, D. D., Favier, R., Rong, S., Dekker, D., Fedoseienko, A., et al. (2016). CCC- and WASH-Mediated Endosomal Sorting of LDLR Is Required for Normal Clearance of Circulating LDL. *Nat. Commun.* 7 (1), 10961. doi:10.1038/ncomms10961

Borén, J., Chapman, M. J., Krauss, R. M., Packard, C. J., Bentzon, J. F., Binder, C. J., et al. (2020). Low-Density Lipoproteins Cause Atherosclerotic Cardiovascular Disease: Pathophysiological, Genetic, and Therapeutic Insights: A Consensus Statement from the European Atherosclerosis Society Consensus Panel. *Eur. Heart J.* 41, 2313–2330. doi:10.1093/eurheartj/ehz962

Boutry, M., and Kim, P. K. (2021). ORP1L Mediated PI(4)P Signaling at ER-Lysosome-Mitochondrion Three-Way Contact Contributes to Mitochondrial Division. *Nat. Commun.* 12 (September), 5354. doi:10.1038/s41467-021-25621-4

Brown, M. S., and Goldstein, J. L. (1986). A Receptor-Mediated Pathway for Cholesterol Homeostasis. *Science* 232 (4746), 34–47. doi:10.1126/science.3513311

Brown, M. S., and Goldstein, J. L. (1979). Receptor-Mediated Endocytosis: Insights from the Lipoprotein Receptor System. *Proc. Natl. Acad. Sci.* 76 (7), 3330–3337. doi:10.1073/pnas.76.7.3330

Burden, J. J., Sun, X.-M., García, A. B. G., and Soutar, A. K. (2004). Ana Bárbara García García, and Anne K. SoutarSorting Motifs in the Intracellular Domain of the Low Density Lipoprotein Receptor Interact with a Novel Domain of Sorting Nexin-17. *J. Biol. Chem.* 279 (16), 16237–16245. doi:10.1074/jbc.M313689200

Chang, C.-L., Hsieh, T.-S., Yang, T. T., Rothberg, K. G., Azizoglu, D. B., Volk, E., et al. (2013). Feedback Regulation of Receptor-Induced Ca2+ Signaling Mediated by E-Syt1 and Nir2 at Endoplasmic Reticulum-Plasma Membrane Junctions. *Cell Rep.* 5 (3), 813–825. doi:10.1016/j.celrep.2013.09.038

Chang, C.-L., and Liou, J. (2015). Phosphatidylinositol 4,5-Bisphosphate Homeostasis Regulated by Nir2 and Nir3 Proteins at Endoplasmic Reticulum-Plasma Membrane Junctions. *J. Biol. Chem.* 290 (23), 14289–14301. doi:10.1074/jbc.M114.621375

Chang, T.-Y., Chang, C. C. Y., Ohgami, N., Yamauchi, Y., and Yamauchi, Yoshio. (2006). Cholesterol Sensing, Trafficking, and Esterification. *Annu. Rev. Cell Dev. Biol.* 22, 129–157. doi:10.1146/annurev.cellbio.22.010305.104656

Charman, M., Kennedy, B. E., Osborne, N., and Karten, B. (2010). MLN64 Mediates Egress of Cholesterol from Endosomes to Mitochondria in the Absence of Functional Niemann-Pick Type C1 Protein. *J. Lipid Res.* 51 (5), 1023–1034. doi:10.1194/jlr.M002345

Chu, B.-B., Liao, Y.-C., Qi, W., Xie, C., Du, X., Wang, J., et al. (2015). Cholesterol Transport through Lysosome-Peroxisome Membrane Contacts. *Cell* 161 (2), 291–306. doi:10.1016/j.cell.2015.02.019

Cuchel, M., Bruckert, E., Ginsberg, H. N., Raal, F. J., Santos, R. D., Hegele, R. A., et al. (2014). Homozygous Familial Hypercholesterolaemia: New Insights and Guidance for Clinicians to Improve Detection and Clinical Management. A Position Paper from the Consensus Panel on Familial Hypercholesterolaemia of the European Atherosclerosis Society. *Eur. Heart J.* 35 (32), 2146–2157. doi:10.1093/eurheartj/ehu274

Davis, C., Goldstein, J. L., Südhof, T. C., Anderson, R. G., Russell, D. W., and Brown, M. S. (1987). Acid-Dependent Ligand Dissociation and Recycling of LDL Receptor Mediated by Growth Factor Homology Region. *Nature* 326 (6115), 760–765. doi:10.1038/326760a0

Derivery, E., Sousa, C., Gautier, J. J., Lombard, B., Loew, D., and Gautreau, A. (2009). The Arp2/3 Activator WASH Controls the Fission of Endosomes through a Large Multiprotein Complex. *Dev. Cell* 17 (5), 712–723. doi:10.1016/j.devcel.2009.09.010

Dong, R., Saheki, Y., Swarup, S., Lucast, L., Harper, J. W., and De Camilli, P. (2016). Endosome-ER Contacts Control Actin Nucleation and Retromer Function through VAP-dependent Regulation of PI4P. *Cell* 166 (2), 408–423. doi:10.1016/j.cell.2016.06.037

Du, X., Kumar, J., Ferguson, C., Schulz, T. A., Ong, Y. S., Hong, W., et al. (2011). A Role for Oxysterol-Binding Protein-Related Protein 5 in Endosomal Cholesterol Trafficking. *J. Cell Biol.* 192 (1), 121–135. doi:10.1083/jcb.201004142

Du, X., Zhou, L., Aw, Y. C., Mak, H. Y., Xu, Y., Rae, J., et al. (2019). ORP5 Localizes to ER-Lipid Droplet Contacts and Regulates the Level of PI(4)P on Lipid Droplets. *J. Cell Biol.* 219 (1), e201905162. doi:10.1083/jcb.201905162

Eden, E. R., Sanchez-Heras, E., Tsapara, A., Sobota, A., Levine, T. P., and Futter, C. E. (2016). Annexin A1 Tethers Membrane Contact Sites that Mediate ER to Endosome Cholesterol Transport. *Dev. Cell* 37 (5), 473–483. doi:10.1016/j.devcel.2016.05.005

Encinar del Dedo, J., Fernández-Golbano, I. M., Pastor, L., Meler, P., Ferrer-Orta, C., Rebollo, E., et al. (2021). Coupled Sterol Synthesis and Transport Machineries at ER-Endocytic Contact Sites. *J. Cell Biol.* 220 (10). doi:10.1083/jcb.202010016

Encinar del Dedo, J., Idrissi, F.-Z., Fernandez-Golbano, I. M., Garcia, P., Rebollo, E., Krzyzanowski, M. K., et al. (2017). ORP-mediated ER Contact with Endocytic Sites Facilitates Actin Polymerization. *Dev. Cell* 43 (5), 588–602. e6. doi:10.1016/j.devcel.2017.10.031

Ercan, B., Naito, T., Koh, D. H. Z., Dharmawan, D., and Saheki, Y. (2021). Molecular Basis of Accessible Plasma Membrane Cholesterol Recognition by the GRAM Domain of GRAMD1b. *Embo J.* 40 (6), e106524. doi:10.15252/embj.2020106524

Ferrari, A., He, C., Kennelly, J. P., Sandhu, J., Xiao, X., Chi, X., et al. (2020). Aster Proteins Regulate the Accessible Cholesterol Pool in the Plasma Membrane, *Mol. Cell Biol*, 40 e00255-20. doi:10.1128/MCB.00255-20

Friedman, J. R., DiBenedetto, J. R., West, M., Rowland, A. A., and Voeltz, G. K. (2013). Endoplasmic Reticulum-Endosome Contact Increases as Endosomes Traffic and Mature. *MBoC* 24 (7), 1030–1040. doi:10.1091/mbc.e12-10-0733

Ghai, R., Du, X., Wang, H., Dong, J., Ferguson, C., Brown, A. J., et al. (2017). ORP5 and ORP8 Bind Phosphatidylinositol-4, 5-Biphosphate (PtdIns(4,5)P 2) and Regulate its Level at the Plasma Membrane. *Nat. Commun.* 8 (1), 757. doi:10.1038/s41467-017-00861-5

Höglinger, D., Burgoyne, T., Sanchez-Heras, E., Hartwig, P., Colaco, A., Newton, J., et al. (2019). NPC1 Regulates ER Contacts with Endocytic Organelles to Mediate Cholesterol Egress. *Nat. Commun.* 10 (1), 4276. doi:10.1038/s41467-019-12152-2

Hoyer, M. J., Chitwood, P. J., Ebmeier, C. C., Striepen, J. F., Qi, R. Z., Old, W. M., et al. (2018). A Novel Class of ER Membrane Proteins Regulates ER-Associated Endosome Fission. *Cell* 175 (1), 254–265.e14. doi:10.1016/j.cell.2018.08.030

Ikonen, E. (2008). Cellular Cholesterol Trafficking and Compartmentalization. *Nat. Rev. Mol. Cell Biol* 9 (2), 125–138. doi:10.1038/nrm2336

Infante, R. E., Wang, M. L., Radhakrishnan, A., Kwon, H. J., Brown, M. S., and Goldstein, J. L. (2008). NPC2 Facilitates Bidirectional Transfer of Cholesterol between NPC1 and Lipid Bilayers, a Step in Cholesterol Egress from Lysosomes. *Proc. Natl. Acad. Sci.* 105 (40), 15287–15292. doi:10.1073/pnas.0807328105

Kaksonen, M., and Roux, A. (2018). Mechanisms of Clathrin-Mediated Endocytosis. *Nat. Rev. Mol. Cell Biol* 19 (5), 313–326. doi:10.1038/nrm.2017.132

Kanerva, K., Uronen, R.-L., Blom, T., Li, S., Bittman, R., Lappalainen, P., et al. (2013). LDL Cholesterol Recycles to the Plasma Membrane via a Rab8a-myosin5b-actin-dependent Membrane Transport Route. *Dev. Cell* 27 (3), 249–262. doi:10.1016/j.devcel.2013.09.016

Kawasaki, A., Sakai, A., Nakanishi, H., Hasegawa, J., Taguchi, T., Sasaki, J., et al. (2021). PI4P/PS Countertransport by ORP10 at ER-Endosome Membrane Contact Sites Regulates Endosome Fission. *J. Cell Biol.* 221 (1), e202103141. doi:10.1083/jcb.202103141

Kim, Y. J., Guzman-Hernandez, M.-L., Wisniewski, E., and Balla, T. (2015). Phosphatidylinositol-Phosphatidic Acid Exchange by Nir2 at ER-PM Contact Sites Maintains Phosphoinositide Signaling Competence. *Dev. Cell* 33 (5), 549–561. doi:10.1016/j.devcel.2015.04.028

Lees, J. A., Messa, M., Sun, E. W., Wheeler, H., Torta, F., Wenk, M. R., et al. (2017). Lipid Transport by TMEM24 at ER-Plasma Membrane Contacts Regulates Pulsatile Insulin Secretion. *Science* 355. doi:10.1126/science.aah6171

Lim, C.-Y., Davis, O. B. Hijai. R. Shin., Shin, H. R., Zhang, J., Berdan, C. A., Jiang, X., et al. (2019). ER-lysosome Contacts Enable Cholesterol Sensing by mTORC1 and Drive Aberrant Growth Signalling in Niemann-Pick Type C. *Nat. Cell Biol* 21 (10), 1206–1218. doi:10.1038/s41556-019-0391-5

Luo, J., Yang, H., and Song, B.-L. (2020). Mechanisms and Regulation of Cholesterol Homeostasis. *Nat. Rev. Mol. Cell Biol* 21 (4), 225–245. doi:10.1038/s41580-019-0190-7

Mach, François., Baigent, Colin., Catapano, Alberico. L., Koskinas, Konstantinos. C., Casula, Manuela., Badimon, Lina., et al. (2019). 2019 ESC/EAS Guidelines for the Management of Dyslipidaemias: Lipid Modification to Reduce Cardiovascular Risk. *Atherosclerosis* 290 (November), 140–205. doi:10.1016/j.atherosclerosis.2019.08.014

Maurer, M. E., and Cooper, J. A. (2006). The Adaptor Protein Dab2 Sorts LDL Receptors into Coated Pits Independently of AP-2 and ARH. *J. Cell Sci.* 119 (Pt 20), 4235–4246. doi:10.1242/jcs.03217

Mesmin, B., Antonny, B., and Drin, G. (2013a). Insights into the Mechanisms of Sterol Transport between Organelles. *Cell. Mol. Life Sci.* 70 (18), 3405–3421. doi:10.1007/s00018-012-1247-3

Mesmin, B., Bigay, J., Moser von Filseck, J., Lacas-Gervais, S., Drin, G., and Antonny, B. (2013b). A Four-step Cycle Driven by PI(4)P Hydrolysis Directs Sterol/PI(4)P Exchange by the ER-Golgi Tether OSBP. *Cell* 155 (4), 830–843. doi:10.1016/j.cell.2013.09.056

Mettlen, M., Loerke, D., Yarar, D., Danuser, G., and Schmid, S. L. (2010). Cargo- and Adaptor-specific Mechanisms Regulate Clathrin-Mediated Endocytosis. *J. Cell Biol.* 188 (6), 919–933. doi:10.1083/jcb.200908078

Naito, T., Ercan, B., Krshnan, L., Triebl, A., Koh, D. H. Z. Koh., Wei, F.-Y., et al. (2019). Movement of Accessible Plasma Membrane Cholesterol by the GRAMD1 Lipid Transfer Protein Complex. *ELife* 8 (November), e51401. doi:10.7554/eLife.51401

Neufeld, E. B., Cooney, A. M., Pitha, J., Dawidowicz, E. A., Dwyer, N. K., Pentchev, P. G., et al. (1996). Intracellular Trafficking of Cholesterol Monitored with a Cyclodextrin. *J. Biol. Chem.* 271 (35), 21604–21613. doi:10.1074/jbc.271.35.21604

Posor, Y., Eichhorn-Grünig, M., and Haucke, V. (2015). Phosphoinositides in Endocytosis. *Biochim. Biophys. Acta (Bba) - Mol. Cell Biol. Lipids* 1851 (6), 794–804. doi:10.1016/j.bbalip.2014.09.014

Rimbert, A., Dalila, N., Wolters, J. C., Huijkman, N., Smit, M., Kloosterhuis, N., et al. (2015). Biobank-Based Integrative Omics Studies Consortium, Frikke-Schmidt R, Burstein E, Tybjærg-Hansen A, van de Sluis B, Kuivenhoven JA. A Common Variant in CCDC93 Protects Against Myocardial Infarction and Cardiovascular Mortality by Regulating Endosomal Trafficking of Low-Density Lipoprotein Receptor. *Eur. Heart J.* 41 (9), 1040–1053. doi:10.1093/eurheartj/ehz727

Rocha, N., Kuijl, C., van der Kant, R., Janssen, L., Houben, D., Janssen, H., et al. (2009). Cholesterol Sensor ORP1L Contacts the ER Protein VAP to Control Rab7-RILP-p150Glued and Late Endosome Positioning. *J. Cell Biol.* 185 (7), 1209–1225. doi:10.1083/jcb.200811005

Rowland, A. A., Chitwood, P. J., Phillips, M. J., and Voeltz, G. K. (2014). ER Contact Sites Define the Position and Timing of Endosome Fission. *Cell* 159 (5), 1027–1041. doi:10.1016/j.cell.2014.10.023

Sandhu, J., Li, S., Fairall, L., Pfisterer, S. G., Gurnett, J. E., Xiao, X., et al. (2018). Aster Proteins Facilitate Nonvesicular Plasma Membrane to ER Cholesterol Transport in Mammalian Cells. *Cell* 175 (2), 514–529.e20. doi:10.1016/j.cell.2018.08.033

Sirinian, M. I., Belleudi, F., Campagna, F., Ceridono, M., Garofalo, T., Quagliarini, F., et al. (2005). Adaptor Protein ARH Is Recruited to the Plasma Membrane by Low Density Lipoprotein (LDL) Binding and Modulates Endocytosis of the LDL/LDL Receptor Complex in Hepatocytes. *J. Biol. Chem.* 280 (46), 38416–38423. doi:10.1074/jbc.M504343200

Sohn, M., Korzeniowski, M., Zewe, J. P., Wills, R. C., Hammond, G. R. V., Humpolickova, J., et al. (2018). PI(4,5)P2 Controls Plasma Membrane PI4P and PS Levels via ORP5/8 Recruitment to ER-PM Contact Sites. *J. Cell Biol.* 217 (5), 1797–1813. doi:10.1083/jcb.201710095

Stefano, G., Renna, L., Wormsbaecher, C., Gamble, J., Zienkiewicz, K., and Brandizzi, F. (2018). Plant Endocytosis Requires the ER Membrane-Anchored Proteins VAP27-1 and VAP27-3. *Cell Rep.* 23 (8), 2299–2307. doi:10.1016/j.celrep.2018.04.091

Stockinger, W. (2002). The PX-Domain Protein SNX17 Interacts with Members of the LDL Receptor Family and Modulates Endocytosis of the LDL Receptor. *EMBO J.* 21 (16), 4259–4267. doi:10.1093/emboj/cdf435

Surdo, P. L., Bottomley, M. J., Calzetta, A., Settembre, E. C., Cirillo, A., Pandit, S., et al. (2011). Mechanistic Implications for LDL Receptor Degradation from the PCSK9/LDLR Structure at Neutral PH. *EMBO Rep.* 12, 1300–1305. doi:10.1038/embor.2011.205

Takahashi, K., Kanerva, K., Vanharanta, L., Almeida-Souza, L., Lietha, D., Olkkonen, V. M., et al. (2021). ORP2 Couples LDL-cholesterol Transport to FAK Activation by Endosomal cholesterol/PI(4,5)P 2 Exchange. *Embo J.* 40 (14), e106871. doi:10.15252/embj.2020106871

Trinder, M., Francis, G. A., and Brunham, L. R. (2020). Association of Monogenic vs Polygenic Hypercholesterolemia with Risk of Atherosclerotic Cardiovascular Disease. *JAMA Cardiol.* 5 (4), 390–399. doi:10.1001/jamacardio.2019.5954

Wijers, M., Kuivenhoven, J. A., and van de Sluis, B. (2015). The Life Cycle of the Low-Density Lipoprotein Receptor. *Curr. Opin. Lipidol.* 26 (2), 82–87. doi:10.1097/MOL.0000000000000157

Wijers, M., Zanoni, P., Liv, N., Vos, D. Y., Jäckstein, M. Y., Smit, M., et al. (2019). The Hepatic WASH Complex is Required for Efficient Plasma LDL and HDL Cholesterol Clearance. *JCI Insight* 4 (11), e126462. doi:10.1172/jci.insight.126462

Wilhelm, L. P., Wendling, C., Védie, B., Kobayashi, T., Chenard, M. P., Tomasetto, C., et al. (2017). STARD 3 Mediates Endoplasmic Reticulum-to-endosome Cholesterol Transport at Membrane Contact sitesSTARD3 Mediates Endoplasmic Reticulum-to-endosome Cholesterol Transport at Membrane Contact Sites. *Embo J.* 36 (10), 1412–1433. doi:10.15252/embj.201695917

Zelcer, N., Hong, C., Boyadjian, R., and Tontonoz, P. (2009). LXR Regulates Cholesterol Uptake through Idol-dependent Ubiquitination of the LDL Receptor. *Science* 325 (5936), 100–104. doi:10.1126/science.1168974

Zhang, D.-W., Lagace, T. A., Garuti, R., Zhao, Z., McDonald, M., Horton, J. D., et al. (2007). Binding of Proprotein Convertase Subtilisin/Kexin Type 9 to Epidermal Growth Factor-like Repeat A of Low Density Lipoprotein Receptor Decreases Receptor Recycling and Increases Degradation. *J. Biol. Chem.* 282 (25), 18602–18612. doi:10.1074/jbc.M702027200

Zhao, K., and Ridgway, N. D. (2017). Oxysterol-Binding Protein-Related Protein 1L Regulates Cholesterol Egress from the Endo-Lysosomal System. *Cell Rep.* 19 (9), 1807–1818. doi:10.1016/j.celrep.2017.05.028

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frontiers in Cell and Developmental Biology

BRIEF RESEARCH REPORT
published: 17 February 2022
doi: 10.3389/fcell.2022.842285

Yeast Vps13 is Crucial for Peroxisome Expansion in Cells With Reduced Peroxisome-ER Contact Sites

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In the yeast *Hansenula polymorpha* the peroxisomal membrane protein Pex11 and three endoplasmic reticulum localized proteins of the Pex23 family (Pex23, Pex24 and Pex32) are involved in the formation of peroxisome-ER contact sites. Previous studies suggested that these contacts are involved in non-vesicular lipid transfer and important for expansion of the peroxisomal membrane. The absence of Pex32 results in a severe peroxisomal phenotype, while cells lacking Pex11, Pex23 or Pex24 show milder defects and still are capable to form peroxisomes and grow on methanol. We performed transposon mutagenesis on *H. polymorpha pex11* cells and selected mutants that lost the capacity to grow on methanol and are severely blocked in peroxisome formation. This strategy resulted in the identification of Vps13, a highly conserved contact site protein involved in bulk lipid transfer. Our data show that peroxisome formation and function is normal in cells of a *vps13* single deletion strain. However, Vps13 is essential for peroxisome biogenesis in *pex11.* Notably, Vps13 is also required for peroxisome formation in *pex23* and *pex24* cells. These data suggest that Vps13 is crucial for peroxisome formation in cells with reduced peroxisome-endoplasmic reticulum contact sites and plays a redundant function in lipid transfer from the ER to peroxisomes.

Keywords: peroxisome, yeast, contact site, VPS13, endoplasmic reticulum

OPEN ACCESS

Edited by:
Michael Schrader,
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Reviewed by:
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *23 December 2021*
Accepted: *28 January 2022*
Published: *17 February 2022*

Citation:
Yuan W, Akşit A, de Boer R, Krikken AM and van der Klei IJ (2022) Yeast Vps13 is Crucial for Peroxisome Expansion in Cells With Reduced Peroxisome-ER Contact Sites. Front. Cell Dev. Biol. 10:842285. doi: 10.3389/fcell.2022.842285

INTRODUCTION

Peroxisomes are ubiquitous organelles. Their function and abundance continuously changes in response to cellular needs (Smith and Aitchison, 2013). During peroxisome growth, the organelles incorporate matrix and membrane proteins a well as membrane lipids. In mammals, membrane contact sites (MCSs) between peroxisomes and the endoplasmic reticulum (ER) function in non-vesicular transport of lipids from the ER to the peroxisomal membrane. These MCSs contain peroxisome bound members of the Acyl-CoA binding domain containing proteins (ACBDs), ACBD5 and ACBD4, and the ER-localized VAP proteins VAPA and VAPB. VAP proteins are highly conserved ER membrane proteins that play a role in various processes, including lipid transport (Lev et al., 2008). At mammalian peroxisome-ER contact sites ACBD5/ACBD4 interact through a FFAT-like motif with both VAP proteins (Costello et al., 2017; Hua et al., 2017; Islinger et al., 2020). Recent studies showed that human VPS13D, a bulk lipid transporter, is also important for peroxisome biogenesis and transport of lipids from the ER to peroxisomes (Baldwin et al., 2021; Guillen-Samander et al., 2021).

Studies in *Saccharomyces cerevisiae* revealed that also in this organism the peroxisomal membrane can receive membrane lipids via non-vesicular transport (Raychaudhuri and Prinz, 2008). These

lipids may derive from various sources including the ER, the vacuole, the mitochondrion and the Golgi apparatus (Rosenberger et al., 2009; Flis et al., 2015). Also, in *S. cerevisiae* peroxisomes form contact sites with many other cellular membranes (Shai et al., 2016; Shai et al., 2018). However, proteins involved in non-vesicular lipid transport to yeast peroxisomes have not been identified yet. In the yeast *Hansenula polymorpha* peroxisomes can form various MCSs. Contacts have been described with the plasma membrane, the ER, mitochondria and vacuoles (Wu et al., 2019). ER-localized peroxins of the Pex23 family (Pex23, Pex24 and Pex32) together with the peroxisomal membrane protein (PMP) Pex11 play a role in the formation of peroxisome-ER contacts (Wu et al., 2020). Similarly, members of the *S. cerevisiae* Pex23 family (called Pex28, Pex29, Pex30, Pex31 and Pex32 (Jansen et al., 2021)) have been implicated in the formation of peroxisome ER contact sites (David et al., 2013; Mast et al., 2016). *S. cerevisiae* Pex23 family proteins also have been implicated in other processes, such as the regulation of pre-peroxisomal vesicle (PPV) formation from the ER (David et al., 2013; Joshi et al., 2016; Mast et al., 2016; Wang et al., 2018) and the biogenesis of lipid bodies (Joshi et al., 2018). Studies in *S. cerevisiae* suggested that Inp1, a protein essential for retention of peroxisomes in yeast mother cells, plays a role in the formation of peroxisome-ER contact sites (Knoblach et al., 2013). However, recent studies showed that Inp1 associates peroxisomes to the plasma membrane (Hulmes et al., 2020; Krikken et al., 2020).

The absence of *H. polymorpha* Pex23, Pex24 or Pex32 leads to reduction, but not a complete loss, of peroxisome-ER contacts. This reduction is accompanied by a decrease in the cellular peroxisomal membrane surface, suggesting that these peroxisome-ER contacts are important for lipid transfer.

The absence of the *H. polymorpha* Pex32 causes the most severe peroxisome-ER MCS defect, which is accompanied by mislocalization of a portion of the peroxisomal matrix proteins to the cytosol. As a consequence *pex32* cells are unable to grown on media containing methanol as sole carbon source (van der Klei et al., 2006). *pex23, pex24* and *pex11* cells show milder peroxisomal defects and still are capable to grow on methanol, although the doubling times are increased (Krikken et al., 2009; Wu et al., 2020).

We hypothesized that these weaker phenotypes are due to functional redundancy of proteins of the peroxisome-ER MCS. To identify these redundant proteins, we performed transposon mutagenesis of *H. polymorpha pex11* cells and selected mutants that fully lost the capacity to grow on methanol. This screen resulted in the identification of Vps13, a highly conserved protein that is responsible for lipid transport and localizes to multiple MCSs in eukaryotic cells. We show that like cells of the *pex11 vps13* double deletion strain, also *pex23 vps13* and *pex24 vps13* cells are unable to utilize methanol. In these double deletion strains very small peroxisomes still occur. PMPs are normally sorted to these organelles, but the bulk of the matrix proteins mislocalize to the cytosol. This suggests that peroxisomes can still form but are unable to grow and incorporate all matrix proteins. Cells of a single *vps13* deletion strain contain normal peroxisomes and grow on methanol like wild type control cells.

These data indicate that Vps13 is essential for peroxisome growth in cells that have reduced peroxisome-ER MCSs.

MATERIALS AND METHODS

Strains and Growth Conditions

The H. polymorpha and *S. cerevisiae* strains used in this study are listed in the **Supplementary Table S1and S2**. *H. polymorpha* cells were grown in batch cultures at 37°C on mineral media (MM) (van Dijken et al., 1976) containing 0.5% glucose, 0.5% methanol or a mixture of 0.5% methanol and 0.05% glycerol (MM-M/G) as carbon sources and 0.25% ammonium sulfate as nitrogen sources. *S. cerevisiae* cells were grown at 30°C on media containing 0.5% glucose and 0.25% ammonium sulfate. When required, amino acids were added to the media to a final concentration of 30 μg/ml. *Escherichia coli* DH5α and DB3.1 were used for cloning.

Plasmids and Molecular Techniques

GFP-SKL and DsRed-SKL are peroxisomal matrix markers appended with the peroxisomal targeting signal -SKL. In *H. polymorpha* the encoding genes were expressed under control of the *TEF1* or *AOX* promoter, in *S. cerevisiae* under control of the *MET25* promoter. For expression of *H. polymorpha* genes encoding various peroxisomal membrane proteins under control of their endogenous promoter, approximately 500 nucleotides upstream from the ORF were included. For the expression under control of a strong promoter the full length gene was cloned. All plasmids were linearized and integrated in the genome as described before (Faber et al., 1994). Plasmids used in this study are listed in **Supplementary Table S3**. All deletions were confirmed by Southern blotting. For DNA and amino acid sequence analysis, the Clone Manager 5 program (Scientific and Educational Software, Durham, NC) was used. Transposon mutagenesis of *H. polymorpha pex11* DsRed-SKL, isolation of total genomic DNA and sequencing of genomic insert was performed as described before (van Dijk et al., 2001).

DNA restriction enzymes were used as recommended by the suppliers (Thermo Scientific or New England Biolabs). Polymerase chain reactions (PCR) for cloning were carried out with Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Colony PCR was carried out using Phire polymerase (Thermo Scientific). For DNA and amino acid sequence analysis, the Clone Manager 5 program (Scientific and Educational Software, Durham, NC) was used.

Microscopy

Fluorescence microscopy images were captured using an Axio Scope A1 (Carl Zeiss) with a 100 × 1.30 NA Plan Neofluar objective, Micro-Manager 1.4 software and a Coolsnap HQ2 camera (Photometrics). GFP was visualized with a 470/40 nm band pass excitation filter, a 495 nm dichromatic mirror, and a 525/50 nm band-pass emission filter. mCherry was visualized with a 587/25 nm band pass excitation filter, a 605 nm dichromatic mirror, and a 647/70 nm band-pass emission

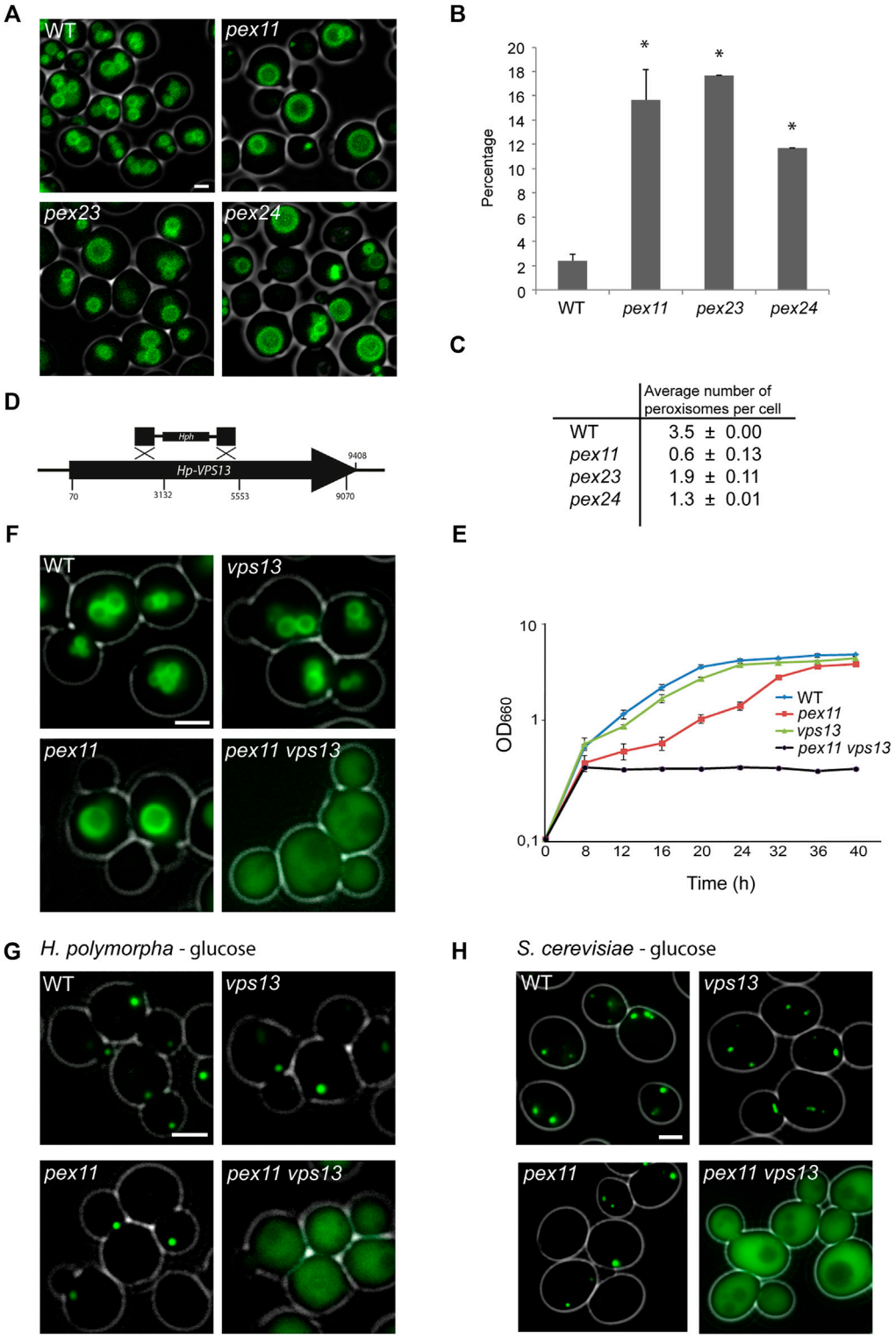

FIGURE 1 | *VPS13* is required for peroxisome formation in *pex11* cells. *H. polymorpha* cells were grown for 16 h on methanol medium unless indicated otherwise. **(A)** FM analysis of the indicated *H. polymorpha* strains producing the peroxisomal membrane marker PMP47-GFP. Scale bar 2 µm. **(B)** Percentage of peroxisomes with a diameter >1 µm. 2 × 660 peroxisomes from two independent cultures were quantified. Two-tailed Student's t test was performed. *, $p < 0.05$. *pex11*: 0.016, *pex23*: 0.003, *pex24*: 0.005. The error bars represent standard deviation (SD). **(C)** Average number of peroxisomes per cell (±SD) of the indicated strains. 2 × 660 cells from two independent cultures were quantified. **(D)** Schematic representation of the *H. polymorpha VPS13* gene, showing the four positions where transposon insertion

(*Continued*)

FIGURE 1 | occurred as well as the region that was replaced (nt 2430–5436) by Zeo^r to disrupt *VPS13*. **(E)** Growth curves of the indicated *H. polymorpha* strains on methanol medium. Error bars represent SD ($n = 2$). **(F)** FM images of the indicated *H. polymorpha* strains producing GFP-SKL. Cells were grown for 16 h on medium containing a mixture of methanol and glycerol. Scale bar: 2 µm. **(G)** FM images of the indicated strains grown on glucose medium (*pex11*.P_{TEF}GFP-SKL, *vps13*P_{TEF}GFP-SKL and *pex11 vps13*P_{TEF}GFP-SKL). **(H)** FM analysis of the indicated *S. cerevisiae* strains producing GFP-SKL and grown on glucose. Scale bars: 2 µm.

filter. DsRed fluorescence was visualized with a 546/12 nm bandpass excitation filter, a 560 nm dichromatic mirror, and a 575–640 nm bandpass emission filter. Image analysis was carried out using ImageJ and Adobe Photoshop CS6 software.

To quantify peroxisomes random images of cells were taken as a stack using a confocal microscope (LSM510, Carl Zeiss) and photomultiplier tubes (Hamamatsu Photonics) and Zen 2009 software (Carl Zeiss). Z-Stack images were made containing 14 optical slices and the GFP signal was visualized by excitation with a 488 nm argon ion laser (Lasos), and a 500–550 nm bandpass emission filter. Peroxisomes were quantified using a custom made plugin for ImageJ (Thomas et al., 2015).

Electron microscopy was performed as described previously (Knoops et al., 2014). For morphological analysis cells were fixed in 1.5% potassium permanganate, post-stained with 0.5% uranyl acetate and embedded in Epon. Immuno-EM was performed as described previously (Knoops et al., 2014) using anti-Pex14 antibodies (Komori et al., 1997) followed by goat-anti-mouse antibodies conjugated to 6 nm gold (Aurion, Netherlands). Ultrathin sections were viewed in a Philips CM12 TEM.

RESULTS

Vps13 is Required for Peroxisome Biogenesis in Yeast *pex11* Cells

Previously, we showed that like *H. polymorpha pex11*, *pex23* and *pex24* cells still contain peroxisomes (Krikken et al., 2009; Wu et al., 2020). However, peroxisome numbers are reduced accompanied by the occurrence of organelles with a relatively large diameter. We confirmed these observations by quantitative analysis of fluorescence microscopy (FM) images of these three deletion strains. The percentage of relatively large peroxisomes (diameter >1 µm) is significantly enhanced in *pex11, pex23* and *pex24* cells (**Figures 1A,B**). The lowest peroxisome numbers were observed in *pex11* cells (**Figures 1A,C**), which we selected to perform transposon mutagenesis.

Transformants were isolated that were still capable to grow on glucose, but not on methanol (Mut$^-$), indicative for severe peroxisome biogenesis defects. FM analysis revealed that out of the 100 Mut$^-$ strains obtained, 42 displayed mislocalization of the red fluorescent peroxisomal matrix marker DsRed-SKL. Sequencing of the genomic regions flanking the integrated pREMI-Z cassette resulted in the identification of 17 different genes (**Supplementary Table S4**). As expected these included various *PEX* genes, because mutations in most *PEX* genes result in a Mut$^-$ phenotype due to mislocalization of matrix proteins. In 9 of the 17 identified mutants the transposon was integrated in *VPS13*. In four mutants the transposon was integrated at different positions in the *VPS13* open reading frame, whereas in the remaining five mutants deletions or truncations of the *VPS13* gene occurred (**Figure 1D**).

To validate this result, we constructed a *pex11 vps13* double mutant (**Figure 1D**). Cells of this strain, but not of the *pex11* or *vps13* single deletion strains, were unable to grow on methanol (**Figure 1E**). Moreover, peroxisomal matrix proteins were mislocalized to the cytosol of cells in the *pex11 vps13* double deletion strain, but not in *pex11* or *vps13* cells. This phenotype was observed both when cells were grown in peroxisome inducing media (methanol; **Figure 1F**) or peroxisome repressing media (glucose; **Figure 1G**).

In *S. cerevisiae* essentially the same observations were made: the peroxisome matrix marker GFP-SKL was properly imported into peroxisomes of *pex11* and *vps13* cells, but mislocalized to the cytosol in *pex11 vps13* double deletion cells (**Figure 1H**).

Summarizing, in *H. polymorpha* and *S. cerevisiae* deletion of *VPS13* in a *pex11* deletion strain strongly affects peroxisome biogenesis.

pex11 vps13 Cells Contain Small, Import Competent Peroxisomes

Electron microscopy (EM) revealed that *H. polymorpha pex11 vps13* cells harbor clusters of very small peroxisomes (**Figures 2AI,II**), which contain the peroxisomal membrane marker Pex14 (**Figure 2AIII**). In the larger organelles small alcohol oxidase (AO) crystalloids could be observed, indicating that these peroxisomes still are capable of importing of matrix protein (**Figure 2AIII**, inset). The bulk of the AO protein mislocalizes to the cytosol, as evident from the large AO crystalloids in the cytosol of the double mutant (**Figure 2AIV**). Fluorescence microscopy showed that Pex14-mCherry localized in spots in *pex11 vps13* cells, which most likely represent the clusters of small peroxisomes observed by EM (**Figure 2B**). All other PMPs, C-terminally tagged with GFP and produced under the control of their endogenous promoters, co-localized with Pex14-mCherry (**Figure 2B**). Based on these data we conclude that PMPs normally localize to the membranes of the small peroxisomes in *pex11 vps13* cells. The small peroxisomes are competent to import a minor fraction of the matrix proteins, but the bulk of the matrix proteins mislocalizes to the cytosol.

VPS13 is also Required for Peroxisome Biogenesis in *H. polymorpha pex23* and *pex24* Cells

Next, we analyzed whether deletion of *VPS13* also affects peroxisome biogenesis in *pex23* and *pex24* cells, two other mutants in which peroxisome-ER MCSs are reduced (Wu et al., 2020). As shown in **Figure 3A**, peroxisomal matrix

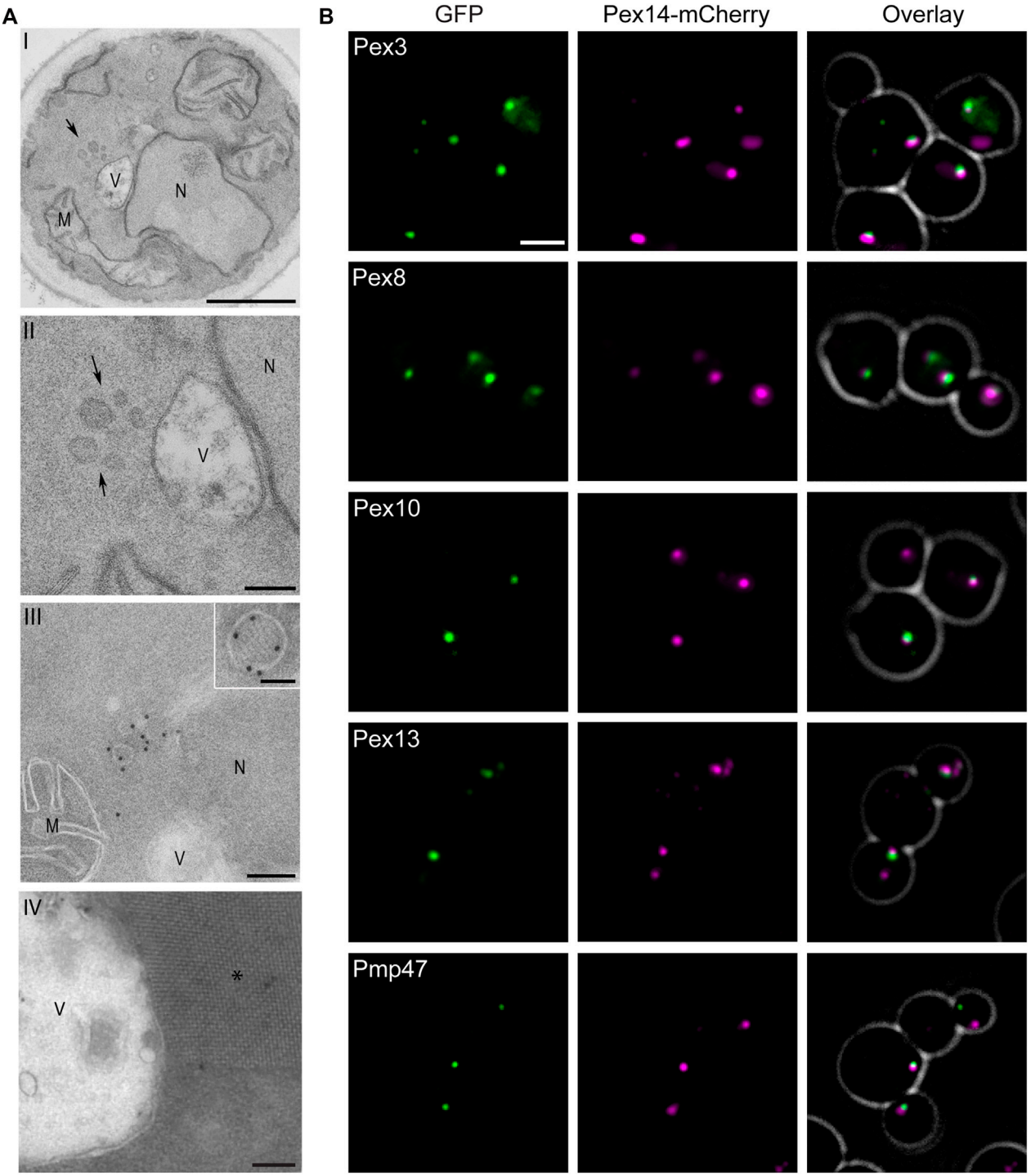

FIGURE 2 | *H. polymorpha pex11 vps13* cells contain small peroxisomes. **(A)** Electron microscopy analysis of thin sections of $KMnO_4$-fixed *pex11 vps13* cells grown for 8 h on a mixture of glycerol and methanol. Cells contain clusters of small peroxisomes (arrows). I—overview, II—magnification. III—Immunolabelling of cryosection of *pex11 vps13* cells using anti-Pex14 antibodies. The inset shows a small peroxisome labelled with anti-Pex14 antibodies, containing an alcohol oxidase crystalloid. IV—Cryosection showing a large, cytosolic alchol oxidase crystalloid. Scale bars: I: 500 nm, II: 100 nm, III 100 nm, inset: 50 nm, IV—100 nm. M-mitochondrion; N—nucleus, V-vacuole. **(B)** FM images of *pex11 vps13* cells producing Pex14-mCherry together with the indicated mGFP fusion proteins, all produced under control of the endogenous promoters. Cells were grown for 8 h on glycerol/methanol medium. Cells producing Pex10-GFP were grown for 4 h on glycerol/methanol. Scale bar: 2 µm.

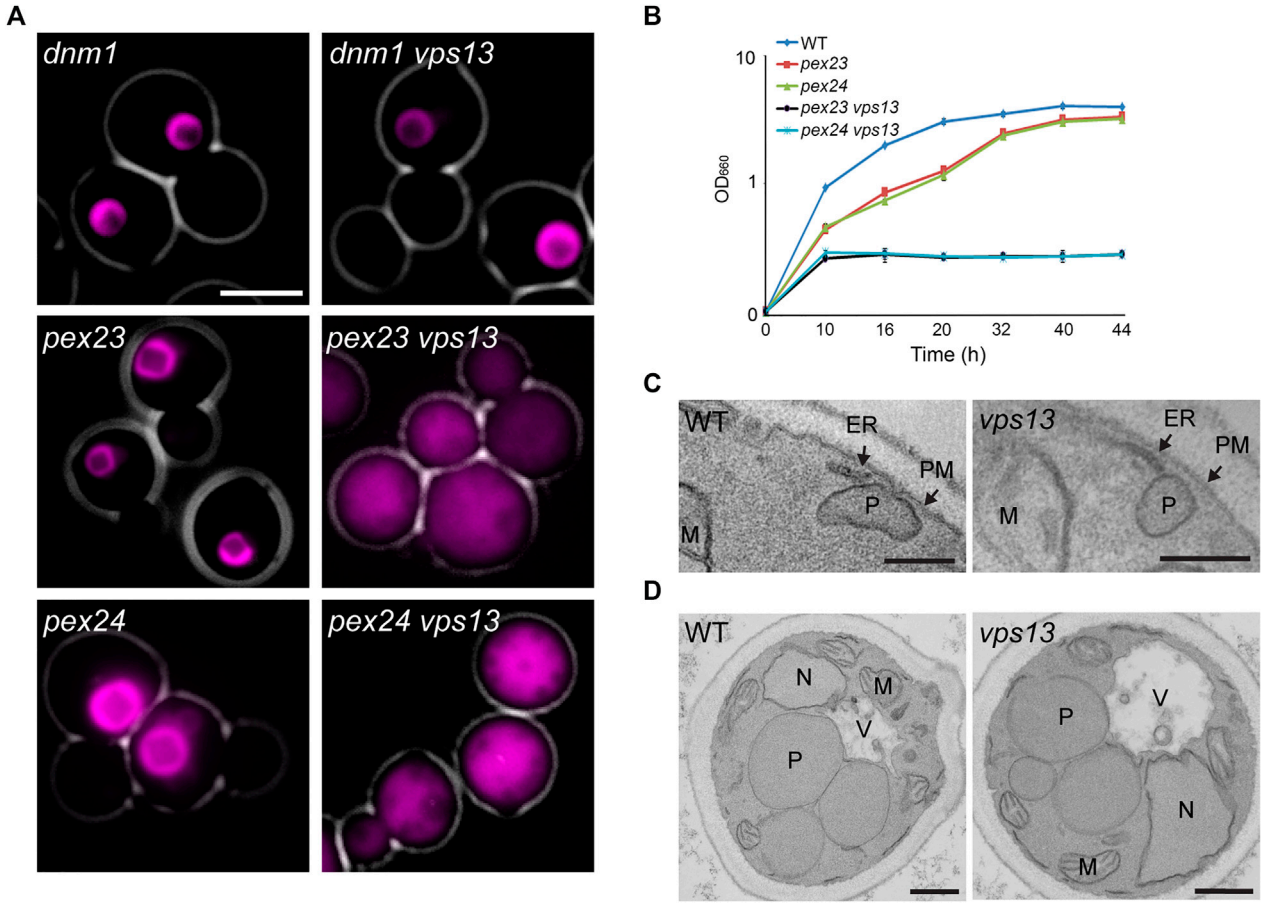

FIGURE 3 | *H. polymorpha pex23 vps13* and *pex24 vps13* cells show a severe peroxisome biogenesis defect. **(A)** FM analysis of the indicated strains producing DsRed-SKL or GFP-SKL, grown for 16 h on a mixture of glycerol and methanol. Scale bar: 2 µm. **(B)** Growth curves of the indicated strains on medium containing methanol. The optical density is expressed as OD_{660}. Error bars represent SD ($n = 2$). **(C,D)** Electron microscopy analysis of KMnO4 fixed WT and *vps13* cells grown on glucose **(C)** or methanol **(D)**. Like the WT controls *vps13* cells still contain peroxisome-ER, peroxisome-plasma membrane (PM), peroxisome-mitochondrion (M) and peroxisome-vacuole (V) contacts. Scale bar in C 200 nm, size bar in D 500 nm. N—nucleus.

markers mislocalized to the cytosol in *pex23 vps13* and *pex24 vps13* cells. In line with these observations, both double deletion strains are unable to grow on methanol, while *pex23* and *pex24* single deletion strains grow on methanol (**Figure 3B**).

The decrease in peroxisome numbers in *pex11*, *pex23* and *pex24* cells is related to reduced ER-peroxisome contacts (Wu et al., 2020). In *H. polymorpha dnm1* cells peroxisome abundance is also decreased, but not due to reduced contacts but caused by a block in peroxisome fission (Nagotu et al., 2008). Hence, we reasoned that deletion of *VPS13* in *dnm1* cells should not lead to enhanced peroxisome biogenesis defects. Indeed, as shown in **Figure 3A**, the peroxisome phenotypes of *dnm1* and *dnm1 vps13* cells are comparable. This observation supports the redundant function of Vps13 with peroxisome-ER contacts. Peroxisome-ER contacts still occur in *vps13* cells (**Figure 3C**). Also, in the absence of Vps13 all other peroxisomal contacts that were identified in *H. polymorpha* wild-type cells are normally present (peroxisome-plasmamembrane, **Figure 3C**; peroxisome-mitochondrion (M) and peroxisome-vacuole (V), **Figure 3D**).

An Artificial Peroxisome-ER Tether Suppresses the *pex11 vps13*, *pex23 vps13* and *pex24 vps13* Phenotypes

The peroxisomal defects that occur in *H. polymorpha pex23* and *pex24* cells are largely suppressed upon introduction of an artificial peroxisome-ER tether (Wu et al., 2020). This tether consists of full-length Pex14 and the tail anchor of the ER protein Ubc6, separated by two heme-agglutinin tags. Deletion of *VPS13* enhances the peroxisomal defects in *pex11, pex23* or *pex24* cells (**Figure 1**). Introduction of the artificial peroxisome-ER tether resulted in partical suppression of the severe peroxisomal phenotypes of the *pex11 vps13*, *pex23 vps13* and *pex24 vps13* double deletion strains (**Figures 4A,B**). This was not observed in control strains in which Pex14, which is part of the artificial tether, was overproduced (Pex14++; **Figures 4A,B**). These data suggest that upon artificially enhancing peroxisome-ER contacts again, Vps13 becomes less important for peroxisome biogenesis in the three indicated double deletion strains.

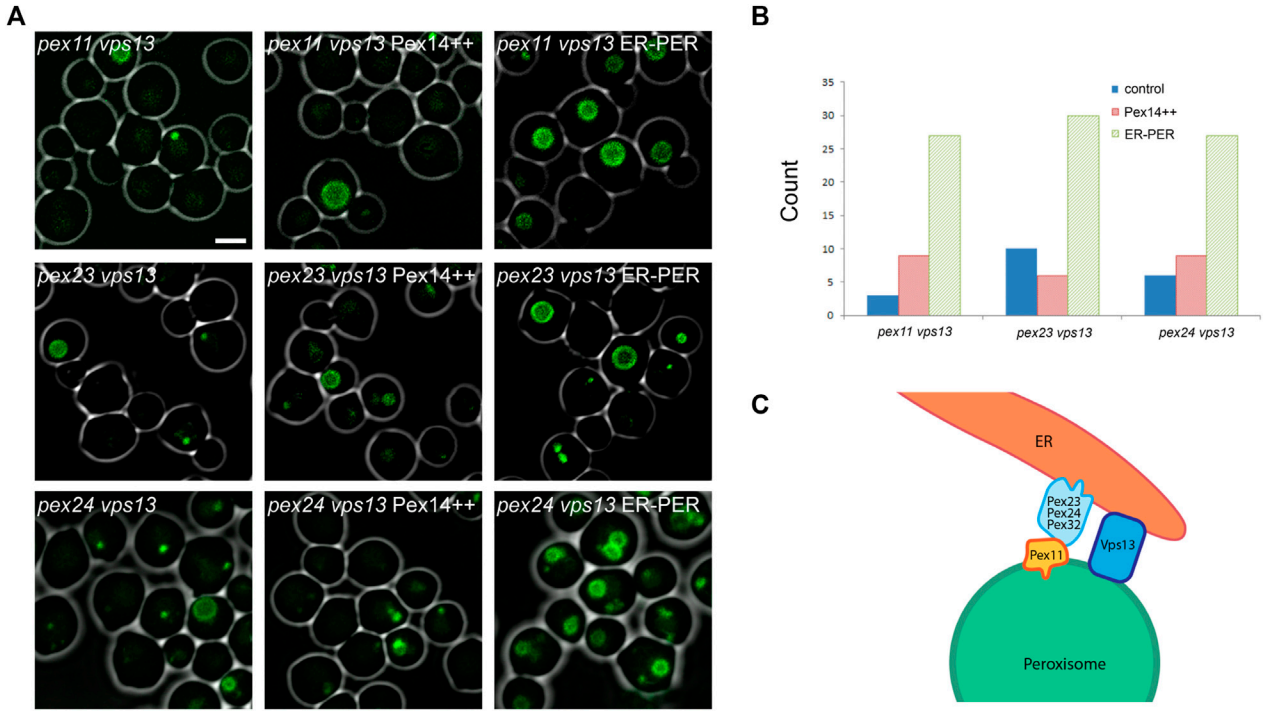

FIGURE 4 | Suppression of peroxisome biogenesis defects by an artificial ER-peroxisome tether. **(A)** FM analysis of methanol-glycerol grown *pex11 vps13*, *pex23 vps13* and *pex24 vps13* cells producing P_{PMP47}*PMP47-GFP* alone or in combination with P_{ADH1}*PEX14* (Pex14++) or P_{ADH1}*PEX14-HAHA-UBC6*^{TA} (ER-PER). Scale bar: 2 µm. **(B)** Quantitative analysis of the presence of peroxisomes in thin sections of methanol-glycerol grown cells of the indicated strains. Thin sections of $KMnO_4$ fixed cells were analyzed by electron microscopy. The number of cell sections containing one or more peroxisomes with a diameter >200 nm were counted. 100 cell sections were analyzed per strain (n = 100). **(C)** Hypothetical model of the peroxisome-ER contact site in *H. polymorpha*, with the currently known components.

DISCUSSION

Here we identified *VPS13* as a gene required for peroxisome biogenesis in *H. polymorpha* cells with disturbed peroxisome-ER contacts. Previous data suggested that these contacts are involved in lipid transfer from the ER to peroxisomes to allow membrane expansion (Wu et al., 2020). This is in line with observations in *S. cerevisiae* indicating that the peroxisomal membrane can receive membrane lipids via non-vesicular transport (Raychaudhuri and Prinz, 2008) and that the ER is one of the lipid sources (Rosenberger et al., 2009; Flis et al., 2015).

Cells of the *H. polymorpha pex11, pex23* and *pex24* single deletion strains still contain peroxisomes, but show reduced growth on methanol media, due to a partial defect in peroxisome function (Wu et al., 2020). We now show that deletion of *VPS13* in each of these mutants results in a complete defect in methanol growth, accompanied by much more severe peroxisome biogenesis defects, including mislocalization of the bulk of the peroxisomal matrix enzymes in the cytosol (**Figures 1E,F**; **Figures 3A,B**). The latter is unlikely to be caused by defects in the importomer. First, peroxisomal membrane proteins, including proteins of the importomer, are still normally sorted to peroxisomes (**Figure 2B**). Second, peroxisomes still can incorporate a minor portion of the matrix proteins (**Figure 2A**). Third, the matrix protein import defect of the double mutants is largely restored by the introduction of an artificial ER-peroxisome tethering protein (**Figure 4**). Hence, the matrix protein import defect is most likely an indirect effect of the inability of the peroxisomes to sufficiently expand as a result of reduced membrane lipid supply.

Like for *H. polymorpha* also the *S. cerevisiae pex11 vps13* double mutant, but not the *pex11* and *vps13* single deletion strains, show severe peroxisome biogenesis defects, indicating that a role for Vps13 in peroxisome biogenesis is conserved in yeast (**Figure 1G**).

Vps13 was initially characterized as an *S. cerevisiae* protein involved in vacuolar protein sorting (Bankaitis et al., 1986). Later studies indicated that ScVps13 is required for many other processes, which all probably relate to a function in lipid transport. Vps13 is a very large (>300 kDa), highly conserved protein. In yeast, there is a single *VPS13* gene, while mammals contain four VPS13 isoforms: VPS13A-D. Protein structure analysis and biochemical studies revealed the presence of a large hydrophilic groove, which can bind and transfer a variety of glycerophospholipids. Most likely Vps13 is responsible for bulk transport of lipids between membranes, because its hydrophilic groove can bind multiple lipids at once (Leonzino et al., 2021).

H. polymorpha Vps13 is very similar to *S. cerevisiae* Vps13 (sequence identity 40%). Also, their length is similar (3135 and 3144 residues, respectively). Like the four human Vps13 homologues and *S. cerevisiae* Vps13, *H. polymorpha* Vps13

contains a chorein domain at the N-terminus and a VAB domain folllowed by an APT1, ATG2_C and plekstrin homology domain at the C-terminus (Dziurdzik and Conibear, 2021). *H. polymorpha* Vps13 has 23% identity with each of the four human Vps13 proteins.

S. cerevisiae Vps13 localizes to multiple MCSs, including the Nuclear Vacuolar Junction (NVJ), the vacuole-mitochondria patch (vCLAMP) and endosome-mitochondrial contact sites (Dziurdzik and Conibear, 2021). In addition, *S. cerevisiae* Vps13 localizes to prospore membranes (Park and Neiman, 2012), the Golgi apparatus (Kolakowski et al., 2021) and peroxisomes (John Peter et al., 2017). Most likely *H. polymorpha* Vps13 also localizes to multiple organelles. Human VPS13 isoforms also localize to different MCSs and cell organelles (Dziurdzik and Conibear, 2021). Moreover, recent studies showed that human VPS13D plays a role in peroxisome formation (Baldwin et al., 2021) and localizes to these organelles (Guillen-Samander et al., 2021).

VPS13D associates to both peroxisomes and mitochondria via Miro proteins that have a dual localization at both organelles. In addition, VPS13D associates to ER localized VAP proteins. In this way VPS13D connects the ER to peroxisomes and mitochondria to mediate bulk lipid transport (Guillen-Samander et al., 2021).

We generated a functional, internally GFP-tagged *H. polymorpha* Vps13 variant, but unfortunately the fluorescence levels were too low to determine its localization. However, given the peroxisomal localization of *S. cerevisiae* Vps13 and human VPS13D it is very likely that *H. polymorpha* Vps13 localizes to peroxisomes as well.

We were unable to detect a peroxisomal phenotype of *H. polymorpha* or *S. cerevisiae vps13* cells. Moreover, electron microscopy revealed that all described peroxisomal MCSs still occur in *H. polymorpha vps13* cells (**Figure 3C**). Similarly, Baldwin and others reported that *S. cerevisiae vps13* cells show no peroxisomal defects (Baldwin et al., 2021). This observation indicates that yeast Vps13 is redundant for peroxisome formation, but becomes important for lipid transport to peroxisomes, when the peroxisome-ER MCS formed by Pex23, Pex24, Pex32 and Pex11 are disturbed (**Figure 4C**). The defects were not only observed in methanol-grown *pex11 vps13* cells, but also when cells were grown on glucose (**Figure 1C**). In these cells peroxisomes mostly form contacts with the plasma-membrane and the ER (Wu et al., 2019). The contacts with the plasma membrane function in peroxisome retention (Krikken et al., 2020). Therefore Vps13 possibly performs its redundant function at peroxisome-ER contacts (**Figure 4C**). However, given the ambiguity of Vps13 function, other models cannot be excluded yet.

Further studies are required to understand the molecular mechanisms of Vps13 function in peroxisome formation. For instance, it would be important to know whether Vps13 plays a role in lipid transport, membrane tethering or both. This could be achieved by the introduction of mutations that affect lipid transfer in Vps13. Such mutations have been reported for *S. cerevisiae* Vps13 (Li et al., 2020). Hence, it would be interesting to learn whether corresponding mutations in *H. polymorpha* Vps13 abolish peroxisome formation in *pex11, pex23* and *pex24* cells. Our data indicate that *H. polymorpha* Vps13 functions together with Pex23 and Pex24 in peroxisomal membrane expansion. Like other protein of the Pex23 family, Pex23 and Pex24 contain a dysferlin (DysF) domain (Jansen et al., 2021). DysF was first identified in dysferlin, a human protein important for membrane repair of the sarcolemma at the site of muscle injury. The function of this domain is still unknown (Bulankina and Thoms, 2020). Interestingly, *S. cerevisiae* Spo73, another yeast DysF domain containing protein, also functions together with Vps13. Spo73 is required for extension of the prospore membrane and interacts with Spo71, which recruits Vps13 to the prospore membrane (Parodi et al., 2015; Okumura et al., 2016). It is tempting to speculate that members of the Pex23 family and Vps13 are components of a protein complex at peroxisome-ER contacts together with a yet unknown Vps13 recruiting protein at the peroxisomal membrane. Possibly the DysF domain is involved in the formation of this complex.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

WY, AA, RB, AK, and IK conceived the project, performed the experiments, analyzed the data and prepared the figures. WY, AA, and IK wrote the original draft. All contributed to reviewing and editing the manuscript.

FUNDING

This work was supported by grants from Netherlands Organization for Scientific Research/Chemical Sciences (NWO/CW) to AA (711.012.002), the CHINA SCHOLARSHIP COUNCIL to WY.

