AUTOPHAGY IN ENDOCRINE-METABOLIC DISEASES ASSOCIATED WITH AGING

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AUTOPHAGY IN ENDOCRINE-METABOLIC DISEASES ASSOCIATED WITH AGING

Topic Editors:
Maria Ines Vaccaro, University of Buenos Aires, Argentina
Vincenzo De Tata, University of Pisa, Italy
Claudio Daniel Gonzalez, Centro de Educación Médica e Investigaciones Clínicas Norberto Quirno (CEMIC), Argentina

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EDITORIAL
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Editorial: Autophagy in Endocrine-Metabolic Diseases Associated With Aging

Maria Ines Vaccaro[1*†], *Vincenzo De Tata*[2] *and Claudio Daniel Gonzalez*[3]

[1] University of Buenos Aires - CONICET, Institute of Biochemistry and Molecular Medicine, Buenos Aires, Argentina,
[2] Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy,
[3] CEMIC University Institute, Buenos Aires, Argentina

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Editorial on the Research Topic

Autophagy in Endocrine-Metabolic Diseases Associated With Aging

Autophagy is a highly regulated self-degradative process of cytoplasmic cellular constituents usually activated under certain conditions such as starvation or other different forms of cell stress that result in breakdown proteins and other cell components to obtain energy. Autophagy is also responsible for removing damaged or aged organelles, eliminating different pathogens and misfolded, aggregated, or altered proteins. Autophagy is an evolutionarily biologically conserved process that sequesters and delivers cytoplasmic components to the lysosome for degradation. It is also involved in the removal of cells that have undergone classical apoptosis. Autophagy is commonly associated with cell survival mechanisms: its dysregulation, however, may be also associated with cell death. In the classical view, according to the pathway that cargo follows to reach the lysosomal compartment, there are three major types of canonical degradative autophagy. These types are: microautophagy/endosomal microautophagy, chaperone mediated autophagy, and macroautophagy, the last one being characterized by the engulfment of cytoplasmic contents by a double membrane vesicle, named autophagosome. However, other non-canonical types of autophagy have been described. One of these unconventional forms is named secretory autophagy, a newly recognized process that is becoming of increasing relevance to explain the non-canonical secretion of a series of cytosolic proteins that have critical biological importance.

Disruption of autophagy is associated with aging and metabolic and degenerative diseases including cancer. This special issue contains a collection of 12 articles covering a broad range of key topics on the interplay of the different types of autophagy alterations with aging, endocrine-metabolic, and degenerative diseases.

Chaperone-mediated autophagy (CMA) represents a major mechanism for degradation of cytosolic proteins. It also plays a significant role in the regulation of lipid and carbohydrate metabolism. Dysregulation of chaperone-mediated autophagy has been found in several models of Parkinson's Disease, Alzheimer's Disease, and Huntington's Disease. Alterations in CMA may also play a role in the pathophysiology of Lateral Amyotrophic Sclerosis and other forms of degenerative disease. Alfaro et al. review in detail the potential involvement of chaperone-mediated autophagy in neurodegeneration as well as in aging, pointing out some critical gaps in knowledge as a rich substrate for further research. CMA consists of the internalization of selected cytosolic substrates into the lysosome by a mechanism that includes recognition of a pentapeptide-like KFERQ in the substrate by the chaperone hsc70, substrate presentation by the chaperone to the receptor LAMP2A, receptor multimerization, and protein internalization for degradation in the lysosome

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Approved by:
Antonello Lorenzini, University of Bologna, Italy

***Correspondence:**
Maria Ines Vaccaro
mvaccaro@ffyb.uba.ar

†ORCID:
Maria Ines Vaccaro
orcid.org/0000-0002-2456-747X

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assisted by a luminal form of hsc70 (1). LAMP2A is the only known lysosomal receptor for CMA. LAMP2A localization is defective and its function impaired in cystinosis (2, 3). Zhang et al. develop and characterize human cystinotic proximal tubule cells and demonstrate that these cells are characterized by CMA defects that affect vesicular trafficking mechanisms regulating the localization in the plasma membrane of the scavenger receptor megalin.

Secretion of some proteins lacking a "signal peptide" (for instance, some cytokines, insulin-degrading enzyme, alpha-Synuclein, etc.) does not follow the canonical secretion pathway. Those proteins are secreted following different unconventional processes. One of these routes relies on autophagy; it is autophagy dependent. "Secretory autophagy" may then explain the secretion of some relevant peptides involved in several pathophysiological processes (4). Some aggregation-prone proteins, like Amyloid beta or alpha-Synuclein are secreted by secretory autophagy. Some pro-inflammatory mediators such as interleukin-1 beta also follow this non-canonical secretory process. Alteration in secretory autophagy, as extensively described by Gonzalez et al. in this special issue, may play a substantial role in degenerative and metabolic diseases and their treatment.

Skeletal muscle atrophy is a common finding in aging and many degenerative diseases. Different degrees of muscle atrophy can be achieved under diverse physiological conditions, exposure to certain drugs, or starvation. Many of the mechanisms associated with muscle atrophy remain obscure. Kretschmar et al. propose a novel role of polycystin-2, a membrane protein of the transient receptor potential family, as a regulator of skeletal muscle atrophy mediated by the modulation of mTOR in myotubes. Polycystin-2 regulates autophagy in several models, under different stimuli and in diverse tissues. However, Kretschmar et al. suggest that the polycystin-2 role in the regulation of muscle atrophy may be independent of autophagy. This article proposes new mechanisms in the field and opens spaces for further investigation in this area. Spinal and bulbar muscle atrophy is a rare disease associated with a mutation in the androgen receptor. A polyglutamine expansion in the N-terminus of the androgen receptor protein is associated with neurotoxicity. These alterations have a critical role in the pathophysiology of the disease. Cicardi et al. provide a detailed characterization of the autophagy activation and involvement in the disease initiation and progression, reveal some novel mechanisms, and suggest some potential targets for therapeutic exploration.

Hypothalamic arcuate neurons can sense the nutrient status of the organism and accordingly regulate food intake and glucose metabolism. Alterations of this neuronal network can contribute to the pathogenesis of obesity-related diseases such as type 2 diabetes mellitus (5). In their paper, Hernández-Cáceres et al. suggest that the inhibition of autophagy could be a potential mechanism for the high fat diet-induced obesity and diabetes. By using the neuronal cell line N43/5 they demonstrate that palmitic acid exposure causes inhibition of autophagy and decreased insulin sensitivity. Their results indicate that hypothalamic autophagy could represent a promising therapeutic target in diabetic patients. It has been proposed that altered autophagy can promote beta cell dysfunction in diabetic patients (6). To further clarify the relationship between autophagy and beta cell damage, Bugliani et al. explore the effects of autophagy modulation (rapamycin induction or 3-methyladenine inhibition) in human islets under conditions of ER stress. Lipotoxic (palmitate) and chemically induced (brefeldin) ER stress are associated with alteration of beta cell survival and function. Interestingly, the authors reported that, in these conditions, the promotion of autophagy by rapamycin ameliorates the function, survival, and ultrastructure of beta cells. Thus, tuning autophagy could be a useful tool for beta cell protection.

Autophagy is an important homeostatic protective mechanism, but, on the other hand, its alterations can be involved in various pathologic processes (7). The paper by Barbosa et al. reviews recent developments on the role of impaired autophagy in age-related diseases. Aging can be defined as a time–dependent deterioration of cell functioning due to damage accumulation. Autophagic activity has been shown to decrease with age, potentially contributing to the accumulation of damaged molecules and organelles. Thus, to clarify the role of dysfunctional autophagy in establishing the hallmarks of aging could help to define new anti-aging therapeutic strategies and to extend longevity.

The mTORC1 signaling pathway couples energy and nutrient abundance to the execution of cell growth, cell division and metabolism (8). Moreover, the activation of mTORC1 inhibits autophagy. The paper of Guillén and Benito reviews the role of mTORC1 in the progression of diabetes. It has been widely demonstrated that autophagy is crucial to maintain pancreatic beta cell homeostasis (9). The progression of the diabetic disease is associated with a chronic overactivation of mTORC1 and consequently with a sustained inhibition of autophagy in pancreatic beta cells. The failure of such an important protective mechanism could induce the apoptosis of pancreatic beta cells and the impairment of compensatory insulin secretion that characterize the transition to prediabetes to clinically evident type 2 diabetes.

Cancer is emerging as a complex disease where metabolic alterations and inflammatory processes play a critical role. Autophagy and autophagy related proteins are involved in cancer pathophysiology. Arroyo et al. report the cases of two patients with progressing chronic lymphocytic leukemia (CLL). They simultaneously detect autophagy protein LC3B and classical phenotypic markers used for identification of tumoral CLL B cell clones. They found that two patients with progressing CLL showed increased expression of the autophagy protein LC3B, in addition to CD38 and ZAP70 positive expression, suggesting that activation of autophagy may correlate with CLL progression even before ibrutinib treatment. Ropolo et al. present evidence of new regulatory pathways involved in autophagy induction in high resistance pancreatic tumor cells. They demonstrated that gemcitabine requires the expression of a pancreatitis associated and autophagy related protein VMP1 (10) to induce autophagy in pancreatic cancer cells PANC-1 and MIAPaCa-2 that carry activated *KRAS*. Ropolo et al. identified E2F1, a molecule regulated by the retinoblastoma pathway, as the transcription factor in gemcitabine-induced VMP1-mediated autophagy of highly resistant pancreatic cancer cells. Finally, Moutinho-Ribeiro et al. discuss the potential role

of exosomes (11) (small circulating extracellular vesicles of 50–150 nm in diameter) in several aspects related to pancreatic cancer, from initiation to tumor progression and its applicability in early detection and treatment. Increasing knowledge on cancer exosomes, whose secretory mechanisms are related to autophagy, provides valuable insights on new therapeutic targets and can potentially open new strategies to treat malignant disease.

Scientific knowledge in the field of autophagy grew exponentially along the last decade. A more precise characterization of the different forms of autophagy, their molecular mechanisms and potential implications in the pathophysiology of many diseases reflect the magnitude of such advances. However, several gaps in knowledge and unmet needs remain uncovered and call for further research. Identification of molecular targets for pharmacological modulation of autophagy and its translation into clinical practice remains among the more urgent ones. The role of autophagy modulation in therapy of metabolic and degenerative diseases challenges our current knowledge and opens potentially promising avenues for future investigation.

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

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REFERENCES

1. Kaushik S, Bandyopadhyay U, Sridhar S, Kiffin R, Martinez-Vicente M, Kon M, et al. Chaperone-mediated autophagy at a glance. *J Cell Sci.* (2011) 124:495–9. doi: 10.1242/jcs.073874
2. Napolitano G, Johnson JL, He J, Rocca CJ, Monfregola J, Pestonjamasp K, et al. Impairment of chaperone-mediated autophagy leads to selective lysosomal degradation defects in the lysosomal storage disease cystinosis. *EMBO Mol Med.* (2015) 7:158–74. doi: 10.15252/emmm.201404223
3. Zhang J, Johnson JL, He J, Napolitano G, Ramadass M, Rocca C, et al. Cystinosin, the small GTPase Rab11, and the Rab7 effector RILP regulate intracellular trafficking of the chaperone-mediated autophagy receptor LAMP2A. *J Biol Chem.* (2017) 292:10328–46. doi: 10.1074/jbc.M116.764076
4. Cavalli G, Cenci S. Autophagy and protein secretion. *J Mol Biol.* (2020) 432:2525–45. doi: 10.1016/j.jmb.2020.01.015
5. Timper K, Brüning JC. Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity. *Dis Model Mech.* (2017) 10: 679–89. doi: 10.1242/dmm.026609
6. Masini M, Bugliani M, Lupi R, del Guerra S, Boggi U, Filipponi F, et al. Autophagy in human type 2 diabetes pancreatic beta cells. *Diabetologia.* (2009) 52:1083–6. doi: 10.1007/s00125-009-1347-2
7. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell.* (2008) 132:27–42. doi: 10.1016/j.cell.2007.12.018
8. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol.* (2011) 12:21–35. doi: 10.1038/nrm3025
9. Lee YH, Kim J, Park K, Lee MS. β-cell autophagy: mechanism and role in β-cell dysfunction. *Mol Metab.* (2019) 27S:S92–103. doi: 10.1016/j.molmet.2019.06.014
10. Ropolo A, Grasso D, Pardo R, Sacchetti ML, Archange C, Lo Re A, et al. The pancreatitis-induced vacuole membrane protein 1 triggers autophagy in mammalian cells. *J Biol Chem.* (2007) 282:37124–33. doi: 10.1074/jbc.M706956200
11. Zhang X, Shi S, Zhang B, Ni Q, Yu X, Xu J. Circulating biomarkers for early diagnosis of pancreatic cancer: facts and hopes. *Am J Cancer Res.* (2018) 8:332–53.

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mTORC1 Overactivation as a Key Aging Factor in the Progression to Type 2 Diabetes Mellitus

Carlos Guillén [1,2]* *and Manuel Benito* [1,2]

[1] *Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain,* [2] *Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Instituto de Salud Carlos III, Madrid, Spain*

Type 2 Diabetes Mellitus (T2DM), a worldwide epidemics, is a progressive disease initially developing an insulin resistant state, with manifest pancreatic beta islet overwork and hyperinsulinemia. As the disease progresses, pancreatic β cells are overwhelmed and fails in their capacity to compensate insulin resistance. In addition, it is usually associated with other metabolic diseases such as hyperlipidemia, obesity and the metabolic syndrome. During the progression to T2DM there is a chronic activation of mTORC1 signaling pathway, which induces aging and acts as an endogenous inhibitor of autophagy. The complex 1 of mTOR (mTORC1) controls cell proliferation, cell growth as well as metabolism in a variety of cell types through a complex signaling network. Autophagy is involved in the recycling of cellular components for energy generation under nutrient deprivation, and serves as a complementary degradation system to the ubiquitin-proteasome pathway. Autophagy represents a protective mechanism for different cell types, including pancreatic β cells, and potentiates β cell survival across the progression to T2DM. Here, we focus our attention on the chronic overactivation of mTORC1 signaling pathway in β islets from prediabetics patients, making these cells more prone to trigger apoptosis upon several cellular stressors and allowing the progression from prediabetes to type 2 diabetes status.

Keywords: T2DM, autophagy, amylin, mitophagy, mTORC1, TSC2

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Edited by:
Maria Ines Vaccaro, Universidad de Buenos Aires, Argentina

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Piero Marchetti, Università degli Studi di Pisa, Italy
James Harper, Sam Houston State University, United States

***Correspondence:**
Carlos Guillén
cguillen@ucm.es

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mTORC1 SIGNALING NETWORK: UPSTREAM AND DOWNSTREAM EFFECTORS

Tuberous sclerosis complex (TSC) is an essential regulator of mTORC1 signaling pathway. TSC is a heterotrimer protein complex formed by the association of TSC1 (hamartin), TSC2 (tuberin) and Tre2-Bub2-Cdc16-1 domain family member 7 (TDC1D7) (1). This complex is the major inhibitory node of the mechanistic target of rapamycin complex 1 (mTORC1) (2). TSC1-TSC2-TBC1D7 complex exerts its inhibitory effect on mTORC1 by a member of the Ras superfamily, called Rheb. TSC2 presents a GTPase activating domain (GAP) favoring the transformation of GTP-Rheb (ras homology enriched in brain) into GDP-Rheb, avoiding mTORC1 activation (2–5). TSC1 and TSC2 are modulated by multiple mechanisms, including phosphorylation. TSC1/TSC2 integrates signals coming from the energetic status of the cell and nutritional availability, with

those from extracellular signaling coming from hormones or growth factors such as insulin or IGF1 respectively (2, 6).

mTOR (mechanistic target of rapamycin) is a serine-threonine kinase, belonging to the phosphatidyl-inositol-3 kinase related kinases (PIKK) (7). Depending on the proteins that are associated with mTOR, it can be found into two different complexes, mTORC1 and mTORC2. mTORC1 is the "rapamycin-sensitive" complex and mTORC2 is the "rapamycin-independent" complex. There are two specific components of mTORC1, the regulatory-associated protein of mTOR (RAPTOR) and the 40-Kda proline-rich Akt substrate (PRAS40) (8–10). However, the rapamycin-independent companion of mTOR, known as RICTOR, the mammalian stress-activated MAP kinase-interacting protein 1 (mSIN1) and the protein observed with RICTOR (PROTOR) are specific members of mTORC2 complex (11). The best characterized substrates of mTORC1 are S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1), controlling protein synthesis and ribosome biogenesis (12). Activation of mTORC2 leads to phosphorylation and activation Akt at serine 473. mTORC2 it is considered as the PDK2 required for the full activation of Akt (13–15).

In the last years, it has been determined that mTORC1 activation is produced on the lysosomal membrane through a complex mechanism involving different proteins (16). Phosphorylation events coming from growth factors, through the Akt and MAPK kinases, directly phosphorylates TSC2 and dissociates it from the surface of the lysosome, avoiding GAP activity toward Rheb and mTORC1 activation (17). Furthermore, nutrients such as aminoacids are capable to activate mTORC1 signaling in a Rag-dependent manner, another group of small G-proteins, acting as heterodimers for mTORC1 activation (18, 19). The presence of aminoacids affects RagA/B-GTP ratio vs. RagA/B-GDP through GAP activity of GATOR1 (20) and the guanine exchange factor activity (GEF) of the complex called "Ragulator" (21). Then, mTORC1 is regulated by two different systems of small G proteins, named Rag and Rheb, on the surface of the lysosome (16). Our group has demonstrated that TSC2, apart from its phosphorylation state, can be regulated by acetylation in different lysine residues (22). Now, we are analyzing the relevance of acetylation status of TSC2 for its recruitment to the membrane of the lysosome.

Another important effector of mTORC1 signaling is AMP-activated protein kinase (AMPK). AMPK is highly conserved kinase in eukaryotes. AMPK acts as an allosteric sensor of cellular energy status and regulates anabolic and catabolic signaling pathways for the maintenance of energy homeostasis (23, 24). In addition, AMPK inhibits mTORC1 signaling directly by TSC2 phosphorylation, favoring TSC1-TSC2 association (25). Furthermore, AMPK modulates mTORC1, independently from TSC2 by raptor phosphorylation and inactivation of mTORC1 (26). Moreover, AMPK and autophagy are connected directly by phosphorylation of ULK1 that leads to autophagy induction. In fact, several residues of ULK1 protein are directly phosphorylated by AMPK (27, 28). It has recently been proposed that ULK1 acetylation is essential for autophagy activation (29, 30).

AUTOPHAGY AND ITS RELEVANCE IN PANCREATIC β CELLS

Autophagy is a process that controls cytoplasm quality by the elimination of protein aggregates or altered organelles (31, 32). Autophagy is involved in the shutdown of energy-consuming pathways and the stimulation of catabolic processes through the degradation of cellular components under nutrient deprivation (33). Alternatively, to the ubiquitin-proteasome system (UPS), cells depend on autophagy as an alternative degradation system. Furthermore, autophagy it is involved in the elimination of misfolded proteins or altered organelles. The term autophagy encompasses several conserved mechanisms in eukaryotes that can be classified as: macroautophagy, chaperone-mediated autophagy and microautophagy. Chaperone-mediated autophagy consists in the recognition of a specific sequence by a chaperone (hsc70) and then migration of the complex to the lysosome. There, the complex interacts with a receptor in the lysosomal membrane, called LAMP-2A, translocates inside and it is finally degraded (34). In microautophagy, the lysosome directly engulfs the components that are going to be degraded (35). As a general process, macroautophagy it is characterized by the production of a double membrane compartment, termed autophagosome, which surrounds cytoplasmic components and then, fuses with lysosomes where proteolytic activity of the later will degrade the engulfed components (36). Autophagy protects pancreatic β cells, increasing pancreatic β cell survival in the progression to T2DM (37, 38). Using a mouse model Atg-7 deletion specifically in pancreatic β cells, it has been determined the essential implication of autophagy in the survival of pancreatic β cells (37). Furthermore, our group has defined this protection of autophagy in ER-stress using a model of insulin secretion-deficient β cells (39). The protective role of autophagy has been determined for avoiding the toxicity of human amylin (hIAPP) (40, 41). In fact, a mouse model with a chronic overactivation of mTORC1 (β-TSC2-/-) showed an increase in pancreatic β cell death and an impairment in autophagy, as we demonstrated in collaboration with Yoshiaki Kido's lab in Kobe University (42). It has recently been observed in human pancreatic β cells an association between dysregulation in autophagy with an increased in cell death (43, 44). Accordingly, this data point to mTORC1 as a signaling pathway that is overactivated in diabetic patients and, at least partially responsible of pancreatic beta cell failure. All the data exposed in the present paper are derived from animal models and not from pre-diabetic patients. There is a clear effect of mTORC1 hyperactivation and the progression to T2DM, but in animal models. The extrapolation to the possible effect in humans has to be assured.

mTORC1 AND MITOPHAGY. CONSEQUENCES ON T2DM

mTORC1 it is an endogenous inhibitor of autophagy. Apart from the bulk autophagy, a non-specific mechanism, all the organelles are submitted to a specific degradation. In the case

of mitochondria, this process is named mitophagy. Additionally, a mitochondrial unfolded protein response (mtUPR) for the degradation of protein aggregates in different locations inside the mitochondria, such as the matrix and in the inner membrane, occurs (45, 46). The inhibition of mTORC1 slows aging by an increased in autophagy, favoring the elimination of misfolded proteins and impaired organelles such as mitochondria, avoiding its accumulation, and associated with aging and different aging-related diseases such as T2DM, or Parkinson disease, or Alzheimer disease (47). In fact, in the progression to T2DM exists an increasing alteration in mitochondrial morphology and function. Then, an increased in mitophagy could be a potential mechanism for delaying the progression to the disease and maintain a preserved β-cell function (48). Mitophagy is a complex process and there are several possible mechanisms. During the development and maturation of red blood cells, it is necessary a removal of mitochondria in the precursor cell or reticulocyte through a NIP3-like protein X (NIX, also known as BNIP3L) mechanism (49). However, in mammals exist another mechanism with involves the system PTEN-induced putative kinase 1 (PINK1)-Parkin pathway. PINK1 is a serine-threonine kinase recruited to the healthy mitochondria, which is imported into the TIM complex of the inner membrane, where it is cleaved by the mitochondrial processing peptidase (MPP) (50) and presenilin-associated rhomboid-like protein (PARL) (51, 52). PINK1 accumulates specifically in damaged mitochondria, because the processing system is inhibited. Then, the E3-ubiquitin ligase Parkin it is recruited to the outer membrane (OM) of the mitochondria, ubiquitinates different substrates, driving the mitophagic process (53). Our group has determined that in MEF TSC2 KO, which presents a hyperactivation of mTORC1, there is an impairment in the PINK1 recruitment in response to a mitophagic inducer such as carbonyl cyanide m-chlorophenylhydrazone (CCCP). Furthermore, in TSC2–/– cells there is a reduction in *Pink1* expression as well as PINK1 protein production. Then, in cells lacking TSC2, an accumulation of damaged and aged mitochondria occurs (54). This situation contributes to the accumulation of reactive species of oxygen (ROS) as occurred in diabetics (48). In addition, amylin accumulates and forms cytotoxic oligomers, which damage the cell membrane and directly affects to the functionality and viability of β cells (55). We have recently published that INS1E overexpressing human amylin (INS1E-hIAPP) presents a hyperactivation of mTORC1 and a diminished level of TSC2. Furthermore, these cells possess an impairment in mitophagic flux with the concomitant accumulation of damaged mitochondria (56). However, the mechanism underlying pancreatic β cell toxicity during the formation of amyloid remains unknown (57). In **Figure 1**, it is depicted the main players in the regulation of pancreatic β cell fate and different drugs acting at different levels.

mTORC1, AGING AND ITS EFFECTS ON T2DM

It is well known that insulin signaling is involved in the control of longevity in a wide spectrum of organisms including worms, flies, and mice (58–62). In addition, the use of rapamycin or knocking-down mTOR or S6K1 can promote life extension in several species (63–66). In addition, TSC1 and TSC2 activation, which negatively controls mTORC1, prolongs life span in *Drosophila* (67). During aging or under a hypercaloric diet exists an mTORC1 hyperactivity, which derives into a disruption in autophagy and, concomitantly an increase in ER stress (16). In this regard, nicotinamide adenine dinucleotide (NAD^+) as well as sirtuins levels can be modulated during caloric restriction, activating autophagy, and directly affecting mammalian longevity (68). Mammalian sirtuins are composed of seven members (SIRT1-7) and can be activated by several stimuli including energy deprivation, caloric restriction and resveratrol (69, 70). Sirtuins are a group of NAD^+-dependent histone deacetylases with homology to the yeast silent information regulator 2 (Sir2) proteins (71). SIRT1-3 catalyzed deacetylation reaction producing a by-product, nicotinamide and O-acetyl ADP-ribose (OAADPr), along with the deacetylated lysine as it has been reviewed in (72). NAD^+ levels declines in different tissues with aging, altering the functionality of sirtuins and hence plays a key role in multiple diseases, such as metabolic disorders, neurodegenerative diseases and many others (73). Then, NAD^+ plays a central role in aging process and longevity (74).

Sirtuins can be activated by different treatments, such as caloric restriction, rapamycin, resveratrol and many others. In fact, all of these treatments are capable to modulate mTORC1 signaling (75). The overactivation of mTORC1 signaling specifically in pancreatic β cells leads to an augmented in β cell mass, which are related to hyperinsulinemia and hypoglycemia (76–79). However, chronic overactivation of mTORC1 signaling pathway develops a progressive hyperglycemia and a diminished islet mass (76). In this regard, we recently published that overactivation of mTORC1 in beta islets from a β-cell specific deletion of Tsc2 (βTsc2 (–/–) mice) induces an impairment in autophagy with an accumulation of damaged mitochondria. Those deleterious effects were partially reverted by the use of rapamycin (42).

In pancreatic β cells, SIRT1 represses the expression of uncoupling protein 2 (UCP2), leading to an increase in mitochondrial ATP production, thus enhancing insulin secretion (80). Accordingly, a β cell-specific SIRT1-overexpressing transgenic mice (BESTO) has a better glucose tolerance and a boost in insulin secretion (81). Nowadays, SIRT1 it is considered a regulator of different metabolic pathways. There are multiple SIRT1-regulated targets, modulating transcriptional activity of different transcription factors, such as peroxisome proliferator-activated receptor gamma (PPARγ), PPARα, PPAR gamma coactivator 1 alpha (PGC-1α), and the forkhead box, subgroup O (FOXO) family. Then, SIRT1 is capable to regulate different processes like insulin secretion, gluconeogenesis and fatty acid oxidation (82).

TYPE 2 DIABETES MELLITUS AND mTORC1

Type 2 diabetes mellitus (T2DM) is a very complicated disorder and it is considered epidemic in the world

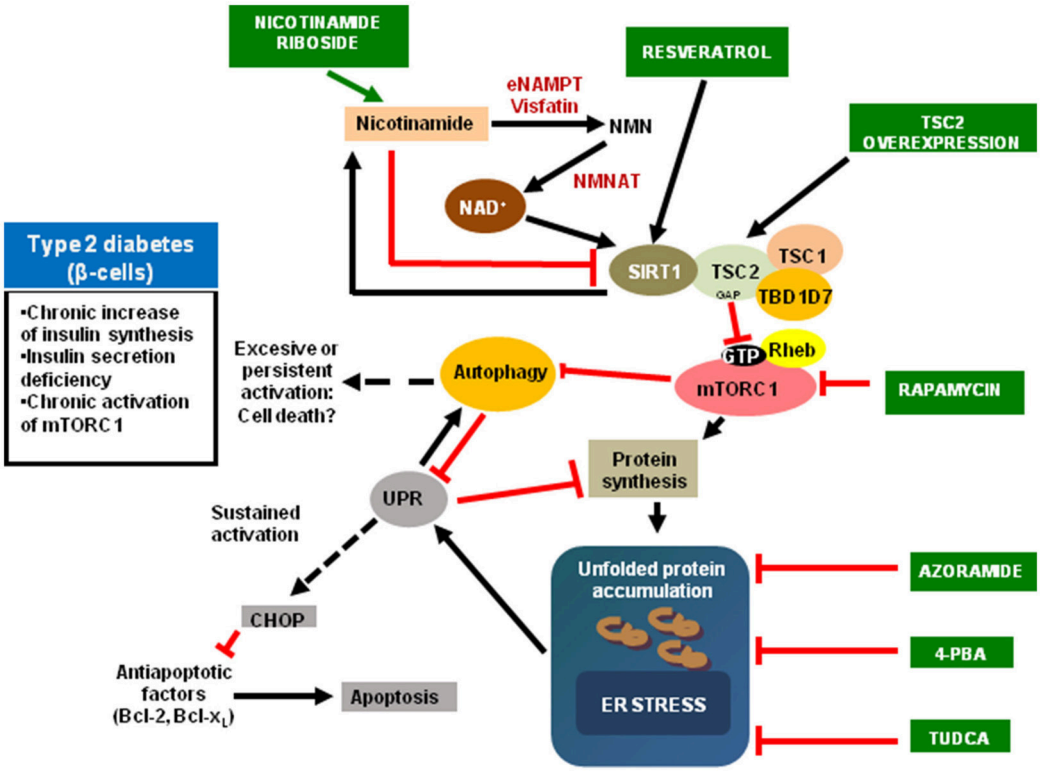

FIGURE 1 | Scheme depicting the main upstream and downstream regulators of mTORC1 signaling pathway. The possible agents that affect aging process are in green boxes. eNAMPT, extracellular nicotinamide phosphoribosyltransferase; NMNAT, Nicotinamide mononucleotide adenylyltransferase; 4-PBA, 4-phenylbutyric acid; TUDCA, Tauroursodeoxycholic acid; CHOP, CCAAT-enhancer-binding protein homologous protein.

(83). T2DM it is a progressive disease including insulin resistance, β-cell hyperplasia and/or β cell hypertrophy, that mediates a compensatory insulin secretion and subsequently hyperinsulinemia, pancreatic β cell dysfunction and a loss of cell identity or de-differentiation (84). However, the underlying mechanism mediating pancreatic β cell fate in type 2 diabetics is not completely known. T2DM is associated with higher production of glucose and lipids facilitating β cell death. Furthermore, advanced glycation end-products (AGEs) have been proposed as pancreatic β cell death inducers (85). The hyperamylinemia found in obese people and in insulin-resistant patients, contributes to its oligomerization inside pancreatic β cells, being deleterious for pancreatic β cells (86). From the whole amount of autopsy from patients with T2DM, around 80% present amyloid deposits in the pancreas. However, the importance of amylin it is not yet completely understood. Thus, at the insulin resistant prediabetic stage, mTORC1 is a key effector for the growth and survival of pancreatic β cells. However, if mTORC1 remains chronically overactivated, pancreatic beta cell death occurs and the compensatory insulin secretion mechanism it is compromised. Then, mTORC1 is a double-edged sword in the progression to T2DM (87). The location where protein synthesis occurs is in the endoplasmic reticulum (ER). When misfolded proteins are accumulated into the ER, the unfolded protein response (UPR) is activated. UPR is a protective mechanism that alleviates the cell from that overload. However, if UPR activation it is maintained for a long period of time, pancreatic β cell death occurs (88). T2DM alters the capacity of ER to manage the increased demand of protein synthesis of pancreatic β cells (89). Increasing the expression of endogenous chaperones such as Bip (90), the use of chemical chaperones, such as taurine-conjugated ursodeoxycholic acid (TUDCA) or 4-Phenyl butyric acid (4-PBA) (91), or increasing the ER protein folding capacity by the use of azoramide (92), diminish β cell failure and facilitate the proper folding and avoiding protein aggregation and accumulation of damaged organelles.

However, mTORC1 hyperactivation is observed in other tissues, such as in the heart muscle contributing to the dysfunction and complication of T2DM (93). Furthermore, not only in cardiac tissue, in the liver, apart from being involved in the regulation of lipid homeostasis, facilitating lipogenesis and inhibiting lipolysis and lipophagy (94), is upregulated in insulin resistant states, such as T2DM contributing to the dysregulation of glucose as well as lipid homeostasis (95). In adipose tissues (96), mTORC1 is also involved in the generation of insulin resistance as it has been reviewed in (16).