ACKNOWLEDGMENTS

We acknowledge the contributions of Srishti Devarajan for assistance in strain construction and of Anita Kram for performing the immunolabeling experiments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.842285/full#supplementary-material

REFERENCES

Baldwin, H. A., Wang, C., Kanfer, G., Shah, H. V., Velayos-Baeza, A., Dulovic-Mahlow, M., et al. (2021). VPS13D Promotes Peroxisome Biogenesis. *J. Cel Biol* 220, e202001188. doi:10.1083/jcb.202001188

Bankaitis, V. A., Johnson, L. M., and Emr, S. D. (1986). Isolation of Yeast Mutants Defective in Protein Targeting to the Vacuole. *Proc. Natl. Acad. Sci.* 83, 9075–9079. doi:10.1073/pnas.83.23.9075

Bulankina, A. V., and Thoms, S. (2020). Functions of Vertebrate Ferlins. *Cells* 9, e9030534. doi:10.3390/cells9030534

Costello, J. L., Castro, I. G., Hacker, C., Schrader, T. A., Metz, J., Zeuschner, D., et al. (2017). ACBD5 and VAPB Mediate Membrane Associations between Peroxisomes and the ER. *J. Cel. Biol.* 216, 331–342. doi:10.1083/jcb.201607055

David, C., Koch, J., Oeljeklaus, S., Laernsack, A., Melchior, S., Wiese, S., et al. (2013). A Combined Approach of Quantitative Interaction Proteomics and Live-Cell Imaging Reveals a Regulatory Role for Endoplasmic Reticulum (ER) Reticulon Homology Proteins in Peroxisome Biogenesis. *Mol. Cell Proteomics* 12, 2408–2425. doi:10.1074/mcp.m112.017830

Dziurdzik, S. K., and Conibear, E. (2021). The Vps13 Family of Lipid Transporters and its Role at Membrane Contact Sites. *Int. J. Mol. Sci.* 22, e22062905. doi:10.3390/ijms22062905

Faber, K. N., Haima, P., Harder, W., Veenhuis, M., and Ab, G. (1994). Highly-efficient Electrotransformation of the Yeast Hansenula Polymorpha. *Curr. Genet.* 25, 305–310. doi:10.1007/bf00351482

Flis, V. V., Fankl, A., Ramprecht, C., Zellnig, G., Leitner, E., Hermetter, A., et al. (2015). Correction: Phosphatidylcholine Supply to Peroxisomes of the Yeast *Saccharomyces cerevisiae*. *PloS one* 10, e0140080. doi:10.1371/journal.pone.0140080

Guillen-Samander, A., Leonzino, M., Hanna, M. G., Tang, N., Shen, H., and De Camilli, P. (2021). VPS13D Bridges the ER to Mitochondria and Peroxisomes via Miro. *J. Cel. Biol.* 220, e202010004. doi:10.1083/jcb.202010004

Hua, R., Cheng, D., Coyaud, É., Freeman, S., Di Pietro, E., Wang, Y., et al. (2017). VAPs and ACBD5 Tether Peroxisomes to the ER for Peroxisome Maintenance and Lipid Homeostasis. *J. Cel. Biol.* 216, 367–377. doi:10.1083/jcb.201608128

Hulmes, G. E., Hutchinson, J. D., Dahan, N., Nuttall, J. M., Allwood, E. G., Ayscough, K. R., et al. (2020). The Pex3-Inp1 Complex Tethers Yeast Peroxisomes to the Plasma Membrane. *J. Cel Biol* 219, e201906021. doi:10.1083/jcb.201906021

Islinger, M., Costello, J. L., Kors, S., Soupene, E., Levine, T. P., Kuypers, F. A., et al. (2020). The Diversity of ACBD Proteins - from Lipid Binding to Protein Modulators and Organelle Tethers. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1867, 118675. doi:10.1016/j.bbamcr.2020.118675

Jansen, R. L. M., Santana-Molina, C., van den Noort, M., Devos, D. P., and van der Klei, I. J. (2021). Comparative Genomics of Peroxisome Biogenesis Proteins: Making Sense of the PEX Proteins. *Front. Cel Dev. Biol.* 9, 654163. doi:10.3389/fcell.2021.654163

John Peter, A. T., Herrmann, B., Antunes, D., Rapaport, D., Dimmer, K. S., and Kornmann, B. (2017). Vps13-Mcp1 Interact at Vacuole-Mitochondria Interfaces and Bypass ER-Mitochondria Contact Sites. *J. Cel. Biol.* 216, 3219–3229. doi:10.1083/jcb.201610055

Joshi, A. S., Huang, X., Choudhary, V., Levine, T. P., Hu, J., and Prinz, W. A. (2016). A Family of Membrane-Shaping Proteins at ER Subdomains Regulates Pre-peroxisomal Vesicle Biogenesis. *J. Cel. Biol.* 215, 515–529. doi:10.1083/jcb.201602064

Joshi, A. S., Nebenfuehr, B., Choudhary, V., Satpute-Krishnan, P., Levine, T. P., Golden, A., et al. (2018). Lipid Droplet and Peroxisome Biogenesis Occur at the Same ER Subdomains. *Nat. Commun.* 9, 2940. doi:10.1038/s41467-018-05277-3

Knoblach, B., Sun, X., Coquelle, N., Fagarasanu, A., Poirier, R. L., and Rachubinski, R. A. (2013). An ER-Peroxisome Tether Exerts Peroxisome Population Control in Yeast. *Embo J.* 32, 2439–2453. doi:10.1038/emboj.2013.170

Knoops, K., Manivannan, S., Cepińska, M. N., Krikken, A. M., Kram, A. M., Veenhuis, M., et al. (2014). Preperoxisomal Vesicles Can Form in the Absence of Pex3. *J. Cel. Biol.* 204, 659–668. doi:10.1083/jcb.201310148

Kolakowski, D., Rzepnikowska, W., Kaniak-Golik, A., Zoladek, T., and Kaminska, J. (2021). The GTPase Arf1 Is a Determinant of Yeast Vps13 Localization to the Golgi Apparatus. *Int. J. Mol. Sci.* 22, 222212274. doi:10.3390/ijms222212274

Komori, M., Rasmussen, S. W., Kiel, J. A., Baerends, R. J., Cregg, J. M., van der Klei, I. J., et al. (1997). The Hansenula Polymorpha PEX14 Gene Encodes a Novel Peroxisomal Membrane Protein Essential for Peroxisome Biogenesis. *EMBO J.* 16, 44–53. doi:10.1093/emboj/16.1.44

Krikken, A. M., Wu, H., de Boer, R., Devos, D. P., Levine, T. P., and van der Klei, I. J. (2020). Peroxisome Retention Involves Inp1-dependent Peroxisome-Plasma Membrane Contact Sites in Yeast. *J. Cel Biol* 219, e20196023. doi:10.1083/jcb.201906023

Krikken, A. M., Veenhuis, M., and van der Klei, I. J. (2009). Hansenula Polymorpha Pex11 Cells Are Affected in Peroxisome Retention. *FEBS J.* 276, 1429–1439. doi:10.1111/j.1742-4658.2009.06883.x

Leonzino, M., Reinisch, K. M., and De Camilli, P. (2021). Insights into VPS13 Properties and Function Reveal a New Mechanism of Eukaryotic Lipid Transport. *Biochim. Biophys. Acta (Bba) - Mol. Cel Biol. Lipids* 1866, 159003. doi:10.1016/j.bbalip.2021.159003

Lev, S., Halevy, D. B., Peretti, D., and Dahan, N. (2008). The VAP Protein Family: from Cellular Functions to Motor Neuron Disease. *Trends Cell Biology* 18, 282–290. doi:10.1016/j.tcb.2008.03.006

Li, P., Lees, J. A., Lusk, C. P., and Reinisch, K. M. (2020). Cryo-EM Reconstruction of a VPS13 Fragment Reveals a Long Groove to Channel Lipids between Membranes. *J. Cel Biol* 219, e1161. doi:10.1083/jcb.202001161

Mast, F. D., Jamakhandi, A., Saleem, R. A., Dilworth, D. J., Rogers, R. S., Rachubinski, R. A., et al. (2016). Peroxins Pex30 and Pex29 Dynamically Associate with Reticulons to Regulate Peroxisome Biogenesis from the Endoplasmic Reticulum. *J. Biol. Chem.* 291, 15408–15427. doi:10.1074/jbc.m116.728154

Nagotu, S., Saraya, R., Otzen, M., Veenhuis, M., and van der Klei, I. J. (2008). Peroxisome Proliferation in Hansenula Polymorpha Requires Dnm1p Which Mediates Fission but Not De Novo Formation. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1783, 760–769. doi:10.1016/j.bbamcr.2007.10.018

Okumura, Y., Nakamura, T. S., Tanaka, T., Inoue, I., Suda, Y., Takahashi, T., et al. (2016). The Dysferlin Domain-Only Protein, Spo73, Is Required for Prospore Membrane Extension in *Saccharomyces cerevisiae*. *mSphere* 1, e15. doi:10.1128/mSphere.00038-15

Park, J. S., and Neiman, A. M. (2012). VPS13 Regulates Membrane Morphogenesis during Sporulation in *Saccharomyces cerevisiae*. *J. Cel Sci* 125, 3004–3011. doi:10.1242/jcs.105114

Parodi, E. M., Roesner, J. M., and Huang, L. S. (2015). SPO73 and SPO71 Function Cooperatively in Prospore Membrane Elongation during Sporulation in *Saccharomyces cerevisiae*. *PloS one* 10, e0143571. doi:10.1371/journal.pone.0143571

Raychaudhuri, S., and Prinz, W. A. (2008). Nonvesicular Phospholipid Transfer between Peroxisomes and the Endoplasmic Reticulum. *Proc. Natl. Acad. Sci.* 105, 15785–15790. doi:10.1073/pnas.0808321105

Rosenberger, S., Connerth, M., Zellnig, G., and Daum, G. (2009). Phosphatidylethanolamine Synthesized by Three Different Pathways Is Supplied to Peroxisomes of the Yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta (Bba) - Mol. Cel Biol. Lipids* 1791, 379–387. doi:10.1016/j.bbalip.2009.01.015

Shai, N., Schuldiner, M., and Zalckvar, E. (2016). No Peroxisome Is an Island - Peroxisome Contact Sites. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1863, 1061–1069. doi:10.1016/j.bbamcr.2015.09.016

Shai, N., Yifrach, E., van Roermund, C. W. T., Cohen, N., Bibi, C., IJlst, L., et al. (2018). Systematic Mapping of Contact Sites Reveals Tethers and a Function for the Peroxisome-Mitochondria Contact. *Nat. Commun.* 9, 1761. doi:10.1038/s41467-018-03957-8

Smith, J. J., and Aitchison, J. D. (2013). Peroxisomes Take Shape. *Nat. Rev. Mol. Cel Biol* 14, 803–817. doi:10.1038/nrm3700

Thomas, A. S., Krikken, A. M., van der Klei, I. J., and Williams, C. P. (2015). Phosphorylation of Pex11p Does Not Regulate Peroxisomal Fission in the Yeast Hansenula Polymorpha. *Sci. Rep.* 5, 11493. doi:10.1038/srep11493

van der Klei, I. J., Yurimoto, H., Sakai, Y., and Veenhuis, M. (2006). The Significance of Peroxisomes in Methanol Metabolism in Methylotrophic Yeast. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1763, 1453–1462. doi:10.1016/j.bbamcr.2006.07.016

van Dijk, R., Faber, K., Hammond, A., Glick, B., Veenhuis, M., and Kiel, J. (2001). Tagging Hansenula Polymorpha Genes by Random Integration of Linear DNA

Fragments (RALF). *Mol. Gen. Genomics* 266, 646–656. doi:10.1007/s004380100584

van Dijken, L. P., Otto, R., and Harder, W. (1976). Growth of Hansenula Polymorpha in a Methanol-Limited Chemostat. *Arch. Microbiol.* 111, 137–144. doi:10.1007/bf00446560

Wang, S., Idrissi, F.-Z., Hermansson, M., Grippa, A., Ejsing, C. S., and Carvalho, P. (2018). Seipin and the Membrane-Shaping Protein Pex30 Cooperate in Organelle Budding from the Endoplasmic Reticulum. *Nat. Commun.* 9, 2939. doi:10.1038/s41467-018-05278-2

Wu, F., de Boer, R., Krikken, A. M., Akşit, A., Bordin, N., Devos, D. P., et al. (2020). Pex24 and Pex32 Are Required to Tether Peroxisomes to the ER for Organelle Biogenesis, Positioning and Segregation in Yeast. *J. Cel Sci* 133, 24683. doi:10.1242/jcs.246983

Wu, H., de Boer, R., Krikken, A. M., Akşit, A., Yuan, W., and van der Klei, I. J. (2019). Peroxisome Development in Yeast Is Associated with the Formation of Pex3-dependent Peroxisome-Vacuole Contact Sites. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1866, 349–359. doi:10.1016/j.bbamcr.2018.08.021

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REVIEW
published: 24 February 2022
doi: 10.3389/fcell.2022.852021

Touch and Go: Membrane Contact Sites Between Lipid Droplets and Other Organelles

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Lipid droplets (LDs) have emerged not just as storage sites for lipids but as central regulators of metabolism and organelle quality control. These critical functions are achieved, in part, at membrane contact sites (MCS) between LDs and other organelles. MCS are sites of transfer of cellular constituents to or from LDs for energy mobilization in response to nutrient limitations, as well as LD biogenesis, expansion and autophagy. Here, we describe recent findings on the mechanisms underlying the formation and function of MCS between LDs and mitochondria, ER and lysosomes/vacuoles and the role of the cytoskeleton in promoting LD MCS through its function in LD movement and distribution in response to environmental cues.

Keywords: membrane contact sites, lipid droplets, mitochondria, endoplasmic reticulum, lysosome, vacuole, cytoskeleton, lipophagy

OPEN ACCESS

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

Received: *10 January 2022*
Accepted: *03 February 2022*
Published: *24 February 2022*

Citation:
Liao P-C, Yang EJ, Borgman T, Boldogh IR, Sing CN, Swayne TC and Pon LA (2022) Touch and Go: Membrane Contact Sites Between Lipid Droplets and Other Organelles. Front. Cell Dev. Biol. 10:852021. doi: 10.3389/fcell.2022.852021

1 INTRODUCTION

Lipid droplets (LDs) have an established function in storing lipids, which are used for energy production, membrane biogenesis and synthesis of signaling molecules. LDs also function in storage of signaling proteins, their precursors and hydrophobic vitamins, and for sequestering toxic lipids, which is critical to reduce lipotoxicity and oxidative stress (Welte and Gould, 2017; Jarc and Petan, 2019; Geltinger et al., 2020; Roberts and Olzmann, 2020; Renne and Hariri, 2021). Finally, recent studies support a role for LDs in ER protein quality control (Garcia et al., 2018; Roberts and Olzmann, 2020).

The physical properties of LDs are distinct from those of other organelles. They consist of neutral lipids, primarily triacylglycerol (TAG) and sterol esters (SE), surrounded by a phospholipid monolayer. Although proteins are associated with LDs, conventional transport proteins that are integrated into lipid bilayers do not take part in transfer of lipids and other constituents from LDs to other organelles. Instead, specialized proteins, such as lipases that associate with the LD boundary membrane, release lipids and vitamin A from LDs (Schreiber et al., 2012; O'Byrne and Blaner, 2013; Grumet et al., 2016; Olzmann and Carvalho, 2019). Moreover, transfer of LD components to other

Abbreviations: ATGL, adipose triglyceride lipase; CMA, chaperone-mediated autophagy; ERAD, ER-associated degradation pathway; ESCRT, endosome sorting complex required for transport; HH, hydrophobic helix; LTD, lipid transfer domain; μLP, macrolipophagy; MCS, membrane contact sites; MVB, multivesicular bodies; VAB, VPS13 adaptor binding.

organelles as well as communication between LDs and other subcellular compartments occurs at membrane contact sites (MCS) between LDs and other organelles.

MCS are sites of close apposition between two organelles. While these contacts may be homotypic (between identical organelles) or heterotypic (between different organelles), the focal point for this review article is heterotypic interactions between LDs and mitochondria, ER, lysosomes (the vacuole in yeast) and the role of the cytoskeleton in promoting contact site formation at LDs. LD MCS are not as well understood as other MCS. Nonetheless, LD MCS are enriched in proteins that mediate specific functions at those sites and are produced and stabilized by tethering proteins. Moreover, in yeast the distance between LDs and other organelles at MCS has been determined by electron microscopy to be <30 nm (Perktold et al., 2007; Binns et al., 2006), which is in the range of that observed in other MCS, typically 10–80 nm (Scorrano et al., 2019; Vance, 2020).

Although the structural components of many LD MCS have not been identified, the function of many LD MCS is well established. The endoplasmic reticulum (ER) constitutes the major site for the biogenesis of LDs and lipids that are incorporated into nascent LDs. Therefore, LD-ER contact sites are essential for LD formation, growth and budding from the ER (Olzmann and Carvalho, 2019; Choudhary and Schneiter, 2021). Recent studies revealed that LDs mediate removal of unfolded or damaged proteins from the ER, and that this occurs at LD-ER contact sites (Vevea et al., 2015; Garcia et al., 2021). At mitochondria, LDs deliver fatty acids, which are produced from neutral lipids that are stored in LDs and oxidized for energy production (Finn and Dice, 2006; Rambold et al., 2015; Wang et al., 2021). Toxic lipids or proteins that are sequestered in LDs can be delivered to lysosomes (the vacuole in yeast) by multiple pathways, including transfer events at LD-lysosome contact sites and piecemeal or wholesale uptake of LDs into the lysosome/vacuolar compartment (Tsuji et al., 2017; Schulze et al., 2020; Garcia et al., 2021; Liao et al., 2021). Finally, contacts between LDs and the cytoskeleton contribute to LD MCS formation through effects on LD movement and positional control (Pfisterer et al., 2017; Valm et al., 2017; Kilwein and Welte, 2019). Here, we review recent findings on the structure and function of LD MCS in yeast and mammalian cells, and how these membrane contacts respond to cellular or environmental cues.

2 LD INTERACTIONS WITH MITOCHONDRIA

Mitochondria are the metabolic centers of the cell. Fatty acids (FAs) that are stored as TAG and other lipids in LDs are used for energy production by β-oxidation in mitochondria. Conversely, mitochondria are the source of ATP and other components that contribute to growth or expansion of LDs. Close contacts between LDs and mitochondria were described in 1959 (Palade, 1959) and have been detected in many cell types (Novikoff et al., 1980; Stemberger et al., 1984). They are the sites for transfer of constituents between mitochondria and LD for LD consumption and expansion and are prominent in tissues with high energy demands such as heart (Kuramoto et al., 2012), skeletal muscle (Shaw et al., 2008), brown adipose tissue (Yu et al., 2015) and liver (Shiozaki et al., 2011; Ma et al., 2021). Although these contact sites have been evident for decades, recent studies have revealed important details of their function and structure.

2.1 LD-Mitochondria MCS Function in Transfer of Fatty Acids From LDs to Mitochondria

During periods of nutrient deprivation, cells reprogram their metabolism from glycolysis to oxidation of FAs for ATP production. During this process, FAs that are stored in TAG in LDs are transferred from LDs to mitochondria (Finn and Dice, 2006). Emerging evidence supports a role for LD-mitochondria MCS in this FA transfer event. First, starvation of cultured mammalian cells results in an increase in contact site formation between LDs and mitochondria (Herms et al., 2015; Rambold et al., 2015; Nguyen et al., 2017; Valm et al., 2017). Live-cell imaging of fluorescent FAs revealed that FAs move from LDs into mitochondria when nutrients are limiting. This process requires close association of mitochondria with LDs. It is also dependent on release of FA from TAG stored in LDs: depletion of an LD-associated neutral lipase, adipose triglyceride lipase (ATGL), or drug-induced inhibition of lipase activity reduces the mitochondrial accumulation of fluorescent FAs (Herms et al., 2015; Rambold et al., 2015; Valm et al., 2017).

Several proteins have been implicated in formation of these LD-mitochondria MCS (**Figure 1**). The SNARE proteins SNAP23 and VAMP4 localize to LDs in mouse fibroblasts (Boström et al., 2005), and SNAP23 has been detected on LDs and mitochondria in skeletal muscle (Strauss et al., 2016). More importantly, deletion of SNAP23 produces a decrease in both LD-mitochondria MCS and β-oxidation of radiolabeled FAs in mouse fibroblasts (Jägerström et al., 2009). A proximity labeling study revealed that ACSL1, a long-chain acyl-CoA synthetase that directs FAs to mitochondria for β-oxidation, interacts with SNAP23 and VAMP4 in hepatocytes (Young et al., 2018). In addition, glucose deprivation, a condition that stimulates FA oxidation, promotes co-immunoprecipitation of SNAP23, VAMP4 and ACSL1 in hepatocytes. (Young et al., 2018). These findings support the notion that increased association of LD and mitochondria contributes to elevated FA oxidation and indicate a role for SNAP23, VAMP4 and ACSL1 in establishing physical and functional interactions between LDs and mitochondria during this process.

Other studies support a role for the vacuolar protein sorting 13D (VPS13D) protein in FA transfer from LD to mitochondria at MCS between these organelles (Wang et al., 2021). VPS13D is a VPS13 family protein (Velayos-Baeza et al., 2004; Wang et al., 2021) that localizes to LD-mitochondria contact sites in response to oleic acid stimulation and starvation in cultured cells (Wang et al., 2021). Structure-function analysis revealed that the N-terminal region of VPS13D is responsible for mitochondrial targeting and that two amphipathic helices in the C-terminal region of the protein target VPS13D to the LDs. Moreover,

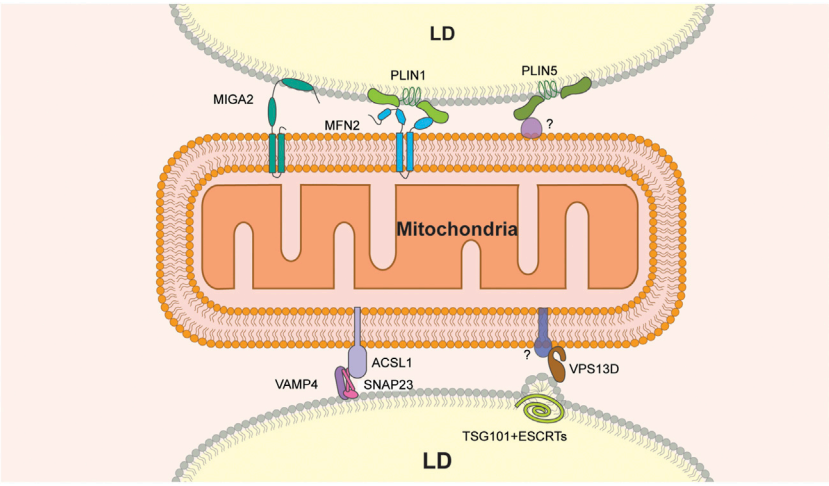

FIGURE 1 | Molecular constituents of LD-mitochondria MCS. MIGA2 is the only LD-mitochondria tether that binds directly to both organelles. Other tethers include the VAMP4-SNAP2-ACSL1 or MFN2-PLIN1 protein complexes. VPS13D and PLIN5 are components of tethers that bind to LDs but have binding partners on mitochondria that have not been identified. In addition, VPS13D interacts with ESCRT on the LD surface and may contribute to membrane remodeling at that site.

VPS13D has a putative lipid transfer domain (LTD) at its N terminus that binds to FAs and is required for VPS13D function in FA transfer from LD to mitochondria. Finally, VPS13D recruits a subunit of the ESCRT (the endosome sorting complex required for transport), a complex that produces changes in membrane curvature (Vietri et al., 2020), to LD-mitochondria MCS. Specifically, the VAB (VPS13 adaptor binding) domain of VPS13D interacts with the ESCRT protein TSG101 and is required for recruitment of TSG101 to LD-mitochondria MCS. Moreover, localization of the VAB domain and TSG101 to this MCS results in the formation of a constricted or tubular structure at the surface of LDs (Wang et al., 2021). Finally, pulse-chase assays of FA transfer from LD to mitochondria revealed that the deletion of VPS13D or TSG101 results in a significant reduction of FA transfer (Wang et al., 2021). Collectively, these findings support a model for VPS13D in energy mobilization by FA oxidation in cells exposed to nutrient limitation. According to this model, VPS13D is recruited to LD-mitochondria junctions in response to starvation, where it contributes to FA transfer from LDs to mitochondria 1) as a lipid transfer protein and 2) by recruiting ESCRT components to LD-mitochondria MCS and facilitating ESCRT-dependent membrane remodeling at those sites.

Finally, the perilipin family protein perilipin 1 (PLIN1) has been implicated in LD-mitochondria contact site formation in brown adipose tissue through interactions with the mitochondrial outer membrane fusion GTPase, mitofusin 2, MFN2 (Boutant et al., 2017). MFN2 and its homolog MFN1 mediate the fusion of mitochondrial outer membranes. In addition, MFN2 is involved in mitochondria-ER contact sites (Giacomello et al., 2020). Nonetheless, depletion or knockout of MFN2 in brown adipose tissue results in fewer LD-mitochondria MCS, altered lipid metabolism and reduced FA oxidation by mitochondria (Boutant et al., 2017). In addition, co-immunoprecipitation studies show that MFN2 directly interacts with PLIN1, and this interaction is enhanced by a treatment with an adrenergic agonist. Finally, PLIN1 expression increases in mice subjected to cold treatment (Yu et al., 2015). These observations suggest that increased mitochondria-LD contacts mediated by MFN2-PLIN1 facilitate the coupling of TAG hydrolysis with FA oxidation upon exposure of brown adipose tissue to cold (Boutant et al., 2017).

2.2 LD-Mitochondria Contact Site Function in LD Expansion

Contact sites between LD and mitochondria can also function in expansion of LD under conditions that promote lipid storage. In brown adipose tissue, a subpopulation of mitochondria is closely associated with large LDs. Benador et al. (2018) developed a method to separate LD-associated mitochondria from LD-free mitochondria and found that these two populations of mitochondria are physically and functionally distinct. LD-associated mitochondria exhibit 1) elevated TCA cycle, ATP synthetic and pyruvate oxidation activities, 2) reduced β-oxidation activity, and 3) increased incorporation of free FAs into TAG in ATP synthase-dependent processes. Thus, contact site formation between LD and mitochondria is associated with lipid storage and generation of energy for this process by oxidation of glucose, not FAs. In contrast, LD-free mitochondria display higher FA oxidation. These observations support the idea that LD-associated mitochondria promote LD expansion and lipid storage by providing ATP for acyl-CoA synthesis during TAG production (Benador et al., 2018).

The LD protein perilipin 5 (PLIN5) has been implicated in LD-mitochondria interactions during LD expansion. PLIN5 is highly expressed in oxidative tissues, such as skeletal and cardiac muscle, brown adipose tissue and liver (Wolins et al., 2006), and is upregulated in response to exercise in muscle tissue (Tarnopolsky et al., 2007). Moreover, PLIN5 overexpression increases the number of LDs and the incorporation of

radiolabeled lipids into TAG in brown adipose tissue and in cultured liver cells (Wang et al., 2011b; Benador et al., 2018). On the other hand, deletion of PLIN5 in mice results in a loss of LDs, and cultured cardiomyocytes from Plin5-null mice exhibit more FA oxidation activity compared to cardiomyocytes from wild-type mice (Kuramoto et al., 2012). Other studies indicate that PLIN5 function in LD expansion may be due to its function in LD-mitochondria MCS. PLIN5 can localize to the mitochondrial surface independent of LD-mitochondria MCS, and localizes to LD-mitochondria interfaces by super-resolution imaging (Gemmink et al., 2018). Moreover, overexpression of PLIN5 in CHO cells induces the recruitment of mitochondria to LD, and this recruitment depends on the presence of 20 amino acids at the C-terminal of the protein (Wang et al., 2011b). This observation supports the notion that PLIN5 is part of a tethering complex that promotes LD expansion at LD-mitochondria MCS.

Interestingly, in hepatocyte-specific Plin5 null mice, the decreased LD-mitochondria interactions resulted in reduced fatty acid oxidation and reduced fatty acid storage into TAGs (Keenan et al., 2019). Therefore, it is possible that even in tissues where PLIN5 is highly expressed, it can promote different aspects of LD-mitochondria interactions. Moreover, PLIN5 has been detected at mitochondria and in the cytoplasm independently of LD (Bosma et al., 2012; Gemmink et al., 2018), suggesting that it may also regulate lipid metabolism. Indeed PLIN5 also regulates the lipolytic activity of ATGL (Granneman et al., 2011; Wang et al., 2011a). These findings raise the possibility that PLIN5 affects TAG production via its regulatory activities on lipolysis independently from its mitochondrial tethering activity.

Mitoguardin 2 (MIGA2) is a mitochondrial outer membrane protein that promotes mitochondrial fusion and modulates body fat in mice by regulating mitochondrial phospholipid metabolism (Zhang et al., 2016). MIGA2 has also been implicated in LD-mitochondria MCS formation in differentiating white adipocytes (Freyre et al., 2019). Overexpression of MIGA2 in adipocytes leads to increased LD-mitochondria MCS formation (Freyre et al., 2019). Structure-function analysis of MIGA2 revealed a direct role for the protein in these MCS: its N-terminal transmembrane domains bind to mitochondria and its C-terminal amphipathic region is exposed to the cytosol and binds directly to LDs (Freyre et al., 2019). Finally, pre-adipocytes lacking MIGA2 exhibit reduced adipocyte differentiation, decreased LD abundance, and diminished TAG synthesis. Consistent with this, radiolabeled glucose is not converted into TAGs in MIGA2-knockout pre-adipocytes (Freyre et al., 2019). Collectively, these data suggest that MIGA2 is a tether that links LDs to mitochondria and raise the possibility that MIGA2 affects LD expansion through effects on *de novo* lipogenesis at MCS in adipocytes.

3 LD-ER CONTACT SITES

LDs form at and bud from the ER in all eukaryotes. LD biogenesis sites are the most complex and best characterized LD MCS. These MCS develop at specialized domains within the ER membrane, are enriched in specific lipids and proteins, and have a well-defined function in LD formation, directional growth and budding. These LD-ER MCS have activities found in other MCS including transfer of lipids and proteins between organelles. However, unlike other MCS in which a pre-existing organelle makes contacts with and is tethered to another organelle, LD-ER MCS develop within the ER membrane during LD biogenesis. While other MCS involve transitory interactions between two physically separate structures, the ER-LD MCS is not so simple. LDs and ER have different membrane and protein composition and different functional characteristics, but the distinction between these two compartments is less stark than, for example, that between ER and mitochondria. There is evidence from electron microscopy (Kassan et al., 2013) and fluorescence imaging (Jacquier et al., 2011; Valm et al., 2017) that in yeast, LDs and ER maintain long-term continuity. Fluorescence and biochemical studies in fly (Wilfling et al., 2013) and mammalian (Zehmer et al., 2009) cells have supported this model, although there are differences among cell types (Hugenroth and Bohnert, 2020).

Here, we describe formation of LD-ER contact sites, their function in LD biogenesis and the environmental cues that modulate these processes.

3.1 Formation of LD-ER MCS at Sites of LD Biogenesis

In light of the critical function of LDs in lipid storage and homeostasis, it is not surprising that LD biogenesis is regulated in response to changes in nutrient availability. Indeed, LD biogenesis is induced by nutrient limitations including the transition from mid-log to stationary phase in yeast, or nitrogen starvation (Jacob, 1987; Kurat et al., 2006; Li et al., 2015). It is also induced by supplementation with oleic acid (Callies et al., 1993; Fujimoto et al., 2006). In contrast, LD biogenesis is required for the survival of nutrient-limited cells (Sandager et al., 2002; Garbarino et al., 2009). One critical step in LD-ER contact site formation during LD synthesis is coalescence of neutral lipids (NL) to form a lens-shaped structure between the leaflets of the ER lipid bilayer. When the NL TAG reaches a threshold concentration (3–5 mol%), it undergoes a phase separation within the ER membrane leading to formation of the TAG lens (Khandelia et al., 2010; Duelund et al., 2013). In yeast, where these structures were first identified, NL lenses are ca. 50 nm in diameter (Choudhary et al., 2015).

The major molecular components and processes in LD-ER biogenesis are illustrated in **Figure 2**. Lens formation is induced by and requires synthesis of TAG and sterol esters (SE). In yeast, TAG is generated by acylation of the precursor diacylglycerol (DAG) by the diacylglycerol acyltransferases Dga1 and Lro1 (Lecithin cholesterol acyl transferase Related Open reading frame 1). SE are generated from sterols by the acyl-CoA:sterol acyltransferases Are1 and Are2. Indeed, inhibition of NL synthesis by deletion of all SE and TAG biosynthetic enzymes (*DGA1*, *LRO1*, *ARE1* and *ARE2*) blocks LD biogenesis (Sandager et al., 2002). Similarly, inhibition of DAG synthesis from phosphatidic acid by deletion of lipin (Pah1, phosphatidic acid phosphohydrolase 1, in yeast) results in reduced LD abundance (Adeyo et al., 2011).

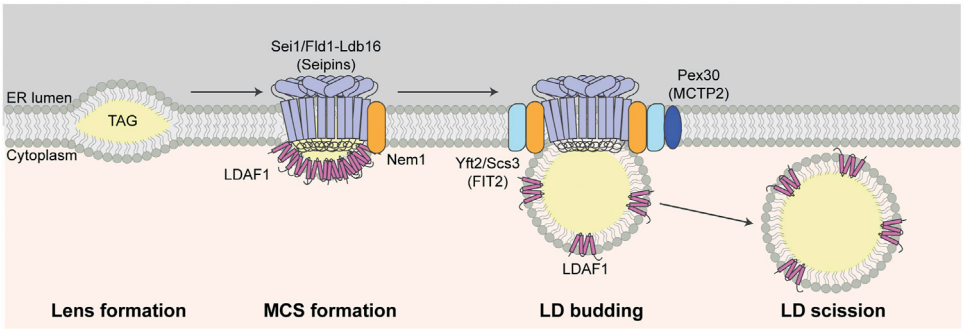

FIGURE 2 | LD biogenesis at LD-ER MCS. TAG accumulates between leaflets of the ER bilayer during lens formation. Seipins, Nem1, and LDAF1 localize to and are required for LD-ER MCS formation at sites of LD biogenesis. Other LD biogenesis proteins including FITM2 and Pex30 are recruited to LD-ER MCS and LDAF1 is later transferred from MD-ER MCS to the surface of LDs during LD budding from the ER membrane. Finally, LDs are separated from ER and released to cytosol during LD scission.

The seipin protein complex determines the site of lens formation, mediates MCS formation between LDs and ER at those sites, and promotes TAG incorporation into lenses and nascent LDs. Seipin is encoded by the *BSCL2* (Berardinelli-Seip Congenital Lipodystrophy 2) gene in humans and *SEI1*/*FLD1* (Seipin/Few LDs) gene in yeast. It is an integral ER membrane protein that localizes to LD-ER contact sites (Szymanski et al., 2007; Fei et al., 2008; Salo et al., 2016; Wang et al., 2016). Seipin contains a highly conserved ER lumen domain, short N- and C-terminal cytosolic domains and two transmembrane domains (Lundin et al., 2006). The luminal domain contains a hydrophobic helix (HH) near the ER bilayer and a β-sandwich fold (Sui et al., 2018; Yan et al., 2018). The β-sandwich fold binds anionic phospholipids such as phosphatidic acid (Yan et al., 2018) and is similar in structure to β-sandwich domains in the sterol-binding Niemann-Pick C2 (NPC2) proteins. Recent, cryo-EM studies revealed that seipin oligomerizes to form a ring-like structure containing 10–12 subunits and that luminal HHs in that ring-like structure bind to TAG, which promotes TAG cluster formation at low concentrations (Prasanna et al., 2021; Zoni et al., 2021). Interestingly, yeast seipin lacks the HH domain found in human or *Drosophila* seipins. However, yeast seipin binds to Ldb16 (low dye binding 16), which contains HH-like regions and supports HH function in the yeast seipin complex (Klug et al., 2021).

Seipin functions in LD-ER MCS and LD formation through its interactions not just with lipids but with proteins including Nem1 (nuclear envelope morphology 1) and LDAF1 (LD activator factor 1), also known as Tmem159 and promethin in mammals, and Ldo45 (LD organization 45 kD protein) in yeast. Seipin-Nem1 interactions promote NL biosynthesis at sites of lens formation. Both proteins localize to and co-localize at punctate structures at sites of lens formation and do so independent of NL biosynthesis or the presence of LDs (Choudhary et al., 2020). Nem1 activates DAG production, and functions with seipin to recruit TAG biosynthetic enzymes (Dga1 and Lro1) at LD-ER MCS during lens initiation and growth (Choudhary et al., 2020).

Interaction of seipin with LDAF1 is also critical for the TAG phase transition during initiation of lens formation. Although small lens-likes structures can form in the ER membrane in the absence of seipin (Salo et al., 2016; Wang et al., 2016), recent studies support the model that seipin and LDAF1 stimulate lens formation by lowering the critical concentration of TAG for phase conversion within membranes. Specifically, deletion of LDAF1 inhibits LD formation during early stages of that process at all TAG concentrations tested, indicating that LDAF1 is required for initiation of LD biogenesis. Notably, it is released from seipin and recruited to the surface of nascent LDs as they mature (Chung et al., 2019). Consistent with this, molecular simulation studies revealed that binding of seipin to TAG promotes its association with LDAF1, which stabilizes nascent lens structures (Prasanna et al., 2021; Zoni et al., 2021). Finally, targeting of LDAF1 to the plasma membrane (PM) results in formation of PM-ER MCS, as well as recruitment of seipin and LD biogenesis at that site. Thus, seipin and LDAF1 can drive lens formation and LD biogenesis *in vivo* (Chung et al., 2019).

3.2 Generation of Lipid and Protein Asymmetry at LD-ER MCS During LD Growth and Budding

LD-ER interactions at sites of LD biogenesis are disrupted when nascent LDs bud from the ER into the cytosol. Budding of LDs from the ER and the size of LDs that are released from ER are influenced by membrane curvature and surface tension at the LD-ER MCS. Phospholipids that promote negative membrane curvature, such as DAG or phosphatidylethanolamine (PE), stabilize the LD-ER contact site and favor retention of LDs in the ER. In contrast, lysolipids, which promote positive membrane curvature, destabilize LD-ER MCS and favor LD budding (Choudhary et al., 2018) and generation of small LDs (Ben M'barek et al., 2017).

Fat storage-inducing transmembrane protein 2 (FITM2) is an evolutionarily conserved ER-localized transmembrane protein that is required for budding of LDs from ER membranes

(Choudhary et al., 2015). Studies in yeast indicate that FITM2 proteins promote this process by regulating the levels of DAG. Although DAG is a precursor for TAG and therefore required for LD biogenesis, DAG can inhibit budding of nascent LDs from LD-ER MCS by promoting negative membrane curvature at those contact sites. Therefore, its levels must be tightly regulated during LD biogenesis. Indeed, lysolipids promote positive membrane curvature and budding of LDs from ER in the absence of FITM2 in yeast. This suggest that the increase of membrane curvature by lysolipids reduces the defects in LD biogenesis caused by high DAG levels in the absence of FITM2 (Choudhary et al., 2018). The FITM2 proteins of yeast (Yft2 and Scs3) are recruited to sites of LD biogenesis by binding to seipin and Nem1 (Choudhary et al., 2018; 2020). Moreover, deletion of both FITM2 proteins in yeast results in increased DAG and this defect is rescued by deletion of *NEM1* (Choudhary et al., 2018). Since Nem1 promotes DAG production, FITM2 proteins may modulate DAG levels though effects on Nem1.

Interactions between seipin and Pex30 (Peroxisome-related 30) have been implicated in modulation of the phospholipid composition at LD-ER MCS during lens formation. This process is downstream of the recruitment of FITM2 proteins to seipin-Nem1 sites (Choudhary et al., 2020). Pex30 is an ER membrane protein with established functions in control of peroxisome size, shape and formation (Joshi et al., 2016; Vizeacoumar et al., 2003; 2004). Interestingly, Pex30 is associated with seipin complexes at LD-ER contact sites during LD formation. Moreover, deletion of Pex30 results in abnormal LD morphology, and deletion of seipin and Pex30 results in inhibition of LD biogenesis, abnormal ER morphology, and growth defects (Joshi et al., 2018; Wang et al., 2018). Notably, the defect in LD biogenesis in *sei1*Δ *pex30*Δ double mutants is rescued by deletion of Pct1 (phosphocholine cytidylyltransferase 1), the rate-limiting enzyme in the phosphatidylcholine (PC) biosynthesis Kennedy pathway. PC is the most abundant phospholipid in the LD membrane. Thus, Pex30 may contribute to LD biogenesis by modulating phospholipid composition in the LD-ER contact site and on the surface of the nascent LD during LD biogenesis (Wang et al., 2018). Interestingly, Pex30 contains membrane-shaping reticulon-like regions (Joshi et al., 2016) and may also contribute to deforming the membrane at LD-ER MCSs and budding of the nascent LDs from the ER membrane.

3.3 Role for ERAD in Removal of Surplus LD Proteins From the ER Membrane

The ER-associated degradation pathway (ERAD) was originally identified as a pathway for degradation of unfolded or damaged proteins in ER membranes. In ERAD, unfolded proteins are ubiquitinated, recognized and extracted by the AAA-ATPase Cdc48 in yeast (p97/VCP in mammals), and degraded by proteasomes (Christianson and Ye, 2014). Recent studies support a novel role for ERAD in degrading LD proteins within the ER membrane.

In mammals, diacylglycerol acyltransferase 2 (DGAT2), an enzyme that catalyzes the conversion of DAG to TAG, is degraded by ERAD with the aid of the ubiquitin ligases gp78 and Hrd1 (Choi et al., 2014; Luo et al., 2018). In yeast, a subset of LD proteins, Pgc1 (phosphatidyl glycerol phospholipase C), Dga1, and Yeh1 (yeast steryl ester hydrolase), are substrates for the ERAD ubiquitin ligase Doa10 and degraded by ERAD. The HH domain of Pgc1 has been implicated as a degron for ERAD: it is both necessary and sufficient for Doa10-dependent degradation (Ruggiano et al., 2016). Interestingly, degradation of Pgc1 by ERAD is accelerated in the absence of yeast FITM2 (Yap et al., 2020). Moreover, the regions for ERAD degradation and for targeting of proteins to LDs overlap (Ruggiano et al., 2016). These findings raise the possibility that proteins that are not incorporated into LDs are degraded in the ER by ERAD.

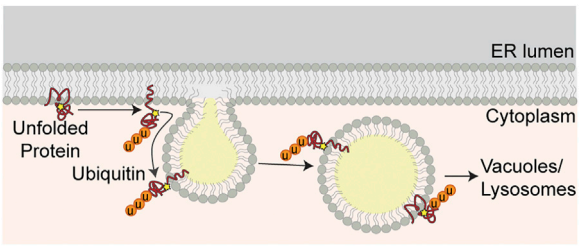

FIGURE 3 | ER proteostasis at LD-ER MCS. Unfolded ER proteins are marked for degradation by ubiquitination. Ubiquitinated proteins are transferred from ER to LDs at LD-ER MCS. LDs containing ubiquitinated proteins bud from the ER and are delivered to vacuoles for degradation by microautophagy.

3.4 LD-ER Contact Sites and ER Proteostasis

As described above, resident LD proteins are recruited to nascent LDs at LD-ER MCS. Recent evidence indicates that unfolded ER proteins, which accumulate in ER under conditions of ER stress and compromise ER and cellular function and fitness, are removed from the ER in LDs by transport from ER to LDs at LD-ER MCS. In contrast to the ERAD system which relieves ER stress by removing individual unfolded proteins from the organelle, this LD-based ER proteostasis mechanism enables high-throughput removal of unfolded ER proteins (**Figure 3**) (Vevea et al., 2015).

Early studies revealed that ER proteins are recovered in isolated LDs. Although these proteins were first interpreted as contaminants in LD preparations, several lines of evidence indicate that ER proteins are recruited to LDs by ER stress. Specifically, treatment of yeast with a reducing agent, dithiothreitol, which inhibits oxidative folding in the ER, results in recruitment of 1) proteins that contain disulfide linkages and undergo oxidative folding in the ER, 2) protein disulfide isomerase (PDI) proteins, multifunctional ER redox chaperones, and 3) other ER chaperones to LDs. Similarly, treatment with tunicamycin, an agent that induces protein misfolding by inhibiting protein glycosylation in the ER, results in recruitment of proteins that are glycosylated in ER and the ER chaperones described above to LDs. Imaging studies revealed that ER proteins that are recovered with LDs also co-

localize with LDs in living yeast exposed to ER stress. These imaging studies also provide documentation of 1) association of LDs with protein aggregates in the ER membrane, 2) co-localization of those protein aggregates with LDs as they bud from ER membranes and move away from the ER, and 3) localization of LDs and their associated ER protein aggregates in the vacuole (yeast lysosome) (Garcia et al., 2021).

Equally important, LD function in ER protein quality control is a physiologically relevant stress response. Indeed, LD biogenesis or abundance is up-regulated in response to ER stressors in yeast (Fei et al., 2009; Vevea et al., 2015; Garcia et al., 2021), in mammalian cells (Lee et al., 2012) and in mouse liver (Yamamoto et al., 2010; Zhang et al., 2011). Furthermore, inhibition of LD biogenesis dramatically reduces cellular growth and survival in yeast challenged by ER stressors (Garcia et al., 2021). Overall, these studies support a model for LD function in ER protein quality control whereby unfolded proteins are transferred from ER membranes to nascent LDs at LD-ER MCS, removed from ER by LDs as they bud from the ER and degraded in response to ER stress.

4 LD-LYSOSOME/VACUOLE MCS

The lysosome (vacuole in yeast) plays a major role in catabolism, recycling of cellular waste, excretion of waste products and cellular signaling. Contact site formation between LDs and lysosomes/vacuoles plays direct and indirect roles in LD autophagy (lipophagy). Lipophagy, in turn, is essential for the mobilization of LD-bound lipids for energy production in response to nutrient limitations and other stressors, and for degradation of excess or toxic lipids or unfolded proteins that are stored and sequestered in LDs during ER stress. Lipophagy is also critical for delivery of sterols and other lipids in LD to the vacuolar membrane in the stationary phase in yeast (Tsuji et al., 2017; Garcia et al., 2018; Jarc and Petan, 2019).

LD-lysosome/vacuole MCS have been implicated in three forms of lipophagy. In LD macroautophagy, which is the primary form of lipophagy in mammalian systems, LDs are encapsulated within autophagosomes, and delivered to the lumen of the lysosome by fusion of autophagosomes with the lysosomal membrane (Singh et al., 2009). In LD microautophagy or microlipophagy (μLP) which is predominantly understood in yeast, LDs make direct contact with the lysosome/vacuole and partial or wholesale uptake of LDs into the lysosome/vacuole at sites of invagination in the lysosome/vacuole membrane (Garcia et al., 2018; Schulze et al., 2020). Finally, in chaperone-mediated autophagy (CMA), specific LD proteins are targeted to the lysosome by chaperones and translocated across the lysosomal membrane by the lysosome-associated membrane protein type 2A (LAMP2A) (Kaushik and Cuervo, 2015; 2016). All three forms of autophagy are induced by nutrient limitation and other environmental cues. Below, we review the two forms of lipophagy that occur by direct contact between LDs and the lysosome/vacuole at MCS between the organelles: LD microlipophagy (**Figures 4I–III**) and CMA (**Figure 4IV**).

4.1 LD-Vacuole MCS During LD Microautophagy in Yeast

Microlipophagy (μLP) was first identified in yeast, and has emerged as the primary mechanism for lipophagy in yeast. μLP can be induced by stressors including nitrogen or glucose limitation, entry into stationary phase, lipid imbalance, and ER stress. Although these conditions all induce μLP, two forms of μLP occur at distinct LD-vacuole MCS and require distinct factors that modulate vacuolar membrane dynamics, invagination and scission (van Zutphen et al., 2014; Wang et al., 2014; Vevea et al., 2015; Oku et al., 2017; Seo et al., 2017; Tsuji et al., 2017; Garcia et al., 2021; Liao et al., 2021). Below, we describe these two mechanisms of μLP at LD-vacuole MCS in yeast and the role of specific proteins and lipids in that process.

4.1.1 LD-Vacuole MCS at Lo Microdomains During μLP in Yeast

In μLP induced by entry into stationary phase or nitrogen starvation, LDs make contacts with the vacuole at liquid ordered (L_o) microdomains in the vacuolar membrane (Tsuji et al., 2017; Wang et al., 2014) (**Figure 4II**). L_o microdomains are lipid raft-like regions that are enriched with sterols and have distinct protein and lipid composition compared to the bulk of the vacuolar membrane, which has been referred to as a liquid disordered (L_d) domain. Transfer of sterols from LDs to vacuoles at LD-vacuole MCS during L_o microdomain formation in stationary-phase yeast cells (Wang et al., 2014) and intravacuolar transfer of sterols to L_o microdomains by Neiman-Pick proteins mediates formation of these microdomains under multiple stress conditions (Tsuji et al., 2017; Liao et al., 2021). These microdomains form in response to various stresses including entry into stationary phase, nitrogen or glucose starvation, osmotic stress, cycloheximide (CHX)-mediated translation inhibition, weak acids, heat, and ER stress induced by lipid imbalance, DTT, or TM (Toulmay and Prinz, 2013; Liao et al., 2021). Thus, Lo microdomain formation is a general stress response (**Figures 4I, II**).

Moreover, vacuolar membrane proteins are enriched in and excluded from vacuolar L_o microdomains. Vph1, a component of vacuolar proton pump ATPase, is excluded from L_o microdomains. In contrast, sterol transporters (LaM6/Ltn1 and Nce102), TORC1 (target of rapamycin complex 1) subunits (Tco89, Tor Complex I 89) and subunits or interactors of the TORC1-regulating EGO/ragulator complex (Ivy1, Interacting with Vps33 and Ypt7; Gtr1 and 2, GTP binding protein resemblance 1 and 2; Iml1, increased minichromosome loss 1) are enriched in L_o microdomains (Toulmay and Prinz, 2013; Wang et al., 2014; Murley et al., 2015, 2017; Numrich et al., 2015; Varlakhanova et al., 2018; Vaskovicova et al., 2020).

The mechanisms underlying LD MCS formation at L_o microdomains and the vacuolar membrane dynamics and invagination at those MCS during release of LDs into the vacuolar lumen are not well understood. However, Ivy1 can bind to Ypt7, the Rab7 GTPase of yeast, and requires Ypt7 for localization to invaginations in the vacuolar membrane in

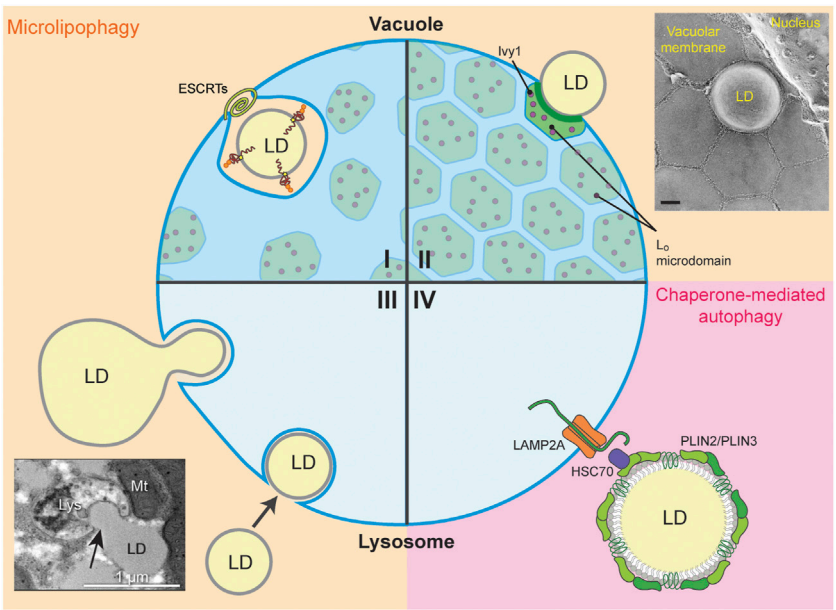

FIGURE 4 | LD-Lysosome/Vacuole MCS. The four quadrants **(I-IV)** display the different types of LD-lysosome/vacuole MCS during microlipophagy (μLP, beige) and chaperone-mediated autophagy (CMA, pink). **(I, II)** MCS between LD and the yeast vacuole form during μLP. **(I)** The ESCRT machinery is required for μLP under DTT-, TM-, or lipid imbalance- induced ER stress and during diauxic shift. Ubiquitinated, unfolded proteins on LDs are engulfed by the vacuole for degradation. Small amounts of L_o microdomains associated with the Ivy1 protein appear in cells under ER stress. However, LDs are not taken up through these L_o microdomains. **(II)** L_o microdomain-dependent uptake of LDs is typical of cells in the stationary phase or under nitrogen starvation, and to some extent in cells under lipid imbalance. Ivy1-containing L_o microdomains are widespread under these conditions. Inset, freeze-fracture EM showing L_o microdomains during stationary phase-induced μLP (image from Tsuji et al. (2017) with permission, scale bar 0.2 μm). **(III, IV)** LD-lysosome MCS in mammalian cells are shown during μLP through partial (piecemeal) or whole-LD uptake and CMA. **(III)** μLP may occur via piecemeal uptake of a larger LD into the lysosome, or by wholesale uptake of smaller LDs into the lysosome. Inset, transmission electron micrograph of rat primary hepatocytes treated with oleic acid to induce LD formation and serum-starved HBSS to induce LD autophagy (image from Schulze et al. (2020) with permission, scale bar 1 μm). (IV) LD-associated proteins Plin2 and Plin3 are degraded via CMA in the lysosome. Hsc70 binds PLIN2 and PLIN3 and delivers these LD-associated proteins to LAMP2A to be translocated from the lysosomal surface to the lumen for degradation.

response to nutrient limitation (Lazar et al., 2002; Numrich et al., 2015). Moreover, as described below, Rab7 has been implicated in LD-lysosome MCS formation in mammalian cells (Schroeder et al., 2015). Thus, Ivy1 may contribute to μLP through effects on MCS formation between LDs and L_o microdomains on the vacuolar membrane. Interestingly, Ivy1 is also a phospholipid-binding protein that contains a putative I-BAR domain, which binds to and stabilizes membranes with negative membrane curvature (Itoh et al., 2016). Therefore, Ivy1 may contribute to the invagination of the vacuolar membrane at contact sites between LDs and vacuolar membrane L_o microdomains (**Figure 4IV**).

4.1.2 Lo Microdomain-Independent, ESCRT-Dependent μLP in Yeast

μLP is induced by the diauxic shift from glycolysis to respiration-driven metabolism during late log phase in yeast (Oku et al., 2017). Moreover, in response to ER stress, LDs that contain unfolded ER proteins are targeted for degradation by μLP (Vevea et al., 2015; Garcia et al., 2021). Although many stressors induce L_o microdomain formation in the vacuolar membrane, LDs do not form MCS with the vacuole at L_o microdomains during μLP induced by ER stressors or the diauxic shift in yeast. Rather, under these conditions, LD-vacuole MCS form at L_d domains in the vacuolar membrane that contain Vph1, which is excluded from L_o microdomains (Vevea et al., 2015; Oku et al., 2017; Garcia et al., 2021). In addition, ESCRT complex proteins are up-regulated and recruited to sites of membrane scission at these LD-vacuole MCS, and are required for L_o microdomain-independent μLP in yeast (Vevea et al., 2015; Oku et al., 2017; Garcia et al., 2021) (**Figure 4I**).

The mechanisms underlying LD-vacuole MCS formation during ER stress-induced μLP are not well understood. However, recent studies indicate that ER stressors induce vacuolar fragmentation in yeast. Moreover, LDs develop persistent interactions with clusters of fragmented vacuoles during L_o microdomain-independent μLP, which supports MCS between LDs and one or more vacuoles during this process. The fragmented vacuoles fuse to form a cup-shaped structure surrounding LDs, and then engulf the LDs. ER stress-induced μLP is blocked by inhibition of this vacuolar fusion (Garcia et al., 2021). Overall, these studies show that vacuolar fragmentation, clustering and fusion around LDs occur during stress-induced μLP, but ongoing studies are needed to determine tmore of the components and regulators of the MCS involved in μLP. Additionally, it has been discovered that the deletion of Rab7, a protein implicated in LD-lysosome MCS, results in accumulation of enlarged, clustered lysosomal compartments

(MVBs) in mammalian cells (Schroeder et al., 2015), so it is possible that the clustering and fusion of degradative compartments is a conserved component of the μLP pathway.

4.2 LD-Lysosome MCS During Microlipophagy (μLP) in Mammalian Cells

LD degradation by macroautophagy has been studied extensively in mammalian cells. However, LD microautophagy (μLP) also occurs in mammalian cells, as revealed in recent studies of nutrient limitation in hepatocytes (Schulze et al., 2020). These studies documented formation of MCS between LDs and lysosomes, and uptake of LD segments or of intact LDs into lysosomes at invaginations in the lysosome membrane. Specifically, live-cell visualization of pH-sensing mRFP1-GFP targeted to the LD marker protein PLIN2 revealed persistent (>60 s) interactions between LDs and lysosomes and uptake of LDs into the acidic lumen of the lysosome under nutrient-limited conditions (**Figure 4III**). Interestingly, nutrient limitation resulted in an increase in the frequency of persistent LD-lysosome contacts. Moreover, silencing of canonical macroautophagy or CMA components has no effect on persistent LD-lysosome contacts, and EM studies revealed that MCS formation between LDs and lysosomes occurs in the absence of double-membrane, autophagosome-like structures. These findings provide the first evidence that LD degradation in response to nutrient limitations can occur by μLP in mammalian cells (Schulze et al., 2020).

The mechanism underlying μLP in mammalian cells is not well understood. However, emerging evidence supports a role for Rab7, a small GTPase and important regulator of endocytic trafficking, in LD-lysosome MCS formation in hepatocytes (Schroeder et al., 2015). Specifically, nutrient limitations result in recruitment of Rab7 to LDs, and an increase in MCS between LDs and degradative compartments including lysosomes, MVBs and late endosomes. Moreover, depletion of Rab7, or inactivating mutation of Rab7, inhibits interactions of LDs and degradative compartments and results in an accumulation of enlarged, clustered MVBs and an overall inhibition of starvation-induced LD degradation. This raises the interesting possibility that Rab7 mediates contact site formation between LDs and lysosomes directly, or by promoting MCS formation between LDs and late endosomes/MVBs (amplisomes) and that late endosomes/MVBs at these MCS mature to form lysosomes (Schroeder et al., 2015). Interestingly, Rab7 has also been implicated in LD activities that may affect LD MCS through effects on vacuolar fusion or LD motility.

4.3 LD-Lysosome MCS During CMA in Mammalian Cells

Although CMA typically targets soluble cytosolic proteins, the LD-associated perilipin proteins PLIN2 and PLIN3 are degraded by CMA at LD-lysosome MCS in cultured mammalian cells. (Kaushik and Cuervo, 2015; 2016; 2018). PLIN2 functions in LD biogenesis, stability and trafficking and serves as a scaffold that regulates association of LDs with the macroautophagy machinery (Tsai et al., 2017). PLIN3 also regulates macroautophagy in a TORC1 (target of rapamycin 1) -dependent manner (Garcia-Macia et al., 2021). Starvation-induced CMA of PLIN2 and PLIN3 is mediated by the 70-kD heat shock protein, hsc70, which binds to the pentapeptide motifs LDRLQ on PLIN2 and SLKVQ on PLIN3, promotes phosphorylation of PLIN2 by 5′ AMP-activated protein kinase (AMPK), and delivers PLIN2 and PLIN3 to the lysosome-associated membrane protein 2A (LAMP2A) (Kaushik and Cuervo, 2015; 2016; 2018), the vacuolar membrane protein that facilitates translocation of CMA substrates from the lysosomal surface to the lumen (Chiang et al., 1989; Salvador et al., 2000; Bandyopadhyay and Cuervo, 2008). Deletion of the pentapeptide CMA recognition motif on PLIN2 results in an increase in PLIN2 levels and a decrease in association of LDs with lysosomes (Schweiger and Zechner, 2015). These findings are consistent with the model that hsc70 binds to LD-associated PLIN2 and that CMA of PLIN2 occurs at MCS between LD and the lysosome (**Figure 4IV**).

CMA of PLIN2 and PLIN3 is triggered by stressors including nutrient limitation, oxidative and lipogenic stresses, and hypoxia (Cuervo et al., 1995; Kiffin et al., 2004; Dohi et al., 2012; Rodriguez-Navarro et al., 2012; Kaushik and Cuervo, 2015), and contributes to stressor-stimulated release of lipids from LDs. Specifically, removal of PLIN2 and PLIN3 from the LD surface promotes association of LDs with 1) cytosolic lipases (e.g., ATGL) that catalyze release of FA from TAG and 2) the LD macroautophagy machinery. In turn, this promotes the release of lipids from LDs after degradation by the lysosome (Kaushik and Cuervo, 2015). These findings support a function of LD-lysosome MCS, and a role for CMA in regulation of lipid homeostasis.

5 CYTOSKELETAL MODULATION OF LD-ORGANELLE INTERACTIONS

As described above, environmental cues including nutrient availability and exposure to stressors induce MCS formation between LDs and organelles including mitochondria, ER and lysosomes. The cytoskeleton plays a fundamental role in this process by controlling the position and movement of LDs and organelles that interact with LDs. For example, in response to nutrient limitation, LDs change from clustered to a dispersed distribution, which allows LDs to make contact with mitochondria for up-regulation of lipid metabolism (Herms et al., 2015; Nguyen et al., 2017; Kong et al., 2020). Although multiple mechanisms have been identified for cytoskeletal control of organelle motility, the best characterized mechanism relies on motor molecule-driven, polarized movement of organelles along actin or microtubule tracks. Here, we summarize cytoskeletal function in LD interactions and contact site formation with other organelles.

5.1 Evidence of Cytoskeleton-Directed LD Distribution and Motility

Cytoskeletal components and motors have been found on LDs in a variety of organisms, including fungi, plants, and mammals.

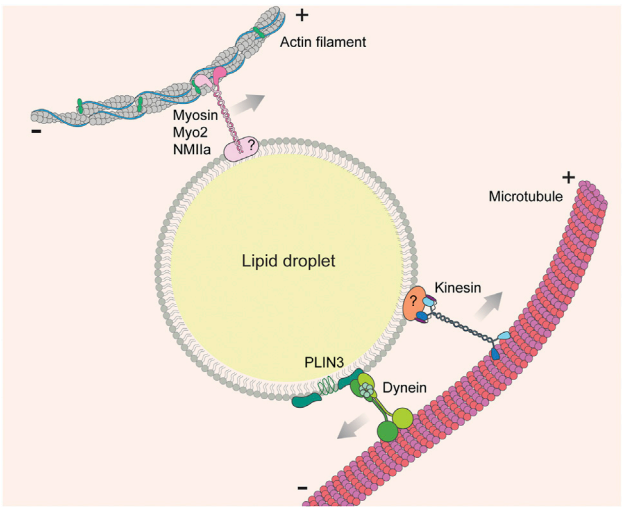

FIGURE 5 | LD-cytoskeleton interaction. Lipid droplets are transported on cytoskeletal fibers (actin filaments or microtubules) by cytoskeleton-associated motor molecules (myosins, kinesins, and dyneins). In most cases the adaptors linking motors to the LD surface are unknown.

Proteomic analysis of LDs revealed actin, tubulin, and motor proteins on LDs (Turró et al., 2006; Weibel et al., 2012; Brocard et al., 2017; Pfisterer et al., 2017; Yu et al., 2017; Zhi et al., 2017; Bersuker et al., 2018). In particular, a high-confidence LD proteome generated from proximity labeling confirmed that actin, tubulin, and a kinesin family protein, KIF16B, are recovered with isolated LDs (Bersuker et al., 2018). Additionally, immunofluorescence staining in rat adrenocortical cells and adipocytes showed that beta-actin is present on the LD surface (Fong et al., 2001).

The actin and microtubule cytoskeletal networks and their associated motor proteins are involved in LD morphology and distribution within the cell. For example, destabilization of the actin cytoskeleton by treatment with either cytochalasin D (CytD) or latrunculin-A decreases the size of LDs in J774 macrophages (Weibel et al., 2012). Destabilizing microtubules by nocodazole treatment also decreases LD size (Boström et al., 2005; van Zutphen et al., 2014; Gu et al., 2019). Presumably, this change in size results from a change in the balance between the addition and removal of LD cargo, which occurs at specific MCS. Consistent with this idea, the position and dynamics of LDs are also dependent on the cytoskeleton. Destabilization of actin filaments prevents LD movement from the vegetal pole to the animal pole in zebrafish embryos (Dutta and Kumar Sinha, 2015). Post-translational modifications of tubulin affect LD motility and distribution. For example, during nutrient deficiency, detyrosinated tubulins accumulate and form networks that promote LD dispersion in Vero cells (Herms et al., 2015). In contrast, acetylated tubulins immobilize LDs in hepatic cells (Groebner et al., 2019).

Although these studies reveal that morphology and distribution of LDs depend on the cytoskeleton, it is not always clear whether the effects observed upon global destabilization of microtubule or actin cytoskeletons are due to direct effects on LD-cytoskeleton interactions. However, the effects of disrupting motor proteins, which drive motility on cytoskeletal tracks, are less ambiguous. Both the actin-based motor myosin and the microtubule-based motors kinesin and dynein drive LD distribution and motility (**Figure 5**) (Gross et al., 2000; Andersson et al., 2006; Shubeita et al., 2008; Knoblach and Rachubinski, 2015; Pfisterer et al., 2017; Rai et al., 2017; Gu et al., 2019; Veerabagu et al., 2020).

In some cases, specific motor proteins that drive LD motility have been identified. In budding yeast, anterograde movement of LDs from mother cells to buds relies on a type V myosin, Myo2p (**Figure 5**) (Knoblach and Rachubinski, 2015). During zebrafish development, inhibiting Myosin-1 with pentachloropseudilin alters the dynamics and distribution of LDs (Gupta et al., 2017). Knockdown of non-muscle myosin IIa (NMIIa) enlarges LDs and promotes their clustering in human osteosarcoma U2OS cells (Pfisterer et al., 2017). Post-translational modification of motor proteins also alters motor-LD interactions. For example, ERK-mediated phosphorylation of dynein increases its affinity for LDs (Andersson et al., 2006). Motor knock-down studies are somewhat more specific than drug-induced cytoskeletal disruption, but still may be subject to pleiotropic effects because motor proteins are shared by multiple cargos. A more specific approach is to target the cargo adaptor proteins that bridge LDs and cytoskeletal/motor proteins, although these adaptors are less well understood. One recently identified adaptor is the LD protein perilipin 3 (PLIN3), which interacts with the dynein intermediate chain subunit, Dync1i1, in AML12 mouse hepatic cells (**Figure 5**) (Gu et al., 2019). Identifying more of these LD-specific cargo adaptor proteins will allow in-depth characterization of the biological function of LD-cytoskeletal interaction.

5.2 Functional Consequences of LD-Cytoskeleton Interactions

MCSs between LD and other organelles play an important role in exchanging metabolites. Therefore, any change in the distribution or dynamics of those sites can affect their function. Indeed, not only the size, but also the lipid composition of LDs in J774 macrophages is changed by actin destabilization (Weibel et al., 2012). Destabilization of the actin cytoskeleton reduces the dissociation of LDs from peroxisomes in *Arabidopsis* (Cui et al., 2016). Microtubules are required for LD autophagy (Boström et al., 2005; van Zutphen et al., 2014; Gu et al., 2019). Nocodazole-treated COS-7 cells have fewer contact sites between LDs and mitochondria or peroxisomes, as well as fewer ternary contacts between LDs, peroxisomes, and Golgi (Valm et al., 2017).

LD-mitochondria interactions are crucial for mobilizing the energy stored in LDs (Rambold et al., 2015). When nutrients are depleted, Vero cells exhibit dispersion of LDs, and a concomitant increase in LD-mitochondria contacts, consistent with the need for lipid exchange and fatty acid metabolism. Starvation-induced LD-mitochondria contacts include both relatively short-lived

interactions ("touch and go") and more stable connections (Herms et al., 2015). Microtubules are required for formation of these contacts (Valm et al., 2017), and the dispersion of LDs from the perinuclear area to the cell periphery specifically depends on detyrosinated microtubules. Detyrosination is promoted by the activation of the energy sensor, AMP protein kinase (AMPK) (Herms et al., 2015). AMPK also phosphorylates PLIN3, which may induce conformational changes of PLIN3 to facilitate LD dispersion (Zhu et al., 2019). Given the link between PLIN3 and dynein and microtubules (Gu et al., 2019), the LD dispersion caused by phosphorylated PLIN3 may be due to the altered interaction between PLIN3 and dynein. Taken together, LD-mitochondria interactions are elevated upon starvation, and this response requires the microtubule network to shuttle LDs to mitochondria and facilitate lipid metabolism in this system.

In another well-characterized system, microtubule-based motor proteins on the surface of LDs stimulate lipid transfer to ER and therefore facilitate lipoprotein assembly in liver cells (Rai et al., 2017). In rat hepatocytes, LDs are actively transported by the motor molecule kinesin-1 on microtubules to the cell periphery, which promotes MCS formation between LDs and smooth endoplasmic reticulum (sER) (Barak et al., 2013; Rai et al., 2017; Kumar et al., 2019). Kinesin-1 is recruited to LDs by directly binding to phosphatidic acid (PA) (Kumar et al., 2019). However, this binding is dependent on the metabolic state of the cells. In nutrient-rich conditions, the GTPase ADP ribosylation factor 1 (ARF1) recruits PA-producing phospholipase-D1 (PLD1) to LDs, which results in elevation of PA levels on LDs, increased association of kinesin-1 with LDs (Wilfling et al., 2014; Rai et al., 2017; Kumar et al., 2019). These LDs are then actively transported to cell periphery to form MCS with sER, which facilitates TAG production in sER and very low density lipoprotein (VLDL) assembly (Thiam et al., 2013; Rai et al., 2017; Kumar et al., 2019). In contrast, in the fasted state, insulin levels decrease, which downregulates the recruitment of ARF1 to the LDs. This diminishes microtubule-dependent LD movement and the formation of LD-sER MCSs at the periphery, resulting in reduced TAG levels (Kumar et al., 2019).

Dynein and microtubules are also involved in LD biogenesis in the alcohol-induced liver damage model (Gu et al., 2019). High-alcohol diets induce accumulation of LDs and elevate the levels of perilipins in liver cells, including the dynein-interacting protein PLIN3. Moreover, immunofluorescence staining revealed that Dync1i1 colocalizes with LDs, and PLIN3 and LDs are partially colocalized with microtubules. Depolymerizing microtubules by nocodazole or knocking down PLIN3 inhibits LD biogenesis from LD-ER contact sites, which reduces the size and distribution of LDs in AML12 cells.

The examples discussed above illustrate the importance of cytoskeletal function in regulating interactions between LDs and other organelles. Cellular modulation of the number and dynamics of these MCS is vital for LD biogenesis, lipid secretion and lipoprotein assembly.

6 CONCLUSION AND FUTURE DIRECTIONS

MCS that form between LDs and organelles including mitochondria, ER and lysosomes/vacuoles function in LD biogenesis and in transfer of lipids, FAs, unfolded proteins and surplus or toxic proteins to or from LDs. Moreover, emerging evidence supports a role for the cytoskeleton in formation of MCS between LDs and other organelles by controlling the position and movement of LDs in response to environmental cues. However, fundamental questions regarding LD MCS remain unanswered. While many tethers that link LDs to mitochondria under conditions of nutrient limitations have been identified, the mechanisms that regulate LD-mitochondria MCS formation and loss are not well understood. Although LD-ER contact sites have an established function in LD biogenesis, the mechanism underlying scission of nascent LDs from ER membranes at LD-ER MCS is not known. The finding that LDs function in ER proteostasis through transfer of unfolded proteins from ER to LDs at LD-ER MCS revealed a novel function for LDs. However, it is not clear whether this process is linked to LD biogenesis. Indeed, if mature LDs can associate with LDs to remove unfolded proteins and mitigate ER stress, the proteins serve as tethers at those LD-ER MCS and mechanisms that promote those MCS remain unknown. Moreover, the proteins that tether LDs to lysosomes/vacuoles; how liquid ordered (L_o) and disordered (L_d) domains in the vacuolar membrane contribute to MCS and vacuolar membrane dynamics at those sites, and the mechanism underlying Rab7 function in LD-lysosome/vacuole MCS in mammalian cells and yeast during μLP are all open questions. Finally, while it is clear that cytoskeleton-dependent LD motility is critical for association of LDs with other organelles in response to nutritional cues, the cytoskeleton may contribute to MCS by other mechanisms including force generation for membrane deformation or scission or for transfer of constituents to and from LDs.

AUTHOR CONTRIBUTIONS

All authors wrote and reviewed the text and bibliography. TS and LP made the final revisions. EY created the figures.

FUNDING

This work was supported by grants from the National Institutes of Health (NIH) (GM122589 and AG051047) to LP and (AG055326 and DK007647) to CS. TS and IB are supported in part by P30 CA013696 (NIH).

REFERENCES

Adeyo, O., Horn, P. J., Lee, S., Binns, D. D., Chandrahas, A., Chapman, K. D., et al. (2011). The Yeast Lipin Orthologue Pah1p Is Important for Biogenesis of Lipid Droplets. *J. Cel. Biol.* 192, 1043–1055. doi:10.1083/jcb.201010111

Andersson, L., Boström, P., Ericson, J., Rutberg, M., Magnusson, B., Marchesan, D., et al. (2006). PLD1 and ERK2 Regulate Cytosolic Lipid Droplet Formation. *J. Cel. Sci.* 119, 2246–2257. doi:10.1242/jcs.02941

Bandyopadhyay, U., and Cuervo, A. M. (2008). Entering the Lysosome through a Transient Gate by Chaperone-Mediated Autophagy. *Autophagy* 4, 1101–1103. doi:10.4161/auto.7150

Barak, P., Rai, A., Rai, P., and Mallik, R. (2013). Quantitative Optical Trapping on Single Organelles in Cell Extract. *Nat. Methods* 10, 68–70. doi:10.1038/nmeth.2287

Ben M'barek, K., Ajjaji, D., Chorlay, A., Vanni, S., Forêt, L., and Thiam, A. R. (2017). ER Membrane Phospholipids and Surface Tension Control Cellular Lipid Droplet Formation. *Dev. Cel.* 41, 591–604. doi:10.1016/j.devcel.2017.05.012

Benador, I. Y., Veliova, M., Mahdaviani, K., Petcherski, A., Wikstrom, J. D., Assali, E. A., et al. (2018). Mitochondria Bound to Lipid Droplets Have Unique Bioenergetics, Composition, and Dynamics that Support Lipid Droplet Expansion. *Cel. Metab.* 27, 869–885. doi:10.1016/j.cmet.2018.03.003

Bersuker, K., Peterson, C. W. H., To, M., Sahl, S. J., Savikhin, V., Grossman, E. A., et al. (2018). A Proximity Labeling Strategy Provides Insights into the Composition and Dynamics of Lipid Droplet Proteomes. *Dev. Cel.* 44, 97–112. doi:10.1016/j.devcel.2017.11.020

Bosma, M., Minnaard, R., Sparks, L. M., Schaart, G., Losen, M., Baets, M. H., et al. (2012). The Lipid Droplet Coat Protein Perilipin 5 Also Localizes to Muscle Mitochondria. *Histochem. Cel. Biol.* 137, 205–216. doi:10.1007/s00418-011-0888-x

Boström, P., Rutberg, M., Ericsson, J., Holmdahl, P., Andersson, L., Frohman, M. A., et al. (2005). Cytosolic Lipid Droplets Increase in Size by Microtubule-dependent Complex Formation. *Atvb* 25, 1945–1951. doi:10.1161/01.ATV.0000179676.41064.d4

Boutant, M., Kulkarni, S. S., Joffraud, M., Ratajczak, J., Valera-Alberni, M., Combe, R., et al. (2017). Mfn2 Is Critical for Brown Adipose Tissue Thermogenic Function. *Embo J.* 36, 1543–1558. doi:10.15252/embj.201694914

Brocard, L., Immel, F., Coulon, D., Esnay, N., Tuphile, K., Pascal, S., et al. (2017). Proteomic Analysis of Lipid Droplets from Arabidopsis Aging Leaves Brings New Insight into Their Biogenesis and Functions. *Front. Plant Sci.* 8, 894. doi:10.3389/fpls.2017.00894

Callies, R., Sri-Pathmanathan, R. M., Ferguson, D. Y. P., and Brindle, K. M. (1993). The Appearance of Neutral Lipid Signals in the1H NMR Spectra of a Myeloma Cell Line Correlates with the Induced Formation of Cytoplasmic Lipid Droplets. *Magn. Reson. Med.* 29, 546–550. doi:10.1002/mrm.1910290418

Chiang, H.-L., Terlecky, S. R., Plant, C. P., and Dice, J. F. (1989). A Role for a 70-kilodalton Heat Shock Protein in Lysosomal Degradation of Intracellular Proteins. *Science* 246, 382–385. doi:10.1126/science.2799391

Choi, K., Kim, H., Kang, H., Lee, S.-Y., Lee, S. J., Back, S. H., et al. (2014). Regulation of Diacylglycerol Acyltransferase 2 Protein Stability by Gp78-Associated Endoplasmic-Reticulum-Associated Degradation. *Febs J.* 281, 3048–3060. doi:10.1111/febs.12841

Choudhary, V., and Schneiter, R. (2021). A Unique Junctional Interface at Contact Sites between the Endoplasmic Reticulum and Lipid Droplets. *Front. Cel. Dev. Biol.* 9, 650186. doi:10.3389/fcell.2021.650186

Choudhary, V., Ojha, N., Golden, A., and Prinz, W. A. (2015). A Conserved Family of Proteins Facilitates Nascent Lipid Droplet Budding from the ER. *J. Cel Biol.* 211, 261–271. doi:10.1083/jcb.201505067

Choudhary, V., Golani, G., Joshi, A. S., Cottier, S., Schneiter, R., Prinz, W. A., et al. (2018). Architecture of Lipid Droplets in Endoplasmic Reticulum Is Determined by Phospholipid Intrinsic Curvature. *Curr. Biol.* 28, 915–926. doi:10.1016/j.cub.2018.02.020

Choudhary, V., El Atab, O., Mizzon, G., Prinz, W. A., and Schneiter, R. (2020). Seipin and Nem1 Establish Discrete ER Subdomains to Initiate Yeast Lipid Droplet Biogenesis. *J. Cel. Biol.* 219, e201910177. doi:10.1083/jcb.201910177

Christianson, J. C., and Ye, Y. (2014). Cleaning up in the Endoplasmic Reticulum: Ubiquitin in Charge. *Nat. Struct. Mol. Biol.* 21, 325–335. doi:10.1038/nsmb.2793

Chung, J., Wu, X., Lambert, T. J., Lai, Z. W., Walther, T. C., and Farese, R. V. (2019). LDAF1 and Seipin Form a Lipid Droplet Assembly Complex. *Dev. Cel.* 51, 551–563. doi:10.1016/j.devcel.2019.10.006

Cuervo, A. M., Knecht, E., Terlecky, S. R., and Dice, J. F. (1995). Activation of a Selective Pathway of Lysosomal Proteolysis in Rat Liver by Prolonged Starvation. *Am. J. Physiol.-Cell Physiol.* 269, C1200–C1208. doi:10.1152/ajpcell.1995.269.5.C1200

Cui, S., Hayashi, Y., Otomo, M., Mano, S., Oikawa, K., Hayashi, M., et al. (2016). Sucrose Production Mediated by Lipid Metabolism Suppresses the Physical Interaction of Peroxisomes and Oil Bodies during Germination of *Arabidopsis thaliana*. *J. Biol. Chem.* 291, 19734–19745. doi:10.1074/jbc.M116.748814

Dohi, E., Tanaka, S., Seki, T., Miyagi, T., Hide, I., Takahashi, T., et al. (2012). Hypoxic Stress Activates Chaperone-Mediated Autophagy and Modulates Neuronal Cell Survival. *Neurochem. Int.* 60, 431–442. doi:10.1016/j.neuint.2012.01.020

Duelund, L., Jensen, G. V., Hannibal-Bach, H. K., Ejsing, C. S., Pedersen, J. S., Pakkanen, K. I., et al. (2013). Composition, Structure and Properties of POPC-Triolein Mixtures. Evidence of Triglyceride Domains in Phospholipid Bilayers. *Biochim. Biophys. Acta (Bba) - Biomembr.* 1828, 1909–1917. doi:10.1016/j.bbamem.2013.03.020

Dutta, A., and Kumar Sinha, D. (2015). Turnover of the Actomyosin Complex in Zebrafish Embryos Directs Geometric Remodelling and the Recruitment of Lipid Droplets. *Sci. Rep.* 5, 13915. doi:10.1038/srep13915

Fei, W., Shui, G., Gaeta, B., Du, X., Kuerschner, L., Li, P., et al. (2008). Fld1p, a Functional Homologue of Human Seipin, Regulates the Size of Lipid Droplets in Yeast. *J. Cel. Biol.* 180, 473–482. doi:10.1083/jcb.200711136

Fei, W., Wang, H., Fu, X., Bielby, C., and Yang, H. (2009). Conditions of Endoplasmic Reticulum Stress Stimulate Lipid Droplet Formation in *Saccharomyces cerevisiae*. *Biochem. J.* 424, 61–67. doi:10.1042/BJ20090785

Finn, P. F., and Dice, J. F. (2006). Proteolytic and Lipolytic Responses to Starvation. *Nutrition* 22, 830–844. doi:10.1016/j.nut.2006.04.008

Fong, T.-H., Wu, C.-H., Liao, E.-W., Chang, C.-Y., Pai, M.-H., Chiou, R.-J., et al. (2001). Association of Globular β-Actin with Intracellular Lipid Droplets in Rat Adrenocortical Cells and Adipocytes. *Biochem. Biophys. Res. Commun.* 289, 1168–1174. doi:10.1006/bbrc.2001.6080

Freyre, C. A. C., Rauher, P. C., Ejsing, C. S., and Klemm, R. W. (2019). MIGA2 Links Mitochondria, the ER, and Lipid Droplets and Promotes De Novo Lipogenesis in Adipocytes. *Mol. Cel.* 76, 811–825. doi:10.1016/j.molcel.2019.09.011

Fujimoto, Y., Onoduka, J., Homma, K. J., Yamaguchi, S., Mori, M., Higashi, Y., et al. (2006). Long-chain Fatty Acids Induce Lipid Droplet Formation in a Cultured Human Hepatocyte in a Manner Dependent of Acyl-CoA Synthetase. *Biol. Pharm. Bull.* 29, 2174–2180. doi:10.1248/bpb.29.2174

Garbarino, J., Padamsee, M., Wilcox, L., Oelkers, P. M., D'Ambrosio, D., Ruggles, K. V., et al. (2009). Sterol and Diacylglycerol Acyltransferase Deficiency Triggers Fatty Acid-Mediated Cell Death. *J. Biol. Chem.* 284, 30994–31005. doi:10.1074/jbc.M109.050443

Garcia, E. J., Vevea, J. D., and Pon, L. A. (2018). Lipid Droplet Autophagy during Energy Mobilization Lipid Homeostasis and Protein Quality Control. *Front. Biosci.* 23, 1552–1563. doi:10.2741/4660

Garcia, E. J., Liao, P.-C., Tan, G., Vevea, J. D., Sing, C. N., Tsang, C. A., et al. (2021). Membrane Dynamics and Protein Targets of Lipid Droplet Microautophagy during ER Stress-Induced Proteostasis in the Budding Yeast, *Saccharomyces cerevisiae*. *Autophagy* 17, 2363–2383. doi:10.1080/15548627.2020.1826691

Garcia-Macia, M., Santos-Ledo, A., Leslie, J., Paish, H. L., Collins, A. L., Scott, R. S., et al. (2021). A Mammalian Target of Rapamycin-Perilipin 3 (mTORC1-Plin3) Pathway Is Essential to Activate Lipophagy and Protects against Hepatosteatosis. *Hepatology* 74, 3441–3459. doi:10.1002/hep.32048

Geltinger, F., Schartel, L., Wiederstein, M., Tevini, J., Aigner, E., Felder, T. K., et al. (2020). Friend or Foe: Lipid Droplets as Organelles for Protein and Lipid Storage in Cellular Stress Response, Aging and Disease. *Molecules* 25, 5053. doi:10.3390/molecules25215053

Gemmink, A., Daemen, S., Kuijpers, H. J. H., Schaart, G., Duimel, H., López-Iglesias, C., et al. (2018). Super-resolution Microscopy Localizes Perilipin 5 at Lipid Droplet-Mitochondria Interaction Sites and at Lipid Droplets Juxtaposing to Perilipin 2. *Biochim. Biophys. Acta (Bba) - Mol. Cel Biol. Lipids* 1863, 1423–1432. doi:10.1016/j.bbalip.2018.08.016

Giacomello, M., Pyakurel, A., Glytsou, C., and Scorrano, L. (2020). The Cell Biology of Mitochondrial Membrane Dynamics. *Nat. Rev. Mol. Cel Biol.* 21, 204–224. doi:10.1038/s41580-020-0210-7

Granneman, J. G., Moore, H.-P. H., Mottillo, E. P., Zhu, Z., and Zhou, L. (2011). Interactions of Perilipin-5 (Plin5) with Adipose Triglyceride Lipase. *J. Biol. Chem.* 286, 5126–5135. doi:10.1074/jbc.M110.180711

Groebner, J. L., Girón-Bravo, M. T., Rothberg, M. L., Adhikari, R., Tuma, D. J., and Tuma, P. L. (2019). Alcohol-induced Microtubule Acetylation Leads to the Accumulation of Large, Immobile Lipid Droplets. *Am. J. Physiol.-Gastrointest. Liver Physiol.* 317, G373–G386. doi:10.1152/ajpgi.00026.2019

Gross, S. P., Welte, M. A., Block, S. M., and Wieschaus, E. F. (2000). Dynein-Mediated Cargo Transport *In Vivo*. A Switch Controls Travel Distance. *J. Cel. Biol.* 148, 945–956. doi:10.1083/jcb.148.5.945

Grumet, L., Taschler, U., and Lass, A. (2016). Hepatic Retinyl Ester Hydrolases and the Mobilization of Retinyl Ester Stores. *Nutrients* 9, 13. doi:10.3390/nu9010013

Gu, Y., Yang, Y., Cao, X., Zhao, Y., Gao, X., Sun, C., et al. (2019). Plin3 Protects against Alcoholic Liver Injury by Facilitating Lipid export from the Endoplasmic Reticulum. *J. Cel. Biochem.* 120, 16075–16087. doi:10.1002/jcb.28889

Gupta, P., Martin, R., Knölker, H.-J., Nihalani, D., and Kumar Sinha, D. (2017). Myosin-1 Inhibition by PClP Affects Membrane Shape, Cortical Actin Distribution and Lipid Droplet Dynamics in Early Zebrafish Embryos. *PLoS ONE* 12, e0180301. doi:10.1371/journal.pone.0180301

Herms, A., Bosch, M., Reddy, B. J. N., Schieber, N. L., Fajardo, A., Rupérez, C., et al. (2015). AMPK Activation Promotes Lipid Droplet Dispersion on Detyrosinated Microtubules to Increase Mitochondrial Fatty Acid Oxidation. *Nat. Commun.* 6, 8176. doi:10.1038/ncomms8176

Hugenroth, M., and Bohnert, M. (2020). Come a Little Bit Closer! Lipid Droplet-ER Contact Sites Are Getting Crowded. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1867, 118603. doi:10.1016/j.bbamcr.2019.118603

Itoh, Y., Kida, K., Hanawa-Suetsugu, K., and Suetsugu, S. (2016). Yeast Ivy1p Is a Putative I-BAR-Domain Protein with pH-Sensitive Filament Forming Ability *In Vitro*. *Cell Struct. Funct.* 41, 1–11. doi:10.1247/csf.15014

Jägerström, S., Polesie, S., Wickström, Y., Johansson, B. R., Schröder, H. D., Højlund, K., et al. (2009). Lipid Droplets Interact with Mitochondria Using SNAP23. *Cell Biol. Int.* 33, 934–940. doi:10.1016/j.cellbi.2009.06.011

Jacob, S. (1987). Lipid Droplet Accumulation in the Heart during Fasting. *Acta Histochem.* 82, 149–152. doi:10.1016/S0065-1281(87)80020-X

Jacquier, N., Choudhary, V., Mari, M., Toulmay, A., Reggiori, F., and Schneiter, R. (2011). Lipid Droplets Are Functionally Connected to the Endoplasmic Reticulum in *Saccharomyces cerevisiae*. *J. Cel. Sci.* 124, 2424–2437. doi:10.1242/jcs.076836

Jarc, E., and Petan, T. (2019). Lipid Droplets and the Management of Cellular Stress. *Yale J. Biol. Med.* 92, 435–452. doi:10.1016/j.bbalip.2017.07.006

Joshi, A. S., Huang, X., Choudhary, V., Levine, T. P., Hu, J., and Prinz, W. A. (2016). A Family of Membrane-Shaping Proteins at ER Subdomains Regulates Pre-peroxisomal Vesicle Biogenesis. *J. Cel. Biol.* 215, 515–529. doi:10.1083/jcb.201602064

Joshi, A. S., Nebenfuehr, B., Choudhary, V., Satpute-Krishnan, P., Levine, T. P., Golden, A., et al. (2018). Lipid Droplet and Peroxisome Biogenesis Occur at the Same ER Subdomains. *Nat. Commun.* 9, 2940. doi:10.1038/s41467-018-05277-3

Kassan, A., Herms, A., Fernández-Vidal, A., Bosch, M., Schieber, N. L., Reddy, B. J. N., et al. (2013). Acyl-CoA Synthetase 3 Promotes Lipid Droplet Biogenesis in ER Microdomains. *J. Cel Biol.* 203, 985–1001. doi:10.1083/jcb.201305142

Kaushik, S., and Cuervo, A. M. (2015). Degradation of Lipid Droplet-Associated Proteins by Chaperone-Mediated Autophagy Facilitates Lipolysis. *Nat. Cel. Biol.* 17, 759–770. doi:10.1038/ncb3166

Kaushik, S., and Cuervo, A. M. (2016). AMPK-dependent Phosphorylation of Lipid Droplet Protein PLIN2 Triggers its Degradation by CMA. *Autophagy* 12, 432–438. doi:10.1080/15548627.2015.1124226

Kaushik, S., and Cuervo, A. M. (2018). The Coming of Age of Chaperone-Mediated Autophagy. *Nat. Rev. Mol. Cel. Biol.* 19, 365–381. doi:10.1038/s41580-018-0001-6

Keenan, S. N., Meex, R. C., Lo, J. C. Y., Ryan, A., Nie, S., Montgomery, M. K., et al. (2019). Perilipin 5 Deletion in Hepatocytes Remodels Lipid Metabolism and Causes Hepatic Insulin Resistance in Mice. *Diabetes* 68, 543–555. doi:10.2337/db18-0670

Khandelia, H., Witzke, S., and Mouritsen, O. G. (2010). Interaction of Salicylate and a Terpenoid Plant Extract with Model Membranes: Reconciling Experiments and Simulations. *Biophys. J.* 99, 3887–3894. doi:10.1016/j.bpj.2010.11.009

Kiffin, R., Christian, C., Knecht, E., and Cuervo, A. M. (2004). Activation of Chaperone-Mediated Autophagy during Oxidative Stress. *MBoC* 15, 4829–4840. doi:10.1091/mbc.e04-06-0477

Kilwein, M. D., and Welte, M. A. (2019). Lipid Droplet Motility and Organelle Contacts. *Contact* 2, 251525641989568. doi:10.1177/2515256419895688

Klug, Y. A., Deme, J. C., Corey, R. A., Renne, M. F., Stansfeld, P. J., Lea, S. M., et al. (2021). Mechanism of Lipid Droplet Formation by the Yeast Sei1/Ldb16 Seipin Complex. *Nat. Commun.* 12, 5892. doi:10.1038/s41467-021-26162-6

Knoblach, B., and Rachubinski, R. A. (2015). Transport and Retention Mechanisms Govern Lipid Droplet Inheritance inSaccharomyces Cerevisiae. *Traffic* 16, 298–309. doi:10.1111/tra.12247

Kong, J., Ji, Y., Jeon, Y. G., Han, J. S., Han, K. H., Lee, J. H., et al. (2020). Spatiotemporal Contact between Peroxisomes and Lipid Droplets Regulates Fasting-Induced Lipolysis via PEX5. *Nat. Commun.* 11, 578. doi:10.1038/s41467-019-14176-0

Kumar, M., Ojha, S., Rai, P., Joshi, A., Kamat, S. S., and Mallik, R. (2019). Insulin Activates Intracellular Transport of Lipid Droplets to Release Triglycerides from the Liver. *J. Cel. Biol.* 218, 3697–3713. doi:10.1083/jcb.201903102

Kuramoto, K., Okamura, T., Yamaguchi, T., Nakamura, T. Y., Wakabayashi, S., Morinaga, H., et al. (2012). Perilipin 5, a Lipid Droplet-Binding Protein, Protects Heart from Oxidative burden by Sequestering Fatty Acid from Excessive Oxidation. *J. Biol. Chem.* 287, 23852–23863. doi:10.1074/jbc.M111.328708

Kurat, C. F., Natter, K., Petschnigg, J., Wolinski, H., Scheuringer, K., Scholz, H., et al. (2006). Obese Yeast: Triglyceride Lipolysis Is Functionally Conserved from Mammals to Yeast. *J. Biol. Chem.* 281, 491–500. doi:10.1074/jbc.M508414200

Lazar, T., Scheglmann, D., and Gallwitz, D. (2002). A Novel Phospholipid-Binding Protein from the Yeast *Saccharomyces cerevisiae* with Dual Binding Specificities for the Transport GTPase Ypt7p and the Sec1-Related Vps33p. *Eur. J. Cel. Biol.* 81, 635–646. doi:10.1078/0171-9335-00290

Lee, J. S., Mendez, R., Heng, H. H., Yang, Z. Q., and Zhang, K. (2012). Pharmacological ER Stress Promotes Hepatic Lipogenesis and Lipid Droplet Formation. *Am. J. Transl. Res.* 4, 102–113.

Li, D., Song, J.-Z., Li, H., Shan, M.-H., Liang, Y., Zhu, J., et al. (2015). Storage Lipid Synthesis Is Necessary for Autophagy Induced by Nitrogen Starvation. *FEBS Lett.* 589, 269–276. doi:10.1016/j.febslet.2014.11.050

Liao, P.-C., Garcia, E. J., Tan, G., Tsang, C. A., and Pon, L. A. (2021). Roles for L O Microdomains and ESCRT in ER Stress-Induced Lipid Droplet Microautophagy in Budding Yeast. *MBoC* 32, br12. doi:10.1091/mbc.E21-04-0179

Lundin, C., Nordström, R., Wagner, K., Windpassinger, C., Andersson, H., von Heijnevon, G., et al. (2006). Membrane Topology of the Human Seipin Protein. *FEBS Lett.* 580, 2281–2284. doi:10.1016/j.febslet.2006.03.040

Luo, H., Jiang, M., Lian, G., Liu, Q., Shi, M., Li, T. Y., et al. (2018). AIDA Selectively Mediates Downregulation of Fat Synthesis Enzymes by ERAD to Retard Intestinal Fat Absorption and Prevent Obesity. *Cel Metab.* 27, 843–853.e6. doi:10.1016/j.cmet.2018.02.021

Ma, X., Qian, H., Chen, A., Ni, H.-M., and Ding, W.-X. (2021). Perspectives on Mitochondria-ER and Mitochondria-Lipid Droplet Contact in Hepatocytes and Hepatic Lipid Metabolism. *Cells* 10, 2273. doi:10.3390/cells10092273

Murley, A., Sarsam, R. D., Toulmay, A., Yamada, J., Prinz, W. A., and Nunnari, J. (2015). Ltc1 Is an ER-Localized Sterol Transporter and a Component of ER-Mitochondria and ER-Vacuole Contacts. *J. Cel. Biol.* 209, 539–548. doi:10.1083/jcb.201502033

Murley, A., Yamada, J., Niles, B. J., Toulmay, A., Prinz, W. A., Powers, T., et al. (2017). Sterol Transporters at Membrane Contact Sites Regulate TORC1 and TORC2 Signaling. *J. Cel. Biol.* 216, 2679–2689. doi:10.1083/jcb.201610032

Nguyen, T. B., Louie, S. M., Daniele, J. R., Tran, Q., Dillin, A., Zoncu, R., et al. (2017). DGAT1-Dependent Lipid Droplet Biogenesis Protects Mitochondrial Function during Starvation-Induced Autophagy. *Dev. Cel.* 42, 9–21. doi:10.1016/j.devcel.2017.06.003

Novikoff, A. B., Novikoff, P. M., Rosen, O. M., and Rubin, C. S. (1980). Organelle Relationships in Cultured 3T3-L1 Preadipocytes. *J. Cel Biol.* 87, 180–196. doi:10.1083/jcb.87.1.180

Numrich, J., Péli-Gulli, M.-P., Arlt, H., Sardu, A., Griffith, J., Levine, T., et al. (2015). The I-BAR Protein Ivy1 Is an Effector of the Rab7 GTPase Ypt7 Involved in Vacuole Membrane Homeostasis. *J. Cel Sci.* 128, 2278–2292. doi:10.1242/jcs.164905

O'Byrne, S. M., and Blaner, W. S. (2013). Retinol and Retinyl Esters: Biochemistry and Physiology. *J. Lipid Res.* 54, 1731–1743. doi:10.1194/jlr.R037648

Oku, M., Maeda, Y., Kagohashi, Y., Kondo, T., Yamada, M., Fujimoto, T., et al. (2017). Evidence for ESCRT- and Clathrin-dependent Microautophagy. *J. Cel Biol.* 216, 3263–3274. doi:10.1083/jcb.201611029

Olzmann, J. A., and Carvalho, P. (2019). Dynamics and Functions of Lipid Droplets. *Nat. Rev. Mol. Cel Biol.* 20, 137–155. doi:10.1038/s41580-018-0085-z

Palade, G. E. (1959). in *Subcellular Particles : A Symposium Held during the Meeting of the Society of General Physiologists at the Marine Biological Laboratory, Woods Hole, Massachusetts, June 9-11, 1958*. Editor T. Hayashi.

Perktold, A., Zechmann, B., Daum, G., and Zellnig, G. (2007). Organelle Association Visualized by Three-Dimensional Ultrastructural Imaging of the Yeast Cell. *FEMS Yeast Res.* 7, 629–638. doi:10.1111/j.1567-1364.2007.00226.x

Pfisterer, S. G., Gateva, G., Horvath, P., Pirhonen, J., Salo, V. T., Karhinen, L., et al. (2017). Role for Formin-like 1-dependent Acto-Myosin Assembly in Lipid Droplet Dynamics and Lipid Storage. *Nat. Commun.* 8, 14858. doi:10.1038/ncomms14858

Prasanna, X., Salo, V. T., Li, S., Ven, K., Vihinen, H., Jokitalo, E., et al. (2021). Seipin Traps Triacylglycerols to Facilitate Their Nanoscale Clustering in the Endoplasmic Reticulum Membrane. *Plos Biol.* 19, e3000998. doi:10.1371/journal.pbio.3000998

Rai, P., Kumar, M., Sharma, G., Barak, P., Das, S., Kamat, S. S., et al. (2017). Kinesin-dependent Mechanism for Controlling Triglyceride Secretion from the Liver. *Proc. Natl. Acad. Sci. U.S.A.* 114, 12958–12963. doi:10.1073/pnas.1713292114

Rambold, A. S., Cohen, S., and Lippincott-Schwartz, J. (2015). Fatty Acid Trafficking in Starved Cells: Regulation by Lipid Droplet Lipolysis, Autophagy, and Mitochondrial Fusion Dynamics. *Dev. Cel.* 32, 678–692. doi:10.1016/j.devcel.2015.01.029

Renne, M. F., and Hariri, H. (2021). Lipid Droplet-Organelle Contact Sites as Hubs for Fatty Acid Metabolism, Trafficking, and Metabolic Channeling. *Front. Cel Dev. Biol.* 9, 726261. doi:10.3389/fcell.2021.726261

Roberts, M. A., and Olzmann, J. A. (2020). Protein Quality Control and Lipid Droplet Metabolism. *Annu. Rev. Cel. Dev. Biol.* 36, 115–139. doi:10.1146/annurev-cellbio-031320-101827

Rodriguez-Navarro, J. A., Kaushik, S., Koga, H., Dall'Armi, C., Shui, G., Wenk, M. R., et al. (2012). Inhibitory Effect of Dietary Lipids on Chaperone-Mediated Autophagy. *Proc. Natl. Acad. Sci.* 109, E705–E714. doi:10.1073/pnas.1113036109

Ruggiano, A., Mora, G., Buxó, L., and Carvalho, P. (2016). Spatial Control of Lipid Droplet Proteins by the ERAD Ubiquitin Ligase Doa10. *Embo J.* 35, 1644–1655. doi:10.15252/embj.201593106

Salo, V. T., Belevich, I., Li, S., Karhinen, L., Vihinen, H., Vigouroux, C., et al. (2016). Seipin Regulates ER -lipid Droplet Contacts and Cargo Delivery. *Embo J.* 35, 2699–2716. doi:10.15252/embj.201695170

Salvador, N., Aguado, C., Horst, M., and Knecht, E. (2000). Import of a Cytosolic Protein into Lysosomes by Chaperone-Mediated Autophagy Depends on its Folding State. *J. Biol. Chem.* 275, 27447–27456. doi:10.1074/jbc.M00139420010.1016/s0021-9258(19)61529-2

Sandager, L., Gustavsson, M. H., Ståhl, U., Dahlqvist, A., Wiberg, E., Banas, A., et al. (2002). Storage Lipid Synthesis Is Non-essential in Yeast. *J. Biol. Chem.* 277, 6478–6482. doi:10.1074/jbc.M109109200

Schreiber, R., Taschler, U., Preiss-Landl, K., Wongsiriroj, N., Zimmermann, R., and Lass, A. (2012). Retinyl Ester Hydrolases and Their Roles in Vitamin A Homeostasis. *Biochim. Biophys. Acta (Bba) - Mol. Cel Biol. Lipids* 1821, 113–123. doi:10.1016/j.bbalip.2011.05.001

Schroeder, B., Schulze, R. J., Weller, S. G., Sletten, A. C., Casey, C. A., and McNiven, M. A. (2015). The Small GTPase Rab7 as a central Regulator of Hepatocellular Lipophagy. *Hepatology* 61, 1896–1907. doi:10.1002/hep.27667

Schulze, R. J., Krueger, E. W., Weller, S. G., Johnson, K. M., Casey, C. A., Schott, M. B., et al. (2020). Direct Lysosome-Based Autophagy of Lipid Droplets in Hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* 117, 32443–32452. doi:10.1073/pnas.2011442117

Schweiger, M., and Zechner, R. (2015). Breaking the Barrier-Chaperone-Mediated Autophagy of Perilipins Regulates the Lipolytic Degradation of Fat. *Cel Metab.* 22, 60–61. doi:10.1016/j.cmet.2015.06.017

Scorrano, L., De Matteis, M. A., Emr, S., Giordano, F., Hajnóczky, G., Kornmann, B., et al. (2019). Coming Together to Define Membrane Contact Sites. *Nat. Commun.* 10, 1287. doi:10.1038/s41467-019-09253-3

Seo, A. Y., Lau, P.-W., Feliciano, D., Sengupta, P., Gros, M. A. L., Cinquin, B., et al. (2017). AMPK and Vacuole-Associated Atg14p Orchestrate μ-lipophagy for Energy Production and Long-Term Survival under Glucose Starvation. *eLife* 6, 1025. doi:10.7554/eLife.21690

Shaw, A. J., Pokorny, L., Shaw, B., Ricca, M., Boles, S., and Szövényi, P. (2008). Genetic Structure and Genealogy in the Sphagnum Subsecundum Complex (Sphagnaceae: Bryophyta). *Mol. Phylogenet. Evol.* 49, 304–317. doi:10.1016/j.ympev.2008.06.009

Shiozaki, M., Hayakawa, N., Shibata, M., Koike, M., Uchiyama, Y., and Gotow, T. (2011). Closer Association of Mitochondria with Lipid Droplets in Hepatocytes and Activation of Kupffer Cells in Resveratrol-Treated Senescence-Accelerated Mice. *Histochem. Cel. Biol.* 136, 475–489. doi:10.1007/s00418-011-0847-6

Shubeita, G. T., Tran, S. L., Xu, J., Vershinin, M., Cermelli, S., Cotton, S. L., et al. (2008). Consequences of Motor Copy Number on the Intracellular Transport of Kinesin-1-Driven Lipid Droplets. *Cell* 135, 1098–1107. doi:10.1016/j.cell.2008.10.021

Singh, R., Kaushik, S., Wang, Y., Xiang, Y., Novak, I., Komatsu, M., et al. (2009). Autophagy Regulates Lipid Metabolism. *Nature* 458, 1131–1135. doi:10.1038/nature07976

Stemberger, B., Walsh, R., and Patton, S. (1984). Morphometric Evaluation of Lipid Droplet Associations with Secretory Vesicles, Mitochondria and Other Components in the Lactating Cell. *Cell Tissue Res.* 236, 471–475. doi:10.1007/BF00214252

Strauss, J. A., Shaw, C. S., Bradley, H., Wilson, O. J., Dorval, T., Pilling, J., et al. (2016). Immunofluorescence Microscopy of SNAP23 in Human Skeletal Muscle Reveals Colocalization with Plasma Membrane, Lipid Droplets, and Mitochondria. *Physiol. Rep.* 4, e12662. doi:10.14814/phy2.12662

Sui, X., Arlt, H., Brock, K. P., Lai, Z. W., DiMaio, F., Marks, D. S., et al. (2018). Cryo-electron Microscopy Structure of the Lipid Droplet-Formation Protein Seipin. *J. Cel Biol.* 217, 4080–4091. doi:10.1083/jcb.201809067

Szymanski, K. M., Binns, D., Bartz, R., Grishin, N. V., Li, W.-P., Agarwal, A. K., et al. (2007). The Lipodystrophy Protein Seipin Is Found at Endoplasmic Reticulum Lipid Droplet Junctions and Is Important for Droplet Morphology. *Proc. Natl. Acad. Sci.* 104, 20890–20895. doi:10.1073/pnas.0704154104

Tarnopolsky, M. A., Rennie, C. D., Robertshaw, H. A., Fedak-Tarnopolsky, S. N., Devries, M. C., and Hamadeh, M. J. (2007). Influence of Endurance Exercise Training and Sex on Intramyocellular Lipid and Mitochondrial Ultrastructure, Substrate Use, and Mitochondrial Enzyme Activity. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* 292, R1271–R1278. doi:10.1152/ajpregu.00472.2006

Thiam, A. R., Farese Jr, R. V., and Walther, T. C. (2013). The Biophysics and Cell Biology of Lipid Droplets. *Nat. Rev. Mol. Cel Biol.* 14, 775–786. doi:10.1038/nrm3699

Toulmay, A., and Prinz, W. A. (2013). Direct Imaging Reveals Stable, Micrometer-Scale Lipid Domains that Segregate Proteins in Live Cells. *J. Cel Biol.* 202, 35–44. doi:10.1083/jcb.201301039

Tsai, T.-H., Chen, E., Li, L., Saha, P., Lee, H.-J., Huang, L.-S., et al. (2017). The Constitutive Lipid Droplet Protein PLIN2 Regulates Autophagy in Liver. *Autophagy* 13, 1130–1144. doi:10.1080/15548627.2017.1319544

Tsuji, T., Fujimoto, M., Tatematsu, T., Cheng, J., Orii, M., Takatori, S., et al. (2017). Niemann-Pick Type C Proteins Promote Microautophagy by Expanding Raft-like Membrane Domains in the Yeast Vacuole. *eLife* 6, e25960. doi:10.7554/eLife.25960

Turró, S., Ingelmo-Torres, M., Estanyol, J. M., Tebar, F., Fernández, M. A., Albor, C. V., et al. (2006). Identification and Characterization of Associated with Lipid Droplet Protein 1: A Novel Membrane-Associated Protein that Resides on Hepatic Lipid Droplets. *Traffic* 7, 1254–1269. doi:10.1111/j.1600-0854.2006.00465.x

Valm, A. M., Cohen, S., Legant, W. R., Melunis, J., Hershberg, U., Wait, E., et al. (2017). Applying Systems-Level Spectral Imaging and Analysis to Reveal the Organelle Interactome. *Nature* 546, 162–167. doi:10.1038/nature22369

van Zutphen, T., Todde, V., de Boer, R., Kreim, M., Hofbauer, H. F., Wolinski, H., et al. (2014). Lipid Droplet Autophagy in the Yeast *Saccharomyces cerevisiae*. *MBoC* 25, 290–301. doi:10.1091/mbc.E13-08-0448

Vance, J. E. (2020). Inter-organelle Membrane Contact Sites: Implications for Lipid Metabolism. *Biol. Direct* 15, 24–28. doi:10.1186/s13062-020-00279-y

Varlakhanova, N. V., Tornabene, B. A., and Ford, M. G. J. (2018). Ivy1 Is a Negative Regulator of Gtr-dependent TORC1 Activation. *J. Cel Sci.* 131, jcs218305. doi:10.1242/jcs.218305

Vaskovicova, K., Vesela, P., Zahumensky, J., Folkova, D., Balazova, M., and Malinsky, J. (2020). Plasma Membrane Protein Nce102 Modulates Morphology and Function of the Yeast Vacuole. *Biomolecules* 10, 1476. doi:10.3390/biom10111476

Veerabagu, M., K Paul, L., Rinne, P. L., and van der Schoot, C. (2020). Plant Lipid Bodies Traffic on Actin to Plasmodesmata Motorized by Myosin XIs. *IJMS* 21, 1422. doi:10.3390/ijms21041422

Velayos-Baeza, A., Vettori, A., Copley, R. R., Dobson-Stone, C., and Monaco, A. P. (2004). Analysis of the Human VPS13 Gene Family. *Genomics* 84, 536–549. doi:10.1016/j.ygeno.2004.04.012

Vevea, J. D., Garcia, E. J., Chan, R. B., Zhou, B., Schultz, M., Di Paolo, G., et al. (2015). Role for Lipid Droplet Biogenesis and Microlipophagy in Adaptation to Lipid Imbalance in Yeast. *Dev. Cel.* 35, 584–599. doi:10.1016/j.devcel.2015.11.010

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

Vizeacoumar, F. J., Torres-Guzman, J. C., Tam, Y. Y. C., Aitchison, J. D., and Rachubinski, R. A. (2003). YHR150w and YDR479c Encode Peroxisomal Integral Membrane Proteins Involved in the Regulation of Peroxisome Number, Size, and Distribution in *Saccharomyces cerevisiae*. *J. Cel. Biol.* 161, 321–332. doi:10.1083/jcb.200210130

Vizeacoumar, F. J., Torres-Guzman, J. C., Bouard, D., Aitchison, J. D., and Rachubinski, R. A. (2004). Pex30p, Pex31p, and Pex32p Form a Family of Peroxisomal Integral Membrane Proteins Regulating Peroxisome Size and Number inSaccharomyces Cerevisiae. *MBoC* 15, 665–677. doi:10.1091/mbc.e03-09-0681

Wang, H., Bell, M., Sreenevasan, U., Hu, H., Liu, J., Dalen, K., et al. (2011a). Unique Regulation of Adipose Triglyceride Lipase (ATGL) by Perilipin 5, a Lipid Droplet-Associated Protein. *J. Biol. Chem.* 286, 15707–15715. doi:10.1074/jbc.M110.207779

Wang, H., Sreenivasan, U., Hu, H., Saladino, A., Polster, B. M., Lund, L. M., et al. (2011b). Perilipin 5, a Lipid Droplet-Associated Protein, Provides Physical and Metabolic Linkage to Mitochondria. *J. Lipid Res.* 52, 2159–2168. doi:10.1194/jlr.M017939

Wang, C.-W., Miao, Y.-H., and Chang, Y.-S. (2014). A Sterol-Enriched Vacuolar Microdomain Mediates Stationary Phase Lipophagy in Budding Yeast. *J. Cel. Biol.* 206, 357–366. doi:10.1083/jcb.201404115

Wang, H., Becuwe, M., Housden, B. E., Chitraju, C., Porras, A. J., Graham, M. M., et al. (2016). Seipin Is Required for Converting Nascent to Mature Lipid Droplets. *eLife* 5, e16582. doi:10.7554/eLife.16582

Wang, S., Idrissi, F.-Z., Hermansson, M., Grippa, A., Ejsing, C. S., and Carvalho, P. (2018). Seipin and the Membrane-Shaping Protein Pex30 Cooperate in Organelle Budding from the Endoplasmic Reticulum. *Nat. Commun.* 9, 2939. doi:10.1038/s41467-018-05278-2

Wang, J., Fang, N., Xiong, J., Du, Y., Cao, Y., and Ji, W.-K. (2021). An ESCRT-dependent Step in Fatty Acid Transfer from Lipid Droplets to Mitochondria through VPS13D–TSG101 Interactions. *Nat. Commun.* 12, 1252. doi:10.1038/s41467-021-21525-5

Weibel, G. L., Joshi, M. R., Jerome, W. G., Bates, S. R., Yu, K. J., Phillips, M. C., et al. (2012). Cytoskeleton Disruption in J774 Macrophages: Consequences for Lipid Droplet Formation and Cholesterol Flux. *Biochim. Biophys. Acta (Bba) - Mol. Cel Biol. Lipids* 1821, 464–472. doi:10.1016/j.bbalip.2011.09.015

Welte, M. A., and Gould, A. P. (2017). Lipid Droplet Functions beyond Energy Storage. *Biochim. Biophys. Acta (Bba) - Mol. Cel Biol. Lipids* 1862, 1260–1272. doi:10.1016/j.bbalip.2017.07.006

Wilfling, F., Wang, H., Haas, J. T., Krahmer, N., Gould, T. J., Uchida, A., et al. (2013). Triacylglycerol Synthesis Enzymes Mediate Lipid Droplet Growth by Relocalizing from the ER to Lipid Droplets. *Dev. Cel.* 24, 384–399. doi:10.1016/j.devcel.2013.01.013

Wilfling, F., Thiam, A. R., Olarte, M.-J., Wang, J., Beck, R., Gould, T. J., et al. (2014). Arf1/COPI Machinery Acts Directly on Lipid Droplets and Enables Their Connection to the ER for Protein Targeting. *eLife* 3, e01607. doi:10.7554/eLife.01607

Wolins, N. E., Quaynor, B. K., Skinner, J. R., Tzekov, A., Croce, M. A., Gropler, M. C., et al. (2006). OXPAT/PAT-1 Is a PPAR-Induced Lipid Droplet Protein that Promotes Fatty Acid Utilization. *Diabetes* 55, 3418–3428. doi:10.2337/db06-0399

Yamamoto, K., Takahara, K., Oyadomari, S., Okada, T., Sato, T., Harada, A., et al. (2010). Induction of Liver Steatosis and Lipid Droplet Formation in ATF6α-Knockout Mice Burdened with Pharmacological Endoplasmic Reticulum Stress. *MBoC* 21, 2975–2986. doi:10.1091/mbc.E09-02-0133

Yan, R., Qian, H., Lukmantara, I., Gao, M., Du, X., Yan, N., et al. (2018). Human SEIPIN Binds Anionic Phospholipids. *Dev. Cel.* 47, 248–256. doi:10.1016/j.devcel.2018.09.010

Yap, W. S., Shyu, P., Gaspar, M. L., Jesch, S. A., Marvalim, C., Prinz, W. A., et al. (2020). Yeast FIT2 Homolog Is Necessary to Maintain Cellular Proteostasis and Membrane Lipid Homeostasis. *J. Cel Sci.* 133, jcs248526. doi:10.1242/jcs.248526

Young, P. A., Senkal, C. E., Suchanek, A. L., Grevengoed, T. J., Lin, D. D., Zhao, L., et al. (2018). Long-chain Acyl-CoA Synthetase 1 Interacts with Key Proteins that Activate and Direct Fatty Acids into Niche Hepatic Pathways. *J. Biol. Chem.* 293, 16724–16740. doi:10.1074/jbc.RA118.004049

Yu, J., Zhang, S., Cui, L., Wang, W., Na, H., Zhu, X., et al. (2015). Lipid Droplet Remodeling and Interaction with Mitochondria in Mouse Brown Adipose Tissue during Cold Treatment. *Biochim. Biophys. Acta (Bba) - Mol. Cel Res.* 1853, 918–928. doi:10.1016/j.bbamcr.2015.01.020

Yu, Y., Li, T., Wu, N., Jiang, L., Ji, X., and Huang, H. (2017). The Role of Lipid Droplets in Mortierella Alpina Aging Revealed by Integrative Subcellular and Whole-Cell Proteome Analysis. *Sci. Rep.* 7, 43896. doi:10.1038/srep43896

Zehmer, J. K., Bartz, R., Bisel, B., Liu, P., Seemann, J., and Anderson, R. G. W. (2009). Targeting Sequences of UBXD8 and AAM-B Reveal that the ER Has a Direct Role in the Emergence and Regression of Lipid Droplets. *J. Cel. Sci.* 122, 3694–3702. doi:10.1242/jcs.054700

Zhang, K., Wang, S., Malhotra, J., Hassler, J. R., Back, S. H., Wang, G., et al. (2011). The Unfolded Protein Response Transducer IRE1α Prevents ER Stress-Induced Hepatic Steatosis. *EMBO J.* 30, 1357–1375. doi:10.1038/emboj.2011.52

Zhang, Y., Liu, X., Bai, J., Tian, X., Zhao, X., Liu, W., et al. (2016). Mitoguardin Regulates Mitochondrial Fusion through MitoPLD and Is Required for Neuronal Homeostasis. *Mol. Cel.* 61, 111–124. doi:10.1016/j.molcel.2015.11.017

Zhi, Y., Taylor, M. C., Campbell, P. M., Warden, A. C., Shrestha, P., El TahchyEl, A., et al. (2017). Comparative Lipidomics and Proteomics of Lipid Droplets in the Mesocarp and Seed Tissues of Chinese Tallow (Triadica Sebifera). *Front. Plant Sci.* 8, 1339. doi:10.3389/fpls.2017.01339

Zhu, J., Xu, M., Liu, Y., Zhuang, L., Ying, K., Liu, F., et al. (2019). Phosphorylation of PLIN3 by AMPK Promotes Dispersion of Lipid Droplets during Starvation. *Protein Cell* 10, 382–387. doi:10.1007/s13238-018-0593-9

Zoni, V., Khaddaj, R., Lukmantara, I., Shinoda, W., Yang, H., Schneiter, R., et al. (2021). Seipin Accumulates and Traps Diacylglycerols and Triglycerides in its Ring-like Structure. *Proc. Natl. Acad. Sci. U.S.A.* 118, e2017205118. doi:10.1073/pnas.2017205118

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frontiers | Frontiers in Cell and Developmental Biology

REVIEW
published: 08 June 2022
doi: 10.3389/fcell.2022.895856

VAP Proteins – From Organelle Tethers to Pathogenic Host Interactors and Their Role in Neuronal Disease

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Vesicle-associated membrane protein (VAMP)-associated proteins (VAPs) are ubiquitous ER-resident tail-anchored membrane proteins in eukaryotic cells. Their N-terminal major sperm protein (MSP) domain faces the cytosol and allows them to interact with a wide variety of cellular proteins. Therefore, VAP proteins are vital to many cellular processes, including organelle membrane tethering, lipid transfer, autophagy, ion homeostasis and viral defence. Here, we provide a timely overview of the increasing number of VAPA/B binding partners and discuss the role of VAPA/B in maintaining organelle-ER interactions and cooperation. Furthermore, we address how viruses and intracellular bacteria hijack VAPs and their binding partners to induce interactions between the host ER and pathogen-containing compartments and support pathogen replication. Finally, we focus on the role of VAP in human disease and discuss how mutated VAPB leads to the disruption of cellular homeostasis and causes amyotrophic lateral sclerosis.

Keywords: VAPB, FFAT motif, membrane contact sites, endoplasmic reticulum, pathogen-host interactions, amyotrophic lateral scelerosis, VAPA, peroxisomes

OPEN ACCESS

Edited by:
Francisco Javier Martin-Romero, University of Extremadura, Spain

Reviewed by:
Fabien Alpy, INSERM U964 Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), France
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Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *14 March 2022*
Accepted: *25 April 2022*
Published: *08 June 2022*

Citation:
Kors S, Costello JL and Schrader M (2022) VAP Proteins – From Organelle Tethers to Pathogenic Host Interactors and Their Role in Neuronal Disease. Front. Cell Dev. Biol. 10:895856. doi: 10.3389/fcell.2022.895856

INTRODUCTION

VAP was initially cloned from the marine mollusk *Aplysia californica* and named vesicle-associated membrane protein (VAMP)-associated protein of 33 kilodaltons (VAP-33) because of its ability to interact with vesicle fusion protein VAMP (also termed synaptobrevin) (Skehel et al., 1995). Since then, VAP proteins have been identified in all eukaryotic cells and reported to interact with a large number of intracellular proteins (Lev et al., 2008; Kamemura and Chihara, 2019). VAPs are C-tail-anchored (or type II) ER membrane proteins with a central coiled-coil domain and N-terminal major sperm (MSP) domain (~125 residues), which faces the cytoplasmic side (Nishimura et al., 1999). This N-terminal domain consists of an immunoglobulin-like β-sheet and is named after the *Ascaris suum* protein MSP due to its 22% sequence identity (Bullock et al., 1996; Kaiser et al., 2005). The highly conserved VAP protein family consists in mammals of VAPA and VAPB, including the VAPB splice variant VAPC which lacks both the C-terminal transmembrane domain (TMD) and the coiled-coil domain (Weir et al., 1998; Nishimura et al., 1999). Five additional VAPB splice variants were

Abbreviations: ALS, amyotrophic lateral sclerosis; FFAT, two phenylalanines (FF) in an acidic tract; MCS, membrane contact sites; MSP, major sperm protein; PI4P, phosphatidylinositol-4-phosphate; TMD, transmembrane domain; VAP, vesicle-associated membrane protein (VAMP)-associated protein.

detected at the mRNA level, although protein levels were undetectable in human tissue lysates by immunoblotting (Nachreiner et al., 2010). VAPA and VAPB share 63% sequence identity, mainly due to similarities in the MSP domain, and a clear functional difference between the paralogues has not been established. Recently, the VAP family was extended with the motile sperm domain-containing proteins 1, 2 and 3 (MOSPD1, MOSPD2 and MOSPD3), which also possess an MSP domain and share binding partners with VAPA/B, though with different affinities (Thaler et al., 2011; Mattia et al., 2018; Cabukusta et al., 2020).

VAP proteins are ubiquitously expressed in mammals (Weir et al., 1998; Nishimura et al., 1999; Skehel et al., 2000), with tissue-specific RNA expression patterns during development (Gabetta et al., 2003). They interact with a wide variety of proteins, imparting them with various functions, including organelle membrane tethering (Costello et al., 2017a), lipid transfer between organelles (Kawano et al., 2006; Ngo and Ridgway, 2009), regulation of calcium homeostasis (De Vos et al., 2012; Lindhout et al., 2019), autophagy (Zhao et al., 2018) and the unfolded protein response (UPR) (Kanekura et al., 2006; Gkogkas et al., 2008). VAP might also have extracellular functions through its cleaved and secreted MSP domain (Deidda et al., 2014), although this has been mainly studied in *C. elegans* and *D. melanogaster* (Tsuda et al., 2008; Han et al., 2012).

In this review, we provide a timely update of VAPA/B binding partners and discuss the role of VAPA/B in maintaining organelle-ER interactions and cooperation. This includes VAP hijacking by viruses and intracellular bacteria to induce interactions between the host ER and pathogen-containing compartments, and the recruitment of host proteins to these sites to support pathogen replication. Finally, we focus on how a mutation in VAPB leads to the disruption of cellular homeostasis, causing amyotrophic lateral sclerosis type 8 (ALS8).

VAP BINDING PARTNERS AND THE FUNCTIONS OF THE ENSUING COMPLEX

The VAP Interaction

The interaction of VAP with a multitude of diverse proteins means that the VAP proteins are important for many cellular processes, including organelle tethering, lipid transfer, autophagy, ion homeostasis and viral defence. **Table 1** provides an overview of the current experimentally confirmed VAP interactors and the proposed functions of the ensuing complex. Many binding partners interact with the MSP domain of VAP via a "two phenylalanines in an acidic tract" (FFAT) motif, which consists of the core consensus sequence ^{1}EFFDA-E^{7} flanked by acidic residues (Loewen et al., 2003). In the proposed interaction model, the acidic tract upstream of the core initially binds in a non-specific manner to the basic electropositive surface of the MSP domain followed by a second step, in which the core residues bind to the FFAT-binding site to form a stable complex (Kaiser et al., 2005; Furuita et al., 2010). The FFAT motif can vary in sequence quite considerably, whilst still allowing binding interaction, potentially giving proteins a different affinity for VAP (Murphy and Levine, 2016). Many known VAP interactors contain only one or no phenylalanine in the FFAT motif, and there is some redundancy in the sequence (*see* **Table 1**). In addition, the acidic tract can vary in length and the number of acidic residues. Interestingly, the VAP family proteins MOSPD1 and MOSPD3 favour motifs with "two phenylalanines in a neutral tract" (FFNT) (Cabukusta et al., 2020). Furthermore, the interaction with VAP can be modulated on multiple levels; for instance, the FFAT-VAP binding can be strengthened and reduced by (de) phosphorylation of the FFAT motif (Kumagai et al., 2014; Johnson et al., 2018; Kirmiz et al., 2018; Di Mattia et al., 2020; Guillén-Samander et al., 2021; Kors et al., 2022). VAP dimerization via the TMD and coiled-coil domains might enhance the recruitment of pre-existing homodimers of FFAT proteins (e.g. OSBP) or bring together two unrelated FFAT-containing proteins, stabilizing the complex (Kaiser et al., 2005; Kim et al., 2010). Additionally, some proteins contain two FFAT motifs, suggesting that the interactor could bind to two VAPs at the same time (e.g. PTPIP51 and ORP3, *see* **Table 1**). However, not all known VAP binding partners possess a FFAT motif–some proteins bind to the MSP in a different, FFAT-independent way, while other proteins mediate the interaction via their and VAP's TMD (**Table 1**).

Organelle Tethering

Organelles form membrane contact sites (MCS) for efficient cooperation (Silva et al., 2020). These MCS are mediated by tethering proteins that cross the two opposing membranes, bringing them in close proximity. Various proteins are attracted to these sites to fulfil and regulate specific functions, e.g. membrane lipid and calcium (Ca^{2+}) transfer between the organelles. In this section, we will describe the organelle tethering function of VAP in more detail, focussing on the FFAT motif-containing binding partners PTPIP51 and ACBD5 as examples. However, other VAP interactors and functions–many of which relate to MCS–are known. There is an abundance of processes involving VAP at other organelle-ER contacts (**Table 1**), e.g. CERT transfers ceramide from the ER to the Golgi apparatus (Kawano et al., 2006); STARD3 transfers cholesterol from the ER to endosomes (Wilhelm et al., 2017; Di Mattia et al., 2020); NIR2 transfers phosphatidylinositol from the ER to both the Golgi and plasma membrane, and phosphatidylcholine from the Golgi to the ER (Peretti et al., 2008; Chang and Liou, 2015); while the interaction of VAP with potassium (K^{+}) channel Kv2 at plasma membrane-ER contacts is important for Kv2 channel clustering and regulation of K^{+} currents (Johnson et al., 2018; Kirmiz et al., 2018; Schulien et al., 2020). Furthermore, other examples will be discussed in the sections on pathogens and amyotrophic lateral sclerosis type 8 (ALS8).

The membrane proteins PTPIP51 (also named RMDN3) and ACBD5 interact with VAPB, mediating mitochondria-ER and peroxisome-ER associations, respectively (**Figure 1**) (De Vos et al., 2012; Costello et al., 2017a; Hua et al., 2017). Knockdown of PTPIP51 or ACBD5 reduced the contacts between the respective organelle and the ER, while overexpression increased the associations (Stoica et al., 2014; Costello et al., 2017a). Whilst VAP itself does not appear to

TABLE 1 | An overview of experimentally confirmed VAP binding partners in mammalian cells.

Complex	Interaction domain		Localisation[a]	MCS	Physiological role of the VAP complex	Reference
	Binding partner	VAP				
ACBD4-VAPB	FFAT motif (score 3.5) $_{173}$RDLDSE **VFCDSLE** QL	MSP	Peroxisomes (TMD)	Peroxisome-ER	Organelle tethering function	Costello et al. (2017b), Kors et al. (2022)
ACBD5-VAPA/B	FFAT motif (score 2.5) $_{259}$SDSDSE **VYCDSME** QF	MSP	Peroxisomes (TMD)	Peroxisome-ER	Organelle tethering function, implicated in: peroxisome motility; peroxisome membrane expansion; plasmalogen synthesis; maintenance of cholesterol levels	Costello et al. (2017a), Hua et al. (2017)
α-Synuclein-VAPB	?	MSP	Cytosol, nucleus, membranes		Disrupts the **PTPIP51**-VAPB interaction, hence mitochondria-ER MCS (affecting Ca^{2+} exchange)	Paillusson et al. (2017)
ASNA1-VAPA/B	FFAT motif (score 2.0) $_{8}$WGVEAE **EFEDAPD** VE	MSP	Cytosol, ER, nucleus		Mediating interaction with the transmembrane-domain recognition complex (TRC; insertion of tail anchored ER proteins)	Baron et al. (2014)
ATF6-VAPA/B	?	MSP	ER (TMD), nucleus (cleaved)		Modulates the activity of ATF6-regulated transcription of genes involved in the unfolded protein response (UPR)	Gkogkas et al. (2008)
CALCOCO1-VAPA/B	FFAT-motif (score 3.0) $_{674}$DHMDGH **FFFSTQD** PF	MSP	Nucleus, cytosol	Autophagosome-ER	Acts as ER-phagy receptor for degradation of the tubular ER, via ATG8 interaction	Nthiga et al. (2020)
CaSR-VAPA	FFAT motif (score 2.5)[b] $_{755}$ELEDEI **IFITCHE** GS	MSP	Plasma membrane (TMD)	Plasma membrane-ER	Ca^{2+} sensing; near surface CaSR expression	Gorkhali et al. (2021)
CERT-VAPA/B	FFAT motif (score 1.0) $_{315}$SLINEE **EFFDAVE** AA	MSP	Cytosol, Golgi (PH domain)	Golgi-ER	Ceramide transfer from the ER to the Golgi apparatus, for sphingomyelin synthesis	Kawano et al. (2006), Saito et al. (2008), Kumagai et al. (2014)
CLN8-VAPA	?	?	ER (TMD), ER-Golgi intermediate compartment (ERGIC; TMD)		*Possibly*: Ceramide metabolism; endo-lysosomal dynamics	Passantino et al. (2013), Adhikari et al. (2019), Pesaola et al. (2021)
CDIP1-VAPA/B	FFAT motif (score 6.0) $_{180}$IPCLIN **DFKDVTH** TC	MSP	Endocytic compartments (MMD)		CDIP1-induced cell death	Inukai et al. (2021)
FAF1-VAPA/B	FFAT motif (score 1.0) $_{289}$SDSDGD **DFEDATE** FG	MSP	Nucleus, cytosol		Binding of ubiquitinated proteins; recruiting p97 to the ER membrane (involved in ER-associated protein degradation (ERAD))	Baron et al. (2014)
FAPP1-VAPA/B	C-terminus	?	Golgi (PH domain)	Golgi-ER	Formation of the SAC1-FAPP1-VAP complex – binding of FAPP1 to the PI4P-phosphatase SAC1 promotes the phosphatase activity	Venditti et al. (2019)
FIP200-VAPA/B	FFAT motif 1 (score 3.0) $_{725}$AESPES **DFMSAVN** EF FFAT motif 2 (score 4.0) $_{206}$ECLTRH **SYRECLG** RL	MSP	Cytosol, (pre-) autophagosomal structures, lysosomes, nucleus	Isolation membrane-ER	Formation/stabilization of the **ULK1**/FIP200-WIPI2 complex during isolation membrane expansion for autophagosome formation	Zhao et al. (2018)
GLTP-VAPA	FFAT motif (score 3.5) $_{26}$AVSHLP **PFFDCLG** SP	MSP	Cytosol		*Possibly*: Glycolipid transfer, glucosylceramide sensor	Tuuf et al. (2009), Backman et al. (2018)
HCN2-VAPA/B	TMD	TMD	ER		Regulation of HCN channel Na^{+}/K^{+} pacemaker currents; dendritic localization of HCN2	Silbernagel et al. (2018)

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TABLE 1 | (*Continued*) An overview of experimentally confirmed VAP binding partners in mammalian cells.

Complex	Interaction domain		Localisation[a]	MCS	Physiological role of the VAP complex	Reference
	Binding partner	VAP				
IFITM3-VAPA	TMD	TMD and CC	Endosomes, lysosomes, plasma membrane		Preventing the VAPA-**OSBP** association, which induces cholesterol accumulation, inhibiting viral entry	Amini-Bavil-Olyaee et al. (2013)
JMY-VAPA	FFAT motif (score 1.5)[b] $_{312}$ETDDPE **EYYES**<u>**L**</u>**S** EL	MSP	Nucleus, cytoskeleton		*Possibly*: Vesicle based transport	Schlüter et al. (2014)
Kv2.1-VAPA/B	FFAT motif (score 3.5) $_{584}$SMSSID **SFI**<u>**S**</u>**CAT** DF	MSP	Plasma membrane (TMD)	Plasma membrane-ER	Kv2 channel clustering; regulating proapoptotic K^+ currents; phosphatidylinositol homeostasis (via **NIR2** recruitment)	Johnson et al. (2018), Kirmiz et al. (2018, 2019), Schulien et al. (2020)
Kv2.2-VAPA/B	FFAT motif (score 3.0) $_{599}$STSSID **SFT**<u>**S**</u>**CAT** DF	MSP	Plasma membrane (TMD)	Plasma membrane-ER	Kv2 channel clustering	Johnson et al. (2018), Kirmiz et al. (2018)
MIGA2-VAPA/B	FFAT motif (score 1.5) $_{286}$SLTSED **SFF**<u>**S**</u>**ATE** LF	MSP	Mitochondria (TMD), lipid droplets	Mitochondria-ER	Linking reactions of *de novo* lipogenesis in mitochondria to triglyceride production in the ER	Freyre et al. (2019)
NIR1-VAPB	FFAT motif (score 0.0) $_{28}$VESSDD **EFFDARE** EM	MSP	Plasma membrane (LNS2 domain), cytosol	Plasma membrane-ER	Promoting **NIR2** recruitment for phosphatidylinositol homeostasis	Amarilio et al. (2005), Quintanilla et al. (2022)
NIR2-VAPB	FFAT motif (score 0.0) $_{343}$ENSSEE **EFFDAHE** GF	MSP	Golgi (LNS2 domain), plasma membrane (LNS2 domain), cytosol	Golgi-ER Plasma membrane-ER	Phosphatidylinositol transfer to the Golgi apparatus and phosphatidylcholine transfer to the ER (important for **CERT** and **OSBP** Golgi targeting/ function) Phosphatidylinositol transfer from the ER to the plasma membrane	Amarilio et al. (2005), Peretti et al. (2008), Chang et al. (2013), Chang and Liou (2015), Kirmiz et al. (2019)
NIR3-VAPB	FFAT motif (score 0.0) $_{338}$DESSDD **EFFDAHE** DL	MSP	Plasma membrane (LNS2 domain), cytosol	Plasma membrane-ER	Phosphatidylinositol transfer from the ER to the plasma membrane Microtubule interaction	Amarilio et al. (2005), Chang and Liou, (2015)
OSBP-VAPA	FFAT motif (score 0.0) $_{352}$DEDDEN **EFFDAPE** II	MSP	Golgi (PH domain), endosomes (PH domain), lysosomes (PH domain), cytosol	Golgi-ER Endosome-ER Lysosome-ER	Cholesterol transfer from the ER to the Golgi apparatus in exchange for PI4P Regulation of PI4P levels on endosomes Cholesterol transfer from the ER to lysosomes, regulating mTORC1 activation	Wyles et al. (2002), Loewen et al. (2003), Mesmin et al. (2013), Dong et al. (2016), Lim et al. (2019)
ORP1L-VAPA/B	FFAT motif (score 1.5) $_{469}$SILSED **EFYDALS** DS	MSP	Late endosomes/lysosomes (PH domain and ankyrin motif), autophagosome, phagolysosome	Late endosome/ lysosome (LEL)-ER Autophagosome-ER Phagolysosome-ER	Cholesterol transport from the LEL to the ER (high cholesterol) and vice versa (low cholesterol); endosome positioning Regulating autopagosome transport and maturation PI4P transfer to the ER, for phagolysosome resolution	Rocha et al. (2009), Eden et al. (2016), Wijdeven et al. (2016), Zhao and Ridgway (2017), Levin-Konigsberg et al. (2019)
ORP2-VAPA	FFAT motif (score 1.5) $_{1}$MNGEE **EFFDAVT** GF	MSP	Lipid droplets, plasma membrane, cytosol	Lipid droplet-ER	Triglyceride metabolism	Weber-Boyvat et al. (2015b)
ORP3-VAPA	FFAT motif 1 (score 1.0) $_{444}$ITDSLS **EFFDAQE** VL FFAT motif 2 (score 4.5) $_{155}$FPHEVN **HFF**<u>**S**</u>**GST** IT	MSP	Plasma membrane (PH domain), cytosol	Plasma membrane-ER Late endosome-nuclear envelope	Stimulating R-Ras signalling The nuclear transfer of extracellular vesicle-derived materials	Lehto et al. (2005), Weber-Boyvat et al. (2015a), Santos et al. (2018)

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TABLE 1 | (*Continued*) An overview of experimentally confirmed VAP binding partners in mammalian cells.

Complex	Interaction domain		Localisation[a]	MCS	Physiological role of the VAP complex	Reference
	Binding partner	VAP				
ORP4L-VAPA	FFAT motif (score 0.0) $_{445}$EEDEDT **EYFDAME** DS	MSP	Golgi (PH domain), plasma membrane	Plasma membrane-ER[c] Golgi-ER	Controlling the localization and activation of the phosphoinositide phospholipases C β3 (PLCβ3) at the plasma membrane The maintenance of Golgi structure	Pan et al. (2018), Pietrangelo and Ridgway, (2018)
ORP6-VAP	FFAT motif (score 1.0)[b] $_{488}$MSESVS **EFFDAQE** VL	MSP	Plasma membrane (PH domain), cytosol	Plasma membrane-ER	PI4P turnover	Wyles and Ridgway (2004), Mochizuki et al. (2018)
ORP7-VAP	FFAT motif (score 1.0)[b] $_{396}$LADSHT **EFFDACE** VL	MSP	Plasma membrane (PH domain), cytosol		?	Wyles and Ridgway (2004), Weber-Boyvat et al. (2015b)
ORP9L-VAPA	FFAT motif (score 1.0) $_{294}$YSSSED **EFYDADE** FH	MSP	Golgi (PH domain), cytosol	Golgi-ER	Golgi organization and protein transport; cholesterol transfer	Wyles and Ridgway (2004), Ngo and Ridgway, (2009)
PP2Cε-VAP	TMD	TMD	ER (TMD)		Dephosphorylating **CERT**, which promotes Golgi localisation of CERT and enhances the CERT-VAPA interaction	Saito et al. (2008)
PRA1-VAP	FFAT motif (score 5.5)[b] $_{66}$RLVRNV **EYYQSNY** VF	MSP	Golgi (TMD), ER (TMD)	Mitochondria-ER	ER retention of PRA1	Abu Irqeba and Ogilvie, (2020)
Prestin-VAPA	?	?	Plasma membrane (TMD)		Prestin translocation to the plasma membrane	Sengupta et al. (2010)
Protrudin-VAPA/B	FFAT motif (score 1.0) $_{280}$EAEPDE **EFKDAIE** ET	MSP, TMD	ER (TMD), endosomes (FYVE domain), plasma membrane (FYVE domain)	Endosome-ER	Endosome trafficking; stimulating process/neurite formation	Saita et al. (2009), Matsuzaki et al. (2011), Raiborg et al. (2015), Petrova et al. (2020)
PTPIP51-VAP	FFAT motif 1 (score 3.0) $_{151}$STGSSS **VYFTASS** GA FFAT motif 2 (score 3.5) $_{160}$TASSGA **TFTDAES** EG	MSP	Mitochondria (TMD)	Mitochondria-ER	Ca^{2+} delivery to mitochondria from ER stores, which regulates autophagy and synaptic function; phosphatidic acid transfer, important for mitochondrial cardiolipin synthesis	De Vos et al. (2012), Stoica et al. (2014), Gómez-Suaga et al. (2017), Gómez-Suaga et al. (2019), Yeo et al. (2021)
RAB3GAP1-VAPA/B	FFAT motif (score 0.5) $_{578}$WSDSEE **EFFECLS** DT	MSP	Cytosol		Regulating nuclear envelope formation through ERGIC	Baron et al. (2014), Hantan et al. (2014)
SCRN1-VAPA/B	FFAT motif (score 2.5) $_{394}$AEVGDL **FYDCVD** TE	MSP	Cytosol		Modulating Ca^{2+} homeostasis and synaptic vesicle cycling; ER dynamics	Lindhout et al. (2019)
SNX2- VAPB	FFAT motif 1 (score 3.5) $_{21}$LEDGED **LFTSTVS** TL FFAT motif 2 (score 2.0) $_{66}$DDDRED **LFAEATE** EV	MSP	Endosomes (PX domain)	Endosome-ER	Retromer-/WASH-dependent actin nucleation (vesicle budding) of endosomes, with a role of PI4P (*see* **OSBP**)	Dong et al. (2016)

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TABLE 1 | (*Continued*) An overview of experimentally confirmed VAP binding partners in mammalian cells.

Complex	Interaction domain		Localisation[a]	MCS	Physiological role of the VAP complex	Reference
	Binding partner	VAP				
STARD3-VAPA/B	FFAT motif (score 5.5) $_{200}$GALSEG **QFYSPPE** SF	MSP	Late endosomes (TMD)	Late endosome-ER	Cholesterol transport from the ER to endosome	Alpy et al. (2013), Wilhelm et al. (2017), Di Mattia et al. (2020)
STARD3NL-VAPA/B	FFAT motif (score 5.5) $_{201}$GGLSDG **QFYSPPE** SE	MSP	Late endosomes (TMD)	Late endosome-ER	Formation of endosomal tubules	Alpy et al. (2013)
TRPC3-VAPB	FFAT motif (score 5.0) $_{140}$QELQDD **DFYAYDE** DG	MSP	Plasma membrane	Plasma membrane-ER	Controls TRPC3's Ca^{2+} current and its receptor-mediated activation	Liu et al. (2022)
TTC39B-VAPB	FFAT motif (score 2.0) $_{76}$LEADED **VFEDALE** TI	MSP	Cytosol		Stabilizing ER-membrane protein SCAP, involved in hepatic lipogenic gene expression	Hsieh et al. (2021)
ULK1-VAPA/B	FFAT motif 1 (score 5.5) $_{87}$SVYLVM **EYCNGGD** LA FFAT motif 2 (score 5.5) $_{74}$NIVALY **DFQEMAN** SV	MSP	Cytosol, pre-autophagosomal structures	Isolation membrane-ER	Formation/stabilization of the ULK1/**FIP200**-WIPI2 complex during isolation membrane expansion for autophagy	Zhao et al. (2018)
Viperin-VAPA	C-terminus	C-terminus	ER, lipid droplets		Restricting Hepatitis C virus replication complex formation by promoting degradation of viral NS5A through VAPA	Wang et al. (2012), Ghosh et al. (2020)
VPS13A-VAPA/B	FFAT motif (score 1.0) $_{836}$EDDSEE **EFFDAPC** SP	MSP	Mitochondria (ATG homology region, PH domain), lipid droplets (PH domain)	Mitochondria-ER Lipid droplet-ER	Mitochondria elongation; glycerolipid transfer between membranes Lipid droplet size and motility; glycerolipid transfer between membranes	Kumar et al. (2018), Yeshaw et al. (2019)
VPS13C-VAPB	FFAT motif (score 0.0) $_{871}$ESESDD **EYFDAED** GE	MSP	Late endosomes/lysosomes (WD40 module), lipid droplets (PH domain)	Endolysosome-ER Lipid droplet-ER	Glycerolipid transfer between membranes Glycerolipid transfer between membranes	Kumar et al. (2018)
VPS13D-VAPB	FFAT motif (score 5.0) $_{761}$TQFSDD **EYKTPLA** TP	MSP	Golgi, mitochondria	Mitochondria-ER	Bridging the organelle membranes via MIRO at the mitochondrial membrane (likely similar with peroxisomes); membrane lipid transfer	Guillén-Samander et al. (2021)
WDR44-VAPA/B	FFAT motif (score 0.5) $_{3}$SESDTE **EFYDAPE** DV	MSP	Cytosol, endosomes, Golgi		Tubular endosome formation and/or stabilization	Baron et al. (2014), Häsler et al. (2020)
YIF1A-VAPB	TMD	MSP	ER-Golgi intermediate compartment (ERGIC; TMD); ER (TMD), Golgi (TMD)		Controls the shuttling of YIF1A between the ERGIC and the ER; promotes intracellular membrane delivery into dendrites	Kuijpers et al. (2013b)

FFAT motif scores were calculated using the FFAT scoring algorithm (best FFAT motif scores zero)[c](Murphy and Levine, 2016). Phosphorylation of serine/threonine at position 4 (double underlined) of the core (bold) of Phospho-FFAT motifs is critical for VAP binding (Di Mattia et al., 2020). Phosphorylation of serine/threonine at position 5 (underlined) of FFAT motifs abolishes VAP binding (Kors et al., 2022). The cellular localisations of the binding partners are listed. The physiological role describes the function of the VAP complex (binding partners may also have been implicated in other non-VAP related processes, or functions might not have been directly linked to VAP yet).