CONCLUDING REMARKS

Diabetes is a multifactorial and progressive disease with two phases; firstly, a prediabetic stage, with an insulin resistance and hyperinsulinemia, and secondly as manifest diabetes associated with hypoinsulinemia and hyperglycemia. Then, it is crucial to understand the transition from prediabetes to type 2-diabetes status and the underlying molecular mechanisms of disease. At this stage, chronic overactivation of mTORC1 signaling pathway in β islets from prediabetics patients leads to on one hand to the expansion of the pancreatic beta cell mass and, on the other to the inhibition of autophagy as protective mechanism of beta cells against the attack of several stressors, making these cells more prone to trigger apoptosis. Thus, the maintenance of a functional autophagy it is an essential component to protect and prolong pancreatic β cell life span precluding chronic hyperglycemia.

AUTHOR CONTRIBUTIONS

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

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REFERENCES

1. Dibble CC, Elis W, Menon S, Qin W, Klekota J, Asara JM, et al. TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1. *Mol Cell* (2012) 47:535–46. doi: 10.1016/j.molcel.2012.06.009
2. Huang J, Manning BD. The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. *Biochem J.* (2008) 412:179–90. doi: 10.1042/BJ20080281
3. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat Cell Biol.* (2003) 5:578–81. doi: 10.1038/ncb999
4. Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J. Tuberous sclerosis complex gene products, tuberin and hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward *Rheb*. *Curr Biol.* (2003) 13:1259–68. doi: 10.1016/S0960-9822(03)00506-2
5. Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev.* (2003) 17:1829–34. doi: 10.1101/gad.1110003
6. Orlova KA, Crino PB. The tuberous sclerosis complex. *Ann N Y Acad Sci.* (2010) 1184:87–105. doi: 10.1111/j.1749-6632.2009.05117.x
7. Lempiäinen H, Halazonetis TD. Emerging common themes in regulation of PIKKs and PI3Ks. *EMBO J.* (2009) 28:3067–73. doi: 10.1038/emboj.2009.281
8. Sabatini DM. mTOR and cancer: insights into a complex relationship. *Nat Rev cancer* (2006) 6:729–34. doi: 10.1038/nrc1974
9. Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, et al. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol Cell* (2007) 25:903–15. doi: 10.1016/j.molcel.2007.03.003
10. Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat Cell Biol.* (2007) 9:316–23. doi: 10.1038/ncb1547
11. Zoncu R, Efekan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol.* (2011) 12:21–35. doi: 10.1038/nrm3025
12. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol.* (2009) 10:307–18. doi: 10.1038/nrm2672
13. Garcia-Martínez JM, Moran J, Clarke RG, Gray A, Cosulich SH, Chresta CM, et al. Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR). *Biochem J.* (2009) 421:29–42. doi: 10.1042/BJ20090489
14. Ikenoue T, Inoki K, Yang Q, Zhou X, Guan KL. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J.* (2008) 27:1919–31. doi: 10.1038/emboj.2008.119
15. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* (2005) 307:1098–101. doi: 10.1126/science.1106148
16. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* (2012) 149:274–93. doi: 10.1016/j.cell.2012.03.017
17. Menon S, Dibble CC, Talbott G, Hoxha G, Valvezan AJ, Takahashi H, et al. Spatial control of the TSC complex integrates insulin and nutrient regulation of mTORC1 at the lysosome. *Cell* (2014) 156:771–85. doi: 10.1016/j.cell.2013.11.049
18. Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL. Regulation of TORC1 by Rag GTPases in nutrient response. *Nat Cell Biol.* (2008) 10:935–45. doi: 10.1038/ncb1753
19. Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, et al. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* (2008) 320:1496–501. doi: 10.1126/science.1157535
20. Bar-Peled L, Chantranupong L, Cherniack AD, Chen WW, Ottina KA, Grabiner BC, et al. A Tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. *Science* (2013) 340:1100–6. doi: 10.1126/science.1232044
21. Bar-Peled L, Schweitzer LD, Zoncu R, Sabatini DM. Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. *Cell* (2012) 150:1196–208. doi: 10.1016/j.cell.2012.07.032
22. García Aguilar A, Guillén C, Nellist M, Bartolomé A, Benito M. TSC2 N-terminal lysine acetylation status affects to its stability modulating mTORC1 signaling and autophagy. BBA. *Mol Cell Res.* (2016) 1863:2658–67. doi: 10.1016/j.bbamcr.2016.08.006
23. Hardie DG. Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. *Endocrinology* (2003) 144:5179–83. doi: 10.1210/en.2003-0982
24. Hardie DG. AMP-activated protein kinase: a cellular energy sensor with a key role in metabolic disorders and in cancer. *Biochem Soc Trans.* (2011) 39:1–13. doi: 10.1042/BST0390001
25. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* (2003) 115:577–90. doi: 10.1016/S0092-8674(03)00929-2
26. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell* (2008) 30:214–26. doi: 10.1016/j.molcel.2008.03.003
27. Egan DF, Shackelford DB, Mihaylova MM, Melino G, Kohnz RA, Mair W, et al. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science* (2011) 331:456–61. doi: 10.1126/science.1196371
28. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature Cell Biol.* (2011) 13:132–41. doi: 10.1038/ncb2152
29. Lin SY, Li TY, Liu Q, Zhang C, Li X, Chen Y, et al. GSK3-TIP60-ULK1 signaling pathway links growth factor deprivation to autophagy. *Science* (2012) 336:477–81. doi: 10.1126/science.1217032
30. Lin SY, Li TY, Liu Q, Zhang C, Li X, Chen Y, et al. Protein phosphorylation-acetylation cascade connects growth factor deprivation to autophagy. *Autophagy* (2012) 8:1385–6. doi: 10.4161/auto.20959

31. Menzies FM, Moreau K, Rubinsztein DC. Protein misfolding disorders and macroautophagy. *Curr Opin Cell Biol.* (2011) 23:190–7. doi: 10.1016/j.ceb.2010.10.010
32. Masiero E, Sandri M. Autophagy inhibition induces atrophy and myopathy in adult skeletal muscles. *Autophagy* (2010) 6:307–9. doi: 10.4161/auto.6.2.11137
33. Stipanuk MH. Macroautophagy and its role in nutrient homeostasis. *Nutr Rev.* (2009) 67:677–89. doi: 10.1111/j.1753-4887.2009.00252.x
34. Kaushik S, Cuervo AM. Chaperone-mediated autophagy: a unique way to enter the lysosome world. *Trends Cell Biol.* (2012) 22:407–17. doi: 10.1016/j.tcb.2012.05.006
35. Li WW, Li J, Bao JK. Microautophagy: lesser-known self-eating. *Cell Mol Life Sci.* (2012) 69:1125–36. doi: 10.1007/s00018-011-0865-5
36. Chen Y, Klionsky DJ. The regulation of autophagy - unanswered questions. *J Cell Sci.* (2011) 124:161–70. doi: 10.1242/jcs.064576
37. Ebato C, Uchida T, Arakawa M, Komatsu M, Ueno T, Komiya K et al. Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. *Cell Metab.* (2008) 8:325–32. doi: 10.1016/j.cmet.2008.08.009
38. Jung HS, Chung KW, Won Kim J, Kim J, Komatsu M, Tanaka K, et al. Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell Metab.* (2008) 8:318–24. doi: 10.1016/j.cmet.2008.08.013
39. Bartolomé A, Guillén C, Benito M. Autophagy plays a protective role in endoplasmic reticulum stress-mediated pancreatic β cell death. *Autophagy* (2012) 8:1757–68. doi: 10.4161/auto.21994
40. Shigihara N, Fukunaka A, Hara A, Komiya K, Honda A, Uchida T, et al. Human IAPP-induced pancreatic β cell toxicity and its regulation by autophagy. *J Clin Invest.* (2014) 124:3634–44. doi: 10.1172/JCI69866
41. Rivera JF, Costes S, Gurlo T, Glabe CG, Butler PC. Autophagy defends pancreatic β cells from human islet amyloid polypeptide-induced toxicity. *J Clin Invest.* (2014) 124:3489–500. doi: 10.1172/JCI71981
42. Bartolomé A, Kimura-Koyanagi M, Asahara S, Guillén C, Inoue H, Teruyama K, et al. Pancreatic β-cell failure mediated by mTORC1 hyperactivity and autophagic impairment. *Diabetes* (2014) 63:2996–3008. doi: 10.2337/db13-0970
43. Yuan T, Rafizadeh S, Gorrepati KD, Lupse B, Oberholzer J, Maedler K, et al. Reciprocal regulation of mTOR complexes in pancreatic islets from humans with type 2 diabetes. *Diabetologia* (2017) 60:668–78. doi: 10.1007/s00125-016-4188-9
44. Masini M, Bugliani M, Lupi R, Del Guerra S, Boggi U, Filipponi F, et al. Autophagy in human type 2 diabetes pancreatic beta cells. *Diabetologia* (2009) 52:1083–6. doi: 10.1007/s00125-009-1347-2
45. Herst PM, Rowe MR, Carson GM, Berridge MV. Functional mitochondria in health and disease. *Front Endocrinol.* (2017) 8:296. doi: 10.3389/fendo.2017.00296
46. Baker MJ, Tatsuta T, Langer T. Quality control of mitochondrial proteostasis. *Cold Spring Harb Perspect Biol.* (2011) 3:a007559. doi: 10.1101/cshperspect.a007559
47. Saxton RA, Sabatini DM. mTOR Signaling in growth, metabolism, and disease. *Cell* (2017) 168:969–76. doi: 10.1016/j.cell.2017.02.004
48. Bhansali S, Bhansali A, Walia R, Saikia UN, Dhawan V. Alterations in mitochondrial oxidative stress and mitophagy in subjects with prediabetes and type 2 diabetes mellitus. *Front Endocrinol.* (2017) 8:347. doi: 10.3389/fendo.2017.00347
49. Youle RJ, Narendra DP. Mechanisms of mitophagy. *Nat Rev Mol Cell Biol.* (2011) 12:9–14. doi: 10.1038/nrm3028
50. Greene AW, Grenier K, Aguileta MA, Muise S, Farazifard R, Haque ME, et al. Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. *EMBO Rep.* (2012) 13:378–85. doi: 10.1038/embor.2012.14
51. Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, Youle RJ. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J Cell Biol.* (2010) 191:933–42. doi: 10.1083/jcb.201008084
52. Meissner C, Lorenz H, Weihofen A, Selke DJ, Lemberg MK. The mitochondrial intramembrane protease PARL cleaves human Pink1 to regulate Pink1 trafficking. *J Neurochem.* (2011) 117:856–67. doi: 10.1111/j.1471-4159.2011.07253.x
53. Pickrell AM, Youle RJ. The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. *Neuron* (2015) 85:257–73. doi: 10.1016/j.neuron.2014.12.007
54. Bartolomé A, García-Aguilar A, Asahara SI, Kido Y, Guillén C, Pajvani UB, et al. MTORC1 regulates both general autophagy and mitophagy induction after oxidative phosphorylation uncoupling. *Mol Cell Biol.* (2017) 37:e00441–17. doi: 10.1128/MCB.00441-17
55. Caillon L, Hoffmann AR, Botz A, Khemtemourian L. Molecular structure, membrane interactions, and toxicity of the islet amyloid polypeptide in type 2 diabetes mellitus. *J Diabetes Res.* (2016) 2016:5639875. doi: 10.1155/2016/5639875
56. García-Hernández M, García Aguilar A, Burillo J, Gómez Oca R, Manca MA, Novials A, et al. Pancreatic β cells overexpressing hIAPP impaired mitophagy and unbalanced mitochondrial dynamics. *Cell Death Dis.* (2018) 9:481. doi: 10.1038/s41419-018-0533-x
57. Zraika S, Hull RL, Verchere CB, Clark A, Potter KJ, Fraser PE, et al. Toxic oligomers and islet beta cell death: guilty by association or convicted by circumstantial evidence? *Diabetologia* (2010) 53:1046–56. doi: 10.1007/s00125-010-1671-6
58. Guarente L, Kenyon C. Genetic pathways that regulate ageing in model organisms. *Nature* (2000) 408:255-62. doi: 10.1038/35041700
59. Kaeberlein M. Longevity and aging. *F1000prime Rep.* (2013) 5:5. doi: 10.12703/P5-5
60. Sun X, Chen WD, Wang YD. DAF-16/FOXO transcription factor in aging and longevity. *Front Pharmacol.* (2017) 8:548. doi: 10.3389/fphar.2017.00548
61. Tatar M, Post S, Yu K. Nutrient control of drosophila longevity. *Trends Endocrinol Metab.* (2014) 25:509–17. doi: 10.1016/j.tem.2014.02.006
62. Fadini GP, Ceolotto G, Pagnin E, de Kreutzenberg S, Avogaro A. At the crossroads of longevity and metabolism: the metabolic syndrome and lifespan determinant pathways. *Aging Cell* (2011) 10:10–7. doi: 10.1111/j.1474-9726.2010.00642.x
63. Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, et al. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. *Cell Metab.* (2010) 11:35–46. doi: 10.1016/j.cmet.2009.11.010
64. Zhang Y, Bokov A, Gelfond J, Soto V, Ikeno Y, Hubbard G, et al. Rapamycin extends life and health in C57BL/6 mice. *J Gerontol A Biol Sci Med Sci.* (2014) 69:119–30. doi: 10.1093/gerona/glt056
65. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* (2009) 460:392–5. doi: 10.1038/nature08221
66. Selman C, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, et al. Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science* (2009) 326:140–4. doi: 10.1126/science.1177221
67. Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. *Curr Biol.* (2004) 14:885–90. doi: 10.1016/j.cub.2004.03.059
68. Morselli E, Maiuri MC, Markaki M, Megalou E, Pasparaki A, Palikaras K, et al. Caloric restriction and resveratrol promote longevity through the Sirtuin-1-dependent induction of autophagy. *Cell Death Dis.* (2010) 1:e10. doi: 10.1038/cddis.2009.8
69. Lam YY, Peterson CM, Ravussin E. Resveratrol vs. calorie restriction: data from rodents to humans. *Exp Gerontol.* (2013) 48:1018–24. doi: 10.1016/j.exger.2013.04.005
70. Greer EL, Brunet A. Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in *C. elegans*. *Aging Cell* (2009) 8:113–27. doi: 10.1111/j.1474-9726.2009.00459.x
71. Imai S, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* (2000) 403:795–800. doi: 10.1038/35001622
72. Knyphausen P, de Boor S, Kuhlmann N, Scislowski L, Extra A, Baldus L, et al. Insights into lysine deacetylation of natively folded substrate proteins by sirtuins. *J Biol Chem.* (2016) 291:14677–94. doi: 10.1074/jbc.M116.726307
73. Johnson S, Imai SI. NAD $^+$ biosynthesis, aging, and disease. *F1000Res.* (2018) 7:132. doi: 10.12688/f1000research.12120.1
74. Imai SI, Guarente L. It takes two to tango: NAD^+ and sirtuins in aging/longevity control. *NPJ Aging Mech Dis.* (2016) 2:16017. doi: 10.1038/npjamd.2016.17

75. Kitada M, Koya D. The use of calorie restriction mimetics to study aging. *Methods Mol Biol.* (2013) 1048:95–107. doi: 10.1007/978-1-62703-556-9_8
76. Shigeyama Y, Kobayashi T, Ido Y, Hashimoto N, Asahara S, Matsuda T, et al. Biphasic response of pancreatic beta-cell mass to ablation of tuberous sclerosis complex 2 in mice. *Mol Cell Biol.* (2008) 28:2971–9. doi: 10.1128/MCB.01695-07
77. Rachdi L, Balcazar N, Osorio-Duque F, Elghazi L, Weiss A, Gould A, et al. Disruption of Tsc2 in pancreatic beta cells induces beta cell mass expansion and improved glucose tolerance in a TORC1-dependent manner. *Proc Natl Acad Sci USA.* (2008) 105:9250–5. doi: 10.1073/pnas.0803047105
78. Mori H, Inoki K, Opland D, Münzberg H, Villanueva EC, Faouzi M, et al. Critical roles for the TSC-mTOR pathway in β-cell function. *Am J Physiol Endocrinol Metab.* (2009) 297:E1013–22. doi: 10.1152/ajpendo.00262.2009
79. Hamada S, Hara K, Hamada T, Yasuda H, Moriyama H, Nakayama R, et al. Upregulation of the mammalian target of rapamycin complex 1 pathway by Ras homolog enriched in brain in pancreatic beta-cells leads to increased beta-cell mass and prevention of hyperglycemia. *Diabetes* (2009) 58:1321–32. doi: 10.2337/db08-0519
80. Bordone L, Motta MC, Picard F, Robinson A, Jhala US, Apfeld J, et al. Sirt1 Regulates Insulin Secretion by Repressing UCP2 in Pancreatic β Cells. *Plos Biol.* (2006) 4:e31. doi: 10.1371/journal.pbio.0040031
81. Moynihan KA, Grimm AA, Plueger MM, Bernal-Mizrachi E, Ford E, Cras-Méneur C, et al. Increased dosage of mammalian Sir2 in pancreatic beta cells enhances glucose-stimulated insulin secretion in mice. *Cell Metab.* (2005) 2:105–17. doi: 10.1016/j.cmet.2005.07.001
82. Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. *Annu Rev Pathol.* (2010)5:293–95. doi: 10.1146/annurev.pathol.4.110807.092250
83. Hu FB. Globalization of diabetes: the role of diet, lifestyle, and genes. *Diabetes Care* (2011) 34:1249–57. doi: 10.2337/dc11-0442
84. Remedi MS, Emfinger C. Pancreatic β-cell identity in diabetes. *Diabetes Obes Metab.* (2016) 18:110–6. doi: 10.1111/dom.12727
85. Nowotny K, Jung T, Höhn A, Weber D, Grune T. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus *Biomolecules* (2015) 5:194–222. doi: 10.3390/biom5010194
86. Akter R, Cao P, Noor H, Ridgway Z, Tu LH, Wang H, et al. Islet amyloid polypeptide: structure, function, and pathophysiology. *J Diabetes Res.* (2016) 2016:1–18. doi: 10.1155/2016/2798269
87. Ardestani A, Lupse B, Kido Y, Leibowitz G, Maedler K. mTORC1 Signaling: a double-edged sword in diabetic β cells. *Cell Metab.* (2018) 27:314–31. doi: 10.1016/j.cmet.2017.11.004
88. Back SH, Kaufman RJ. Endoplasmic reticulum stress and type 2 diabetes. *Annu Rev Biochem.* (2012) 81:767–93. doi: 10.1146/annurev-biochem-072909-095555
89. Mukherjee A, Morales-Scheihing D, Butler PC, Soto C. Type 2 diabetes as a protein misfolding disease. *Trends Mol Med.* (2015) 21:439–49. doi: 10.1016/j.molmed.2015.04.005
90. Fritz JM, Dong M, Apsley KS, Marin EP, Na CL, Sitamaran S, et al. Deficiency of the BiP cochaperone ERdj4 causes constitutive endoplasmic reticulum stress and metabolic defects. *Mol Biol Cell* (2014) 25:431–40. doi: 10.1091/mbc.e13-06-0319
91. Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, et al. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* (2006) 313:1137–40. doi: 10.1126/science.1128294
92. Fu S, Yalcin A, Lee GY, Li P, Fan J, Arruda AP, et al. Phenotypic assays identify azoramide as a small-molecule modulator of the unfolded protein response with antidiabetic activity. *Sci Transl Med.* (2015) 7:292ra98. doi: 10.1126/scitranslmed.aaa9134.
93. Suhara T, Baba Y, Shimada BK, Higa JK, Matsui T. The mTOR signaling pathway in myocardial dysfunction in type 2 diabetes mellitus. *Curr Diab Rep.* (2017) 17:38. doi: 10.1007/s11892-017-0865-4
94. Han J, Wang Y. mTORC1 signaling in hepatic lipid metabolism. *Protein Cell* (2018)c 9:145–51. doi: 10.1007/s13238-017-0409-3
95. Titchenell PM, Lazar MA, Birnbaum MJ. Unraveling the regulation of hepatic metabolism by insulin.*Trends Endocrinol Metab.* (2017) 28:497–505. doi: 10.1016/j.tem.2017.03.003
96. Lee P, Jung SM, Guertin DA. The complex roles of mTOR in adipocytes and beyond. *Trends Endocrinol Metab.* (2017):28:319–39. doi: 10.1016/j.tem.2017.01.004

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REVIEW
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Pancreatic Cancer Diagnosis and Management: Has the Time Come to Prick the Bubble?

Pedro Moutinho-Ribeiro[1,2], *Guilherme Macedo*[1,2*] *and Sónia A. Melo*[2,3,4*]

[1] Department of Gastroenterology, Centro Hospitalar São João, Porto, Portugal, [2] Faculty of Medicine of the University of Porto, Porto, Portugal, [3] Institute for Research Innovation in Health (i3S), Porto, Portugal, [4] Institute of Pathology and Molecular Immunology of the University of Porto, Porto, Portugal

Pancreatic cancer (PC) is associated with poor prognosis and very dismal survival rates. The most effective possibility of cure is tumor resection, which is only possible in about 15% of patients diagnosed at early stages of disease progression. Recent whole-genome sequencing studies pointed genetic alterations in 12 core signaling pathways in PC. These observations hint at the possibility that the initial mutation in PC might appear nearly 20 years before any symptoms occur, suggesting that a large window of opportunity may exist for early detection. Biomarkers with the potential to identify pre-neoplastic disease or very early stages of cancer are of great promise to improve patient survival. The concept of liquid biopsy refers to a minimally invasive sampling and analysis of liquid biomarkers that can be isolated from body fluids, primarily blood, urine and saliva. A myriad of circulating molecules may be useful as tumor markers, including cell-free DNA (cfDNA), cell-free RNA (cfRNA), circulating tumor cells (CTC), circulating tumor proteins, and extracellular vesicles, more specifically exosomes. In this review, we discuss with more detail the potential role of exosomes in several aspects related to PC, from initiation to tumor progression and its applicability in early detection and treatment. Exosomes are small circulating extracellular vesicles of 50–150 nm in diameter released from the plasma membrane by almost all cells and exhibit some advantages over other biomarkers. Exosomes are central players of intercellular communication and they have been implicated in a series of biological process, including tumorigenesis, migration and metastasis. Several exosomal microRNAs and proteins have been observed to distinguish PC from benign pancreatic diseases and healthy controls. Besides their possible role in diagnosis, understanding exosomes functions in cancer has clarified the importance of microenvironment in PC progression as well as its influence in proliferation, metastasis and resistance to chemotherapy. Increasing knowledge on cancer exosomes provides valuable insights on new therapeutic targets and can potentially open new strategies to treat this disease. Continuous research is needed to ascertain the reliability of using exosomes and their content as potential biomarkers, so that, hopefully, in the near future, they will provide the opportunity for early diagnosis, treatment intervention and increase survival of PC patients.

Keywords: pancreatic cancer, early diagnosis, liquid biopsy, biomarkers, exosomes

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***Correspondence:**
Guilherme Macedo
guilhermemacedo59@gmail.com
Sónia A. Melo
smelo@ipatimup.pt

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC), the most frequent type of pancreatic cancer (PC), is associated with poor prognosis, with 53,670 new cases and 43,090 estimated deaths in 2017 (1). It represents around 3% of the new cancer cases each year, but it is the fourth most common cause of cancer mortality (2). It is expected to become the second cause of death by cancer by 2020 in the USA (2). On the contrary to the death rates for many other cancer, such as lung, colorectal, breast, and prostate, the death rate for PDAC patients has increased (3) as well as its incidence, that has raised in about 30% (2).

Pancreatic cancer is typically asymptomatic in its first stages of development, and as a consequence of the late diagnosis, this disease presents a very low survival rate. Combining all stages, the overall 5-year survival rate is 5% (4).Tumor resection is the only possibility of cure, but recurrence often happens and, therefore, the 5-year survival rate in resected patients is only up to 25% (1). Its unique tumor biology contributes to early recurrence, metastasis, and a subpar response to conventional therapies (5).

Since lifetime risk of PC in general population is low (1.3%), a population-based screening is not recommended (6). Meanwhile, individuals with family history or genetic predisposition have been identified as risk groups. According to the International Cancer of the Pancreas Screening Consortium (CAPS) it has been proposed that these individuals at higher risk for PC should be considered for screening (6, 7). The referred Consortium defined eligible individuals to be those with more than 5% of lifetime risk or with a 5-fold increased relative risk for PC (6, 7). Nonetheless, this group of patients represents only 10% of the total spectrum of PC, the other 90% of cases being sporadic (8).

PANCREATIC CANCER PRECURSOR LESIONS: A WINDOW FOR OPPORTUNITY

Recent studies based on whole-genome sequencing indicated 12 important signaling pathways altered in PC. It was also suggested that the initial mutation occurred nearly 20 years before the first symptoms. This evidence has offered a time frame for pancreatic carcinogenesis, suggesting that a large window of opportunity may exist for early detection, which could improve the prognosis of this lethal disease (9, 10).

In recent years, a myriad of biomarkers have been investigated with specificity for PC detection (8). The ideal biomarker should be able to detect the disease in its early stages, when patients are still amenable for a curative treatment, or even in a more favorable scenario, its premalignant precursor lesions. Pancreatic cancer precursor lesions are intraductal papillary mucinous neoplasms (IPMNs), the mucinous cystic neoplasms (MCNs), and the pancreatic intraepithelial neoplasias (PanINs), the first two being macroscopic cystic alterations and the last one representing microscopic non-invasive epithelial proliferations within the pancreatic ducts (5). Intraductal papillary mucinous neoplasms (IPMNs) are papillary proliferations inside the pancreatic ducts that usually secrete thick mucus, leading to its focal dilation. They represent up to 10% of the neoplasms of the pancreas, and the ones harboring high-grade dysplasia carry an important risk of malignant transformation (11, 12). Depending on the extent of ductal involvement, three subtypes can be recognized: the main-duct intraductal papillary mucinous neoplasm (MD-IPMN), the branch duct intraductal papillary mucinous neoplasm (BD-IPMN), and the mixed type. Malignant transformation is more frequent in main duct and mixed types IPMNs, while BD-IPMNs are considered indolent lesions (11, 12).

In relation to cytoarchitectural and immunophenotypic features, four histological subtypes of IPMNs are considered: the gastric type (49–63%), the intestinal type (18–36%), the pancreaticobiliary type (7–18%), and the oncocytic (1–8%) type (11, 13). Main-duct intraductal papillary mucinous neoplasm (MD-IPMNs) are more frequently of the intestinal type, a combination that carries the highest risk of invasive transformation, usually giving rise to a colloid type carcinoma. On the other hand, the branch duct intraductal papillary mucinous neoplasm (BD-IPMN) are predominantly of the gastric type, which are characterized by an insignificant risk of malignant transformation. The pancreatobiliary type usually harbors high-grade dysplasia and is considered the aggressive evolution of the gastric type. Finally, the rarer oncocytic type presents with cytological atypia, frequently with high-grade dysplasia (14).

MCNs, unlike IPMNs, are neoplasms that develop in the pancreatic parenchyma, without involvement of the ductal system. Characteristically they are seen in women, rarely occurring in men, with a preferential location in the body and tail of the gland (15).

An ovarian-type stroma is an essential feature for the diagnosis of MCNs that clearly separates them from the much more frequent IPMNs. Depending on the grade of cytologic atypia in epithelial lining (low, intermediate or high-grade dysplasia), which has been associated with point mutations in KRAS and p53 genes, mucinous cystic neoplasms (MCNs) may exhibit different risks of malignant potential (15–17). Interestingly, the observation that in invasive MCNs, the inactivation of SMAD4/DPC4 suppressor gene complex occurs only in the epithelial lining but not at the stroma level, suggests that the typical ovarian-type stroma of these lesions is not involved in the process of malignant transformation (18). The reported incidence of invasive carcinoma among MCNs is variable according to different series, ranging from 6 to 36% (15, 19, 20).

With the development and widespread of imaging modalities, the diagnosis of both of these macroscopic cystic PC precursor lesions is increasing. As the majority of them are asymptomatic and discovered incidentally, its proper management is not consensual and is a matter of debate by many International Societies worldwide. In fact, despite the availability of a significant number of guidelines and recommendations (21), there is still a lack of consensus in the decision of which one(s) to follow.

The vast majority of carcinomas originate from microscopic non-invasive epithelial proliferations within the pancreatic ducts, described as PanIN (22). These lesions are considered precursors

in the stepwise progression from intraepithelial to invasive neoplasia. This morphological progression is accompanied by accumulation of genetic changes, in which activating KRAS mutations are thought to be the driving force (8, 12). PanIN lesions are characteristically asymptomatic and are composed of columnar to cuboidal cells with varying amounts of mucin and different degrees of cytological and architectural atypia (23). These lesions are classified into 3 grades: PanIN-1A (flat) and PanIN-1B (papillary) are low-grade lesions with minimal cytological and architectural atypia, while high-grade PanINs (PanIN-3), also described as "carcinoma *in situ*," are characterized by severe cytological and architectural atypia (23). In cadavers of patients over the age of 80 the prevalence of PanIN lesions is about 55%. Also, these lesions are very frequent in patients with concomitant PC and in familial PC kindreds. The major problem concerning these very frequent lesions is their identification and the evaluation of its malignant potential. Currently, there is no imaging technique capable of an accurate diagnosis of a PanIN lesion and many promising biomarkers are being investigated for this purpose.

CONVENTIONAL DIAGNOSTIC TOOLS ARE INSUFFICIENT FOR EARLY DETECTION

Early diagnosis of PC is very challenging with the currently available methods (**Figure 1**) (24). Unlike colonoscopies for colorectal cancer and serum prostate-specific antigen (PSA) levels for prostate cancer, there is currently no standardized PC screening strategy, even for high-risk populations. Pancreatic cancer diagnosis and staging depends, substantially, on imaging modalities including ultrasonography (US) and endoscopic ultrasonography (EUS), multidetector computed tomography (MDCT), magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography (MRCP), endoscopic retrograde cholangiopancreatography (ERCP), and positron emission tomography (PET) (25–30).

Nowadays, multidetector computed tomography (MDCT) is the imaging technique of choice for pancreatic diseases, especially in the setting of solid tumors, where it has high accuracy to detect and to stage pancreatic malignancies (31). However, its sensitivity may be suboptimal as it misses some target lesions in the PC screening context. Even when considering thin-section, triple-phase helical CT, the sensitivity to detect lesions smaller than 2 cm is only up to 80% (32). Moreover, ionizing radiation exposition is also an important drawback, precluding CT to be an ideal screening and/or surveillance imaging technique.

In high-risk individuals, magnetic resonance imaging (MRI) can be used as a non-invasive screening imaging test considering the possibility to scan the entire abdomen and pelvis, avoiding radiation exposure. Considering the MRCP, this technique offers, in a non-invasive way (in contrast to ERCP), the capacity to characterize the ductal anatomy of the pancreas and diagnose small cystic lesions such as IPMNs. Preliminary data from CAPS3 study, that included high-risk patients submitted to surgical resection, suggests that MRI/MRCP may be superior to CT particularly for detection of IPMNs (71 vs. 14%, $p < 0.001$) (33). Some MRI features have been recently added to this technique improving its diagnostic capacity. In fact, diffusion-weighted imaging (DWI), a technique based on the Brownian motion of water molecules in tissue (34), has brought functional aspects into conventional anatomic evaluation, allowing higher contrast resolution and the identification of very small PC lesions.

In diagnosing PC, the advantage of EUS over MDCT has been reported for more than 10 years (35, 36). Despite being an imaging technique operator-dependent, with inherent risks of invasiveness and sedation, EUS has progressively being considered as the most accurate tool to investigate pancreatic diseases. It is also a non-radiation technique that can offer high-resolution images and can accurately characterize solid and cystic lesions. Moreover, it can evaluate cystic wall features associated with increased risk of malignancy, namely mural nodules, and other focal thickenings. The data analysis from a screening program involving high-risk individuals that were submitted to surgical resection confirmed the superiority of EUS, as it was able to detect almost twice as many neoplastic lesions comparing to MRI/MRCP or CT (33). Also, past studies (37, 38) that used EUS as a screening tool showed its accuracy in detecting asymptomatic precancerous branch duct IPMNs, large PanINs, incidental pancreatic endocrine tumors and ductal adenocarcinomas. The chance to add some recent techniques such as EUS-guided elastography and contrast-enhanced imaging has expanded new and promising fields of investigation (37–40).