ACBD4/5, acyl-CoA-binding domain-containing protein 4/5; ASNA1 (TRC40), arsenite-stimulated ATPase; ATF6, activating transcription factor 6; CALCOCO1, calcium-binding and coiled-coil domain-containing protein 1; CaSR, calcium-sensing receptor; CERT, ceramide transfer protein; CLN8, ceroid-lipofuscinosis neuronal protein 8; CDIP1, cell death-inducing p53-target protein 1; FAF1, FAS-associated factor 1 (ubiquitin-binding protein); FAPP1, phosphatidylinositol-four-phosphate adapter protein 1; FIP200, FAK family kinase-interacting protein of 200 kDa; GLTP, glycolipid transfer protein; HCN2, hyperpolarization-activated cyclic nucleotide-gated channel 2; IFITM3, interferon-inducible transmembrane protein 3; JMY, junction-mediating and -regulatory protein; Kv2, potassium voltage-gated channel subfamily B; MCS, membrane contact site; MIGA2, mitoguardin 2; MMD, monotopic integral membrane domain; MSP, major sperm protein; NIR, PYK2 N-terminal domain-interacting receptor; ORP, oxysterol-binding protein-related protein; OSBP, oxysterol-binding protein; PP2Cε, protein phosphatase 2Cε; PRA1, prenylated Rab acceptor 1; PTPIP51, protein tyrosine phosphatase-interacting protein 51; RAB3GAP1, RAB3 GTPase-activating protein catalytic subunit; SCRN1, secernin-1; SNX2, sorting nexin-2; STARD3, StAR-related lipid transfer protein 3; STARD3NL, STARD3 N-terminal like; TMD, transmembrane domain; TRPC3, transient receptor potential channel 3; TTC39B, tetratricopeptide repeat domain containing protein 39 B; ULK1, UNC-51-like autophagy-activating kinase 1; Viperin, Virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible; VPS13, Vacuolar protein sorting-associated protein 13; WDR44, WD repeat-containing protein 44; YIF1A, YIP1-interacting factor homologue A.

[a]Due to their interaction with VAP, the proteins also localise at the ER (ER is only mentioned if the protein contains another ER targeting domain, e.g. TMD).

[b]Predicted FFAT motif, but not confirmed.

[c]The FFAT score does not indicate the definite binding strength.

possess lipid binding capacity, many of its interacting partners have lipid binding properties, including PTPIP51 and ACBD5. PTPIP51 has a tetratricopeptide repeat (TPR) domain with which it can bind and transfer phosphatidic acid (PA) (Ito et al., 2021; Yeo et al., 2021). PA supply to mitochondria from the ER is required for the synthesis of cardiolipin, an important phospholipid of the inner mitochondrial membrane, which was decreased upon depletion of PTPIP51. This function was independent of the tethering function of PTPIP51 (Yeo et al., 2021). Mitochondria-ER contacts are also important for cellular Ca^{2+} homeostasis, with transport between the organelles mediated by the IP3R-GRP75-VDAC1 complex. Although not directly involved in Ca^{2+} transfer, the PTPIP51-VAPB interaction plays an important tethering role to allow the Ca^{2+} uptake by mitochondria from ER stores (De Vos et al., 2012). This PTPIP51-VAPB-regulated Ca^{2+} delivery modulates autophagosome formation and synaptic activity (Gómez-Suaga et al., 2017, 2019). Additionally, PTPIP51 was shown to be involved in the mitochondrial Ca^{2+} overload during cardiac ischemia/reperfusion, by increasing the mitochondria-sarcoplasmic reticulum contacts (Qiao et al., 2017).

ACBD5 has an acyl-CoA binding (ACB) domain, which has been shown to have lipid/fatty acid binding capacity *in vitro* (Yagita et al., 2017), but it is not yet clear if it directly transfers lipids between peroxisomes and the ER. However, as ACBD5 deficient patients present with accumulation of very long chain fatty acids (VLCFA), it is suggested that ACBD5 facilitates VLCFA transport into peroxisomes for degradation via the peroxisomal ABC transporter for VLCFA (Ferdinandusse et al., 2017; Yagita et al., 2017; Herzog et al., 2018). The ACBD5-VAPB mediated peroxisome-ER contacts have also been implicated in the regulation of peroxisome motility and positioning, and the delivery of lipids for peroxisomal membrane expansion to maintain peroxisome biogenesis (**Figure 1**) (Costello et al., 2017a; Hua et al., 2017; Darwisch et al., 2020). ACBD5 and VAPB are also required to support the transfer of plasmalogen precursors, of which the synthesis is initiated in peroxisomes and completed in the ER, and for the maintenance of cholesterol levels (Hua et al., 2017; Herzog et al., 2018).

The examples above illustrate some of the various processes that occur at mitochondria-ER and peroxisome-ER contact sites. These processes appear to require contacts to be in a dynamic equilibrium, with reduced contacts reducing the required substrate transfer but increased contacts potentially also resulting in an excess of exchange. For example, whilst loss of PTPIP51-VAPB stimulates autophagy, increased PTPIP51-VAPB inhibits autophagy implying that dynamism in the mitochondria-ER interaction is required for this process (Gómez-Suaga et al., 2017)). In a similar way, whilst loss of ACBD5-VAPB tethering appears to limit peroxisomal membrane expansion, increased ACBD5 levels lead to peroxisomal elongation, potentially implying an excess membrane expansion (Costello et al., 2017a; Kors et al., 2022). Overall, this suggests that these organelle interactions involving VAP protein tethers are highly regulated. One way to regulate tethers would be to modulate the level of interaction between VAP and its interaction partners. In line with this, we revealed that the ACBD5-VAPB tether can be modulated by phosphorylation of serine/threonine residues within the acidic tract of the FFAT motif (Kors et al., 2022), a mechanism initially described for CERT (Kumagai et al., 2014). Phosphorylation of these residues mimics the canonical aspartic and glutamic acid residues, supporting the acidic environment and enhancing binding to VAPB. Notably, the acidic tract of PTPIP51 is also mainly composed of serine/threonine residues, suggesting that phosphorylation of these residues could as well modulate the binding of PTPIP51 to VAPB. Indeed, *in vitro* studies with PTPIP51 FFAT peptide and VAPB protein revealed a low affinity suggesting a minor contribution to mitochondria-ER tethering (Yeo et al., 2021). Although this may be different *in vivo*, phosphorylation of the acidic tract and the FFAT core of PTPIP51 (*see* below) could stengthen the interaction.

In addition to the acidic tract, phosphorylation of the core FFAT motif of both PTPIP51 and ACBD5 also regulates their interaction to VAPB. However, the different positions of the phosphorylated residues have opposing effects on the binding. Phosphorylation of PTPIP51 at position 4 of the FFAT core (^{1}VYFTASS^{7}) is critical for VAPB binding *in vitro* (Di Mattia et al., 2020), while phosphorylation of ACBD5 at position 5 of the FFAT core (^{1}VYCDSME^{7}) abolishes the interaction with VAPB (Kors et al., 2022). The canonical FFAT motif possesses aspartic acid (D) at position 4, which could be mimicked by phosphorylated threonine (T) at this position in PTPIP51 to enhance the VAPB binding. The residue at position 5 of the FFAT core–alanine (A) in the canonical motif–binds the VAP MSP domain in a hydrophobic pocket (Kaiser et al., 2005; Furuita et al., 2010). Adding a phosphate group to the serine (S) at this position in ACBD5 likely causes steric hindrance, blocking the interaction. We recently showed that GSK3β can directly phosphorylate this serine residue of the ACBD5 FFAT core (**Figure 1**). Accordingly, increased GSK3β activity inhibited the ACBD5-VAPB interaction and hence peroxisome-ER contacts, while reduced GSK3β activity increased the organelle associations (Kors et al., 2022). Interestingly, GSK3β also negatively regulates the PTPIP51-VAPB interaction and mitochondria-ER associations, although the precise mechanism is not known (Stoica et al., 2014, 2016; Gómez-Suaga et al., 2022). It was shown that the ALS-associated proteins TDP-43, FUS and C9orf72-derived dipeptide repeat polypeptides (DPR) activate GSK3β, causing disruption of the mitochondria-ER tether and membrane contacts. This suggests altered mitochondria-ER and peroxisome-ER MCS in TDP-43/FUS/C9orf72-induced pathologies.

Overall, there are three regulation mechanisms involving phosphorylation of FFAT motifs: *1*) phosphorylation of residues in the acidic tract enhances the interaction with VAP, acting as a potential fine-tuning mechanism (Kumagai et al., 2014; Di Mattia et al., 2020; Kors et al., 2022); *2*) phosphorylation of S/T in position 4 acts as a switch mechanism (OFF/ON), being critical for VAP binding and defines the so-called "Phospho-FFAT motif" (Kirmiz et al., 2018; Di Mattia et al., 2020; Guillén-Samander et al., 2021); and *3*) phosphorylation of S/T in position 5 also acts as a switch mechanism (ON/OFF), but in this case the phosphorylated FFAT motif is not able to interact with VAP (Mikitova and Levine, 2012; Kors et al., 2022).

VAP HIJACKING BY VIRUSES AND BACTERIA

The VAP proteins are exploited by various viruses and intracellular bacteria for their replication. Some pathogens hijack VAP via

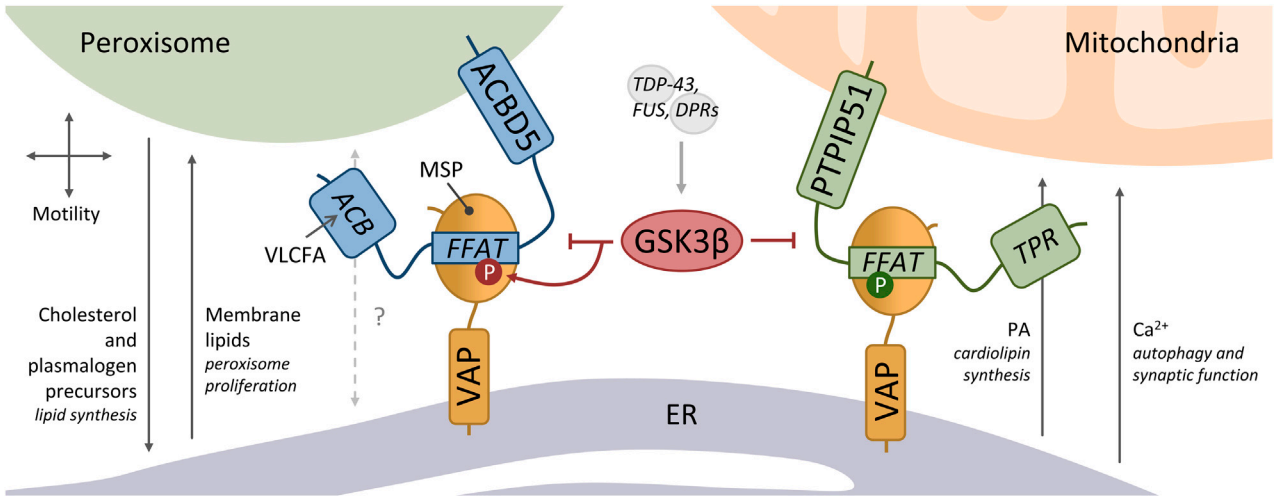

FIGURE 1 | Peroxisome-ER and mitochondria-ER membrane contacts tethered by VAP. ACBD5 interacts via its FFAT motif to the major sperm (MSP domain) of VAP to mediate peroxisome-ER contacts. These peroxisome-ER contacts have been implicated in peroxisome motility, the transfer of cholesterol and plasmalogen precursors for further synthesis in the ER, and the transfer of membrane lipids for peroxisome proliferation. ACBD5 has an acyl-CoA binding (ACB) domain which likely binds very long chain fatty acids (VLCFA). PTPIP51 also binds to the VAP-MSP domain via a FFAT motif, which mediates mitochondria-ER contacts. PTPIP51 has a tetratricopeptide repeat (TPR) domain with which it can bind and transfer phosphatidic acid (PA) to the mitochondria - required for the synthesis of cardiolipin. Ca^{2+} uptake by mitochondria from ER stores at these contacts modulates autophagosome formation and synaptic activity. GSK3β negatively regulates both peroxisome-ER and mitochondria-ER associations. GSK3β acts on the ACBD5-VAP tether by directly phosphorylating the serine residue (S) at position 5 of the ACBD5 FFAT core (^{1}VYCDSME^{7}). GSK3β can be activated by the ALS-associated proteins TDP-43, FUS and C9orf72-derived dipeptide repeat polypeptides (DPR). Phosphorylation of PTPIP51 at position 4 of the FFAT core (^{1}VYFTASS^{7}) is critical for binding to VAP.

FFAT motif mimicry, while others express pathogenic proteins that interact with VAP or VAP-interactors in other ways. Below we describe how different viruses and bacteria make use of the many functions of VAP for membrane remodelling, the formation of MCS between the host ER and pathogen-containing compartments, and targeting host MCS components to rewire the host lipid metabolism and other processes.

FFAT Motif-Containing Pathogenic Proteins

Chlamydia trachomatis

The bacterium *Chlamydia trachomatis* is an intracellular pathogen, causing non-congenital blindness, and is the most common sexually transmitted infection worldwide. The bacterium proliferates inside the cell in a membranous compartment, called an inclusion. The integral inclusion membrane protein IncV has been found to directly interact with VAPA/B via two FFAT motifs upon *C. trachomatis* infection ($_{280}$DSSSSS EYMDALE TV; $_{256}$ESSSSS SFHTPPN SD; **Figure 2A**) (Stanhope et al., 2017). Overexpression of IncV in *C. trachomatis*-infected cells enhanced the recruitment of VAPA and the ER to the inclusion membrane, suggesting that IncV promotes the formation of inclusion-ER MCS. However, depletion of IncV had only a moderate impact on VAPA enrichment at the inclusion membrane, suggesting that other proteins contribute to the stability of inclusion-ER MCS (e.g. IncD-CERT-VAPA/B complex, *see* below). The two FFAT motifs of IncV both have an acidic tract consisting of multiple serine residues, suggesting that, like ACBD5, its interaction with VAPA/B could be regulated by phosphorylation of these residues, to mimic the negative charge of the conventional acidic residues (Stanhope et al., 2017). The exact contribution of the IncV-VAP interaction in *Chlamydia* pathogenesis remains to be determined.

In mammalian cells, ceramide is transported from the ER to the Golgi complex at MCS by transport protein CERT for the synthesis of the membrane lipid sphingomyelin (**Table 1**). Another *C. trachomatis* integral inclusion membrane protein, IncD, has been found to interact with host CERT, recruiting CERT and thus its binding partner VAPA/B to the inclusion membrane (**Figure 2A**) (Derré et al., 2011; Agaisse and Derré, 2014; Kumagai et al., 2018). In this way the IncD-CERT-VAPA/B complex may facilitate ceramide transfer from the ER to the inclusion membrane, where it is converted to sphingomyelin with the use of host and/or bacterial sphingomyelin synthases (Elwell et al., 2011; Tachida et al., 2020). This CERT-dependent sphingomyelin pathway is critical for *C. trachomatis* replication.

Another example of how *C. Trachomatis* hijacks components usually present at host ER-organelle MCS is STIM1. This ER-resident Ca^{2+} sensor protein colocalized with VAPB at inclusion-ER MCS (Agaisse and Derré, 2015). However, the plasma membrane Ca^{2+} channel ORAI1, the interaction partner of STIM1 at PM-ER MCS, did not associate with the inclusion membrane. Instead, STIM1 may work with another Ca^{2+} channel: IP3R, an ER protein present at mitochondria-ER MCS. IP3R has been found to bind both STIM1 (Santoso et al., 2011) and the inclusion membrane protein MrcA, presumably forming a Ca^{2+} signalling complex at the inclusion-ER MCS (Nguyen et al., 2018). Both STIM1 and ITPR3 are required for chlamydial release via extrusion of the inclusion. The regulation of local Ca^{2+} levels may influence the myosin motor complex, which promotes the extrusion.

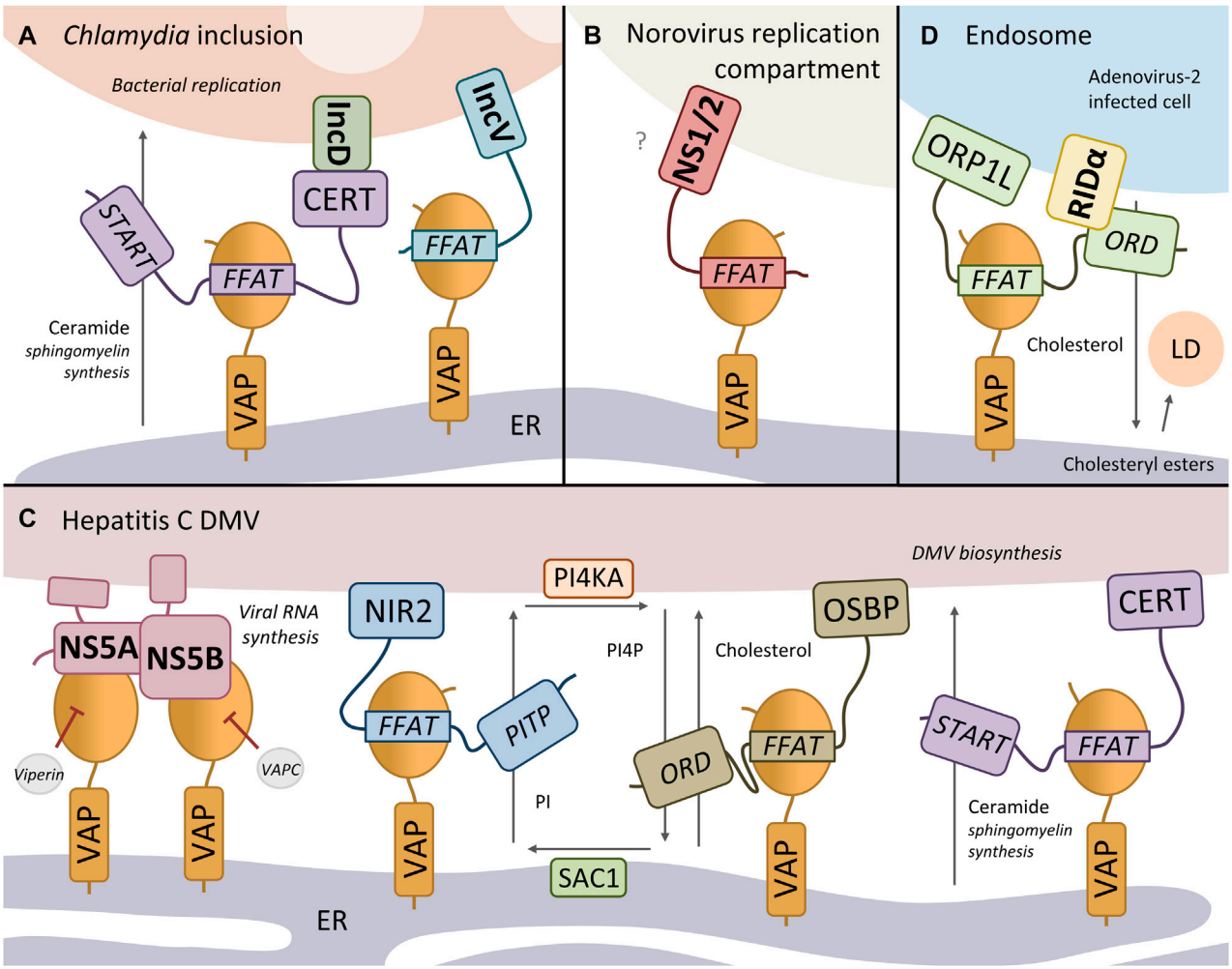

FIGURE 2 | VAP hijacking by bacteria and viruses. **(A)** The Chlamydia integral inclusion membrane proteins IncV and IncD are both found in a complex with VAP. While IncV binds VAP directly via a FFAT motif, IncD interacts with VAP-interactor CERT, forming an IncD-CERT-VAP complex at inclusion-ER contact sites. CERT may facilitate ceramide transfer from the ER to the inclusion membrane for the synthesis of sphingomyelin, important for *C. trachomatis* replication. **(B)** The murine norovirus protein NS1/2 binds VAP via its FFAT motif, critical for viral replication. The NS1/2-VAP may tether the replication membrane to the ER. **(C)** VAP binds to the hepatitis C virus proteins NS5A and NS5B, important for viral RNA replication at double-membrane vesicles (DMVs). VAP supports the viral replication by recruiting host NIR2, OSBP, CERT to the DMV-ER contact site. NIR2 transfers PI from the ER membrane to the DMV membrane, where it is converted to PI4P by the PI4P-kinase PI4KA. The PI4P is then exchanged for cholesterol from the ER by OSBP. The PI4P-phosphatase SAC1 converts PI4P back to PI. CERT transports ceramide from the ER to the DMV, where it is converted to sphingomyelin, important for the biosynthesis of DMVs. **(D)** The Adenovirus-2 protein RIDα directly interacts and recruits ORP1L to maturing early endosomes to form endosome-ER MCS via ORP1L-VAP binding, which facilitates the transport of cholesterol from endosomes to the ER. Here, cholesterol is converted into cholesteryl esters, which are stored in lipid droplets (LD). Bacterial/viral proteins are indicated in bold.

Overall, the interaction of *Chlamydia* membrane protein IncV with VAPA/B promotes the formation of inclusion-ER MCS within cells. At these sites, *C. trachomatis* redirects several host proteins for sphingomyelin synthesis (e.g. CERT via IncD; important for bacterial replication) and Ca^{2+} signalling (e.g. STIM1 via MrcA; bacterial extrusion) to assist its pathogenicity.

Norovirus

Noroviruses are non-enveloped RNA viruses and the primary cause of gastroenteritis. The murine and human (GI) norovirus protein NS1/2 has been reported to interact with VAPA/B (**Figure 2B**) (Ettayebi and Hardy, 2003; McCune et al., 2017). Structural analysis revealed that the murine NS1/2-VAP interaction is mediated by a FFAT-motif mimic located in the N-terminal NS1 domain of NS1/2 ($_{40}$ESEDEV NYMTPPE QE) (McCune et al., 2017). The FFAT-motif is conserved across murine norovirus strains, although the inherently disordered NS1 domain itself is not well conserved in contrast to the NS2 domain (Baker et al., 2012). Interestingly, NS1/2 has been found to form dimers, a property of many FFAT motif-containing proteins, which could stabilize the interaction with VAP-

dimers. It would be interesting to determine whether the human NS1/2^{GI}-VAP interaction is also mediated via a FFAT motif.

Strikingly, mutagenesis of the NS1/2 FFAT residues critical for VAP binding eliminated virus replication (McCune et al., 2017). Additionally, VAPA depletion in cells showed that VAPA was important in the early stage of norovirus replication. However, it is not clear how the NS1/2-VAP interaction contributes to the viral replication cycle. Localisation of NS1/2 to the ER might contribute to the formation of the membranous viral replication compartment, possibly by bridging the ER and replication membrane via its interaction with VAP and putative transmembrane domain (Baker et al., 2012).

VAP-Exploiting Pathogens

Hepatitis C Virus

Hepatitis C virus (HCV) is an enveloped RNA virus that predominantly infects liver cells, and can cause liver cirrhosis and cancer. Upon HCV infection, a so-called membranous web, consisting primarily of double-membrane vesicles (DMVs), is formed, that is thought to be the site of viral RNA replication. Three HCV proteins have been reported to associate with VAP. While a direct interaction for the viral NS3/4A protease was not examined (Ramage et al., 2015), structural studies have looked into the binding domains of HCV proteins NS5A and NS5B. The viral RNA-dependent RNA polymerase NS5B interacts via its C-terminal auto-regulatory motif with the MSP domain of VAPA/B (**Figure 2C**) (Gupta and Song, 2016). This C-terminal motif seems to define whether NS5B is in a folded, auto-inhibitory state, or in a disordered, active state that binds to VAP and initiates RNA synthesis. Additionally, several studies report an interaction between HCV protein NS5A and VAPB, although they attribute the interaction to different domains. One study reveals that NS5A forms a dynamic complex with VAP-MSP by interacting via its disordered C-terminal D3 domain (Gupta et al., 2012). However, other studies report that the coiled-coil domain and transmembrane domain of VAPA/B and other residues of NS5A are essential for NS5A-VAP binding (Tu et al., 1999; Hamamoto et al., 2005; Goonawardane et al., 2017; Wang and Tai, 2019). Phosphorylation of NS5A has been reported to regulate the interaction with VAP (Evans et al., 2004; Goonawardane et al., 2017).

Overexpression and knockdown studies show that the VAP proteins play an important, but yet undefined role in the formation of the HCV replication complex and in RNA replication (Gao et al., 2004; Hamamoto et al., 2005). Although the function of NS5A/B-VAP binding in HCV infection is not fully understood, recent studies are starting to decipher how VAPA/B supports the viral replication. It has been suggested that VAP, NIR2 and OSBP operate in a phosphoinositide cycle between the ER and HCV DMV membrane (**Figure 2C**). Both VAP and the VAP-interactor NIR2 are required to upregulate phosphatidylinositol-4-phosphate (PI4P) levels during HCV infection (Wang and Tai, 2019), indicating that the phosphatidylinositol (PI) transfer protein NIR2 transfers PI from the ER membrane to the DMV membrane, which is then used to generate PI4P by phosphatidylinositol 4-kinase III α (PI4KA) (Berger et al., 2011). Interestingly, NS5A was shown to associate with and stimulate PI4KA activity. The PI4P is then exchanged for cholesterol from the ER by the VAP-interactor OSBP (Wang et al., 2014). The PI4P enrichment of the DMV membrane can also recruit other PI4P-interacting proteins to the DMV-ER MCS such as the VAP-interactor CERT, which transports ceramide from the ER to the DMV, where it can be converted to sphingomyelin, important for the biosynthesis of DMVs (Gewaid et al., 2020). NIR2, OSBP and CERT normally function at the Golgi-ER MCS (**Table 1**).

To inhibit the replication of HCV, the cell has mechanisms to disrupt the NS5A/B-VAPA/B binding. The ER-associated virus inhibitory protein Viperin, which binds to both NS5A and the C-terminal region of VAPA (**Table 1**), promotes the degradation of NS5A, an effect that is enhanced by VAPA (Wang et al., 2012; Ghosh et al., 2020). VAPC, an unstructured VAPB splice variant, acts as an endogenous inhibitor by binding to NS5B, interrupting the interaction of NS5B with VAPA/B (Kukihara et al., 2009; Goyal et al., 2012). The ability of VAPC to negatively regulate HCV replication has been of interest in anti-HCV drug development (Wen et al., 2011). Another potential anti-HCV drug also acts via disrupting the viral-host protein interaction; bicyclol restricts HCV replication by upregulating FFAT-motif containing protein GLTP (**Table 1**), which interrupted the interaction between VAPA and NS5A (Huang et al., 2019).

Overall, it seems that the VAP proteins anchor the viral RNA replication machinery to the ER membrane via viral NS5A/B interaction, and recruit host VAP interactors (e.g. NIR2, OSBP, CERT) for the synthesis of cholesterol and sphingomyelin, important for HCV replication. Targeting VAP in this way allows pathogens to use a single degenerate and potentially regulatable FFAT motif to interact with a range of useful host proteins.

Other Pathogens and Strategies for Utilisation of VAP

In addition to HCV, several other viruses hijack cholesterol trafficking within the cell. The Aichi virus (AiV) proteins 2B, 2BC, 2C, 3A, and 3AB are found in a complex with VAPA/B, OSBP and other components of the cholesterol transport machinery at Golgi-ER MCS such as the PI4P-phosphatase SAC1 and ACBD3 (which recruits PI4KB) (Sasaki et al., 2012; Ishikawa-Sasaki et al., 2018). The proteins are recruited to AiV genome replication sites at the replication organelle (RO)-ER MCS, where cholesterol accumulates in the RO membrane. Knockdown of each component resulted in inhibition of AiV RNA replication. Other viruses that utilise the OSBP-cholesterol transport to facilitate RNA synthesis at RO membranes include poliovirus (Arita, 2014), rhinovirus (Roulin et al., 2014) and encephalomyocarditis virus (EMCV) (Dorobantu et al., 2015). Although no virus-VAP (interactor) complexes have been reported for these viruses, poliovirus and EMCV proteins bind to PI4KB and PI4KA respectively, which stimulates PI4P production and leads to recruitment of OSBP.

The Adenovirus-2 (Ad2) adopts a different mechanism to employ the host cholesterol transport pathway. The Ad2 membrane protein RIDα directly interacts and recruits sterol-

binding protein ORP1L to maturing early endosomes to form endosome-ER MCS via ORP1L-VAP binding (Cianciola et al., 2017) (**Figure 2D**). RIDα stabilizes the interaction between ORP1L and VAP, which supports the transport of cholesterol from maturing endosomes to the ER under high cholesterol conditions. The RIDα-ORPL1-VAP interaction induces the conversion of cholesterol into cholesteryl esters, which are stored in lipid droplets. This change in cholesterol trafficking attenuates proinflammatory TLR4 signalling involved in the innate immune response. ORP1L is also hijacked by the intracellular bacterium *Coxiella burnetii*, which forms a lysosome-like parasitophorous vacuole (PV) in the host cell for its replication (Justis et al., 2017). ORP1L is recruited to the PV by an unknown PV membrane protein, while also associating with ER-VAP. Although the function of ORP1L at PV-ER MCS in *C. burnetii* pathogenicity is unclear, ORP1L is important for PV expansion.

Herpes simplex virus type-1 (HSV-1) replicates its DNA and assembles its capsids in the host cell nucleus. The virion then crosses the nuclear envelope for further maturation in the cytoplasm. VAPB contributes to this nuclear egress as knockdown led to nuclear virion accumulation, however its exact role in this is still unclear (Saiz-Ros et al., 2019). VAP also plays a role in the replication of another DNA virus, the human papillomavirus 16 (HPV-16). However, instead of a role in nuclear egress, VAP is important in the nuclear entry pathway of HPV-16 (Siddiqa et al., 2018). Virus particles enter the cell via endocytic uptake, disassemble into protein complexes that traffic to the *trans*-Golgi-network (TGN) and then access the nucleus during mitosis when the nuclear envelope breaks down. VAP is required for the endosome-to-Golgi viral protein delivery, as it is essential for the formation of endosomal tubules induced upon HPV-16 infection. Whether these viruses exploit VAP directly via viral protein interactions or via other mechanism needs to be further elucidated.

The genetic disease cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR) protein. Patients have an increased susceptibility to bacterial infections such as *Pseudomonas aeruginosa* infection, which aggravates CF. *P. aeruginosa* exploits VAPB's mitochondrial tethering function for infection (Rimessi et al., 2020). The bacteria induced increased VAPB and PTPIP51 expression in CF bronchial cells, but not in non-CF cells. The consequent increase in mitochondria-ER contacts caused impairment of autophagy, inducing inflammation and disease progression.

Overall, a variety of different pathogens utilise VAP interaction and modulation to allow them increased access to host resources. This likely reflects the multifunctionality of VAP as a versatile access point (Murphy and Levine, 2016) to the ER membrane and also the diversity of its interaction partners, which have roles in many different cellular functions. Therapeutic strategies which attempt to prevent pathogen access to VAP could perhaps be feasible but would need to be carefully targeted as inhibition of VAP function itself has a dramatic effect on cellular function and is linked to numerous neuronal disorders, as addressed in the following section.

THE ROLE OF VAPB IN NEURONAL DISORDERS

VAPB has been linked to several neurological disorders, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and the α-synucleinopathies, Parkinson's disease (PD) and multiple system atrophy (MSA). This is via mutations in VAPB (ALS, PD) (Nishimura et al., 2004a; Kun-Rodrigues et al., 2015), disruption of VAPB's interaction with PTPIP51 and hence ER-mitochondria contacts (ALS, AD, and PD via α-Synuclein binding, *see* **Table 1**) (Paillusson et al., 2017; Lau et al., 2020; Gómez-Suaga et al., 2022) or reduced VAPB levels (ALS, AD, MSA) (Anagnostou et al., 2010; Lau et al., 2020; Mori et al., 2021). Recent findings on the role of mutated VAPB in the pathogenesis of ALS are discussed in more detail below.

Clinical Features of Amyotrophic Lateral Sclerosis Type 8 (ALS8)

An autosomal dominant missense mutation in VAPB, resulting in a substitution of proline to serine at codon 56 (P56S), was initially found in several Brazilian families (Nishimura et al., 2004a, 2004b; Marques et al., 2006). The patients presented with a heterogeneous phenotype of typical ALS, atypical ALS and late onset spinal muscular atrophy (SMA), and was termed ALS8 (OMIM 608627) (Nishimura et al., 2004a). Patients with ALS8 have predominant lower motor neuron involvement, with symptoms including progressive muscle weakness (mainly in the lower limbs), muscle atrophy, cramp, tremor, fasciculations, pain, abdominal protrusion, autonomic dysfunction (e.g. choking, constipation), and subtle cognitive and behavioural impairments (Nishimura et al., 2004a, 2004b; Marques et al., 2006; Funke et al., 2010; Kosac et al., 2013; Di et al., 2016; Chadi et al., 2017; Sun et al., 2017; Guber et al., 2018; de Alcântara et al., 2019; Trilico et al., 2020; Nunes Gonçalves et al., 2021; Temp et al., 2021; Leoni et al., 2022). ALS8's clinical heterogeneity manifests not only in the symptoms but also in the age of onset (reported at 20–57 years) and the disease progression (rapid [<5 years] to slow [30+ years]). To understand the mechanisms behind this phenotypic variability, researchers compared gene expression profiles of iPSC (induced pluripotent stem cells)-derived motor neurons from mild and severe ALS8 patients (Oliveira et al., 2020). VAPB mRNA and protein levels were equally downregulated in mild and severe patients. The differentially expressed genes found in the study were associated with pathways involved in protein translation and protein targeting to the ER; pathways that may mitigate neurodegeneration in the mild ALS8 patients by maintaining proteostasis. Interestingly, a reduction in VAPB mRNA and protein levels was also observed in the spinal cord of sporadic and familiar (superoxide dismutase 1 (SOD1)-linked) ALS patients and mice, suggesting a role of VAPB in the pathogenesis of non-VAPB linked ALS as well (Teuling et al., 2007; Anagnostou et al., 2010). These reduced levels might be associated with SNPs (single-nucleotide polymorphisms) within the VAPB gene (Chen et al., 2010). It has even been suggested that VAPB aggregates can be used as a pathologic marker in the

screening of sporadic non-VAPB linked ALS, as VAPB clusters were detected in peripheral blood mononuclear cells (PBMCs) and fibroblasts isolated from these patients (Cadoni et al., 2020).

Haplotype analysis showed a common Portuguese ancestor of the Brazilian families, with a founding event 23 generations ago, resulting in about 200 affected family members (Nishimura et al., 2005). Mutations in VAPB have not been associated with sporadic ALS (Conforti et al., 2006; Kirby et al., 2007) and the frequency of VAPB mutations is low in other populations (Tsai et al., 2011; Ingre et al., 2013; Kenna et al., 2013). However, the P56S mutation has also been identified in German, Japanese, Chinese and North American families displaying ALS8 symptoms, and have arisen independently from the Brazilian patients (Funke et al., 2010; Millecamps et al., 2010; Di et al., 2016; Guber et al., 2018). Another mutation in codon 56 of VAPB, in which proline is substituted for histidine (P56H), has also been found, in a Chinese family with similar clinical features as patients with P56S (Sun et al., 2017). Other mutations located in VAPB and associated with ALS are T46I, A145V and V234I (see *Other Mutations in VAPB*) (Chen et al., 2010; van Blitterswijk et al., 2012; Kabashi et al., 2013).

Although VAPB is ubiquitously expressed in the body and fulfils functions important for basal cell performance, it is mainly motor neuron dysfunction that is reported in the VAPB P56S/H patients. Electromyography and muscle/nerve biopsies revealed neurogenic damage with chronic denervation of muscles and reduced numbers of myelinated axons (Nishimura et al., 2004a; Marques et al., 2006; Kosac et al., 2013; Di et al., 2016; Sun et al., 2017; Guo et al., 2020). Additionally, neuroanatomical abnormalities were observed in ALS8 patients, including atrophy in the brainstem, globi pallida and upper cervical spinal cord (Leoni et al., 2022). The reason why VAPB mutations lead specifically to neurodegeneration is not well understood, although VAPB has been found to be highly abundant in motor neurons and different regions of the brain (Teuling et al., 2007; Larroquette et al., 2015; Leoni et al., 2022).

VAPB Aggregates

VAPB Aggregate Features and Formation

Several studies have reported that overexpression of VAPB P56S induces the formation of insoluble cytosolic aggregates in neuronal and non-neuronal cells (Nishimura et al., 2004b; Kanekura et al., 2006; Teuling et al., 2007), in culture as well as in transgenic mice and *Drosophila* ALS models (Chai et al., 2008; Ratnaparkhi et al., 2008; Qiu et al., 2013). The aggregation-prone VAPB P56S recruits wild-type VAPB and, to a lesser extent, VAPA to the aggregates, having a dominant-negative effect on normal VAP function (Kanekura et al., 2006; Teuling et al., 2007; Chai et al., 2008; Ratnaparkhi et al., 2008; Suzuki et al., 2009). The VAPB mutant has also been shown to sequesters ER-Golgi recycling protein YIF1A (via its TMD) to the aggregates, depleting the protein from these organelles (Kuijpers et al., 2013b). Nevertheless, VAPB P56S does not seem to induce "classical protein aggregates," formed of insoluble fibrils, a hallmark of other neurodegenerative disorders like Huntington's disease (huntingtin), PD (α-synuclein) and ALS (SOD1). For example, VAPB P56S forms aggregates rapidly after expression (<2 h), while the formation of SOD1 aggregates takes hours to days (Matsumoto et al., 2005; Fasana et al., 2010). Additionally, ultrastructural studies showed that overexpression of the mutant VAPB protein caused accumulation of large membranous aggregates, consisting of ribbons of stacked ER cisternae (Teuling et al., 2007; Fasana et al., 2010; Papiani et al., 2012). Live cell photobleaching experiments, using ER membrane-targeted GFP, revealed that VAPB P56S-ER subdomain inclusions are continuous with the rest of the ER (Fasana et al., 2010). However, there is some discrepancy between overexpression studies about the presence of proteins from the secretory pathway in the VAPB aggregates; for instance, ER luminal proteins calreticulin and PDI, and ER membrane protein calnexin associate with mutant VAPB aggregates is some studies, whilst others observed exclusion of these proteins (Kanekura et al., 2006; Teuling et al., 2007; Prosser et al., 2008; Fasana et al., 2010; Kuijpers et al., 2013a). This may be attributed to differences in cell lines, VAPB expression levels, and the exclusion of some (rough) ER membrane proteins from the aggregates (Fasana et al., 2010).

To understand how VAPB P56S induces aggregate formation, we will first discuss how the mutation affects the protein structure. VAPB proline 56 is conserved in VAPA, but mutating this residue does not seem to have such a significant effect, with some studies suggesting that no aggregation was observed whilst others observe minor levels of aberrant aggregation for VAPA P56S, notably in HeLa cells (Teuling et al., 2007; Prosser et al., 2008; Suzuki et al., 2009). VAPA P56S's resistance to aggregation seems to rely on two other proline residues present in this region, whereas VAPB P56S has only one remaining proline residue (Nakamichi et al., 2011). Substituting one of the prolines in VAPA P56S to the equivalent in VAPB P56S (VAPA P56S/P63A), resulted in the formation of membranous aggregates indistinguishable from those observed with VAPB P56S. The three proline residues of VAPA are conserved in the yeast VAP protein Scs2p, which is also resistant to the ALS8-causing mutation, showing that the proline distribution is an important feature in the pathophysiology of ALS8 (Nakamichi et al., 2011).

P56 is located in the MSP domain of VAPB and is critical for the correct folding of the seven β-strands of the MSP domain (**Figure 3A**) (Shi et al., 2010). P56 stabilizes the *cis*-peptide bond within the S-shaped loop that connects strands D1 and D2 (Kaiser et al., 2005; Teuling et al., 2007; Shi et al., 2010). The P56S mutation induces a conformational change within the recombinant MSP domain, resulting in the exposure of hydrophobic patches, which may enhance oligomerization of the mutant VAPB protein under physiological conditions (**Figure 3B**) (Kim et al., 2010). However, studies with recombinant MSP P56S domains show differences in structural stability and solubility (Kim et al., 2010; Shi et al., 2010). P56S eliminates the native β-sheet structure in water, and the exposed hydrophobic patches seem to drive aggregation of recombinant MSP P56S, making the structure highly insoluble in various buffers (Shi et al., 2010; Qin et al., 2013a). This makes it difficult to understand exactly how the VAPB P56S structure behaves under physiological conditions. Nevertheless, it has been

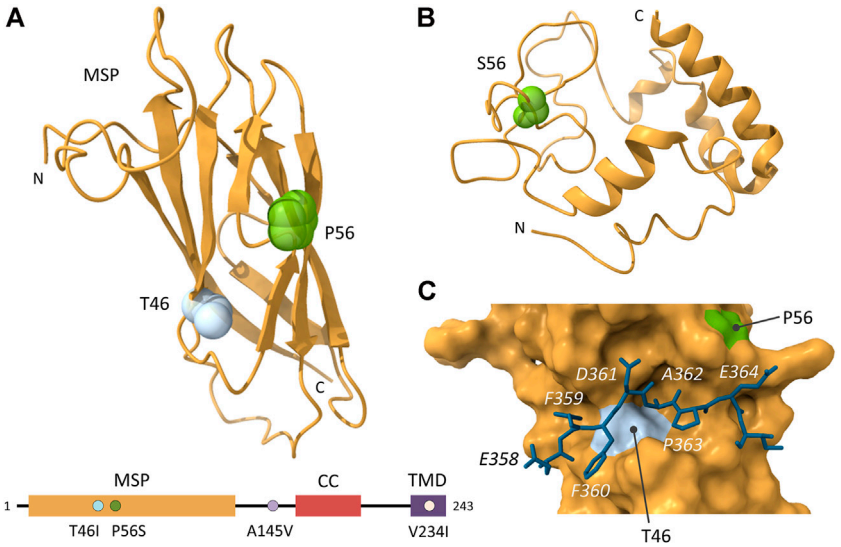

FIGURE 3 | Structure of the VAP MSP domain. **(A)** Structure of the MSP domain of VAPB (PDB ID: 3IKK) and schematic representation of the domain architecture of VAPB, with the ALS-related mutations indicated. The two ALS-related residues that are located in the MSP domain (T46 and P56) are mapped onto the structure. **(B)** Structure of the MSP domain of VAPB P56S (PDB ID: 2MDK). The ALS-related mutation S56 is mapped onto the structure. **(C)** Structure of the MSP domain of VAPA in complex with the OSBP FFAT motif ($_{358}$EFFDAPE I) (PDB ID: 2RR3). MSP residues T46 and P56 are indicated. The FFAT core residues of OSBP are written in *italic*. Images created with UCSF ChimeraX (Pettersen et al., 2021).

shown that MSP P56S retains its ability to bind to FFAT motif-containing proteins in HeLa cells, but the FFAT-binding of full-length VAPB P56S is perturbed (Kim et al., 2010). The aberrant oligomerization of full-length VAPB P56S may interfere with the binding of FFAT motifs to the MSP domain. In line with this, no FFAT-motif containing proteins were observed in pull-down assays using biotinylation-tagged VAPB P56S (Teuling et al., 2007). However, overexpression of a FFAT motif peptide rescued the aggregation phenotype of the mutant, suggesting protein stabilisation via FFAT motif-binding (Prosser et al., 2008). Additionally, VAPB P56S induces clustering of mitochondria and peroxisomes that colocalise with the VAPB aggregates (De Vos et al., 2012; Hua et al., 2017). The clustering of peroxisomes was dependent on the presence of ACBD5, suggesting that the mutant VAPB can sequester FFAT-motif containing proteins such as peroxisomal ACBD5 and possibly mitochondrial PTPIP51 (De Vos et al., 2012; Hua et al., 2017). However, FFAT-proteins ORP9 and NIR2 were not detectable in the VAPB aggregates (Kuijpers et al., 2013a). In summary, the P56S mutation causes conformational changes in the MSP domain and although this does not affect FFAT-binding to the domain on its own, in the presence of full-length VAPB, exposed hydrophobic patches cause enhanced oligomerization of the protein, which seems to reduce accessibility to the FFAT-binding site.

The disordered MSP P56S domain, but not the wild-type MSP domain, is able to interact with dodecylphosphocholine, a lipid commonly used to resemble membrane lipids, transforming the domain into a highly helical conformation (Qin et al., 2013b). This allows MSP P56S to be inserted into membrane environments (Qin et al., 2013a). Therefore, the interaction of VAPB P56S with lipids from the ER membranes could provide a mechanism for the formation of the membranous aggregates. The presence of membrane structures within the aggregates could also be attributed to VAPB being a tail-anchored protein. VAPB P56S has been shown to be efficiently post-translationally inserted into the ER membrane, after which it rapidly clusters (Fasana et al., 2010). This is further confirmed by a study showing that co-expression of a FFAT-containing peptide with VAPB P56S, partially restored the characteristic reticular ER pattern of VAPB (Prosser et al., 2008), suggesting that FFAT binding can maybe stabilise the mutant MSP structure and that the MSP domain/FFAT-interaction plays an important role in the formation of (membranous) aggregates.

Overall, VAPB P56S seems prone to aggregation due to instability of its MSP structure caused by the mutation. Because of its unaffected TMD, the mutant protein is still targeted to the ER membrane (Fasana et al., 2010), where it forms clusters, recruits wild-type VAPA/B and VAP interactors, and induces the formation of membranous clusters (Teuling et al., 2007; Fasana et al., 2010). Conceivably, newly synthesized mutant VAPB could also aggregate in the cytosol before its insertion into the ER membrane. This may depend on the rate of protein synthesis and levels/capacity of the chaperone machinery, which may differ between different cell types, but could then give rise to two different types of aggregates, cytosolic and membranous, which may explain some discrepancy between studies.

VAPB Aggregate Clearance

While overexpression studies show that VAPB P56S is aggregation prone, aggregate formation was also induced at

physiological conditions at low levels of mutant VAPB, comparable to endogenous wild-type protein, in HeLa cells (Fasana et al., 2010; Papiani et al., 2012). However, more research is required to clarify in what extend these aggregates form in patients; it has been shown that iPSC-derived motor neurons from ALS8 patients have reduced levels of VAPB and no signs of aggregate accumulation (Mitne-Neto et al., 2011; Oliveira et al., 2020), while ALS8 patient-derived muscle biopsy and fibroblasts revealed VAPB aggregates (Tripathi et al., 2021). As HeLa cells also displayed aggregated VAPA P56S (Teuling et al., 2007), which was not observed in other cell types, it seems likely that different cell types show altered VAP aggregate accumulation.

Discrepancy in detection of aggregates in patients might be due to differences in clearance of mutant VAPB. VAPB P56S has been reported to be less stable than the wild-type protein in both cultured cells and transgenic mice (Papiani et al., 2012; Aliaga et al., 2013; Genevini et al., 2014). VAPB P56S was polyubiquitinated shortly after synthesis and degraded by the proteasome in inducibly-expressing HeLa and NSC34 (motoneuronal) cells, with no evident involvement of basal autophagy (although it can be targeted by stimulated autophagy) (Kanekura et al., 2006; Papiani et al., 2012; Genevini et al., 2014). Ubiquitination of VAPB P56S has also been observed in motor neurons and muscle of transgenic mice and flies (Ratnaparkhi et al., 2008; Tsuda et al., 2008; Tudor et al., 2010). The data further indicates that in the HeLa cells and transgenic mice, the mutant protein initially avoids degradation, clusters and is then cleared by the proteasome (Papiani et al., 2012; Kuijpers et al., 2013a). This comprises the involvement of ER membrane chaperone BAP31 and the ATPase chaperone p97/VCP, proteins involved in ER-associated protein degradation (ERAD), likely by extracting mutant VAPB from the ER membrane. However, a study reported that overexpression of both wild-type and mutant VAPB impaired proteasome activity, possibly by inducing ER stress (see below) (Moumen et al., 2011), although this might be attributed to the high levels of expressed VAPB in comparison to the inducible system. Interestingly, it has also been reported that VAPB P56S is resistant to proteolysis by an unidentified protease that releases the MSP domain from wild-type VAPB (Gkogkas et al., 2011).

Disruption of Cellular Homeostasis

Below we highlight some of the functions of VAPB and the effects that VAPB P56S has on ER stress responses and autophagy. But since VAPB has many functions and binding partners (*see* **Table 1**), and the P56S mutation impacts the protein properties (*see* above), it is plausible that most processes involving VAP are in some extent impacted by mutant VAPB, including organelle tethering (Yamanaka et al., 2020) and regulation of PI4P levels (Wilson et al., 2021). We focus on how VAPB P56S affects motor neurons specifically. However, in addition to neurological problems, ALS8 patients also exhibit altered metabolic functions, such as dyslipidemia with increased cholesterol and triglyceride levels (Marques et al., 2006). VAPB P56S was found to suppresses adipocyte differentiation (Tokutake et al., 2015a) and VAPB is involved in different cholesterol and triglyceride pathways via its binding partners, as shown in **Table 1**.

ER Stress

The P56S mutant VAPB causes ER stress (Aliaga et al., 2013; Larroquette et al., 2015), altered ER domain properties (Fasana et al., 2010; Papiani et al., 2012; Yamanaka et al., 2020) and malfunction of the unfolded protein response (UPR), a physiological reaction to suppress accumulation of misfolded proteins in the ER (Kanekura et al., 2006; Suzuki et al., 2009). In mammalian cells, the three main signalling pathways of UPR are IRE1, ATF6, and PERK–with all three shown to be affected by mutant VAPB. VAPB P56S suppress the IRE1-XBP1 pathway that activates expression of UPR target genes, such as chaperones and ERAD components (Kanekura et al., 2006; Suzuki et al., 2009; Tokutake et al., 2015b). VAPB directly interacts with the ER-localized transcription factor ATF6 which, by acting as an ER stress sensor, regulates the transcription of genes encoding chaperones and other UPR transcription factors (Gkogkas et al., 2008). VAPB P56S was shown to attenuate the ATF6-mediated UPR transcription. On the other hand, VAPB P56S activates UPR via PERK-ATF4 which, by promoting the expression of the pro-apoptotic gene CHOP, initiates the cell apoptotic pathway under prolonged ER stress (Aliaga et al., 2013; Tokutake et al., 2015a). Increased basal ER stress and UPR activation has also been reported in the ALS8 patient-derived fibroblasts (Guber et al., 2018). Overall, if the UPR impacted by VAPB P56S cannot restore proteostasis, it might lead to apoptosis.

Autophagy

Mutant VAPB has been linked with dysfunctional autophagy (Zhao et al., 2018; Tripathi et al., 2021). The P56S mutation reduced VAPB's interaction with early autophagy proteins ULK1 and FIP200, impairing autophagosome biogenesis (*see* **Table 1**) (Zhao et al., 2018). Additionally, VAPB P56S accumulates in autophagosomes and impairs their clearance, showing that VAPB acts at different stages of autophagy (Larroquette et al., 2015; Tripathi et al., 2021). An accumulation and sequestering of autophagic markers p62 and LC3 at VAPB P56S aggregates was also observed in ALS8 patient fibroblasts and muscle biopsies (Tripathi et al., 2021). Impairment of autophagy by mutant VAPB can result in the aggregation of FUS, TDP-43 and Matrin 3 – mutations in which are associated with familial ALS–leading to the formation of stress granules (Tudor et al., 2010; Tripathi et al., 2021). Overexpression of FUS and TDP-43 have both been linked with disruption of the PTPIP51-VAPB association and hence, mitochondria-ER contacts (Stoica et al., 2014, 2016) (**Figure 1**). Loosening mitochondria-ER contacts via PTPIP51 or VAPB knockdown has been shown to stimulate autophagosome formation by disrupting the Ca^{2+} delivery to mitochondria from ER stores (Gómez-Suaga et al., 2017). VAPB P56S and TDP-43 may also co-operate in the pathogenesis of ALS by activating the mitochondrial apoptotic pathway (Suzuki and Matsuoka, 2011). VAPB is also involved in ER-phagy, a selective form of autophagy for degradation of the ER, via interaction with the soluble ER-phagy receptor CALCOCO1 which, via ATG8

binding, connects the ER and autophagosome membranes (*see* **Table 1**) (Nthiga et al., 2020).

VAP in Neurones

Although VAPB is ubiquitously expressed and hence disruption caused by the P56S mutation would affect all cells in the body, ALS8 patients mainly present with (lower) motor neuron dysfunction and neurodegeneration. The large size and complex morphology of motor neurons make the maintenance of protein homeostasis and the distribution of organelles a greater challenge. Hence, motor neurons may be more vulnerable to the overall homeostatic disruption caused by aberrant VAPB. Several studies illustrate how VAPB P56S can affect neuron-specific processes and morphology. For instance, the mutant VAPB disrupts anterograde mitochondrial axonal transport by disrupting Ca^{2+} homeostasis in neurons (Mórotz et al., 2012). Peroxisomal movement in hippocampal neurones has also been shown to resemble that of mitochondria and be altered by levels of the peroxisome-ER tethering protein ACBD5 (Wang et al., 2018). However, unlike for mitochondria, this did not appear to be dependent upon VAPB interaction. A loss of the VAPB orthologue in *Drosophila* also resulted in abnormal organelle distribution in neuronal axons and dendrites, including mitochondria and the Golgi apparatus, which may have contributed to the altered dendrite morphology (Kamemura et al., 2021), indicating the importance of ER-tethering in organelle distribution. Furthermore, mitochondria-ER contacts, mediated by the PTPIP51-VAPB interaction, are present at synapses and regulate synaptic function (Gómez-Suaga et al., 2019). Loss of PTPIP51 or VAPB reduced synaptic function and altered dendritic morphology. VAPB P56S also sequesters VAP-interactor YIF1A (**Table 1**), which regulates membrane trafficking into dendrites and dendritic morphology (Kuijpers et al., 2013b). VAPB is also important for neurite extension of motor neurons (Genevini et al., 2014), possibly via its interaction with protrudin (**Table 1**) (Saita et al., 2009). Additionally, VAPB P56S led to a loss of HCN channel activity, important for neuronal and cardiac pacemaker currents (Silbernagel et al., 2018). These alterations in motor neurons may partly explain the neurodegeneration and muscle-related symptoms observed in ALS8 patients. VAPB P56S may also affect muscle cells more directly; the VAPB mutation disrupted the formation of multinuclear myotubes (muscle fibres) by mouse skeletal muscle cells (Tokutake et al., 2015b) and caused accumulation of ER Ca^{2+} sensor STIM1 at neuromuscular junctions (NMJ) in muscle fibres of ALS8 patients, suggesting altered intracellular Ca^{2+} homeostasis (Goswami et al., 2015). Interestingly, in *Drosophila*, VAPB regulates the number and size of synaptic boutons at NMJ (Pennetta et al., 2002; Chai et al., 2008). Additionally, VAPB deficient mice showed abnormal skeletal muscle energy metabolism upon fasting (Han et al., 2013). Impaired degradation pathways, accumulation/aggregation of misfolded proteins and disrupted Ca^{2+} homeostasis in motor neurons and muscle fibres may all contribute to ALS8 pathogenesis.

Acknowledging the various roles VAPB plays in many important physiological pathways, it is not surprising that disruption of the protein has a major effect on cellular homeostasis. Nevertheless, it is still under debate whether the P56S mutation in VAPB induces the symptoms of ALS8 patients by a loss of function (lost/reduced protein interactions), a toxic gain of function (aggregate formation, protein sequestering), or a dominant negative effect (wild-type VAP recruitment). VAPB P56S aggregates in the nervous system of transgenic mice did not cause motor neuron dysfunction, suggesting that aggregates are not sufficient to initiate pathogenesis (Tudor et al., 2010; Qiu et al., 2013), although, with a higher fold increase of VAPB P56S protein expression, mice developed abnormal motor behaviour and progressive degeneration of corticospinal motor neurons (Aliaga et al., 2013). A study using both homozygous and heterozygous VAPB P56S knock-in mice showed defects in motor behaviours, with accumulation of cytoplasmic inclusions selectively in motor neurons before onset of the defects, though the homozygous knock-in mice presented with a more severe phenotype, reflecting a dose-dependent effect of the mutant protein (Larroquette et al., 2015). On the other hand, VAPB knockdown was sufficient to lead to motor deficits in zebrafish and mild, late-onset motor deficits were observed in VAPB knockout mice, however, VAPB depletion was unable to induce a complete ALS phenotype (Kabashi et al., 2013). Thus, VAPB P56S abnormalities might be a combination of gained and lost functions, in a dominant and dose-dependent manner.

Other Mutations in VAPB

A second mutation located in the MSP domain of VAPB has also been associated with familial ALS. An amino acid change from threonine to isoleucine at codon 46 (T46I) was identified in a patient from the United Kingdom, with non-Brazilian kindred–affected family members were not available to screen (Chen et al., 2010). The patient presented with typical ALS, with onset of symptoms at the age of 73 years. Unlike the P65S mutation that completely eliminates the native MSP structure in various buffers (Shi et al., 2010), MSP T46I retains a structure highly similar to the native MSP domain, although with reduced stability (Lua et al., 2011). This makes the MSP domain more easily accessible to unfolded intermediates that are prone to aggregation as shown *in vitro*, in cultured cells as well as *in vivo* (Chen et al., 2010; Lua et al., 2011). T46 is part of the hydrophobic pocket that binds the side chain of FFAT motif residue 5 (A) and forms hydrogen bonds with the side chains of FFAT motif residues 2 (F) and 3 (F) (**Figure 3C**) (Kaiser et al., 2005; Furuita et al., 2010). The threonine to isoleucine substitution induced some dynamic changes of local regions within the MSP domain (Lua et al., 2011). These alterations seem to affect the ability of VAPB to bind FFAT-motif containing proteins, as illustrated with the NIR2 FFAT motif that showed a 3-fold decrease in binding affinity (Chen et al., 2010). Analysis of VAPB T46I in neuronal cells and *D. melanogaster* indicates similar cellular abnormalities as with the P56S mutation, such as wild-type VAPB sequestering, ER fragmentation and neurodegeneration.

Two VAPB mutations outside of the MSP domain have also been identified in ALS patients. An alanine to valine substitution at codon 145 (A145V) was identified (Kabashi et al., 2013), which is located in the region between the MSP and TMD of VAPB but little else is known about the pathogenicity of A145V. Furthermore, V234I was identified in a patient of Dutch

origin, who also harboured a repeat expansion in C9orf72, an ALS causative gene (van Blitterswijk et al., 2012). Transgenic expression of the VAPB V234I orthologue in *D. melanogaster* was able to induce ALS hallmarks (Sanhueza et al., 2014). The valine to isoleucine substitution is located in the transmembrane domain of VAPB, and although it is close to the dimerization motif, it did not affect VAPB dimerization (Chattopadhyay and Sengupta, 2014). However, the V234I mutation seems to affect the ER-targeting of VAPB as it did not localize with ER-marker PDI. The V234I mutated VAPB did not form typical P56S aggregates, but formed small aggregates/granules in HeLa cells, which may lead to cell death (Chattopadhyay and Sengupta, 2014).

CONCLUSION

Here, we provided a timely summary of the constantly growing number of VAP interacting proteins, their FFAT motifs (if present) and interaction domains, which will present a helpful overview for future studies on VAP binding partners. We discussed new findings on the regulation of VAP binding by phosphorylation of the FFAT motif core, and the role of GSK3β in the regulation of both mitochondria-ER and peroxisome-ER membrane contact sites. How the interaction of VAP with tether proteins and other interaction partners is regulated, is still not well explored. Future studies may shed light on the regulation of those interactions and their impact on the multiple cellular functions of VAP proteins. An intriguing aspect is also the hijacking of VAP by bacteria and viruses and its role in pathogen infection. It will be interesting to investigate if and how the organelle-specific binding partners are influenced, and if those proteins are suitable new therapeutic targets to combat pathogen infection. Furthermore, the impact of VAP mutations on neurological disorders deserves further investigation. Although our knowledge about VAP and its binding partners at membrane contacts has increased, we do not yet fully understand the (patho)physiological consequences of altered ER-organelle contacts and how this would impact on neurological functions. Thus, VAP proteins and their interacting proteins will remain in the focus of fundamental, discovery-based research as well as biomedical studies.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

AUTHOR CONTRIBUTIONS

SK wrote the manuscript and created the figures and table. JC and MS conceived the project and wrote the manuscript.

FUNDING

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) (BB/T002255/1, BB/V018167/1 to MS and JC), an UK Research and Innovation Future Leader Fellowship Award (MR/T019409/1 to JC) and in part by grant MR/N0137941/1 for the GW4 BIOMED MRC DTP, awarded to the Universities of Bath, Bristol, Cardiff and Exeter from the Medical Research Council (MRC)/UKRI (PhD studentship SK). For the purpose of open access, the author has applied a Creative Commons Attribution (CC BY) licence to any Author Accepted Manuscript version arising.

ACKNOWLEDGMENTS

The authors would like to thank R. E. Carmichael (University of Exeter) for critical reading of the manuscript.