Considering EUS-guided fine-needle aspiration (EUS-FNA), when performed in suspected lesions, it shows diagnostic accuracy for malignancy of more than 85–90%. Apart from its role in the study of solid lesions, the investigation of pancreatic cystic lesions by EUS-FNA can also be very useful, allowing a cytological diagnosis of IPMN and MCN in up to 70% of the cases (41). "Cell-block preparation" and "core tissue sampling" are two developments of this technique (42), which might be useful not only in providing more material for histological evaluation, but also for recently developed ancillary diagnostic techniques, namely: microRNA profiling, KRAS mutation detection and chemo sensitivity testing (43–45).

Lately, confocal laser endomicroscopy has surged as a technological improvement to EUS with a particular interest in cystic lesions characterization. In this technique, a dedicated miniprobe is introduced through a 19-gauge needle previously inserted into the cystic lesion, allowing a real time direct visualization of the epithelial lining at a microscopic level, permitting the identification of suspicious architectural changes (46).

Nowadays, no biomarker exists with adequate sensitivity and specificity for routine clinical diagnosis or screening of PC (8). Carbohydrate antigen (CA) 19-9 and carcinoembryonic antigen (CEA) are the most commonly used blood-based tumor biomarkers in clinical practice. The only biomarker approved by the US FDA for monitoring the progression and therapeutic response of PC is CA 19-9, which is also the only one recommended by the NCCN guidelines for clinical management of PC patients (47). Nevertheless, it is not specific and can be found in normal pancreatic and biliary duct cells, as well as in gastric, endometrial, colonic, and salivary epithelia. In

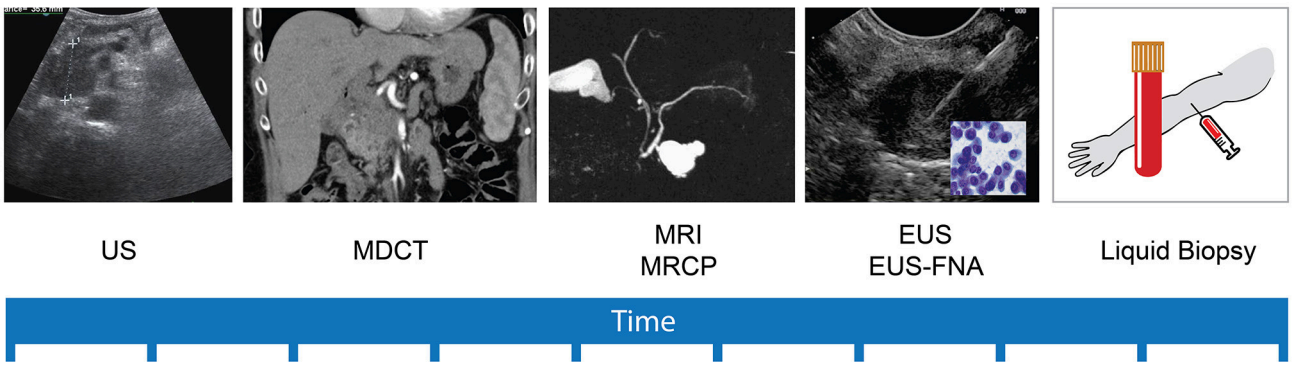

FIGURE 1 | Timeline of development of pancreatic cancer diagnostic and staging modalities. Pancreatic cancer (PC) diagnosis and staging depends, substantially, on imaging modalities. Abdominal Ultrasonography (US) was the first to appear, but lacks sensitivity to detect small treatable lesions. Multidetector computed tomography (MDCT) is nowadays frequently used to detect and stage pancreatic masses, with good accuracy specially for 2 cm and larger lesions. Magnetic resonance imaging (MRI) and its variant magnetic resonance cholangiopancreatography (MRCP) improved the sensitivity for characterization small cystic lesions. More recently, endoscopic ultrasonography (EUS) with the possibility to perform fine needle aspiration (FNA) constitutes a prime modality for precise diagnosis and local staging of small (<2 cm) solid and cystic pancreatic lesions. The innovative concept of liquid biopsy refers to a simple and painless collection of a body fluid sample (usually blood), in order to study proved and anticipated biomarkers with the potential to detect PC in its early non detectable stages and even the premalignant precursors.

fact, depending on the series, its specificity ranges from 30 to 100% (48–50), so the extremely high rate of false positive results prohibits its routine use for diagnosis. Moreover, CA 19-9 sensitivity is also imperfect, ranging from 41 to 86% (48–50). Additionally, up to 15% of general population do not express Lewis antigen and, consequently, CA 19-9 levels cannot be measured (50–53). Another clinically relevant issue is the fact that only 65% of patients with resectable tumors have increased levels of CA 19-9. In relation to its ability to differentiate PC from chronic pancreatitis, this biomarker is also inadequate, as up to 40% of patients harboring this last condition exhibit CA 19-9 levels above the normal range (52, 54). Considering all these pitfalls, serum CA 19-9 is used primarily as a prognostic tool in monitoring patients for recurrence or managing those with late stage disease (52, 55).

Taking the aforementioned aspects in account, it is understandable that most patients present with advanced disease, with only up to 25% having resectable tumors (56–58). We should also have in mind that even if these 25% of patients are identified and properly treated under the current standards of care, their survival, at the best, is up to 24 months (4). So, the focus should be, on one side, in aiming to detect the "real" curable lesions, that is the premalignant and the very small malignant ones, and, on the other side, the development of better therapeutic options. For the early detection scenario, the conventional diagnostic tools are far from being competent. In fact, available non-biopsy tests (serum CA19-9) and imaging techniques lack the sensitivity and specificity necessary to detect tumors smaller than 1 cm in the context of a 1.5% lifetime risk disease (59–65).

In consequence, it is crucial to develop new and improved strategies which can overtake all the obstacles described and diagnose primary tumors that can be resectable at very early stage. The development of markers with high sensitivities and specificities for PC and for its precursor conditions should be a priority issue, with extreme importance mainly in the context of high-risk individuals (66).

LIQUID BIOPSY, A HORIZON FOR EARLY DETECTION AND SURVIVAL IMPROVEMENT

As mentioned before, given the typical late stage of disease at the time of presentation, when treatment is disappointing, PC remains one of the most dismal diseases worldwide, with incidence nearly parallel to mortality. There has been much effort invested in identifying accurate tumor markers, ideally present in the timeframe between cancer onset and invasion, to allow diagnosing PC in early curable stages, to ultimately improve patient's survival (67, 68). In this setting, the ideal biomarker should be easily detected with satisfactory sensitivity and specificity and should distinguish PC from other benign pancreatic lesions. In the context of early detection, the identification of preneoplastic conditions, such as PanINs, IPMNs and MCNs, is of great importance (69).

The concept of liquid biopsy refers to the analysis of biomarkers present in a sample of a body fluid collected through a simple and painless, minimally invasive technique. The body fluids mostly used for biomarker isolation are essentially blood, urine and saliva (70–72). A myriad of circulating molecules may be used as tumor markers, including cell-free DNA (cfDNA), cell-free RNA (cfRNA), circulating tumor cells (CTC), circulating tumor proteins, and extracellular vesicles, more specifically exosomes (66, 73). Blood is easily accessible and relatively stable, making serum an ideal specimen to explore potential biomarkers. However, biomarkers secreted into serum are extremely diluted and probably obscured by other more-abundant serum proteins (74). Technological advances in the last decade have provided more opportunities to discover circulating biomarkers based

on "omics" analyses, including methods focused on proteins, nucleic acids, CTCs, and exosomes. Numerous proteins of low abundance can be analyzed by mass spectrometry-based approaches and proteomic technologies (24).

In recent years, based on the expression of transcriptional profiles and structural variations, different molecular subtypes of PC have been described thought genomic analyses (75–78). The early detection of mutant genes that identify PC and its subtypes is essential for an effective strategy for the management of the disease.

In PDAC, there are four major driver genes (one oncogene and three tumor suppressor genes) implicated in tumorigenesis (5). KRAS is the most frequently altered oncogene that encodes a GTPase which mediates downstream signaling from growth factor receptors; somatic mutations, clustered in specific hotspots (most in codon 12), are identified in more than 90% of PC (79). CDKN2A, by turn, is the most frequently altered tumor suppressor gene, with loss of function in more than 90% of tumors; it encodes an important cell-cycle regulator (79). TP53 is another tumor suppressor gene, with essential role in cellular response to stress, also exhibits frequent somatic mutations (79). Lastly, SMAD4, a tumor suppressor gene mediating downstream signaling of the transforming growth factor β (TGFβ) receptor is inactivated in about half of the PC cases (79).

Since Mandel and Metais, in 1948, (80) first described circulating free DNA in body fluids, an exponential interest in non-invasive technology for disease monitoring has been the focus of research in many centers worldwide.

Recently, due to the possibility to pair genomic tests with tests on CTCs, circulating tumor nucleic acids (ctNAs) and tumor-derived exosomes, liquid biopsies have gained increased value for clinical application (71, 81, 82).

During PC initiation and progression, many different genetic modifications take place, including genetic diversification, amplifications and homozygous deletions, an increase in duplicate chromosomal number, recapitulation of clonal expansion, clonal selection, driver mutations and losses of heterozygosity (10, 83–90).

Next-generation sequencing techniques provide deeper insight into somatic mutations and epigenetics analysis of the genome and broaden the characterization of circulating tumor DNA (ctDNA) and (cfRNA). With the development of cell tracking techniques and flow cytometry, it is now possible to capture and analyze CTCs and exosomes (24).

In this manner, the capabilities of liquid biopsy are enormous, allowing the characterization of tumor biomarkers in the same way tissue biopsy does, favoring improvement of the knowledge of tumor heterogeneity and, most importantly, contributing to early detection, monitoring of disease progression and response to treatment (**Figure 2**) (71, 82, 91–96).

EXOSOMES: THE BUBBLES OF THE FUTURE?

Exosomes are small cup-shaped extracellular vesicles (50–150 nm in size) released from the plasma membrane by almost all cells, including cancer cells (**Figure 1**) (24). They play an important role in intercellular communication, tumorigenesis and cancer metastasis (97–100). Structurally, they are enveloped by a lipid bilayer membrane with tissue-specific content instead of cellular organelles, such as pathogenic mRNA, microRNA, DNA fragments, and proteins (101, 102). After release, exosomes are stable in the extracellular environment or enter the circulation and can be taken up by neighbor or distant cells (103, 104). Exosomes allow the exchange of material and information between cells, altering gene expression or mediating RNA silencing (105).

Considering their extensive distribution and functions, exosomes are ideal candidates to find circulating biomarkers for PC detection and management (67, 104, 106). Moreover, exosomes have some advantages over other biomarkers (106, 107). First, they are widely distributed in nearly all body fluids, including serum, and are relatively stable when stored for long term at −80°C. Second, cancer cells secrete more exosomes than normal healthy cells. One of the reasons for this can be the acidic conditions of the tumor microenvironment that enhances the release of exosomes (108–110). Third, exosomes contain a combination of proteins, DNA, coding and non-coding RNAs, and lipids that can be used as a natural panel of biomarkers for simultaneous evaluation. Fourth, PC-derived exosomes enter the circulation at an early stage of cancer development and are related to metastasis. In fact, on the contrary of some other cancer markers that are released into the blood only after necrosis-related cell death occurs, a phenomenon usually associated with advanced stage disease with large volume tumor mass, cell-secreted exosomal nucleic acids (DNA fragments, mRNA, miRNAs, and others) are released in circulation during the initial phases of the tumorigenesis process (66, 91, 107). This aspect is critical for the early detection of PC because PC cells are able to metastasize at an early stage, with great impact on prognosis.

Recent studies have implicated PC-derived exosomes in the early development of PC (103) and showed that they contribute for establishing a premetastatic niche in the liver (98, 111) and that they can promote tumor formation and proliferation (112). In relation to exosomes' content, a main focus of research has been on RNA and microRNA profiling, in part due to the established utility of a variety of some of these molecules in cancer screening and also due to the relative ease feasibility of its amplification (99, 113–118). By turn, in order to determine their cellular origin, exosomal proteomic profiling is being an important focus of research in recent years (114).

Besides their possible role in diagnosis, the study of exosomal function has contributed to the improvement of the comprehension of the microenvironment related to PC and progression of the disease. The ultimate aim of understanding the way exosomes can influence tumor initiation, proliferation and metastasis is improving the knowledge on PC pathophysiology and patient prognosis. Moreover, its role in the development of some paraneoplastic conditions, such as diabetes *mellitus* and cachexia, and in the resistance to chemotherapy, can provide insights for future therapeutic targets (119–125).

The extraction of exosomes from body fluids has been described using various methods and technologies. Commonly

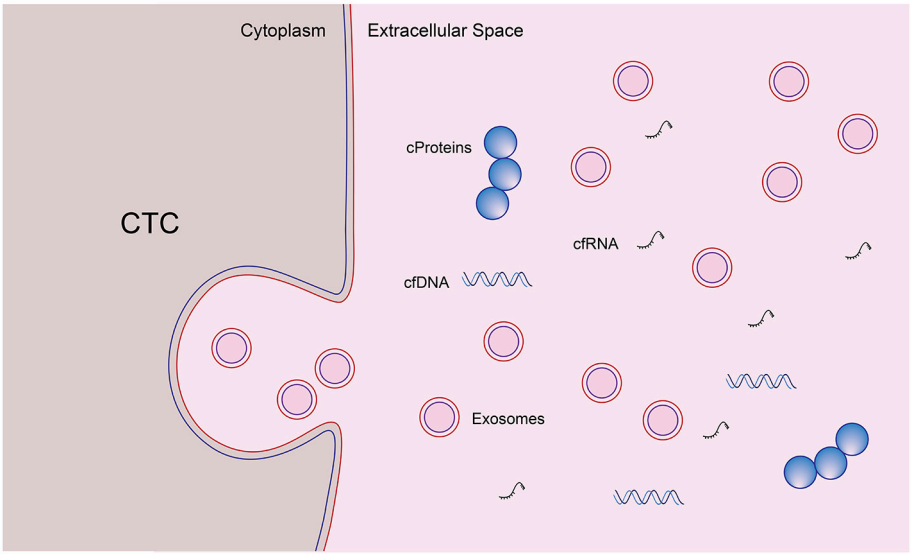

FIGURE 2 | Current understanding of molecular biomarkers for pancreatic cancer. A myriad of circulating molecules may be used as tumor markers, including cell-free DNA (cfDNA), cell-free RNA (cfRNA), circulating tumor cells (CTC), circulating tumor proteins, and extracellular vesicles, more specifically exosomes. The characterization of these tumor biomarkers favors improvement of the knowledge of tumor heterogeneity and, most importantly, contributes to early detection, monitoring of disease progression and response to treatment. Moreover, some of these molecules, in the near future, can play a role as therapeutic targets, hopefully allowing the control of the disease during its early curable stages.

used methods for isolation are ultracentrifugation, precipitation, size, immunoaffinity, and microfluidics (126).

Strong efforts have focused on developing sensitive diagnostics tools improving early detection of PC via identifying pancreatic cancer-associated exosomal markers (67, 104, 127). The first case–control study on PC exosomes was conducted by Que et al. (128), where four exosomal microRNAs were evaluated as candidates. In this study, a moderate discrimination of cases from controls was seen with miR-21 and miR-17-5p. In 2015, in a study involving patients with PC, chronic pancreatitis and controls, a higher expression of exosomal miR-10b was shown only in cancer patients (129). Although this cohort was a small one, the strength of this study was the use of new technology of label-free nanoplasmonic-based short non-coding RNA sensing. In the same year, a more extended cohort including PC patients, patients with chronic pancreatitis, individuals with benign pancreatic neoplasms and controls was evaluated by Madhavan et al. (130). In this well conducted study, a combination of four miRNAs (miR-1246, miR-4644, miR-3976, and miR-4306) and five proteins (CD44v6, Tspan8, EpCAM, MET and CD104) in circulating exosomes was able to distinguish PC patients from non-cases ones, with a sensitivity of 1.0 (CI: 0.95-1) and a specificity of 0.80 (CI: 0.67-0.90).

In the past years, the number of exosomal miRNAs studied in this context was considerable. Examples of these molecules are miR-21, miR-17-5p, miR-155, miR-34, miR- 196a, miR-181a, miR-181b, miR-138-5p, miR-494, miR- 542-3p, miR-31, and miR-205, that have been implicated for several studies to have the capacity, when upregulated, to promote cellular proliferation and angiogenesis, metastasis and disease progression and even chemo-resistance in PC patients (113, 128, 131–138).

These studies emphasize the importance of exosomal miRNAs not only as diagnostic and prognostic biomarkers, but also open a field of investigation of its use as potential targets for treatment PC.

Recently, Melo et al. showed that glypican-1 (GPC1), a membrane anchored protein, in circulating exosomes may distinguish with 100% specificity patients with PC or precancerous pancreatic lesions from patients with benign pancreatic diseases (139). Melo et al. reported that GPC1 expression patterns in exosomes secreted by PC could be utilized to identify subjects with PC early and offer considerable insights into the disease progress and tumor load. A comparison of exosomes from PC and control cell lines indicated that the exosomes from cancer exhibited enhanced levels of GPC1. In serum specimens from subjects with PC ($n = 190$), a significantly larger amount of GPC1+ circulating exosomes was present compared with normal controls ($n = 100$). Interestingly, direct analysis of GPC1 in serum itself revealed lower sensitivity and specificity than measurement of GPC1 in purified serum exosomes. Furthermore, GPC1+ exosomes were also confirmed to contain identical KRAS mutations, which frequently are present in PC and precancerous lesions and have been considered a fundamental mutation (139). Moreover, higher levels of GPC1 positive circulating exosomes were seen in both PC and PC precursor lesions, such as IPMNs, when compared to other benign diseases of the pancreas and healthy controls, with a perfect area under the receiver-operating characteristic curve (AUC) of 1.0. The authors concluded that given the high sensitivity and specificity of exosomal GPC1 in differentiating PC, independently of its stage, from non-PC controls, this biomarker could have a promising role

in PC early detection. Not surprisingly, when compared to the tumor marker CA 19-9 (AUC: ranging between 0.69 and 0.79), exosomal GPC1 was found to be significantly superior ($p < 0.001$) (139). Additionally, using a genetically engineered mouse that progressively developed into PC, the identification of GPC1+ exosomes exhibited positive results prior to pancreatic lesions being detectable by MRI. Tumor burden was associated positively with levels of GPC1+ circulating exosomes. In most subjects, the exosome levels reduced following the removal of the solid tumor. In this manner, circulating exosomal GPC1 may be seen as a prognostic indicator too, reflecting tumor load and monitoring disease progression and patients' survival. Moreover, evolving research will address if this biomarker can play a role in the treatment of PC as a potential pharmacological target (139). Many others since than have reported findings related to GPC1 detection in exosomes, not only in PC but also breast and colorectal cancer cases (140–148). In any event, in the future, further independent confirmation of exosomal GPC1 performance is needed, assessing its role as a more reliable marker in predicting diagnosis and prognosis of PC when compared to CA19-9, but also, and as it seems to be overexpressed in some precursor lesions such as IPMNs, its potential role in individualizing the management of such conditions (149).

Also interestingly, a paper from Hoshino et al. (98) has described that integrin αvβ5-expressing PC exosomes can determine liver metastasis and that targeting that molecule reduced exosome absorption by resident cells and inhibited liver involvement. The levels of integrin αvβ5 were noticeably enhanced in exosomes isolated from PC individuals with liver metastasis compared with those with no distant metastasis. Together, these findings have raised the possibility that GPC1+ and integrin αvβ5-expressing circulating exosomes may be used as indicators of PC progression and liver metastization.

Taking in account the excellent biodistribution and biocompatibility of exosomes, the idea of its utilization as vehicles for drug, genes or nucleic acids delivery has gained increased acceptance for continuous research in the field of PC treatment (124, 150, 151), especially when considering the fact that they can be particularly targeted to specific cell types by engineered exosomes-producer cells (124, 150, 152). This is important especially when dealing with a tumor with such difficulties in therapy delivery due to the intrinsic resistance of its microenvironment and its dense stroma (153).

CONCLUSION AND NEW PATHWAYS OF INVESTIGATION

Given the timeframe from pancreatic tumor initiation to invasion and metastatic capacity, there is a large window of opportunity for early management of this lethal disease. An ideal diagnostic method for PC should definitively distinguish malignant lesions from benign ones and detect early-stage disease and preneoplastic conditions, such as PanINs and cystic mucinous lesions with risk of progression.

There are many challenges in the early detection of PC, including its asymptomatic nature, the lack of imaging exams able to detect minimal lesions and the absence so far of sensitive and specific molecules in body fluids. Recently described circulating biomarkers associated with PC initiation and progression, easily detectable in blood, followed by confirmative diagnosis based on imaging and pathologic results might be the future ideal strategy for screening and diagnosing PC.

A number of circulating biomarkers have been widely studied, but validation for routine clinical use is still needed. The lack of sufficient samples from non-invasive precursor lesions and early-stage PC must be addressed, and animal models are important tools for research. In fact, extensive understanding of the fundamentals of PC development and the nature of precursor lesions is crucial prerequisite toward discovering and applying novel biomarkers.

Exosomes have been proving to be reliable candidates as PC biomarkers, as its contents, namely DNA, RNASs, proteins, lipids, and metabolites are largely derived from tumor cells. In this way, tracking these molecules will conduct to the knowledge of cell-type of origin, and also importantly the specificity for pre-metastatic niches formation and distant colonization.

Its potential utilization as biomarkers, besides the ultimate goal of early detection of PC, can also play an important role in monitoring disease progression.

Moreover, considering its excellent biodistribution, biocompatibility and cell-specific nature, exosomes can be used, in the future, as drug delivery vehicles.

The majority of studies addressing exosomes as biomarkers have been based on patients with established diagnosis of PC. However, and having in mind that when facing this dismal disease, the earlier the diagnosis the better the chance for cure, it would be of great interest to access the performance of these biomarkers in the context of high-risk individuals screening.

Continuous investigation is needed to ascertain the potential of these biomarkers, so that, hopefully, in the near future, they will provide the opportunity for early diagnosis, treatment intervention and increasing survival of PC patients.

AUTHOR CONTRIBUTIONS

PM-R did the bibliographic search and wrote the initial manuscript. GM and SM critically revised the manuscript and actively contributed to it.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin.* (2017) 67:7–30. doi: 10.3322/caac.21387
2. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res.* (2014) 74:2913–21. doi: 10.1158/0008-5472.CAN-14-0155
3. Siegel R, Ward E, Brawley O, Jemal A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin.* (2011) 61:212–36. doi: 10.3322/caac.20121
4. Hruban RH, Canto MI, Goggins M, Schulick R, Klein AP. Update on familial pancreatic cancer. *Adv Surg.* (2010) 44:293–311. doi: 10.1016/j.yasu.2010.05.011
5. Kamisawa T, Wood LD, Itoi T, Takaori K. Pancreatic cancer. *Lancet* (2016) 388:73–85. doi: 10.1016/S0140-6736(16)00141-0
6. Canto MI, Harinck F, Hruban RH, Offerhaus GJ, Poley JW, Kamel I, et al. International Cancer of the Pancreas Screening (CAPS) Consortium summit on the management of patients with increased risk for familial pancreatic cancer. *Gut* (2013) 62:339–47. doi: 10.1136/gutjnl-2012-303108
7. Wang W, Chen S, Brune KA, Hruban RH, Parmigiani G, Klein AP. PancPRO: risk assessment for individuals with a family history of pancreatic cancer. *J Clin Oncol.* (2007) 25:1417–22. doi: 10.1200/JCO.2006.09.2452
8. Moutinho-Ribeiro P, Coelho R, Giovannini M, Macedo G. Pancreatic cancer screening: Still a delusion? *Pancreatology* (2017) 17:754–65. doi: 10.1016/j.pan.2017.07.001
9. Iacobuzio-Donahue CA. Genetic evolution of pancreatic cancer: lessons learnt from the pancreatic cancer genome sequencing project. *Gut* (2012) 61:1085–94. doi: 10.1136/gut.2010.236026
10. Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* (2010) 467:1114–7. doi: 10.1038/nature09515
11. Machado NO, Al Qadhi H, Al Wahibi K. Intraductal papillary mucinous neoplasm of pancreas. *N Am J Med Sci.* (2015) 7:160–75. doi: 10.4103/1947-2714.157477
12. Shi C, Klein AP, Goggins M, Maitra A, Canto M, Ali S, et al. Increased prevalence of precursor lesions in familial pancreatic cancer patients. *Clin Cancer Res.* (2009) 15:7737–43. doi: 10.1158/1078-0432.CCR-09-0004
13. Castellano-Megias VM, Andres CI, Lopez-Alonso G, Colina-Ruizdelgado F. Pathological features and diagnosis of intraductal papillary mucinous neoplasm of the pancreas. *World J Gastrointest Oncol.* (2014) 6:311–24. doi: 10.4251/wjgo.v6.i9.311
14. Paini M, Crippa S, Partelli S, Scopelliti F, Tamburrino D, Baldoni A, et al. Molecular pathology of intraductal papillary mucinous neoplasms of the pancreas. *World J Gastroenterol.* (2014) 20:10008–23. doi: 10.3748/wjg.v20.i29.10008
15. Reddy RP, Smyrk TC, Zapiach M, Levy MJ, Pearson RK, Clain JE, et al. Pancreatic mucinous cystic neoplasm defined by ovarian stroma: demographics, clinical features, and prevalence of cancer. *Clin Gastroenterol Hepatol.* (2004) 2:1026–31. doi: 10.1016/S1542-3565(04)00450-1
16. Campbell F, Azadeh B. Cystic neoplasms of the exocrine pancreas. *Histopathology* (2008) 52:539–51. doi: 10.1111/j.1365-2559.2007.02856.x
17. Yoshizawa K, Nagai H, Sakurai S, Hironaka M, Morinaga S, Saitoh K, et al. Clonality and K-ras mutation analyses of epithelia in intraductal papillary mucinous tumor and mucinous cystic tumor of the pancreas. *Virchows Arch.* (2002) 441:437–43. doi: 10.1007/s00428-002-0645-6
18. Iacobuzio-Donahue CA, Wilentz RE, Argani P, Yeo CJ, Cameron JL, Kern SE, et al. Dpc4 protein in mucinous cystic neoplasms of the pancreas: frequent loss of expression in invasive carcinomas suggests a role in genetic progression. *Am J Surg Pathol.* (2000) 24:1544–8. doi: 10.1097/00000478-200011000-00011
19. Crippa S, Salvia R, Warshaw AL, Dominguez I, Bassi C, Falconi M, et al. Mucinous cystic neoplasm of the pancreas is not an aggressive entity: lessons from 163 resected patients. *Ann Surg.* (2008) 247:571–9. doi: 10.1097/SLA.0b013e31811f4449
20. Sarr MG, Carpenter HA, Prabhakar LP, Orchard TF, Hughes S, van Heerden JA, et al. Clinical and pathologic correlation of 84 mucinous cystic neoplasms of the pancreas: can one reliably differentiate benign from malignant (or premalignant) neoplasms? *Ann Surg.* (2000) 231:205–12. doi: 10.1097/00000658-200002000-00009
21. Brugge WR. Pancreatic cyst surveillance: Threat or opportunity? *Gastrointest Endosc.* (2016) 83:1118–20. doi: 10.1016/j.gie.2016.02.027
22. Hruban RH, Adsay NV, Albores-Saavedra J, Compton C, Garrett ES, Goodman SN, et al. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. *Am J Surg Pathol.* (2001) 25:579–86. doi: 10.1097/00000478-200105000-00003
23. Distler M, Aust D, Weitz J, Pilarsky C, Grutzmann R. Precursor lesions for sporadic pancreatic cancer: PanIN, IPMN, and MCN. *Biomed Res Int.* (2014) 2014:474905. doi: 10.1155/2014/474905
24. Zhang X, Shi S, Zhang B, Ni Q, Yu X, Xu J. Circulating biomarkers for early diagnosis of pancreatic cancer: facts and hopes. *Am J Cancer Res.* (2018) 8:332–53.
25. Appel BL, Tolat P, Evans DB, Tsai S. Current staging systems for pancreatic cancer. *Cancer J.* (2012) 18:539–49. doi: 10.1097/PPO.0b013e318278c5b5
26. Conrad C, Fernandez-Del Castillo C. Preoperative evaluation and management of the pancreatic head mass. *J Surg Oncol.* (2013) 107:23–32. doi: 10.1002/jso.23165
27. Fusaroli P, Kypraios D, Caletti G, Eloubeidi MA. Pancreatico-biliary endoscopic ultrasound: a systematic review of the levels of evidence, performance and outcomes. *World J Gastroenterol.* (2012) 18:4243–56. doi: 10.3748/wjg.v18.i32.4243
28. Karmazanovsky G, Fedorov V, Kubyshkin V, Kotchatkov A. Pancreatic head cancer: accuracy of CT in determination of resectability. *Abdom Imaging* (2005) 30:488–500. doi: 10.1007/s00261-004-0279-z
29. Lee ES, Lee JM. Imaging diagnosis of pancreatic cancer: a state-of-the-art review. *World J Gastroenterol.* (2014) 20:7864–77. doi: 10.3748/wjg.v20.i24.7864
30. Mohammad Alizadeh AH, Shahrokh S, Hadizadeh M, Padashi M, Zali MR. Diagnostic potency of EUS-guided FNA for the evaluation of pancreatic mass lesions. *Endosc Ultrasound.* (2016) 5:30–4. doi: 10.4103/2303-9027.175879
31. Canto MI. Screening and surveillance approaches in familial pancreatic cancer. *Gastrointest Endosc Clin N Am.* (2008) 18:535–553. doi: 10.1016/j.giec.2008.05.012
32. Bronstein YL, Loyer EM, Kaur H, Choi H, David C, DuBrow RA, et al. Detection of small pancreatic tumors with multiphasic helical CT. *AJR Am J Roentgenol.* (2004) 182:619–23. doi: 10.2214/ajr.182.3.1820619
33. Canto MI, Hruban RH, Fishman EK, Kamel IR, Schulick R, Zhang Z, et al. Frequent detection of pancreatic lesions in asymptomatic high-risk individuals. *Gastroenterology* (2012) 142:796–804. doi: 10.1053/j.gastro.2012.01.005
34. Balci NC, Perman WH, Saglam S, Akisik F, Fattahi R, Bilgin M. Diffusion-weighted magnetic resonance imaging of the pancreas. *Top Magn Reson Imaging* (2009) 20:43–7. doi: 10.1097/RMR.0b013e3181b48667
35. Agarwal B, Abu-Hamda E, Molke KL, Correa AM, Ho L. Endoscopic ultrasound-guided fine needle aspiration and multidetector spiral CT in the diagnosis of pancreatic cancer. *Am J Gastroenterol.* (2004) 99:844–50. doi: 10.1111/j.1572-0241.2004.04177.x
36. DeWitt J, Devereaux B, Chriswell M, McGreevy K, Howard T, Imperiale TF, et al. Comparison of endoscopic ultrasonography and multidetector computed tomography for detecting and staging pancreatic cancer. *Ann Intern Med.* (2004) 141:753–63. doi: 10.7326/0003-4819-141-10-200411160-00006
37. D'Onofrio M, Canestrini S, De Robertis R, Crosara S, Demozzi E, Ciaravino V, et al. CEUS of the pancreas: Still research or the standard of care. *Eur J Radiol.* (2015) 84:1644–9. doi: 10.1016/j.ejrad.2015.02.021
38. Larino Noia J. [Pancreatic tumors: recent developments]. *Gastroenterol Hepatol.* (2014) 37(Suppl. 3):98–106. doi: 10.1016/S0210-5705(14)70089-5
39. Giovannini M. Endoscopic ultrasound elastography. *Pancreatology* (2011) 11(Suppl. 2):34–9. doi: 10.1159/000323496
40. Giovannini M, Thomas B, Erwan B, Christian P, Fabrice C, Benjamin E, et al. Endoscopic ultrasound elastography for evaluation of lymph nodes and pancreatic masses: a multicenter study. *World J Gastroenterol.* (2009) 15:1587–93. doi: 10.3748/wjg.15.1587
41. Emerson RE, Randolph ML, Cramer HM. Endoscopic ultrasound-guided fine-needle aspiration cytology diagnosis of intraductal papillary mucinous