REFERENCES

Abu Irqeba, A., and Ogilvie, J. M. (2020). Di-arginine and FFAT-like Motifs Retain a Subpopulation of PRA1 at ER-Mitochondria Membrane Contact Sites. *PLoS One* 15, e0243075. doi:10.1371/JOURNAL.PONE.0243075

Adhikari, B., De Silva, B., Molina, J. A., Allen, A., Peck, S. H., and Lee, S. Y. (2019). Neuronal Ceroid Lipofuscinosis Related ER Membrane Protein CLN8 Regulates PP2A Activity and Ceramide Levels. *Biochimica Biophysica Acta (BBA) - Mol. Basis Dis.* 1865, 322–328. doi:10.1016/j.bbadis.2018.11.011

Agaisse, H., and Derré, I. (2014). Expression of the Effector Protein IncD in *Chlamydia trachomatis* Mediates Recruitment of the Lipid Transfer Protein CERT and the Endoplasmic Reticulum-Resident Protein VAPB to the Inclusion Membrane. *Infect. Immun.* 82, 2037–2047. doi:10.1128/IAI.01530-14

Agaisse, H., and Derré, I. (2015). STIM1 Is a Novel Component of ER-Chlamydia trachomatis Inclusion Membrane Contact Sites. *PLoS One* 10, e0125671. doi:10.1371/journal.pone.0125671

Aliaga, L., Lai, C., Yu, J., Chub, N., Shim, H., and Sun, L. (2013). Amyotrophic Lateral Sclerosis-Related VAPB P56S Mutation Differentially Affects the Function and Survival of Corticospinal and Spinal Motor Neurons. *Hum. Mol. Genet.* 22, 4293–4305. doi:10.1093/hmg/ddt279

Alpy, F., Rousseau, A., Schwab, Y., Legueux, F., Stoll, I., Wendling, C., et al. (2013). STARD3 or STARD3NL and VAP Form a Novel Molecular Tether between Late Endosomes and the ER. *J. Cell Sci.* 126, 5500–5512. doi:10.1242/jcs.139295

Amarilio, R., Ramachandran, S., Sabanay, H., and Lev, S. (2005). Differential Regulation of Endoplasmic Reticulum Structure through VAP-Nir Protein Interaction. *J. Biol. Chem.* 280, 5934–5944. doi:10.1074/jbc.M409566200

Amini-Bavil-Olyaee, S., Choi, Y. J., Lee, J. H., Shi, M., Huang, I. C., Farzan, M., et al. (2013). The Antiviral Effector IFITM3 Disrupts Intracellular Cholesterol Homeostasis to Block Viral Entry. *Cell Host Microbe* 13, 452–464. doi:10.1016/j.chom.2013.03.006

Anagnostou, G., Akbar, M. T., Paul, P., Angelinetta, C., Steiner, T. J., and de Belleroche, J. (2010). Vesicle Associated Membrane Protein B (VAPB) Is Decreased in ALS Spinal Cord. *Neurobiol. Aging* 31, 969–985. doi:10.1016/j.neurobiolaging.2008.07.005

Arita, M. (2014). Phosphatidylinositol-4 Kinase III Beta and Oxysterol-Binding Protein Accumulate Unesterified Cholesterol on Poliovirus-Induced Membrane Structure. *Microbiol. Immunol.* 58, 239–256. doi:10.1111/1348-0421.12144

Backman, A. P. E., Halin, J., Nurmi, H., Möuts, A., Kjellberg, M. A., and Mattjus, P. (2018). Glucosylceramide Acyl Chain Length Is Sensed by the Glycolipid Transfer Protein. *PLoS One* 13, e0209230. doi:10.1371/journal.pone.0209230

Baker, E. S., Luckner, S. R., Krause, K. L., Lambden, P. R., Clarke, I. N., and Ward, V. K. (2012). Inherent Structural Disorder and Dimerisation of Murine Norovirus NS1-2 Protein. *PLoS One* 7, e30534. doi:10.1371/journal.pone.0030534

Baron, Y., Pedrioli, P. G., Tyagi, K., Johnson, C., Wood, N. T., Fountaine, D., et al. (2014). VAPB/ALS8 Interacts with FFAT-like Proteins Including the P97 Cofactor FAF1 and the ASNA1 ATPase. *BMC Biol.* 12, 39. doi:10.1186/1741-7007-12-39

Berger, K. L., Kelly, S. M., Jordan, T. X., Tartell, M. A., and Randall, G. (2011). Hepatitis C Virus Stimulates the Phosphatidylinositol 4-Kinase III Alpha-dependent Phosphatidylinositol 4-Phosphate Production that Is Essential for its Replication. *J. Virol.* 85, 8870–8883. doi:10.1128/JVI.00059-11

Bullock, T. L., Roberts, T. M., and Stewart, M. (1996). 2.5 Å Resolution Crystal Structure of the Motile Major Sperm Protein (MSP) ofAscaris Suum. *J. Mol. Biol.* 263, 284–296. doi:10.1006/JMBI.1996.0575

Cabukusta, B., Berlin, I., van Elsland, D. M., Forkink, I., Spits, M., de Jong, A. W. M., et al. (2020). Human VAPome Analysis Reveals MOSPD1 and MOSPD3 as Membrane Contact Site Proteins Interacting with FFAT-Related FFNT Motifs. *Cell Rep.* 33, 108475. doi:10.1016/j.celrep.2020.108475

Cadoni, M. P. L., Biggio, M. L., Arru, G., Secchi, G., Orrù, N., Clemente, M. G., et al. (2020). VAPB ER-Aggregates, a Possible New Biomarker in ALS Pathology. *Cells* 9, 164. doi:10.3390/cells9010164

Chadi, G., Maximino, J. R., Jorge, F. M. de. H., Borba, F. C. de., Gilio, J. M., Callegaro, D., et al. (2017). Genetic Analysis of Patients with Familial and Sporadic Amyotrophic Lateral Sclerosis in a Brazilian Research Center. *Amyotroph. Lateral Scler. Front. Degener.* 18, 249–255. doi:10.1080/21678421.2016.1254245

Chai, A., Withers, J., Koh, Y. H., Parry, K., Bao, H., Zhang, B., et al. (2008). hVAPB, the Causative Gene of a Heterogeneous Group of Motor Neuron Diseases in Humans, Is Functionally Interchangeable with its Drosophila Homologue DVAP-33A at the Neuromuscular Junction. *Hum. Mol. Genet.* 17, 266–280. doi:10.1093/hmg/ddm303

Chang, C. L., Hsieh, T. S., Yang, T. T., Rothberg, K. G., Azizoglu, D. B., Volk, E., et al. (2013). Feedback Regulation of Receptor-Induced Ca2+ Signaling Mediated by E-Syt1 and Nir2 at Endoplasmic Reticulum-Plasma Membrane Junctions. *Cell Rep.* 5, 813–825. doi:10.1016/j.celrep.2013.09.038

Chang, C. L., and Liou, J. (2015). Phosphatidylinositol 4, 5-bisphosphate Homeostasis Regulated by Nir2 and Nir3 Proteins at Endoplasmic Reticulum-Plasma Membrane Junctions. *J. Biol. Chem.* 290, 14289–14301. doi:10.1074/jbc.M114.621375

Chattopadhyay, D., and Sengupta, S. (2014). First Evidence of Pathogenicity of V234I Mutation of hVAPB Found in Amyotrophic Lateral Sclerosis. *Biochem. Biophys. Res. Commun.* 448, 108–113. doi:10.1016/j.bbrc.2014.04.102

Chen, H. J., Anagnostou, G., Chai, A., Withers, J., Morris, A., Adhikaree, J., et al. (2010). Characterization of the Properties of a Novel Mutation in VAPB in Familial Amyotrophic Lateral Sclerosis. *J. Biol. Chem.* 285, 40266–40281. doi:10.1074/jbc.M110.161398

Cianciola, N. L., Chung, S., Manor, D., and Carlin, C. R. (2017). Adenovirus Modulates Toll-like Receptor 4 Signaling by Reprogramming ORP1L-VAP Protein Contacts for Cholesterol Transport from Endosomes to the Endoplasmic Reticulum. *J. Virol.* 91, 1904–1920. doi:10.1128/JVI.01904-16

Conforti, F. L., Sprovieri, T., Mazzei, R., Ungaro, C., Tessitore, A., Tedeschi, G., et al. (2006). Sporadic ALS Is Not Associated with VAPB Gene Mutations in Southern Italy. *J. Negat. Results Biomed.* 5, 7. doi:10.1186/1477-5751-5-7

Costello, J. L., Castro, I. G., Hacker, C., Schrader, T. A., Metz, J., Zeuschner, D., et al. (2017a). ACBD5 and VAPB Mediate Membrane Associations between Peroxisomes and the ER. *J. Cell Biol.* 216, 331–342. doi:10.1083/jcb.201607055

Costello, J. L., Castro, I. G., Schrader, T. A., Islinger, M., and Schrader, M. (2017b). Peroxisomal ACBD4 Interacts with VAPB and Promotes ER-Peroxisome Associations. *Cell Cycle* 16, 1039–1045. doi:10.1080/15384101.2017.1314422

Darwisch, W., von Spangenberg, M., Lehmann, J., Singin, Ö., Deubert, G., Kühl, H., et al. (2020). Cerebellar and Hepatic Alterations in ACBD5-Deficient Mice Are Associated with Unexpected, Distinct Alterations in Cellular Lipid Homeostasis. *Commun. Biol.* 3, 1–19. doi:10.1038/s42003-020-01442-x

de Alcântara, C., Cruzeiro, M. M., França, M. C., Camargos, S. T., and de Souza, L. C. (2019). Amyotrophic Lateral Sclerosis Type 8 Is Not a Pure Motor Disease: Evidence from a Neuropsychological and Behavioural Study. *J. Neurol.* 266, 1980–1987. doi:10.1007/s00415-019-09369-y

De Vos, K. J., Mórotz, G. M., Stoica, R., Tudor, E. L., Lau, K. F., Ackerley, S., et al. (2012). VAPB Interacts with the Mitochondrial Protein PTPIP51 to Regulate Calcium Homeostasis. *Hum. Mol. Genet.* 21, 1299–1311. doi:10.1093/hmg/ddr559

Deidda, I., Galizzi, G., Passantino, R., Cascio, C., Russo, D., Colletti, T., et al. (2014). Expression of Vesicle-Associated Membrane-Protein-Associated Protein B Cleavage Products in Peripheral Blood Leukocytes and Cerebrospinal Fluid of Patients with Sporadic Amyotrophic Lateral Sclerosis. *Eur. J. Neurol.* 21, 478–485. doi:10.1111/ENE.12334

Derré, I., Swiss, R., and Agaisse, H. (2011). The Lipid Transfer Protein CERT Interacts with the Chlamydia Inclusion Protein IncD and Participates to ER-Chlamydia Inclusion Membrane Contact Sites. *PLoS Pathog.* 7, e1002092. doi:10.1371/journal.ppat.1002092

Di, L., Chen, H., Da, Y., Wang, S., and Shen, X. M. (2016). Atypical Familial Amyotrophic Lateral Sclerosis with Initial Symptoms of Pain or Tremor in a Chinese Family Harboring VAPB-P56s Mutation. *J. Neurol.* 263, 263–268. doi:10.1007/s00415-015-7965-3

Di Mattia, T., Martinet, A., Ikhlef, S., McEwen, A. G., Nominé, Y., Wendling, C., et al. (2020). FFAT Motif Phosphorylation Controls Formation and Lipid Transfer Function of Inter-organelle Contacts. *EMBO J.* 39, e104369. doi:10.15252/embj.2019104369

Dong, R., Saheki, Y., Swarup, S., Lucast, L., Harper, J. W., and De Camilli, P. (2016). Endosome-ER Contacts Control Actin Nucleation and Retromer Function through VAP-dependent Regulation of PI4P. *Cell* 166, 408–423. doi:10.1016/j.cell.2016.06.037

Dorobantu, C. M., Albulescu, L., Harak, C., Feng, Q., van Kampen, M., Strating, J. R. P. M., et al. (2015). Modulation of the Host Lipid Landscape to Promote RNA Virus Replication: The Picornavirus Encephalomyocarditis Virus Converges on the Pathway Used by Hepatitis C Virus. *PLoS Pathog.* 11, e1005185. doi:10.1371/JOURNAL.PPAT.1005185

Eden, E. R., Sanchez-Heras, E., Tsapara, A., Sobota, A., Levine, T. P., and Futter, C. E. (2016). Annexin A1 Tethers Membrane Contact Sites that Mediate ER to Endosome Cholesterol Transport. *Dev. Cell* 37, 473–483. doi:10.1016/J.DEVCEL.2016.05.005

Elwell, C. A., Jiang, S., Kim, J. H., Lee, A., Wittmann, T., Hanada, K., et al. (2011). *Chlamydia trachomatis* Co-opts GBF1 and CERT to Acquire Host Sphingomyelin for Distinct Roles during Intracellular Development. *PLoS Pathog.* 7, e1002198. doi:10.1371/journal.ppat.1002198

Ettayebi, K., and Hardy, M. E. (2003). Norwalk Virus Nonstructural Protein P48 Forms a Complex with the SNARE Regulator VAP-A and Prevents Cell Surface Expression of Vesicular Stomatitis Virus G Protein. *J. Virol.* 77, 11790–11797. doi:10.1128/jvi.77.21.11790-11797.2003

Evans, M. J., Rice, C. M., and Goff, S. P. (2004). Phosphorylation of Hepatitis C Virus Nonstructural Protein 5A Modulates its Protein Interactions and Viral RNA Replication. *Proc. Natl. Acad. Sci.* 101, 13038–13043. doi:10.1073/PNAS.0405152101

Fasana, E., Fossati, M., Ruggiano, A., Brambillasca, S., Hoogenraad, C. C., Navone, F., et al. (2010). A VAPB Mutant Linked to Amyotrophic Lateral Sclerosis Generates a Novel Form of Organized Smooth Endoplasmic Reticulum. *FASEB J.* 24, 1419–1430. doi:10.1096/fj.09-147850

Ferdinandusse, S., Falkenberg, K. D., Koster, J., Mooyer, P. A., Jones, R., van Roermund, C. W. T., et al. (2017). ACBD5 Deficiency Causes a Defect in Peroxisomal Very Long-Chain Fatty Acid Metabolism. *J. Med. Genet.* 54, 330–337. doi:10.1136/jmedgenet-2016-104132

Freyre, C. A. C., Rauher, P. C., Ejsing, C. S., and Klemm, R. W. (2019). MIGA2 Links Mitochondria, the ER, and Lipid Droplets and Promotes De Novo Lipogenesis in Adipocytes. *Mol. Cell* 76, 811–825. e14. doi:10.1016/j.molcel.2019.09.011

Funke, A. D., Esser, M., Krüttgen, A., Weis, J., Mitne-Neto, M., Lazar, M., et al. (2010). The p.P56S Mutation in the VAPB Gene Is Not Due to a Single Founder: The First European Case. *Clin. Genet.* 77, 302–303. doi:10.1111/j.1399-0004.2009.01319.x

Furuita, K., Jee, J. G., Fukada, H., Mishima, M., and Kojima, C. (2010). Electrostatic Interaction between Oxysterol-Binding Protein and VAMP-Associated Protein a Revealed by NMR and Mutagenesis Studies. *J. Biol. Chem.* 285, 12961–12970. doi:10.1074/jbc.M109.082602

Gabetta, V., Trzyna, W., Phiel, C., and McHugh, K. M. (2003). Vesicle-associated Protein-A Is Differentially Expressed during Intestinal Smooth Muscle Cell Differentiation. *Dev. Dyn.* 228, 11–20. doi:10.1002/dvdy.10349

Gao, L., Aizaki, H., He, J.-W., and Lai, M. M. C. (2004). Interactions between Viral Nonstructural Proteins and Host Protein hVAP-33 Mediate the Formation of Hepatitis C Virus RNA Replication Complex on Lipid Raft. *J. Virol.* 78, 3480–3488. doi:10.1128/JVI.78.7.3480-3488.2004

Genevini, P., Papiani, G., Ruggiano, A., Cantoni, L., Navone, F., and Borgese, N. (2014). Amyotrophic Lateral Sclerosis-Linked Mutant VAPB Inclusions Do Not Interfere with Protein Degradation Pathways or Intracellular Transport in a Cultured Cell Model. *PLoS One* 9, e113416. doi:10.1371/journal.pone.0113416

Gewaid, H., Aoyagi, H., Arita, M., Watashi, K., Suzuki, R., Sakai, S., et al. (2020). Sphingomyelin Is Essential for the Structure and Function of the Double-Membrane Vesicles in Hepatitis C Virus RNA Replication Factories. *J. Virol.* 94, e01080–20. doi:10.1128/JVI.01080-20

Ghosh, S., Patel, A. M., Grunkemeyer, T. J., Dumbrepatil, A. B., Zegalia, K., Kennedy, R. T., et al. (2020). Interactions between Viperin, Vesicle-Associated Membrane Protein A, and Hepatitis C Virus Protein NS5A Modulate Viperin Activity and NS5A Degradation. *Biochemistry* 59, 780–789. doi:10.1021/acs.biochem.9B01090

Gkogkas, C., Middleton, S., Kremer, A. M., Wardrope, C., Hannah, M., Gillingwater, T. H., et al. (2008). VAPB Interacts with and Modulates the Activity of ATF6. *Hum. Mol. Genet.* 17, 1517–1526. doi:10.1093/hmg/ddn040

Gkogkas, C., Wardrope, C., Hannah, M., and Skehel, P. (2011). The ALS8-Associated Mutant VAPBP56S Is Resistant to Proteolysis in Neurons. *J. Neurochem.* 117, 286–294. doi:10.1111/j.1471-4159.2011.07201.x

Gómez-Suaga, P., Gábor, |, Mórotz, M., Markovinovic, A., Martín-Guerrero, S. M., Preza, E., et al. (2022). Disruption of ER-Mitochondria Tethering and Signalling in C9orf72-Associated Amyotrophic Lateral Sclerosis and Frontotemporal Dementia. *Aging Cell* 00, e13549. doi:10.1111/ACEL.13549

Gómez-Suaga, P., Paillusson, S., Stoica, R., Noble, W., Hanger, D. P., and Miller, C. C. J. (2017). The ER-Mitochondria Tethering Complex VAPB-PTPIP51 Regulates Autophagy. *Curr. Biol.* 27, 371–385. doi:10.1016/J.CUB.2016.12.038

Gómez-Suaga, P., Pérez-Nievas, B. G., Glennon, E. B., Lau, D. H. W., Paillusson, S., Mórotz, G. M., et al. (2019). The VAPB-PTPIP51 Endoplasmic Reticulum-Mitochondria Tethering Proteins Are Present in Neuronal Synapses and Regulate Synaptic Activity. *Acta Neuropathol. Commun.* 7, 35. doi:10.1186/S40478-019-0688-4

Goonawardane, N., Gebhardt, A., Bartlett, C., Pichlmair, A., and Harris, M. (2017). Phosphorylation of Serine 225 in Hepatitis C Virus NS5A Regulates Protein-Protein Interactions. *J. Virol.* 91, e00805–17. doi:10.1128/JVI.00805-17

Gorkhali, R., Tian, L., Dong, B., Bagchi, P., Deng, X., Pawar, S., et al. (2021). Extracellular Calcium Alters Calcium-Sensing Receptor Network Integrating Intracellular Calcium-Signaling and Related Key Pathway. *Sci. Rep.* 11, 20576. doi:10.1038/S41598-021-00067-2

Goswami, A., Jesse, C. M., Chandrasekar, A., Bushuven, E., Vollrath, J. T., Dreser, A., et al. (2015). Accumulation of STIM1 Is Associated with the Degenerative Muscle Fibre Phenotype in ALS and Other Neurogenic Atrophies. *Neuropathol. Appl. Neurobiol.* 41, 304–318. doi:10.1111/NAN.12164

Goyal, S., Gupta, G., Qin, H., Upadya, M. H., Tan, Y. J., Chow, V. T. K., et al. (2012). VAPC, an Human Endogenous Inhibitor for Hepatitis C Virus (HCV) Infection, Is Intrinsically Unstructured but Forms a "Fuzzy Complex" with HCV NS5B. *PLoS One* 7, e40341. doi:10.1371/JOURNAL.PONE.0040341

Guber, R. D., Schindler, A. B., Budron, M. S., Chen, K. L., Li, Y., Fischbeck, K. H., et al. (2018). Nucleocytoplasmic Transport Defect in a North American Patient with ALS8. *Ann. Clin. Transl. Neurol.* 5, 369–375. doi:10.1002/ACN3.515

Guillén-Samander, A., Leonzino, M., Hanna, M. G., Tang, N., Shen, H., and De Camilli, P. (2021). VPS13D Bridges the ER to Mitochondria and Peroxisomes via Miro. *J. Cell Biol.* 220, e202010004. doi:10.1083/jcb.202010004

Guo, X., Gang, Q., Meng, L., Li, J., Li, F., Liu, J., et al. (2020). Peripheral Nerve Pathology in VAPB-Associated Amyotrophic Lateral Sclerosis with Dysautonomia in a Chinese Family. *Clin. Neuropathol.* 39, 282–287. doi:10.5414/NP301281

Gupta, G., Qin, H., and Song, J. (2012). Intrinsically Unstructured Domain 3 of Hepatitis C Virus NS5A Forms a "Fuzzy Complex" with VAPB-MSP Domain Which Carries ALS-Causing Mutations. *PLoS One* 7, e39261. doi:10.1371/JOURNAL.PONE.0039261

Gupta, G., and Song, J. (2016). C-terminal Auto-Regulatory Motif of Hepatitis C Virus NS5B Interacts with Human VAPB-MSP to Form a Dynamic Replication Complex. *PLoS One* 11, e0147278. doi:10.1371/JOURNAL.PONE.0147278

Hamamoto, I., Nishimura, Y., Okamoto, T., Aizaki, H., Liu, M., Mori, Y., et al. (2005). Human VAP-B Is Involved in Hepatitis C Virus Replication through Interaction with NS5A and NS5B. *J. Virol.* 79, 13473–13482. doi:10.1128/JVI.79.21.13473-13482.2005

Han, S. M., El Oussini, H., Scekic-Zahirovic, J., Vibbert, J., Cottee, P., Prasain, J. K., et al. (2013). VAPB/ALS8 MSP Ligands Regulate Striated Muscle Energy Metabolism Critical for Adult Survival in *Caenorhabditis elegans*. *PLoS Genet.* 9, e1003738. doi:10.1371/JOURNAL.PGEN.1003738

Han, S. M., Tsuda, H., Yang, Y., Vibbert, J., Cottee, P., Lee, S. J., et al. (2012). Secreted VAPB/ALS8 Major Sperm Protein Domains Modulate Mitochondrial Localization and Morphology via Growth Cone Guidance Receptors. *Dev. Cell* 22, 348–362. doi:10.1016/J.DEVCEL.2011.12.009

Hantan, D., Yamamoto, Y., and Sakisaka, T. (2014). VAP-B Binds to Rab3GAP1 at the ER: Its Implication in Nuclear Envelope Formation through the ER-Golgi Intermediate Compartment. *Kobe J. Med. Sci.* 60, 48–56. doi:10.24546/81008726

Häsler, S. L. A., Vallis, Y., Pasche, M., and McMahon, H. T. (2020). GRAF2, WDR44, and MICAL1 Mediate Rab8/10/11–dependent Export of E-Cadherin, MMP14, and CFTR ΔF508. *J. Cell Biol.* 219, e201811014. doi:10.1083/JCB.201811014/151714

Herzog, K., Pras-Raves, M. L., Ferdinandusse, S., Vervaart, M. A. T., Luyf, A. C. M., van Kampen, A. H. C., et al. (2018). Functional Characterisation of Peroxisomal β-oxidation Disorders in Fibroblasts Using Lipidomics. *J. Inherit. Metab. Dis.* 41, 479–487. doi:10.1007/s10545-017-0076-9

Hsieh, J., Molusky, M. M., McCabe, K. M., Fotakis, P., Xiao, T., Tascau, L., et al. (2021). TTC39B Destabilizes Retinoblastoma Protein Promoting Hepatic Lipogenesis in a Sex-specific Fashion. *J. Hepatol.* 76, 383–393. doi:10.1016/J.JHEP.2021.09.021

Hua, R., Cheng, D., Coyaud, É., Freeman, S., Di Pietro, E., Wang, Y., et al. (2017). VAPs and ACBD5 Tether Peroxisomes to the ER for Peroxisome Maintenance and Lipid Homeostasis. *J. Cell Biol.* 216, 367–377. doi:10.1083/jcb.201608128

Huang, M. H., Li, H., Xue, R., Li, J., Wang, L., Cheng, J., et al. (2019). Up-regulation of Glycolipid Transfer Protein by Bicyclol Causes Spontaneous Restriction of Hepatitis C Virus Replication. *Acta Pharm. Sin. B* 9, 769–781. doi:10.1016/J.APSB.2019.01.013

Ingre, C., Pinto, S., Birve, A., Press, R., Danielsson, O., De Carvalho, M., et al. (2013). No Association between VAPB Mutations and Familial or Sporadic ALS in Sweden, Portugal and Iceland. *Amyotroph. Lateral Scler. Front. Degener.* 14, 620–627. doi:10.3109/21678421.2013.822515

Inukai, R., Mori, K., Kuwata, K., Suzuki, C., Maki, M., Takahara, T., et al. (2021). The Novel ALG-2 Target Protein CDIP1 Promotes Cell Death by Interacting with ESCRT-I and VAPA/B. *Int. J. Mol. Sci.* 22, 1175. doi:10.3390/ijms22031175

Ishikawa-Sasaki, K., Nagashima, S., Taniguchi, K., and Sasaki, J. (2018). Model of OSBP-Mediated Cholesterol Supply to Aichi Virus RNA Replication Sites Involving Protein-Protein Interactions Among Viral Proteins, ACBD3, OSBP, VAP-A/B, and SAC1. *J. Virol.* 92, 1952–1969. doi:10.1128/JVI.01952-17

Ito, N., Takahashi, T., Shiiba, I., Nagashima, S., Inatome, R., and Yanagi, S. (2021). MITOL Regulates Phosphatidic Acid-Binding Activity of RMDN3/PTPIP51. *J. Biochem.* 1, mvab153. doi:10.1093/JB/MVAB153

Johnson, B., Leek, A. N., Solé, L., Maverick, E. E., Levine, T. P., and Tamkun, M. M. (2018). Kv2 Potassium Channels Form Endoplasmic Reticulum/plasma Membrane Junctions via Interaction with VAPA and VAPB. *Proc. Natl. Acad. Sci.* 115, E7331–E7340. doi:10.1073/pnas.1805757115

Justis, A. V., Hansen, B., Beare, P. A., King, K. B., Heinzen, R. A., and Gilk, S. D. (2017). Interactions between the Coxiella Burnetii Parasitophorous Vacuole and the Endoplasmic Reticulum Involve the Host Protein ORP1L. *Cell. Microbiol.* 19, e12637. doi:10.1111/CMI.12637

Kabashi, E., El Oussini, H., Bercier, V., Gros-Louis, F., Valdmanis, P. N., Mcdearmid, J., et al. (2013). Investigating the Contribution of VAPB/ALS8 Loss of Function in Amyotrophic Lateral Sclerosis. *Hum. Mol. Genet.* 22, 2350–2360. doi:10.1093/HMG/DDT080

Kaiser, S. E., Brickner, J. H., Reilein, A. R., Fenn, T. D., Walter, P., and Brunger, A. T. (2005). Structural Basis of FFAT Motif-Mediated ER Targeting. *Structure* 13, 1035–1045. doi:10.1016/j.str.2005.04.010

Kamemura, K., Chen, C. A., Okumura, M., Miura, M., and Chihara, T. (2021). Amyotrophic Lateral Sclerosis-Associated Vap33 Is Required for Maintaining Neuronal Dendrite Morphology and Organelle Distribution in Drosophila. *Genes Cells* 26, 230–239. doi:10.1111/GTC.12835

Kamemura, K., and Chihara, T. (2019). Multiple Functions of the ER-Resident VAP and its Extracellular Role in Neural Development and Disease. *J. Biochem.* 165, 391–400. doi:10.1093/jb/mvz011

Kanekura, K., Nishimoto, I., Aiso, S., and Matsuoka, M. (2006). Characterization of Amyotrophic Lateral Sclerosis-Linked P56S Mutation of Vesicle-Associated Membrane Protein-Associated Protein B (VAPB/ALS8). *J. Biol. Chem.* 281, 30223–30233. doi:10.1074/jbc.M605049200

Kawano, M., Kumagai, K., Nishijima, M., and Hanada, K. (2006). Efficient Trafficking of Ceramide from the Endoplasmic Reticulum to the Golgi Apparatus Requires a VAMP-Associated Protein-Interacting FFAT Motif of CERT. *J. Biol. Chem.* 281, 30279–30288. doi:10.1074/jbc.M605032200

Kenna, K. P., McLaughlin, R. L., Byrne, S., Elamin, M., Heverin, M., Kenny, E. M., et al. (2013). Delineating the Genetic Heterogeneity of ALS Using Targeted High-Throughput Sequencing. *J. Med. Genet.* 50, 776–783. doi:10.1136/jmedgenet-2013-101795

Kim, S. H., Leal, S. S., Halevy, D. Ben., Gomes, C. M., and Lev, S. (2010). Structural Requirements for VAP-B Oligomerization and Their Implication in Amyotrophic Lateral Sclerosis-Associated VAP-B(P56S) Neurotoxicity. *J. Biol. Chem.* 285, 13839–13849. doi:10.1074/jbc.M109.097345

Kirby, J., Hewamadduma, C. A. A., Hartley, J. A., Nixon, H. C., Evans, H., Wadhwa, R. R., et al. (2007). Mutations in VAPB Are Not Associated with Sporadic ALS. *Neurology* 68, 1951–1953. doi:10.1212/01.wnl.0000263195.50981.a6

Kirmiz, M., Gillies, T. E., Dickson, E. J., and Trimmer, J. S. (2019). Neuronal ER-Plasma Membrane Junctions Organized by Kv2-VAP Pairing Recruit Nir Proteins and Affect Phosphoinositide Homeostasis. *J. Biol. Chem.* 294, 17735–17757. doi:10.1074/jbc.RA119.007635

Kirmiz, M., Vierra, N. C., Palacio, S., and Trimmer, J. S. (2018). Identification of VAPA and VAPB as Kv2 Channel-Interacting Proteins Defining Endoplasmic Reticulum-Plasma Membrane Junctions in Mammalian Brain Neurons. *J. Neurosci.* 38, 7562–7584. doi:10.1523/JNEUROSCI.0893-18.2018

Kors, S., Hacker, C., Bolton, C., Maier, R., Reimann, L., Kitchener, E. J. A., et al. (2022). Regulating Peroxisome–ER Contacts via the ACBD5-VAPB Tether by FFAT Motif Phosphorylation and GSK3β. *J. Cell Biol.* 221, e202003143. doi:10.1083/JCB.202003143

Kosac, V., de Freitas, M. R. G., Prado, F. M., Nascimento, O. J. M., and Bittar, C. (2013). Familial Adult Spinal Muscular Atrophy Associated with the VAPB Gene: Report of 42 Cases in Brazil. *Arq. Neuropsiquiatr.* 71, 788–790. doi:10.1590/0004-282X20130123

Kuijpers, M., van Dis, V., Haasdijk, E. D., Harterink, M., Vocking, K., Post, J. A., et al. (2013a). Amyotrophic Lateral Sclerosis (ALS)-associated VAPB-P56s Inclusions Represent an ER Quality Control Compartment. *Acta Neuropathol. Commun.* 2, 1–19. doi:10.1186/2051-5960-1-24

Kuijpers, M., YuLou, K., Teuling, E., Akhmanova, A., Jaarsma, D., and Hoogenraad, C. C. (2013b). The ALS8 Protein VAPB Interacts with the ER–Golgi Recycling Protein YIF1A and Regulates Membrane Delivery into Dendrites. *EMBO J.* 32, 2056–2072. doi:10.1038/emboj.2013.131

Kukihara, H., Moriishi, K., Taguwa, S., Tani, H., Abe, T., Mori, Y., et al. (2009). Human VAP-C Negatively Regulates Hepatitis C Virus Propagation. *J. Virol.* 83, 7959–7969. doi:10.1128/JVI.00889-09

Kumagai, K., Elwell, C. A., Ando, S., Engel, J. N., and Hanada, K. (2018). Both the N- and C- Terminal Regions of the Chlamydial Inclusion Protein D (IncD) Are Required for Interaction with the Pleckstrin Homology Domain of the Ceramide Transport Protein CERT. *Biochem. Biophys. Res. Commun.* 505, 1070–1076. doi:10.1016/j.bbrc.2018.09.168

Kumagai, K., Kawano-Kawada, M., and Hanada, K. (2014). Phosphoregulation of the Ceramide Transport Protein CERT at Serine 315 in the Interaction with VAMP-Associated Protein (VAP) for Inter-organelle Trafficking of Ceramide in Mammalian Cells. *J. Biol. Chem.* 289, 10748–10760. doi:10.1074/jbc.M113.528380

Kumar, N., Leonzino, M., Hancock-Cerutti, W., Horenkamp, F. A., Li, P. Q., Lees, J. A., et al. (2018). VPS13A and VPS13C Are Lipid Transport Proteins Differentially Localized at ER Contact Sites. *J. Cell Biol.* 217, 3625–3639. doi:10.1083/JCB.201807019

Kun-Rodrigues, C., Ganos, C., Guerreiro, R., Schneider, S. A., Schulte, C., Lesage, S., et al. (2015). A Systematic Screening to Identify De Novo Mutations Causing Sporadic Early-Onset Parkinson's Disease. *Hum. Mol. Genet.* 24, 6711–6720. doi:10.1093/HMG/DDV376

Larroquette, F., Seto, L., Gaub, P. L., Kamal, B., Wallis, D., Larivière, R., et al. (2015). Vapb/Amyotrophic Lateral Sclerosis 8 Knock-In Mice Display Slowly Progressive Motor Behavior Defects Accompanying ER Stress and Autophagic Response. *Hum. Mol. Genet.* 24, 6515–6529. doi:10.1093/HMG/DDV360

Lau, D. H. W., Paillusson, S., Hartopp, N., Rupawala, H., Mórotz, G. M., Gomez-Suaga, P., et al. (2020). Disruption of Endoplasmic Reticulum-Mitochondria Tethering Proteins in Post-mortem Alzheimer's Disease Brain. *Neurobiol. Dis.* 143, 105020. doi:10.1016/J.NBD.2020.105020

Lehto, M., Hynynen, R., Karjalainen, K., Kuismanen, E., Hyvärinen, K., and Olkkonen, V. M. (2005). Targeting of OSBP-Related Protein 3 (ORP3) to Endoplasmic Reticulum and Plasma Membrane Is Controlled by Multiple Determinants. *Exp. Cell Res.* 310, 445–462. doi:10.1016/j.yexcr.2005.08.003

Leoni, T. B., Rezende, T. J. R., Peluzzo, T. M., Martins, M. P., Neto, A. R. C., Gonzalez-Salazar, C., et al. (2022). Structural Brain and Spinal Cord Damage in Symptomatic and Pre-symptomatic VAPB-Related ALS. *J. Neurol. Sci.* 434, 120126. doi:10.1016/J.JNS.2021.120126

Lev, S., Ben Halevy, D., Peretti, D., and Dahan, N. (2008). The VAP Protein Family: From Cellular Functions to Motor Neuron Disease. *Trends Cell Biol.* 18, 282–290. doi:10.1016/j.tcb.2008.03.006

Levin-Konigsberg, R., Montaño-Rendón, F., Keren-Kaplan, T., Li, R., Ego, B., Mylvaganam, S., et al. (2019). Phagolysosome Resolution Requires Contacts with the Endoplasmic Reticulum and Phosphatidylinositol-4-Phosphate Signalling. *Nat. Cell Biol.* 21, 1234–1247. doi:10.1038/s41556-019-0394-2

Lim, C. Y., Davis, O. B., Shin, H. R., Zhang, J., Berdan, C. A., Jiang, X., et al. (2019). ER–lysosome Contacts Enable Cholesterol Sensing by mTORC1 and Drive Aberrant Growth Signalling in Niemann–Pick Type C. *Nat. Cell Biol.* 21, 1206–1218. doi:10.1038/s41556-019-0391-5

Lindhout, F. W., Cao, Y., Kevenaar, J. T., Bodzęta, A., Stucchi, R., Boumpoutsari, M. M., et al. (2019). VAP-SCRN1 Interaction Regulates Dynamic Endoplasmic Reticulum Remodeling and Presynaptic Function. *EMBO J.* 38, e101345. doi:10.15252/embj.2018101345

Liu, H., Lin, W.-Y., Leibow, S. R., Morateck, A. J., Ahuja, M., and Muallem, S. (2022). TRPC3 Channel Gating by Lipids Requires Localization at the ER/PM Junctions Defined by STIM1. *J. Cell Biol.* 221, e202107120. doi:10.1083/JCB.202107120

Loewen, C. J. R., Roy, A., and Levine, T. P. (2003). A Conserved ER Targeting Motif in Three Families of Lipid Binding Proteins and in Opi1p Binds VAP. *EMBO J.* 22, 2025–2035. doi:10.1093/emboj/cdg201

Lua, S., Qin, H., Lim, L., Shi, J., Gupta, G., and Song, J. (2011). Structural, Stability, Dynamic and Binding Properties of the ALS-Causing T46I Mutant of the hVAPB MSP Domain as Revealed by NMR and MD Simulations. *PLoS One* 6, e27072. doi:10.1371/journal.pone.0027072

Marques, V. D., Barreira, A. A., Davis, M. B., Abou-Sleiman, P. M., Silva, W. A., Zago, M. A., et al. (2006). Expanding the Phenotypes of the Pro56SerVAPB Mutation: Proximal SMA with Dysautonomia. *Muscle Nerve* 34, 731–739. doi:10.1002/mus.20657

Matsumoto, G., Stojanovic, A., Holmberg, C. I., Kim, S., and Morimoto, R. I. (2005). Structural Properties and Neuronal Toxicity of Amyotrophic Lateral Sclerosis-Associated Cu/Zn Superoxide Dismutase 1 Aggregates. *J. Cell Biol.* 171, 75–85. doi:10.1083/jcb.200504050

Matsuzaki, F., Shirane, M., Matsumoto, M., and Nakayama, K. I. (2011). Protrudin Serves as an Adaptor Molecule that Connects KIF5 and its Cargoes in Vesicular Transport during Process Formation. *Mol. Biol. Cell* 22, 4602–4620. doi:10.1091/mbc.e11-01-0068

Mattia, T., Wilhelm, L. P., Ikhlef, S., Wendling, C., Spehner, D., Nominé, Y., et al. (2018). Identification of MOSPD2, a Novel Scaffold for Endoplasmic Reticulum Membrane Contact Sites. *EMBO Rep.* 19, e45453. doi:10.15252/EMBR.201745453

McCune, B. T., Tang, W., Lu, J., Eaglesham, J. B., Thorne, L., Mayer, A. E., et al. (2017). Noroviruses Co-opt the Function of Host Proteins VAPA and VAPB for Replication via a Phenylalanine–Phenylalanine- Acidic-Tract-Motif Mimic in Nonstructural Viral Protein NS1/2. *MBio* 8, 668–685. doi:10.1128/mBio.00668-17

Mesmin, B., Bigay, J., Moser Von Filseck, J., Lacas-Gervais, S., Drin, G., and Antonny, B. (2013). A Four-step Cycle Driven by PI(4)P Hydrolysis Directs sterol/PI(4)P Exchange by the ER-Golgi Tether OSBP. *Cell* 155, 830–843. doi:10.1016/j.cell.2013.09.056

Mikitova, V., and Levine, T. P. (2012). Analysis of the Key Elements of FFAT-like Motifs Identifies New Proteins that Potentially Bind VAP on the ER, Including Two AKAPs and FAPP2. *PLoS One* 7, e30455. doi:10.1371/journal.pone.0030455

Millecamps, S., Salachas, F., Cazeneuve, C., Gordon, P., Bricka, B., Camuzat, A., et al. (2010). SOD1, ANG, VAPB, TARDBP, and FUS Mutations in Familial Amyotrophic Lateral Sclerosis: Genotype-Phenotype Correlations. *J. Med. Genet.* 47, 554–560. doi:10.1136/jmg.2010.077180

Mitne-Neto, M., Machado-Costa, M., Marchetto, M. C. N., Bengtson, M. H., Joazeiro, C. A., Tsuda, H., et al. (2011). Downregulation of VAPB Expression in Motor Neurons Derived from Induced Pluripotent Stem Cells of ALS8 Patients. *Hum. Mol. Genet.* 20, 3642–3652. doi:10.1093/hmg/ddr284

Mochizuki, S., Miki, H., Zhou, R., Kido, Y., Nishimura, W., Kikuchi, M., et al. (2018). Oxysterol-binding Protein-Related Protein (ORP) 6 Localizes to the ER and ER-Plasma Membrane Contact Sites and Is Involved in the Turnover of PI4P in Cerebellar Granule Neurons. *Exp. Cell Res.* 370, 601–612. doi:10.1016/J.YEXCR.2018.07.025

Mori, F., Miki, Y., Tanji, K., Kon, T., Tomiyama, M., Kakita, A., et al. (2021). Role of VAPB and Vesicular Profiles in α-synuclein Aggregates in Multiple System Atrophy. *Brain Pathol.* 31, e13001. doi:10.1111/BPA.13001

Mórotz, G. M., De Vos, K. J., Vagnoni, A., Ackerley, S., Shaw, C. E., and Miller, C. C. J. (2012). Amyotrophic Lateral Sclerosis-Associated Mutant VAPBP56S Perturbs Calcium Homeostasis to Disrupt Axonal Transport of Mitochondria. *Hum. Mol. Genet.* 21, 1979–1988. doi:10.1093/HMG/DDS011

Moumen, A., Virard, I., and Raoul, C. (2011). Accumulation of Wildtype and ALS-Linked Mutated VAPB Impairs Activity of the Proteasome. *PLoS One* 6, e26066. doi:10.1371/journal.pone.0026066

Murphy, S. E., and Levine, T. P. (2016). VAP, a Versatile Access Point for the Endoplasmic Reticulum: Review and Analysis of FFAT-like Motifs in the VAPome. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1861, 952–961. doi:10.1016/j.bbalip.2016.02.009

Nachreiner, T., Esser, M., Tenten, V., Troost, D., Weis, J., and Krüttgen, A. (2010). Novel Splice Variants of the Amyotrophic Lateral Sclerosis-Associated Gene VAPB Expressed in Human Tissues. *Biochem. Biophys. Res. Commun.* 394, 703–708. doi:10.1016/j.bbrc.2010.03.055

Nakamichi, S., Yamanaka, K., Suzuki, M., Watanabe, T., and Kagiwada, S. (2011). Human VAPA and the Yeast VAP Scs2p with an Altered Proline Distribution Can Phenocopy Amyotrophic Lateral Sclerosis-Associated VAPB(P56S). *Biochem. Biophys. Res. Commun.* 404, 605–609. doi:10.1016/j.bbrc.2010.12.011

Ngo, M., and Ridgway, N. D. (2009). Oxysterol Binding Protein-Related Protein 9 (ORP9) Is a Cholesterol Transfer Protein that Regulates Golgi Structure and Function. *Mol. Biol. Cell* 20, 1388–1399. doi:10.1091/mbc.E08-09-0905

Nguyen, P. H., Lutter, E. I., and Hackstadt, T. (2018). *Chlamydia trachomatis* Inclusion Membrane Protein MrcA Interacts with the Inositol 1,4,5-trisphosphate Receptor Type 3 (ITPR3) to Regulate Extrusion Formation. *PLoS Pathog.* 14, e1006911. doi:10.1371/journal.ppat.1006911

Nishimura, A. L., Al-Chalabi, A., and Zatz, M. (2005). A Common Founder for Amyotrophic Lateral Sclerosis Type 8 (ALS8) in the Brazilian Population. *Hum. Genet.* 118, 499–500. doi:10.1007/s00439-005-0031-y

Nishimura, A. L., Mitne-Neto, M., Silva, H. C. A., Oliveira, J. R. M., Vainzof, M., and Zatz, M. (2004a). A Novel Locus for Late Onset Amyotrophic Lateral Sclerosis/motor Neurone Disease Variant at 20q13. *J. Med. Genet.* 41, 315–320. doi:10.1136/jmg.2003.013029

Nishimura, A. L., Mitne-Neto, M., Silva, H. C. A., Richieri-Costa, A., Middleton, S., Cascio, D., et al. (2004b). A Mutation in the Vesicle-Trafficking Protein VAPB Causes Late-Onset Spinal Muscular Atrophy and Amyotrophic Lateral Sclerosis. *Am. J. Hum. Genet.* 75, 822–831. doi:10.1086/425287

Nishimura, Y., Hayashi, M., Inada, H., and Tanaka, T. (1999). Molecular Cloning and Characterization of Mammalian Homologues of Vesicle-Associated Membrane Protein-Associated (VAMP-Associated) Proteins. *Biochem. Biophys. Res. Commun.* 254, 21–26. doi:10.1006/bbrc.1998.9876

Nthiga, T. M., Kumar Shrestha, B., Sjøttem, E., Bruun, J., Bowitz Larsen, K., Bhujabal, Z., et al. (2020). CALCOCO 1 Acts with VAMP -associated Proteins to Mediate ER -phagy. *EMBO J.* 39, e103649. doi:10.15252/embj.2019103649

Nunes Gonçalves, J. P., Leoni, T. B., Martins, M. P., Peluzzo, T. M., Dourado, M. E. T., Saute, J. A. M., et al. (2021). Genetic Epidemiology of Familial ALS in Brazil. *Neurobiol. Aging* 102, 227. doi:10.1016/j.neurobiolaging.2021.01.007

Oliveira, D., Morales-Vicente, D. A., Amaral, M. S., Luz, L., Sertié, A. L., Leite, F. S., et al. (2020). Different Gene Expression Profiles in iPSC-Derived Motor Neurons from ALS8 Patients with Variable Clinical Courses Suggest Mitigating Pathways for Neurodegeneration. *Hum. Mol. Genet.* 29, 1465–1475. doi:10.1093/hmg/ddaa069

Paillusson, S., Gomez-Suaga, P., Stoica, R., Little, D., Gissen, P., Devine, M. J., et al. (2017). α-Synuclein Binds to the ER–Mitochondria Tethering Protein VAPB to Disrupt Ca2+ Homeostasis and Mitochondrial ATP Production. *Acta Neuropathol.* 134, 129–149. doi:10.1007/s00401-017-1704-z

Pan, G., Cao, X., Liu, B., Li, C., Li, D., Zheng, J., et al. (2018). OSBP-related Protein 4L Promotes Phospholipase Cβ3 Translocation from the Nucleus to the Plasma Membrane in Jurkat T-Cells. *J. Biol. Chem.* 293, 17430–17441. doi:10.1074/jbc.RA118.005437

Papiani, G., Ruggiano, A., Fossati, M., Raimondi, A., Bertoni, G., Francolini, M., et al. (2012). Restructured Endoplasmic Reticulum Generated by Mutant Amyotrophic Lateral Sclerosis-Linked VAPB Is Cleared by the Proteasome. *J. Cell Sci.* 125, 3601–3611. doi:10.1242/jcs.102137

Passantino, R., Cascio, C., Deidda, I., Galizzi, G., Russo, D., Spedale, G., et al. (2013). Identifying Protein Partners of CLN8, an ER-Resident Protein Involved in Neuronal Ceroid Lipofuscinosis. *Biochim. Biophys. Acta - Mol. Cell Res.* 1833, 529–540. doi:10.1016/j.bbamcr.2012.10.030

Pennetta, G., Hiesinger, P. R., Fabian-Fine, R., Meinertzhagen, I. A., and Bellen, H. J. (2002). Drosophila VAP-33A Directs Bouton Formation at Neuromuscular Junctions in a Dosage-dependent Manner. *Neuron* 35, 291–306. doi:10.1016/S0896-6273(02)00769-9

Peretti, D., Dahan, N., Shimoni, E., Hirschberg, K., and Lev, S. (2008). Coordinated Lipid Transfer between the Endoplasmic Reticulum and the Golgi Complex Requires the VAP Proteins and Is Essential for Golgi-Mediated Transport. *Mol. Biol. Cell* 19, 3871–3844. doi:10.1091/mbc.E0810.1091/mbc.e08-05-0498

Pesaola, F., Quassollo, G., Venier, A. C., De Paul, A. L., Noher, I., and Bisbal, M. (2021). The Neuronal Ceroid Lipofuscinosis-Related Protein CLN8 Regulates Endo-Lysosomal Dynamics and Dendritic Morphology. *Biol. Cell* 113, 419–437. doi:10.1111/BOC.202000016

Petrova, V., Pearson, C. S., Ching, J., Tribble, J. R., Solano, A. G., Yang, Y., et al. (2020). Protrudin Functions from the Endoplasmic Reticulum to Support Axon Regeneration in the Adult CNS. *Nat. Commun.* 11, 1–15. doi:10.1038/s41467-020-19436-y

Pettersen, E. F., Goddard, T. D., Huang, C. C., Meng, E. C., Couch, G. S., Croll, T. I., et al. (2021). UCSF ChimeraX: Structure Visualization for Researchers, Educators, and Developers. *Protein Sci.* 30, 70–82. doi:10.1002/PRO.3943

Pietrangelo, A., and Ridgway, N. D. (2018). Golgi Localization of Oxysterol Binding Protein-Related Protein 4L (ORP4L) Is Regulated by Ligand Binding. *J. Cell Sci.* 131, jcs215335. doi:10.1242/jcs.215335

Prosser, D. C., Tran, D., Gougeon, P. Y., Verly, C., and Ngsee, J. K. (2008). FFAT Rescues VAPA-Mediated Inhibition of ER-To-Golgi Transport and VAPB-Mediated ER Aggregation. *J. Cell Sci.* 121, 3052–3061. doi:10.1242/jcs.028696

Qiao, X., Jia, S., Ye, J., Fang, X., Zhang, C., Cao, Y., et al. (2017). PTPIP51 Regulates Mouse Cardiac Ischemia/reperfusion through Mediating the Mitochondria-SR Junction. *Sci. Rep.* 7, 1–14. doi:10.1038/srep45379

Qin, H., Lim, L., Wei, Y., Gupta, G., and Song, J. (2013a). Resolving the Paradox for Protein Aggregation Diseases: NMR Structure and Dynamics of the Membrane-Embedded P56S-MSP Causing ALS Imply a Common Mechanism for Aggregation-Prone Proteins to Attack Membranes. *F1000Research* 2, 221. doi:10.12688/f1000research.2-221.v2

Qin, H., Wang, W., and Song, J. (2013b). ALS-causing P56S Mutation and Splicing Variation on the hVAPB MSP Domain Transform its β-sandwich Fold into Lipid-Interacting Helical Conformations. *Biochem. Biophys. Res. Commun.* 431, 398–403. doi:10.1016/j.bbrc.2013.01.039

Qiu, L., Qiao, T., Beers, M., Tan, W., Wang, H., Yang, B., et al. (2013). Widespread Aggregation of Mutant VAPB Associated with ALS Does Not Cause Motor

Neuron Degeneration or Modulate Mutant SOD1 Aggregation and Toxicity in Mice. *Mol. Neurodegener.* 8, 1–16. doi:10.1186/1750-1326-8-1

Quintanilla, C. G., Lee, W.-R., and Liou, J. (2022). Nir1 Constitutively Localizes at ER-PM Junctions and Promotes Nir2 Recruitment for PIP 2 Homeostasis. *Mol. Biol. Cell* 33 (3), br2. doi:10.1091/MBC.E21-07-0356

Raiborg, C., Wenzel, E. M., Pedersen, N. M., Olsvik, H., Schink, K. O., Schultz, S. W., et al. (2015). Repeated ER-Endosome Contacts Promote Endosome Translocation and Neurite Outgrowth. *Nature* 520, 234–238. doi:10.1038/nature14359

Ramage, H. R., Kumar, G. R., Verschueren, E., Johnson, J. R., VonDollen, J., Johnson, T., et al. (2015). A Combined Proteomics/Genomics Approach Links Hepatitis C Virus Infection with Nonsense-Mediated mRNA Decay. *Mol. Cell* 57, 329–340. doi:10.1016/J.MOLCEL.2014.12.028

Ratnaparkhi, A., Lawless, G. M., Schweizer, F. E., Golshani, P., and Jackson, G. R. (2008). A Drosophila Model of ALS: Human ALS-Associated Mutation in VAP33A Suggests a Dominant Negative Mechanism. *PLoS One* 3, e2334. doi:10.1371/journal.pone.0002334

Rimessi, A., Pozzato, C., Carparelli, L., Rossi, A., Ranucci, S., de Fino, I., et al. (2020). Pharmacological Modulation of Mitochondrial Calcium Uniporter Controls Lung Inflammation in Cystic Fibrosis. *Sci. Adv.* 6, eaax9093. doi:10.1126/sciadv.aax9093

Rocha, N., Kuijl, C., Van Der Kant, R., Janssen, L., Houben, D., Janssen, H., et al. (2009). Cholesterol Sensor ORP1L Contacts the ER Protein VAP to Control Rab7-RILP-p150Glued and Late Endosome Positioning. *J. Cell Biol.* 185, 1209–1225. doi:10.1083/JCB.200811005/VIDEO-8

Roulin, P. S., Lötzerich, M., Torta, F., Tanner, L. B., Van Kuppeveld, F. J. M., Wenk, M. R., et al. (2014). Rhinovirus Uses a Phosphatidylinositol 4-Phosphate/Cholesterol Counter-current for the Formation of Replication Compartments at the ER-Golgi Interface. *Cell Host Microbe* 16, 677–690. doi:10.1016/J.CHOM.2014.10.003

Saita, S., Shirane, M., Natume, T., Iemura, S. I., and Nakayama, K. I. (2009). Promotion of Neurite Extension by Protrudin Requires its Interaction with Vesicle-Associated Membrane Protein-Associated Protein. *J. Biol. Chem.* 284, 13766–13777. doi:10.1074/jbc.M807938200

Saito, S., Matsui, H., Kawano, M., Kumagai, K., Tomishige, N., Hanada, K., et al. (2008). Protein Phosphatase 2Cε Is an Endoplasmic Reticulum Integral Membrane Protein that Dephosphorylates the Ceramide Transport Protein CERT to Enhance its Association with Organelle Membranes. *J. Biol. Chem.* 283, 6584–6593. doi:10.1074/jbc.M707691200

Saiz-Ros, N., Czapiewski, R., Epifano, I., Stevenson, A., Swanson, S. K., Dixon, C. R., et al. (2019). Host Vesicle Fusion Protein VAPB Contributes to the Nuclear Egress Stage of Herpes Simplex Virus Type-1 (HSV-1) Replication. *Cells* 8, 120. doi:10.3390/CELLS8020120

Sanhueza, M., Zechini, L., Gillespie, T., and Pennetta, G. (2014). Gain-of-function Mutations in the ALS8 Causative Gene VAPB Have Detrimental Effects on Neurons and Muscles. *Biol. Open* 3, 59–71. doi:10.1242/BIO.20137070

Santos, M. F., Rappa, G., Karbanová, J., Kurth, T., Corbeil, D., and Lorico, A. (2018). VAMP-associated Protein-A and Oxysterol-Binding Protein–Related Protein 3 Promote the Entry of Late Endosomes into the Nucleoplasmic Reticulum. *J. Biol. Chem.* 293, 13834–13848. doi:10.1074/jbc.RA118.003725

Santoso, N. G., Cebotaru, L., and Guggino, W. B. (2011). Polycystin-1, 2, and STIM1 Interact with IP_3R to Modulate ER Ca^{2+} Release through the PI3K/Akt Pathway. *Cell. Physiol. biochem.* 27, 715–726. doi:10.1159/000330080

Sasaki, J., Ishikawa, K., Arita, M., and Taniguchi, K. (2012). ACBD3-mediated Recruitment of PI4KB to Picornavirus RNA Replication Sites. *EMBO J.* 31, 754–766. doi:10.1038/emboj.2011.429

Schlüter, K., Waschbüsch, D., Anft, M., Hügging, D., Kind, S., Hänisch, J., et al. (2014). JMY Is Involved in Anterograde Vesicle Trafficking from the Trans-golgi Network. *Eur. J. Cell Biol.* 93, 194–204. doi:10.1016/j.ejcb.2014.06.001

Schulien, A. J., Yeh, C. Y., Orang, B. N., Pa, O. J., Hopkin, M. P., Moutal, A., et al. (2020). Targeted Disruption of Kv2.1-VAPA Association Provides Neuroprotection against Ischemic Stroke in Mice by Declustering Kv2.1 Channels. *Sci. Adv.* 6, eaaz8110. doi:10.1126/sciadv.aaz8110

Sengupta, S., Miller, K. K., Homma, K., Edge, R., Cheatham, M. A., Dallos, P., et al. (2010). Interaction between the Motor Protein Prestin and the Transporter Protein VAPA. *Biochim. Biophys. Acta - Mol. Cell Res.* 1803, 796–804. doi:10.1016/j.bbamcr.2010.03.017

Shi, J., Lua, S., Tong, J. S., and Song, J. (2010). Elimination of the Native Structure and Solubility of the hVAPB MSP Domain by the Pro56Ser Mutation that Causes Amyotrophic Lateral Sclerosis. *Biochemistry* 49, 3887–3897. doi:10.1021/bi902057a

Siddiqa, A., Massimi, P., Pim, D., Broniarczyk, J., and Banks, L. (2018). Human Papillomavirus 16 Infection Induces VAP-dependent Endosomal Tubulation. *J. Virol.* 92, e01514–17. doi:10.1128/JVI.01514-17

Silbernagel, N., Walecki, M., Schäfer, M. K. H., Kessler, M., Zobeiri, M., Rinné, S., et al. (2018). The VAMP-Associated Protein VAPB Is Required for Cardiac and Neuronal Pacemaker Channel Function. *FASEB J.* 32, 6159–6173. doi:10.1096/FJ.201800246R

Silva, B. S. C., DiGiovanni, L., Kumar, R., Carmichael, R. E., Kim, P. K., and Schrader, M. (2020). Maintaining Social Contacts: The Physiological Relevance of Organelle Interactions. *Biochim. Biophys. Acta - Mol. Cell Res.* 1867, 118800. doi:10.1016/j.bbamcr.2020.118800

Skehel, P. A., Fabian-Fine, R., and Kandel, E. R. (2000). Mouse VAP33 Is Associated with the Endoplasmic Reticulum and Microtubules. *Proc. Natl. Acad. Sci. U. S. A.* 97, 1101–1106. doi:10.1073/pnas.97.3.1101

Skehel, P. A., Martin, K. C., Kandel, E. R., and Bartsch, D. (1995). A VAMP-Binding Protein from Aplysia Required for Neurotransmitter Release. *Sci. (80-* 269, 1580–1583. doi:10.1126/science.7667638

Stanhope, R., Flora, E., Bayne, C., and Derré, I. (2017). IncV, a FFAT Motif-Containing Chlamydia Protein, Tethers the Endoplasmic Reticulum to the Pathogen-Containing Vacuole. *Proc. Natl. Acad. Sci.* 114, 12039–12044. doi:10.1073/pnas.1709060114

Stoica, R., De Vos, K. J., Paillusson, S., Mueller, S., Sancho, R. M., Lau, K.-F., et al. (2014). ER-mitochondria Associations Are Regulated by the VAPB-PTPIP51 Interaction and Are Disrupted by ALS/FTD-associated TDP-43. *Nat. Commun.* 5, 3996. doi:10.1038/ncomms4996

Stoica, R., Paillusson, S., Gomez-Suaga, P., Mitchell, J. C., Lau, D. H., Gray, E. H., et al. (2016). ALS/FTD-associated FUS Activates GSK-3β to Disrupt the VAPB-PTPIP51 Interaction and ER-Mitochondria Associations. *EMBO Rep.* 17, 1326–1342. doi:10.15252/embr.201541726

Sun, Y. M., Dong, Y., Wang, J., Lu, J. H., Chen, Y., and Wujun, J. (2017). A Novel Mutation of VAPB in One Chinese Familial Amyotrophic Lateral Sclerosis Pedigree and its Clinical Characteristics. *J. Neurol.* 264, 2387–2393. doi:10.1007/s00415-017-8628-3

Suzuki, H., Kanekura, K., Levine, T. P., Kohno, K., Olkkonen, V. M., Aiso, S., et al. (2009). ALS-linked P56S-VAPB, an Aggregated Loss-Of-Function Mutant of VAPB, Predisposes Motor Neurons to ER Stress-Related Death by Inducing Aggregation of Co-expressed Wild-type VAPB. *J. Neurochem.* 108, 973–985. doi:10.1111/j.1471-4159.2008.05857.x

Suzuki, H., and Matsuoka, M. (2011). Amyotrophic Lateral Sclerosis-Linked Mutant VAPB Enhances TDP-43-Induced Motor Neuronal Toxicity. *J. Neurochem.* 119, 1099–1107. doi:10.1111/J.1471-4159.2011.07491.X

Tachida, Y., Kumagai, K., Sakai, S., Ando, S., Yamaji, T., and Hanada, K. (2020). *Chlamydia trachomatis* -infected Human Cells Convert Ceramide to Sphingomyelin without Sphingomyelin Synthases 1 and 2. *FEBS Lett.* 594, 519–529. doi:10.1002/1873-3468.13632

Temp, A. G. M., Dyrba, M., Kasper, E., Teipel, S., and Prudlo, J. (2021). Case Report: Cognitive Conversion in a Non-brazilian VAPB Mutation Carrier (ALS8). *Front. Neurol.* 12, 668772. doi:10.3389/fneur.2021.668772

Teuling, E., Ahmed, S., Haasdijk, E., Demmers, J., Steinmetz, M. O., Akhmanova, A., et al. (2007). Motor Neuron Disease-Associated Mutant Vesicle-Associated Membrane Protein-Associated Protein (VAP) B Recruits Wild-type VAPs into Endoplasmic Reticulum-Derived Tubular Aggregates. *J. Neurosci.* 27, 9801–9815. doi:10.1523/JNEUROSCI.2661-07.2007

Thaler, R., Rumpler, M., Spitzer, S., Klaushofer, K., and Varga, F. (2011). Mospd1, a New Player in Mesenchymal versus Epidermal Cell Differentiation. *J. Cell. Physiol.* 226, 2505–2515. doi:10.1002/JCP.22595

Tokutake, Y., Gushima, K., Miyazaki, H., Shimosato, T., and Yonekura, S. (2015a). ALS-associated P56S-VAPB Mutation Restrains 3T3-L1 Preadipocyte Differentiation. *Biochem. Biophys. Res. Commun.* 460, 831–837. doi:10.1016/J.BBRC.2015.03.118

Tokutake, Y., Yamada, K., Ohata, M., Obayashi, Y., Tsuchiya, M., and Yonekura, S. (2015b). ALS-linked P56S-VAPB Mutation Impairs the Formation of Multinuclear Myotube in C2C12 Cells. *Int. J. Mol. Sci.* 16, 18628–18641. doi:10.3390/IJMS160818628

Trilico, M. L. C., Lorenzoni, P. J., Kay, C. S. K., Ducci, R. D. P., Fustes, O. J. H., Werneck, L. C., et al. (2020). Characterization of the Amyotrophic Lateral Sclerosis-Linked

P56S Mutation of the *VAPB* Gene in Southern Brazil. *Amyotroph. Lateral Scler. Front. Degener.* 21, 286–290. doi:10.1080/21678421.2020.1738495

Tripathi, P., Guo, H., Dreser, A., Yamoah, A., Sechi, A., Jesse, C. M., et al. (2021). Pathomechanisms of ALS8: Altered Autophagy and Defective RNA Binding Protein (RBP) Homeostasis Due to the VAPB P56S Mutation. *Cell Death Dis.* 125 (12), 1–18. doi:10.1038/s41419-021-03710-y

Tsai, C. P., Soong, B. W., Lin, K. P., Tu, P. H., Lin, J. L., and Lee, Y. C. (2011). FUS, TARDBP, and SOD1 Mutations in a Taiwanese Cohort with Familial ALS. *Neurobiol. Aging* 32, 553. doi:10.1016/j.neurobiolaging.2010.04.009

Tsuda, H., Han, S. M., Yang, Y., Tong, C., Lin, Y. Q., Mohan, K., et al. (2008). The Amyotrophic Lateral Sclerosis 8 Protein VAPB Is Cleaved, Secreted, and Acts as a Ligand for Eph Receptors. *Cell* 133, 963–977. doi:10.1016/j.cell.2008.04.039

Tu, H., Gao, L., Shi, S. T., Taylor, D. R., Yang, T., Mircheff, A. K., et al. (1999). Hepatitis C Virus RNA Polymerase and NS5A Complex with a SNARE-like Protein. *Virology* 263, 30–41. doi:10.1006/VIRO.1999.9893

Tudor, E. L., Galtrey, C. M., Perkinton, M. S., Lau, K. F., De Vos, K. J., Mitchell, J. C., et al. (2010). Amyotrophic Lateral Sclerosis Mutant Vesicle-Associated Membrane Protein-Associated Protein-B Transgenic Mice Develop TAR-DNA-Binding Protein-43 Pathology. *Neuroscience* 167, 774–785. doi:10.1016/j.neuroscience.2010.02.035

Tuuf, J., Wistbacka, L., and Mattjus, P. (2009). The Glycolipid Transfer Protein Interacts with the Vesicle-Associated Membrane Protein-Associated Protein VAP-A. *Biochem. Biophys. Res. Commun.* 388, 395–399. doi:10.1016/j.bbrc.2009.08.023

van Blitterswijk, M., van Es, M. A., Koppers, M., van Rheenen, W., Medic, J., Schelhaas, H. J., et al. (2012). VAPB and C9orf72 Mutations in 1 Familial Amyotrophic Lateral Sclerosis Patient. *Neurobiol. Aging* 33, 2950e1–2950. e4. doi:10.1016/j.neurobiolaging.2012.07.004

Venditti, R., Masone, M. C., Rega, L. R., Tullio, G., Santoro, M., Polishchuk, E., et al. (2019). The Activity of Sac1 across ER–TGN Contact Sites Requires the Four-Phosphate-Adaptor-Protein-1. *J. Cell Biol.* 218, 783–797. doi:10.1083/JCB.201812021/VIDEO-1

Wang, H., Perry, J. W., Lauring, A. S., Neddermann, P., De Francesco, R., and Tai, A. W. (2014). Oxysterol-Binding Protein Is a Phosphatidylinositol 4-Kinase Effector Required for HCV Replication Membrane Integrity and Cholesterol Trafficking. *Gastroenterology* 146, 1373–1385. e11. doi:10.1053/J.GASTRO.2014.02.002

Wang, H., and Tai, A. W. (2019). Nir2 Is an Effector of VAPs Necessary for Efficient Hepatitis C Virus Replication and Phosphatidylinositol 4-Phosphate Enrichment at the Viral Replication Organelle. *J. Virol.* 93, e00742–19. doi:10.1128/JVI.00742-19

Wang, S., Wu, X., Pan, T., Song, W., Wang, Y., Zhang, F., et al. (2012). Viperin Inhibits Hepatitis C Virus Replication by Interfering with Binding of NS5A to Host Protein hVAP-33. *J. Gen. Virol.* 93, 83–92. doi:10.1099/VIR.0.033860-0/CITE/REFWORKS

Wang, Y., Metz, J., Costello, J. L., Passmore, J., Schrader, M., Schultz, C., et al. (2018). Intracellular Redistribution of Neuronal Peroxisomes in Response to ACBD5 Expression. *PLoS One* 13, e0209507. doi:10.1371/journal.pone.0209507

Weber-Boyvat, M., Kentala, H., Lilja, J., Vihervaara, T., Hanninen, R., Zhou, Y., et al. (2015a). OSBP-related Protein 3 (ORP3) Coupling with VAMP-Associated Protein A Regulates R-Ras Activity. *Exp. Cell Res.* 331, 278–291. doi:10.1016/j.yexcr.2014.10.019

Weber-Boyvat, M., Kentala, H., Peränen, J., and Olkkonen, V. M. (2015b1987). Ligand-dependent Localization and Function of ORP-VAP Complexes at Membrane Contact Sites. *Cell. Mol. Life Sci.* 72, 1967. doi:10.1007/s00018-014-1786-x

Weir, M. L., Klip, A., and Trimble, W. S. (1998). Identification of a Human Homologue of the Vesicle-Associated Membrane Protein (VAMP)-associated Protein of 33 kDa (VAP-33): A Broadly Expressed Protein that Binds to VAMP. *Biochem. J.* 333, 247–251. doi:10.1042/bj3330247

Wen, X., Abe, T., Kukihara, H., Taguwa, S., Mori, Y., Tani, H., et al. (2011). Elimination of Hepatitis C Virus from Hepatocytes by a Selective Activation of Therapeutic Molecules. *PLoS One* 6, e15967. doi:10.1371/JOURNAL.PONE.0015967

Wijdeven, R. H., Janssen, H., Nahidiazar, L., Janssen, L., Jalink, K., Berlin, I., et al. (2016). Cholesterol and ORP1L-Mediated ER Contact Sites Control Autophagosome Transport and Fusion with the Endocytic Pathway. *Nat. Commun.* 71 (7), 1–14. doi:10.1038/ncomms11808

Wilhelm, L. P., Wendling, C., Védie, B., Kobayashi, T., Chenard, M.-P., Tomasetto, C., et al. (2017). STARD3 Mediates Endoplasmic Reticulum-To-Endosome Cholesterol Transport at Membrane Contact Sites. *EMBO J.* 36, 1412–1433. doi:10.15252/EMBJ.201695917

Wilson, C., Venditti, R., and De Matteis, M. A. (2021). Deregulation of Phosphatidylinositol-4-Phosphate in the Development of Amyotrophic Lateral Sclerosis 8. *Adv. Biol. Regul.* 79, 100779. doi:10.1016/J.JBIOR.2020.100779

Wyles, J. P., McMaster, C. R., and Ridgway, N. D. (2002). Vesicle-associated Membrane Protein-Associated Protein-A (VAP-A) Interacts with the Oxysterol-Binding Protein to Modify Export from the Endoplasmic Reticulum. *J. Biol. Chem.* 277, 29908–29918. doi:10.1074/jbc.M201191200

Wyles, J. P., and Ridgway, N. D. (2004). VAMP-associated Protein-A Regulates Partitioning of Oxysterol-Binding Protein-Related Protein-9 between the Endoplasmic Reticulum and Golgi Apparatus. *Exp. Cell Res.* 297, 533–547. doi:10.1016/j.yexcr.2004.03.052

Yagita, Y., Shinohara, K., Abe, Y., Nakagawa, K., Al-Owain, M., Alkuraya, F. S., et al. (2017). Deficiency of a Retinal Dystrophy Protein, Acyl-Coa Binding Domain-Containing 5 (ACBD5), Impairs Peroxisomal β-oxidation of Very-Long-Chain Fatty Acids. *J. Biol. Chem.* 292, 691–705. doi:10.1074/jbc.M116.760090

Yamanaka, T., Nishiyama, R., Shimogori, T., and Nukina, N. (2020). Proteomics-Based Approach Identifies Altered ER Domain Properties by ALS-Linked VAPB Mutation. *Sci. Rep.* 10, 1–16. doi:10.1038/s41598-020-64517-z

Yeo, H. K., Park, T. H., Kim, H. Y., Jang, H., Lee, J., Hwang, G.-S., et al. (2021). Phospholipid Transfer Function of PTPIP51 at Mitochondria-Associated ER Membranes. *EMBO Rep.* 22, e51323. doi:10.15252/EMBR.202051323

Yeshaw, W. M., van der Zwaag, M., Pinto, F., Lahaye, L. L., Faber, A. I. E., Gómez-Sánchez, R., et al. (2019). Human VPS13A Is Associated with Multiple Organelles and Influences Mitochondrial Morphology and Lipid Droplet Motility. *Elife* 8, e43561. doi:10.7554/eLife.43561

Zhao, K., and Ridgway, N. D. (2017). Oxysterol-Binding Protein-Related Protein 1L Regulates Cholesterol Egress from the Endo-Lysosomal System. *Cell Rep.* 19, 1807–1818. doi:10.1016/J.CELREP.2017.05.028

Zhao, Y. G., Liu, N., Miao, G., Chen, Y., Zhao, H., and Zhang, H. (2018). The ER Contact Proteins VAPA/B Interact with Multiple Autophagy Proteins to Modulate Autophagosome Biogenesis. *Curr. Biol.* 28, 1234–1245. e4. doi:10.1016/j.cub.2018.03.002

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frontiers | Frontiers in Cell and Developmental Biology

TYPE Brief Research Report
PUBLISHED 09 August 2022
DOI 10.3389/fcell.2022.957871

OPEN ACCESS

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

RECEIVED 31 May 2022
ACCEPTED 12 July 2022
PUBLISHED 09 August 2022

CITATION
Wu F and van der Klei IJ (2022), Structure–function analysis of the ER-peroxisome contact site protein Pex32. *Front. Cell Dev. Biol.* 10:957871. doi: 10.3389/fcell.2022.957871

Structure–function analysis of the ER-peroxisome contact site protein Pex32

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In the yeast *Hansenula polymorpha*, the ER protein Pex32 is required for associating peroxisomes to the ER. Here, we report on a structure–function analysis of Pex32. Localization studies of various Pex32 truncations showed that the N-terminal transmembrane domain of Pex32 is responsible for sorting. Moreover, this part of the protein is sufficient for the function of Pex32 in peroxisome biogenesis. The C-terminal DysF domain is required for concentrating Pex32 at ER-peroxisome contact sites and has the ability to bind to peroxisomes. In order to better understand the role of Pex32 in peroxisome biogenesis, we analyzed various peroxisomal proteins in *pex32* cells. This revealed that Pex11 levels are strongly reduced in *pex32* cells. This may explain the strong reduction in peroxisome numbers in *pex32* cells, which also occurs in cells lacking Pex11.

KEYWORDS

peroxisome, endoplasmic reticulum, pex32, PEX11, contact site

Introduction

Proteins of the Pex23 family exclusively occur in yeast and filamentous fungi (Jansen et al., 2021). Members of this family contain an N-terminal domain with several predicted transmembrane (TM) helices and a DysF motif at the extreme C-terminus. So far, little is known about the function of both domains. For *Saccharomyces cerevisiae* Pex30 and Pex31, two members of the Pex23 family, a reticulon-like region in the membrane-bound domain was shown to display membrane tubulation activity (Joshi et al., 2016).

All yeast species contain several members of the Pex23 family. *S. cerevisiae* has five (Pex28, Pex29, Pex30, Pex31, and Pex32), while *Hansenula polymorpha* has four (Pex23, Pex24, Pex29, and Pex32) (Jansen et al., 2021). The absence of a member of the Pex23 family generally results in abnormal peroxisome numbers and/or size, explaining why these proteins were designated Pex. Some members also play a role in the formation of lipid bodies (Joshi et al., 2018; Wang et al., 2018) or accumulate at nuclear vacuole junctions (NVJs) (Wu et al., 2020; Ferreira and Carvalho, 2021). The function of Pex23 proteins at NVJs is still unknown.