neoplasm of the pancreas is highly predictive of pancreatic neoplasia. *Diagn Cytopathol.* (2006) 34:457–62. doi: 10.1002/dc.20446
42. Qin SY, Zhou Y, Li P, Jiang HX. Diagnostic efficacy of cell block immunohistochemistry, smear cytology, and liquid-based cytology in endoscopic ultrasound-guided fine-needle aspiration of pancreatic lesions: a single-institution experience. *PLoS ONE* (2014) 9:e108762. doi: 10.1371/journal.pone.0108762
43. Brand RE, Adai AT, Centeno BA, Lee LS, Rateb G, Vignesh S, et al. A microRNA-based test improves endoscopic ultrasound-guided cytologic diagnosis of pancreatic cancer. *Clin Gastroenterol Hepatol.* (2014) 12:1717–23. doi: 10.1016/j.cgh.2014.02.038
44. Fuccio L, Hassan C, Laterza L, Correale L, Pagano N, Bocus P, et al. The role of K-ras gene mutation analysis in EUS-guided FNA cytology specimens for the differential diagnosis of pancreatic solid masses: a meta-analysis of prospective studies. *Gastrointest Endosc.* (2013) 78:596–608. doi: 10.1016/j.gie.2013.04.162
45. Jabbar KS, Verbeke C, Hyltander AG, Sjovall H, Hansson GC, Sadik R. Proteomic mucin profiling for the identification of cystic precursors of pancreatic cancer. *J Natl Cancer Inst.* (2014) 106:djt439. doi: 10.1093/jnci/djt439
46. Robles-Medranda C. Confocal endomicroscopy: Is it time to move on? *World J Gastrointest Endosc.* (2016) 8:1–3. doi: 10.4253/wjge.v8.i1.1
47. Tempero MA, Malafa MP, Al-Hawary M, Asbun H, Bain A, Behrman SW, et al. Pancreatic adenocarcinoma, version 2.2017, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw.* (2017) 15:1028–61. doi: 10.6004/jnccn.2017.0131
48. Brentnall TA, Bronner MP, Byrd DR, Haggitt RC, Kimmey MB. Early diagnosis and treatment of pancreatic dysplasia in patients with a family history of pancreatic cancer. *Ann Intern Med.* (1999) 131:247–55. doi: 10.7326/0003-4819-131-4-199908170-00003
49. Bunger S, Laubert T, Roblick UJ, Habermann JK. Serum biomarkers for improved diagnostic of pancreatic cancer: a current overview. *J Cancer Res Clin Oncol.* (2011) 137:375–89. doi: 10.1007/s00432-010-0965-x
50. Ritts RE, Pitt HA. CA 19-9 in pancreatic cancer. *Surg Oncol Clin N Am.* (1998) 7:93–101. doi: 10.1016/S1055-3207(18)30286-2
51. Safi F, Schlosser W, Falkenreck S, Beger HG. CA 19-9 serum course and prognosis of pancreatic cancer. *Int J Pancreatol.* (1996) 20:155–61. doi: 10.1007/BF02803763
52. Goggins M. Identifying molecular markers for the early detection of pancreatic neoplasia. *Semin Oncol.* (2007) 34:303–10. doi: 10.1053/j.seminoncol.2007.05.003
53. Mann DV, Edwards R, Ho S, Lau WY, Glazer G. Elevated tumour marker CA19-9: clinical interpretation and influence of obstructive jaundice. *Eur J Surg Oncol.* (2000) 26:474–9. doi: 10.1053/ejso.1999.0925
54. Rosty C, Goggins M. Early detection of pancreatic carcinoma. *Hematol Oncol Clin North Am.* (2002) 16:37–52. doi: 10.1016/S0889-8588(01)00007-7
55. Lamerz R. Role of tumour markers, cytogenetics. *Ann Oncol.* (1999) 10(Suppl. 4):145–9. doi: 10.1093/annonc/10.suppl_4.S145
56. American gastroenterological association medical position statement: epidemiology, diagnosis, and treatment of pancreatic ductal adenocarcinoma. *Gastroenterology* (1999) 117:1463–84. doi: 10.1016/S0016-5085(99)70297-0
57. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev.* (2006) 20:1218–49. doi: 10.1101/gad.1415606
58. Pitt HA. Curative treatment for pancreatic neoplasms. Standard resection. *Surg Clin North Am.* (1995) 75:891–904. doi: 10.1016/S0039-6109(16)46734-4
59. Brenner DJ, Hall EJ. Computed tomography–an increasing source of radiation exposure. *N Engl J Med.* (2007) 357:2277–84. doi: 10.1056/NEJMra072149
60. Giovinazzo F, Turri G, Zanini S, Butturini G, Scarpa A, Bassi C. Clinical implications of biological markers in Pancreatic Ductal Adenocarcinoma. *Surg Oncol.* (2012) 21:e171–182. doi: 10.1016/j.suronc.2012.07.004
61. Guo J, Wang W, Liao P, Lou W, Ji Y, Zhang C, et al. Identification of serum biomarkers for pancreatic adenocarcinoma by proteomic analysis. *Cancer Sci.* (2009) 100:2292–301. doi: 10.1111/j.1349-7006.2009.01324.x
62. Hidalgo M. Pancreatic cancer. *N Engl J Med.* (2010) 362:1605–17. doi: 10.1056/NEJMra0901557
63. Kaur S, Baine MJ, Jain M, Sasson AR, Batra SK. Early diagnosis of pancreatic cancer: challenges and new developments. *Biomark Med.* (2012) 6:597–612. doi: 10.2217/bmm.12.69
64. Kelly KA, Hollingsworth MA, Brand RE, Liu CH, Singh VK, Srivastava S, et al. Advances in biomedical imaging, bioengineering, and related technologies for the development of biomarkers of pancreatic disease: summary of a National Institute of Diabetes and Digestive and Kidney Diseases and National Institute of Biomedical Imaging and Bioengineering Workshop. *Pancreas* (2015) 44:1185–94. doi: 10.1097/MPA.0000000000000552
65. Sahani DV, Shah ZK, Catalano OA, Boland GW, Brugge WR. Radiology of pancreatic adenocarcinoma: current status of imaging. *J Gastroenterol Hepatol.* (2008) 23:23–33. doi: 10.1111/j.1440-1746.2007.05117.x
66. Lu L, Risch HA. Exosomes: potential for early detection in pancreatic cancer. *Future Oncol.* (2016) 12:1081–90. doi: 10.2217/fon-2015-0005
67. Nuzhat Z, Kinhal V, Sharma S, Rice GE, Joshi V, Salomon C. Tumour-derived exosomes as a signature of pancreatic cancer - liquid biopsies as indicators of tumour progression. *Oncotarget* (2017) 8:17279–91. doi: 10.18632/oncotarget.13973
68. Zhou B, Xu JW, Cheng YG, Gao JY, Hu SY, Wang L, et al. Early detection of pancreatic cancer: Where are we now and where are we going? *Int J Cancer* (2017) 141:231–41. doi: 10.1002/ijc.30670
69. Steinberg WM, Barkin JS, Bradley EL 3rd, DiMagno E, Layer P, Canto MI, et al. Should patients with a strong family history of pancreatic cancer be screened on a periodic basis for cancer of the pancreas? *Pancreas* (2009) 38:e137–50. doi: 10.1097/MPA.0b013e3181a86b2c
70. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. *Nat Rev Clin Oncol.* (2013) 10:472–84. doi: 10.1038/nrclinonc.2013.110
71. Diaz LA Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol.* (2014) 32:579–86. doi: 10.1200/JCO.2012.45.2011
72. Yadav DK, Bai X, Yadav RK, Singh A, Li G, Ma T, et al. Liquid biopsy in pancreatic cancer: the beginning of a new era. *Oncotarget* (2018) 9:26900–33. doi: 10.18632/oncotarget.24809
73. Zhou X, Lu Z, Wang T, Huang Z, Zhu W, Miao Y. Plasma miRNAs in diagnosis and prognosis of pancreatic cancer: a miRNA expression analysis. *Gene* (2018) 673:181–93. doi: 10.1016/j.gene.2018.06.037
74. Pan S, Chen R, Crispin DA, May D, Stevens T, McIntosh MW, et al. Protein alterations associated with pancreatic cancer and chronic pancreatitis found in human plasma using global quantitative proteomics profiling. *J Proteome Res.* (2011) 10:2359–76. doi: 10.1021/pr101148r
75. Bailey P, Chang DK, Nones K, Johns AL, Patch AM, Gingras MC, et al. Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature* (2016) 531:47–52. doi: 10.1038/nature16965
76. Collisson EA, Sadanandam A, Olson P, Gibb WJ, Truitt M, Gu S, et al. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med.* (2011) 17:500–3. doi: 10.1038/nm.2344
77. Moffitt RA, Marayati R, Flate EL, Volmar KE, Loeza SG, Hoadley KA, et al. Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma. *Nat Genet.* (2015) 47:1168–78. doi: 10.1038/ng.3398
78. Waddell N, Pajic M, Patch AM, Chang DK, Kassahn KS, Bailey P, et al. Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature* (2015) 518:495–501. doi: 10.1038/nature14169
79. Wood LD, Hruban RH. Pathology and molecular genetics of pancreatic neoplasms. *Cancer J.* (2012) 18:492–501. doi: 10.1097/PPO.0b013e31827459b6
80. Anker P, Mulcahy H, Chen XQ, Stroun M. Detection of circulating tumour DNA in the blood (plasma/serum) of cancer patients. *Cancer Metastasis Rev.* (1999) 18:65–73. doi: 10.1023/A:1006260319913
81. Cheng F, Su L, Qian C. Circulating tumor DNA: a promising biomarker in the liquid biopsy of cancer. *Oncotarget* (2016) 7:48832–41. doi: 10.18632/oncotarget.9453
82. Rahbari M, Rahbari N, Reissfelder C, Weitz J, Kahlert C. Exosomes: novel implications in diagnosis and treatment of gastrointestinal cancer. *Langenbecks Arch Surg.* (2016) 401:1097–110. doi: 10.1007/s00423-016-1468-2

83. Beroukhim R, Lin M, Park Y, Hao K, Zhao X, Garraway LA, et al. Inferring loss-of-heterozygosity from unpaired tumors using high-density oligonucleotide SNP arrays. *PLoS Comput Biol.* (2006) 2:e41. doi: 10.1371/journal.pcbi.0020041
84. Calhoun ES, Hucl T, Gallmeier E, West KM, Arking DE, Maitra A, et al. Identifying allelic loss and homozygous deletions in pancreatic cancer without matched normals using high-density single-nucleotide polymorphism arrays. *Cancer Res.* (2006) 66:7920–8. doi: 10.1158/0008-5472.CAN-06-0721
85. Campbell PJ, Yachida S, Mudie LJ, Stephens PJ, Pleasance ED, Stebbings LA, et al. The patterns and dynamics of genomic instability in metastatic pancreatic cancer. *Nature* (2010) 467:1109–13. doi: 10.1038/nature09460
86. Greaves M, Maley CC. Clonal evolution in cancer. *Nature* (2012) 481:306–13. doi: 10.1038/nature10762
87. Harada T, Chelala C, Bhakta V, Chaplin T, Caulee K, Baril P, et al. Genome-wide DNA copy number analysis in pancreatic cancer using high-density single nucleotide polymorphism arrays. *Oncogene* (2008) 27:1951–60. doi: 10.1038/sj.onc.1210832
88. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* (2008) 321:1801–6. doi: 10.1126/science.1164368
89. Karhu R, Mahlamaki E, Kallioniemi A. Pancreatic adenocarcinoma – genetic portrait from chromosomes to microarrays. *Genes Chromosomes Cancer* (2006) 45:721–30. doi: 10.1002/gcc.20337
90. Makohon-Moore A, Iacobuzio-Donahue CA. Pancreatic cancer biology and genetics from an evolutionary perspective. *Nat Rev Cancer* (2016) 16:553–65. doi: 10.1038/nrc.2016.66
91. Allenson K, Castillo J, San Lucas FA, Scelo G, Kim DU, Bernard V, et al. High prevalence of mutant KRAS in circulating exosome-derived DNA from early-stage pancreatic cancer patients. *Ann Oncol.* (2017) 28:741–7. doi: 10.1093/annonc/mdx004
92. Lianidou ES, Mavroudis D, Sotiropoulou G, Agelaki S, Pantel K. What's new on circulating tumor cells? A meeting report. *Breast Cancer Res.* (2010) 12:307. doi: 10.1186/bcr2601
93. Lim SH, Becker TM, Chua W, Caixeiro NJ, Ng WL, Kienzle N, et al. Circulating tumour cells and circulating free nucleic acid as prognostic and predictive biomarkers in colorectal cancer. *Cancer Lett.* (2014) 346:24–33. doi: 10.1016/j.canlet.2013.12.019
94. Shigeyasu K, Toden S, Zumwalt TJ, Okugawa Y, Goel A. Emerging role of MicroRNAs as liquid biopsy biomarkers in gastrointestinal cancers. *Clin Cancer Res.* (2017) 23:2391–9. doi: 10.1158/1078-0432.CCR-16-1676
95. Thiele JA, Bethel K, Kralickova M, Kuhn P. Circulating tumor cells: fluid surrogates of solid tumors. *Annu Rev Pathol.* (2017) 12:419–47. doi: 10.1146/annurev-pathol-052016-100256
96. Vendrell JA, Mau-Them FT, Beganton B, Godreuil S, Coopman P, Solassol J. Circulating cell free tumor DNA detection as a routine tool for lung cancer patient management. *Int J Mol Sci.* (2017) 18:264. doi: 10.3390/ijms18020264
97. Barros FM, Carneiro F, Machado JC, Melo SA. Exosomes and immune response in cancer: friends or foes? *Front Immunol.* (2018) 9:730. doi: 10.3389/fimmu.2018.00730
98. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, et al. Tumour exosome integrins determine organotropic metastasis. *Nature* (2015) 527:329–35. doi: 10.1038/nature15756
99. Melo SA, Sugimoto H, O'Connell JT, Kato N, Villanueva A, Vidal A, et al. Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. *Cancer Cell* (2014) 26:707–21. doi: 10.1016/j.ccell.2014.09.005
100. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol.* (2014) 14:195–208. doi: 10.1038/nri3622
101. Ruivo CF, Adem B, Silva M, Melo SA. The biology of cancer exosomes: insights and new perspectives. *Cancer Res.* (2017) 77:6480–8. doi: 10.1158/0008-5472.CAN-17-0994
102. Yang S, Che SP, Kurywchak P, Tavormina JL, Gansmo LB, Correa de Sampaio P, et al. Detection of mutant KRAS and TP53 DNA in circulating exosomes from healthy individuals and patients with pancreatic cancer. *Cancer Biol Ther.* (2017) 18:158–65. doi: 10.1080/15384047.2017.1281499
103. Azmi AS, Bao B, Sarkar FH. Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. *Cancer Metastasis Rev.* (2013) 32:623–42. doi: 10.1007/s10555-013-9441-9
104. Qiu J, Yang G, Feng M, Zheng S, Cao Z, You L, et al. Extracellular vesicles as mediators of the progression and chemoresistance of pancreatic cancer and their potential clinical applications. *Mol Cancer* (2018) 17:2. doi: 10.1186/s12943-017-0755-z
105. Lo Cicero A, Stahl PD, Raposo G. Extracellular vesicles shuffling intercellular messages: for good or for bad. *Curr Opin Cell Biol.* (2015) 35:69–77. doi: 10.1016/j.ceb.2015.04.013
106. Zhao C, Gao F, Weng S, Liu Q. Pancreatic cancer and associated exosomes. *Cancer Biomarkers* (2017) 20:357–67. doi: 10.3233/CBM-170258
107. Castillo J, Bernard V, San Lucas FA, Allenson K, Capello M, Kim DU, et al. Surfaceome profiling enables isolation of cancer-specific exosomal cargo in liquid biopsies from pancreatic cancer patients. *Ann Oncol.* (2018) 29:223–9. doi: 10.1093/annonc/mdx542
108. Armstrong EA, Beal EW, Chakedis J, Paredes AZ, Moris D, Pawlik TM, et al. Exosomes in pancreatic cancer: from early detection to treatment. *J Gastrointest Surg.* (2018) 22:737–50. doi: 10.1007/s11605-018-3693-1
109. Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, et al. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem.* (2009) 284:34211–22. doi: 10.1074/jbc.M109.041152
110. Smyth TJ, Redzic JS, Graner MW, Anchordoquy TJ. Examination of the specificity of tumor cell derived exosomes with tumor cells in vitro. *Biochim Biophys Acta* (2014) 1838:2954–65. doi: 10.1016/j.bbamem.2014.07.026
111. Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol.* (2015) 17:816–26. doi: 10.1038/ncb3169
112. Charrier A, Chen R, Chen L, Kemper S, Hattori T, Takigawa M, et al. Connective tissue growth factor (CCN2) and microRNA-21 are components of a positive feedback loop in pancreatic stellate cells (PSC) during chronic pancreatitis and are exported in PSC-derived exosomes. *J Cell Commun Signal.* (2014) 8:147–56. doi: 10.1007/s12079-014-0220-3
113. Goto T, Fujiya M, Konishi H, Sasajima J, Fujibayashi S, Hayashi A, et al. An elevated expression of serum exosomal microRNA-191, - 21,−451a of pancreatic neoplasm is considered to be efficient diagnostic marker. *BMC Cancer* (2018) 18:116. doi: 10.1186/s12885-018-4006-5
114. Schey KL, Luther JM, Rose KL. Proteomics characterization of exosome cargo. *Methods* (2015) 87:75–82. doi: 10.1016/j.ymeth.2015.03.018
115. Silva M, Melo SA. Non-coding RNAs in exosomes: new players in cancer biology. *Curr Genomics* (2015) 16:295–303. doi: 10.2174/1389202916666150707154719
116. Ayars M, Goggins M. Pancreatic cancer: classifying pancreatic cancer using gene expression profiling. *Nat Rev Gastroenterol Hepatol.* (2015) 12:613–4. doi: 10.1038/nrgastro.2015.180
117. Bastos N, Melo SA. Quantitative analysis of precursors MicroRNAs and their respective mature MicroRNAs in cancer exosomes overtime. *Methods Mol Biol.* (2018) 1733:137–43. doi: 10.1007/978-1-4939-7601-0_11
118. Zeringer E, Li M, Barta T, Schageman J, Pedersen KW, Neurauter A, et al. Methods for the extraction and RNA profiling of exosomes. *World J Methodol.* (2013) 3:11–8. doi: 10.5662/wjm.v3.i1.11
119. Andre Mdo R, Pedro A, Lyden D. Cancer exosomes as mediators of drug resistance. *Methods Mol Biol.* (2016) 1395:229–39. doi: 10.1007/978-1-4939-3347-1_13
120. Bastos N, Ruivo CF, da Silva S, Melo SA. Exosomes in cancer: use them or target them? *Semin Cell Dev Biol.* (2018) 78:13–21. doi: 10.1016/j.semcdb.2017.08.009
121. Friedman A, Hao W. The role of exosomes in pancreatic cancer microenvironment. *Bull Math Biol.* (2018) 80:1111–33. doi: 10.1007/s11538-017-0254-9
122. Javeed N, Sagar G, Dutta SK, Smyrk TC, Lau JS, Bhattacharya S, et al. Pancreatic cancer-derived exosomes cause paraneoplastic beta-cell dysfunction. *Clin Cancer Res.* (2015) 21:1722–33. doi: 10.1158/1078-0432.CCR-14-2022
123. Jin H, Wu Y, Tan X. The role of pancreatic cancer-derived exosomes in cancer progress and their potential application as biomarkers. *Clin Transl Oncol.* (2017) 19:921–30. doi: 10.1007/s12094-017-1625-2

124. Krantz BA, O'Reilly EM. Biomarker-based therapy in pancreatic ductal adenocarcinoma: an emerging reality? *Clin Cancer Res.* (2018) 24:2241–50. doi: 10.1158/1078-0432.CCR-16-3169
125. Masamune A, Yoshida N, Hamada S, Takikawa T, Nabeshima T, Shimosegawa T. Exosomes derived from pancreatic cancer cells induce activation and profibrogenic activities in pancreatic stellate cells. *Biochem Biophys Res Commun.* (2018) 495:71–7. doi: 10.1016/j.bbrc.2017.10.141
126. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in exosome isolation techniques. *Theranostics* (2017) 7:789–804. doi: 10.7150/thno.18133
127. Yan Y, Fu G, Ming L. Role of exosomes in pancreatic cancer. *Oncol Lett.* (2018) 15:7479–88. doi: 10.3892/ol.2018.8348
128. Que R, Ding G, Chen J, Cao L. Analysis of serum exosomal microRNAs and clinicopathologic features of patients with pancreatic adenocarcinoma. *World J Surg Oncol.* (2013) 11:219. doi: 10.1186/1477-7819-11-219
129. Joshi GK, Deitz-McElyea S, Liyanage T, Lawrence K, Mali S, Sardar R, et al. Label-free nanoplasmonic-based short noncoding RNA sensing at attomolar concentrations allows for quantitative and highly specific assay of MicroRNA-10b in biological fluids and circulating exosomes. *ACS Nano* (2015) 9:11075–89. doi: 10.1021/acsnano.5b04527
130. Madhavan B, Yue S, Galli U, Rana S, Gross W, Muller M, et al. Combined evaluation of a panel of protein and miRNA serum-exosome biomarkers for pancreatic cancer diagnosis increases sensitivity and specificity. *Int J Cancer* (2015) 136:2616–27. doi: 10.1002/ijc.29324
131. Ali S, Dubaybo H, Brand RE, Sarkar FH. Differential expression of MicroRNAs in tissues and plasma co-exists as a biomarker for pancreatic cancer. *J Cancer Sci Ther.* (2015) 7:336–46. doi: 10.4172/1948-5956.1000372
132. Mikamori M, Yamada D, Eguchi H, Hasegawa S, Kishimoto T, Tomimaru Y, et al. MicroRNA-155 controls exosome synthesis and promotes gemcitabine resistance in pancreatic ductal adenocarcinoma. *Sci Rep.* (2017) 7:42339. doi: 10.1038/srep42339
133. Pang W, Su J, Wang Y, Feng H, Dai X, Yuan Y, et al. Pancreatic cancer-secreted miR-155 implicates in the conversion from normal fibroblasts to cancer-associated fibroblasts. *Cancer Sci.* (2015) 106:1362–9. doi: 10.1111/cas.12747
134. Rana S, Malinowska K, Zoller M. Exosomal tumor microRNA modulates premetastatic organ cells. *Neoplasia* (2013) 15:281–95. doi: 10.1593/neo.122010
135. Taucher V, Mangge H, Haybaeck J. Non-coding RNAs in pancreatic cancer: challenges and opportunities for clinical application. *Cell Oncol.* (2016) 39:295–318. doi: 10.1007/s13402-016-0275-7
136. Tian S, Guo X, Yu C, Sun C, Jiang J. miR-138-5p suppresses autophagy in pancreatic cancer by targeting SIRT1. *Oncotarget* (2017) 8:11071–82. doi: 10.18632/oncotarget.14360
137. Yu C, Wang M, Li Z, Xiao J, Peng F, Guo X, et al. MicroRNA-138-5p regulates pancreatic cancer cell growth through targeting FOXC1. *Cell Oncol.* (2015) 38:173–81. doi: 10.1007/s13402-014-0200-x
138. Zoller M. Pancreatic cancer diagnosis by free and exosomal miRNA. *World J Gastrointest Pathophysiol.* (2013) 4:74–90. doi: 10.4291/wjgp.v4.i4.74
139. Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* (2015) 523:177–82. doi: 10.1038/nature14581
140. Frampton AE, Prado MM, Lopez-Jimenez E, Fajardo-Puerta AB, Jawad ZAR, Lawton P, et al. Glypican-1 is enriched in circulating-exosomes in pancreatic cancer and correlates with tumor burden. *Oncotarget* (2018) 9:19006–13. doi: 10.18632/oncotarget.24873
141. Qian JY, Tan YL, Zhang Y, Yang YF, Li XQ. Prognostic value of glypican-1 for patients with advanced pancreatic cancer following regional intra-arterial chemotherapy. *Oncol Lett.* (2018) 16:1253–8. doi: 10.3892/ol.2018.8701
142. Etayash H, McGee AR, Kaur K, Thundat T. Nanomechanical sandwich assay for multiple cancer biomarkers in breast cancer cell-derived exosomes. *Nanoscale* (2016) 8:15137–41. doi: 10.1039/C6NR03478K
143. Hu J, Sheng Y, Kwak KJ, Shi J, Yu B, Lee LJ. A signal-amplifiable biochip quantifies extracellular vesicle-associated RNAs for early cancer detection. *Nat Commun.* (2017) 8:1683. doi: 10.1038/s41467-017-01942-1
144. Lewis JM, Vyas AD, Qiu Y, Messer KS, White R, Heller MJ. Integrated Analysis of Exosomal Protein Biomarkers on Alternating Current Electrokinetic Chips Enables Rapid Detection of Pancreatic Cancer in Patient Blood. *ACS Nano* (2018) 12:3311–20. doi: 10.1021/acsnano.7b08199
145. Li J, Chen Y, Guo X, Zhou L, Jia Z, Peng Z, et al. GPC1 exosome and its regulatory miRNAs are specific markers for the detection and target therapy of colorectal cancer. *J Cell Mol Med.* (2017) 21:838–47. doi: 10.1111/jcmm.12941
146. Li J, Li B, Ren C, Chen Y, Guo X, Zhou L, et al. The clinical significance of circulating GPC1 positive exosomes and its regulative miRNAs in colon cancer patients. *Oncotarget* (2017) 8:101189–202. doi: 10.18632/oncotarget.20516
147. Lu H, Niu F, Liu F, Gao J, Sun Y, Zhao X. Elevated glypican-1 expression is associated with an unfavorable prognosis in pancreatic ductal adenocarcinoma. *Cancer Med.* (2017) 6:1181–91. doi: 10.1002/cam4.1064
148. Yang KS, Im H, Hong S, Pergolini I, Del Castillo AF, Wang R, et al. Multiparametric plasma EV profiling facilitates diagnosis of pancreatic malignancy. *Sci Transl Med.* (2017) 9:eaal3226. doi: 10.1126/scitranslmed.aal3226
149. Moutinho-Ribeiro P, Melo S, Macedo G. Glypican-1 circulating exosomes: a promising clue to individualize surveillance of pancreatic cysts? *Eur Radiol.* (2018) 28:3018–9. doi: 10.1007/s00330-018-5321-6
150. Kamerkar S, LeBleu VS, Sugimoto H, Yang S, Ruivo CF, Melo SA, et al. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* (2017) 546:498–503. doi: 10.1038/nature22341
151. van den Boorn JG, Dassler J, Coch C, Schlee M, Hartmann G. Exosomes as nucleic acid nanocarriers. *Adv Drug Deliv Rev.* (2013) 65:331–5. doi: 10.1016/j.addr.2012.06.011
152. Bellavia D, Raimondo S, Calabrese G, Forte S, Cristaldi M, Patinella A, et al. Interleukin 3- receptor targeted exosomes inhibit *in vitro* and *in vivo* Chronic Myelogenous Leukemia cell growth. *Theranostics* (2017) 7:1333–45. doi: 10.7150/thno.17092
153. Kourembanas S. Exosomes: vehicles of intercellular signaling, biomarkers, and vectors of cell therapy. *Annu Rev Physiol.* (2015) 77:13–27. doi: 10.1146/annurev-physiol-021014-071641

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Hallmarks of Aging: An Autophagic Perspective

María Carolina Barbosa[1], *Rubén Adrián Grosso*[1] *and Claudio Marcelo Fader*[1,2]*

[1] *Laboratorio de Biología Celular y Molecular, Instituto de Histología y Embriología (IHEM), Universidad Nacional de Cuyo, CONICET, Mendoza, Argentina,* [2] *Facultad de Odontología, Universidad Nacional de Cuyo, Mendoza, Argentina*

Autophagy is a major protein turnover pathway by which cellular components are delivered into the lysosomes for degradation and recycling. This intracellular process is able to maintain cellular homeostasis under stress conditions, and its dysregulation could lead to the development of physiological alterations. The autophagic activity has been found to decrease with age, likely contributing to the accumulation of damaged macromolecules and organelles during aging. Interestingly, failure of the autophagic process has been reported to worsen aging-associated diseases, such as neurodegeneration or cancer, among others. Likewise, it has been proposed in different organisms that maintenance of a proper autophagic activity contributes to extending longevity. In this review, we discuss recent papers showing the impact of autophagy on cell activity and age-associated diseases, highlighting the relevance of this process to the hallmarks of aging. Thus, understanding how autophagy plays an important role in aging opens new avenues for the discovery of biochemical and pharmacological targets and the development of novel anti-aging therapeutic approaches.

Keywords: autophagy, ROS, aging, hallmarks of aging, mitophagy

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***Correspondence:**
Claudio Marcelo Fader
cfader@fcm.uncu.edu.ar

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THE AUTOPHAGIC PROCESS

Autophagy, literally meaning "self-eating," is an evolutionarily conserved catabolic process in eukaryotic cells by means of which intracellular components and extracellular incorporated material are delivered into lysosomes, where their degradation occurs (1). Since its discovery, autophagy has been associated with the maintenance of cellular homeostasis, as well as the cytoplasmic quality control process (1, 2). Its dysregulation has being related to a diversity of pathological or physiological processes such as neurodegenerative, infectious, and metabolic disorders, as well as cancer and aging, among others (3–5). Several studies have demonstrated that autophagy can be very selective in targeting its cargo for degradation. Three major types of autophagy have been identified: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy (hereafter referred to as autophagy) begins with the extension of a specialized membrane, known as the phagophore, derived from the endoplasmic reticulum (ER), the mitochondria, and the Golgi cisternae (6, 7). The phagophore engulfs the molecules and organelles to be eliminated, forming a double membrane vesicle called autophagosome (7, 8). Finally, autophagosomes are targeted to lysosomes and fusion occurs, the sequestered material is degraded and released back into the cytosol (8). In microautophagy, the lysosome picks up cytosolic components directly via invagination of the lysosomal membrane (9). On the other hand, CMA is a process involving the direct transport of cytosolic components across the lysosomal membrane via chaperone proteins. Several studies have demonstrated that CMA is a highly regulated and degradative event, involving HSC70 (heat shock protein 70 complex) and multimerization of

the LAMP2A receptor (lysosome-associated membrane protein type 2A). Interestingly, not all proteins are able to be CMA substrates. To undergo CMA degradation, proteins must contain a KFERQ motif in their amino acid sequences, which is necessary to bind the chaperone HSC70 (10, 11). Substrate and the HSC70 complex can bind a 12-amino-acid cytosolic tail of LAMP2A for lysosomal docking. In addition, LAMP2A multimerization is necessary for substrate translocation into the lysosomal lumen. Cytosolic HSC70 is released from the multimeric complex, and then a chaperone HSP90 (located at the lumen of the lysosomal membrane) interacts with LAMP2A, stabilizing it during the substrate translocation. Finally, a luminal chaperone HSC70 is required to end the translocation process, and once inside the targeted protein is degraded by the lysosomal enzymes (12) (**Figure 1**).

Autophagy can be induced by a variety of stressors, and nutrient restriction is one of the major stimuli, capable of rapidly activating the autophagic process with the concomitant inhibition of protein synthesis (1). Studies in both yeasts and mammals have characterized at least 40 autophagy-related genes (Atg), which encode proteins that participate in autophagy (13). In addition, the canonical autophagy pathway includes the inactivation of mammalian target of rapamycin complex 1 (mTORC1), allowing the phosphorylation and activation of the Unc-51-like kinase complex (Ulk1/2), with the subsequent cascade activation of the other ULK complex members such as FIP200 and ATG13 (14, 15). Another important complex that is activated is the BECLIN1, in which VPS34, one of its members, is translocated into the ER membranes and it produces high levels of phosphatidylinositol-3-phosphate, which is necessary for the recruitment of other effectors such as WIPI2b (16). Next, this effector interacts and recruits ATG16L, which binds ATG5-ATG12 conjugated to generate the ATG12-ATG5-ATG16L complex. This complex is required for the lipidation of LC3 (Microtubule-associated protein light chain 3), because it determines the site where LC3 will be conjugated and activated to LC3-II (17). Moreover, ATG3 (an E2-like protein) is associated with LC3-I and it binds to the complex through ATG12, allowing the conjugation of LC3-I with phosphatidylethanolamine to generate LC3-II. LC3-II, which is present in both inner and outer membranes of autophagosomal structures and is necessary for phagophore extension, cargo engulfment, and vesicle closure to form the autophagosome. Additionally, the targeted cargo is able to bind receptor/adaptor molecules like p62, NDP52, and NIX. These proteins contain a LC3 interacting region (LIR), which allows the recognition of elements to be engulfed by the phagophore and eliminated in an autophagic manner (18, 19).

In addition to degradation, autophagy, or part of its machinery, can mediate a regulated cell death, named autophagy-dependent cell death (ADCD). Moreover, autophagy can participate in other cell death types [reviewed in reference (20)]. Interestingly, despite the fact that regulated cell death of malignant cells is a pro-survival mechanism at the whole organism level, it can also lead to tissue degeneration and function loss, and this can reduce the fitness of the aged individual (21).

AGING

Aging, the natural event occurring in all living organisms, can be defined as a deterioration of the cell functioning due to damage accumulation over time (22–25). This is an important biological, demographic and socio-economic issue all over the world. Dr. Barja points out that all living organisms have different longevity, indicating that evolution has played an important role in regulation and flexibilization of aging between species, in a relatively fast process (26). The understanding of the molecular basis of aging and longevity could let us manipulate it somehow in the future. In this regard, in the last 50 years numerous investigations related to aging have emerged, trying to explain this unstoppable process.

Despite the general accepted concept that aging is a multifactorial process, several theories have emerged in an attempt to explain it as a single predominant age-related change. A popular aging theory is the "Stochastic Theory," which suggests that aging results from random damage accumulation. This can be due to external and internal sources over time, in addition to a failure of the repairing capacity. On the other hand, other theories support the idea that aging is a regulated process, mainly by the genetic code, such as the telomere length, the number of divisions that a somatic cell can go through (the "Hayflick limit") and spatio-temporal regulation of gene expression (27, 28). Nevertheless, one of the most popular theories is the Free Radicals (or Oxidative Stress) Theory of Aging, which hypothesizes that an accumulation of Reactive Oxygen Species (hereafter ROS) falls into an oxidative damage of biomolecules, with the consequent cell functioning decline (27–29). A considerable body of evidence supports this theory, because it points to an increase in ROS cellular levels as we age, due to a higher production of them as well as a failure in the anti-oxidant systems (30, 31).

AUTOPHAGY IN AGING

Several animal models have contributed to our understanding of how the impairment of autophagy and redox homeostasis can result in age-related diseases. In the same way, numerous studies involving genetic ablation or induction of autophagic genes have revealed the importance of this process in aging of yeast, nematodes, flies, and mammals (32). The most important work that links an overexpression of a single Atg gene with an increment in mammals' lifespan was conducted by Pyo and collaborators. The authors overexpressed Atg5 in mice and found an enhancement of the autophagy process and anti-aging features, compared with the wild type mice. The mean lifespan was also incremented, suggesting the importance of autophagy in the longevity of mice (33). Another approach that demonstrates the importance of autophagy in aging has been done in Ana María Cuervo's laboratory. In aged mice, they generated a double transgenic mouse model, in which it was possible to modulate the expression of the lysosomal receptor for CMA. The results revealed that the enhancement of this receptor can prevent features of aging at cellular and organ levels (34). In addition, mice overexpressing Atg5 showed a better resistance to

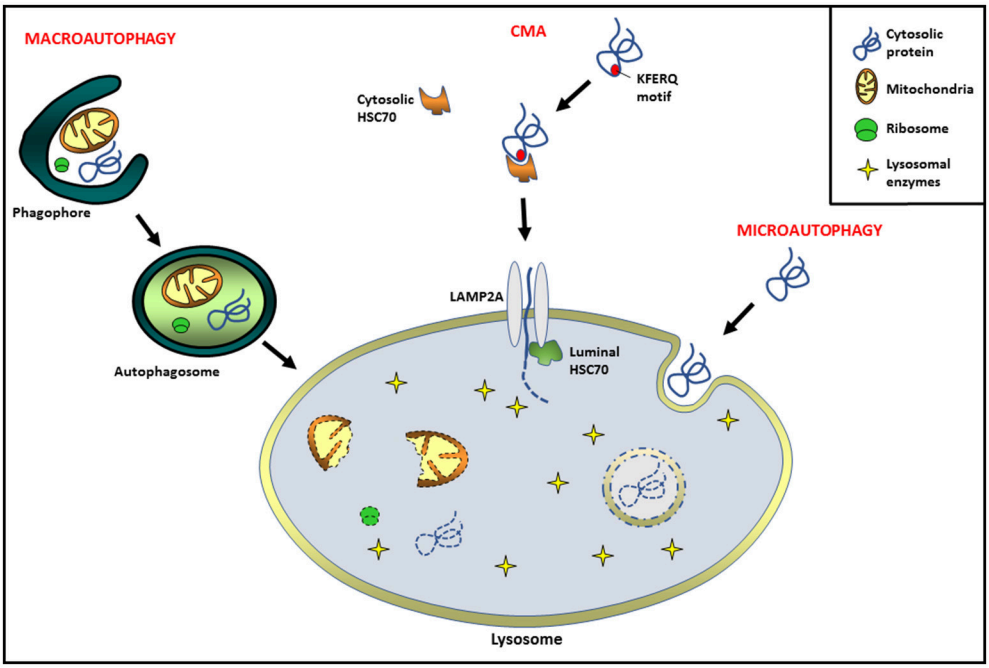

FIGURE 1 | Different types of autophagy pathways in mammals. Macroautophagy: extension of a specialized membrane (phagophore) surrounds molecules and organelles, forming a double membrane vesicle called autophagosome. Finally, the fusion of autophagosomes with lysosomes leads to cargo degradation. Chaperone-mediated autophagy (CMA): proteins containing a KFERQ motif are delivered to lysosome via cytosolic HSC70 chaperone complex. The receptor lysosome-associated membrane protein type 2A (LAMP2A) is necessary for substrate translocation into the lysosomal lumen, where the degradation occurs. Microautophagy: invagination of the lysosomal membrane engulfs cytosolic cargo in small vesicles for its degradation inside.

age-related obesity and enhanced insulin sensitivity, exhibiting an improved metabolism in aged individuals (33). Despite the mentioned studies, several others failed to demonstrate that upregulation of a single autophagic component can extend lifespan (32). Moreover, several KO mouse models have been shown to have extended lifespan, although the molecular mechanisms behind it and the connection with aging are not yet clear (35).

Notably, another relationship between autophagy augmentation and extended lifespan has been reported in exceptionally healthy centenarian humans, who have increased levels of BECLIN1, compared to young people (36). We hope that in the next years these preliminary studies in humans will be more advanced, providing insights into our species longevity mechanisms from clinical case studies.

The knockout for essential Atg genes is lethal in mice, and tissue-specific ablation has a less-dramatic phenotype, manifesting premature signs of aging (37). Specific-Atg5 or Atg7 KO leads to neurodegeneration or tissue abnormalities in most of the cases available in the literature [for a more detailed summary see reference (37)].

Finally, as we age, the incidence of cancer rises, probably because of the decline of homeostatic processes and the increase in the accumulation of potentially harmful molecules such as ROS and protein aggregates. Autophagy has been proposed to have a dual role in tumorigenesis, being important both in suppression as well as in tumor progression and surveillance (38, 39).

ROS GENERATION AND AGING

The ROS are considered metabolites of molecular oxygen during cellular respiration, being very reactive due to an unpaired electron (40). Mitochondria are the major ROS producers and perhaps the organelle most affected by them. In order to avoid detrimental effects of ROS, two important processes arise: Mitophagy and antioxidant system. Mitochondrial ROS can activate the autophagic pathway upon starvation by the activation of ATG4 (41), and this in turn leads to autophagic degradation of mitochondria (mitophagy) in order to reduce the ROS levels by limiting the number of mitochondria per cell (42). In addition, hypoxia and exercise can also trigger redox-dependent autophagy, suggesting that ROS might regulate the autophagic response to several stresses (43). Regarding the second process, the antioxidant system consists of several enzymes and molecules that react with ROS and neutralize them somehow, but the connection of antioxidants with lifespan is controversial (44). Notably, overexpression of a mitochondrial-targeted catalase in mice extends lifespan and reduces overall ROS, reinforcing the Free Radicals Theory of Aging in such model (45). Moreover, these mice showed a reduction in age-related pathologies (46). Additionally, Mn-superoxide dismutase (SOD2) heterozygous mice showed a life-long reduction, but surprisingly they did not have an accelerated aging phenotype. Nevertheless, this $Sod2^{+/-}$ mice showed a higher oxidative damage to DNA and had higher cancer incidence compared with wild type individuals (47). By contrast, knockout of 17 genes involved in the antioxidant system

exhibited no effect in lifespan: Only the knockout for Cu/Zn-superoxide dismutase (Sod1) resulted in a decrease in longevity and premature aging as well (48). This mouse model showed an increase in senescent markers, suggesting that the oxidative stress that $Sod1^{-/-}$ mice suffer leads to DNA damage, promoting an irreversible state of quiescence (49). In addition, these $Sod1^{-/-}$ mice showed an accelerated sarcopenia, manifesting muscular mass loss and altered neuromuscular junctions (50). Despite these controversial and unexpected results in mice, the relationship between antioxidants and their role in healthy or pathologic aging needs to be deeply studied in the future.

Finally, it is important to highlight that ROS have been proposed to be implicated in proliferation and survival signaling in certain conditions (42). A new concept has emerged recently in the aging field, termed "hormesis," according to which low doses of a stressor can improve the cell response for a more detrimental condition in the future (32, 51). This could increase lifespan and cellular fitness (52). In this context, low levels of ROS can be beneficial due to the trigger of homeostatic responses, but its disproportional augmentation can lead to damage or aging (42, 53). From an autophagic perspective, an augmentation in ROS levels and a decline in mitophagy occur simultaneously, leading to aging (43, 52, 54).

HALLMARKS OF AGING: AN AUTOPHAGIC VIEW

In the last years, aging has begun to be seen as an active and highly regulated process (55). Age-related changes at cellular level include an increase in ROS, loss of proteostasis, genome instability, and telomere exhaustion, among others (23, 56, 57). These characteristic features of aging were termed "hallmarks of aging" by López-Otín et al. (52). In the following sections, we discuss how autophagy plays an important role in some of these hallmarks of aging, in both health and disease.

Loss of Proteostasis

Proteostasis is one of the major functions of autophagy in normal tissues. Imbalance of proteostasis due to aging leads to protein aggregation, accumulation of misfolded proteins and in the end to cellular dysfunction, among others (23, 56, 57). Notably, carbonylation due to oxidative stress is one of the changes that leads to loss of proteostasis (44). To avoid cell death or dysfunction, numerous homeostatic mechanisms turn on, mainly autophagy (58) and the Ubiquitin-Proteasome-System (UPS). Because autophagy is considered one of the most important intracellular homeostatic processes, an alteration or deterioration of this pathway could modify the normal cell functioning, including a variety of diseases and normal cell physiology declination. Autophagosomes and lysosomes decline in an age-dependent manner in muscles (59), heart (43), and several other tissues. Moreover, CMA has also been implicated in removing oxidized and potentially dangerous proteins by direct lysosomal degradation (60).

The UPS is mostly implicated in the degradation of misfolded proteins, as well as short- and long-lived proteins by their ubiquitination. This process is achieved thanks to three major proteins that sequentially activate the ubiquitin tag (E1), transfer it to a second enzyme (E2), and finally ligate the ubiquitin tag to the target molecule (by E3 ligase), which eventually reaches the proteasome for degradation (61, 62). It is important to note that almost all regulatory proteins are substrates for this system (61, 63), and UPS declines with age [reviewed in (64)]. Interestingly, mTORC1 was found to regulate not only lysosomal protein degradation, but also proteasomal proteolysis of long-lived proteins, independently of protein synthesis, suggesting a common regulation of both proteolytic systems by nutrient-sensing (63). In addition, overexpression of a sole subunit of the proteasome enhanced its activity and the survival against several oxidants in two cell lines as well as primary culture of human fibroblasts (65). Moreover, proteasome activity decreased in an age-dependent manner (66). Overexpression of proteasome subunits in aged dermal human fibroblasts ameliorated the aged phenotype and restored the oxidized and ubiquitinated proteins to young levels (66). In the same way, transgenic mice with reduced proteasomal activity accumulated oxidized and ubiquitinated proteins, accelerating the aging phenotype and the age-related metabolic diseases (67). Besides, inhibition of proteasome activity impaired cell proliferation and shortened lifespan (68), reinforcing the importance of a correct proteostasis in healthy aging and longevity.

Several studies have been done on neurodegenerative diseases related to aging and autophagy, including those most relevant for their high impact on human population. Most of them share the accumulation of ROS, misfolded proteins, and damaged organelles, aging being the main risk factor (69, 70). This accumulation interferes with proper axonal traffic, enhancing neurotoxicity. Both autophagy and CMA impairment hamper the correct protein-aggregates degradation and the remodeling of dendrites and axons, thus diminishing the nervous plasticity (71, 72). In Parkinson's disease (PD), the cytoplasmic aggregates are formed by α-synuclein and ubiquitin (or Lewy bodies) in dopaminergic neurons of substantia nigra, leading to their death (32). Alterations in UPS (71) and also in CMA can develop the disease too. It is worth noting that overexpression of Lamp2A improved CMA performance and decreased α-synuclein cytoplasmic levels (73). In the same way, Alzheimer's disease (AD) is characterized by intracellular accumulation of tau protein as well as β-amyloids (Aβ), derived from the amyloid precursor protein (APP). This aggregate formation impairs normal cell function, finally leading to cell death (32). Also, extracellular Aβ-plaques secreted by autophagosomes can interrupt intercellular communication (72), another hallmark of aging (52). Besides, Apolipoprotein E4 (ApoE4) is the main genetic risk for sporadic AD and was found to promote the disruption of the lysosomal membrane together with Aβ, leading to neuronal degeneration (72, 74). Mutations in Presenilin1 or 2 (PS1 and PS2, two transmembrane subunits of gamma-secretase), as well as in tau protein or in APP are common causes of the familial AD (72). Other neurodegenerative diseases implicate alterations in the autophagic process, i.e., SENDA, Huntington, Amyotrophic Lateral Sclerosis, and Frontotemporal Dementia disorders [reviewed in (70, 75, 76)]. In all these cases, the lack

of proper degradation by autophagy promotes the aggregation of several proteins and the consequent malfunctioning of axonal transport.

Regarding metabolic diseases and autophagy, it is well known that islet amyloid deposition leads to type 2 diabetes in humans due to the amyloidogenic property of human islet amyloid polypeptide (hIAPP). It is important to note that mice do not develop such aggregation. In order to bypass this model difference, Kim et al. developed transgenic mice expressing hIAPP specifically in β-cells and bred them with $Atg7^{\Delta\beta-cell}$ mice. Male mice had premature diabetes, while females had hyperglycemia but never developed the disease, suggesting a synergism between autophagy deficiency and human amyloid overexpression. Moreover, primary culture of monkey islet cells overexpressing precursors of hIAPP showed that autophagic inhibition by 3-methyladenine (3-MA) increased pro-hIAPP dimer or trimer accumulation, blocking the autophagic activity in these pancreatic cells (77). It is also important to highlight that diabetes or glucose handling deficiencies are risk factors for the AD, as the amyloids properties of proteins implicated in metabolic diseases and AD are similar and probably interconnected (78). More studies regarding the connection between metabolic and neurodegenerative diseases are required for a better understanding of the molecular basis of such relationships at systemic level.

Finally, sarcopenia is characterized by a progressive loss of muscle mass and strength thanks to an imbalance between production and degradation of proteins (79). Aged-related declination of autophagy (both mitophagy as well as CMA) promotes sarcopenia by protein accumulation interference with normal myofibers functioning, but an exacerbation of autophagy can also result in cellular stress and finally death (79). Thus, an age-related imbalance of proteostasis could drive a variety of diseases involving both protein accumulation and degradation.

Mitochondrial Dysfunction

Mitophagy is a basal process involved in the autophagic degradation of mitochondria (76, 80, 81). It is necessary in normal differentiation of certain cell types such as red blood cells (82), in embryogenesis, immune response, cell programming, and cell death (80). Mitophagy is required not only to remove damaged mitochondria, but also to promote the biosynthesis of new ones, supporting the mitochondrial quality control (76, 80). Given that mitochondria are implicated in bioenergetics and ROS production, the mitophagy plays an important role in cell homeostasis. Additionally, a decrease in mitophagy is observed in aged animals and this contributes to aging phenotype (81).

Canonically, mitophagy is triggered by the cytosolic exposition of mitochondrial outer membrane (MOM) proteins, which have a LIR domain. The mitophagy is tightly regulated by several molecules, NIX and BNIP being two of the most widely characterized mitochondrial adaptors for autophagic machinery (83). NIX activation is associated with an increment in mitochondrial degradation in HeLa cells, protecting them against cellular stress (84). Interestingly, NIX has a LIR domain which binds LC3 once it is activated by phosphorylation (84). Additionally, PINK1 and PARKIN have been involved in the regulation of mitophagy when the mitochondrion loses its membrane potential (80). These proteins have been considered as key components in controlling the activation of mitophagy (85) and also as participants of mitophagy-associated cancer resistance. PINK1 and PARKIN are activated in response to an increment of intracellular ROS levels, which stimulate the MAPK and ERK1/2 signaling cascades, triggering parapoptosis in non-malignant cells, which bypass the caspases activation and, thus, the apoptosis (86) (**Figure 2**).

Additionally, Mitofusin 2 (MFN2) is a mitochondrial membrane fusing protein involved in several processes, including mitochondria fusion and mitophagy. Its expression declines with age, and its deficiency provokes precocious sarcopenia, accumulation of damaged mitochondria, and metabolic disorders in young mice (87). In addition, Humanin, an antiapoptotic mitochondrial protein, is capable of activating the CMA machinery, thus protecting several cell types from oxidative stress (88). Interestingly, both CMA and Humanin decline with age (89, 90), contributing to the age-related deterioration of proteostasis and mitochondrial functionality. As can be seen, several proteins regulate the mitophagy and contribute to the mitochondria homeostasis. As almost all of them decline with age, the modulation of mitophagy regulatory proteins could be a novel anti-aging therapeutic approach in the future. Despite this, more studies are needed in order to understand the complex regulation of mitophagy and the relationships between the players.

Different compounds, intracellular changes or stimuli could drive an activation of mitochondrial dysfunction. Normally, ROS oxidative stress, loss of membrane potential, MOM permeability, and aging are able to cause mitochondrial dysregulation. This

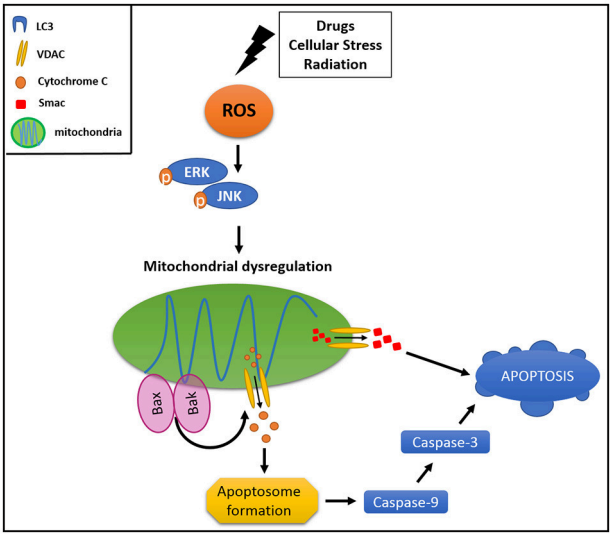

FIGURE 2 | Mitophagy protects cancer cell from apoptosis. Different stimuli could drive an activation of mitochondrial dysregulation, triggering signaling pathways involved in activation of pro-apoptotic proteins (BAK and BAX). This results in MOM damage and the consequent cytochrome c and SMAC release to the cytoplasm, activating intrinsic apoptotic pathway.

imbalance triggers signaling pathways involving activation of pro-apoptotic proteins of the BCL-2 family such as BAK and BAX, resulting in an MOM damage and the consequent release of cytochrome c and SMAC (second mitochondrial-derived activator of caspase) to the cytoplasm (91), activating the intrinsic apoptotic pathway through caspase 9 (92, 93) (**Figure 2**). Furthermore, hypoxia inducible factor 1α (HIF1α) is able to trigger mitophagy by stabilization and activation of NIX protein, and is also responsible for autophagic activation through VMP1 (vacuole membrane protein 1) promoter, causing colon cancer resistance to photodynamic therapy (94).

Disruption in mitophagy, and thus in redox homeostasis, can produce different cardiovascular pathologies (43). PINK1-KO mice developed a decline in cardiac function due to dysfunctional mitochondria and an increase in oxidative stress (95). Notably, the same features were observed in human end-stage heart failure samples, where diminished PINK1 levels were also found (95). Despite the ubiquitous KO model, the authors were capable of demonstrating the importance of PINK1 in heart functioning. Additionally, altered mitophagy due to elevated ROS production has been linked to Alzheimer Disease (AD), but there is controversy as to whether this disturbance in the autophagic pathway is a cause or a consequence of AD (72). Remarkably, PARKIN and PINK1 have been found mutated in Parkinson Disease (PD) patients, suggesting the importance of this pathway in dysfunctional mitochondria clearance by autophagy (76, 96). In 2015, Sun et al. published a new approach for measuring mitophagy *in vivo*, using a transgenic mouse model consisting in the mitochondria-targeted overexpression of a fluorescence reporter named Keima (81). We think that this tool could be very interesting for the *in vivo* study of mitophagy and its regulation under a wide variety of conditions. Deeper studies are then required to fully understand this process and its role in healthy and pathologic aging.

Deregulated Nutrient Sensing

Cellular response to nutrient privation implies some kind of intracellular sensor, capable of triggering the corresponding survival mechanisms. It has been proved that nutrient sensing is a highly conserved process across eukaryotes (97). Several nutrient-related signaling pathways converge on mTOR [mammalian Target Of Rapamycin; (97)], which triggers the response to growth factors, energy, glucose or amino acid changes (38, 97–100). Interestingly, nutrient sensors can also be activated under oxidative stress conditions, suggesting a common regulatory mechanism linking redox homeostasis and nutrients availability [reviewed in (101)].

The kinase mTOR is capable of linking environmental conditions with reproduction and somatic maintenance, thus influencing the individual lifespan (102). In addition, mTOR and mslt8 (a positive regulator of mTORC1) haploinsufficient female mice showed an increment in lifespan, compared to wild type mice (103). Mice carrying hypomorphic alleles of mTOR also had an increase in lifespan, a reduction in aging biomarkers, and a normal metabolism (104), showing the importance of nutrient sensing in aging. Moreover, Ribosomal S6 kinase 1 (S6K1) knockout mice showed an extended lifespan, compared with wild type counterparts, demonstrating again the importance of the mTOR pathway inhibition in longevity (105).

Besides, mTOR forms two distinct complexes, the Complex 1 (mTORC1) being capable of integrating different responses depending on nutrients availability (99, 106, 107). Upstream mTORC1, there are multiple regulating complexes, responding to each amino acid, glucose or growth factor input (99, 106, 107). Notably, p62 is one of the mTOR interactors upon amino acid stimulation, and it has been proposed to specifically stabilize the activated mTORC1 at the lysosome surface (108). It is well known that p62 is an autophagic adaptor protein whose role in nutrient sensing pathway might be another connection between mTOR and the regulation of autophagy.

Because autophagy is a catabolic mechanism, it can be assumed to be implicated in cellular and systemic metabolism. Metabolic stress responses could be compromised due to a decline in autophagic activity (109). As an important process regulating the general cellular status, autophagy can also crosslink metabolic pathways to maintain the homeostasis under a variety of conditions (43). In this sense, it has been demonstrated that, after nutrient or growth factor deprivation, ULK1 and ULK2 are activated, and these kinases phosphorylate and activate several glycolytic enzymes as well as autophagic proteins. This makes it possible to obtain metabolites thanks to glucose uptake, gluconeogenic pathway blockage, and autophagic degradation of cytosolic components (110). Supporting this, mTOR hyperactivation was found in several diseases such as obesity, metabolic syndrome, and type 2 diabetes (100), which highlights the importance of a tight regulation of autophagy as well as the nutrient sensing pathway.

Given that mTOR is capable of sensing the nutritional state of the cell, it was proposed to play an important role in Caloric Restriction (CR) therapy. Indeed, mTOR signaling network was shown to mediate lifespan extension by CR. Notably, the sole amino acid-restriction is enough to promote CR-response (111). Sirtuins 1 and 3 (NAD+ deacetylases) are activated in response to CR, as well as SOD1, in order to change the metabolism accordingly and prevent oxidative damage, respectively. Such ROS regulation rules out the Free Radicals Theory of Aging, at least partially (101, 112, 113). It is important to highlight the fact that inhibition of mTOR (specifically mTORC1) in embryogenesis is lethal, while the ablation of these pathways in adulthood can extend life [reviewed in (111)]. In fact, weight loss is suggested as prevention as well as therapy for a variety of age-related diseases (98).

Genomic Instability

In the last decade, several studies have demonstrated that autophagy or autophagic-related molecules act as a "safeguard" of genome stability both directly (DNA repair modulation) and indirectly (by acting as a homeostatic response) (114). Several mouse models have provided substantial information regarding genomic instability and its connection with healthy and pathological aging (55).

Regarding oxidative stress and DNA damage, ROS increase is thought to be mainly harmful for the mitochondrial

DNA (mtDNA), generating the mutagenic 8-hydroxy-20-deoxyguanosine (8-OHdG), as well as mutations and deletions in mtDNA that result in a dysfunctional mitochondrion (69). Moreover, mitochondrial dysfunction promotes telomere attrition, telomere loss, and chromosome alterations, culminating in apoptosis in mouse embryos (115). Besides, Donati and collaborators demonstrated that, upon autophagic stimulation with an anti-lipolytic agent in 16-month-old rats, 8-OHdG accumulation in liver was successfully supressed, reaching the values obtained from young animals in only 6 h. When they measured the cytochrome c oxidase activity, they found that this decrease was not associated with lower mitochondrial enzyme activity, demonstrating the selective mitophagy of a small population of 8-OHdG-positive mitochondria and the importance of this proteostatic process in anti-aging mechanisms (54, 116). In the same way, dietary restriction reduced 8-OHdG levels in mitochondrial DNA (mtDNA) of aged rats and mice compared with those fed *ad libitum* (117), supporting the importance of dietary restriction in prevention of mtDNA damage by ROS in aged animals. Notably, $Sod2^{-/-}$ mice accumulated high levels of 8-OHdG both in nuclear and mitochondrial DNA, compared with wild type mice. Nevertheless, they showed no changes in lifespan or age biomarkers (47). On the other hand, $Atg7^{-/-}$ mouse keratinocytes presented premature aging after oxidative stress induction, supporting the importance of autophagy in healthy aging (118). In addition, Bender et al. found high levels of mtDNA deletions in dopaminergic neurons of PD patients, compared to controls (119). As we have already mentioned, PARKIN and PINK1 are mutated in PD, thus altered mitophagy can explain, in part, the accumulation of mtDNA damage in PD patients.

Autophagy has emerged as an important process in genome maintenance. After treatment with several cell cycle blockers, human osteosarcoma cells (U2OS) increased the micronuclei frequency as well as autophagosomes. Importantly, the authors observed a small but significant colocalization between them. Knockdown of Atg5 or Atg7 abolished this colocalization. P62/SQSTM1 also colocalized with micronuclei, indicating that micronuclei can be degraded by autophagy and this may contribute to genome stability (120). Moreover, NDP52 and p62-dependent autophagy can degrade retrotransposon RNA, preventing new insertions into the genome of long and short interspersed elements (121). Additionally, autophagy deficiency leads to an accumulation of RHOA with p62. This phenotype drives cytokinesis failure, aneuploidy, and multinucleation due to inappropriate formation of contractile ring (122). Furthermore, allelic loss of Beclin1 promotes tumorigenesis and activation of DNA-damage response in neoplasic cells. In this context, autophagy deficiency leads to genome instability under metabolic stress in these mouse mammary epithelial cells (123). Artificially aneuploid mouse cells showed increased autophagy to protect cells from genome instability (124).

Autophagic adaptor p62 has been found to be implicated in genome instability in several studies. Accumulation of p62 led to the activation of DNA-damage response (125). By contrast, overexpression of p62 (or autophagy deficiency) suppressed DNA-damage response by its direct inhibitory interaction with RNF168, an important E3 ligase for histone H2A ubiquitination and DNA-damage response (126). In this regard, p62 downregulates the protein levels of several molecules involved in homologous recombination (HR) of damaged DNA, inducing at the same time non-homologous end-joining (NHEJ), stressing the importance of p62 nuclear accumulation upon several stresses (127). More studies are needed to completely elucidate the role of p62 and other autophagic components in genome maintenance throughout life.

Epigenetic Alterations

Epigenetic changes due to external or internal factors drive several processes, including development and aging (128). In muscular and hematopoietic stem cells, different histone modifications help to establish the quiescence state with age (129). Besides, epigenetic alterations lead to different responses in aging and longevity in several mouse models [reviewed in (55)]. Notably, macrophages of old mice had hypermethylated LC3 and Atg5 promoter regions, compared with those of young mice, downregulating the protein levels and promoting aging decline of autophagy (130).

The natural polyphenol resveratrol, an autophagic inducer, has been proved to exert its action by inositol 1,4,5-triphosphate receptor (IP_3R) and by protein acetylation decrease (131, 132). In this sense, a balance between different degrees of protein acetylation is presumed to be important for autophagy (32). High levels of acetyl-CoA, which serves as an acetyl group donor, were found to promote histone hyperacetylation, downregulating the expression of Atg genes, thus inhibiting autophagy and shortening lifespan in aged yeast (133). In addition, these authors found that the knockout of a certain acetyl-CoA in *Drosophila melanogaster* brain enhances autophagy and prolongs lifespan. Morselli et al. used specific siRNA knockdown of Sirtuin1, a NAD^+ deacetylase in HCT116 cell line, which resulted in a suppression of the autophagic response to resveratrol. Importantly, resveratrol induced autophagy by AMP-dependent kinase/mTOR-independent pathway and changed the acetylation of 375 proteins, half of them involved in autophagy. On the other hand, deacetylations were often observed in metabolism-related proteins, thus activating autophagy (131). Another autophagic inducer, spermidine, is a well-known pro-longevity polyamine (56), largely studied as an anti-aging agent. Like resveratrol, spermidine can induce both acetylation and deacetylation changes that promote the autophagic pathways via AMP-dependent kinase signaling. In this regard, low doses of this polyamine together with low doses of resveratrol synergistically caused the same autophagic response as higher doses of each inducer separately (131).

Taken together, organismal models as well as *in vitro* studies highlight the importance of epigenetics throughout life. The relationship between epigenetic changes and autophagy needs to be deeply studied in order to understand the regulatory loop that seems to be involved in development and aging.

Telomere Attrition

Telomeres are specific repetitive sequences at the end of chromosomes, the telomerase is the special polymerase involved in the elongation of this protective zone. It is important to note that somatic cells of mammalian species lack the expression of telomerase. Thus, telomerase is highly regulated in a spatio-temporal manner (134). In each cell cycle, the telomere becomes shorter and shorter, leading to a vulnerability of the chromosomal end. This process is called telomere attrition and is considered another hallmark of aging (52).