Although Pex23 family proteins were initially reported to be peroxisomal membrane proteins, recent studies showed that they localize to ER (Mast et al., 2016; Wu et al., 2020). Often, Pex23 proteins accumulate at specialized ER regions, where contact sites are formed with

other organelles, such as peroxisomes or the nucleus. These specialized ER regions are also implicated in the formation of pre-peroxisomal vesicles and lipid bodies (Joshi et al., 2018).

We recently showed that *H. polymorpha* Pex23, Pex24, and Pex32, but not Pex29, play important roles in the formation of ER-peroxisome contact sites (Wu et al., 2020). In the absence of HpPex23, HpPex24, or HpPex32, fewer peroxisome-ER contact sites occur, paralleled by a reduction in the average peroxisomal membrane surface and decreased peroxisome numbers (Wu et al., 2020; Yuan et al., 2022). The reduction in peroxisomal membrane surface suggests that contact sites may play a role in the transfer of lipids from ER to peroxisomes to allow organellar expansion. Recent data indicated that the bulk lipid transporter protein Vps13 may contribute to lipid transfer at these contacts (Yuan et al., 2022).

Why peroxisome numbers are decreased in cells lacking Pex23 family proteins is still unknown. Intriguingly, *H. polymorpha pex23* and *pex24* cells have very similar peroxisome abnormalities as *H. polymorpha pex11* cells, namely reduced peroxisome abundance together with an increase in organellar size (Krikken et al., 2009; Yuan et al., 2022). Like in *pex23*, *pex24*, and *pex32* cells, also in *pex11* cells, peroxisome-ER contact sites are disrupted (Wu et al., 2020), suggesting that Pex23 family proteins at ER together with Pex11 at the peroxisomal membrane are involved in peroxisome-ER contact site formation. Pex11 is an abundant peroxisomal membrane protein (PMP), well known for its role in peroxisome multiplication (Erdmann and Blobel, 1995; Krikken et al., 2009; Carmichael and Schrader, 2022). Intriguingly, *S. cerevisiae* Pex11 was shown to be important for the formation of peroxisome-mitochondria contacts, suggesting that it may be a general contact site resident protein (Mattiazzi Ušaj et al., 2015; Wu et al., 2020).

H. polymorpha Pex32 is a crucial Pex23 family protein for peroxisome biogenesis because of absence of Pex32 results in most severe peroxisomal defects (Wu et al., 2020). Here, we investigated the function of different domains of HpPex32. We show that the second transmembrane (TM) helix harbors ER targeting information. The DysF domain has the capacity to associate with peroxisomes but is not essential for the Pex32 function. Unexpectedly, Pex11 levels are very low in *pex32* cells. This may explain why peroxisome numbers are low in *pex32* cells, like in *pex11* cells.

Results

The overproduced Pex32 N-terminal domain localizes to the ER, while the C-terminal DysF domain can associate to peroxisomes

Sequence analysis of the *H. polymorpha* Pex32 protein revealed four predicted TM helices in the N-terminus and a DysF motif at the extreme C-terminus (Wu et al., 2020) (Figure 1A). To analyze which part of the protein is important for sorting to ER, we constructed several truncated variants containing GFP at the C-terminus. Considering the very low endogenous Pex32 levels (Wu et al., 2020), all truncations were produced under the control of the relatively strong *ADH1* promoter (P_{ADH1}). We previously showed that overproduced full-length Pex32-GFP localizes to ER similar to the endogenously produced protein (Wu et al., 2020). The constructs were introduced in a *pex32* strain, also producing BiP-mCherry-HDEL as the ER marker, and analyzed by confocal laser scanning microscopy (CLSM, Airy Scan). The western blot analysis using anti-GFP antibodies confirmed that all Pex32 variants were present at the expected molecular weight (Supplementary Figure S1).

In line with our earlier report overproduced full-length Pex32-GFP localizes to the peripheral ER and nuclear envelope [Figure 1B; (Wu et al., 2020)]. Removal of the extreme N-terminal 31 residues, which precede the first predicted TM helix ($Pex32^{\Delta 31}$) or removal of the C-terminal DysF domain ($Pex32^{TM(I–IV)}$) did not affect sorting to ER, indicating that ER sorting information is present in the region containing four predicted TM helices, as expected.

To study which region in the membrane-bound domain of Pex32 is required for sorting to ER, the location of several truncated variants was determined by fluorescence microscopy. Of these constructs, a truncation consisting of only the first TM helix [$P_{ADH1}Pex32^{TM(I)}$] was mainly cytosolic, with only very little fluorescence detectable at the nuclear envelope. All other constructs are fully co-localized with the ER marker. These include a construct consisting of only the second TM helix [$Pex32^{TM(II)}$], the first second TM helices [$Pex32^{TM(I–II)}$] or the first third TM helices [$Pex32^{TM(I–III)}$]. Similarly, a construct lacking the first TM helix [$Pex32^{TM(II–IV)}$] is localized to the ER. The protein level of a construct consisting of the third and fourth TM helix [without the DysF domain; $Pex32^{TM(III–IV)}$] was below the limit of detection by fluorescence microscopy and therefore could not be localized.

To summarize, our study revealed that all constructs containing TM(II) localized to ER, indicating that TM(II) contains ER sorting information. Whether ER sorting information is also present in TM(III) or TM(IV) could not be established.

Overexpression of the soluble DysF domain containing a C-terminal GFP in *pex32* cells ($Pex32^{DysF}$-GFP) resulted in some spots of higher intensity in addition to cytosolic fluorescence (Figure 1C). Co-localization experiments showed that these spots frequently overlapped with the Pex14-mKate2 peroxisomal membrane marker (Figure 1C), indicating that the soluble DysF domain is capable to associate with peroxisomes. Note that not all *pex32* cells contain peroxisomes, therefore many cells lack a Pex14-mKate2 spot (Wu et al., 2020).

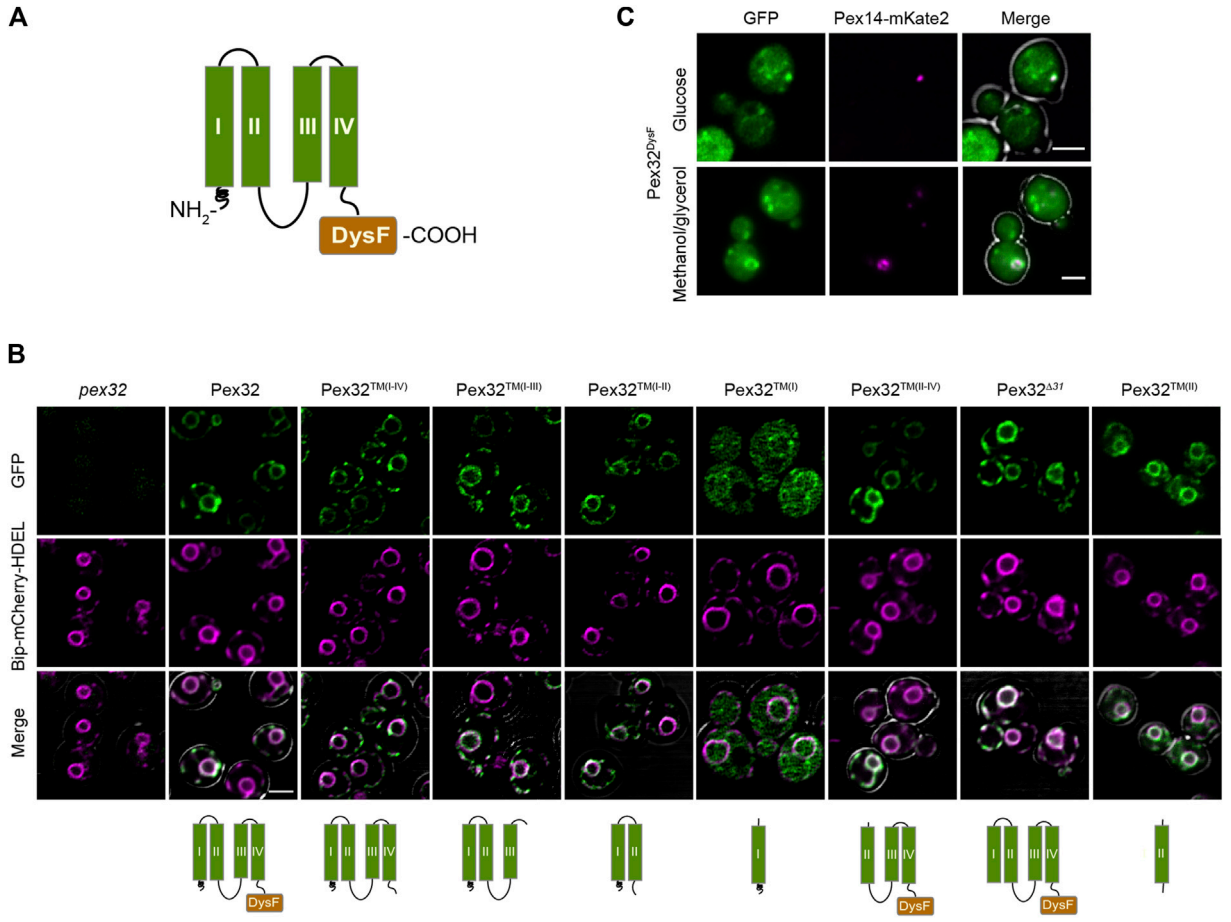

FIGURE 1
Pex32 TM helices contain ER sorting information, while the DysF domain has the capacity to associate with peroxisomes. **(A)** Predicted Pex32 structure. Transmembrane helixes (TMs) are numbered I, II, III and IV. **(B)** Confocal Laser Scanning Microscopy (CLSM) Airy-scan analysis of glucose-grown pex32 cells producing the ER marker Bip-mCherry-HDEL and the indicated Pex32 truncations fused to GFP and produced under control of the PADH1. Images were processed differently, in order to visualize the GFP signal optimally. Scale bars: 2 µm. **(C)** CLSM Airy-scan images of glucose-grown cells (top) and fluorescence microscopy (FM) images of methanol/glycerol-grown pex32 cells producing Pex14-mKate2 and PADH1Pex32DysF-mGFP (bottom). Scale bars: 2 µm.

The DysF domain is not essential for Pex32 function in peroxisome biogenesis

To analyze the role of the different domains of Pex32 in peroxisome biology, we quantified peroxisome numbers in *pex32* cells producing Pex32 truncations containing GFP at the C-terminus. To rule out overproduction artifacts, all truncations were produced under the control of the endogenous promoter (P_{PEX32}). A WT strain producing full-length Pex32 containing GFP at the C-terminus (P_{PEX32}Pex32^{FL}-GFP) was used as a positive control, while *pex32* was included as a negative control. Peroxisomes were marked with PMP47-mKate2 and images were obtained by wide-field microscopy. Cells were grown on a mixture of glycerol and methanol to induce peroxisome proliferation. In line with our previous observations, the absence of Pex32 resulted in a strong decrease in peroxisome numbers (Figures 2A,B). Peroxisome numbers are restored to WT levels upon introduction of a construct that contains the N-terminal domain [*pex32*:Pex32$^{TM(I–IV)}$], also including first 31 N-terminal residues (Figures 2A,B). Similarly, the growth defect of *pex32* cells on glycerol/methanol was fully rescued upon introduction of the entire Pex32 N-terminal domain [*pex32*:Pex32$^{TM(I–IV)}$; Figure 2C]. None of other smaller constructs fully complemented *pex32* in terms of peroxisome numbers or growth on glycerol/methanol (Figures 2B,C). These findings indicate that the complete N-terminus (extreme N-terminal 31 residues together with the four predicted TM helices) is required and sufficient for Pex32 function. Consequently, the DysF domain is not required for the function of *H. polymorpha* Pex32 in peroxisome biology.

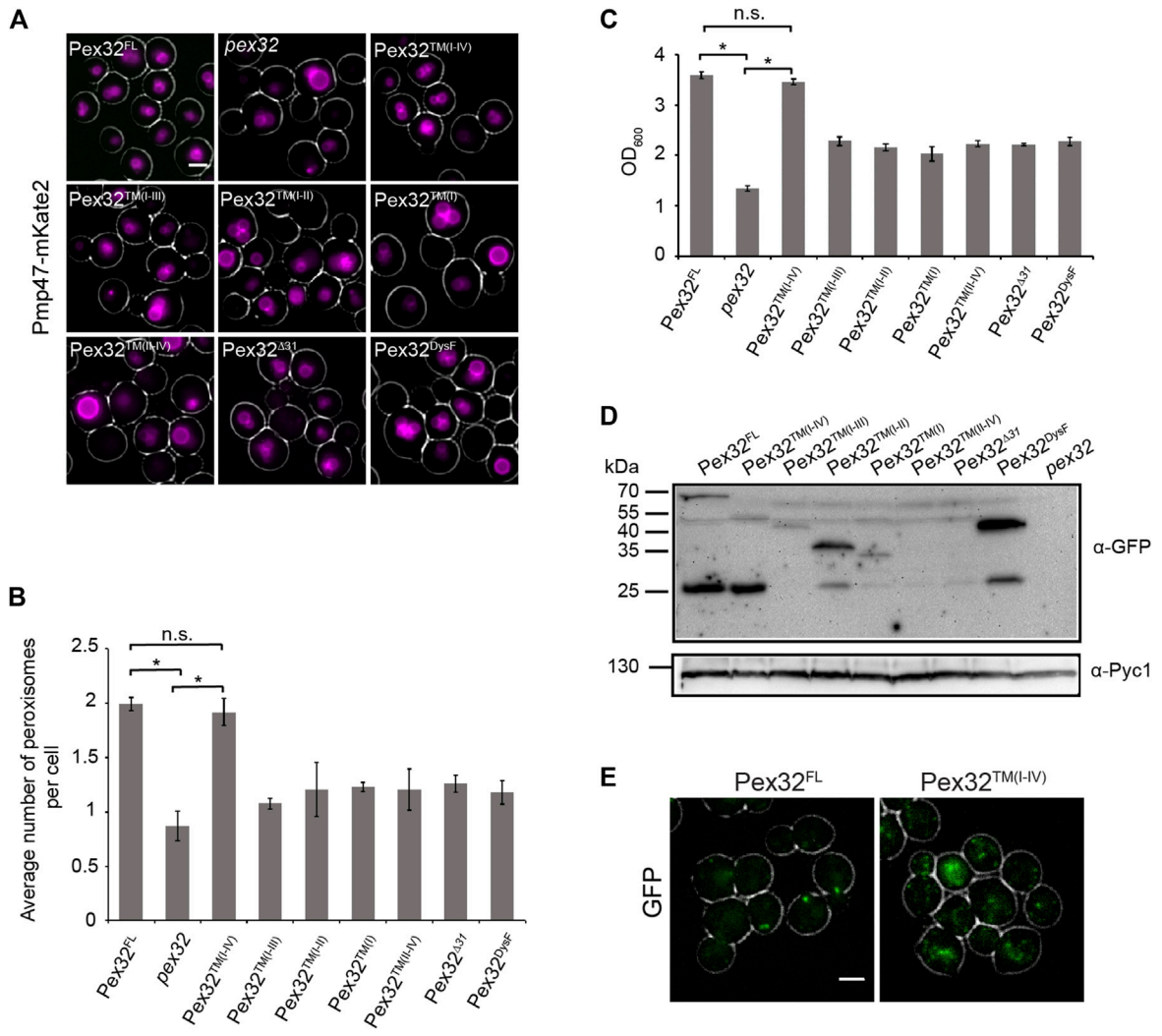

FIGURE 2
N-terminal domain of Pex32 is important for its function, while the DysF domain is required to accumulate Pex32 at peroxisome-ER contact sites. **(A)** FM analysis of methanol/glycerol-grown pex32 cells producing Pmp47-mKate2 and indicated Pex32 truncations containing GFP at the C-terminus and produced under the control of PPEX32. Scale bar: 2 µm. **(B)** Quantification of the average number of peroxisomes per cell in the indicated strains. Error bars represent s.d. from three independent experiments (n = 3 using 200 cells from each experiment). Significance indications: n. s. = $p > 0.05$, * = $p < 0.01$. **(C)** Optical densities of the indicated strains upon growth for 16 h on methanol/glycerol medium. Significance indications: n. s. = $p > 0.05$, * = $p < 0.01$. Error bars represent s.d. from three independent experiments. **(D)** Western blot analysis of the indicated strains. Cells were grown in a methanol/glycerol medium for 16 h. Blots were decorated with anti-GFP or anti-Pyc1 antibodies. Pyc1 was used as a loading control. **(E)** FM analysis of glucose-grown WT cells producing Pex32-GFP (left) or pex32 cells producing Pex32TM(I-IV) (right). Scale bar: 2 µm.

The western blot analysis using antibodies against GFP revealed that all Pex32 constructs were present at the expected size, except for Pex32$^{™(II–IV)}$ and Pex32$^{\Delta 31}$, which were below the limit of detection (Figure 2D). Therefore, the inability of the latter two constructs to complement *pex32* cells maybe due to insufficient protein levels. For full-length Pex32 as well as for some of the truncations also a band of approx. 27 kDa was observed. This band most likely represents free GFP.

In glucose-grown WT cells Pex32-GFP, produced under the control of the endogenous promoter, accumulates in a single spot per cell, which represents the peroxisome-ER contact site (Wu et al., 2020) (Figure 2E, Pex32^{FL}). However, upon removal of the DysF domain, GFP fluorescence was no longer concentrated in a single spot [*pex32*:Pex32$^{™(I–IV)}$] (Figure 2E). This suggests that the DysF domain of Pex32 contributes to concentrating Pex32 at peroxisome-ER contacts.

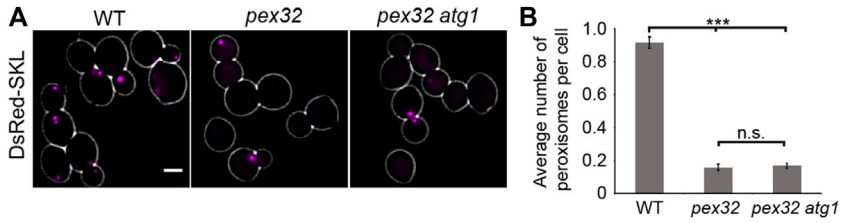

FIGURE 3
Reduced peroxisome abundance in pex32 cells is not caused by autophagy. **(A)** FM images of DsRed-SKL produced in WT, pex32, and pex32 atg1 cells grown on glucose for 4 h. Scale bar: 2 µm. **(B)** Quantification of the average number of peroxisome per cell in indicated strains. Error bars represent s.d. from three independent experiments (n = 3 using 200 cells from each experiment). Significance indications: n. s. = $p > 0.05$, *** = $p < 0.001$.

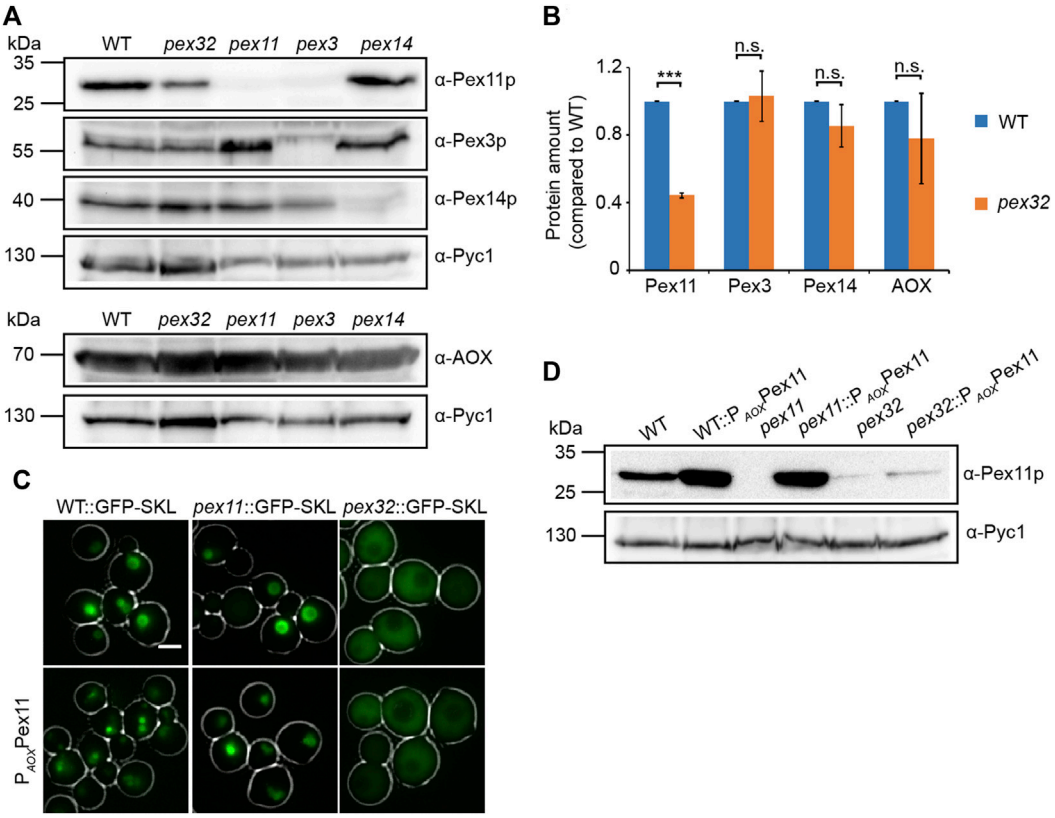

FIGURE 4
Pex11 levels are low in *pex32* cells. **(A)** Western blot analysis and **(B)** quantification of indicated proteins in WT, *pex32*, and indicated negative control cells grown for 16 h on methanol/glycerol. Blots were decorated with anti-Pex11p, anti-Pex3p, anti-Pex14p, anti-AOX, or anti-Pyc1 antibodies. Pyc1 was used as a loading control. In B, the protein levels of WT cells were set to 1. Significance indications: n. s. = $p > 0.05$, ***p = < 0.001. The error bars represent s.d. from three independent experiments. **(C)** FM images of WT, *pex11*, and *pex32* cells producing GFP-SKL grown on methanol/glycerol for 6 h. Scal e bar: 2 µm. **(D)** Western blot analysis of the indicated strains grown on methanol/glycerol for 6 h. Blots were decorated with anti-Pex11p or anti-Pyc1 antibodies. Pyc1 was used as a loading control.

In contrast to the DysF domain of Pex32, the DysF domain of *H. polymorpha* Pex23 is essential for its function (Supplementary Figure S2). Cells lacking Pex23 (*pex23*) or only producing the N-terminal membrane-bound domain of Pex23 without the DysF domain showed a similar phenotype as *pex23* cells (Supplementary Figure S2). Replacing the DysF domain of Pex23 with the same domain of Pex32 did not restore peroxisome formation (Supplementary

Figure S2), indicating that there is no functional redundancy among the DysF domains of Pex23 and Pex32.

The reduction of peroxisome abundance in *pex32* cells is not caused by enhanced autophagy

Deletion of *PEX32* results in a strong reduction of peroxisome numbers (Wu et al., 2020). To block autophagy, we deleted *ATG1* in *pex32*. The quantitative analysis of FM images of cells producing the peroxisomal matrix marker DsRed-SKL revealed that peroxisome numbers were similar in *pex32* and *pex32 atg1* cells (Figures 3A,B). This indicates that the reduced peroxisome numbers in *pex32* are not due to autophagy.

Deletion of *PEX32* results in reduced Pex11 levels

To understand why peroxisome numbers are reduced in *pex32* cells, we analyzed the levels of several peroxisomal proteins by western blotting. As shown in Figures 4A,B the levels of the peroxisomal matrix protein alcohol oxidase (AOX) were similar in *pex32* and WT cells. Also, PMPs Pex3 and Pex14 were similar in both strains. However, Pex11 levels were strongly reduced in *pex32* cells. As reported previously, Pex11 levels are also reduced in *H. polymorpha pex3* cells, which were included as a negative control for the Pex3 blot (Knoops et al., 2014).

To investigate whether the peroxisome phenotype was restored by artificially increasing Pex11 levels, *PEX11* was overexpressed using the strong *AOX* promoter (P_{AOX}) in *pex32* cells producing the peroxisomal matrix protein GFP-SKL. As expected, *PEX11* overexpression resulted in enhanced peroxisome proliferation in WT and *pex11* controls (Figure 4C). The western blot analysis confirmed the increase in Pex11 protein levels in these two strains. In contrast, *pex32*:P_{AOX}*PEX11* cells still showed mislocalization of GFP-SKL to the cytosol, like in the *pex32* control (Figure 4C). Also, Pex11 levels were not increased in *pex32* cells upon overexpression of *PEX11* (Figure 4D).

Discussion

Here, we show that the second predicted TM in the N-terminal domain of *H. polymorpha* Pex32 is important for sorting the protein to ER, while the C-terminal DysF domain is capable to associate with peroxisomes. DysF is important to concentrate Pex32 at peroxisome-ER contact sites. Unexpectedly, the DysF domain is redundant for the function of Pex32 in peroxisome biogenesis. Finally, we show that Pex11 levels are strongly reduced in *pex32*, which explains low peroxisome abundance, as observed in *pex11* cells.

Localization studies of various truncated Pex32 variants revealed that removal of the first N-terminal 31 residues or the DysF domain had no effect on ER sorting, indicating that the region with the four predicted TM helices contains ER sorting information. The western blot analysis revealed that deletion of the first 31 N-terminal residues caused a strong decrease in protein levels (Supplementary Figure S1; Figure 2D). Possibly, the absence of this part of the protein makes Pex32 more susceptible to proteolytic degradation.

A construct consisting of only TM(I) was mainly cytosolic, while the levels of the construct consisting of TM(III-IV) were too low to allow its localization. All constructs containing TM(II) localized to ER, indicating that this domain contains ER sorting information.

We show that the DysF domain is redundant for the role of *H. polymorpha* Pex32 in peroxisome biology because peroxisome defects are restored upon introduction of a Pex32 construct lacking the DysF domain in *pex32* cells. This observation differs from earlier findings in *P. pastoris* and *S. cerevisiae*. In *P. pastoris*, DysF domains of Pex30 and Pex31 are essential for the regulation of peroxisome number and size (Yan et al., 2008). Similarly, the DysF domain of *S. cerevisiae* Pex30 was shown to be essential for normal peroxisome biology (Ferreira and Carvalho, 2021). Moreover, removal of the ScPex30 DysF domain results in defects in the NVJ organization and lipid body clustering (Ferreira and Carvalho, 2021).

Our data show that accumulation of HpPex32-GFP at peroxisome-ER contact sites requires the DysF domain because GFP fluorescence no longer is present in a single spot per cell when the DysF motif was removed. Moreover, we showed that the Pex32 DysF domain has the ability to associate with peroxisomes. Most likely the Pex32 DysF domain has a binding partner at the peroxisomal membrane, which keeps the protein concentrated at peroxisome-ER contact sites. This accumulation apparently is not essential, because a construct lacking the DysF domain, which does not accumulate at contact sites, still can functionally complement a *pex32* deletion strain. Possibly in these cells, sufficient Pex32 is present at the contact sites for their function.

We show that cells lacking Pex32 have strongly reduced Pex11 levels, while the levels of other peroxisomal proteins tested were normal. Moreover, upon placing *PEX11* under the control of a strong promoter in *pex32* cells, Pex11 protein levels were not enhanced, suggesting that Pex32 is required to maintain normal Pex11 levels. This observation may explain why in *pex32* cells peroxisome numbers are strongly reduced as observed for *pex11* cells. Why Pex11 levels are low in *pex32* is not yet understood and requires further analysis.

Materials and methods

Strains and growth conditions

H. polymorpha cells were grown in batch cultures at 37°C on mineral media (Van Dijken et al., 1976) supplemented with 0.5% glucose or 0.5% methanol, or a mixture of 0.5% methanol and 0.05%

glycerol as carbon source. When required leucine was added to a final concentration of 60 μg/ml. For growth on plates, YPD media (1% yeast extract, 1% peptone, and 1% glucose) or YND media (0.67% yeast nitrogen base without amino acids (YNB; Difco; BD) and 0.5% glucose) were supplemented with 2% agar. Resistant transformants were selected using 100 μg/ml zeocin (Invitrogen), 100 μg/ml nourseothricin (WERNER BioAgents), or 300 μg/ml hygromycin (Invitrogen).

Escherichia coli strain DH5α was used for cloning. *E. coli* cells were grown at 37°C in Luria broth (LB) media (1% Bactotryptone, 0.5% Yeast Extract, and 0.5% NaCl) supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin. 2% agar was added in LB medium for growth on plates.

Construction of *H. polymorpha* strains

All strains, plasmids, and primers used in this study are listed in Supplementary Tables S1, S2, and S3, respectively. Plasmid integration was performed as described previously (Faber et al., 1994). All integrations were confirmed by PCR. Gene deletions were confirmed by PCR and Southern blotting.

Construction of strains expressing GFP-tagged Pex32 truncations

Plasmids encoding P_{ADH1}Pex32$^{™(I–IV)}$-mGFP and P_{ADH1}Pex32^{DysF}-mGFP were constructed as follows: a PCR fragment encoding the N-terminus (all 4 TMs) of *PEX32* was obtained using primers Fw $Pex32_{1\text{-}696}$ and Rv $Pex32_{1\text{-}696}$ with *H. polymorpha yku80* genomic DNA as a template. Similarly, a PCR fragment encoding the DysF domain of *PEX32* was obtained with primers Fw $Pex32_{697\text{-}1062}$ and Rv $Pex32_{697\text{-}1062}$. The obtained PCR fragments were digested with *Hin*dIII and *Bgl*II and separately inserted between the *Hin*dIII and *Bgl*II sites of plasmid pHIPZ18-*INP1*-GFP, resulting in pHIPZ18-*PEX32*$^{™(I–IV)}$-mGFP and pHIPZ18-*PEX32*^{DysF}-mGFP. Both plasmids and pAMK106 were digested by *Hin*dIII and *Sal*I separately, and ligated, resulting in pHIPN18-*PEX32*$^{™(I–IV)}$-mGFP and pHIPN18-*PEX32*^{DysF}-mGFP.

By using pHIPN18-*PEX32*$^{™(I–IV)}$-mGFP as the template, primer pairs: 1) Fw $Pex32_{1\text{-}696}$/Rev $Pex32_{1\text{-}501}$, 2) Fw $Pex32_{1\text{-}696}$/Rev $Pex32_{1\text{-}312}$, and 3) Fw $Pex32_{1\text{-}696}$/Rev $Pex32_{1\text{-}177}$ were used to amplify constructs containing: 1) *PEX32*$^{™(I–III)}$, 2) *PEX32*$^{™(I–II)}$, and 3) *PEX32*$^{™(I)}$, respectively. PCR products were digested with *Hin*dIII and *Bgl*II, and inserted between the *Hin*dIII and *Bgl*II sites of pHIPN18-*PEX32*$^{™(I–IV)}$-mGFP separately to obtain pHIPN18-*PEX32*$^{™(I–III)}$-mGFP, pHIPN18-*PEX32*$^{™(I–II)}$-mGFP, and pHIPN18-*PEX32*$^{™(I)}$-mGFP.

Plasmids pHIPN18-*PEX32*$^{™(II–IV)}$-mGFP and pHIPN18-*PEX32*^{Δ31}-mGFP were constructed by using the same method. *H. polymorpha* Pex32-mGFP genomic DNA was used as the template, using primer pairs Fw $Pex32_{(169–1062)}$/Rv $DysF_{PEX32\text{-}mGFP}$ and Fw $Pex32_{(94–1062)}$/Rv $DysF_{PEX32\text{-}mGFP}$ to amplify fragments containing *PEX32*$^{™(II–IV)}$-mGFP and *PEX32*^{Δ31}-mGFP, respectively. Both PCR products and pHIPN18-*PEX32*$^{™(I–IV)}$-GFP were restricted by *Hin*dIII and *Xho*I separately, resulting in pHIPN18-*PEX32*$^{™(II–IV)}$-mGFP and pHIPN18-*PEX32*^{Δ31}-mGFP.

The plasmid for *PEX32* overexpression was constructed as follows: a PCR fragment containing full-length *PEX32* was obtained using primers Fw $Pex32_{1\text{-}696}$ and Rv $DysF_{PEX32\text{-}mGFP}$ with Pex32-mGFP strain as a template. The PCR product and pHIPN18-Pex32$^{™(I–IV)}$-mGFP were digested by *Hin*dIII and *Xho*I and ligated resulting in pHIPN18-*PEX32*-mGFP.

All aforementioned plasmids were linearized with *Aat*II and integrated into *pex32* strains separately to produce P_{ADH1}Pex32-mGFP, P_{ADH1}Pex32$^{™(I–IV)}$-mGFP, P_{ADH1}Pex32$^{™(I–III)}$-mGFP, P_{ADH1}Pex32$^{™(I–II)}$-mGFP, P_{ADH1}Pex32$^{™(I)}$-mGFP, P_{ADH1}Pex32$^{™(II–IV)}$-mGFP, P_{ADH1}Pex32^{Δ31}-mGFP, or P_{ADH1}Pex32^{DysF}-mGFP. *Dra*I-linearized pHIPX7-BiP_{N30}-mCherry-HDEL was integrated into various truncations independently to express BiP-mCherry-HDEL.

To obtain pHIPN18-*PEX32*$^{™(II)}$-mGFP and pHIPN18-*PEX32*$^{™(III–IV)}$-mGFP, plasmid pHIPN18-*PEX32*$^{™(I–IV)}$-mGFP was used as a template, and primer pairs Fw $Pex32_{o2'TM}$/Rev $Pex32_{1\text{-}312}$, Fw $Pex32_{TM3+4}$/Rv $Pex32_{1\text{-}696}$ were used to amplify fragments containing *PEX32*$^{™(II)}$ and *PEX32*$^{™(II–IV)}$, respectively. These PCR products and pHIPN18-*PEX32*$^{™(I–IV)}$-mGFP were digested with *Hin*dIII and *Bgl*II and ligated to obtain pHIPN18-*PEX32*$^{™(II)}$-mGFP and pHIPN18-*PEX32*$^{™(III–IV)}$-mGFP. *Aat*II-linearized plasmids were integrated into *pex32*:BiP-mCherry-HDEL separately to produce P_{ADH1}Pex32$^{™(II)}$-mGFP and P_{ADH1}Pex32$^{™(III–IV)}$-mGFP.

Plasmids for producing various Pex32 truncations under the control of the *PEX32* promoter were constructed as follows: PCR was performed on *yku80* genomic DNA to amplify the *PEX32* promoter using primers P_{PEX32} fw and P_{PEX32} rev. The obtained PCR fragment was digested with *Not*I and *Hin*dIII, and then replaced the *ADH1* promoter (P_{ADH1}) in *Not*I/*Hin*dIII digested variants of Pex32 truncations. All constructions under the control of P_{PEX32} were linearized with *Eco*RV and integrated into *pex32*:Pmp47-mKate2 cells separately to produce Pex32$^{™(I–IV)}$-mGFP, Pex32$^{™(I–III)}$-mGFP, Pex32$^{™(I–II)}$-mGFP, Pex32$^{™(I)}$-mGFP, Pex32$^{™(II–IV)}$-mGFP, Pex32^{Δ31}-mGFP, and Pex32^{DysF}-mGFP.

Construction of the *pex32 atg1* double deletion strain

To construct *pex32 atg1*, a PCR fragment containing the *ATG1* deletion cassette was amplified with primers pDEL-ATG1-fwd and pDEL-ATG1-rev using plasmid pARM011 as a template. The resulting *ATG1* deletion cassette was transformed into *pex32* cells to get a double mutant of *pex32 atg1*. *Dra*I-linearized pAMK15 plasmid was transformed into *pex32 atg1* cells to produce DsRed-SKL.

Construction of strains expressing *PEX11* under control of the alcohol oxidase promoter (P_{AOX})

Plasmid pHIPH4-*PEX11* was produced by ligation of *Not*I and *Sma*I digested pHIPX4-*PEX11* and pHIPH7-*PEX11*. The plasmid pHIPX4-*PEX11* was constructed as follows: a PCR fragment containing *PEX11* was obtained using primers Pex11-3 and Pex11-4 with WT genomic DNA as a template. The PCR product and pHIPX4 were restricted by *Hin*dIII and *Sal*I, ligated which result in pHIPX4-*PEX11*. pHIPH7-*PEX11* was constructed from the ligation of *Bam*HI and *Xma*I digested pHIPH5-*PEX11* and pHIPH7-DsRed-SKL. To get pHIPH5-*PEX11*, the *PEX11* gene was amplified with primers PEX11-01 and PEX11-02 by using the WT genomic DNA as templates, *Bam*HI and *Xma*I digested PCR fragment was inserted between *Bam*HI and *Xma*I sites of pSEM04. *Nsi*I-linearized pHIPH4-*PEX11* was integrated into WT: GFP-SKL, *pex11*:GFP-SKL, and *pex32*:GFP-SKL strains, respectively, to produce P_{AOX}Pex11.

Preparation of yeast TCA lysates, SDS-PAGE, and western blotting

Cell extracts of TCA-treated cells were prepared for SDS-PAGE as described previously (Baerends et al., 2000). Equal amounts of protein were loaded per lane and blots were probed with anti-mGFP antibodies (sc-9996, Santa Cruz Biotech; 1:2000 dilution), anti-Pex11 antibodies (Knoops et al., 2014; 1:2,000 dilution), anti-Pex14 antibodies (Komori et al., 1997; 1:10,000 dilution), anti-Pex3 antibodies (Baerends et al., 1997; 1:5,000 dilution), anti-AOX antibodies (van der Klei et al., 1995; 1:10,000 dilution), or anti-pyruvate carboxylase 1 (Pyc1) antibodies (Ozimek et al., 2007; 1: 10,000 dilution). Secondary goat anti-rabbit (31,460) or goat anti-mouse (31,430) antibodies conjugated to horseradish peroxidase (HRP) (Thermo Scientific; 1:5,000 dilution) were used for detection. Pyc1 was used as a loading control.

Quantification of western blots

Blots were scanned using a densitometer (Bio-Rad, GS-710) and protein levels were quantified using ImageJ software. The intensity of each band measured was normalized by dividing by the intensity of the corresponding Pyc1 band (loading control). Normalized values obtained for Pex11, Pex3, Pex14, and AOX levels in WT cells were set to one and levels in *pex32* cells were displayed relative to WT control. Standard deviations were calculated using Excel. Significance was determined using two-tailed Student's *t*-test. n. s. represents p-values > 0.05 and *** represents p-values < 0.001. The data presented are derived from three independent experiments.

Fluorescence microscopy

Wide-field FM images were captured at room temperature using a 100 × 1.30 NA objective (Carl Zeiss, Oberkochen, Germany). Images were acquired using a Zeiss Axioscope A1 fluorescence microscope (Carl Zeiss), Micro-Manager 1.4 software, and a CoolSNAP HQ^2 digital camera. GFP fluorescence was visualized with a 470/40 nm band-pass excitation filter, a 495 nm dichromatic mirror, and a 525/50 nm band-pass emission filter. DsRed fluorescence was visualized with a 546/12 nm band-pass excitation filter, a 560 nm dichromatic mirror, and a 575–640 nm band-pass emission filter. mCherry and mKate2 fluorescence were visualized with a 587/25 nm band-pass excitation filter, a 605 nm dichromatic mirror, and a 670/70 nm band-pass emission filter.

Airy-scan images were captured with a confocal microscope (LSM800; Carl Zeiss) equipped with a 32-channel gallium arsenide phosphide photomultiplier tube (GaAsP-PMT), Zen 2009 software (Carl Zeiss) and a 63 × 1.40 NA objective (Carl Zeiss, Oberkochen, Germany). The GFP, mKate2, and mCherry fluorescence were visualized with a 488, 561, and 587 nm laser, respectively.

Image analysis was performed using ImageJ. Bright field images have been adjusted to only show cell outlines. Figures were prepared using Adobe Illustrator software.

Quantification of peroxisomes numbers

Peroxisome numbers were quantified using 200 randomly selected cells from three independent cultures. Numbers correspond to the average number of peroxisomes per cell. Standard deviations were calculated using Excel. Significance was determined using two-tailed Student's *t*-test. n. s. represents p-values > 0.05 and *** represents p-values < 0.001.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

FW and IK conceived the project and reviewed and edited the manuscript. FW performed the experiments, analyzed the data, prepared the figures, and wrote the original draft.

Funding

This work was supported by a grant from the China Scholarship Council (CSC) to FW.

Acknowledgments

The authors thank Eline de Lange for strain construction and for performing the DysF domain swap experiment in Supplementary Figure S2. They thank Rinse de Boer for fruitful discussions and help with image analysis.

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.

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Supplementary material

The Supplementary Material for this article can be foundSupplementary Material online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.957871/full#supplementary-material

References

Baerends, R. J. S., Salomons, F. A., Faber, K. N., Kiel, J. A. K. W., Van der Klei, I. J., Veenhuis, M., et al. (1997). Deviant Pex3p levels affect normal peroxisome formation in Hansenula polymorpha : High steady-state levels of the protein fully abolish matrix protein import. *Yeast* 13 (15), 1437–1448. doi:10.1002/(SICI)1097-0061

Baerends, R. J., Faber, K. N., Kram, A. M., Kiel, J. A., van der Klei, I. J., and VeenhuisM. (2000). A stretch of positively charged amino acids at the N terminus of Hansenula polymorpha Pex3p is involved in incorporation of the protein into the peroxisomal membrane. *J. Biol. Chem.* 275 (14), 9986–9995. doi:10.1074/jbc.275.14.9986

Carmichael, R. E., and Schrader, M. (2022). Determinants of peroxisome membrane dynamics. *Front. Physiol.* 13, 834411. doi:10.3389/fphys.2022.834411

Erdmann, R., and Blobel, G. (1995). Giant peroxisomes in oleic acid-induced *Saccharomyces cerevisiae* lacking the peroxisomal membrane protein Pmp27p. *J. Cell Biol.* 128 (4), 509–523. doi:10.1083/jcb.128.4.509

Faber, K. N., Haima, P., Harder, W., VeenhuisM.and Ab, G. (1994). Highly-efficient electrotransformation of the yeast Hansenula polymorpha. *Curr. Genet.* 25 (4), 305–310. doi:10.1007/BF00351482

Ferreira, J. V., and Carvalho, P. (2021). Pex30-like proteins function as adaptors at distinct ER membrane contact sites. *J. Cell Biol.* 220 (10), e202103176. doi:10.1083/jcb.202103176

Jansen, R. L. M., Santana-Molina, C., van den Noort, M., Devos, D. P., and van der Klei, I. J. (2021). Comparative genomics of peroxisome biogenesis proteins: Making sense of the PEX proteins. *Front. Cell Dev. Biol.* 9, 654163. doi:10.3389/fcell.2021.654163

Joshi, A. S., Huang, X., Choudhary, V., Levine, T. P., Hu, J., and Prinz, W. A. (2016). A family of membrane-shaping proteins at ER subdomains regulates pre-peroxisomal vesicle biogenesis. *J. Cell Biol.* 215 (4), 515–529. doi:10.1083/jcb.201602064

Joshi, A. S., Nebenfuehr, B., Choudhary, V., Satpute-Krishnan, P., Levine, T. P., Golden, A., et al. (2018). Lipid droplet and peroxisome biogenesis occur at the same ER subdomains. *Nat. Commun.* 9 (1), 2940. doi:10.1038/s41467-018-05277-3

Knoops, K., Manivannan, S., Cepinska, M. N., Krikken, A. M., Kram, A. M., Veenhuis, M., et al. (2014). Preperoxisomal vesicles can form in the absence of Pex3. *J. Cell Biol.* 204 (5), 659–668. doi:10.1083/jcb.201310148

Komori, M., Rasmussen, S. W., Kiel, J. A., Baerends, R. J., Cregg, J. M., van der Klei, I. J., et al. (1997). The Hansenula polymorpha PEX14 gene encodes a novel peroxisomal membrane protein essential for peroxisome biogenesis. *EMBO J.* 16 (1), 44–53. doi:10.1093/emboj/16.1.44

Krikken, A. M., Veenhuis, M., and van der Klei, I. J. (2009). Hansenula polymorpha pex11 cells are affected in peroxisome retention. *FEBS J.* 276 (5), 1429–1439. doi:10.1111/j.1742-4658.2009.06883.x

Mast, F. D., Jamakhandi, A., Saleem, R. A., Dilworth, D. J., Rogers, R. S., Rachubinski, R. A., et al. (2016). Peroxins Pex30 and Pex29 dynamically associate with reticulons to regulate peroxisome biogenesis from the endoplasmic reticulum. *J. Biol. Chem.* 291 (30), 15408–15427. doi:10.1074/jbc.M116.728154

Ozimek, P. Z., Klompmaker, S. H., Visser, N., Veenhuis, M., and van der Klei, I. J. (2007). The transcarboxylase domain of pyruvate carboxylase is essential for assembly of the peroxisomal flavoenzyme alcohol oxidase. *FEMS Yeast Res.* 7 (7), 1082–1092. doi:10.1111/j.1567-1364.2007.00214.x

Ušaj, M., Brloznik, M., Kaferle, P., Zitnik, M., Wolinski, H., Leitner, F., et al. (2015). Genome-wide localization study of yeast pex11 identifies peroxisome-mitochondria interactions through the ERMES complex. *J. Mol. Biol.* 427 (11), 2072–2087. doi:10.1016/j.jmb.2015.03.004

van der Klei, I. J., Hilbrands, R. E., Swaving, G. J., Waterham, H. R., Vrieling, E. G., Titorenko, V. I., et al. (1995). The Hansenula polymorpha PER3 gene is essential for the import of PTS1 proteins into the peroxisomal matrix. *J. Biol. Chem.* 270 (29), 17229–17236. doi:10.1074/jbc.270.29.17229

Van Dijken, L. P., Otto, R., and Harder, W. (1976). Growth of Hansenula polymorpha in a methanol-limited chemostat. Physiological responses due to the involvement of methanol oxidase as a key enzyme in methanol metabolism. *Arch. Microbiol.* 111 (1–2), 137–144. doi:10.1007/BF00446560

Wang, S., Idrissi, F. Z., Hermansson, M., Grippa, A., Ejsing, C. S., and Carvalho, P. (2018). Seipin and the membrane-shaping protein Pex30 cooperate in organelle budding from the endoplasmic reticulum. *Nat. Commun.* 9 (1), 2939. doi:10.1038/s41467-018-05278-2

Wu, F., de Boer, R., Krikken, A. M., Aksit, A., Bordin, N., Devos, D. P., et al. (2020). Pex24 and Pex32 are required to tether peroxisomes to the ER for organelle biogenesis, positioning and segregation in yeast. *J. Cell Sci.* 133 (16), jcs246983. doi:10.1242/jcs.246983

Yan, M., Rachubinski, D. A., Joshi, S., Rachubinski, R. A., and Subramani, S. (2008). Dysferlin domain-containing proteins, Pex30p and Pex31p, localized to two compartments, control the number and size of oleate-induced peroxisomes in Pichia pastoris. *Mol. Biol. Cell* 19 (3), 885–898. doi:10.1091/mbc.e07-10-1042

Yuan, W., Aksit, A., de Boer, R., Krikken, A. M., and van der Klei, I. J. (2022). Yeast Vps13 is crucial for peroxisome expansion in cells with reduced peroxisome-ER contact sites. *Front. Cell Dev. Biol.* 10, 842285. doi:10.3389/fcell.2022.842285

frontiers | Frontiers in Cell and Developmental Biology

TYPE Mini Review
PUBLISHED 30 August 2022
DOI 10.3389/fcell.2022.992235

OPEN ACCESS

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

RECEIVED 12 July 2022
ACCEPTED 10 August 2022
PUBLISHED 30 August 2022

CITATION
Sargsyan Y, Kalinowski J and Thoms S (2022), Calcium in peroxisomes: An essential messenger in an essential cell organelle.
Front. Cell Dev. Biol. 10:992235.
doi: 10.3389/fcell.2022.992235

Calcium in peroxisomes: An essential messenger in an essential cell organelle

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Calcium is a central signal transduction element in biology. Peroxisomes are essential cellular organelles, yet calcium handling in peroxisomes has been contentious. Recent advances show that peroxisomes are part of calcium homeostasis in cardiac myocytes and therefore may contribute to or even shape their calcium-dependent functionality. However, the mechanisms of calcium movement between peroxisomes and other cellular sites and their mediators remain elusive. Here, we review calcium handling in peroxisomes in concert with other organelles and summarize the most recent knowledge on peroxisomal involvement in calcium dynamics with a focus on mammalian cells.

KEYWORDS

peroxisomes, calcium, Ca^{2+}, cell organelle, cardiomyocyte, FRET sensor

Introduction

Calcium ions (Ca^{2+}) are among the most important intracellular second messengers with essential roles in various cellular processes such as embryonic development, muscle contraction, neuron excitability, and cell death (Berridge et al., 2000; Giorgi et al., 2018). Ca^{2+} is the only form of calcium with biological relevance and no mechanisms of its degradation or synthesis are known. Ca^{2+} is biologically active by two main mechanisms: The movement of charge along electrical currents across membranes, and binding and unbinding of target proteins translocate Ca^{2+} within cells (Görlach et al., 2015). In this context, calmodulin (CaM) is of particular importance as a Ca^{2+}-binding messenger protein that acts on a wide range of cellular pathways (Cheung, 1980; Kahl and Means, 2003).

Calcium signaling can be initiated by calcium influx through the plasma membrane (PM), and by efflux from the endoplasmic reticulum (ER) (or sarcoplasmic reticulum (SR) in muscle cells), the major intracellular calcium store. ER calcium is released either into the cytosol, or through specialized compartments and membrane contact sites to juxtaposed organelles (Paupe and Prudent, 2018). Calcium release from intracellular stores activates store operated calcium entry (SOCE) from the extracellular space. Cytosolic calcium is either constantly pumped back to the ER through the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) or exits the cell by the plasma membrane calcium ATPase (PMCA) (Raffaello et al., 2016).

Peroxisomes are membrane-bound organelles originally identified as sites for production and degradation of hydrogen peroxide, and fatty acid metabolism (Wanders and Waterham, 2006; Islinger et al., 2018). Mutations in any of the 15 genes encoding essential peroxisomal biogenesis factors (peroxins) can cause rhizomelic chondrodysplasia punctata (RCDP) or disorders of the Zellweger syndrome spectrum (ZSS), a group of rare multisystem disorders marked by peroxisomal dysfunction and concomitant metabolic abnormalities (Klouwer et al., 2015). Peroxisomes are spherical or tubular with diameters ranging from 100 nm to 1 μM (Soliman et al., 2018; Sograte-Idrissi et al., 2020). The large range is due to species differences and depends on the methods used. The smallest diameters are detected by super-resolution microscopy (Soliman et al., 2018). Peroxisomes contain over 130 proteins participating in a large variety of metabolic pathways (Wanders, 2014). Peroxisomes play a crucial role, e.g., in ether lipid and bile acid biosynthesis, the metabolism of D-amino acids, reactive oxygen species (ROS), and the degradation of purines, polyamines and L-pipecolic acid in mammals (Wanders and Waterham, 2006; Sargsyan and Thoms, 2020). Furthermore, peroxisomes cooperate with mitochondria for the efficient β-oxidation of several fatty acid species and virtually all peroxisomal metabolic pathways require intimate communication of peroxisomes with other organelles (Sargsyan and Thoms, 2020).

In electron micrographs of rodent hearts, peroxisomes are found in immediate vicinity of T-tubules and with junctional SR (Hicks and Fahimi, 1977). T-tubule and SR interaction sites are the main determinants of excitation-contraction coupling and effective Ca^{2+} handling in cardiomyocytes (CMs) (Flucher et al., 1994). The defined localization of peroxisomes at these sites suggests that Ca^{2+} may be important for peroxisomes, and that peroxisomes may take up Ca^{2+} and are part of Ca^{2+} homeostasis in CMs (Sargsyan et al., 2021).

Calcium presence in peroxisomes

Peroxisomes are highly dynamic organelles capable of fast adaptation to nutritional and environmental changes (Islinger et al., 2012). The multiple interconnections of peroxisomal and extraperoxisomal metabolic pathways imply that peroxisomes may be involved in the regulation of cellular processes and be a part of signaling pathways (Islinger et al., 2012; Sargsyan and Thoms, 2020). Recently, peroxisomal ether lipid metabolism was found to be essential under hypoxic conditions (Jain et al., 2020). At the same time, peroxisomal ROS regulate the activity of mTOR signaling and autophagy (Zhang et al., 2013), suggesting peroxisomes are fine-tuning cellular homeostasis at different levels.

In plants, Ca^{2+}-sensitive targets involved in peroxisomal metabolism have been described. In *Arabidopsis* and tobacco, ROS-scavenging efficiency increases with Ca^{2+}-mediated activation of peroxisomal catalase 3 (Yang and Poovaiah, 2002; Costa et al., 2010). Furthermore, the Ca^{2+}-dependent protein kinase AtCPK1 is targeted to peroxisomes (Dammann et al., 2003) and peroxisomal Ca^{2+} and CaM are essential for protein import and functionality of peroxisomal enzymes (Corpas and Barroso, 2018), including nitric oxide (NO) synthase, which, in plants, has an inducible peroxisomal isoform and is associated with pathogen defense (Barroso et al., 1999; Corpas et al., 2004).

Peroxisomal calcium in mammalian cells

The study of purified hamster liver peroxisomes suggested that peroxisomes store Ca^{2+} and carry a vanadate-sensitive Ca^{2+}-ATPase on the peroxisomal membrane (Raychaudhury et al., 2006). Drago et al. (2008) and Lasorsa et al. (2008) were the first to measure peroxisomal Ca^{2+} in intact mammalian cells. These studies gave conflicting results about the levels of Ca^{2+} in peroxisomes and the kinetics of peroxisomal Ca^{2+} dynamics. Lasorsa et al. (2008) did not find Ca^{2+}-ATPase activity in resting peroxisomes. Using an aequorin sensor, they concluded that peroxisomal Ca^{2+} concentration in steady state is around 20-fold higher than in the cytosol, rise up to 50–100 μM depending on the cell type and reach 70 μM in HeLa cells. On the other side, Drago et al. (2008) showed that peroxisomal Ca^{2+} levels are similar to cytosolic Ca^{2+} and rise slowly when the concentration of the latter rises. Differences in measurement techniques and biophysical properties of the sensors can partially explain these contradicting results (Costa et al., 2013).

In our recent study, three genetically encoded ratiometric Ca^{2+} indicators covering a broad Ca^{2+} sensitivity range—K_d 0.6, 1.7, and 60 μM—were used to reassess the results of the aforementioned papers (Sargsyan et al., 2021). D1cpV-px with the highest K_d had the lowest dynamic range and only minimal calcium-dependent increase in fluorescence resonance energy transfer (FRET) could be detected upon maximal stimulation. The response of D3cpV-px (FRET sensor) and pericam-px (ratiometric sensor) were comparable and were not saturated, suggesting that peroxisomal Ca^{2+} levels are in the optimal detection range of these sensors (Sargsyan et al., 2021). Pericam as a classical EYFP-based sensor may be pH-sensitive in an acidic environment (Nagai et al., 2001). We did not detect signal changes of YFP variants that could be attributed to Ca^{2+}-independent changes of the sensor, suggesting measurements with the more pH-sensitive pericam-px are also reliable.

Parts of the results of our work are based on measurements with the same sensor (D3cpV-KVK-SKL) as Drago et al. (2008), the only difference is a stronger PTS1 (peroxisomal targeting signal 1) signal in D3cpV-px. In this manner we could overcome

the problem of unspecific targeting of the sensor described by Drago et al. (2008), which these authors solved by adding a linker before the PTS1 tripeptide.

In HeLa cells, we found basal peroxisomal Ca^{2+} levels with a mean value of 600 nM and increase upon stimulation up to 2.4 μM (Sargsyan et al., 2021). Of note, 7% of the analyzed cells had basal peroxisomal Ca^{2+} higher than 1 μM, which would be over 10-fold higher than the expected cytosolic level and would partially correspond to the findings of Lasorsa et al. (2008). Upon stimulation, again some rare cells showed an extremely high increase of peroxisomal Ca^{2+} up to 6.5 μM and higher. The absence of correlation between the high maximal response and basal Ca^{2+} values speaks against the hypothesis that peroxisomes have a strictly limited Ca^{2+} uptake capacity and may rather imply that Ca^{2+} increase in peroxisomes highly depends on the cell state and current cellular needs. Our findings are integrated in an updated overview of organellar calcium concentrations (Figure 1).

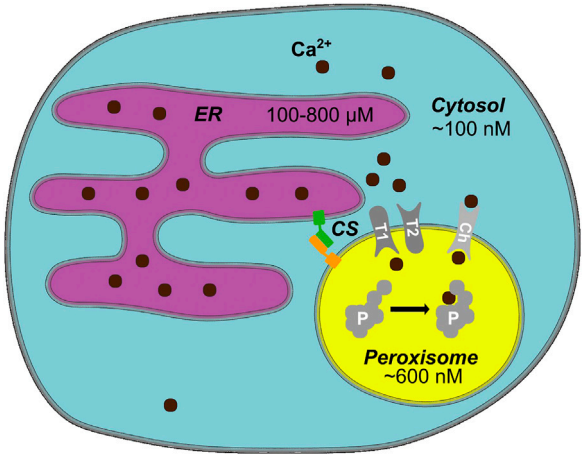

FIGURE 1
Peroxisomal Calcium—an Overview. In mammalian cells, cytosolic Ca^{2+} levels are around 100 nM, whereas the endoplasmic reticulum (ER) as a main cellular Ca^{2+} store has several hundred micromolar Ca^{2+} (Samtleben et al., 2013). An average peroxisome contains around 600 nM Ca^{2+} (Sargsyan et al., 2021). Ca^{2+} likely enters peroxisomes either through a channel/pore or a transporter (Ch). The entry and exit mechanism of Ca^{2+}, however, may differ (T1/T2 hypothetical importer/exporter). Known protein tethers form contact sites (CS) between peroxisomes and the ER, potentially create microdomains that facilitate the exchange of Ca^{2+}. Ca^{2+} may bind to a yet unknown intraperoxisomal protein (P) or membrane protein and affect its function, e.g., by inducing a conformational change when bound to Ca^{2+}. Hypothetical elements of the model (channel, transporter, intraperoxisomal Ca^{2+}-sensitive protein) are shown in gray.

Peroxisomal calcium in cardiomyocytes

The role of Ca^{2+} is even broader for CMs than in other tissues. Here, Ca^{2+} interconnects the electrical stimulation of cardiac myocytes and their contraction—a process termed excitation-contraction coupling (Bers, 2008). The specific crucial molecular players of Ca^{2+} handling in CMs are ryanodine receptors (RyR) on the SR, voltage-operated Ca^{2+} channels in the T-tubules and Na^{+}/Ca^{2+}-exchanger on the plasma membrane. Strict control of cellular Ca^{2+} levels is of particular importance in cardiomyocytes. Ca^{2+} overload in CMs results in malfunction of the heart by affecting both the electrical and contractile properties of cardiomyocytes (Vassalle and Lin, 2004). Electrical abnormalities of CMs present as arrhythmias with varying severity from relatively harmless to life-threatening (Vassalle and Lin, 2004; Landstrom et al., 2017).

The localization of peroxisomes in proximity to the T-tubular system and SR in cardiac myocytes (Hicks and Fahimi, 1977) hinted at a role for peroxisomes in Ca^{2+} handling. Intracellular store-depletion by the activation of RyRs on the SR through Ca^{2+} from the T-tubule localized L-type Ca^{2+} channel (LTCC, Ca^{2+}-induced Ca^{2+}-release) is the main source of Ca^{2+} increase in CMs in the excitation-contraction coupling (Bers and Perez-Reyes, 1999). Using chemical stimulation, we have shown that Ca^{2+} enters peroxisomes upon intracellular Ca^{2+}-store depletion in neonatal rat CMs (NRCMs) and human induced pluripotent stem cell-derived CMs (hiPSC-CMs) (Sargsyan et al., 2021). We hypothesized that cardiac peroxisomes take up Ca^{2+} on a beat-to-beat basis. Indeed, we showed that upon electrical field stimulation with 1 Hz frequency, peroxisomes in NRCM take up Ca^{2+} in beat-to-beat manner (Sargsyan et al., 2021).

Peroxisomes in hiPSC-CMs occasionally localize in vicinity of ER protein RyR2 and rarely to T-tubular system and LTCC (Sargsyan et al., 2021). The striation pattern is not well-developed in hiPSC-CMs and therefore the relative localization of RyR2, LTCC, and peroxisomes in hiPSC-CMs may differ from that in CMs in the beating heart. It is known from monkey kidney fibroblast-like COS-7 cell line that over 90% of peroxisomes are in contact with the ER (Valm et al., 2017). ER-peroxisome contact is dependent on ACBD4/5-VAPB, and on Miro1v4-VPS13D-VAP (Costello et al., 2017a, 2017b; Hua et al., 2017; Guillén-Samander et al., 2021). However, it is not known whether membrane contact sites are relevant for Ca^{2+} entry to peroxisomes, if there are tissue or cell type-specific molecular composition and/or abundance of contact sites, and which of these are relevant for CMs.

As peroxisomes take up Ca^{2+} from the SR and localize in the proximity of RyR receptors in CMs, it is plausible that peroxisomes may contribute to or even be essential for normal excitation-contraction coupling. This hypothesis is supported by the fact that patients with mild forms of ZSS occasionally present with cardiac arrhythmias that may become the cause of lethal outcome (Wanders and Komen, 2007). The metabolic role of peroxisomes could be the reason for these arrhythmias (Colasante et al., 2015).

However, a direct contribution to efficient Ca^{2+} handling by the peroxisomes is another plausible reason.

Candidates of peroxisomal calcium channels

Although peroxisomal Ca^{2+} changes largely follow cytosolic Ca^{2+}, our experiments with maximal Ca^{2+} mobilization through ionophore addition showed a slower Ca^{2+} increase and even slower decline in peroxisomes compared to the cytosol (Sargsyan et al., 2021). The slow but constant increase in peroxisomal Ca^{2+} when the cytosolic Ca^{2+} rises suggests that the transfer mechanism may have limited capacity and can be saturated. Potential peroxisomal Ca^{2+} channels and pores are PEX11 (Mindthoff et al., 2016), PXMP2 (Rokka et al., 2009), PMP34 (Wylin et al., 1998), or any other peroxisomal membrane protein with a transport function (Chornyi et al., 2020). PEX11 and PXMP2 are reported to be unspecific peroxisomal channel-forming proteins with permeability to small solutes in *in vitro* experiments on artificial membranes (Rokka et al., 2009; Mindthoff et al., 2016). PMP34 has been suggested as a coenzyme A, FAD, and NAD^+ transporter across peroxisomal membranes using liposomes with reconstituted recombinant protein (Agrimi et al., 2012). An *in vivo* study investigating the channel function of PXMP2 and PEX11 examined their role in hydrogen peroxide transport across peroxisomal membrane (Lismont et al., 2019). Judging from the molecular weight of the hydrogen peroxide it could freely pass through both PXMP2 and PEX11. Nonetheless, a fluorescent biosensor for H_2O_2 in PXMP2- and/or PEX11-deficient cells, showed that neither PXMP2 nor PEX11 are essential for H_2O_2 trafficking across the peroxisomal membrane (Lismont et al., 2019). Altogether, this suggests that the search for peroxisomal Ca^{2+} transport machinery may present a challenging task.

Biological relevance of peroxisomal calcium

Highly localized calcium dynamics play a central role in controlling cellular processes. At the same time, excessive increase of intracellular Ca^{2+} to levels that cannot be handled by the cell is known as Ca^{2+} overload and can have detrimental consequences (Vassalle and Lin, 2004). For example, mitochondrial calcium stimulates energy gain from Krebs cycle and respiratory chain but can also induce cell death in case of mitochondrial Ca^{2+} overload (Görlach et al., 2015). Thus, the subcellular sequestration of Ca^{2+} in different compartments is vital for the regulation of physiological processes.

The interplay of redox and Ca^{2+} signaling is well described for mitochondria, where Ca^{2+}-dependent opening of calcium channels is regulated by interaction with the oxidoreductase Mia40 (Petrungaro et al., 2015). Additionally, metabolic processes also regulate Ca^{2+} uptake by mitochondria (Nemani et al., 2018). Whether redox or metabolic processes influence peroxisomal Ca^{2+} is not known. There is also no consensus about the drivers of Ca^{2+} transport to peroxisomes and the role of known ion transporters like Ca^{2+}/H^+ antiporter, Ca^{2+}/Na^+ exchanger, and V-ATPase for peroxisomal Ca^{2+} levels (Drago et al., 2008; Lasorsa et al., 2008).

Presently, in contrast to plant catalases and kinases (Yang and Poovaiah, 2002; Dammann et al., 2003), no mammalian peroxisomal enzymes are known to bind Ca^{2+}. Weber et al. (1997) suggested the presence of a Ca^{2+}-dependent mitochondrial solute carrier *Efinal* (gene *SLC25A24*) also on peroxisomes based on immunoelectron microscopy in rabbit small intestinal tissue. Along with peroxisomal malate dehydrogenase and lactate dehydrogenase (Schueren et al., 2014; Hofhuis et al., 2016; Schueren and Thoms, 2016), they would serve as crucial components of malate and lactate shuttles across peroxisomal membranes for reduction equivalent reoxidation (McClelland et al., 2003). However, in human cell lines, *Efinal* homologues—identified as members of the short calcium-binding mitochondrial carriers (SCaMC) protein subfamily—were found exclusively in mitochondria (del Arco and Satrústegui, 2004).

Direct evidence for a functional role of intra-peroxisomal Ca^{2+} is still missing. For the mammalian peroxisome, however, Ca^{2+} appears to be important. Particularly, Ca^{2+} channel blockers nifedipine, diltiazem and nicardipine suppress peroxisomal fatty acid oxidation enzymes and peroxisome proliferation (Watanabe and Suga, 1988; Itoga et al., 1990; Zhang et al., 1996). These findings might be due to direct regulation of a peroxisomal enzyme through Ca^{2+} or by indirect regulation of peroxisomal functions by extraperoxisomal Ca^{2+}.

In case of the latter, peroxisomal membrane protein Miro1v4 could be a potential target. Miro1v4 is a peroxisomal variant of mitochondrial the Miro1 protein that form ER contact sites through VPS13D to exchange lipids and has been shown to mediate the linkage of mitochondria to motor proteins in a calcium-dependent manner (MacAskill et al., 2009). Miro1v4 has two potentially Ca^{2+} binding EF-hands which are essential for Miro-VPS13D interaction on peroxisome-ER contact sites (Guillén-Samander et al., 2021). Whether Ca^{2+} binding really regulates peroxisome-ER contact was not studied experimentally. Still, no Ca^{2+}-dependent interaction of the mitochondrial-ER contact site mediated by Miro-VPS13D could be found in experiments (Guillén-Samander et al., 2021). Other lipid transfer mechanisms, like the vesicle-based lipid exchange mediated by synaptotagmin, have been shown to be Ca^{2+}-dependent (Yu et al., 2016). Similarly to the findings for

mitochondria, peroxisomal Miro isoforms have been implicated in peroxisome motility (Castro and Schrader, 2018) although there is no evidence on involvement of Ca^{2+} in peroxisome motility in this context.

The role of peroxisomal Ca^{2+} might not necessarily be the regulation of peroxisomal processes. Drago et al. (2008) have suggested that peroxisomes may serve as an additional cytosolic Ca^{2+} buffer compartment. This idea is supported by the fact that peroxisomal Ca^{2+} rises after ER-store depletion in case of knockdown of mitochondrial calcium uniporter (MCU) beyond its initial maximum (Sargsyan et al., 2021). This suggests that at least in some situations of cellular Ca^{2+} overload peroxisomes may take up more Ca^{2+} than under near-physiological standard conditions, therefore buffering potentially deleterious effects of excess Ca^{2+} on the cell. Consequently, the protective effect of peroxisomal Ca^{2+} uptake may be necessary only in some special situations such as Ca^{2+} overload.

As Ca^{2+} concentration in the peroxisome is higher than in the cytosol, peroxisomes may also in extreme situations play a role of additional Ca^{2+} store for the cytosol. The buffering function of peroxisomes may be protective in some cases of arrhythmia, such as catecholaminergic polymorphic ventricular tachycardia, when increased predisposition to inadequate SR Ca^{2+} release events (so called Ca^{2+} sparks) occur. On the other hand, the absence of peroxisomes may contribute to cardiac pathogenesis and promote the development or extent of arrhythmias.

Conclusion

Ca^{2+} enters peroxisomes of non-excitable and excitable mammalian cells upon near-physiological electrical and chemical stimulation. Peroxisomal Ca^{2+} handling presents an exciting research area with many open questions: For example, the existence of Ca^{2+}-dependent transporters in peroxisomes as suggested (Weber et al., 1997) is still unclear. Similarly, Ca^{2+}-sensitive targets in the mammalian peroxisome, as known for the plant peroxisome, may yet be identified. Furthermore, it is conceivable that peroxisomal Ca^{2+} homeostasis may be important in the absence of luminal Ca^{2+} binding proteins, by buffering local calcium. In excitable cells, peroxisomal Ca^{2+} dynamics might be of special importance, also with regard to their pathophysiology. The cellular function of peroxisomal Ca^{2+} and the role of peroxisomal Ca^{2+} in pathology remain to be studied further.