Telomere shortening can cause genomic instability, and thus contributes to cancer or age-related diseases (115). This progressive diminishment in telomere length can influence gene expression and provoke several pathologies without damaging DNA (134). Moreover, ROS can trigger telomere attrition or loss and genomic instability, which can be prevented by using an antioxidant (115). In addition, telomerase activity can support cell cycle progression by preventing the arrest due to short telomeres, leading to a putative malignancy. Remarkably, overexpression of Beclin1 in HeLa cells revealed that telomerase activity is reduced after autophagy induction (39). This approach argues in favor of the hypothesis that autophagy plays an important tumor suppressor role by the modulation of telomerase activity in somatic cells. Similarly, Guanosine-rich zones, like telomeres, can suffer the formation of G-quadruplexes, interfering with gene expression and cell growth (135). In this regard, melanoma cells treated with an anthracene-based ligand (Ant 1,5, capable of stabilizing G-quadruplexes) showed an increase in genome instability and telomere dysfunction. Furthermore, Ant 1,5 induces a p21-dependent augmentation of autophagy levels. This autophagic response arises in order to avoid genome instability and telomeric dysfunction, thus promoting cell survival (135).

Other Hallmarks of Aging

Cellular senescence is considered another hallmark of aging (52). Senescence can be induced by DNA damage, telomere attrition or other stress signals, with the consequent cell cycle arrest. Several mouse models have contributed to the current knowledge of senescence and its characteristics (55). For example, Apolipoprotein E (ApoE) knockout mice present atherosclerotic plaques, which can be prevented by the addition of rapamycin. Furthermore, autophagy regulates the senescence of vascular smooth muscle cells (VSMCs) of ApoE$^{-/-}$ mice, involving the mTORC1/ULK1/ATG13 pathway in atherosclerosis progression (101). In addition, senescent MEF cells accumulate copper due to higher import and lower export, enhancing antioxidant defense mechanisms. In addition, rapamycin treatment can prevent and reverse copper accumulation, suggesting that autophagy mediates the copper homeostasis (136). Along the same lines, primary cultures from human fibroblasts depleted for Atg7, Atg12, or Lamp2 showed cell cycle arrest and high levels of SA-β-Gal staining, a characteristic feature of replicative senescence cells (137). Intriguingly, autophagy can mediate the transition to a senescent phenotype in IMR90 human diploid oncogene-induced senescence fibroblasts, making possible the protein remodeling needed to establish the senescent phenotype under oncogene activation (138). An interesting review conducted by Kwon et al. put forward a "toolkit" of differential diagnosis to resolve the apparent contradiction of autophagy in the cellular senescence. They proposed that type of autophagy, the exact moment when it acts, and the place where it occurs can define the pro or anti-senescence role of autophagy (139).

Another hallmark of aging, according to López-Otín et al., is stem cell (SC) exhaustion, with the consequent decline in tissue regenerative potential (52). Self-renewal is important to maintain the population of tissue-specific stem cells throughout life. Importantly, as we age, stem-cell activity decreases (140). In addition, SCs function is highly regulated in response to external stimuli (129). Ho et al. have shown that autophagy is necessary for preservation and quiescence of hematopoietic stem cells (HSCs). The authors demonstrated, in an Atg12-KO mice model, that autophagy-defective HSCs resemble old HSCs, in terms of accumulation of mitochondria, augmentation of myeloid-to-lymphoid ratio, diminishment of the regenerative potential, and decrease in self-renewal (141). This study supports the importance of autophagy in blood system homeostasis (142). Autophagy is also important to maintain stemness in bone marrow-derived mesenchymal stem cells (BMMSCs), and induction with rapamycin restores the biological properties of BMMSCs (143). In addition, Atg7 loss in aged muscle stem cells (satellite cells) of transgenic mice caused altered mitophagy and an accumulation of ROS, all features of senescence that diminish the regenerative potential of aged satellite cells (144). It is important to note that autophagic modulation could be an interesting therapeutic approach to prevent stem cell senescence and decline. Due to SC complexity, more studies are required to fully elucidate the role of autophagy in maintenance of stem cells.

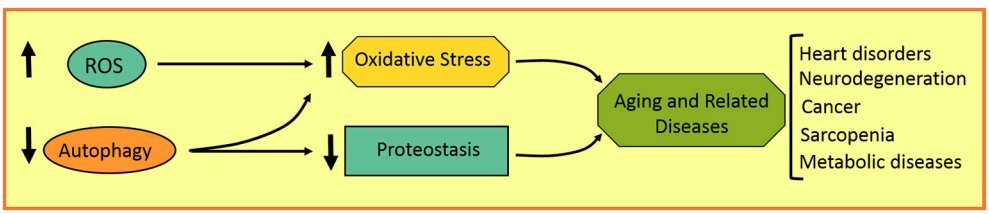

FIGURE 3 | Schematic representation of aging-related disorders in autophagy and redox imbalance.

CURRENT AND FUTURE AUTOPHAGIC TREATMENTS IN AGING AND AGE-RELATED DISEASES

It can be presumed that induction or restoration of autophagy and antioxidant cellular systems could alleviate aging symptoms. Three major anti-aging therapies were evaluated over the last 30 years: Autophagic inducers, antioxidant (polyamine-rich) consumption, and caloric restriction.

Rapamycin, an immunosuppressive macrolide, is a well-known autophagic activator via mTORC1 inhibition of the mTOR complex. This pharmacological treatment has proved to increase lifespan in flies, nematodes, yeast, and mice [reviewed in (111)]. Moderate doses of this drug can alleviate atherosclerosis, achieving the same effects as higher concentrations (101). Furthermore, rapamycin added during reperfusion after heart infarction in a C57 myocardial ischemia mouse model improved the survival and cardiac functioning, reducing the infarcted zone size as well as apoptosis post-ischemia/reperfusion. In addition, AKT phosphorylation increased after treatment, suggesting that AKT-ERK pathways were selectively activated by rapamycin (145). Finally, rapamycin improves whole metabolism in several ways, strengthening the importance of regulating autophagy activity by external compounds in order to ameliorate metabolic diseases at cellular and whole organism levels (100, 109).

In addition, exogenously administered spermidine extends lifespan in mice treated throughout life or at pre-aged adulthood, and in hypertensive rats as well. In addition, spermidine dietary intake was inversely correlated with cardiovascular pathologies in a human population-based cohort (146). Spermidine, thus, has been proposed to be a neuro and cardioprotector in aging models and humans, highlighting the importance of polyamine-rich diets (56).

Caloric restriction (CR) is the reduction of total calorie intake by 30-40% without malnutrition. There is strong evidence supporting this therapy as one of the most effective in reducing oxidative stress in rats and mice, prolonging lifespan (31, 111). CR has been found to provoke a decrease in ROS generation (69) and a diminishment of DNA damage (31). Regarding CR and autophagy, it was demonstrated that 8%-CR in combination with exercise or 8%-CR alone were capable of upregulating Atg7, LC3, and LAMP2 in type II skeletal muscle in rats (147). The authors also found that aging augmented BECLIN1 protein expression and oxidative stress, but CR alone or with exercise diminished this modification as well as the apoptotic index, both correlating negatively with LAMP2 gene expression (147). $Sod^{-/-}$ mice treated with CR attenuated the age-related-like phenotype of this knockout in terms of DNA damage, cellular senescence, and inflammation (49). CR could be beneficial to human health, according to epidemiological studies (37). As CR regulates several pathways, more integrative studies are required in order to fully understand its anti-aging effect.

Finally, several reviews and research studies highlight the importance of autophagic modulation as an anti-aging therapy for the future (32, 37, 43). Rapamycin, resveratrol, polyamines, and CR are possible candidates to be tested more carefully in order to improve the putative treatments for human age-related diseases.

CONCLUDING REMARKS

Aging involves several features that can promote the development of a variety of disorders in aged individuals, such as neurodegenerative, heart, and metabolic diseases, as well as cancer. These age-related characteristics involve a gradual increase in ROS production and genome instability, and a progressive decrease in antioxidant, DNA repairing, and proteostatic systems, among others (**Figure 3**). Autophagy, as a homeostatic process, plays an important role in the maintenance of cell physiology and avoidance of any internal or external damage that could eventually appear. Several attempts were made to improve age-related features, such as caloric restriction as well as antioxidants and autophagy inducers. Rapamycin, resveratrol, and polyamines are autophagic inductors clinically available that could improve aging and some age-related disorders. It is important to highlight the fact that chemotherapies combined with autophagic inhibitors (i.e., chloroquine and derivatives) could be more effective in cancer treatment. Further studies are required to make autophagy modulation a more promising anti-aging and anti-tumoral therapy in the next decades.

AUTHOR CONTRIBUTIONS

MB analyzed the data, wrote the manuscript, and critically read the manuscript. RG wrote the manuscript. CF designed the research, analyzed the data, wrote the manuscript, produced the figures and critically read the manuscript.

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REFERENCES

1. Boya P, Reggiori F, Codogno P. Emerging regulation and functions of autophagy. *Nat Cell Biol.* (2013) 15:713–20. doi: 10.1038/ncb2788
2. Galluzzi L, Baehrecke EH, Ballabio A, Boya P, Bravo-San Pedro JM, Cecconi F, et al. Molecular definitions of autophagy and related processes. *EMBO J.* (2017) 36:1811–36. doi: 10.15252/embj.201796697
3. Mehrpour M, Esclatine A, Beau I, Codogno P. Autophagy in health and disease. 1. Regulation and significance of autophagy: an overview. *Am J Physiol Cell Physiol.* (2010) 298:C776–85. doi: 10.1152/ajpcell.00507.2009
4. White E. The role for autophagy in cancer. *J Clin Invest.* (2015) 125:42–46. doi: 10.1172/JCI73941
5. Yang Z, Goronzy JJ, Weyand CM. Autophagy in autoimmune disease. *J Mol Med.* (2015) 93:707–17. doi: 10.1007/s00109-015-1297-8

6. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol.* (2008) 182:685–701. doi: 10.1083/jcb.200803137
7. Lamb CA, Yoshimori T, Tooze SA. The autophagosome: origins unknown, biogenesis complex. *Nat Rev Mol Cell Biol.* (2013) 14:759–74. doi: 10.1038/nrm3696
8. Militello RD, Colombo MI. A membrane is born: origin of the autophagosomal compartment. *Curr Mol Med.* (2011) 11:197–203. doi: 10.2174/156652411795243441
9. Jiang ZF, Shao LJ, Wang WM, Yan XB, Liu RY. Decreased expression of Beclin-1 and LC3 in human lung cancer. *Mol Biol Rep.* (2012) 39:259–267. doi: 10.1007/s11033-011-0734-1
10. Dice JF. *Altered Degradation of Proteins Microinjected into Senescent Human Fibroblasts*. (1982). Available online at: at: http://www.jbc.org/content/257/24/14624.full.pdf (Accessed July 27, 2018).
11. Dice JF. Peptide sequences that target proteins for lysosomal degradation. *J Control Release* (1990) 11:291–6. doi: 10.1016/0168-3659(90)90141-F
12. Kaushik S, Cuervo AM. The coming of age of chaperone-mediated autophagy. *Nat Rev Mol Cell Biol.* (2018) 19:365–81. doi: 10.1038/s41580-018-0001-6
13. Shibutani ST, Saitoh T, Nowag H, Münz C, Yoshimori T. Autophagy and autophagy-related proteins in the immune system. *Nat Immunol.* (2015) 16:1014–24. doi: 10.1038/ni.3273
14. Wirth M, Joachim J, Tooze SA. Autophagosome formation—the role of ULK1 and Beclin1–PI3KC3 complexes in setting the stage. *Semin Cancer Biol.* (2013) 23:301–9. doi: 10.1016/J.SEMCANCER.2013.05.007
15. Karanasios E, Stapleton E, Manifava M, Kaizuka T, Mizushima N, Walker SA, et al. Dynamic association of the ULK1 complex with omegasomes during autophagy induction. *J Cell Sci.* (2013) 126:5224–38. doi: 10.1242/jcs.132415
16. Fader CM, Aguilera MO, Colombo MI. Autophagy response: manipulating the mTOR-controlled machinery by amino acids and pathogens. *Amino Acids* (2015) 47:2101–12. doi: 10.1007/s00726-014-1835-7
17. Nishimura T, Kaizuka T, Cadwell K, Sahani MH, Saitoh T, Akira S, et al. FIP200 regulates targeting of Atg16L1 to the isolation membrane. *EMBO Rep.* (2013) 14:284–91. doi: 10.1038/embor.2013.6
18. Novak I, Kirkin V, McEwan DG, Zhang J, Wild P, Rozenknop A, et al. Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO Rep.* (2010) 11:45–51. doi: 10.1038/embor.2009.256
19. Zhu Y, Wang C, Yu M, Cui J, Liu L, Xu Z. ULK1 and JNK are involved in mitophagy incurred by LRRK2 G2019S expression. *Protein Cell* (2013) 4:711–721. doi: 10.1007/s13238-013-3910-3
20. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ.* (2018) 25:486–541. doi: 10.1038/s41418-017-0012-4
21. Zinger A, Cho WC, Ben-Yehuda A. Cancer and aging - the inflammatory connection. *Aging Dis.* (2017) 8:611–27. doi: 10.14336/AD.2016.1230
22. Vellai T, Takács-Vellai K, Sass M, Klionsky DJ. The regulation of aging: does autophagy underlie longevity? *Trends Cell Biol.* (2009) 19:487–94. doi: 10.1016/J.TCB.2009.07.007
23. Chuang SY, Lin CH, Fang JY. Natural compounds and aging: between autophagy and inflammasome. *Biomed Res Int.* (2014) 2014:297293. doi: 10.1155/2014/297293
24. Nakamura S, Yoshimori T. Autophagy and longevity. *Mol Cells* (2018) 41:65–72. doi: 10.14348/molcells.2018.2333
25. *Ageing and Health*. Available online at: http://www.who.int/news-room/fact-sheets/detail/ageing-and-health (Accessed July 27, 2018)
26. Barja G. The Cell Aging Regulation System (CARS). *Barja G React Oxyg Species* (2017) 148:148–83. doi: 10.20455/ros.2017.829
27. Kamel HK, Mooradian AD, Mir T. Biological theories of aging. *Disease-a-Month* (2015) 61:460–6. doi: 10.1016/J.DISAMONTH.2015.09.005
28. Wahab Pathath A. Theories of aging. *Artic Int J Indian Psychol.* (2017) 4:15–22. doi: 10.25215/0403.142
29. Harman D. The free radical theory of aging. *Antioxid Redox Signal.* (2003) 5:557–61. doi: 10.1089/152308603770310202
30. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* (2000) 408:239–47. doi: 10.1038/35041687
31. Bokov A, Chaudhuri A, Richardson A. The role of oxidative damage and stress in aging. *Mech Ageing Dev.* (2004) 125:811–26. doi: 10.1016/J.MAD.2004.07.009
32. Markaki M, Metaxakis A, Tavernarakis N. The role of autophagy in aging: molecular mechanisms. *Autoph Cancer Other Pathol Inflamm Immunity Infect Aging* (2017) 123–38. doi: 10.1016/B978-0-12-812146-7.00002-0
33. Pyo JO, Yoo SM, Ahn HH, Nah J, Hong SH, Kam TI, et al. Overexpression of Atg5 in mice activates autophagy and extends lifespan. *Nat Commun.* (2013) 4:2300. doi: 10.1038/ncomms3300
34. Zhang C, Cuervo AM. Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function. *Nat Med.* (2008) 14:959–65. doi: 10.1038/nm.1851
35. Liang H, Masoro EJ, Nelson JF, Strong R, McMahan CA, Richardson A. Genetic mouse models of extended lifespan. *Exp Gerontol.* (2003) 38:1353–64. doi: 10.1016/J.EXGER.2003.10.019
36. Emanuele E, Minoretti P, Sanchis-Gomar F, Pareja-Galeano H, Yilmaz Y, Garatachea N, et al. Can enhanced autophagy be associated with human longevity? Serum levels of the autophagy biomarker Beclin-1 are increased in healthy centenarians. *Rejuvenation Res.* (2014) 17:518–24. doi: 10.1089/rej.2014.1607
37. Rubinsztein DC, Mariño G, Kroemer G. Autophagy and aging. *Cell* (2011) 146:682–95. doi: 10.1016/J.CELL.2011.07.030
38. Jiang X, Overholtzer M, Thompson CB. Autophagy in cellular metabolism and cancer. *J Clin Invest.* (2015) 125:47–54. doi: 10.1172/JCI73942
39. Taji F, Kouchesfahani HM, Sheikholeslami F, Romani B, Baesi K, Vahabpour R, et al. Autophagy induction reduces telomerase activity in HeLa cells. *Mech Ageing Dev.* (2017) 163:40–5. doi: 10.1016/J.MAD.2016.12.011
40. Abbas G, Salman A, Rahman SU, Ateeq MK, Usman M, Sajid S, et al. Aging Mechanisms: Linking oxidative stress, obesity and inflammation. *Matrix Sci Med.* (2017) 1:30–3. doi: 10.26480/msm.01.2017.30.33
41. Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J.* (2007) 26:1749–60. doi: 10.1038/sj.emboj.7601623
42. Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. *Mol Cell* (2012) 48:158–67. doi: 10.1016/J.MOLCEL.2012.09.025
43. Yan Y, Finkel T. Autophagy as a regulator of cardiovascular redox homeostasis. *Free Radic Biol Med.* (2017) 109:108–13. doi: 10.1016/J.FREERADBIOMED.2016.12.003
44. Santos AL, Sinha S, Lindner AB. The good, the bad, and the ugly of ROS: new insights on aging and aging-related diseases from eukaryotic and prokaryotic model organisms. *Oxid Med Cell Longev.* (2018) 2018:1–23. doi: 10.1155/2018/1941285
45. Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, et al. Q2: extension of murine life span by overexpression of catalase targeted to mitochondria. *Science* (2003) 299:1342–6. doi: 10.1126/science.1077991
46. Treuting PM, Linford NJ, Knoblaugh SE, Emond MJ, Morton JF, Martin GM, et al. Reduction of age-associated pathology in old mice by overexpression of catalase in mitochondria. *J Gerontol Ser A Biol Sci Med Sci.* (2008) 63:813–22. doi: 10.1093/gerona/63.8.813
47. Van Remmen H, Ikeno Y, Hamilton M, Pahlavani M, Wolf N, Thorpe SR, et al. Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol Genomics* (2003) 16:29–37. doi: 10.1152/physiolgenomics.00122.2003
48. Pérez VI, Bokov A, Van Remmen H, Mele J, Ran Q, Ikeno Y, et al. Is the oxidative stress theory of aging dead? *Biochim Biophys Acta* (2009) 1790:1005–14. doi: 10.1016/J.BBAGEN.2009.06.003
49. Zhang Y, Unnikrishnan A, Deepa SS, Liu Y, Li Y, Ikeno Y, et al. A new role for oxidative stress in aging: the accelerated aging phenotype in $Sod1^{-/-}$ mice is correlated to increased cellular senescence. *Redox Biol.* (2017) 11:30–7. doi: 10.1016/J.REDOX.2016.10.014
50. Deepa SS, Van Remmen H, Brooks SV, Faulkner JA, Larkin L, McArdle A, et al. Accelerated sarcopenia in Cu/Zn superoxide dismutase knockout mice. *Free Radic Biol Med.* (in press) doi: 10.1016/J.FREERADBIOMED.2018.06.032
51. Calabrese EJ, Baldwin LA. Defining hormesis. *Hum Exp Toxicol.* (2002) 21:91–7. doi: 10.1191/0960327102ht217oa

52. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell* (2013) 153:1194–217. doi: 10.1016/J.CELL.2013.05.039
53. Hekimi S, Lapointe J, Wen Y. Taking a "good" look at free radicals in the aging process. *Trends Cell Biol.* (2011) 21:569–76. doi: 10.1016/J.TCB.2011.06.008
54. Cavallini G, Donati A, Taddei M, Bergamini E. Evidence for selective mitochondrial autophagy and failure in aging. *Autophagy* (2007) 3:26–7. doi: 10.4161/auto.3268
55. Folgueras AR, Freitas-Rodríguez S, Velasco G, López-Otín C. Mouse models to disentangle the hallmarks of human aging. *Circ Res.* (2018) 123:905–24. doi: 10.1161/CIRCRESAHA.118.312204
56. Bhukel A, Madeo F, Sigrist SJ. Spermidine boosts autophagy to protect from synapse aging. *Autophagy* (2017) 13:444–5. doi: 10.1080/15548627.2016.1265193
57. Gong Z, Tasset I. Humanin enhances the cellular response to stress by activation of chaperone-mediated autophagy. *Oncotarget* (2018) 9:10832–3. doi: 10.18632/oncotarget.24396
58. Amm I. Protein quality control and elimination of protein waste: the role of the ubiquitin–proteasome system. *Biochim Biophys Acta Mol Cell Res.* (2014) 1843:182–96. doi: 10.1016/J.BBAMCR.2013.06.031
59. Jiao J, Demontis F. Skeletal muscle autophagy and its role in sarcopenia and organismal aging. *Curr Opin Pharmacol.* (2017) 34:1–6. doi: 10.1016/J.COPH.2017.03.009
60. Kiffin R, Christian C, Knecht E, Cuervo AM. Activation of chaperone-mediated autophagy during oxidative stress. *Mol Biol Cell* (2004) 15:4829–40. doi: 10.1091/mbc.e04-06-0477
61. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev.* (2002) 82:373–428. doi: 10.1152/physrev.00027.2001
62. Callis J. The ubiquitination machinery of the ubiquitin system. *Arab B* (2014) 12:e0174. doi: 10.1199/tab.0174
63. Zhao J, Zhai B, Gygi SP, Goldberg AL. mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy. *Proc Natl Acad Sci USA.* (2015) 112:15790–7. doi: 10.1073/pnas.1521919112
64. Höhn A, Weber D, Jung T, Ott C, Hugo M, Kochlik B, et al. Happily (n)ever after: Aging in the context of oxidative stress, proteostasis loss and cellular senescence. *Redox Biol.* (2017) 11:482–501. doi: 10.1016/J.REDOX.2016.12.001
65. Chondrogianni N, Tzavelas C, Pemberton AJ, Nezis IP, Rivett AJ, Gonos ES. *Overexpression of Proteasome β 5 Subunit Increases Amount of Assembled Proteasome and Confers Ameliorated Response to Oxidative Stress and Higher Survival Rates.* (2005). Available online at: http://www.jbc.org/ (Accessed November 15, 2018).
66. Hwang JS, Hwang JS, Chang I, Kim S. Age-associated decrease in proteasome content and activities in human dermal fibroblasts: restoration of normal level of proteasome subunits reduces aging markers in fibroblasts from elderly persons. *J Gerontol Ser A Biol Sci Med Sci.* (2007) 62:490–9. doi: 10.1093/gerona/62.5.490
67. Tomaru U, Takahashi S, Ishizu A, Miyatake Y, Gohda A, Suzuki S, et al. Decreased proteasomal activity causes age-related phenotypes and promotes the development of metabolic abnormalities. *Am J Pathol.* (2012) 180:963–72. doi: 10.1016/J.AJPATH.2011.11.012
68. Torres C, Lewis L, Cristofalo VJ. Proteasome inhibitors shorten replicative life span and induce a senescent-like phenotype of human fibroblasts. *J Cell Physiol.* (2006) 207:845–853. doi: 10.1002/jcp.20630
69. Barja G. Free radicals and aging. *Trends Neurosci.* (2004) 27:595–600. doi: 10.1016/J.TINS.2004.07.005
70. Nassif M, Woehlbier U, Manque PA. The delicate balance of autophagy in neurodegeneration. *Autoph Cancer, Other Pathol Inflamm Immunity Infect Aging* (2017)387–99. doi: 10.1016/B978-0-12-812146-7.00019-6
71. Rezzani R, Stacchiotti A, Rodella LF. Morphological and biochemical studies on aging and autophagy. *Ageing Res Rev.* (2012) 11:10–31. doi: 10.1016/J.ARR.2011.09.001
72. Li Q, Liu Y, Sun M. Autophagy and Alzheimer's disease. *Cell Mol Neurobiol.* (2017) 37:377–88. doi: 10.1007/s10571-016-0386-8
73. Xilouri M, Brekk OR, Landeck N, Pitychoutis PM, Papasilekas T, Papadopoulou-Daifoti Z, et al. Boosting chaperone-mediated autophagy *in vivo* mitigates α-synuclein-induced neurodegeneration. *Brain* (2013) 136:2130–46. doi: 10.1093/brain/awt131
74. Ji ZS, Müllendorff K, Cheng IH, Dennis Miranda R, Huang Y, Mahley RW. Reactivity of apolipoprotein E4 And Amyloid β peptide: lysosomal stability and neurodegeneration. *J Biol Chem.* (2005) 281:2683–92 doi: 10.1074/jbc.M506646200
75. Filfan M, Elena Sandu R, Zăvăleanu A, Greşită A, Glăvan D, Olaru D, et al. Autophagy in aging and disease. *Rom J Morphol Embryol.* (2017):27–31.
76. Shi R, Guberman M, Kirshenbaum LA. Mitochondrial quality control: the role of mitophagy in aging. *Trends Cardiovasc Med.* (2018) 28:246–60. doi: 10.1016/J.TCM.2017.11.008
77. Kim J, Cheon H, Jeong YT, Quan W, Kim KH, Cho JM, et al. Amyloidogenic peptide oligomer accumulation in autophagy-deficient β cells induces diabetes. *J Clin Invest.* (2014) 124:3311–24. doi: 10.1172/JCI69625
78. Loos B, Klionsky DJ, Wong E. Augmenting brain metabolism to increase macro- and chaperone-mediated autophagy for decreasing neuronal proteotoxicity and aging. *Prog Neurobiol.* (2017) 156:90–106. doi: 10.1016/J.PNEUROBIO.2017.05.001
79. Fan J, Kou X, Jia S, Yang X, Yang Y, Chen N. Autophagy as a potential target for sarcopenia. *J Cell Physiol.* (2016) 231:1450–9. doi: 10.1002/jcp.25260
80. Um JH, Yun J. Emerging role of mitophagy in human diseases and physiology. *BMB Rep.* (2017) 50:299–307. doi: 10.5483/BMBREP.2017.50.6.056
81. Sun N, Yun J, Liu J, Malide D, Liu C, Rovira II, et al. Measuring *in vivo mitophagy*. *Mol Cell* (2015) 60:685–96. doi: 10.1016/J.MOLCEL.2015.10.009
82. Grosso R, Fader CM, Colombo MI. Autophagy: a necessary event during erythropoiesis. *Blood Rev.* (2017) 31:300–5. doi: 10.1016/J.BLRE.2017.04.001
83. Sandoval H, Thiagarajan P, Dasgupta SK, Schumacher A, Prchal JT, Chen M, et al. Essential role for Nix in autophagic maturation of erythroid cells. *Nature* (2008) 454:232–5. doi: 10.1038/nature07006
84. Rogov VV, Suzuki H, Marinković M, Lang V, Kato R, Kawasaki M, et al. Phosphorylation of the mitochondrial autophagy receptor Nix enhances its interaction with LC3 proteins. *Sci Rep.* (2017) 7:1131. doi: 10.1038/s41598-017-01258-6
85. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, et al. PINK1 is selectively stabilized on impaired mitochondria to activate parkin. *PLoS Biol.* (2010) 8:e1000298. doi: 10.1371/journal.pbio.1000298
86. Han H, Chou CC, Li R, Liu J, Zhang L, Zhu W, et al. Chalcomoracin is a potent anticancer agent acting through triggering oxidative stress via a mitophagy- and paraptosis-dependent mechanism. *Sci Rep.* (2018) 8:9566. doi: 10.1038/s41598-018-27724-3
87. Sebastián D, Sorianello E, Segalés J, Irazoki A, Ruiz-Bonilla V, Sala D, et al. Mfn2 deficiency links age-related sarcopenia and impaired autophagy to activation of an adaptive mitophagy pathway. *EMBO J.* (2016) 35:1677–93. doi: 10.15252/embj.201593084
88. Gong Z, Tasset I, Diaz A, Anguiano J, Tas E, Cui L, et al. Humanin is an endogenous activator of chaperone-mediated autophagy. *J Cell Biol.* (2018) 217:635–47. doi: 10.1083/jcb.201606095
89. Cuervo AM, Dice JF. Age-related decline in chaperone-mediated autophagy. *J Biol Chem.* (2000) 275:31505–13. doi: 10.1074/jbc.M002102200
90. Muzumdar RH, Huffman DM, Atzmon G, Buettner C, Cobb LJ, Fishman S, et al. Humanin: a novel central regulator of peripheral insulin action. *PLoS ONE* (2009) 4:e6334. doi: 10.1371/journal.pone.0006334
91. Tait SWG, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol.* (2010) 11:621–32. doi: 10.1038/nrm2952
92. Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, Vandenabeele P. Toxic proteins released from mitochondria in cell death. *Oncogene* (2004) 23:2861–74. doi: 10.1038/sj.onc.1207523
93. Danish L, Imig D, Allgöwer F, Scheurich P, Pollak N. Bcl-2-mediated control of TRAIL-induced apoptotic response in the non-small lung cancer cell line NCI-H460 is effective at late caspase processing steps. *PLoS ONE* (2018) 13:e0198203. doi: 10.1371/journal.pone.0198203
94. Rodríguez ME, Catrinacio C, Ropolo A, Rivarola VA, Vaccaro MI. A novel HIF-1α/VMP1-autophagic pathway induces resistance to photodynamic