Author contributions

YS and ST conceived the article, YS wrote the first draft, JK and ST contributed to the writing, YS drafted the figure, JK and ST edited the figure. All authors edited the article.

Funding

This work was supported by the following grants: Deutsche Forschungsgemeinschaft TH 1538/3-1 to ST, Collaborate Research Council "Modulatory units in heart failure" SFB 1002/3 TP A10 to ST, "Shared expertise" funding by the DZHK (German Centre for Cardiovascular Research), and a PhD stipend by the DAAD program 57381412 ID 91572398 to YS. We acknowledge the financial support of the German Research Foundation (DFG) and the Open Access Publication Fund of Bielefeld University for the article processing charge.

Acknowledgments

We thank Leonie Burghardt, Anna-Lena Toschke, and Dennis Lebeda for comments. We also thank the reviewers for their constructive comments on the manuscript.

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

Agrimi, G., Russo, A., Scarcia, P., and Palmieri, F. (2012). The human gene SLC25A17 encodes a peroxisomal transporter of coenzyme A, FAD and NAD+. *Biochem. J.* 443, 241–247. doi:10.1042/BJ20111420

Barroso, J. B., Corpas, F. J., Carreras, A., Sandalio, L. M., Valderrama, R., Palma, JoséM., et al. (1999). Localization of nitric-oxide synthase in plant peroxisomes. *J. Biol. Chem.* 274, 36729–36733. doi:10.1074/jbc.274.51.36729

Berridge, M. J., Lipp, P., and Bootman, M. D. (2000). The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 1, 11–21. doi:10.1038/35036035

Bers, D. M. (2008). Calcium cycling and signaling in cardiac myocytes. *Annu. Rev. Physiol.* 70, 23–49. doi:10.1146/annurev.physiol.70.113006.100455

Bers, D. M., and Perez-Reyes, E. (1999). Ca channels in cardiac myocytes: Structure and function in Ca influx and intracellular Ca release. *Cardiovasc. Res.* 42, 339–360. doi:10.1016/s0008-6363(99)00038-3

Castro, I. G., and Schrader, M. (2018). Miro1 – The missing link to peroxisome motility. *Commun. Integr. Biol.* 11, e1526573. doi:10.1080/19420889.2018.1526573

Cheung, W. Y. (1980). Calmodulin plays a pivotal role in cellular regulation. *Science* 207, 19–27. doi:10.1126/science.6243188

Chornyi, S., IJlst, L., van Roermund, C. W. T., Wanders, R. J. A., and Waterham, H. R. (2020). Peroxisomal metabolite and cofactor transport in humans. *Front. Cell Dev. Biol.* 8, 613892. doi:10.3389/fcell.2020.613892

Colasante, C., Chen, J., Ahlemeyer, B., and Baumgart-Vogt, E. (2015). Peroxisomes in cardiomyocytes and the peroxisome/peroxisome proliferator-activated receptor-loop. *Thromb. Haemost.* 113, 452–463. doi:10.1160/TH14-06-0497

Corpas, F. J., and Barroso, J. B. (2018). Calmodulin antagonist affects peroxisomal functionality by disrupting both peroxisomal Ca2+ and protein import. *J. Cell Sci.* 131, jcs201467. doi:10.1242/jcs.201467

Corpas, F. J., Barroso, J. B., Carreras, A., Quirós, M., León, A. M., Romero-Puertas, M. C., et al. (2004). Cellular and subcellular localization of endogenous nitric oxide in young and senescent pea plants. *Plant Physiol.* 136, 2722–2733. doi:10.1104/pp.104.042812

Costa, A., Drago, I., Behera, S., Zottini, M., Pizzo, P., Schroeder, J. I., et al. (2010). H2O2 in plant peroxisomes: An *in vivo* analysis uncovers a Ca2+-dependent scavenging system. *Plant J.* 62, 760–772. doi:10.1111/j.1365-313X.2010.04190.x

Costa, A., Drago, I., Zottini, M., Pizzo, P., and Pozzan, T. (2013). Peroxisome Ca(2+) homeostasis in animal and plant cells. *Subcell. Biochem.* 69, 111–133. doi:10.1007/978-94-007-6889-5_7

Costello, J. L., Castro, I. G., Hacker, C., Schrader, T. A., Metz, J., Zeuschner, D., et al. (2017a). ACBD5 and VAPB mediate membrane associations between peroxisomes and the ER. *J. Cell Biol.* 216, 331–342. doi:10.1083/jcb.201607055

Costello, J. L., Castro, I. G., Schrader, T. A., Islinger, M., and Schrader, M. (2017b). Peroxisomal ACBD4 interacts with VAPB and promotes ER-peroxisome associations. *Cell cycle* 16, 1039–1045. doi:10.1080/15384101.2017.1314422

Dammann, C., Ichida, A., Hong, B., Romanowsky, S. M., Hrabak, E. M., Harmon, A. C., et al. (2003). Subcellular targeting of nine calcium-dependent protein kinase isoforms from Arabidopsis. *Plant Physiol.* 132, 1840–1848. doi:10.1104/pp.103.020008

del Arco, A., and Satrústegui, J. (2004). Identification of a novel human subfamily of mitochondrial carriers with calcium-binding domains. *J. Biol. Chem.* 279, 24701–24713. doi:10.1074/jbc.M401417200

Drago, I., Giacomello, M., Pizzo, P., and Pozzan, T. (2008). Calcium dynamics in the peroxisomal lumen of living cells. *J. Biol. Chem.* 283, 14384–14390. doi:10.1074/jbc.M800600200

Flucher, B. E., Andrews, S. B., and Daniels, M. P. (1994). Molecular organization of transverse tubule/sarcoplasmic reticulum junctions during development of excitation-contraction coupling in skeletal muscle. *Mol. Biol. Cell* 5, 1105–1118. doi:10.1091/mbc.5.10.1105

Giorgi, C., Danese, A., Missiroli, S., Patergnani, S., and Pinton, P. (2018). Calcium dynamics as a machine for decoding signals. *Trends Cell Biol.* 28, 258–273. doi:10.1016/j.tcb.2018.01.002

Görlach, A., Bertram, K., Hudecova, S., and Krizanova, O. (2015). Calcium and ROS: A mutual interplay. *Redox Biol.* 6, 260–271. doi:10.1016/j.redox.2015.08.010

Guillén-Samander, A., Leonzino, M., Hanna, M. G., Tang, N., Shen, H., and De Camilli, P. (2021). VPS13D bridges the ER to mitochondria and peroxisomes via Miro. *J. Cell Biol.* 220, e202010004. doi:10.1083/jcb.202010004

Hicks, L., and Fahimi, H. D. (1977). Peroxisomes (microbodies) in the myocardium of rodents and primates. A comparative Ultrastructural cytochemical study. *Cell Tissue Res.* 175, 467–481. doi:10.1007/BF00222413

Hofhuis, J., Schueren, F., Nötzel, C., Lingner, T., Gärtner, J., Jahn, O., et al. (2016). The functional readthrough extension of malate dehydrogenase reveals a modification of the genetic code. *Open Biol.* 6, 160246. doi:10.1098/rsob.160246

Hua, R., Cheng, D., Coyaud, É., Freeman, S., Di Pietro, E., Wang, Y., et al. (2017). VAPs and ACBD5 tether peroxisomes to the ER for peroxisome maintenance and lipid homeostasis. *J. Cell Biol.* 216, 367–377. doi:10.1083/jcb.201608128

Islinger, M., Grille, S., Fahimi, H. D., and Schrader, M. (2012). The peroxisome: An update on mysteries. *Histochem. Cell Biol.* 137, 547–574. doi:10.1007/s00418-012-0941-4

Islinger, M., Voelkl, A., Fahimi, H. D., and Schrader, M. (2018). The peroxisome: An update on mysteries 2.0. *Histochem. Cell Biol.* 150, 443–471. doi:10.1007/s00418-018-1722-5

Itoga, H., Tamura, H., Watanabe, T., and Suga, T. (1990). Characteristics of the suppressive effect of nicardipine on peroxisome induction in rat liver. *Biochim. Biophys. Acta* 1051, 21–28. doi:10.1016/0167-4889(90)90169-e

Jain, I. H., Calvo, S. E., Markhard, A. L., Skinner, O. S., To, T.-L., Ast, T., et al. (2020). Genetic screen for cell fitness in high or low oxygen highlights mitochondrial and lipid metabolism. *Cell* 181, 716–727. e11. doi:10.1016/j.cell.2020.03.029

Kahl, C. R., and Means, A. R. (2003). Regulation of cell cycle progression by calcium/calmodulin-dependent pathways. *Endocr. Rev.* 24, 719–736. doi:10.1210/er.2003-0008

Klouwer, F. C. C., Berendse, K., Ferdinandusse, S., Wanders, R. J. A., Engelen, M., and Poll-The, B. T. (2015). Zellweger spectrum disorders: Clinical overview and management approach. *Orphanet J. Rare Dis.* 10, 151. doi:10.1186/s13023-015-0368-9

Landstrom, A. P., Dobrev, D., and Wehrens, X. H. T. (2017). Calcium signaling and cardiac arrhythmias. *Circ. Res.* 120, 1969–1993. doi:10.1161/CIRCRESAHA.117.310083

Lasorsa, F. M., Pinton, P., Palmieri, L., Scarcia, P., Rottensteiner, H., Rizzuto, R., et al. (2008). Peroxisomes as novel players in cell calcium homeostasis. *J. Biol. Chem.* 283, 15300–15308. doi:10.1074/jbc.M800648200

Lismont, C., Koster, J., Provost, S., Baes, M., Van Veldhoven, P. P., Waterham, H. R., et al. (2019). Deciphering the potential involvement of PXMP2 and PEX11B in hydrogen peroxide permeation across the peroxisomal membrane reveals a role for PEX11B in protein sorting. *Biochim. Biophys. Acta. Biomembr.* 1861, 182991. doi:10.1016/j.bbamem.2019.05.013

MacAskill, A. F., Rinholm, J. E., Twelvetrees, A. E., Arancibia-Carcamo, I. L., Muir, J., Fransson, A., et al. (2009). Miro1 is a calcium sensor for glutamate receptor-dependent localization of mitochondria at synapses. *Neuron* 61, 541–555. doi:10.1016/j.neuron.2009.01.030

McClelland, G. B., Khanna, S., González, G. F., Butz, C. E., and Brooks, G. A. (2003). Peroxisomal membrane monocarboxylate transporters: Evidence for a redox shuttle system? *Biochem. Biophys. Res. Commun.* 304, 130–135. doi:10.1016/s0006-291x(03)00550-3

Mindthoff, S., Grunau, S., Steinfort, L. L., Girzalsky, W., Hiltunen, J. K., Erdmann, R., et al. (2016). Peroxisomal Pex11 is a pore-forming protein homologous to TRPM channels. *Biochim. Biophys. Acta* 1863, 271–283. doi:10.1016/j.bbamcr.2015.11.013

Nagai, T., Sawano, A., Park, E. S., and Miyawaki, A. (2001). Circularly permuted green fluorescent proteins engineered to sense Ca2+. *Proc. Natl. Acad. Sci. U. S. A.* 98, 3197–3202. doi:10.1073/pnas.051636098

Nemani, N., Shanmughapriya, S., and Madesh, M. (2018). Molecular regulation of MCU: Implications in physiology and disease. *Cell Calcium* 74, 86–93. doi:10.1016/j.ceca.2018.06.006

Paupe, V., and Prudent, J. (2018). New insights into the role of mitochondrial calcium homeostasis in cell migration. *Biochem. Biophys. Res. Commun.* 500, 75–86. doi:10.1016/j.bbrc.2017.05.039

Petrungaro, C., Zimmermann, K. M., Küttner, V., Fischer, M., Dengjel, J., Bogeski, I., et al. (2015). The Ca(2+)-dependent release of the mia40-induced MICU1-MICU2 dimer from MCU regulates mitochondrial Ca(2+) uptake. *Cell Metab.* 22, 721–733. doi:10.1016/j.cmet.2015.08.019

Raffaello, A., Mammucari, C., Gherardi, G., and Rizzuto, R. (2016). Calcium at the center of cell signaling: Interplay between endoplasmic reticulum, mitochondria, and lysosomes. *Trends biochem. Sci.* 41, 1035–1049. doi:10.1016/j.tibs.2016.09.001

Raychaudhury, B., Gupta, S., Banerjee, S., and Datta, S. C. (2006). Peroxisome is a reservoir of intracellular calcium. *Biochim. Biophys. Acta* 1760, 989–992. doi:10.1016/j.bbagen.2006.02.022

Rokka, A., Antonenkov, V. D., Soininen, R., Immonen, H. L., Pirilä, P. L., Bergmann, U., et al. (2009). Pxmp2 is a channel-forming protein in Mammalian peroxisomal membrane. *PloS one* 4, e5090. doi:10.1371/journal.pone.0005090

Samtleben, S., Jaepel, J., Fecher, C., Andreska, T., Rehberg, M., and Blum, R. (2013). Direct imaging of ER calcium with targeted-esterase induced dye loading (TED). *J. Vis. Exp.* (75), e50317. doi:10.3791/50317

Sargsyan, Y., Bickmeyer, U., Gibhardt, C. S., Streckfuss-Bömeke, K., Bogeski, I., and Thoms, S. (2021). Peroxisomes contribute to intracellular calcium dynamics in cardiomyocytes and non-excitable cells. *Life Sci. Alliance* 4, e202000987. doi:10.26508/lsa.202000987

Sargsyan, Y., and Thoms, S. (2020). Staying in healthy contact: How peroxisomes interact with other cell organelles. *Trends Mol. Med.* 26, 201–214. doi:10.1016/j.molmed.2019.09.012

Schueren, F., Lingner, T., George, R., Hofhuis, J., Dickel, C., Gärtner, J., et al. (2014). Peroxisomal lactate dehydrogenase is generated by translational readthrough in mammals. *eLife* 3, e03640. doi:10.7554/eLife.03640

Schueren, F., and Thoms, S. (2016). Functional translational readthrough: A systems Biology perspective. *PLoS Genet.* 12, e1006196. doi:10.1371/journal.pgen.1006196

Sograte-Idrissi, S., Schlichthaerle, T., Duque-Afonso, C. J., Alevra, M., Strauss, S., Moser, T., et al. (2020). Circumvention of common labelling artefacts using secondary nanobodies. *Nanoscale* 12, 10226–10239. doi:10.1039/d0nr00227e

Soliman, K., Göttfert, F., Rosewich, H., Thoms, S., and Gärtner, J. (2018). Super-resolution imaging reveals the sub-diffraction phenotype of Zellweger Syndrome ghosts and wild-type peroxisomes. *Sci. Rep.* 8, 7809. doi:10.1038/s41598-018-24119-2

Valm, A. M., Cohen, S., Legant, W. R., Melunis, J., Hershberg, U., Wait, E., et al. (2017). Applying systems-level spectral imaging and analysis to reveal the organelle interactome. *Nature* 546, 162–167. doi:10.1038/nature22369

Vassalle, M., and Lin, C.-I. (2004). Calcium overload and cardiac function. *J. Biomed. Sci.* 11, 542–565. doi:10.1007/BF02256119

Wanders, R. J. A., and Komen, J. C. (2007). Peroxisomes, Refsum's disease and the alpha- and omega-oxidation of phytanic acid. *Biochem. Soc. Trans.* 35, 865–869. doi:10.1042/BST0350865

Wanders, R. J. A. (2014). Metabolic functions of peroxisomes in health and disease. *Biochimie* 98, 36–44. doi:10.1016/j.biochi.2013.08.022

Wanders, R. J. A., and Waterham, H. R. (2006). Biochemistry of mammalian peroxisomes revisited. *Annu. Rev. Biochem.* 75, 295–332. doi:10.1146/annurev.biochem.74.082803.133329

Watanabe, T., and Suga, T. (1988). Suppression of clofibrate-induced peroxisome proliferation in rat liver by nicardipine, a calcium antagonist. *FEBS Lett.* 232, 293–297. doi:10.1016/0014-5793(88)80756-7

Weber, F. E., Minestrini, G., Dyer, J. H., Werder, M., Boffelli, D., Compassi, S., et al. (1997). Molecular cloning of a peroxisomal Ca2+-dependent member of the mitochondrial carrier superfamily. *Proc. Natl. Acad. Sci. U. S. A.* 94, 8509–8514. doi:10.1073/pnas.94.16.8509

Wylin, T., Baes, M., Brees, C., Mannaerts, G. P., Fransen, M., and Van Veldhoven, P. P. (1998). Identification and characterization of human PMP34, a protein closely related to the peroxisomal integral membrane protein PMP47 of Candida boidinii. *Eur. J. Biochem.* 258, 332–338. doi:10.1046/j.1432-1327.1998.2580332.x

Yang, T., and Poovaiah, B. W. (2002). Hydrogen peroxide homeostasis: Activation of plant catalase by calcium/calmodulin. *Proc. Natl. Acad. Sci. U. S. A.* 99, 4097–4102. doi:10.1073/pnas.052564899

Yu, H., Liu, Y., Gulbranson, D. R., Paine, A., Rathore, S. S., and Shen, J. (2016). Extended synaptotagmins are Ca^{2+}-dependent lipid transfer proteins at membrane contact sites. *Proc. Natl. Acad. Sci. U. S. A.* 113, 4362–4367. doi:10.1073/pnas.1517259113

Zhang, H., Tamura, H., Yamada, J., Watanabe, T., and Suga, T. (1996). Effect of nicardipine, a calcium antagonist, on induction of peroxisomal enzymes by dehydroepiandrosterone sulfate in cultured rat hepatocytes. *J. Toxicol. Sci.* 21, 235–241. doi:10.2131/jts.21.4_235

Zhang, J., Kim, J., Alexander, A., Cai, S., Tripathi, D. N., Dere, R., et al. (2013). A tuberous sclerosis complex signalling node at the peroxisome regulates mTORC1 and autophagy in response to ROS. *Nat. Cell Biol.* 15, 1186–1196. doi:10.1038/ncb2822

frontiers | Frontiers in Cell and Developmental Biology

TYPE Review
PUBLISHED 26 September 2022
DOI 10.3389/fcell.2022.934331

OPEN ACCESS

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

RECEIVED 02 May 2022
ACCEPTED 27 July 2022
PUBLISHED 26 September 2022

CITATION
Bittner E, Stehlik T and Freitag J (2022), Sharing the wealth: The versatility of proteins targeted to peroxisomes and other organelles.
Front. Cell Dev. Biol. 10:934331.
doi: 10.3389/fcell.2022.934331

Sharing the wealth: The versatility of proteins targeted to peroxisomes and other organelles

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Peroxisomes are eukaryotic organelles with critical functions in cellular energy and lipid metabolism. Depending on the organism, cell type, and developmental stage, they are involved in numerous other metabolic and regulatory pathways. Many peroxisomal functions require factors also relevant to other cellular compartments. Here, we review proteins shared by peroxisomes and at least one different site within the cell. We discuss the mechanisms to achieve dual targeting, their regulation, and functional consequences. Characterization of dual targeting is fundamental to understand how peroxisomes are integrated into the metabolic and regulatory circuits of eukaryotic cells.

KEYWORDS

dual targeting, Endoplasmic reticulum, peroxisome, targeting signal, Mitochondria

Origin of peroxisomes

Peroxisomes are eukaryotic organelles containing enzymes for the breakdown of reactive oxygen species and fatty acids (Poirier et al., 2006; Wanders, 2014). Peroxisomes may have no direct endosymbiotic origin, are unlike mitochondria and plastids devoid of nucleic acids but show a certain degree of autonomy, and contain dedicated systems for protein import (Gabaldón, 2010; Smith and Aitchison, 2013). One scenario is their emergence from the endoplasmic reticulum (ER) to reduce the detrimental effects of reactive oxygen species on ER protein homeostasis (Gabaldón, 2014). This idea is supported by findings indicating that the import machinery for peroxisomal matrix proteins is similar to the ERAD system known to control the export of misfolded proteins from the ER (Gabaldón et al., 2006; Schlüter et al., 2006).

According to a different opinion, peroxisomes are derivatives of mitochondria, since both organelles are sites of fatty acid breakdown. Peroxisomes may have emerged during the evolution of ancient eukaryotic cells to reduce the ROS burden of their progenitor (Speijer, 2017). Phylogenetic analysis revealed a bacterial origin of three out of four β-oxidation enzymes (Bolte et al., 2015). This led the authors to propose that at least the present form of peroxisomes emerged after mitochondria since a major catabolic pathway of peroxisomes likely originated from this organelle (Bolte et al., 2015). Mitochondrial

enzymes probably became retargeted to peroxisomes over time, and some of these still remained dually localized (Gabaldón, 2018). Indeed, recent work shows that the early diverged cryptophyte *Guillardia theta* contains peroxisomes, but enzymes for fatty acid oxidation seem to be only mitochondrial (Vasilev et al., 2022). Independent of the actual evolutionary scenario, both the ER and mitochondria likely contributed to the proteome of modern peroxisomes and still sustain their biogenesis.

Peroxisome functions

The biochemical functions of peroxisomes are versatile. We only provide an overview and mainly refer to review articles focused on a more detailed explanation of peroxisomes from different species. The coupled degradation of fatty acids and H_2O_2 is a prominent task, and peroxisomes owe their name to this process (De Duve and Baudhuin, 1966; Poirier et al., 2006). Remarkably, in several parasitic species including trypanosomes peroxisomes house the enzymes for glycolysis (Haanstra et al., 2016). This already highlights a fascinating feature of the organelle—it is highly adaptable to a specific lifestyle. Another example is the metabolism of methylotrophic yeasts that break down methanol and harbor this oxidative process inside of the peroxisome (van der Klei and Veenhuis, 2006b). Yeasts and filamentous fungi contain a large variety of biosynthetic pathways inside of peroxisomes including enzymes involved in the production of antibiotics, biotin, surface-active glycolipids, and siderophores (Meijer et al., 2010; Magliano et al., 2011; Tanabe et al., 2011; Gründlinger et al., 2013; Freitag et al., 2014; Stehlik et al., 2014). Furthermore, peroxisomes are important for virulence of several human- and plant-pathogenic fungi (Kretschmer et al., 2012a; Falter and Reumann, 2022).

Peroxisomes are essential for the regular development of humans, and mutations in peroxisomal proteins are associated with severe diseases including the Zellweger syndrome (Wanders, 2014). Besides their prominent function in fatty acid catabolism, mammalian peroxisomes are required for further processes such as the production of ether lipids and bile acids or metabolism of amino acids including D-amino acids (Wanders and Waterham, 2006; Wanders et al., 2016). Furthermore, peroxisomes play an important role in the development of the brain and their dysfunction may contribute to neurological pathologies including amyotrophic lateral sclerosis and Alzheimer's disease (Berger et al., 2016). More recent evidence supports a major function of peroxisomes in regulating the response of the immune system to pathogenic attack in several animals (Odendall et al., 2014; Di Cara et al., 2017; Di Cara, 2020). Plant peroxisomes are critical for oil mobilization during early seedling development and play a role in the metabolism of the toxic by-product 2-phosphoglycolate derived from an O_2-consuming side reaction of RuBisCO. 2-Phosphoglycolate needs to be removed from the plastid, where it can inhibit several enzymes and is recycled into 3-phosphoglycerate in a series of reactions involving enzymatic reactions in peroxisomes and mitochondria (Reumann and Weber, 2006; Hu et al., 2012; Dellero et al., 2016; Pan et al., 2020). Besides their metabolic tasks, peroxisomes are also emerging as cellular signaling platforms (Tripathi and Walker, 2016). In addition, they can act as proviral and antiviral organelles depending on the type of virus (Ferreira et al., 2022). Given this remarkable variability, it is likely that many more functions of peroxisomes remain elusive, which may often be specific to a particular organism.

Protein targeting to peroxisomes

To attach to a defined organelle, proteins usually possess targeting signals, which act as a molecular zip code (Blobel and Sabatini, 1971). Proteins designated for the peroxisomal matrix mostly contain C-terminal or N-terminal sequence motifs termed peroxisomal targeting signal type I (PTS1) or type II (PTS2) (Kunze et al., 2015; Francisco et al., 2017; Walter and Erdmann, 2019; Kunze, 2020; Bürgi et al., 2021). These signals are recognized and bound by soluble targeting factors in the cytosol. Cargo proteins can be imported in a folded state and even as oligomers (Francisco et al., 2017; Walter and Erdmann, 2019). Very large particles have been shown to be imported into the lumen of the organelle revealing a flexible import pore (Walton et al., 1995; Yang et al., 2018). Proteins without a defined signal can enter the peroxisome in complex with canonical cargo (Islinger et al., 2009; Schueren et al., 2014; Effelsberg et al., 2015; Al-Saryi et al., 2017a; Gabay-Maskit et al., 2020). This piggyback import may be a hallmark of import pathways, which accept folded or partially folded clients such as the nucleus and the peroxisome. Several proteins are known to contain elements that enable binding to the targeting factor Pex5 in the cytosol although they lack a classical PTS1 (van der Klei and Veenhuis, 2006a; Kempiński et al., 2020; Rosenthal et al., 2020; Yifrach et al., 2021). Peroxisomal membrane proteins (PMPs) can be directly inserted into the peroxisomal membrane aided by the chaperone Pex19 and the transmembrane protein Pex3, but some can also be sorted *via* the ER (for a review, see Kim and Hettema, 2015).

Mechanisms to achieve the dual localization of proteins

We have previously discussed mechanisms involved in dual targeting of peroxisomal proteins in greater detail (Ast et al.,

2013). Therefore, they are only briefly discussed, and instead, the focus of this work is on more recent findings on the plethora of dually targeted proteins and their potential function. We provide single chapters focusing on proteins targeted to peroxisomes and at least one other organelle with a major focus on yeasts and filamentous fungi. In addition, we showcase parallels to other eukaryotes.

Dual targeting can be achieved through very different mechanisms. Gene duplication and subsequent development of isoforms with import signals for only one cellular compartment are often found in *Saccharomyces cerevisiae*, presumably due to the genome duplications, which happened during its evolution (Kellis et al., 2004; Yogev et al., 2011; Ast et al., 2013). Polypeptides with different targeting signals can also be generated from a single gene (Yogev et al., 2011; Ast et al., 2013), e.g., from alternative transcripts, alternative splicing (Natsoulis et al., 1986; Clausmeyer et al., 1999; Freitag et al., 2012; Strijbis et al., 2012), programmed readthrough of stop codons (for a review, see Schueren and Thoms, 2016), and noncanonical translation initiation (Monteuuis et al., 2019; Kremp et al., 2020). In addition, proteins can contain ambiguous targeting signals at their N-termini, which enable sorting into two cellular compartments. This is particularly prominent in plants for those proteins required in mitochondria and plastids—two organelles, which use a related protein import pathway involving N-terminal targeting signals and translocation of unfolded proteins (Carrie et al., 2009). We also discuss the dual targeting of proteins that contain N-terminal targeting signals, e.g., for mitochondria or the ER in combination with a C-terminal PTS1. It was suggested previously that the localization of these proteins might be dictated by the N-terminal signal since it can be bound by respective targeting factors during translation before the C-terminal PTS1 becomes accessible (Ast et al., 2013; Kunze and Berger, 2015). However, a recent study uncovered that proteins with competing N-terminal and C-terminal targeting signals localize in peroxisomes and mitochondria (Stehlik et al., 2020). Low efficiency or unusual peroxisomal targeting signals often provoke partial cytosolic retention due to low import rates or other reasons, e.g., the modification of the peroxisomal targeting machinery (Ast et al., 2013; Okumoto et al., 2020). Another focus of our review is on peroxisomal membrane proteins (PMPs) and membrane-associated proteins, which follow variable transit routes.

Typical enzymes with a role in multiple cellular compartments

One critical type of enzymes required inside of peroxisomes and in other compartments are NADH-dependent dehydrogenases. They can assist peroxisomal NAD^+ regeneration, e.g., during β-oxidation. The reduced substrate can be translocated into the cytosol and exchanged with an oxidized molecule giving rise to a redox shuttle system (Visser et al., 2007). The existence of a peroxisomal redox shuttle was first demonstrated in *S. cerevisiae*—a peroxisomal isoenzyme of malate dehydrogenase (Mdh3p) containing a PTS1 was shown to be involved (Van Roermund et al., 1995). Malate can be generated from oxaloacetate enabling the reoxidation of NADH. The small molecules are thought to pass the peroxisomal membrane, albeit it is not fully understood if transporters or size-selective pore-forming proteins are involved (for a review, see Chornyi et al., 2021). Recently, it was shown that a second malate dehydrogenase Mdh2p from *S. cerevisiae* involved in glyoxylate metabolism binds Mdh3p and can enter peroxisomes *via* piggyback import resulting in dual localization (Gabay-Maskit et al., 2020). It is yet unclear how cells exactly benefit from having two MDH enzymes inside of peroxisomes, but a possible answer is their different activities (Steffan and McAlister-Henn, 1992; Gabay-Maskit et al., 2020). In addition to the described malate–oxaloacetate shuttle, *S. cerevisiae* contains a second shuttle system relying on the glycerol-3-phosphate dehydrogenase Gpd1p, which catalyzes the interconversion of glycerol-3-phosphate and dihydroxyacetone phosphate. Gpd1p is dually localized in the cytosol and in peroxisomes (Jung et al., 2010) (Figure 1). Mdh3p and Gpd1p exhibit redundant functions for NAD shuttling in conditions that require biosynthesis of lysine, the last step of which occurs inside of yeast peroxisomes (Al-Saryi et al., 2017a). Why cells employ multiple shuttle systems for NAD^+/NADH and how this functionally connects peroxisomal metabolism to the metabolism of the entire cell requires further investigation. The presence of multiple systems, however, seems to be common. Many fungi contain a peroxisomal isoform of the NAD^+-dependent glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) derived from alternative splicing or stop codon readthrough. Genetic data point to overlapping functions of peroxisomal GAPDH, GPD, and MDH presumably for the regulation of NAD^+/NADH balance (Freitag et al., 2012).

In mammalian cells, programmed stop codon readthrough triggers the synthesis of extended PTS1-containing isoforms of lactate dehydrogenase LDHB and malate dehydrogenase MDH1 (Schueren et al., 2014; Stiebler et al., 2014). This is in accordance with at least two pathways to control the NAD^+/NADH ratio. Stop codon readthrough is a widespread mechanism to regulate the dual localization of central metabolic enzymes in fungi and animals; e.g., a peroxisomal isoform of the glycolytic enzyme phosphoglycerate kinase contains a readthrough-derived PTS1 (Freitag et al., 2012; Figure 1). Other prominent enzymes occurring in many cellular compartments are inorganic pyrophosphatases—several enzymes harbor putative PTS1-containing extensions, which can be activated by stop codon readthrough. These include the *Caenorhabditis elegans* Pyp-1 protein and PAP1 or PAP2 from different mammals (Stiebler

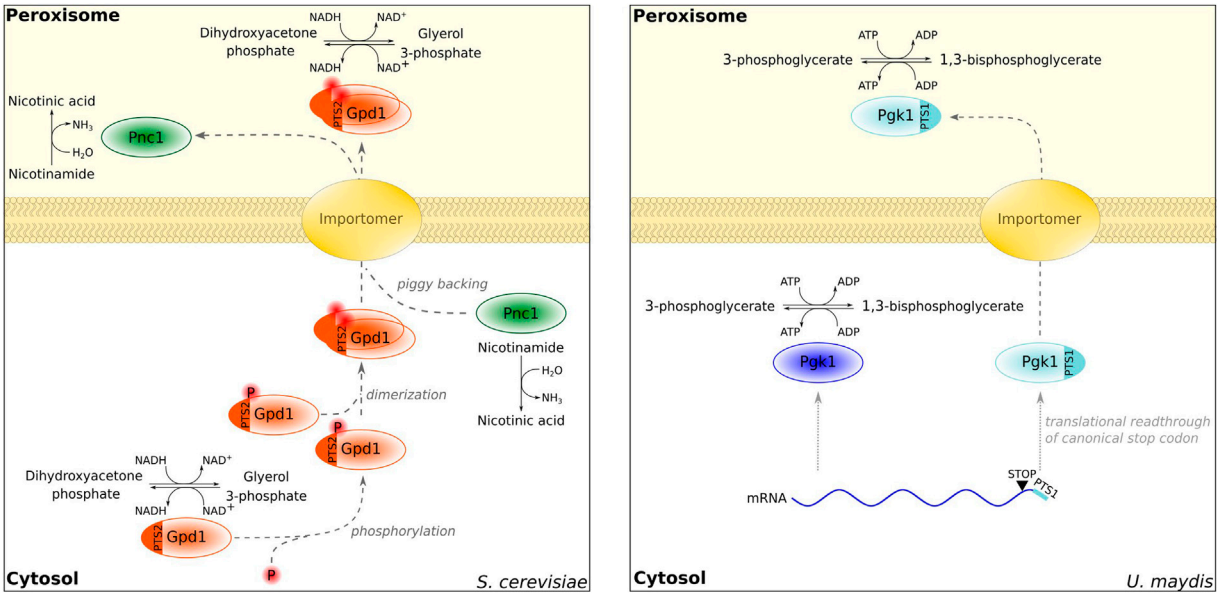

FIGURE 1
Examples of proteins localized in the cytosol and peroxisomes. Gpd1 distribution is controlled by phosphorylation [left; Jung et al. (2010)]. In addition, Gpd1 regulates the sorting of Pnc1 *via* piggybacking (Effelsberg et al. (2015), Kumar et al. (2016), Al-Saryi et al. (2017b)). An extended isoform of Pgk1 derived from translational readthrough is localized in peroxisomes in *Ustilago maydis* [right; Freitag et al. (2012)].

et al., 2014). Inorganic pyrophosphatase is a central enzyme rendering energy-dependent reactions throughout the cell virtually irreversible (Kornberg, 1962) and may also speed up peroxisomal metabolism.

Why is stop codon readthrough such a prominent mechanism for the generation of peroxisomal isoforms? First, a trivial reason is the C-terminal position of PTS1—a simple prerequisite for this mechanism to activate a hidden PTS1. Furthermore, readthrough rates are low but seem to be sufficient to enable enzyme supply for the relatively small peroxisomal compartment in concentrations high enough to satisfy demands. Finally, the region behind the stop codon could be regarded as a playground for evolution and partial peroxisomal localization can be easily tested and rejected again. This may explain the previously observed patchy distribution of readthrough sites coupled to PTS1 motifs among different eukaryotic species (Freitag et al., 2012; Dunn et al., 2013; Stiebler et al., 2014; Hofhuis et al., 2016).

Peroxisomal NADPH turnover is involved in the oxidation of fatty acids with a cis double bond at an even position (Poirier et al., 2006). Early work in *S. cerevisiae* demonstrated a role of an isocitrate dehydrogenase as part of a peroxisomal NADPH shuttle (van Roermund et al., 1998). Isocitrate is oxidized to alpha-ketoglutarate and CO_2 leading to the formation of NADPH. Idp1p is the mitochondrial isoform, Idp2p is the cytosolic isoform, and Idp3p is the peroxisomal isoform (Haselbeck and McAlister-Henn, 1991; Haselbeck and McAlister-Henn, 1993; van Roermund et al., 1998). In contrast, peroxisomal, mitochondrial, and cytosolic isocitrate dehydrogenases are derived from a single gene in the filamentous fungus *Neurospora crassa* (Szewczyk et al., 2001). In *Arabidopsis thaliana*, several enzymes of the pentose phosphate pathway including an isoform of the NADPH-dependent enzyme 6-phosphogluconate dehydrogenase (PGD) reside in peroxisomes and are critical for development (Corpas et al., 1998; Meyer et al., 2011; Hölscher et al., 2016). Dually targeted peroxisomal isoforms of PGD can be generated by alternative splicing or noncanonical translation initiation in different fungi (Strijbis et al., 2012; Kremp et al., 2020). Recently, in *A. thaliana* a transport protein for glucose-6-phosphate has been identified that reaches peroxisomes *via* the ER but is also found in plastids (Baune et al., 2020).

Additional carbohydrate-metabolizing enzymes contain cryptic or low-efficiency peroxisomal targeting signals in several fungi including not only glycolytic/gluconeogenetic enzymes but also enzymes of the non-oxidative part of the pentose phosphate pathway (Freitag et al., 2012; Freitag et al., 2018). This points to many molecular links of the peroxisomal metabolism to the central carbohydrate metabolism. In several fungal species, optimal growth on glucose medium requires intact peroxisomes—even on full medium in the logarithmic growth phase (Idnurm et al., 2007; Freitag et al., 2012; Camões et al., 2015; Ast et al., 2022). Whether these growth phenotypes

are directly related to the dual targeting of glycolytic enzymes or pentose phosphate pathway enzymes is yet elusive.

Dually targeted proteins—the dark matter of the peroxisomal proteome

Minor destinations of proteins are very likely to be missed. A recent systematic study in *S. cerevisiae* uncovered many novel proteins residing in peroxisomes, as well as in other organelles or in the cytosol (Yifrach et al., 2021). Using systematic metabolomics analysis of mutants and overexpression strains, the authors suggest that several of these have potential peroxisomal functions. Among the newly identified dually localized proteins are direct substrates of Pex5, which lack a classical PTS1. This reveals a greater substrate repertoire of this targeting factor (Yifrach et al., 2021). Other work also points to the occurrence of many unusual variations of the PTS1 motif, which are recognized by Pex5 in several fungi (Freitag et al., 2012; Camões et al., 2015; Nötzel et al., 2016). An unexpected example is sequence motifs resembling a PTS1, which are located near the C-terminus of a protein rather than at its end. This type of signal was suggested to be responsible for peroxisomal localization of catalase from pumpkin and for human ataxia telangiectasia–mutated (ATM) kinase (Kamigaki et al., 2003; Zhang J. et al., 2015). It is still unclear how PTS1 motifs, which do not reside at the C-terminus, are recognized by Pex5 as this is thought to be a structural prerequisite for binding (Fodor et al., 2015).

An interesting example identified by Yifrach et al. (2021) is dually localized subunits of the GID (glucose-induced degradation-deficient) complex, which regulate the stability of fructose-1,6-bisphosphatase (FBP). The targeting of GID complex proteins to peroxisomes was found to increase FBP levels probably enhancing gluconeogenesis (Yifrach et al., 2021). These data indicate a novel function of peroxisomes in regulating carbohydrate mechanism upon glucose limitation. Similarly, in different yeasts nicotinamidase Pnc1 is targeted to peroxisomes more efficiently in response to certain stresses—this is achieved through hitchhiking on the PTS2 protein Gpd1 (Anderson et al., 2003; Effelsberg et al., 2015; Kumar et al., 2016; Al-Saryi et al., 2017b) (Figure 1). Sequestration of a protein inside of the peroxisomal lumen to remove it from its site of action anywhere in the cell may be a more widespread function of peroxisomes. They seem to be promiscuous sites among the cellular organelles and tolerate many different proteins and activities. Even the efficient production of toxic compounds was successfully engineered aided by the peroxisomal targeting of respective enzymes (Grewal et al., 2021). For each of the dually localized proteins, it will be critical to assess whether they fulfill a specific function inside of peroxisomes or whether their localization reflects a strategy to sequester them from a different cellular compartment.

Many functions of peroxisomes relying on dually targeted proteins are likely to remain established. Surprisingly, in *Aspergillus nidulans* the microtubule-organizing protein ApsB is partially localized to a subpopulation of peroxisomes, but its role at this location has not been understood (Zekert et al., 2010). Several proteins from *A. thaliana* show a dual localization to peroxisomes and to other compartments, often due to PTS1 motifs with low import efficiency (Reumann et al., 2007; Kataya and Reumann, 2010; Lingner et al., 2011). Interestingly, protein kinases were identified that often contain unusual but functional PTS1 motifs (Kataya et al., 2022). These findings are of particular interest as little is known about the phosphorylation-dependent regulation of enzymes inside of the peroxisomal lumen. More data are needed to assess which kinases play a crucial role in regulating the activity of the peroxisomal proteome. Particularly, the fact that some kinases contain transmembrane domains is puzzling with regard to the classical view of peroxisomal matrix protein import (Walter and Erdmann, 2019). Recent data, however, point to a function of the peroxisomal matrix protein import machinery in membrane protein translocation (Martenson et al., 2020). Together, all the mentioned studies emphasize a plethora of diverse peroxisomal proteins, for which a functional characterization is lacking so far. We expect more surprises concerning the proteome composition of peroxisomes in the future.

Peroxisomes—organelles in a twilight zone

Peroxisomes have been adapted for multiple purposes during evolution, e.g., as a seal for septal pores in fungal hyphae, as sites for methanol breakdown in different yeast, or as glycolytic organelles in trypanosomes (Jedd and Chua, 2000; van der Klei and Veenhuis, 2006b; Liu et al., 2011; Haanstra et al., 2016). Why do peroxisomes show this high degree of functional flexibility? For many species, cellular survival does not strictly depend on peroxisomal functions; hence, their repertoire of proteins could change without detrimental consequences. Furthermore, peroxisomes represent a cellular one-way road. They import cargo, grow, divide, and are degraded but probably do not fuse regularly in their mature form (Islinger et al., 2012; Kim and Hettema, 2015; Germain and Kim, 2020). This may explain their tolerance to a plethora of proteins and the coexistence of multiple peroxisome variants in a single cell as evident for the Woronin body biogenesis in ascomycetes.

Peroxisomes and mitochondria—striking similarities

Dually targeted soluble proteins

Mitochondria and peroxisomes cooperate in versatile metabolic processes, exchange many molecules, and also share proteins required for fission and quality control (Schrader et al., 2015; Wanders et al., 2016). This peroxisome–mitochondria connection has been excellently discussed before (Fransen et al., 2017; Costello et al., 2018), and we only focus on several examples. Both organelles can be cellular sites for β-oxidation of fatty acids—while in several fungi and plants, β-oxidation occurs exclusively inside of peroxisomes, other fungi and animals harbor full sets of β-oxidation enzymes inside of both organelles (Maggio-Hall and Keller, 2004; Poirier et al., 2006; Goepfert and Poirier, 2007; Kretschmer et al., 2012b; Camões et al., 2015). An important difference between the compartments is the mechanisms they use for the import of proteins: While mitochondrial proteins often rely on N-terminal targeting signals and can be imported in an unfolded or flexible state (Backes and Herrmann, 2017), import into the peroxisomal matrix is different.

To exchange intermediates of the β-oxidation pathway, substitution of a coenzyme A moiety with carnitine is one possibility to shuttle acetyl and acyl groups as acetyl- or acylcarnitine units (Antonenkov and Hiltunen, 2012). Acetylcarnitine transferases are dually localized enzymes, and isoforms with different targeting signals have been reported, which can be generated *via* alternative transcriptional and translational start sites or differential splicing (Corti et al., 1994; Elgersma et al., 1995; Ueda et al., 1998; Houten et al., 2020). Within mitochondria, carnitine is replaced by coenzyme A to enable further metabolization (Houten et al., 2020).

A second cellular pathway enables the exchange of C2 units between peroxisomes and mitochondria, albeit more indirectly. Citrate can be generated from oxaloacetate *via* citrate synthases, which are not only part of the Krebs cycle but also part of the glyoxylate cycle, partially located inside of peroxisomes (Kim et al., 1986; Kunze et al., 2006). The citrate synthase Cit2 has overlapping functions with acetylcarnitine transferase Cat2p in *S. cerevisiae* and is contained in peroxisomes (Van Roermund et al., 1995; van Roermund et al., 1999; Swiegers et al., 2001; Shai et al., 2018). Of interest, Cit2p can be targeted to mitochondria, as well as to peroxisomes, and can compensate for the absence of mitochondrial citrate synthase Cit1p (Kim et al., 1986; Hoppins et al., 2011; Lee et al., 2011; Morgenstern et al., 2017). Citrate can be shuttled *via* dedicated transport proteins linking the mitochondrial matrix to the cytosol (Kaplan et al., 1995; Palmieri, 2004).

How are proteins distributed that do not come in different isoforms but contain a mitochondrial targeting signal at the N-terminus and a PTS1 at the C-terminus? Previously, it was discussed that these usually end up in mitochondria, because the N-terminal signal will exit the ribosome first, can directly interact with the mitochondrial import machinery, and thus will dominate a PTS1—a hierarchy of targeting signals is likely to exist (Kunze and Berger, 2015). Indeed, the mitochondrial ribosomal protein Mrp7p contains a functional PTS1 in *S. cerevisiae* but does not localize in peroxisomes presumably because the N-terminal signal is recognized first (Neuberger et al., 2004). However, several *bona fide* peroxisomal proteins such as the thiolase Tes1p or the catalase Cta1p have been identified in highly purified mitochondria of *S. cerevisiae* (Morgenstern et al., 2017).

Another PTS1-containing protein—the protein phosphatase Ptc5p—has been recently shown to first target mitochondria where its N-terminus is proteolytically removed. Subsequently, the protein is translocated into the peroxisomal lumen *via* interaction with Pex5p (Stehlik et al., 2020) (Figure 2). Further proteins were also shown to be retargeted from mitochondria to the cytosol, e.g., *S. cerevisiae* fumarase (Stein et al., 1994; Yogev et al., 2010). The sorting of Ptc5p is different as the presence of Pex5p is critical for export from mitochondria, indicating an interaction between Ptc5p and Pex5p prior to full mitochondrial import. One target of Ptc5p in peroxisomes is Gpd1p (Stehlik et al., 2020), and dephosphorylation of Gpd1p increases its activity (Lee et al., 2012). In line with these data, a synthetic growth defect was observed for strains deleted for *PTC5* and *MDH3* (Costanzo et al., 2016; see the previous chapter on the redundancy of Gpd1p and Mdh3p). Together, these data suggest a network consisting of Ptc5p, Gpd1p, and Mdh3p to regulate peroxisomal NADH metabolism presumably in a manner adapting the different compartments to each other. In *A. thaliana*, the sorting of NAD(P)H dehydrogenases may resemble Ptc5p from *S. cerevisiae* as competing N- and C-terminal targeting signals for mitochondria and peroxisomes are involved (Carrie et al., 2008).

Targeting of proteins with competing targeting signals may represent a more generic approach to regulate the interaction and communication of compartments—the overexpression of several of these proteins increases the fraction of peroxisomes associated with mitochondria (Stehlik et al., 2020). In mammalian cells, the overexpression of the dual affinity protein ACBD2/ECI2 also enhances the proximity of peroxisomes and mitochondria (Fan et al., 2016). The protein Cnm1p with competing targeting signals at its termini can increase the attachment of mitochondria to the nuclear envelope in *S. cerevisiae*, suggesting a similar mechanism occurring between another pair of organelles (Eisenberg-Bord et al., 2021).

Dual targeting of mitochondrial membrane proteins

In addition to luminal proteins, peroxisomes and mitochondria share a number of membrane proteins or membrane-associated proteins with various tasks (also see

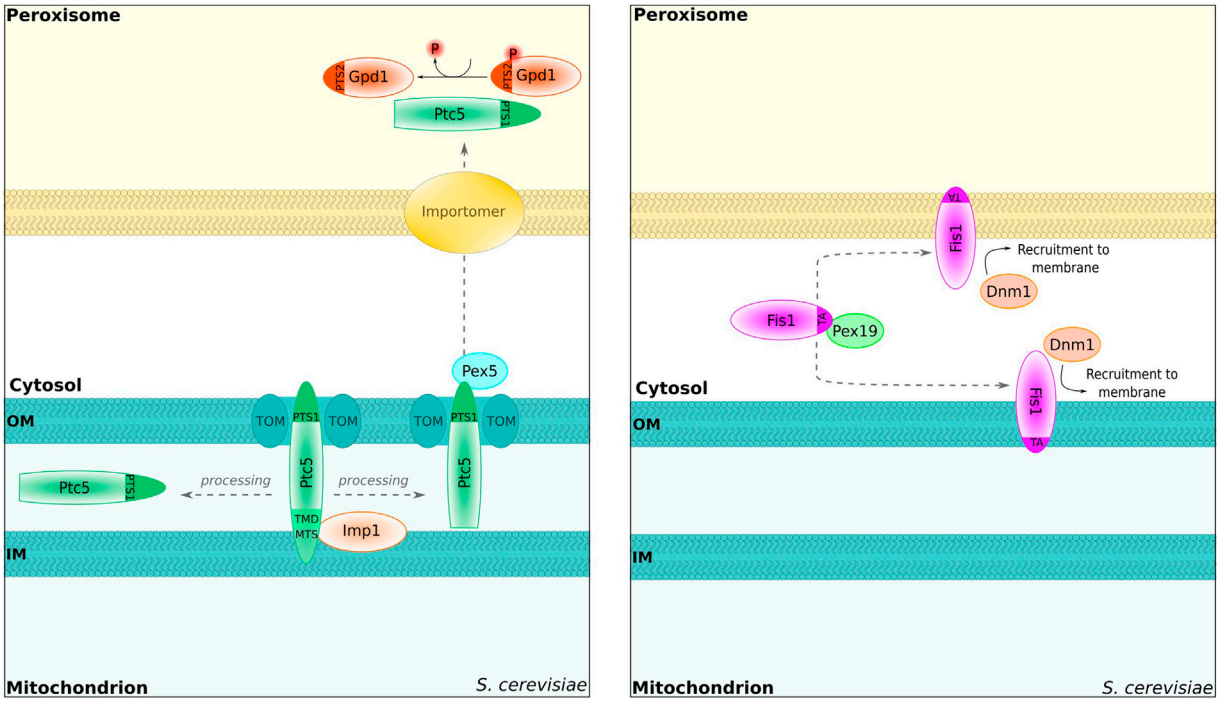

FIGURE 2
Proteins shared between peroxisomes and mitochondria. *S. cerevisiae* Ptc5p can reach the peroxisome *via* mitochondrial transit. The protein is proteolytically processed inside of the inner mitochondrial membrane by the peptidase Imp1 and subsequently translocated to peroxisomes in a Pex5-dependent manner [left; Stehlik et al. (2020)]. Inside of the peroxisome, Ptc5 dephosphorylates Gpd1 [left; Stehlik et al. (2020)]. TOM, translocator of the outer mitochondrial membrane. Importomer, complex for peroxisomal matrix protein import [Walter and Erdmann (2019)]. The tail-anchored protein Fis1 is involved in the fission process of peroxisomes and mitochondria [right; Kuravi et al. (2006)]. Targeting is regulated by Pex19 [Cichocki et al. (2018)]. Dnm1, dynamin-related GTPase for peroxisome fission Kuravi et al. (2006).

Fransen et al., 2017; Costello et al., 2018). A key process controlled by overlapping factors is fission. Both in *S. cerevisiae* and in mammalian cells, the C-terminally tail-anchored protein Fis1p/FIS1, dynamin-related GTPases, and additional shared proteins such as the WD40 repeat-containing protein Caf4p belong to the factors involved in the fission process (Koch et al., 2003; Praefcke and McMahon, 2004; Koch et al., 2005; Kuravi et al., 2006; Motley et al., 2008; Motley et al., 2015; Castro et al., 2018). C-terminally tail-anchored membrane proteins such as FIS1 tend to be dually localized, and it was shown that the hydrophobicity of the transmembrane domain together with changes in the charge of the luminal tail determines targeting efficiency and hence the subcellular distribution (Costello et al., 2017a). Remarkably, the peroxisomal targeting factor Pex19p is required for the correct sorting of Fis1p to mitochondria and to peroxisomes in *S. cerevisiae* (Cichocki et al., 2018) (Figure 2). How the distribution of proteins such as Fis1p is adapted to the needs of both organelles is not known yet.

Further proteins operating at mitochondria and peroxisomes are ATPases termed Msp1p in *S. cerevisiae* or ATAD in mammals, which can extract superfluous or mistargeted tail-anchored proteins (Chen Y.-C. et al., 2014; Okreglak and Walter, 2014; Weir et al., 2017). Of interest, a variety of PMPs can be removed from mitochondria *via* Msp1p/ATAD, suggesting a broader range of substrates (Nuebel et al., 2021). Furthermore, these authors observed that many peroxisomal proteins are not degraded or downregulated in the absence of preexisting peroxisomes, but functional PMP-containing protein complexes assemble on mitochondria, which causes unfavorable consequences such as the import of PTS1 cargo into mitochondria (Nuebel et al., 2021). Mitochondrial dysfunction in the absence of functional peroxisomes is emerging as one cause of symptoms in patients lacking peroxisomes (Wanders, 2014; Lismont et al., 2019; Schrader et al., 2020; Nuebel et al., 2021). The ubiquitin ligase MARCH5—another protein for quality control—was shown to localize to peroxisomes in addition to its known location at mitochondria in mammals. At peroxisomes, MARCH5 is involved in controlling selective autophagy (Zheng et al., 2021).

A multi-subunit protein structure associated with both organelles in fungi is the ERMES (ER–mitochondria encounter structure) complex, which has a role in tethering of mitochondria to the ER and to peroxisomes to form putative

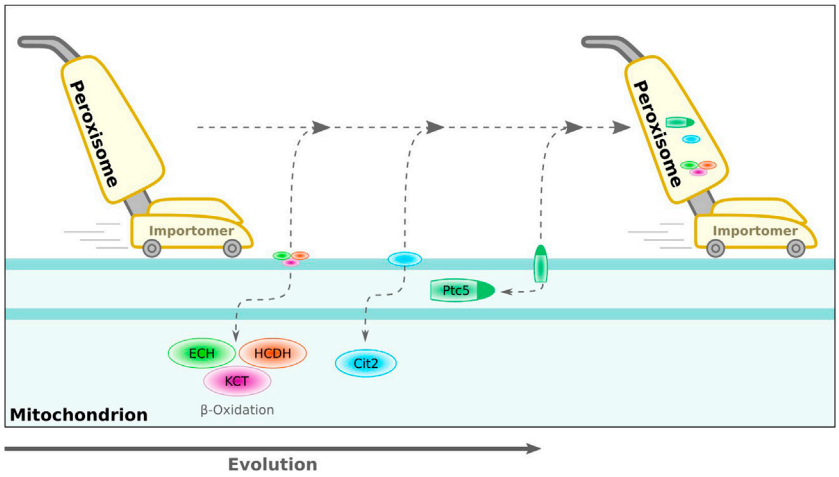

FIGURE 3
Hypothetical model for translocation of proteins from mitochondria to peroxisomes during evolution. Peroxisomes are indicated by a vacuum cleaner—this parallel is inferred from their unique import mode for folded proteins and the possible evolutionary origin of the importomer from an ER-derived quality control system (Gabaldón et al. (2006), Schlüter et al. (2006)). We speculate that over time, various mitochondrial enzymes developed into peroxisomal enzymes *via* a dually targeted intermediate. Sorting of the phosphatase Ptc5p from *S. cerevisiae* shows hallmarks for such an evolutionary scenario. ECH, enoyl-CoA hydratase; HCDH, 3-hydroxyacyl-CoA dehydrogenase; KCT, 3-ketoacyl-CoA-thiolase; Cit2, citrate synthase 2 (*S. cerevisiae*); Ptc5, PP2C-type phosphatase (*S. cerevisiae*).

three-way junctions (Kornmann et al., 2009; Cohen et al., 2014; Ušaj et al., 2015; Kundu and Pasrija, 2020). Moreover, the ERMES regulating GTPase Gem1p can be found in peroxisomes and mitochondria of *S. cerevisiae* (Kornmann et al., 2011; Cichocki et al., 2018). In mammalian cells, the Gem1p ortholog MIRO1 is dually localized as well and has a role in recruiting the lipid transfer protein VPS13D probably involved in sustaining organellar growth and lipid exchange (Castro et al., 2018; Baldwin et al., 2021; Guillén-Samander et al., 2021). Thus, mitochondria and peroxisomes use an overlapping set of proteins to connect to the ER.

A different mode of transport to accomplish the dual localization of selected proteins is vesicular trafficking from one organelle to the other. Dynamin-independent carriers were proposed to transport the ubiquitin ligase MAPL from mitochondria to peroxisomes (Neuspiel et al., 2008). More recently, it was suggested that *de novo* peroxisome formation involves mitochondria-derived vesicles (Sugiura et al., 2017).

Dual targeting and evolution of peroxisomes

All these data point to a close relationship of mitochondria and peroxisomes not only in terms of metabolism but also in terms of biogenesis, quality control, and turnover. This remarkable overlap might reflect peroxisome evolution as partially mitochondria-derived organelles (Bolte et al., 2015; Speijer, 2017). Are peroxisomes indeed outposts of mitochondria, and how can this be in line with the key role of the endoplasmic reticulum for peroxisome biogenesis (Joshi et al., 2017; see also in the next chapter)?

We speculate that peroxisomes may have emerged from an ancient ER-derived quality control compartment involved in clearing specific mitochondrial proteins, especially under conditions of oxidative stress. In this perspective, the ERAD-related peroxisomal import machinery (Schliebs et al., 2010) could be regarded as an extraction machine for mitochondrial proteins working *in trans*. Particularly, the oxidative enzymes of the β-oxidation pathway may be a major burden for mitochondrial metabolism, which may explain why this pathway has been completely transferred to peroxisomes of several species, e.g., *S. cerevisiae* (Hiltunen et al., 2003; Poirier et al., 2006; Speijer, 2017). The indirect targeting of the phosphatase Ptc5p *via* mitochondrial transit (Stehlik et al., 2020) could be a snapshot or remnant of this evolutionary scenario. In this effect, peroxisomes may represent ancient molecular vacuum cleaners of the mitochondrial surface (Figure 3), which became autonomous over time by hitchhiking the mitochondrial division and quality control machinery. Further shared proteins might still be obscure—one study indicated a role for the dually localized mitofusin ortholog Fzo1 from *S. cerevisiae* for tethering peroxisomes to mitochondria (Shai et al., 2018).

Proteins localized to peroxisomes and the endoplasmic reticulum

The endoplasmic reticulum as a source for peroxisomal membrane

The endoplasmic reticulum (ER) is the major source of cellular lipids and has a key role in supporting the growth of the entire cell (Ferro-Novick et al., 2013). Early work already revealed intimate connections between this large-supply organelle and peroxisomes (Novikoff and Shin, 1964). Metabolites are exchanged between both compartments, e.g., during the biosynthesis of ether lipids (Wanders et al., 2016). In *S. cerevisiae*, the sorting of the multifunctional PMP Pex3—a key factor in regulating PMP import into peroxisomes—from the ER to peroxisomes was described (Hoepfner et al., 2005; Jansen and van der Klei, 2019). ER-derived vesicular carriers were reported to contain peroxisomal proteins, and machinery was uncovered that is relevant for the emergence of peroxisomal vesicles from the ER in yeasts (Titorenko et al., 2000; Lam et al., 2010; Agrawal et al., 2011; Van der Zand et al., 2012; Agrawal et al., 2016; Mast et al., 2018). Hence, vesicular trafficking from the ER represents one road for lipids and proteins to the peroxisome and also operates in mammalian cells (Kim et al., 2006; Aranovich et al., 2014; Sugiura et al., 2017). In *S. cerevisiae* and other yeasts, ER-resident microdomain-forming reticulon proteins play a critical role in tethering of preexisting peroxisomes (David et al., 2013; Mast et al., 2016; Wu et al., 2020; Ferreira and Carvalho, 2021). Interestingly, the same domains may be required for the budding of peroxisome precursors (Joshi et al., 2016; Joshi et al., 2018; Wang et al., 2018). As an alternative to vesicle formation, direct lipid transfer promotes the growth of peroxisomes and recruitment of lipid transfer proteins such as VPS13 is involved in peroxisome biogenesis both in yeasts and in mammals (Raychaudhuri and Prinz, 2008; Baldwin et al., 2021; Guillén-Samander et al., 2021; Yuan et al., 2022). How these mechanisms for lipid supply each contribute to peroxisome formation is an exciting question for future work. An intimate physical connection between peroxisomes and the ER is probably required for the correct activity of both mechanisms. This can, e.g., be supported through an interaction between the peroxisomal acyl-CoA binding protein ACBD5 and the ER membrane-associated protein VAPB in mammalian cells (Costello et al., 2017b; Hua et al., 2017).

Proteins found in the endoplasmic reticulum and peroxisomes

Many PMPs are synthesized in the vicinity of the peroxisome of *S. cerevisiae* (Zipor et al., 2009; Dahan et al., 2022), and direct insertion into the peroxisomal membrane was demonstrated for several of them (Sacksteder et al., 2000; Fang et al., 2004; Jones et al., 2004; Matsuzaki and Fujiki, 2008; Yagita et al., 2013; Chen Y. et al., 2014). Interestingly, other PMPs are synthesized proximal to the ER and may reach the peroxisome *via* ER transit (Jan et al., 2014). For Pex3, dual localization to peroxisomes and the ER of *S. cerevisiae* was shown by fractionation experiments (Mast et al., 2016). Hence, the ER is not only an intermediate but also a relevant steady-state location for certain PMPs, indicating functions beyond ER transit. Indeed, in mammalian cells ER-localized PEX3 is involved in the sorting of a protein designated for lipid droplets (Schrul and Kopito, 2016). In addition to PEX3, the soluble targeting factor PEX19 is associated with the ER and involved in targeting reticulon-homology proteins to this organelle besides its prominent function in peroxisome biogenesis (Yamamoto and Sakisaka, 2018; Zimmermann et al., 2021).

The coat protein I (COPI) complex localizes primarily along the organelles of the secretory pathway where it is involved in retrograde transport (Barlowe and Miller, 2013). In addition, this protein assembly can be associated with peroxisomes and also with mitochondria, but the molecular function of the localization at these organelles is not fully understood (Passreiter et al., 1998; Barlowe and Miller, 2013; David et al., 2013; Zabezhinsky et al., 2016). Remarkably, COPI-dependent sorting of a viral protein from peroxisomes to the ER was reported (McCartney et al., 2005). The small GTPase Arf1p—a major regulator of COPI—shows multiple subcellular localizations including peroxisomes and mitochondria in *S. cerevisiae* (Ackema et al., 2014; Yofe et al., 2017). So far, the role of COPI-dependent vesicular trafficking for peroxisome biogenesis is elusive although in *S. cerevisiae,* COPI components can be purified together with the subdomain-forming protein Pex30p, which is involved in peroxisome formation (David et al., 2013). Pex30p is also found at sites of lipid droplet formation in *S. cerevisiae*, and COPI was shown to be involved in formation of this organelle in *Drosophila* cells (Wilfling et al., 2014; Joshi et al., 2018; Wang et al., 2018; Ferreira and Carvalho, 2021). Further research is required to understand how key factors of the secretory pathway are dynamically distributed between the different organelles and how this coordinates organelle biogenesis at different sites. Nevertheless, the discussed examples emphasize that the ER and peroxisomes are possible alternative destinations of several factors required for the proper maintenance of each.

Another interesting example of dual targeting was described in *A. thaliana*—a purple acid phosphatase-containing competing targeting signals is localized to the ER and to peroxisomes (Kataya et al., 2016). Various other phosphatases with noncanonical PTS1 motifs were identified in this study that may reside inside of peroxisomes and in additional compartments including the nucleus (Kataya et al., 2016).

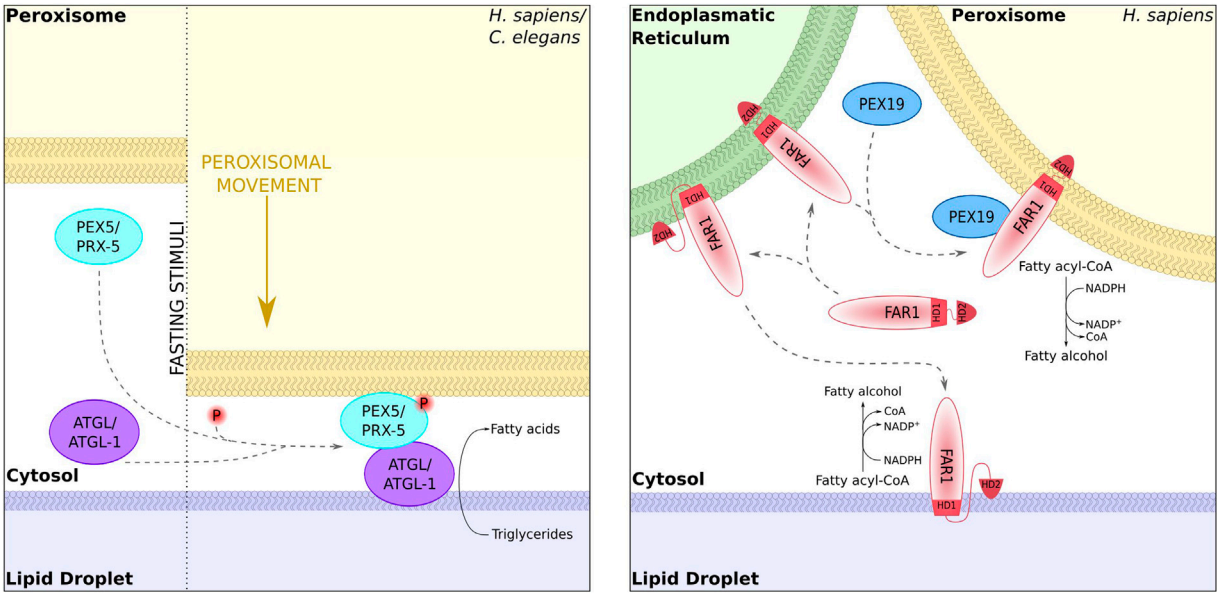

FIGURE 4
Dual targeting to peroxisomes and lipid droplets. The lipases ATGL-1 (*Caenorhabditis elegans*) and ATGL (mammalian cells) can target lipid droplets in a Pex5-dependent manner at sites of organelle contact [left; Kong et al. (2020)]. The fatty acid reductase Far1 is dually localized to peroxisomes and lipid droplets. Localization to one or the other organelle requires different topologies of the hydrophobic domains [right; Exner et al. (2019)].

Dual targeting to the nucleus and peroxisomes

Different types of pathogenic attack trigger the nuclear localization of plant catalases (Inaba et al., 2011; Zhang M. et al., 2015). A recent study in *A. thaliana* uncovered that catalase is localized both in peroxisomes and in the nucleus even in the absence of any infection (Al-Hajaya et al., 2022). Regulation of dual targeting *via* metabolites such as H_2O_2 is likely to operate in plants similar to what was observed in mammals (Okumoto et al., 2020). Peroxisomes together with mitochondria have a key role in controlling ROS production, which is important for cellular signaling and needs to be maintained on a level acceptable for the integrity of cells, especially of the nucleus (Fransen et al., 2017; Sies, 2017; Li et al., 2021; Paradis et al., 2021).

Interestingly, the hypoxia-inducible transcription factor Hif1 and hydroxylases regulating its activity are found in peroxisomes and mitochondria (Khan et al., 2006; Li et al., 2019). Sequestration upon reoxygenation was suggested as a possible biological function of peroxisomal targeting (Khan et al., 2006).

Proteins shared by lipid droplets and peroxisomes

Lipid droplets are sites of cellular fat storage and mobilization. Their biosynthesis and function are both intricately linked to peroxisomes. Both organelles require overlapping proteins for biogenesis, e.g., the lipodystrophy protein seipin and other factors including Pex30p (Szymanski et al., 2007; Salo et al., 2016; Joshi et al., 2017; Joshi et al., 2018; Wang et al., 2018). The targeting factor PEX19 and ER-localized PEX3 facilitate the insertion of the membrane protein UBXD8 into a subdomain of the ER membrane, which turned out as a prerequisite for subsequent transfer to lipid droplets in mammalian cells (Schrul and Kopito, 2016). Upon depletion of PEX19, UBXD8 appeared predominantly in mitochondria. Of interest, a proper targeting of UBXD8 requires C-terminal farnesylation of PEX19 (Schrul and Kopito, 2016).

Peroxisomes and lipid droplets are physically connected, and membrane protrusions emerging from peroxisomes have been shown to attach to lipid droplets in *S. cerevisiae* (Binns et al., 2006). In *A. thaliana*, a dually localized lipase can be sorted from peroxisomes to lipid droplets *via* similar protrusions in the course of fat mobilization during seed germination (Thazar-Poulot et al., 2015). In a similar way, in fasting animals triglyceride lipases are translocated from peroxisomes to lipid droplets depending on the targeting factor PEX5. This process was proposed to involve sites of organelle contact (Kong et al., 2020) (Figure 4).

Since peroxisomes are enclosed by a lipid bilayer and lipid droplets only by a monolayer, the dual localization of membrane proteins is likely to depend on dual topology. This was demonstrated for mammalian fatty acyl-CoA reductase 1, which is targeted to peroxisomes and lipid droplets and transits *via* the ER (Exner et al., 2019) (Figure 4).

What about the remaining cell?

Knowledge about proteins destined for peroxisomes and the plasma membrane or peroxisomes and endosomes is limited. It has been described that fungal peroxisomes hitchhike on motile endosomes to move through fungal hyphae (Guimaraes et al., 2015; Salogiannis et al., 2016). In *A. nidulans*, the association between endosomes and peroxisomes is mediated by the linker protein PxdA (Salogiannis et al., 2016). Proteins required for the maturation of endosomes such as members of the ESCRT (endosomal sorting complex required for transport) pathway (Henne et al., 2011) are involved in peroxisome biogenesis (Mast et al., 2018). Of interest, recent work revealed intraluminal vesicles inside of plant peroxisomes, which might also require the function of the ESCRT pathway, hinting at some parallels to multi-vesicular bodies (Wright and Bartel, 2020).