therapy in colon cancer cells. *Photochem Photobiol Sci.* (2017) 16:1631–42. doi: 10.1039/C7PP00161D
95. Billia F, Hauck L, Konecny F, Rao V, Shen J, Mak TW. PTEN-inducible kinase 1 (PINK1)/Park6 is indispensable for normal heart function. *Proc Natl Acad Sci USA.* (2011) 108:9572–7. doi: 10.1073/pnas.1106291108
96. Ryan BJ, Hoek S, Fon EA, Wade-Martins R. Mitochondrial dysfunction and mitophagy in Parkinson's: from familial to sporadic disease. *Trends Biochem Sci* (2015) 40:200–10. doi: 10.1016/J.TIBS.2015.02.003
97. Kume S, Thomas MC, Koya D. Nutrient sensing, autophagy, and diabetic nephropathy. *Diabetes* (2012) 61:23–9. doi: 10.2337/db11-0555
98. Blagosklonny M V. Calorie restriction: decelerating mTOR-driven aging from cells to organisms (including humans). *Cell Cycle* (2010) 9:683–8. doi: 10.4161/cc.9.4.10766
99. González A, Hall MN. Nutrient sensing and TOR signaling in yeast and mammals. *EMBO J.* (2017) 36:397–408. doi: 10.15252/embj.201696010
100. Das A, Reis F, Maejima Y. Cai Z, Ren J. *mTOR Signaling in Cardiometabolic Disease, Cancer, and Aging.* (2017). Available online at: http://downloads.hindawi.com/journals/omcl/2017/6018675.pdf (Accessed July 19, 2018).
101. Luo Z, Xu W, Ma S, Qiao H, Gao L, Zhang R, et al. Moderate autophagy inhibits vascular smooth muscle cell senescence to stabilize progressed atherosclerotic plaque via the mTORC1/ULK1/ATG13 signal pathway. *Oxid Med Cell Longev* (2017) 2017:1–9. doi: 10.1155/2017/3018190
102. Templeman NM, Murphy CT. Regulation of reproduction and longevity by nutrient-sensing pathways. *J Cell Biol.* (2018) 217:93–106. doi: 10.1083/jcb.201707168
103. Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, et al. Rapamycin-induced insulin resistance. *Science* (2012) 335 1638–43. doi: 10.1126/science.1215135
104. Wu JJ, Liu J, Chen EB, Wang JJ, Cao L, Narayan N, et al. Increased mammalian lifespan and a segmental and tissue-specific slowing of aging after genetic reduction of mtor expression. *Cell Rep.* (2013) 4:913–20. doi: 10.1016/J.CELREP.2013.07.030
105. Selman C, Tullet JMA, Wieser D, Irvine E, Lingard SJ, Choudhury AI, et al. Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science* (2009) 326:140–4. doi: 10.1126/science.1177221
106. Gu X, Orozco JM, Saxton RA, Condon KJ, Liu GY, Krawczyk PA, et al. SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway. *Science* (2017) 358:813–8. doi: 10.1126/science.aao3265
107. Efeyan A, Comb WC, Sabatini DM. Nutrient-sensing mechanisms and pathways. *Nature* (2015) 517:302–10. doi: 10.1038/nature14190
108. Duran A, Amanchy R, Linares JF, Joshi J, Abu-Baker S, Porollo A, et al. p62 is a key regulator of nutrient sensing in the mTORC1 pathway. *Mol Cell* (2011) 44:134–46. doi: 10.1016/J.MOLCEL.2011.06.038
109. Kim J, Lim YM, Lee MS. The role of autophagy in systemic metabolism and human-type diabetes. *Mol Cells* (2018) 41:11–7. doi: 10.14348/molcells.2018.2228
110. Li TY, Sun Y, Liang Y, Liu Q, Shi Y, Zhang CS, et al. ULK1/2 constitute a bifurcate node controlling glucose metabolic fluxes in addition to autophagy. *Mol Cell* (2016) 62:359–70. doi: 10.1016/J.MOLCEL.2016.04.009
111. Kapahi P, Chen D, Rogers AN, Katewa SD, Li PWL, Thomas EL, et al. With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. *Cell Metab.* (2010) 11:453–65. doi: 10.1016/J.CMET.2010.05.001
112. Imai S, Guarente L. Ten years of NAD-dependent SIR2 family deacetylases: implications for metabolic diseases. *Trends Pharmacol Sci.* (2010) 31:212–20. doi: 10.1016/J.TIPS.2010.02.003
113. Tsang CK, Chen M, Cheng X, Qi Y, Chen Y, Das I, et al. SOD1 phosphorylation by mTORC1 couples nutrient sensing and redox regulation. *Mol Cell* (2018) 70:502–15.e8. doi: 10.1016/J.MOLCEL.2018.03.029
114. Hewitt G, Korolchuk VI. Repair, reuse, recycle: the expanding role of autophagy in genome maintenance. *Trends Cell Biol.* (2017) 27:340–51. doi: 10.1016/J.TCB.2016.11.011
115. Liu L, Trimarchi JR, Smith PJS, Keefe DL. Mitochondrial dysfunction leads to telomere attrition and genomic instability. *Aging Cell* (2002) 1:40–6. doi: 10.1046/j.1474-9728.2002.00004.x
116. Donati A, Taddei M, Cavallini G, Bergamini E. Stimulation of macroautophagy can rescue older cells from 8-OHdG mtDNA accumulation: a safe and easy way to meet goals in the SENS agenda. *Rejuvenation Res.* (2006) 9:408–12. doi: 10.1089/rej.2006.9.408
117. Van Remmen H, Hamilton ML, Richardson A. Oxidative damage to DNA and aging. *Exerc Sport Sci Rev.* (2003) 31:149–53. doi: 10.1097/00003677-200307000-00009
118. Song X, Narzt MS, Nagelreiter IM, Hohensinner P, Terlecki-Zaniewicz L, Tschachler E, et al. Autophagy deficient keratinocytes display increased DNA damage, senescence and aberrant lipid composition after oxidative stress *in vitro* and *in vivo*. *Redox Biol.* (2017) 11:219–30. doi: 10.1016/J.REDOX.2016.12.015
119. Bender A, Krishnan KJ, Morris CM, Taylor GA, Reeve AK, Perry RH, et al. High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nat Genet.* (2006) 38:515–7. doi: 10.1038/ng1769
120. Rello-Varona S, Lissa D, Shen S, Niso-Santano M, Senovilla L, Mariño G, et al. Autophagic removal of micronuclei. *Cell Cycle* (2012) 11:170–6. doi: 10.4161/cc.11.1.18564
121. Guo H, Chitiprolu M, Gagnon D, Meng L, Perez-Iratxeta C, Lagace D, et al. Autophagy supports genomic stability by degrading retrotransposon RNA. *Nat Commun.* (2014) 5:5276. doi: 10.1038/ncomms6276
122. Belaid A, Cerezo M, Chargui A, Corcelle-Termeau E, Pedeutour F, Giuliano S, et al. Autophagy plays a critical role in the degradation of active RHOA, the control of cell cytokinesis, and genomic stability. *Cancer Res.* (2013) 73:4311–22. doi: 10.1158/0008-5472.CAN-12-4142
123. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, et al. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes Dev.* (2007) 21:1621–35. doi: 10.1101/gad.1565707
124. Ariyoshi K, Miura T, Kasai K, Fujishima Y, Oshimura M, Yoshida MA. Induction of genomic instability and activation of autophagy in artificial human aneuploid cells. *Mutat Res Mol Mech Mutagen.* (2016) 790:19–30. doi: 10.1016/J.MRFMMM.2016.06.001
125. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, et al. Autophagy Suppresses Tumorigenesis through Elimination of p62. *Cell* (2009) 137:1062–75. doi: 10.1016/J.CELL.2009.03.048
126. Wang Y, Zhang N, Zhang L, Li R, Fu W, Ma K, et al. Autophagy regulates chromatin ubiquitination in DNA damage response through elimination of SQSTM1/p62. *Mol Cell* (2016) 63:34–48. doi: 10.1016/J.MOLCEL.2016.05.027
127. Hewitt G, Carroll B, Sarallah R, Correia-Melo C, Ogrodnik M, Nelson G, et al. SQSTM1/p62 mediates crosstalk between autophagy and the UPS in DNA repair. *Autophagy* (2016) 12:1917–30. doi: 10.1080/15548627.2016.1210368
128. Gabbianelli R, Malavolta M. Epigenetics in ageing and development. *Mech Ageing Dev.* (2018) 174:1–2. doi: 10.1016/j.mad.2018.05.005
129. Brunet A, Rando TA. Interaction between epigenetic and metabolism in aging stem cells. *Curr Opin Cell Biol.* (2017) 45:1–7. doi: 10.1016/J.CEB.2016.12.009
130. Khalil H, Tazi M, Caution K, Ahmed A, Kanneganti A, Assani K, et al. Aging is associated with hypermethylation of autophagy genes in macrophages. *Epigenetics* (2016) 11:381–8. doi: 10.1080/15592294.2016.1144007
131. Morselli E, Mariño G, Bennetzen MV, Eisenberg T, Megalou E, Schroeder S, et al. Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome. *J Cell Biol.* (2011) 192:615–29. doi: 10.1083/jcb.201008167
132. Luyten T, Welkenhuyzen K, Roest G, Kania E, Wang L, Bittremieux M, et al. Resveratrol-induced autophagy is dependent on IP3Rs and on cytosolic Ca2+. *Biochim Biophys Acta Mol Cell Res.* (2017) 1864:947–56. doi: 10.1016/J.BBAMCR.2017.02.013
133. Eisenberg T, Schroeder S, Andryushkova A, Pendl T, Küttner V, Bhukel A, et al. Nucleocytosolic depletion of the energy metabolite acetyl-coenzyme a stimulates autophagy and prolongs lifespan. *Cell Metab.* (2014) 19:431–44. doi: 10.1016/J.CMET.2014.02.010
134. Shay JW. Telomeres and aging. *Curr Opin Cell Biol.* (2018) 52:1–7. doi: 10.1016/J.CEB.2017.12.001
135. Orlotti NI, Cimino-Reale G, Borghini E, Pennati M, Sissi C, Perrone F, et al. Autophagy acts as a safeguard mechanism against G-quadruplex ligand-mediated DNA damage. *Autophagy* (2012) 8:1185–96. doi: 10.4161/auto.20519

136. Masaldan S, Clatworthy SAS, Gamell C, Smith ZM, Francis PS, Denoyer D, et al. Copper accumulation in senescent cells: interplay between copper transporters and impaired autophagy. *Redox Biol.* (2018) 16:322–31. doi: 10.1016/J.REDOX.2018.03.007
137. Kang HT, Lee KB, Kim SY, Choi HR, Park SC. Autophagy impairment induces premature senescence in primary human fibroblasts. *PLoS ONE* (2011) 6:e23367. doi: 10.1371/journal.pone.0023367
138. Young ARJ, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JFJ, et al. Autophagy mediates the mitotic senescence transition. *Genes Dev.* (2009) 23:798–803. doi: 10.1101/gad.519709
139. Kwon Y, Kim JW, Jeoung JA, Kim MS, Kang C. Autophagy is pro-senescence when seen in close-up, but anti-senescence in long-shot. *Mol Cells* (2017) 40:607–12. doi: 10.14348/molcells.2017.0151
140. Rossi DJ, Jamieson CHM, Weissman IL. Stems cells and the pathways to aging and cancer. *Cell* (2008) 132:681–96. doi: 10.1016/J.CELL.2008.01.036
141. Ho TT, Warr MR, Adelman ER, Lansinger OM, Flach J, Verovskaya EV, et al. Autophagy maintains the metabolism and function of young and old stem cells. *Nature* (2017) 543:205–10. doi: 10.1038/nature21388
142. Doulatov S, Daley GQ. Autophagy: it's in your blood. *Dev Cell* (2017) 40:518–20. doi: 10.1016/J.DEVCEL.2017.03.011
143. Ma Y, Qi M, An Y, Zhang L, Yang R, Doro DH, et al. Autophagy controls mesenchymal stem cell properties and senescence during bone aging. *Aging Cell* (2018) 17:e12709. doi: 10.1111/acel.12709
144. García-Prat L, Martínez-Vicente M, Perdiguero E, Ortet L, Rodríguez-Ubreva J, Rebollo E, et al. Autophagy maintains stemness by preventing senescence. *Nature* (2016) 529:37–42. doi: 10.1038/nature16187
145. Filippone SM, Samidurai A, Roh SK, Cain CK, He J, Salloum FN, et al. Reperfusion therapy with rapamycin attenuates myocardial infarction through activation of AKT and ERK. *Oxid Med Cell Longev.* (2017) 2017:1–16. doi: 10.1155/2017/4619720
146. Eisenberg T, Abdellatif M, Schroeder S, Primessnig U, Stekovic S, Pendl T, et al. Cardioprotection and lifespan extension by the natural polyamine spermidine. *Nat Med.* (2016) 22:1428–38. doi: 10.1038/nm.4222
147. Wohlgemuth SE, Seo AY, Marzetti E, Lees HA, Leeuwenburgh C. Skeletal muscle autophagy and apoptosis during aging: effects of calorie restriction and life-long exercise. *Exp Gerontol.* (2010) 45:138–48. doi: 10.1016/J.EXGER.2009.11.002

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REVIEW
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Chaperone Mediated Autophagy in the Crosstalk of Neurodegenerative Diseases and Metabolic Disorders

Iván E. Alfaro[1], Amelina Albornoz[1], Alfredo Molina[2], José Moreno[2], Karina Cordero[2], Alfredo Criollo[2,3,4] and Mauricio Budini[2,3]**

[1] Fundación Ciencia & Vida, Santiago, Chile, [2] Dentistry Faculty, Institute in Dentistry Sciences, University of Chile, Santiago, Chile, [3] Autophagy Research Center (ARC), Santiago, Chile, [4] Advanced Center for Chronic Diseases (ACCDiS), University of Chile, Santiago, Chile

Chaperone Mediated Autophagy (CMA) is a lysosomal-dependent protein degradation pathway. At least 30% of cytosolic proteins can be degraded by this process. The two major protein players of CMA are LAMP-2A and HSC70. While LAMP-2A works as a receptor for protein substrates at the lysosomal membrane, HSC70 specifically binds protein targets and takes them for CMA degradation. Because of the broad spectrum of proteins able to be degraded by CMA, this pathway has been involved in physiological and pathological processes such as lipid and carbohydrate metabolism, and neurodegenerative diseases, respectively. Both, CMA, and the mentioned processes, are affected by aging and by inadequate nutritional habits such as a high fat diet or a high carbohydrate diet. Little is known regarding about CMA, which is considered a common regulation factor that links metabolism with neurodegenerative disorders. This review summarizes what is known about CMA, focusing on its molecular mechanism, its role in protein, lipid and carbohydrate metabolism. In addition, the review will discuss how CMA could be linked to protein, lipids and carbohydrate metabolism within neurodegenerative diseases. Furthermore, it will be discussed how aging and inadequate nutritional habits can have an impact on both CMA activity and neurodegenerative disorders.

Keywords: CMA, neurodegeneration, lipids, carbohydrates, metabolism

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****Correspondence:***
Iván E. Alfaro
ialfaro@cienciavida.org
Mauricio Budini
mbudini@u.uchile.cl

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INTRODUCTION

Autophagy

Autophagy is a cellular process in which proteins and organelles become degraded through lysosomes in order to maintain an adequate cellular homeostasis. In mammals, three different types of autophagy pathways have been described, (i) macroautophagy (mostly known as "autophagy"), (ii) microautophagy, and (iii) chaperone-mediated autophagy (CMA).

Macroautophagy and CMA, together with proteasome, are involved in the recycling of cellular proteins, including those that can compromise the normal physiology of the cell by adopting aberrant folded forms which are able to generate aggregates or inclusions (1). Macroautophagy is a multistep pathway that starts with the formation of an autophagosome, a double membrane vesicular structure that includes the cargo to be degraded. Autophagosome formation involves the participation of different factors like the Atg protein family, LC3-II, p62, Beclin, among others.After its formation, the autophagosome fuses to the lysosome where it releases its proteolytic material to form an autolysosome structure that finally degrades the cargo (1).

In addition to proteins, macroautophagy targets can also include damaged and/or oxidized organelles such as mitochondria (mitophagy), endoplasmic reticulum (ERphagy), peroxisomes (pexophagy), lipid droplets (lipophagy), ferritin (ferritinophagy) and zymogen granules (zymophagy) (2–5).

CMA, which will be further described below, involves the participation of HSC70 (heat shock-cognate chaperone 70 KDa) and LAMP-2A (lysosomal associated-membrane protein 2A) proteins. With respect to macro- and microautophagy, CMA has been reported only to degrade cytosolic proteins previously bound to HSC70-specific sequence (see below) (6, 7). In the case of microautophagy, it carries the degradation of proteins and organelles out, by their direct engulfment of the lysosome. In microautophagy, whereas some of the proteins can be incorporated into the lysosome through an "unspecific way", others, like those in CMA, also need the interaction with the Hsc70 chaperone. The similarities and differences between CMA and microautophagy have recently been discussed in the review of Tekiradg and Cuervo (8).

Chaperone Mediated Autophagy (CMA)

CMA Overview

Chaperone-mediated autophagy (CMA) is a specific lysosomal-dependent protein degradation pathway (9). It differs from macroautophagy because, (i) it does not involve the formation of autophagosomes and autolysosomes, (ii) its targets are cellular proteins and not organelles, (iii) the protein cargo is directly delivered into the lysosomal lumen through the interaction with HSC70 and LAMP-2A (10). HSC70 belongs to the Hsp70 protein family and it has a constitutive expression, participating principally in the CMA pathway but also in microautophagy (8). LAMP-2A protein is the A isoform of Lamp-2 (lysosome associated-membrane protein type 2) and restricts the CMA degradation process. Importantly, in CMA, selectivity resides in the fact that all CMA substrate proteins contain at least one amino acidic motif biochemically related to the penta-peptide KFERQ (11, 12). In the cell cytoplasm, HSC70 recognizes the KFERQ sequence in target proteins to form the complex HSC70-substrate. Although the temporality of this process is not well known, the complex HSC70-substrate interacts with the cytosolic tail of LAMP-2A that, in turns, drives the translocation of the target protein into the lysosome lumen (13). All cells have a basal CMA activity which helps to maintain the homeostasis of many cellular proteins. However, under certain stimulus, like nutrient starvation, serum deprivation or cellular stress (e.g., protein aggregation), CMA activity increases. This up-regulation condition can be visualized by mRNA LAMP-2A overexpression, HSC70 and LAMP-2A co-localization and LAMP-2A positive lysosomes with increased perinuclear distribution (14–16).

CMA Target Recognition

In 1985 Dice et al. found a pentapeptide in the Ribonuclease A (RNase A) which was necessary for the degradation of this protein by lysosomes in fibroblasts (11, 17–20). When different proteins were analyzed, it was found that the best aminoacidic sequence, to be present in a protein that is going to be degraded through CMA, corresponds to the pentapeptide KFERQ (11). The general rule, is that this sequence is composed of an amino acid Glutamine (Q), that must contain in one side (right of left) a tetrapeptide, including basic-, acidic- and hydrophobic- amino acids (11). Target proteins for CMA should contain at least one KFERQ-like domain, however some of them include more than one (21). Proteins containing a KFERQ-like domain do not enter the lysosomes by themselves, they first need to be recognized and bound by the chaperone HSC70 (22). This protein, also known as HspA8, is a 73 kDa protein belonging to the Hsp70 protein family (23). Thus, HSC70 specifically recognize the KFERQ-like motive in cytosolic proteins to target them for lysosomal degradation through CMA (22). In some cases, other co-factors, or covalent modifications have been found to be necessary for the binding between the complex HSC70-protein substrate and LAMP-2A. For example, STUB1/CHIP (a ubiquitin E3 ligase) assists the degradation of HIF1A through CMA by the ubiquitination of Lysin 63 (K63) (24) and also by supporting the interaction of LAMP-2A through its chaperon binding domain (24). In addition, covalent modifications, like acetylation and phosphorylation have been described to create KFERQ-like domains capable of targeting proteins for CMA degradation (25–29).

CMA Substrate Translocation and Degradation

In the lysosome membrane, the HSC70-substrate complex interacts with the lysosome receptor LAMP-2A. The protein LAMP-2A was first described by Dr. Ana María Cuervo and Dr. J. Fred Dice (30). It was identified as the LGP96 (lysosome membrane glycoprotein 96 kDa) and it was found to be a necessary receptor for the specific degradation of GAPDH (Glyceraldehyde- 3-phosphate dehydrogenase) and RNase I (Ribonuclease I) through lysosomes (30). The authors showed that both GAPDH and RNase I, were able to bind the LGP96, which were isolated from rat liver (30). The Lysosome-associated membrane glycoprotein 2 (LAMP-2) is able to generate three variants through its pre-mRNA alternative splicing, (LAMP-2A, Lamp-2B, and Lamp-2C) but, to date, it is well established that only the variant A is responsible for the CMA activity (31). Compared to variants B and C, LAMP-2A has four positively charged residues in its C-terminal domain that specifically allow its interaction with target proteins (31). Characterization of LAMP-2A showed that multimerization of this variant (and not the others) in the lysosomal membrane is directly correlated with CMA activity (13, 31). The substrate up-take by the lysosome is a step that depends on LAMP-2A (32). Monomeric LAMP-2A is able to interact with substrates, however, substrate translocation is dependent on the formation of LAMP-2A oligomers (32). Further evidence showed that LAMP-2A arranges in a stable homotrimer, with helical transmembrane domains bound by a coiled-coil conformation, and with the cytosolic tails interacting with the complex HSC70-substrate protein (13). In addition, LAMP-2A oligomerization was shown to be regulated by different proteins. Glial fibrillary acidic protein (GFAP) helps to stabilize the translocation complex in an EF1α dependent manner. The stabilizing effect of GFAP is disrupted by the association of EF1α to GTP, which in turn is released from the translocation complex and allows the self-association between

GFAP molecules. The self-interaction between GFAP molecules have a negative impact on the stabilization of the translocation complex. Thus, GTP acts as an inhibitor of CMA activity (33). Phosphorylating and dephosphorylating signals also regulate CMA activity at the level of the translocation complex (34). For example, mTORC2 (mammalian target of rapamycin complex 2) has been shown to activate, through phosphorylation, Akt kinase, which in turn inhibits the CMA activity. The mechanism by which active Akt decreases the CMA activity is not yet well established. However, it has been postulated that active Akt phosphorylates GFAP, destabilizing its binding to the LAMP-2A translocation complex. In this scenario, both, mTORC2 and Akt act as negative regulators of CMA activity. On the contrary, Pleckstrin homology (PH) domain and leucine-rich repeat protein phosphatase 1 (PHLPP1), has been shown to be recruited to the lysosome membrane in a Rac1 dependent manner. On the lysosomal membrane, PHLPP1 induces CMA activation by dephosphorylating Akt at the same residues previously phosphorylated by mTORC2. Thus, PHLPP1 is a positive regulator of CMA activity (34). In addition, the binding and up-take of the CMA substrates through the translocation complex is assisted by HSC70 and Hsp90 (heat shock protein 90) chaperones. In the case of Hsp90, it was shown that this protein stabilizes the binding of LAMP-2A at the lysosomal membrane (32). HSC70, apart from its important role in binding to the KFERQ domain, participates in two additional steps of the CMA process. One step is related to the recycling of LAMP-2A in the absence of substrates, where HSC70 has been proposed to support the destabilization of LAMP-2A from the translocation complex (32). The other step occurs at the lumen of the lysosome, where a lysosomal HSC70 (lys-HSC70) is also involved in the up-take process of the substrate. The blockage of lys-HSC70, by a specific antibody, has been directly correlated with an inhibition of the CMA activity (35). **Figure 1** summarizes the main aspects of the CMA mechanism.

CMA Activation

Koga et al. (36) showed that basal CMA activity is present in a variety of cell types. However, an up-regulation in this pathway can be observed under different stimulus or conditions. The most common stimulus for CMA activation is nutrient deprivation (or starvation). Starvation activates CMA both *in vitro* and *in vivo* (11, 20, 36) and, although the exact mechanism has not been described yet, at least *in vivo,* it has been proposed that it depends on the circulating ketone bodies (37). CMA over-activation has also been observed under DNA damage. Under this condition, CMA activity is up-regulated with the purpose to degrade the checkpoint protein kinase 1 (Chk1). The accelerated degradation of Chk1 by CMA reduces its nuclear entrance and consequently decreases the phosphorylation and destabilization of the MRN (Mre11–Rad50–Nbs1), a complex that participates in the early steps of particular DNA repair pathways (38). In line with this result, DNA irradiation of a hepatocellular carcinoma cell line also over-activates CMA. In this cell line, CMA up-regulation was responsible for the degradation of HMGB1 (high-mobility group box 1 protein) degradation, which in turn, provoked the down-regulation of the p53 protein and the consequent decrease in the apoptotic rate. In this case, CMA activation could be considered a mechanism by which this type of carcinoma is able to resist the irradiation treatment (39). Oxidative stress also activates CMA constitutively. Both, mRNA LAMP-2A levels and the recruitment of LAMP-2A protein to the lysosomal membrane (40, 41) were augmented under oxidative stress. The reasons by which CMA is activated under oxidative stress are unknown.

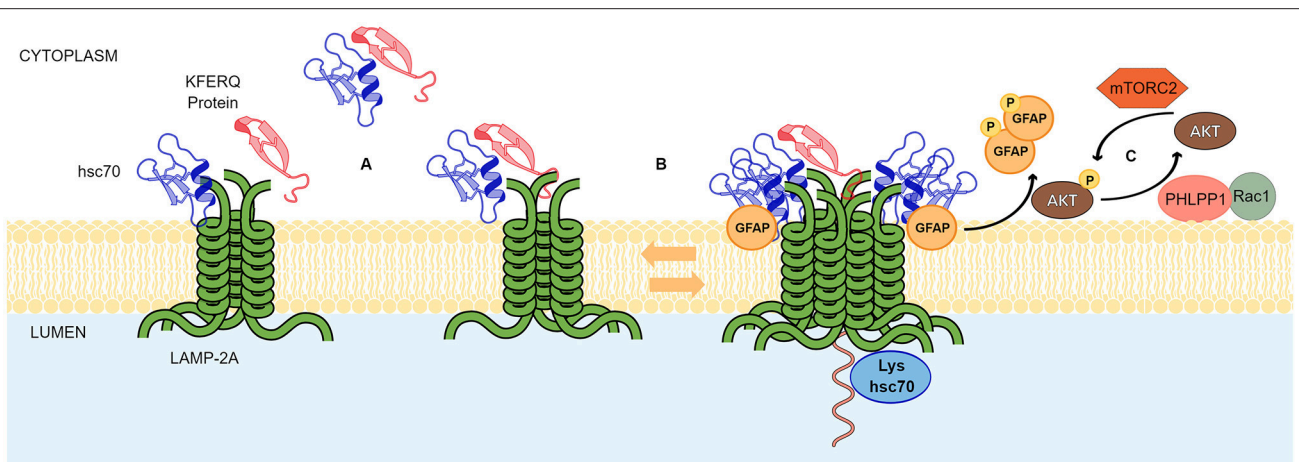

FIGURE 1 | Chaperone mediated autophagy mechanism. The scheme shows the principal events in CMA mediated translocation of proteins through the lysosomal membrane and known regulatory mechanisms. **(A)** LAMP-2A exist as an inactive oligomer at the lysosomal membrane. CMA protein substrates containing KFERQ motifs and HSC70 can bind to the cytosolic tail of LAMP-2A with similar affinity. The short C-terminal tail of LAMP-2A exposed to the cytoplasm and conformed by 12 amino acids probably binds to the chaperone and the substrate in a competitive manner. However, on the substrate, binding sites for HSC70 and LAMP-2A do not overlap allowing the HSC70-substrate complexes to interact with LAMP-2A oligomer in a substrate dependent manner. **(B)** In a dynamic fashion, GFAP favors the formation of high molecular weight aggregates of LAMP2A. Somehow, coupled and simultaneous binding of the substrate to HSC70 and LAMP-2A is sensed by the transmembrane domains of the LAMP-2A for rearrangements in the supramolecular complexes. Next, substrate unfolding and translocation to the lysosomal lumen occurs. A lysosomal-HSC70 (Lys-hsc70) collaborates in this process. **(C)** A signaling pathway involving phosphorylating and dephosphorylating signals, where AKT, MTORC2, PHLPP1, RAC1, and GFAP, regulate the stabilization of the LAMP-2A complex at lysosomal membranes.

However, a possibility could be that CMA acts as a degradative backup system for an inhibited macroautophagy pathway (42). With respect to the molecular mechanism, recent work showed that under hydrogen peroxide treatment, the transcription factor NFE2L2/NRF2 (nuclear factor, erythroid derived 2, like 2) can drive the expression of the lamp2 gene. Pharmacological activation of NFE2L2/NRF2 further supported the role of this transcription factor on lamp-2A gene overexpression (43). However, under oxidative stress, CMA up-regulation could also have a toxic effect. It was shown that CMA over-activity strongly reduces the levels of MEF2A (myocyte enhancer factor 2a), a protein necessary for the adaptive response of the cells to oxidative stress (41). Another stressor that up-regulates CMA activity is hypoxia. Over-activation of CMA was directly correlated with the survival of neuronal cells exposed to different hypoxic conditions and, on the contrary, the down-regulation of this pathway sensitized the cells to the stress (44). On the other hand, it was reported that HIF-1α, a protein that activates HIF-1 (Hypoxia-inducible factor-1) and mediates an adaptive response to hypoxia, is degraded by CMA in cells exposed to hypoxic conditions (45). Therefore, the exact mechanisms by which CMA protects cells against hypoxia, needs to be further elucidated. A constitutive activation of CMA has also been observed in different models of neurodegenerative diseases. For example, in cellular and mouse models of Huntington Disease, CMA was activated as a compensatory mechanism for the inhibition of macroautophagy. In this case, both lys-HSC70 and LAMP-2A protein levels were markedly augmented. In particular, there was an observed stability in LAMP-2A proteins at the lysosomal membrane and a transcriptional upregulation of this splice variant (46). Up-regulation of CMA was also observed in molecular mechanisms related with Parkinson's disease. For example, recent studies performed with a dopaminergic neuronal cell line, using an endoplasmic reticulum (ER) stress mouse model, showed that ER stressors are able to induce CMA activation. The mechanism for such activation involved the recruitment of MKK4 protein kinase to the lysosomal membrane and the subsequent phosphorylation of LAMP-2A by p38 MAPK protein kinase (47). This regulatory mechanism for CMA up-regulation is relevant as neurotoxins associated with PD are directly correlated with an activation of the ER-p38 MAPK–CMA response pathway (48). Additionally, in PD models, LAMP-2A and HSC70 were observed to be up-regulated when α-syn was over-expressed *in vivo*, indicating that a CMA over-activation is triggered in response to a α-syn pathological condition (49)

CMA and Metabolism

Participation of CMA in the Regulation of General Protein Metabolism

Protein homeostasis is regulated by a series of interconnected signaling pathways that sense amino acid availability, among them, the integrated signaling pathway (ISR) through the general control nonderepressible-2 protein (GCN2) and the V-ATPase/Ragulator/mTOR pathway located in lysosomes (50, 51). GCN2 is a protein kinase that is activated in the presence of accumulated deacetylated tRNAs, sensing amino acid depletion. The main target of GCN2 is the eukaryotic initiation factor 2 (eIF2), whose phosphorylation induces the inhibition of general translation and favors the synthesis of a specific set of mRNAs that regulates cell survival and promotion of macroautophagy (52). On the other hand, mTORC1 kinase is activated in response to amino acid availability, promoting translation and inhibiting macroautophagy. The mechanism of amino acid sensing, coupled with mTOR activation, involves the lysosomal amino acid transporter SLC38A9, and mTOR-binding proteins, Sestrin2 and Castor1 (50). These factors that regulate mTOR activation or inhibition, also modulate mTOR activity and thus mTOR-downstream targets involved in promotion of protein translation, cell growth and anabolism, and inhibition of macroautophagy initiation. In response to amino acid deprivation, mTOR activity is attenuated, protein translation is inhibited and macroautophagy is maximally activated to restore amino acid levels.

These non-selective mechanisms of protein homeostasis, added to a variety of mechanisms of stabilization and destabilization of protein pools, define the average life time of every protein in the cell, in nutrient rich conditions and in response to nutritional stress. Selective mechanisms of protein degradation are in contrast, generally linked to the modulation of the function of specific proteins or to the degradation of misfolded proteins that have been targeted for efficient elimination. Selective and non-selective mechanisms of proteasomal and macroautophagy protein degradation pathways have been extensively reviewed in several articles (53–56). Whereas proteins degraded by CMA need to be recognized specifically by HSC70 protein through its KFERQ domain, CMA can be considered a selective protein degradation process. An initial analysis indicated that at least 30% of cytosolic proteins contain CMA KFERQ-like motifs (7). These proteins mainly correspond to a fraction of long-lived proteins that were more rapidly degraded by lysosomes in response to serum starvation or amino acids withdrawal. Indeed, at least 90% of enhanced proteolysis observed during serum withdrawal in fibroblast cells is thought to be part of the protein pools targeted for degradation by CMA (20). In comparison to macroautophagy, the increase in degradation rates of the pool of proteins targeted to CMA is observed in response to prolonged periods of serum or nutrient deprivation (7, 12, 57). Indeed, macroautophagy activity decreases in activity after 6–12 h of nutrient removal, but CMA progressively increases in response to prolonged periods of serum or nutrient deprivation (57). Thus, CMA is a protein degradation pathway specifically activated during long term starvation. The pool of proteins degraded by CMA is very specific and different to the protein pools degraded by other protein catabolic neutral or acid proteolytic pathways. Additionally, CMA-targeted proteins accumulate in lysosomal membranes under starvation and, probably, because of this reason, this pool is excluded for degradation by proteasome or macroautophagy.

The pool of proteins targeted by CMA depends, most likely, on the periods of deprivation, type of deprivation (glucose, amino acids, serum or specific growth factors) and the cell type in the study (12). In addition, the selective pool of proteins degraded by CMA would also be associated with certain functions capable of overcoming metabolic changes in response to starvation.