How to choose between the different destinations?

Insights into the molecular mechanisms that regulate the distribution of proteins among their different destinations are of interest to understand communication and homeostasis of cellular compartments. Studies on the dual localization of catalase in mammalian cells could represent a blueprint—it was shown that an elevated cytosolic concentration of H_2O_2 provokes cytosolic retention of this detoxifying enzyme (Walton et al., 2017). Recent studies showed phosphorylation of the peroxisomal membrane Pex14 in response to oxidative stress and during mitosis, which reduces the peroxisomal import of catalase to a greater extent than the import of other tested matrix proteins (Okumoto et al., 2020; Yamashita et al., 2020). Modification of the peroxisomal import machinery, hence, has an important role to adapt the peroxisomal and cytosolic protein composition to cellular demands. In fungi, the peroxisomal import of catalase is exceptional, and it relies on a noncanonical PTS1 and a second unusual peroxisomal targeting signal closer to the N-terminus (Kragler et al., 1993; Williams et al., 2012). Substitution of the C-terminus with a canonical targeting signal lowers catalase activity and leads to the aggregation of the enzyme (Williams et al., 2012). In *S. cerevisiae*, peroxisomal localization of the glyoxylate cycle enzyme citrate synthase 2 is reduced upon expression of a version of Pex14 mimicking a phosphorylated state (Schummer et al., 2020). Phosphorylation of the targeting factor Pex5 has been implicated in the regulation of pexophagy upon stress exposure in mammalian cells and occurs in different species (Zhang J. et al., 2015; Oeljeklaus et al., 2016). How phosphorylation of Pex5 modulates the import of specific cargo besides regulating peroxisome breakdown remains to be established. Redox regulation at an N-terminal cysteine residue is another way to control Pex5 activity (Ma et al., 2013).

Phosphorylation of dually localized cargo proteins is also involved in regulating their intracellular distribution (Figure 1). *S. cerevisiae* glycerol-3-phosphate dehydrogenase Gpd1 is phosphorylated in the vicinity of the PTS2 (Jung et al., 2010). While phosphomimetic variants of Gpd1 are efficiently targeted to the peroxisome, variants resembling non-phosphorylated Gpd1 are retained in the cytosol.

Many fungal species encode at least two versions of the PTS1 receptor Pex5 (Kiel et al., 2006). The expression of the Pex5 paralog Pex9 is induced upon incubation of *S. cerevisiae* cells in oleic acid medium and regulates the import of a subset of peroxisomal matrix proteins with specific targeting signals (Effelsberg et al., 2016; Yifrach et al., 2016; Yifrach et al., 2022). In the corn smut fungus *Ustilago maydis*, two Pex5 paralogs with different cargo selectivity are required for peroxisome function (Ast et al., 2022). The concentration or availability of different targeting factors at peroxisomes and at other organelles may emerge as an additional regulatory device to control the subcellular distribution of proteins.

Conclusion

Knowledge about proteins with various cellular destinations significantly increased in the last years. It probably will continue to grow rapidly as sophisticated approaches become more and more available, which allow the tracking of minor or transient destinations. Careful investigation to confirm whether dual or multiple localizations are of biological significance will be required. Targeted disruption of sorting signals leaving the overall protein function intact is one approach to reveal a function inside of a particular organelle (Kunze et al., 2002; Freitag et al., 2012).

The mechanisms to achieve dual or multiple targeting are highly variable, and how they are embedded in the regulatory circuits of the cell is not established in many of the cases. Proteins with different destinations may further emerge as critical factors regulating organellar interplay in terms of metabolism and molecule exchange, as well as in terms of biogenesis and proliferation. Thus, a better characterization of how the shared proteins are distributed and how this is regulated will improve our understanding of eukaryotic cells.

Author contributions

EB designed the figures. All authors contributed to literature searches and conceptualization. JF wrote the manuscript with inputs from EB and TS.

Funding

TS was supported by a fellowship from DAAD and by the Center for Synthetic Microbiology, Marburg. JF acknowledges funding from the DFG (Grant ID FR-3586/2-1). Open Access funding provided by the Open Acess Publication Fund of Philipps-Universität Marburg with support of the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation).

Acknowledgments

We thank Björn Sandrock for critical reading of the manuscript and Michael Bölker for support. We apologize to all scientists whose excellent work was not cited in this article.

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.

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References

Ackema, K. B., Hench, J., Böckler, S., Wang, S. C., Sauder, U., Mergentaler, H., et al. (2014). The small GTPase Arf1 modulates mitochondrial morphology and function. *EMBO J.* 33, 2659–2675. doi:10.15252/embj.201489039

Agrawal, G., Fassas, S. N., Xia, Z.-J., and Subramani, S. (2016). Distinct requirements for intra-ER sorting and budding of peroxisomal membrane proteins from the ER. *J. Cell Biol.* 212, 335–348. doi:10.1083/jcb.201506141

Agrawal, G., Joshi, S., and Subramani, S. (2011). Cell-free sorting of peroxisomal membrane proteins from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U. S. A.* 108, 9113–9118. doi:10.1073/pnas.1018749108

Al-Hajaya, Y., Karpinska, B., Foyer, C. H., and Baker, A. (2022). Nuclear and peroxisomal targeting of catalase. *Plant Cell Environ.* 45, 1096–1108. doi:10.1111/pce.14262

Al-Saryi, N. A., Al-Hejjaj, M. Y., van Roermund, C. W. T., Hulmes, G. E., Ekal, L., Payton, C., et al. (2017a). Two NAD-linked redox shuttles maintain the peroxisomal redox balance in *Saccharomyces cerevisiae*. *Sci. Rep.* 7, 11868. doi:10.1038/s41598-017-11942-2

Al-Saryi, N. A., Hutchinson, J. D., Al-hejjaj, M. Y., Sedelnikova, S., Baker, P., and Hettema, E. H. (2017b). Pnc1 piggy-back import into peroxisomes relies on Gpd1 homodimerisation. *Sci. Rep.* 7, 42579. doi:10.1038/srep42579

Anderson, R. M., Bitterman, K. J., Wood, J. G., Medvedik, O., and Sinclair, D. A. (2003). Nicotinamide and PNC1 govern lifespan extension by calorie restriction in *Saccharomyces cerevisiae*. *Nature* 423, 181–185. doi:10.1038/nature01578

Antonenkov, V. D., and Hiltunen, J. K. (2012). Transfer of metabolites across the peroxisomal membrane. *Biochim. Biophys. Acta* 1822, 1374–1386. doi:10.1016/j.bbadis.2011.12.011

Aranovich, A., Hua, R., Rutenberg, A. D., and Kim, P. K. (2014). PEX16 contributes to peroxisome maintenance by constantly trafficking PEX3 via the ER. *J. Cell Sci.* 127, 3675–3686. doi:10.1242/jcs.146282

Ast, J., Bäcker, N., Bittner, E., Martorana, D., Ahmad, H., Bölker, M., et al. (2022). Two Pex5 proteins with different cargo specificity are critical for peroxisome function in Ustilago maydis. *Front. Cell Dev. Biol.* 10, 858084. doi:10.3389/fcell.2022.858084

Ast, J., Stiebler, A. C., Freitag, J., and Bölker, M. (2013). Dual targeting of peroxisomal proteins. *Front. Physiol.* 4, 297. doi:10.3389/fphys.2013.00297

Backes, S., and Herrmann, J. M. (2017). Protein translocation into the intermembrane space and matrix of mitochondria: Mechanisms and driving forces. *Front. Mol. Biosci.* 4, 83. doi:10.3389/fmolb.2017.00083

Baldwin, H. A., Wang, C., Kanfer, G., Shah, H. V., Velayos-Baeza, A., Dulovic-Mahlow, M., et al. (2021). VPS13D promotes peroxisome biogenesis. *J. Cell Biol.* 220, e202001188. doi:10.1083/jcb.202001188

Barlowe, C. K., and Miller, E. A. (2013). Secretory protein biogenesis and traffic in the early secretory pathway. *Genetics* 193, 383–410. doi:10.1534/genetics.112.142810

Baune, M.-C., Lansing, H., Fischer, K., Meyer, T., Charton, L., Linka, N., et al. (2020). The Arabidopsis plastidial glucose-6-phosphate transporter GPT1 is dually targeted to peroxisomes via the endoplasmic reticulum. *Plant Cell* 32, 1703–1726. doi:10.1105/tpc.19.00959

Berger, J., Dorninger, F., Forss-Petter, S., and Kunze, M. (2016). Peroxisomes in brain development and function. *Biochim. Biophys. Acta* 1863, 934–955. doi:10.1016/j.bbamcr.2015.12.005

Binns, D., Januszewski, T., Chen, Y., Hill, J., Markin, V. S., Zhao, Y., et al. (2006). An intimate collaboration between peroxisomes and lipid bodies. *J. Cell Biol.* 173, 719–731. doi:10.1083/jcb.200511125

Blobel, G., and Sabatini, D. D. (1971). "Ribosome-membrane interaction in eukaryotic cells," in *Biomembranes*. Editor L. A. Manson (Boston, MA: Springer), 193–195.

Bolte, K., Rensing, S. A., and Maier, U.-G. (2015). The evolution of eukaryotic cells from the perspective of peroxisomes: Phylogenetic analyses of peroxisomal beta-oxidation enzymes support mitochondria-first models of eukaryotic cell evolution. *BioEssays* 37, 195–203. doi:10.1002/bies.201400151

Bürgi, J., Ekal, L., and Wilmanns, M. (2021). Versatile allosteric properties in Pex5-like tetratricopeptide repeat proteins to induce diverse downstream function. *Traffic* 22 (5), 140–152. doi:10.1111/tra.12785

Camões, F., Islinger, M., Guimarães, S. C., Kilaru, S., Schuster, M., Godinho, L. F., et al. (2015). New insights into the peroxisomal protein inventory: Acyl-CoA oxidases and-dehydrogenases are an ancient feature of peroxisomes. *Biochim. Biophys. Acta* 1853, 111–125. doi:10.1016/j.bbamcr.2014.10.005

Carrie, C., Giraud, E., and Whelan, J. (2009). Protein transport in organelles: Dual targeting of proteins to mitochondria and chloroplasts. *FEBS J.* 276, 1187–1195. doi:10.1111/j.1742-4658.2009.06876.x

Carrie, C., Murcha, M. W., Kuehn, K., Duncan, O., Barthet, M., Smith, P. M., et al. (2008). Type II NAD(P)H dehydrogenases are targeted to mitochondria and chloroplasts or peroxisomes in *Arabidopsis thaliana*. *FEBS Lett.* 582, 3073–3079. doi:10.1016/j.febslet.2008.07.061

Castro, I. G., Richards, D. M., Metz, J., Costello, J. L., Passmore, J. B., Schrader, T. A., et al. (2018). A role for Mitochondrial Rho GTPase 1 (MIRO1) in motility and membrane dynamics of peroxisomes. *Traffic* 19, 229–242. doi:10.1111/tra.12549

Chen, Y.-C., Umanah, G. K. E., Dephoure, N., Andrabi, S. A., Gygi, S. P., Dawson, T. M., et al. (2014). Msp1/ATAD1 maintains mitochondrial function by facilitating the degradation of mislocalized tail-anchored proteins. *EMBO J.* 33, 1548–1564. doi:10.15252/embj.201487943

Chen, Y., Pieuchot, L., Loh, R. A., Yang, J., Kari, T. M. A., Wong, J. Y., et al. (2014). Hydrophobic handoff for direct delivery of peroxisome tail-anchored proteins. *Nat. Commun.* 5, 5790. doi:10.1038/ncomms6790

Chornyi, S., IJlst, L., van Roermund, C. W. T., Wanders, R. J. A., and Waterham, H. R. (2021). Peroxisomal metabolite and cofactor transport in humans. *Front. Cell Dev. Biol.* 8, 613892. doi:10.3389/fcell.2020.613892

Cichocki, B. A., Krumpe, K., Vitali, D. G., and Rapaport, D. (2018). Pex19 is involved in importing dually targeted tail-anchored proteins to both mitochondria and peroxisomes. *Traffic* 19, 770–785. doi:10.1111/tra.12604

Clausmeyer, S., Stürzebecher, R., and Peters, J. (1999). An alternative transcript of the rat renin gene can result in a truncated prorenin that is transported into adrenal mitochondria. *Circ. Res.* 84, 337–344. doi:10.1161/01.res.84.3.337

Cohen, Y., Klug, Y. A., Dimitrov, L., Erez, Z., Chuartzman, S. G., Elinger, D., et al. (2014). Peroxisomes are juxtaposed to strategic sites on mitochondria. *Mol. Biosyst.* 10 (7), 1742–1748. doi:10.1039/c4mb00001c

Corpas, F., Barroso, J., Sandalio, L., Distefano, S., Palma, J., Luoiannez, J., et al. (1998). A dehydrogenase-mediated recycling system of NADPH in plant peroxisomes. *Biochem. J.* 330, 777–784. doi:10.1042/bj3300777

Corti, O., DiDonato, S., and Finocchiaro, G. (1994). Divergent sequences in the 5'region of cDNA suggest alternative splicing as a mechanism for the generation of carnitine acetyltransferases with different subcellular localizations. *Biochem. J.* 303, 37–41. doi:10.1042/bj3030037

Costanzo, M., VanderSluis, B., Koch, E. N., Baryshnikova, A., Pons, C., Tan, G., et al. (2016). A global genetic interaction network maps a wiring diagram of cellular function. *Science* 353 (6306), aaf1420. doi:10.1126/science.aaf1420

Costello, J. L., Castro, I. G., Camões, F., Schrader, T. A., McNeall, D., Yang, J., et al. (2017a). Predicting the targeting of tail-anchored proteins to subcellular compartments in mammalian cells. *J. Cell Sci.* 130, 1675–1687. doi:10.1242/jcs.200204

Costello, J. L., Castro, I. G., Hacker, C., Schrader, T. A., Metz, J., Zeuschner, D., et al. (2017b). ACBD5 and VAPB mediate membrane associations between peroxisomes and the ER. *J. Cell Biol.* 216, 331–342. doi:10.1083/jcb.201607055

Costello, J. L., Passmore, J. B., Islinger, M., and Schrader, M. (2018). Multi-localized proteins: The peroxisome-mitochondria connection. *Subcell. Biochem.* 89, 383–415. doi:10.1007/978-981-13-2233-4_17

Dahan, N., Bykov, Y. S., Boydston, E. A., Fadel, A., Gazi, Z., Hochberg-Laufer, H., et al. (2022). Peroxisome function relies on organelle-associated mRNA translation. *Sci. Adv.* 8, eabk2141. doi:10.1126/sciadv.abk2141

David, C., Koch, J., Oeljeklaus, S., Laernsack, A., Melchior, S., Wiese, S., et al. (2013). A combined approach of quantitative interaction proteomics and live-cell imaging reveals a regulatory role for endoplasmic reticulum (ER) reticulon homology proteins in peroxisome biogenesis. *Mol. Cell. Proteomics* 12, 2408–2425. doi:10.1074/mcp.M112.017830

De Duve, C., and Baudhuin, P. (1966). Peroxisomes (microbodies and related particles). *Physiol. Rev.* 46, 323–357. doi:10.1152/physrev.1966.46.2.323

Dellero, Y., Jossier, M., Schmitz, J., Maurino, V. G., and Hodges, M. (2016). Photorespiratory glycolate-glyoxylate metabolism. *J. Exp. Bot.* 67 (10), 3041–3052. doi:10.1093/jxb/erw090

Di Cara, F. (2020). Peroxisomes in host defense. *PLoS Pathog.* 16, e1008636. doi:10.1371/journal.ppat.1008636

Di Cara, F., Sheshachalam, A., Braverman, N. E., Rachubinski, R. A., and Simmonds, A. J. (2017). Peroxisome-mediated metabolism is required for immune response to microbial infection. *Immunity* 47, 93–106. e7. doi:10.1016/j.immuni.2017.06.016

Dunn, J. G., Foo, C. K., Belletier, N. G., Gavis, E. R., and Weissman, J. S. (2013). Ribosome profiling reveals pervasive and regulated stop codon readthrough in *Drosophila melanogaster*. *Elife* 2, e01179. doi:10.7554/eLife.01179

Effelsberg, D., Cruz-Zaragoza, L. D., Schliebs, W., and Erdmann, R. (2016). Pex9p is a new yeast peroxisomal import receptor for PTS1-containing proteins. *J. Cell Sci.* 129, 4057–4066. doi:10.1242/jcs.195271

Effelsberg, D., Cruz-Zaragoza, L. D., Tonillo, J., Schliebs, W., and Erdmann, R. (2015). Role of Pex21p for piggyback import of Gpd1p and Pnc1p into peroxisomes of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 290, 25333–25342. doi:10.1074/jbc.M115.653451

Eisenberg-Bord, M., Zung, N., Collado, J., Drwesh, L., Fenech, E. J., Fadel, A., et al. (2021). Cnm1 mediates nucleus--mitochondria contact site formation in response to phospholipid levels. *J. Cell Biol.* 220, e202104100. doi:10.1083/jcb.202104100

Elgersma, Y., van Roermund, C. W., Wanders, R. J., and Tabak, H. F. (1995). Peroxisomal and mitochondrial carnitine acetyltransferases of *Saccharomyces cerevisiae* are encoded by a single gene. *EMBO J.* 14, 3472–3479. doi:10.1002/j.1460-2075.1995.tb07353.x

Exner, T., Romero-Brey, I., Yifrach, E., Rivera-Monroy, J., Schrul, B., Zouboulis, C. C., et al. (2019). An alternative membrane topology permits lipid droplet localization of peroxisomal fatty acyl-CoA reductase 1. *J. Cell Sci.* 132, jcs223016. doi:10.1242/jcs.223016

Falter, C., and Reumann, S. (2022). The essential role of fungal peroxisomes in plant infection. *Mol. Plant Pathol.* 23 (6), 781–794. doi:10.1111/mpp.13180

Fan, J., Li, X., Issop, L., Culty, M., and Papadopoulos, V. (2016). ACBD2/ECI2-Mediated peroxisome-mitochondria interactions in leydig cell steroid biosynthesis. *Mol. Endocrinol.* 30, 763–782. doi:10.1210/me.2016-1008

Fang, Y., Morrell, J. C., Jones, J. M., and Gould, S. J. (2004). PEX3 functions as a PEX19 docking factor in the import of class I peroxisomal membrane proteins. *J. Cell Biol.* 164, 863–875. doi:10.1083/jcb.200311131

Ferreira, A. R., Marques, M., Ramos, B., Kagan, J. C., and Ribeiro, D. (2022). Emerging roles of peroxisomes in viral infections. *Trends Cell Biol.* 32, 124–139. doi:10.1016/j.tcb.2021.09.010

Ferreira, J. V., and Carvalho, P. (2021). Pex30-like proteins function as adaptors at distinct ER membrane contact sites. *J. Cell Biol.* 220, e202103176. doi:10.1083/jcb.202103176

Ferro-Novick, S., Rapoport, T. A., and Schekman, R. (2013). *The endoplasmic reticulum*. New York: Cold Spring Harbor Lab Press.

Fodor, K., Wolf, J., Reglinski, K., Passon, D. M., Lou, Y., Schliebs, W., et al. (2015). Ligand-induced compaction of the PEX5 receptor-binding cavity impacts protein import efficiency into peroxisomes. *Traffic* 16, 85–98. doi:10.1111/tra.12238

Francisco, T., Rodrigues, T. A., Dias, A. F., Barros-Barbosa, A., Bicho, D., and Azevedo, J. E. (2017). Protein transport into peroxisomes: Knowns and unknowns. *BioEssays* 39, 1700047. doi:10.1002/bies.201700047

Fransen, M., Lismont, C., and Walton, P. (2017). The peroxisome-mitochondria connection: How and why? *Int. J. Mol. Sci.* 18, 1126. doi:10.3390/ijms18061126

Freitag, J., Ast, J., and Bölker, M. (2012). Cryptic peroxisomal targeting via alternative splicing and stop codon read-through in fungi. *Nature* 485, 522–525. doi:10.1038/nature11051

Freitag, J., Ast, J., Linne, U., Stehlik, T., Martorana, D., Bölker, M., et al. (2014). Peroxisomes contribute to biosynthesis of extracellular glycolipids in fungi. *Mol. Microbiol.* 93, 24–36. doi:10.1111/mmi.12642

Freitag, J., Stehlik, T., Stiebler, A. C., and Bölker, M. (2018). "The obvious and the hidden: Prediction and function of fungal peroxisomal matrix proteins," in *Proteomics of peroxisomes, Subcellular Biochemistry*, Editor L. del Río and M. Schrader (Singapore: Springer), 89. doi:10.1007/978-981-13-2233-4_6

Gabaldón, T. (2014). A metabolic scenario for the evolutionary origin of peroxisomes from the endomembranous system. *Cell. Mol. Life Sci.* 71, 2373–2376. doi:10.1007/s00018-013-1424-z

Gabaldón, T. (2018). "*Evolution of the peroxisomal proteome BT - proteomics of peroxisomes: Identifying novel functions and regulatory networks*," in, eds. L. A. del Río and M. Schrader (Singapore: Springer Singapore), 221–233.

Gabaldón, T. (2010). Peroxisome diversity and evolution. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 365, 765–773. doi:10.1098/rstb.2009.0240

Gabaldón, T., Snel, B., van Zimmeren, F., Hemrika, W., Tabak, H., and Huynen, M. A. (2006). Origin and evolution of the peroxisomal proteome. *Biol. Direct* 1, 8. doi:10.1186/1745-6150-1-8

Gabay-Maskit, S., Cruz-Zaragoza, L. D., Shai, N., Eisenstein, M., Bibi, C., Cohen, N., et al. (2020). A piggybacking mechanism enables peroxisomal localization of the glyoxylate cycle enzyme Mdh2 in yeast. *J. Cell Sci.* 133, jcs244376. doi:10.1242/jcs.244376

Germain, K., and Kim, P. K. (2020). Pexophagy: A model for selective autophagy. *Int. J. Mol. Sci.* 21, E578. doi:10.3390/ijms21020578

Goepfert, S., and Poirier, Y. (2007). Beta-oxidation in fatty acid degradation and beyond. *Curr. Opin. Plant Biol.* 10, 245–251. doi:10.1016/j.pbi.2007.04.007

Grewal, P. S., Samson, J. A., Baker, J. J., Choi, B., and Dueber, J. E. (2021). Peroxisome compartmentalization of a toxic enzyme improves alkaloid production. *Nat. Chem. Biol.* 17, 96–103. doi:10.1038/s41589-020-00668-4

Gründlinger, M., Yasmin, S., Lechner, B. E., Geley, S., Schrettl, M., Hynes, M., et al. (2013). Fungal siderophore biosynthesis is partially localized in peroxisomes. *Mol. Microbiol.* 88 (5), 862–875. doi:10.1111/mmi.12225

Guillén-Samander, A., Leonzino, M., Hanna IV, M. G., Tang, N., Shen, H., and De Camilli, P. (2021). VPS13D bridges the ER to mitochondria and peroxisomes via Miro. *J. Cell Biol.* 220, e202010004. doi:10.1083/jcb.202010004

Guimaraes, S. C., Schuster, M., Bielska, E., Dagdas, G., Kilaru, S., Meadows, B. R. A., et al. (2015). Peroxisomes, lipid droplets, and endoplasmic reticulum "hitchhike" on motile early endosomes. *J. Cell Biol.* 211 (5), 945–954. doi:10.1083/jcb.201505086

Haanstra, J. R., González-Marcano, E. B., Gualdrón-López, M., and Michels, P. A. M. (2016). Biogenesis, maintenance and dynamics of glycosomes in trypanosomatid parasites. *Biochim. Biophys. Acta* 1863, 1038–1048. doi:10.1016/j.bbamcr.2015.09.015

Haselbeck, R. J., and McAlister-Henn, L. (1993). Function and expression of yeast mitochondrial NAD-and NADP-specific isocitrate dehydrogenases. *J. Biol. Chem.* 268, 12116–12122. doi:10.1016/s0021-9258(19)50315-5

Haselbeck, R. J., and McAlister-Henn, L. (1991). Isolation, nucleotide sequence, and disruption of the *Saccharomyces cerevisiae* gene encoding mitochondrial NADP(H)-specific isocitrate dehydrogenase. *J. Biol. Chem.* 266, 2339–2345. doi:10.1016/s0021-9258(18)52249-3

Henne, W. M., Buchkovich, N. J., and Emr, S. D. (2011). The ESCRT pathway. *Dev. Cell* 21, 77–91. doi:10.1016/j.devcel.2011.05.015

Hiltunen, J. K., Mursula, A. M., Rottensteiner, H., Wierenga, R. K., Kastaniotis, A. J., and Gurvitz, A. (2003). The biochemistry of peroxisomal β-oxidation in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 27, 35–64. doi:10.1016/S0168-6445(03)00017-2

Hoepfner, D., Schildknegt, D., Braakman, I., Philippsen, P., and Tabak, H. F. (2005). Contribution of the endoplasmic reticulum to peroxisome formation. *Cell* 122, 85–95. doi:10.1016/j.cell.2005.04.025

Hofhuis, J., Schueren, F., Nötzel, C., Lingner, T., Gärtner, J., Jahn, O., et al. (2016). The functional readthrough extension of malate dehydrogenase reveals a modification of the genetic code. *Open Biol.* 6, 160246. doi:10.1098/rsob.160246

Hölscher, C., Lutterbey, M.-C., Lansing, H., Meyer, T., Fischer, K., and von Schaewen, A. (2016). Defects in peroxisomal 6-phosphogluconate dehydrogenase isoform PGD2 prevent gametophytic interaction in *Arabidopsis thaliana*. *Plant Physiol.* 171, 192–205. doi:10.1104/pp.15.01301

Hoppins, S., Collins, S. R., Cassidy-Stone, A., Hummel, E., DeVay, R. M., Lackner, L. L., et al. (2011). A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. *J. Cell Biol.* 195, 323–340. doi:10.1083/jcb.201107053

Houten, S. M., Wanders, R. J. A., and Ranea-Robles, P. (2020). Metabolic interactions between peroxisomes and mitochondria with a special focus on acylcarnitine metabolism. *Biochim. Biophys. Acta. Mol. Basis Dis.* 1866, 165720. doi:10.1016/j.bbadis.2020.165720

Hu, J., Baker, A., Bartel, B., Linka, N., Mullen, R. T., Reumann, S., et al. (2012). Plant peroxisomes: Biogenesis and function. *Plant Cell* 24, 2279–2303. doi:10.1105/tpc.112.096586

Hua, R., Cheng, D., Coyaud, É., Freeman, S., Di Pietro, E., Wang, Y., et al. (2017). VAPs and ACBD5 tether peroxisomes to the ER for peroxisome maintenance and lipid homeostasis. *J. Cell Biol.* 216, 367–377. doi:10.1083/jcb.201608128

Idnurm, A., Giles, S. S., Perfect, J. R., and Heitman, J. (2007). Peroxisome function regulates growth on glucose in the basidiomycete fungus Cryptococcus neoformans. *Eukaryot. Cell* 6, 60–72. doi:10.1128/EC.00214-06

Inaba, J., Kim, B. M., Shimura, H., and Masuta, C. (2011). Virus-induced necrosis is a consequence of direct protein-protein interaction between a viral RNA-silencing suppressor and a host catalase. *Plant Physiol.* 156, 2026–2036. doi:10.1104/pp.111.180042

Islinger, M., Grille, S., Fahimi, H. D., and Schrader, M. (2012). The peroxisome: An update on mysteries. *Histochem. Cell Biol.* 137, 547–574. doi:10.1007/s00418-012-0941-4

Islinger, M., Li, K. W., Seitz, J., Völkl, A., and Lüers, G. H. (2009). Hitchhiking of Cu/Zn superoxide dismutase to peroxisomes--evidence for a natural piggyback import mechanism in mammals. *Traffic* 10, 1711–1721. doi:10.1111/j.1600-0854.2009.00966.x

Jan, C. H., Williams, C. C., and Weissman, J. S. (2014). Principles of ER cotranslational translocation revealed by proximity-specific ribosome profiling. *Science* 346, 1257521. doi:10.1126/science.1257521

Jansen, R. L. M., and van der Klei, I. J. (2019). The peroxisome biogenesis factors Pex3 and Pex19: Multitasking proteins with disputed functions. *FEBS Lett.* 593, 457–474. doi:10.1002/1873-3468.13340

Jedd, G., and Chua, N. H. (2000). A new self-assembled peroxisomal vesicle required for efficient resealing of the plasma membrane. *Nat. Cell Biol.* 2, 226–231. doi:10.1038/35008652

Jones, J. M., Morrell, J. C., and Gould, S. J. (2004). PEX19 is a predominantly cytosolic chaperone and import receptor for class 1 peroxisomal membrane proteins. *J. Cell Biol.* 164, 57–67. doi:10.1083/jcb.200304111

Joshi, A. S., Huang, X., Choudhary, V., Levine, T. P., Hu, J., and Prinz, W. A. (2016). A family of membrane-shaping proteins at ER subdomains regulates pre-peroxisomal vesicle biogenesis. *J. Cell Biol.* 215 (4), 515–529. doi:10.1083/jcb.201602064

Joshi, A. S., Nebenfuehr, B., Choudhary, V., Satpute-Krishnan, P., Levine, T. P., Golden, A., et al. (2018). Lipid droplet and peroxisome biogenesis occur at the same ER subdomains. *Nat. Commun.* 9, 2940. doi:10.1038/s41467-018-05277-3

Joshi, A. S., Zhang, H., and Prinz, W. A. (2017). Organelle biogenesis in the endoplasmic reticulum. *Nat. Cell Biol.* 19, 876–882. doi:10.1038/ncb3579

Jung, S., Marelli, M., Rachubinski, R. A., Goodlett, D. R., and Aitchison, J. D. (2010). Dynamic changes in the subcellular distribution of Gpd1p in response to cell stress. *J. Biol. Chem.* 285, 6739–6749. doi:10.1074/jbc.M109.058552

Kamigaki, A., Mano, S., Terauchi, K., Nishi, Y., Tachibe-Kinoshita, Y., Nito, K., et al. (2003). Identification of peroxisomal targeting signal of pumpkin catalase and the binding analysis with PTS1 receptor. *Plant J.* 33, 161–175. doi:10.1046/j.0960-7412.2003.001605.x

Kaplan, R. S., Mayor, J. A., Gremse, D. A., and Wood, D. O. (1995). High level expression and characterization of the mitochondrial citrate transport protein from the yeast *Saccharomyces cerevisiae* (*). *J. Biol. Chem.* 270, 4108–4114. doi:10.1074/jbc.270.8.4108

Kataya, A., Gautam, N., Jamshed, M., Muench, D. G., Samuel, M. A., Thelen, J. J., et al. (2022). Identification of Arabidopsis protein kinases that harbor functional type 1 peroxisomal targeting signals. *Front. Cell Dev. Biol.* 10, 745883. doi:10.3389/fcell.2022.745883

Kataya, A. R. A., and Reumann, S. (2010). Arabidopsis glutathione reductase 1 is dually targeted to peroxisomes and the cytosol. *Plant Signal. Behav.* 5, 171–175. doi:10.4161/psb.5.2.10527

Kataya, A., Schei, E., and Lillo, C. (2016). Towards understanding peroxisomal phosphoregulation in *Arabidopsis thaliana*. *Planta* 243, 699–717. doi:10.1007/s00425-015-2439-5

Kellis, M., Birren, B. W., and Lander, E. S. (2004). Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*. *Nature* 428, 617–624. doi:10.1038/nature02424

Kempiński, B., Chełstowska, A., Poznański, J., Król, K., Rymer, Ł., Frydzińska, Z., et al. (2020). The peroxisomal targeting signal 3 (PTS3) of the budding yeast acyl-CoA oxidase is a signal patch. *Front. Cell Dev. Biol.* 8, 198. doi:10.3389/fcell.2020.00198

Khan, Z., Michalopoulos, G. K., and Stolz, D. B. (2006). Peroxisomal localization of hypoxia-inducible factors and hypoxia-inducible factor regulatory hydroxylases in primary rat hepatocytes exposed to hypoxia-reoxygenation. *Am. J. Pathol.* 169, 1251–1269. doi:10.2353/ajpath.2006.060360

Kiel, J. A. K. W., Veenhuis, M., and Van Der Klei, I. J. (2006). PEX genes in fungal genomes: Common, rare or redundant. *Traffic* 7, 1291–1303. doi:10.1111/j.1600-0854.2006.00479.x

Kim, K.-S., Rosenkrantz, M. S., and Guarente, L. (1986). *Saccharomyces cerevisiae* contains two functional citrate synthase genes. *Mol. Cell. Biol.* 6, 1936–1942. doi:10.1128/mcb.6.6.1936

Kim, P. K., and Hettema, E. H. (2015). Multiple pathways for protein transport to peroxisomes. *J. Mol. Biol.* 427, 1176–1190. doi:10.1016/j.jmb.2015.02.005

Kim, P. K., Mullen, R. T., Schumann, U., and Lippincott-Schwartz, J. (2006). The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. *J. Cell Biol.* 173, 521–532. doi:10.1083/jcb.200601036

Koch, A., Thiemann, M., Grabenbauer, M., Yoon, Y., McNiven, M. A., and Schrader, M. (2003). Dynamin-like protein 1 is involved in peroxisomal fission. *J. Biol. Chem.* 278, 8597–8605. doi:10.1074/jbc.M211761200

Koch, A., Yoon, Y., Bonekamp, N. A., McNiven, M. A., and Schrader, M. (2005). A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. *Mol. Biol. Cell* 16 (11), 5077–5086. doi:10.1091/mbc.e05-02-0159

Kong, J., Ji, Y., Jeon, Y. G., Han, J. S., Han, K. H., Lee, J. H., et al. (2020). Spatiotemporal contact between peroxisomes and lipid droplets regulates fasting-induced lipolysis via PEX5. *Nat. Commun.* 11, 578. doi:10.1038/s41467-019-14176-0

Kornberg, A. (1962). "On the metabolic significance of phosphorolytic and pyrophosphorolytic reactions," in *Horizons in biochemistry*. Editors H. Kasha, and B. Pullman (New York, USA: Academic Press), 251–264.

Kornmann, B., Currie, E., Collins, S. R., Schuldiner, M., Nunnari, J., Weissman, J. S., et al. (2009). An ER-mitochondria tethering complex revealed by a synthetic biology screen. *Science* 325, 477–481. doi:10.1126/science.1175088

Kornmann, B., Osman, C., and Walter, P. (2011). The conserved GTPase Gem1 regulates endoplasmic reticulum-mitochondria connections. *Proc. Natl. Acad. Sci. U. S. A.* 108, 14151–14156. doi:10.1073/pnas.1111314108

Kragler, F., Langeder, A., Raupachova, J., Binder, M., and Hartig, A. (1993). Two independent peroxisomal targeting signals in catalase A of *Saccharomyces cerevisiae*. *J. Cell Biol.* 120, 665–673. doi:10.1083/jcb.120.3.665

Kremp, M., Bittner, E., Martorana, D., Klingenberger, A., Stehlik, T., Bölker, M., et al. (2020). Non-AUG translation initiation generates peroxisomal isoforms of 6-phosphogluconate dehydrogenase in fungi. *Front. Cell Dev. Biol.* 8, 251. doi:10.3389/fcell.2020.00251

Kretschmer, M., Klose, J., and Kronstad, J. W. (2012a). Defects in mitochondrial and peroxisomal β-oxidation influence virulence in the maize pathogen Ustilago maydis. *Eukaryot. Cell* 11, 1055–1066. doi:10.1128/EC.00129-12

Kretschmer, M., Wang, J., and Kronstad, J. W. (2012b). Peroxisomal and mitochondrial β-oxidation pathways influence the virulence of the pathogenic fungus Cryptococcus neoformans. *Eukaryot. Cell* 11, 1042–1054. doi:10.1128/EC.00128-12

Kumar, S., Singh, R., Williams, C. P., and van der Klei, I. J. (2016). Stress exposure results in increased peroxisomal levels of yeast Pnc1 and Gpd1, which are imported via a piggy-backing mechanism. *Biochim. Biophys. Acta* 1863, 148–156. doi:10.1016/j.bbamcr.2015.10.017

Kundu, D., and Pasrija, R. (2020). The ERMES (endoplasmic reticulum and mitochondria encounter structures) mediated functions in fungi. *Mitochondrion* 52, 89–99. doi:10.1016/j.mito.2020.02.010

Kunze, M., and Berger, J. (2015). The similarity between N-terminal targeting signals for protein import into different organelles and its evolutionary relevance. *Front. Physiol.* 6, 259. doi:10.3389/fphys.2015.00259

Kunze, M., Kragler, F., Binder, M., Hartig, A., and Gurvitz, A. (2002). Targeting of malate synthase 1 to the peroxisomes of *Saccharomyces cerevisiae* cells depends on growth on oleic acid medium. *Eur. J. Biochem.* 269, 915–922. doi:10.1046/j.0014-2956.2001.02727.x

Kunze, M., Malkani, N., Maurer-Stroh, S., Wiesinger, C., Schmid, J. A., and Berger, J. (2015). Mechanistic insights into PTS2-mediated peroxisomal protein import: The co-receptor PEX5L drastically increases the interaction strength between the cargo protein and the receptor PEX7. *J. Biol. Chem.* 290 (8), 4928–4940. doi:10.1074/jbc.M114.601575

Kunze, M., Pracharoenwattana, I., Smith, S. M., and Hartig, A. (2006). A central role for the peroxisomal membrane in glyoxylate cycle function. *Biochim. Biophys. Acta* 1763, 1441–1452. doi:10.1016/j.bbamcr.2006.09.009

Kunze, M. (2020). The type-2 peroxisomal targeting signal. *Biochim. Biophys. Acta. Mol. Cell Res.* 1867, 118609. doi:10.1016/j.bbamcr.2019.118609

Kuravi, K., Nagotu, S., Krikken, A. M., Sjollema, K., Deckers, M., Erdmann, R., et al. (2006). Dynamin-related proteins Vps1p and Dnm1p control peroxisome abundance in *Saccharomyces cerevisiae*. *J. Cell Sci.* 119, 3994–4001. doi:10.1242/jcs.03166

Lam, S., Yoda, N., and Schekman, R. (2010). A vesicle carrier that mediates peroxisome protein traffic from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U. S. A.* 107 (50), 21523–21528. doi:10.1073/pnas.1013397107

Lee, Y. J., Jang, J. W., Kim, K. J., and Maeng, P. J. (2011). TCA cycle-independent acetate metabolism via the glyoxylate cycle in *Saccharomyces cerevisiae*. *Yeast* 28, 153–166. doi:10.1002/yea.1828

Lee, Y. J., Jeschke, G. R., Roelants, F. M., Thorner, J., and Turk, B. E. (2012). Reciprocal phosphorylation of yeast glycerol-3-phosphate dehydrogenases in adaptation to distinct types of stress. *Mol. Cell. Biol.* 32, 4705–4717. doi:10.1128/MCB.00897-12

Li, H.-S., Zhou, Y.-N., Li, L., Li, S.-F., Long, D., Chen, X.-L., et al. (2019). HIF-1α protects against oxidative stress by directly targeting mitochondria. *Redox Biol.* 25, 101109. doi:10.1016/j.redox.2019.101109

Li, H., Lismont, C., Revenco, I., Hussein, M. A. F., Costa, C. F., and Fransen, M. (2021). The peroxisome-autophagy redox connection: A double-edged sword? *Front. Cell Dev. Biol.* 9, 814047. doi:10.3389/fcell.2021.814047

Lingner, T., Kataya, A. R., Antonicelli, G. E., Benichou, A., Nilssen, K., Chen, X.-Y., et al. (2011). Identification of novel plant peroxisomal targeting signals by a combination of machine learning methods and *in vivo* subcellular targeting analyses. *Plant Cell* 23, 1556–1572. doi:10.1105/tpc.111.084095

Lismont, C., Revenco, I., and Fransen, M. (2019). Peroxisomal hydrogen peroxide metabolism and signaling in health and disease. *Int. J. Mol. Sci.* 20, 3673. doi:10.3390/ijms20153673

Liu, F., Lu, Y., Pieuchot, L., Dhavale, T., and Jedd, G. (2011). Import oligomers induce positive feedback to promote peroxisome differentiation and control organelle abundance. *Dev. Cell* 21 (3), 457–468. doi:10.1016/j.devcel.2011.08.004

Ma, C., Hagstrom, D., Polley, S. G., and Subramani, S. (2013). Redox-regulated cargo binding and release by the peroxisomal targeting signal receptor, Pex5. *J. Biol. Chem.* 288, 27220–27231. doi:10.1074/jbc.M113.492694

Maggio-Hall, L. A., and Keller, N. P. (2004). Mitochondrial β-oxidation in Aspergillus nidulans. *Mol. Microbiol.* 54, 1173–1185. doi:10.1111/j.1365-2958.2004.04340.x

Magliano, P., Flipphi, M., Arpat, B. A., Delessert, S., and Poirier, Y. (2011). Contributions of the peroxisome and β-oxidation cycle to biotin synthesis in fungi. *J. Biol. Chem.* 286, 42133–42140. doi:10.1074/jbc.M111.279687

Martenson, J. S., Tam, H., McQuown, A. J., Reif, D., Zhou, J., and Denic, V. (2020). The importomer is a peroxisomal membrane protein translocase. *bioRxiv*. bioarchives preprints.

Mast, F. D., Herricks, T., Strehler, K. M., Miller, L. R., Saleem, R. A., Rachubinski, R. A., et al. (2018). ESCRT-III is required for scissioning new peroxisomes from the endoplasmic reticulum. *J. Cell Biol.* 217, 2087–2102. doi:10.1083/jcb.201706044

Mast, F. D., Jamakhandi, A., Saleem, R. A., Dilworth, D. J., Rogers, R. S., Rachubinski, R. A., et al. (2016). Peroxins Pex30 and Pex29 dynamically associate with reticulons to regulate peroxisome biogenesis from the endoplasmic reticulum. *J. Biol. Chem.* 291, 15408–15427. doi:10.1074/jbc.M116.728154

Matsuzaki, T., and Fujiki, Y. (2008). The peroxisomal membrane protein import receptor Pex3p is directly transported to peroxisomes by a novel Pex19p- and Pex16p-dependent pathway. *J. Cell Biol.* 183, 1275–1286. doi:10.1083/jcb.200806062

McCartney, A. W., Greenwood, J. S., Fabian, M. R., White, K. A., and Mullen, R. T. (2005). Localization of the tomato bushy stunt virus replication protein p33 reveals a peroxisome-to-endoplasmic reticulum sorting pathway. *Plant Cell* 17, 3513–3531. doi:10.1105/tpc.105.036350

Meijer, W. H., Gidijala, L., Fekken, S., Kiel, J. A. K. W., van den Berg, M. A., Lascaris, R., et al. (2010). Peroxisomes are required for efficient penicillin biosynthesis in Penicillium chrysogenum. *Appl. Environ. Microbiol.* 76, 5702–5709. doi:10.1128/AEM.02327-09

Meyer, T., Hölscher, C., Schwöppe, C., and von Schaewen, A. (2011). Alternative targeting of Arabidopsis plastidic glucose-6-phosphate dehydrogenase G6PD1 involves cysteine-dependent interaction with G6PD4 in the cytosol. *Plant J.* 66, 745–758. doi:10.1111/j.1365-313X.2011.04535.x

Monteuuis, G., Miścicka, A., Świrski Michałand Zenad, L., Niemitalo, O., Wrobel, L., Alam, J., et al. (2019). Non-canonical translation initiation in yeast generates a cryptic pool of mitochondrial proteins. *Nucleic Acids Res.* 47, 5777–5791. doi:10.1093/nar/gkz301

Morgenstern, M., Stiller, S. B., Lübbert, P., Peikert, C. D., Dannenmaier, S., Drepper, F., et al. (2017). Definition of a high-confidence mitochondrial proteome at quantitative scale. *Cell Rep.* 19, 2836–2852. doi:10.1016/j.celrep.2017.06.014

Motley, A. M., Galvin, P. C., Ekal, L., Nuttall, J. M., and Hettema, E. H. (2015). Reevaluation of the role of Pex1 and dynamin-related proteins in peroxisome membrane biogenesis. *J. Cell Biol.* 211, 1041–1056. doi:10.1083/jcb.201412066

Motley, A. M., Ward, G. P., and Hettema, E. H. (2008). Dnm1p-dependent peroxisome fission requires Caf4p, Mdv1p and Fis1p. *J. Cell Sci.* 121, 1633–1640. doi:10.1242/jcs.026344

Natsoulis, G., Hilger, F., and Fink, G. R. (1986). The HTS1 gene encodes both the cytoplasmic and mitochondrial histidine tRNA synthetases of *S. cerevisiae*. *Cell* 46 (2), 235–243. doi:10.1016/0092-8674(86)90740-3

Neuberger, G., Kunze, M., Eisenhaber, F., Berger, J., Hartig, A., and Brocard, C. (2004). Hidden localization motifs: Naturally occurring peroxisomal targeting signals in non-peroxisomal proteins. *Genome Biol.* 5, R97. doi:10.1186/gb-2004-5-12-r97

Neuspiel, M., Schauss, A. C., Braschi, E., Zunino, R., Rippstein, P., Rachubinski, R. A., et al. (2008). Cargo-selected transport from the mitochondria to peroxisomes is mediated by vesicular carriers. *Curr. Biol.* 18, 102–108. doi:10.1016/j.cub.2007.12.038

Nötzel, C., Lingner, T., Klingenberg, H., and Thoms, S. (2016). Identification of new fungal peroxisomal matrix proteins and revision of the PTS1 consensus. *Traffic* 17, 1110–1124. doi:10.1111/tra.12426

Novikoff, A. B., and Shin, W.-Y. (1964). The endoplasmic reticulum in the Golgi zone and its relations to microbodies, Golgi apparatus and autophagic vacuoles in rat liver cells. *J. Microsc.* 3, 187–206.

Nuebel, E., Morgan, J. T., Fogarty, S., Winter, J. M., Lettlova, S., Berg, J. A., et al. (2021). The biochemical basis of mitochondrial dysfunction in Zellweger Spectrum Disorder. *EMBO Rep.* 22, e51991. doi:10.15252/embr.202051991

Odendall, C., Dixit, E., Stavru, F., Bierne, H., Franz, K. M., Durbin, A. F., et al. (2014). Diverse intracellular pathogens activate type III interferon expression from peroxisomes. *Nat. Immunol.* 15, 717–726. doi:10.1038/ni.2915

Oeljeklaus, S., Schummer, A., Mastalski, T., Platta, H. W., and Warscheid, B. (2016). Regulation of peroxisome dynamics by phosphorylation. *Biochim. Biophys. Acta* 1863, 1027–1037. doi:10.1016/j.bbamcr.2015.12.022

Okreglak, V., and Walter, P. (2014). The conserved AAA-ATPase Msp1 confers organelle specificity to tail-anchored proteins. *Proc. Natl. Acad. Sci. U. S. A.* 111, 8019–8024. doi:10.1073/pnas.1405755111

Okumoto, K., El Shermely, M., Natsui, M., Kosako, H., Natsuyama, R., Marutani, T., et al. (2020). The peroxisome counteracts oxidative stresses by suppressing catalase import via Pex14 phosphorylation. *Elife* 9, e55896. doi:10.7554/eLife.55896

Palmieri, F. (2004). The mitochondrial transporter family (SLC25): Physiological and pathological implications. *Pflügers Arch.* 447, 689–709. doi:10.1007/s00424-003-1099-7

Pan, R., Liu, J., Wang, S., and Hu, J. (2020). Peroxisomes: Versatile organelles with diverse roles in plants. *New Phytol.* 225, 1410–1427. doi:10.1111/nph.16134

Paradis, M., Kucharowski, N., Edwards, G., Palacios, S. M., Meyer, C., Stümpges, B., et al. (2021). The ER protein Creld regulates ER-mitochondria

contact dynamics and respiratory complex 1 activity. *ResearchSquare*. preprint. doi:10.21203/rs.3.rs-911428/v1

Passreiter, M., Anton, M., Lay, D., Frank, R., Harter, C., Wieland, F. T., et al. (1998). Peroxisome biogenesis: Involvement of ARF and coatomer. *J. Cell Biol.* 141, 373–383. doi:10.1083/jcb.141.2.373

Poirier, Y., Antonenkov, V. D., Glumoff, T., and Hiltunen, J. K. (2006). Peroxisomal β-oxidation—A metabolic pathway with multiple functions. *Biochim. Biophys. Acta* 1763, 1413–1426. doi:10.1016/j.bbamcr.2006.08.034

Praefcke, G. J. K., and McMahon, H. T. (2004). The dynamin superfamily: Universal membrane tubulation and fission molecules? *Nat. Rev. Mol. Cell Biol.* 5, 133–147. doi:10.1038/nrm1313

Raychaudhuri, S., and Prinz, W. A. (2008). Nonvesicular phospholipid transfer between peroxisomes and the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U. S. A.* 105, 15785–15790. doi:10.1073/pnas.0808321105

Reumann, S., Babujee, L., Ma, C., Wienkoop, S., Siemsen, T., Antonicelli, G. E., et al. (2007). Proteome analysis of Arabidopsis leaf peroxisomes reveals novel targeting peptides, metabolic pathways, and defense mechanisms. *Plant Cell* 19, 3170–3193. doi:10.1105/tpc.107.050989

Reumann, S., and Weber, A. P. M. (2006). Plant peroxisomes respire in the light: Some gaps of the photorespiratory C2 cycle have become filled—others remain. *Biochim. Biophys. Acta* 1763, 1496–1510. doi:10.1016/j.bbamcr.2006.09.008

Rosenthal, M., Metzl-Raz, E., Bürgi, J., Yifrach, E., Drwesh, L., Fadel, A., et al. (2020). Uncovering targeting priority to yeast peroxisomes using an in-cell competition assay. *Proc. Natl. Acad. Sci. U. S. A.* 117, 21432–21440. doi:10.1073/pnas.1920078117

Sacksteder, K. A., Jones, J. M., South, S. T., Li, X., Liu, Y., and Gould, S. J. (2000). PEX19 binds multiple peroxisomal membrane proteins, is predominantly cytoplasmic, and is required for peroxisome membrane synthesis. *J. Cell Biol.* 148, 931–944. doi:10.1083/jcb.148.5.931

Salo, V. T., Belevich, I., Li, S., Karhinen, L., Vihinen, H., Vigouroux, C., et al. (2016). Seipin regulates ER–lipid droplet contacts and cargo delivery. *EMBO J.* 35, 2699–2716. doi:10.15252/embj.201695170

Salogiannis, J., Egan, M. J., and Reck-Peterson, S. L. (2016). Peroxisomes move by hitchhiking on early endosomes using the novel linker protein PxdA. *J. Cell Biol.* 212, 289–296. doi:10.1083/jcb.201512020

Schliebs, W., Girzalsky, W., and Erdmann, R. (2010). Peroxisomal protein import and ERAD: Variations on a common theme. *Nat. Rev. Mol. Cell Biol.* 11, 885–890. doi:10.1038/nrm3008

Schlüter, A., Fourcade, S., Ripp, R., Mandel, J. L., Poch, O., and Pujol, A. (2006). The evolutionary origin of peroxisomes: An ER-peroxisome connection. *Mol. Biol. Evol.* 23, 838–845. doi:10.1093/molbev/msj103

Schrader, M., Costello, J., Godinho, L. F., and Islinger, M. (2015). Peroxisome-mitochondria interplay and disease. *J. Inherit. Metab. Dis.* 38, 681–702. doi:10.1007/s10545-015-9819-7

Schrader, M., Kamoshita, M., and Islinger, M. (2020). Organelle interplay—Peroxisome interactions in health and disease. *J. Inherit. Metab. Dis.* 43, 71–89. doi:10.1002/jimd.12083

Schrul, B., and Kopito, R. R. (2016). Peroxin-dependent targeting of a lipid-droplet-destined membrane protein to ER subdomains. *Nat. Cell Biol.* 18, 740–751. doi:10.1038/ncb3373

Schueren, F., Lingner, T., George, R., Hofhuis, J., Dickel, C., Gärtner, J., et al. (2014). Peroxisomal lactate dehydrogenase is generated by translational readthrough in mammals. *Elife* 3, e03640. doi:10.7554/eLife.03640

Schueren, F., and Thoms, S. (2016). Functional translational readthrough: A systems biology perspective. *PLoS Genet.* 12, e1006196. doi:10.1371/journal.pgen.1006196

Schummer, A., Maier, R., Gabay-Maskit, S., Hansen, T., Mühlhäuser, W. W. D., Suppanz, I., et al. (2020). Pex14p phosphorylation modulates import of citrate synthase 2 into peroxisomes in *Saccharomyces cerevisiae*. *Front. Cell Dev. Biol.* 8, 549451. doi:10.3389/fcell.2020.549451

Shai, N., Yifrach, E., Roermund, C. W. T., Cohen, N., Bibi, C., IJlst, L., et al. (2018). Systematic mapping of contact sites reveals tethers and a function for the peroxisome-mitochondria contact. *Nat. Commun.* 9, 1761. doi:10.1038/s41467-018-03957-8

Sies, H. (2017). Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. *Redox Biol.* 11, 613–619. doi:10.1016/j.redox.2016.12.035

Smith, J. J., and Aitchison, J. D. (2013). Peroxisomes take shape. *Nat. Rev. Mol. Cell Biol.* 14, 803–817. doi:10.1038/nrm3700

Speijer, D. (2017). Evolution of peroxisomes illustrates symbiogenesis. *BioEssays* 39, 1700050. doi:10.1002/bies.201700050

Steffan, J. S., and McAlister-Henn, L. (1992). Isolation and characterization of the yeast gene encoding the MDH3 isozyme of malate dehydrogenase. *J. Biol. Chem.* 267, 24708–24715. doi:10.1016/s0021-9258(18)35822-8

Stehlik, T., Kremp, M., Kahnt, J., Bölker, M., and Freitag, J. (2020). Peroxisomal targeting of a protein phosphatase type 2C via mitochondrial transit. *Nat. Commun.* 11, 2355. doi:10.1038/s41467-020-16146-3

Stehlik, T., Sandrock, B., Ast, J., and Freitag, J. (2014). Fungal peroxisomes as biosynthetic organelles. *Curr. Opin. Microbiol.* 22, 8–14. doi:10.1016/j.mib.2014.09.011

Stein, I., Peleg, Y., Even-Ram, S., and Pines, O. (1994). The single translation product of the FUM1 gene (fumarase) is processed in mitochondria before being distributed between the cytosol and mitochondria in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 14, 4770–4778. doi:10.1128/mcb.14.7.4770

Stiebler, A. C., Freitag, J., Schink, K. O., Stehlik, T., Tillmann, B. A. M., Ast, J., et al. (2014). Ribosomal readthrough at a short UGA stop codon context triggers dual localization of metabolic enzymes in fungi and animals. *PLoS Genet.* 10, e1004685–9. doi:10.1371/journal.pgen.1004685

Strijbis, K., den Burg, J., F Visser, W., den Berg, M., and Distel, B. (2012). Alternative splicing directs dual localization of Candida albicans 6-phosphogluconate dehydrogenase to cytosol and peroxisomes. *FEMS Yeast Res.* 12 (1), 61–68. doi:10.1111/j.1567-1364.2011.00761.x

Sugiura, A., Mattie, S., Prudent, J., and McBride, H. M. (2017). Newly born peroxisomes are a hybrid of mitochondrial and ER-derived pre-peroxisomes. *Nature* 542, 251–254. doi:10.1038/nature21375

Swiegers, J. H., Dippenaar, N., Pretorius, I. S., and Bauer, F. F. (2001). Carnitine-dependent metabolic activities in *Saccharomyces cerevisiae*: Three carnitine acetyltransferases are essential in a carnitine-dependent strain. *Yeast* 18, 585–595. doi:10.1002/yea.712

Szewczyk, E., Andrianopoulos, A., Davis, M. A., and Hynes, M. J. (2001). A single gene produces mitochondrial, cytoplasmic, and peroxisomal NADP-dependent isocitrate dehydrogenase inAspergillus nidulans. *J. Biol. Chem.* 276, 37722–37729. doi:10.1074/jbc.M105645200

Szymanski, K. M., Binns, D., Bartz, R., Grishin, N. V., Li, W.-P., Agarwal, A. K., et al. (2007). The lipodystrophy protein seipin is found at endoplasmic reticulum lipid droplet junctions and is important for droplet morphology. *Proc. Natl. Acad. Sci. U. S. A.* 104, 20890–20895. doi:10.1073/pnas.0704154104

Tanabe, Y., Maruyama, J., Yamaoka, S., Yahagi, D., Matsuo, I., Tsutsumi, N., et al. (2011). Peroxisomes are involved in biotin biosynthesis in Aspergillus and Arabidopsis. *J. Biol. Chem.* 286, 30455–30461. doi:10.1074/jbc.M111.247338

Thazar-Poulot, N., Miquel, M., Fobis-Loisy, I., and Gaude, T. (2015). Peroxisome extensions deliver the Arabidopsis SDP1 lipase to oil bodies. *Proc. Natl. Acad. Sci. U. S. A.* 112 (13), 4158–4163. doi:10.1073/pnas.1403322112

Titorenko, V. I., Chan, H., and Rachubinski, R. A. (2000). Fusion of small peroxisomal vesicles *in vitro* reconstructs an early step in the *in vivo* multistep peroxisome assembly pathway of yarrowia lipolytica. *J. Cell Biol.* 148, 29–44. doi:10.1083/jcb.148.1.29

Tripathi, D. N., and Walker, C. L. (2016). The peroxisome as a cell signaling organelle. *Curr. Opin. Cell Biol.* 39, 109–112. doi:10.1016/j.ceb.2016.02.017

Ueda, M., Kawachi, H., Atomi, H., and Tanaka, A. (1998). Peroxisomal and mitochondrial carnitine acetyltransferase isozymes of the n-alkane-assimilating yeast, *Candida tropicalis*, occurred by alternative initiation of translation from the transcripts of a single gene. *Biochim. Biophys. Acta* 1397, 213–222. doi:10.1016/s0167-4781(98)00009-8

Ušaj, M. M., Brložnik, M., Kaferle, P., Žitnik, M., Wolinski, H., Leitner, F., et al. (2015). Genome-wide localization study of yeast Pex11 identifies peroxisome--mitochondria interactions through the ERMES complex. *J. Mol. Biol.* 427, 2072–2087. doi:10.1016/j.jmb.2015.03.004

van der Klei, I. J., and Veenhuis, M. (2006a). PTS1-independent sorting of peroxisomal matrix proteins by Pex5p. *Biochim. Biophys. Acta* 1763, 1794–1800. doi:10.1016/j.bbamcr.2006.08.013

van der Klei, I. J., and Veenhuis, M. (2006b). Yeast and filamentous fungi as model organisms in microbody research. *Biochim. Biophys. Acta* 1763, 1364–1373. doi:10.1016/j.bbamcr.2006.09.014

Van der Zand, A., Gent, J., Braakman, I., and Tabak, H. (2012). Biochemically distinct vesicles from the endoplasmic reticulum fuse to form peroxisomes. *Cell* 149, 397–409. doi:10.1016/j.cell.2012.01.054

Van Roermund, C. W., Elgersma, Y., Singh, N., Wanders, R. J., and Tabak, H. F. (1995). The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD (H) and acetyl-CoA under *in vivo* conditions. *EMBO J.* 14, 3480–3486. doi:10.1002/j.1460-2075.1995.tb07354.x

van Roermund, C. W. T., Hettema, E. H., Kal, A. J., van den Berg, M., Tabak, H. F., and Wanders, R. J. A. (1998). Peroxisomal β-oxidation of polyunsaturated fatty

acids in *Saccharomyces cerevisiae*: Isocitrate dehydrogenase provides NADPH for reduction of double bonds at even positions. *EMBO J.* 17, 677–687. doi:10.1093/emboj/17.3.677

van Roermund, C. W. T., Hettema, E. H., van den Berg, M., Tabak, H. F., and Wanders, R. J. A. (1999). Molecular characterization of carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria in *Saccharomyces cerevisiae* and identification of a plasma membrane carnitine transporter, Agp2p. *EMBO J.* 18, 5843–5852. doi:10.1093/emboj/18.21.5843

Vasilev, J., Mix, A. K., Heimerl, T., Maier, U. G., and Moog, D. (2022). Inferred subcellular localization of peroxisomal matrix proteins of Guillardia theta suggests an important role of peroxisomes in cryptophytes. *Front. Plant Sci.* 13, 889662. doi:10.3389/fpls.2022.889662

Visser, W. F., Van Roermund, C. W. T., Ijlst, L., Waterham, H. R., and Wanders, R. J. A. (2007). Metabolite transport across the peroxisomal membrane. *Biochem. J.* 401, 365–375. doi:10.1042/BJ20061352

Walter, T., and Erdmann, R. (2019). Current advances in protein import into peroxisomes. *Protein J.* 38, 351–362. doi:10.1007/s10930-019-09835-6

Walton, P. A., Brees, C., Lismont, C., Apanasets, O., and Fransen, M. (2017). The peroxisomal import receptor PEX5 functions as a stress sensor, retaining catalase in the cytosol in times of oxidative stress. *Biochim. Biophys. Acta. Mol. Cell Res.* 1864, 1833–1843. doi:10.1016/j.bbamcr.2017.07.013

Walton, P. A., Hill, P. E., and Subramani, S. (1995). Import of stably folded proteins into peroxisomes. *Mol. Biol. Cell* 6, 675–683. doi:10.1091/mbc.6.6.675

Wanders, R. J. A. (2014). Metabolic functions of peroxisomes in health and disease. *Biochimie* 98, 36–44. doi:10.1016/j.biochi.2013.08.022

Wanders, R. J. A., and Waterham, H. R. (2006). Biochemistry of mammalian peroxisomes revisited. *Annu. Rev. Biochem.* 75, 295–332. doi:10.1146/annurev.biochem.74.082803.133329

Wanders, R. J. A., Waterham, H. R., and Ferdinandusse, S. (2016). Metabolic interplay between peroxisomes and other subcellular organelles including mitochondria and the endoplasmic reticulum. *Front. Cell Dev. Biol.* 3, 83. doi:10.3389/fcell.2015.00083

Wang, S., Idrissi, F.-Z., Hermansson, M., Grippa, A., Ejsing, C. S., and Carvalho, P. (2018). Seipin and the membrane-shaping protein Pex30 cooperate in organelle budding from the endoplasmic reticulum. *Nat. Commun.* 9, 2939. doi:10.1038/s41467-018-05278-2

Weir, N. R., Kamber, R. A., Martenson, J. S., and Denic, V. (2017). The AAA protein Msp1 mediates clearance of excess tail-anchored proteins from the peroxisomal membrane. *Elife* 6, e28507. doi:10.7554/eLife.28507

Wilfling, F., Thiam, A. R., Olarte, M.-J., Wang, J., Beck, R., Gould, T. J., et al. (2014). Arf1/COPI machinery acts directly on lipid droplets and enables their connection to the ER for protein targeting. *Elife* 3, e01607. doi:10.7554/eLife.01607

Williams, C., Bener Aksam, E., Gunkel, K., Veenhuis, M., and van der Klei, I. J. (2012). The relevance of the non-canonical PTS1 of peroxisomal catalase. *Biochim. Biophys. Acta* 1823, 1133–1141. doi:10.1016/j.bbamcr.2012.04.006

Wright, Z. J., and Bartel, B. (2020). Peroxisomes form intralumenal vesicles with roles in fatty acid catabolism and protein compartmentalization in Arabidopsis. *Nat. Commun.* 11, 6221. doi:10.1038/s41467-020-20099-y

Wu, F., de Boer, R., Krikken, A. M., Akşit, A., Bordin, N., Devos, D. P., et al. (2020). Pex24 and Pex32 are required to tether peroxisomes to the ER for organelle biogenesis, positioning and segregation in yeast. *J. Cell Sci.* 133, jcs246983. doi:10.1242/jcs.246983

Yagita, Y., Hiromasa, T., and Fujiki, Y. (2013). Tail-anchored PEX26 targets peroxisomes via a PEX19-dependent and TRC40-independent class I pathway. *J. Cell Biol.* 200, 651–666. doi:10.1083/jcb.201211077

Yamamoto, Y., and Sakisaka, T. (2018). The peroxisome biogenesis factors posttranslationally target reticulon homology domain-containing proteins to the endoplasmic reticulum membrane. *Sci. Rep.* 8, 2322. doi:10.1038/s41598-018-20797-0

Yamashita, K., Tamura, S., Honsho, M., Yada, H., Yagita, Y., Kosako, H., et al. (2020). Mitotic phosphorylation of Pex14p regulates peroxisomal import machinery. *J. Cell Biol.* 219, e202001003. doi:10.1083/jcb.202001003

Yang, J., Pieuchot, L., and Jedd, G. (2018). Artificial import substrates reveal an omnivorous peroxisomal importomer. *Traffic* 19, 786–797. doi:10.1111/tra.12607

Yifrach, E., Chuartzman, S. G., Dahan, N., Maskit, S., Zada, L., Weill, U., et al. (2016). Characterization of proteome dynamics during growth in oleate reveals a new peroxisome-targeting receptor. *J. Cell Sci.* 129, 4067–4075. doi:10.1242/jcs.195255

Yifrach, E., Holbrook-Smith, D., Bürgi, J., Othman, A., Eisenstein, M., Van Roermund, C. W. T., et al. (2021). Systematic multi-level analysis of an organelle proteome reveals new peroxisomal functions. *bioRxiv*. bioarchives preprints.

Yifrach, E., Rudowitz, M., Cruz-Zaragoza, L. D., Tirosh, A., Gazi, Z., Peleg, Y., et al. (2022). Determining the targeting specificity of the selective peroxisomal targeting factor Pex9. *bioRxiv*. bioarchives preprints.

Yofe, I., Soliman, K., Chuartzman, S. G., Morgan, B., Weill, U., Yifrach, E., et al. (2017). Pex35 is a regulator of peroxisome abundance. *J. Cell Sci.* 130 (4), 791–804. doi:10.1242/jcs.187914

Yogev, O., Naamati, A., and Pines, O. (2011). Fumarase: A paradigm of dual targeting and dual localized functions. *FEBS J.* 278, 4230–4242. doi:10.1111/j.1742-4658.2011.08359.x

Yogev, O., Singer, E., Shaulian, E., Goldberg, M., Fox, T. D., Pines, O., et al. (2010). Fumarase: A mitochondrial metabolic enzyme and a cytosolic/nuclear component of the DNA damage response. *PLoS Biol.* 8, e1000328. doi:10.1371/journal.pbio.1000328

Yuan, W., Akşit, A., de Boer, R., Krikken, A. M., and van der Klei, I. J. (2022). Yeast Vps13 is crucial for peroxisome expansion in cells with reduced peroxisome-ER contact sites. *Front. Cell Dev. Biol.* 10, 842285. doi:10.3389/fcell.2022.842285

Zabezhinsky, D., Slobodin, B., Rapaport, D., and Gerst, J. E. (2016). An essential role for COPI in mRNA localization to mitochondria and mitochondrial function. *Cell Rep.* 15, 540–549. doi:10.1016/j.celrep.2016.03.053

Zekert, N., Veith, D., and Fischer, R. (2010). Interaction of the Aspergillus nidulans microtubule-organizing center (MTOC) component ApsB with gamma-tubulin and evidence for a role of a subclass of peroxisomes in the formation of septal MTOCs. *Eukaryot. Cell* 9, 795–805. doi:10.1128/EC.00058-10

Zhang, J., Tripathi, D. N., Jing, J., Alexander, A., Kim, J., Powell, R. T., et al. (2015). ATM functions at the peroxisome to induce pexophagy in response to ROS. *Nat. Cell Biol.* 17, 1259–1269. doi:10.1038/ncb3230

Zhang, M., Li, Q., Liu, T., Liu, L., Shen, D., Zhu, Y., et al. (2015). Two cytoplasmic effectors of Phytophthora sojae regulate plant cell death via interactions with plant catalases. *Plant Physiol.* 167, 164–175. doi:10.1104/pp.114.252437

Zheng, J., Chen, X., Liu, Q., Zhong, G., and Zhuang, M. (2021). Ubiquitin ligase MARCH5 localizes to peroxisomes to regulate pexophagy. *J. Cell Biol.* 221, e202103156. doi:10.1083/jcb.202103156

Zimmermann, R., Lang, S., Lerner, M., Förster, F., Nguyen, D., Helms, V., et al. (2021). Quantitative proteomics and differential protein abundance analysis after the depletion of PEX3 from human cells identifies additional aspects of protein targeting to the ER. *Int. J. Mol. Sci.* 22, 13028. doi:10.3390/ijms222313028

Zipor, G., Haim-Vilmovsky, L., Gelin-Licht, R., Gadir, N., Brocard, C., and Gerst, J. E. (2009). Localization of mRNAs coding for peroxisomal proteins in the yeast, *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U. S. A.* 106, 19848–19853. doi:10.1073/pnas.0910754106

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