This has been observed in the regulation of glycolytic flux, through the degradation of glycolytic enzymes by CMA, in order to protect cells from apoptosis (7, 21). On the other hand, proteins that do not contain KFERQ-like regions would most likely be protected against degradation, to sustain the critical functions of cells under nutritional stress. It has also been suggested that activation of non-selective macroautophagy in the first stages of starvation, help to provide metabolite building blocks (as amino acids) for the continuous synthesis of macromolecules. However, at prolonged times of starvation, a relief of macroautophagy by selective CMA-dependent protein degradation, would be necessary to avoid the elimination of essential proteins for cell survival (57). It is unknown whether nutrient deprivation directly controls the CMA activity, as with macroautophagy. The main hypothesis is that an indirect regulation of CMA by nutrient deprivation would be carried out by translational and transcriptional up-regulation of lysosomal proteins and genes involved in lysosome biogenesis (58–60). However, a recent report indicates that upregulation of CMA was responsible for mTOR activation by a mechanism that sensed the augmentation of the free amino acid content dependent on CMA activation (61). In addition, increased CMA activity, in response to LAMP2-A overexpression, was also shown to down-regulate macroautophagy through mTOR activation (62). Overall, this latter evidence reveals that CMA activation functions as a negative feedback loop for mTOR and, thus, would regulate the general amino acid and protein homeostasis signaling.

As mentioned before, basal CMA activity can be detected in different tissues, suggesting that this pathway has a constant role in the regulation of certain proteins, even under conditions of nutrient or growth factor availability (57). However, whether the degradation of these target proteins increases under CMA activation (e.g., starvation), or another specific cell response, remains to be clarified. Most likely, the latter will depend on the different affinities that these proteins would have for HSC70 and LAMP-2A, and/or on the availability of HSC70 and LAMP-2A (63).

As a consequence of its important role in controlling protein homeostasis, CMA is able to regulate metabolic pathways that are crucial to maintain balance in cellular and systemic physiology.

CMA and Regulation of Lipid Metabolism

Neutral cytosolic lipolysis and lysosome-associated acid lipolysis were classically classified in two different pathways. However, both are now recognized as synergistic, cooperative and interconnected mechanisms that contribute to lipid catabolism (64). Lysosomal lipolysis involves the degradation of lipids derived from endocytosis, as well as from cytosolic lipid stores (lipid droplets) through an autophagic process known as Lipophagy (65). Lipid droplets (LDs) are specialized organelles that store neutral lipids during fatty acid availability, mainly triglycerides (TG) and cholesterol esters, for their posterior use as energy or precursors for lipids and steroids (66). LDs consist of a core of neutral lipids separated from the aqueous cytoplasm by a phospholipid monolayer decorated by a set of proteins. These proteins are enzymes involved in lipid synthesis and lipid hydrolysis, membrane-trafficking proteins and a set of specialized LDs-associated structural proteins called perilipins (PLINs). PLINs cover a family of 5 major proteins and some additional splice variants expressed in lower amounts. Perilipin 1 (PLIN1) and 2 (PLIN2) are exclusively associated with LDs whereas perilipins 3–5 are found in the cytoplasm, associated with LDs, as well as microdroplets and lipoprotein particles (67). Perilipins 1, 2 and 5 control, at the surface of the lipid droplets, the access of cytosolic neutral lipases. For example, phosphorylation of PLIN1 and PLIN5 by protein kinase A (PKA), in response to hormones or growth factors, trigger the recruitment of neutral lipases like hormone sensitive lipase (HSL) and triacyl-glycerol lipase (ATGL) (68, 69). In contrast to PLN1 and PLIN5, PLN2 is not phosphorylated by PKA and does not recruit lipases at LDs (67). Similar to PLIN2, PLIN3 and PLIN4 protect LDs from degradation and they seem to have a tissue-specific function in the regulation of LDs size, maturation and mitochondrial association (70–72).

The involvement of autophagy in lipid turnover was first demonstrated for macroautophagy (65). Electron-microscopy studies indicated that autophagosomes engulf LDs or small portions of large LDs for degradation, forming autolipophagosomes in a process now known as macrolipophagy. In hepatocytes, macrolipophagy and the presence of lipid-containing autophagolysosomes, is stimulated by nutrient starvation and low fatty acid treatments (65). Members of the Rab GTPase family, RAB7 and RAB10 have been involved in the early steps of autophagosome recruitment to LDs (73). Moreover, the Rab effectors, EHBP1 and EH2, have been suggested to help in the elongation of autophagosomal membranes around LDs (74, 75). Neutral lipases ATGL and HSL contain LIR motifs (LC3-interaction region) and interact with the cytosolic face of autophagosomes through LC3-II, being recruited to LDs in response to macrolipophagy activation (64, 76).

In addition to macroautophagy, CMA also plays a central role in lipid homeostasis. In a mouse model, where LAMP-2A was specifically down-regulated in liver by deleting the exon 9 of lamp2 gene, it was shown that the constitutive blockade of CMA activity induces pronounced steatosis without a substantial increase in TG synthesis (77). In addition, LAMP-2A knock-out cells were insensitive to lipolysis induced by starvation and displayed increased density, size and occupancy area of LDs (78). Interestingly, in this model, non-selective macroautophagy was intact, indicating a specific and new role of CMA in lipophagy (77). In line with this evidence, further studies found the presence of KFERQ-like motifs in PLIN2 and PLIN3 proteins, and identified these two proteins as substrates for CMA lysosomal degradation. In addition, lysosomes actives in CMA isolated from rat liver, were enriched in PLIN2 and PLIN3, supporting the fact that these proteins are CMA substrates. This evidence was further complemented by experiments indicating that PLIN2 and PLIN3 degradation was reduced in LAMP-2A knockout cells (78). With regards to the mechanism, the degradation of PLIN2 through CMA in response to nutrient starvation and lipid challenges, was correlated with an increment in the association between HSC70

and perilipins (78). Reduced degradation of PLIN2 in cells that are deficient for the CMA pathway, leads to reduced lipolysis, likely due to an impairment in the association of ATGL with LDs as well as due to the defective recruitment of autophagosomal proteins and Rab7 to LDs (25, 78). Moreover, PLIN2 phosphorylation by AMPK was found to be a necessary modification for PLIN2 association with HSC70 in a form dependent on CMA activation (25, 78). Therefore, the role of CMA in lipophagy not only relays in the regulation of the recruitment of neutral lipases to LDs trough perlipin degradation, but also in the integration of a regulatory step that involves a post-transcriptional modification guided by AMPK (25). Thus, CMA itself could be considered a nutrient sensing pathway supporting the fact that lysosomes can play a role as initial sensors and integrators of lipid homeostasis (79). Additional research, however, is needed to determine how cellular pathways involved in cellular lipid homeostasis communicate with CMA and lipophagy, and how CMA-dependent lipolysis affects different tissues.

CMA and Regulation of Carbohydrate Metabolism

As mentioned before, glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was one of the first characterized as a CMA substrate (80). Additionally, at least 8 of 10 glycolytic enzymes contain CMA-targeting motifs (77). Substrates of CMA that participate in glycolysis, validated by LAMP2-A loss of function or lysosome-transport assays, also include hexokinase-2 (HK-2) (28), the M2 isoform of pyruvate kinase (81), aldolase-A, enolase-1, 3-phosphoglyceric phosphokinase, phosphoglycerate mutase, glucose-6-phosphate-dehydrogenase and the mitochondrial proteins glutamate dehydrogenase, ornithine transcarbamoylase and malate dehydrogenase (77). Other related substrates that have been suggested to be regulated by CMA in their function include phosphofructokinase-2 (82) and aldolase B (83). Glycolytic flux regulation by CMA has been confirmed *in vivo* in a mouse model with specific down-regulation of LAMP-2A in hepatocytes (77). These mice displayed higher protein levels of glycolytic enzymes and enzymes from the tricarboxylic acid cycle (TCA), a reduction in hepatic gluconeogenesis, lower glycogen synthesis and an increase in lactate production and TCA intermediates (77). This metabolic profile suggests a switch in hepatic metabolism to carbohydrate consumption as a source of energy vs. glucose biosynthesis in response to low CMA activity (77). On the other hand, classical inhibition of hepatic glycolysis caused by serum starvation (84) was not observed in mice with liver-specific CMA down-regulation (77). These results suggest that CMA activity would be necessary for a metabolic adaptive mechanism that triggers glucose production in liver to support peripheral organs under nutritional stress conditions.

The mechanism regulating CMA in response to changes in glucose availability are not fully understood. Pointing to a central role of the lysosome in sensing glucose homeostasis, new evidence indicates that glucose starvation induces changes in lysosomal acidification in an AMPK activity dependent-manner (84, 85). The mechanisms implicated in this regulation may involve a glucose-dependent regulation of the lysosome biogenesis through the transcription factor EB (TFEB) (86). Additional research is needed to elucidate how these lysosomal changes, induced by carbohydrate availability, regulate the CMA activity and, in turn, how this affects the cellular glycolytic flux.

CMA and Neurodegenerative Diseases

There is increasing evidence supporting the idea that dysregulation in the CMA pathway plays a crucial role in neurodegeneration.

Parkinson's Disease (PD)

Evidence indicates that a dysregulation in CMA could impact on the onset or progression of Parkinson's Disease (PD). As mentioned above, the main protein associated with this neurogenerative disorder, alpha-synuclein protein (α-syn), has been identified as a CMA substrate (87). More specifically, reduced α-syn degradation was observed when its KFERQ motif was mutated and the expression of LAMP-2A was knocked-down. The involvement of CMA in α-syn degradation was confirmed in different neuronal cell lines (PC12 and SH-SY5Y) and primary cultures of cortical and midbrain neurons (87). One of the hallmarks of PD is the neurotoxicity caused by the abnormal aggregation of α-syn. In this context, mutations in the protein impair its degradation through a CMA pathway, causing the accumulation α-syn oligomers that are unable to be degraded by the lysosome. This event blocks the entire CMA pathway, enhancing the oligomers formation and compromising the degradation of other CMA substrates (88, 89). As mentioned above, LAMP-2A and HSC70 were observed to be up-regulated when α-syn was over-expressed *in vivo* (49). In line with these results, it was shown that the down-regulation of LAMP-2A in adult rat substantia nigra, via an adeno-associated virus, induced intracellular accumulation of α-syn puncta. In addition, LAMP-2A down-regulation was also correlated with a progressive loss of dopaminergic neurons, severe reduction in striatal dopamine levels/terminals, increased astro- and microgliosis and relevant motor deficits (90). Furthermore, studies using the *Drosophila melanogaster* model, showed that the overexpression of human LAMP-2A protein protected the flies from progressive locomotor and oxidative defects induced by neuronal expression of a human pathological form of α-syn (91). Also, other PD related proteins seem to be regulated by CMA activity. This is the case of PARK7/DJ-1 and MEF2D. PARK7/DJ-1 is an autosomal recessive familial PD gene that plays a critical role in the antioxidative response and its dysfunction leads to mitochondrial defects. CMA was shown to degrade, preferentially, an oxidized and altered form of PARK7, providing protection from mitochondrial damage and impairing cell viability (92). For MEF2D (myocyte-specific enhancer factor 2D protein), it was shown that an inhibition of CMA provoked the accumulation of this protein in the cell cytoplasm (93). These results are in line with the observation that MEF2D levels are increased in brains of α-syn transgenic mice and in samples from patients with PD (93). Thus, an impairment in CMA activity, caused by the α-syn oligomers, could be the reason for MEF2D accumulation in PD. Beyond the studies performed in cell cultures and *in*

vivo models, strong evidence indicates that CMA could also be dysregulated in individuals affected by PD. In this regard, LAMP-2A and HSC70 were observed to be down-regulated in PD patients, suggesting that a CMA dysregulation can occur before the appearance of α-syn aggregation and other PD associated disorders (94, 95). Although the mechanisms that lead to a reduction in LAMP-2A and HSC70 levels in PD remain unknown, some studies suggest that genetic variations in the promoter of the lamp-2A gene and the up-regulation of different microRNAs that target both LAMP-2A and HSC70, could be implicated in their down-regulation (96, 97).

Alzheimer Disease (AD)

The association between CMA and Alzheimer's Disease (AD) can be described by the degradation of the RCAN1 protein (Regulator of calcineurin 1) through this pathway. Two KFERQ-like motifs identified in RCAN1 were responsible for its degradation by the CMA pathway (98). In addition, the inhibition of CMA pathway increased RCAN1 protein levels and consequently reduced the NFAT transcription factor activity (98). Interestingly, NFAT has been involved in the transcriptional regulation of the lamp2 gene (99). Thus, inhibition of CMA activity could be further enhanced by increased levels of RCAN1, that will subsequently impair NFAT-dependent transcription of lamp2. On the other hand, the Tau protein, one of the principal factors associated with AD, has also shown to be a CMA substrate (100, 101). The Tau protein is degraded by the autophagy–lysosomal system producing different fragments that, in turn, bind to HSC70 and become CMA substrates. Although these fragments are able to reach the lysosomes, they remain bound to the lysosomal membrane, causing the formation of pathological Tau aggregates that cause lysosomal damage and block the degradation of other CMA targets (100). Additionally, the amyloid precursor protein (APP), can be processed to produce another pathogenic molecule associated with AD, the β-amyloid peptide (Aβ), which contains a KFERQ related motif (102). However, deletion of KFERQ did not affect its binding to HSC70, but did somehow keep the APP and its C-terminal fragments (CTFs) away from lysosomes. Thus, the KFERQ-like domain of APP is relevant to preclude the accumulation of APP and its CTFs but, in this case, in an CMA degradation independent manner (102).

Huntington Disease (HD)

As previously mentioned, a constitutive activation of CMA has been observed in cell lines and mouse models of HD (46, 103). In a mouse model for this disease, a strong co-localization between LAMP-2A and HSC70 was correlated with augmented lysosomal degradation of the native and aberrant huntingtin protein (Htt) (46). Furthermore, additional studies performed *in vivo* also showed that Htt aggregates could be forced to be degraded through CMA, by targeting them with a polyQ binding protein (QBP1) including a KFERQ domain in its amino acidic sequence (104). The data indicated that modulation of the CMA pathway can be a plausible strategy for HD treatment.

Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Lobar Degeneration (FTLD)

Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Lobar Degeneration (FTLD) are two important neurodegenerative diseases where the implication of CMA has been poorly studied. So far, only two studies have connected these disorders with CMA and both focus on the ribonuclear protein TDP-43. Huang et al. described an KFERQ-like domain in TDP-43 that was responsible for the interaction of this protein with HSC70 under ubiquitination condition. Mutation of the KFERQ-like domain disrupted the ubiquitin-dependent binding of TDP-43 with HSC70. In addition, the down-regulation LAMP-2A by siRNA treatment, seems to increase the level of the pathologically-related 25-KDa and 35-KDa TDP-43 C-terminal fragments, but not the full-length protein (105). On the other hand, in a recent study, Tamaki, Y., *et al* designed an antibody able to recognize abnormal (unfolded or mislocated) TDP-43. The addition of the KFERQ sequence to this antibody, was able to promote the degradation of abnormal TDP-43 through the CMA pathway. Although in this case the connection of TDP-43 with CMA was not in a physiological context, this work indicates that a forced CMA target degradation of TDP-43 can be used as a strategy to ameliorate neurodegenerative diseases associated with this protein (106).

CMA in the Crosstalk With Metabolic Disorders and Neurodegeneration

Reduced CMA Activity With Aging

Aging can be considered as a physiological process that is associated with metabolic and neurodegenerative disorders via mechanisms that in many cases are completely unknown. With regards to autophagy, it has been observed that this lysosomal degradative mechanism also declines with age (107). In the case of CMA, this was determined by isolating lysosomes from the liver of young and old rats (108). Lysosomes from old rats had reduced LAMP-2A protein levels at both, total and lysosomal levels. The decrease of the LAMP-2A protein in lysosomes is associated with changes in lipid composition of these organelles that accelerate the degradation rate of the protein (108). With respect to the HSC70 protein, although the total protein levels remained unchanged, lysosomal HSC70 levels were augmented, probably to compensate the decline observed in the CMA activity (108). Further experiments demonstrated that, although the levels of Lamp2A mRNA were unchanged, there was a problem in the localization of LAMP-2A at the lysosomal membrane in old rats, consequently reducing the CMA activity (109). Furthermore, by using an animal model with specific down-regulation of CMA in the liver, it was possible to demonstrate that reduced CMA activity in old animals had an impact on the ability to overcome proteostasis induced by different stressors like oxidative stress, lipid challenging and aging (110). The mentioned evidences clearly demonstrate that CMA activity is reduced in older individuals and that this protein degradative pathway helps to stabilize the different disorders associated with aging.

Thus, considering that CMA activity is reduced with age, it is possible to argue that a decline in CMA activity during aging can be a risk factor for the development of neurodegenerative disorders associated with adult and senior people. In fact, beyond of what it can be concluded from *in vitro* and *in vivo* models, more evidence is coming out revealing that a dysregulation in CMA activity can be present in human neurodegeneration. Most of the evidence is a result of studies on PD. For example, a study performed with peripheral blood mononuclear cells from PD patients, showed that affected people had reduced HSC70 protein levels (111). Another study, analyzing CSF (cerebrospinal fluid) of PD patients with mutations in the repeat kinase 2 (LRRK2) gene, confirmed that LAMP-2A protein levels were reduced in affected females, compared to healthy people (112).

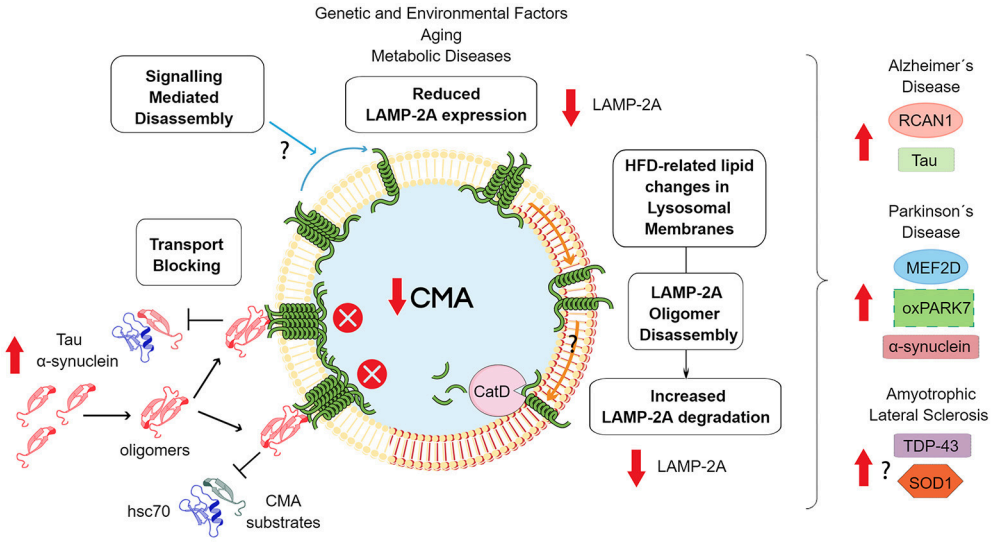

FIGURE 2 | Consequences of downregulated CMA activity. The scheme shows the principal mechanisms of CMA downregulation. Reduced expression of the lamp2 gene has been related with genetic polymorphisms (96), aging (108) and metabolic disorders (132). In relation to the latter situation, HFD has been shown to modify the lipid composition of lysosomal membranes, including increased levels of cholesterol and ceramide (132). These modifications induces the formation of organized lipid microdomains, which prevent LAMP-2A multimerization and stimulate subsequent degradation through proteolysis by Cathepsin D (CatD) (133). Independently of the mechanism, a reduction in CMA activity can induce an increase in the levels of some proteins related with the development of neurodegenerative diseases. In the case of tau, α-synuclein or other less characterized CMA protein substrates that are able to aggregate, increases the total mass that these proteins would contribute to protein oligomerization, an event associated with a blockade of CMA transport. In addition, oxidative damage, would enhance the aggregation process. Additionally, changes in signaling pathways that control LAMP2A oligomerization and membrane stabilization can alter LAMP-2A membrane dynamics and CMA activity. Finally, CMA down-regulation can alter a series of other specific proteins related with neuronal survival or neuroinflammation, that participate in the development of neurodegenerative diseases.

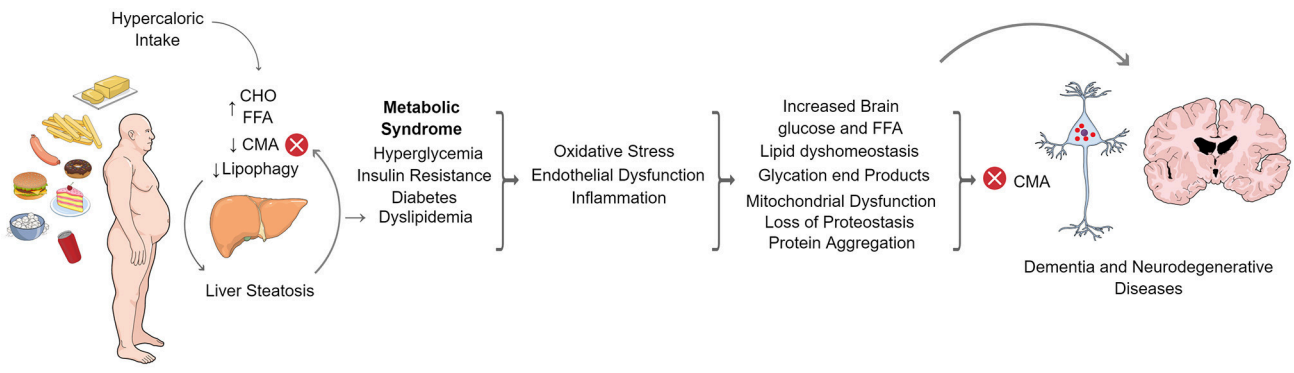

FIGURE 3 | CMA in the crosstalk of metabolic dysfunction and neurodegenerative diseases. Increased caloric intake in humans produces elevated circulating levels of carbohydrates (CHO) and free fatty acids (FFA). In the liver, FFAs accumulate in lipid droplets leading to hepatic steatosis. FFAs also inhibit CMA and lipophagy. The latter contributes to hepatic fat accumulation and systemic dyslipidemia that, together with increased levels of glucose, leads to metabolic syndrome characterized by insulin resistance and diabetes. Metabolic syndrome is a general status also characterized by oxidative stress, inflammation and endothelial dysfunction. This condition is present throughout the body and is also capable of affecting the nervous system, resulting in lipid dyshomeostasis, mitochondrial dysfunction, loss of proteostasis and protein aggregation in the brain. All of these processes have been shown to alter proteostatic mechanisms, including CMA. Loss of neuronal proteostasis finally alters neuronal function which leads to dementia or the development of neurodegenerative diseases.

However, studies focused on analyzing the levels of LAMP-2A and HSC70 in healthy people during normal aging, failed to find any positive association (113). Thus, future studies need to focus on whether changes in CMA activity (or CMA protein players) are altered during normal aging in healthy people, or if they are only associated with patients affected by neurodegeneration. The latter will help to elucidate whether observed changes in CMA protein players are a cause or a consequence of these disorders.

CMA Activity, Metabolic Disorders and Neurodegenerative Diseases

In vivo models strongly support the hypothesis that CMA activity decreases with aging. Considering this evidence, which of these could be the consequence of CMA activity in people with a high fat diet (HFD)? Rodriguez-Navarro et al. showed that chronic exposure of rats to an HFD or cholesterol-enriched diet, provoked a decrease in CMA activity (114). Further characterization of these animals showed that isolated lysosomes from liver have reduced LAMP-2A protein at lysosomal membranes. The latter was triggered by an accelerated degradation of lysosomal LAMP-2A, something also observed in older animals. Interestingly, authors also found that lysosomal membranes from animals with a HFD presented changes in lipid composition, in a similar way than that was observed with age (114). Thus, a dysregulation of CMA activity produced by aging, could impact on the LDs composition, affecting the aggregation of some proteins involved in neurodegenerative diseases.

In addition, a systemic decrease in CMA activity, and the onset or progression of neurodegenerative disorders, could be enhanced by an HFD. Different works demonstrate that a HFD can be a risk factor for the development of neurodegenerative diseases. For example, in a mouse model of PD, it was observed that HFD provoked a reduction in Parkin protein levels (115). In addition, similar results were obtained with a α-syn transgenic mouse model of PD. Compared with wild type mice, an HFD causes obesity and glucose intolerance in transgenic mice. Furthermore, transgenic mice also had an accelerated onset of PD disease and premature movement phenotype and death (116). The same situation was observed in transgenic mice models for AD. The model with an HFD, presented exacerbated neuropathology, defects in synaptic stability/plasticity, apoptotic neuronal cell death (117) and increased levels of insoluble amyloid-β (AB) and Tau (118). On the other hand, LDs have been found to play a role in neurodegenerative diseases. For example, as reviewed by Pennetta et al. LDs seem to be important for the progression of motor neuron diseases (MND) (119). The mechanisms by which LDs are affecting neurodegeneration processes are unknown, however it has been observed that they can affect protein aggregation. For example, in cells treated with an HFD, α-syn accumulated in triglyceride-riche LDs (120). In a similar way, amyloidal fibrils from amyloidotic polyneuropathy (FAP), were observed to be colocalizing with a high density lipoprotein (HDL) (121). Altogether, these data support the idea that LDs could be involved with the aggregation process of neurodegenerative disease associated proteins.

On the other hand, there is increasing evidence which now supports the idea that a high carbohydrate diet (HCD) is a risk factor for certain neurodegenerative diseases (122). Probably, because of a dysregulation in protein glycation, which can control the balance between protein solubility and aggregation (123, 124). Moreover, many reports indicate that there is a strong association between diabetes and people affected by AD (125, 126), suggesting that a dysregulation in glucose metabolism could impact on the onset of this and other neurodegenerative diseases (127). Although the mechanisms are not clear, evidence has shown that there is an increase in the activity of glycolytic enzymes in AD patients (128). In addition, α-syn was observed to bind GAPDH, increasing the activity of this enzyme (129). Also, analyses performed with postmortem human brain tissue, reported a reduction of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in AD and PD (127).

Carbohydrate metabolism dysregulation has also been found in different models of HD. Gouarné et al. found that a rat model of HD had a deficit in glycolysis in striatal neurons (130). In a mouse model of HD, it was observed that glycolysis inhibition decreases the levels of glutamate transport and provoked neurotoxicity (131). On the contrary, in two different models of HD, it was found that the activity of some glycolytic enzymes was higher. The latter results were not correlated with the enhanced conversion of glucose to lactate and increased ATP in the brain and tissue, respectively. Altogether, this evidence indicates that in neurodegenerative diseases, an imbalance in a glycolytic pathway could play an important role in the onset or progression of the disease. Considering that CMA regulates the homeostasis of glycolytic enzymes, changes in the activity of this pathway along with aging could strongly impact on the risk to develop a neurodegenerative disease. In addition, the risk can also be augmented by aging and by a continuous intake of a high carbohydrate diet. **Figure 2** shows the main mechanisms that can influence CMA down-regulation and its impact in neurodegenerative diseases.

CONCLUDING REMARKS

As part of the autophagic pathways, CMA has been involved in regulating the metabolism of different proteins. Many of these proteins are strongly associated with neurodegenerative diseases affecting humans, while others are involved in the regulation of metabolic pathways such as lipids and carbohydrate metabolism. In addition, neurodegenerative diseases and metabolic disorders are common features of aged humans and many reports associate the dysregulation in lipids or carbohydrate metabolism, with the risk of developing some type of neurodegenerative disease. It has also been reported that a high fat, or high carbohydrate diet (or glucose reach diet), can also increase neurodegeneration prevalence or progression. On the other hand, CMA activity, has been observed to decline with age and with a non-balanced dietary intake such as a high fat diet. Overall, CMA can be considered a common factor in the regulation of metabolic and neurodegenerative pathways

and a dysregulation in CMA, provoked by normal aging, or by metabolic disorders induced by deficient nutritional habits, which could tilt the balance toward a pathological situation. **Figure 3** shows an overview of how CMA could be connected with metabolic dysfunctions and neurodegenerative disorders.

AUTHOR CONTRIBUTIONS

AA and MB wrote the article. IEA wrote the article and created the figures. AM, JM, KC, and AC made important contributions, participated in discussions and provided corrections to the manuscript.

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REFERENCES

1. Kaushik S, Cuervo AM. The coming of age of chaperone-mediated autophagy. *Nat Rev Mol Cell Biol.* (2018) 19:365–81. doi: 10.1038/s41580-018-0001-6
2. Iwata J, Ezaki J, Komatsu M, Yokota S, Ueno T, Tanida I, et al. Excess peroxisomes are degraded by autophagic machinery in mammals. *J Biol Chem.* (2006) 281:4035–41. doi: 10.1074/jbc.M512283200
3. Lamb CA, Yoshimori T, Tooze SA. The autophagosome: origins unknown, biogenesis complex. *Nat Rev Mol Cell Biol.* (2013) 14:759–74. doi: 10.1038/nrm3696
4. Narendra D, Tanaka A, Suen DF, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol.* (2008) 183:795–803. doi: 10.1083/jcb.200809125
5. Grasso D, Ropolo A, Lo Re A, Boggio V, Molejon MI, Iovanna JL, et al. Zymophagy, a novel selective autophagy pathway mediated by VMP1-USP9x-p62, prevents pancreatic cell death. *J Biol Chem.* (2011) 286:8308–24. doi: 10.1074/jbc.M110.197301
6. Agarraberes FA, Dice JF. A molecular chaperone complex at the lysosomal membrane is required for protein translocation. *J Cell Sci.* (2001) 114:2491–9.
7. Cuervo AM, Knecht E, Terlecky SR, Dice JF. Activation of a selective pathway of lysosomal proteolysis in rat liver by prolonged starvation. *Am J Physiol.* (1995) 269:C1200–8. doi: 10.1152/ajpcell.1995.269.5.C1200
8. Tekirdag K, Cuervo AM. Chaperone-mediated autophagy and endosomal microautophagy: joint by a chaperone. *J Biol Chem.* (2018) 293:5414–24. doi: 10.1074/jbc.R117.818237
9. Isenman LD, Dice JF. Secretion of intact proteins and peptide fragments by lysosomal pathways of protein degradation. *J Biol Chem*. (1989) 264:21591–6.
10. Arias E, Cuervo AM. Chaperone-mediated autophagy in protein quality control. *Curr Opin Cell Biol*. (2011) 23:184–9. doi: 10.1016/j.ceb.2010.10.009
11. Dice JF. Peptide sequences that target cytosolic proteins for lysosomal proteolysis. *Trends Biochem Sci.* (1990) 15:305–9. doi: 10.1016/0968-0004(90)90019-8
12. Wing SS, Chiang HL, Goldberg AL, Dice JF. Proteins containing peptide sequences related to Lys-Phe-Glu-Arg-Gln are selectively depleted in liver and heart, but not skeletal muscle, of fasted rats. *Biochem J.* (1991) 275(Pt 1):165–9. doi: 10.1042/bj2750165
13. Rout AK, Strub MP, Piszczek G, Tjandra N. 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*. (2014) 289:35111–23. doi: 10.1074/jbc.M114.609446
14. Arias E. Methods to study chaperone-mediated autophagy. *Methods Enzymol*. (2017) 588:283–305. doi: 10.1016/bs.mie.2016.10.009
15. Kaushik S, Cuervo AM. Chaperone-mediated autophagy. *Methods Mol Biol*. (2008) 445:227–44. doi: 10.1007/978-1-59745-157-4_15
16. Patel B, Cuervo AM. Methods to study chaperone-mediated autophagy. *Methods* (2015) 75:133–40. doi: 10.1016/j.ymeth.2015.01.003
17. McElligott MA, Miao P, Dice JF. Lysosomal degradation of ribonuclease A and ribonuclease S-protein microinjected into the cytosol of human fibroblasts. *J Biol Chem.* (1985) 260:11986–93.
18. McElligott MA, Dice JF. Degradation of microinjected ribonuclease A and ribonuclease S-protein by lysosomal pathways. *Prog Clin Biol Res*. (1985) 180:471–3.
19. Dice JF, Backer JM, Miao P, Bourret L, McElligott MA. Regulation of catabolism of ribonuclease A microinjected into human fibroblasts. *Prog Clin Biol Res*. (1985) 180:385–94.
20. Chiang HL, Dice JF. Peptide sequences that target proteins for enhanced degradation during serum withdrawal. *J Biol Chem.* (1988) 263:6797–805.
21. Cuervo AM, Gomes AV, Barnes JA, Dice JF. Selective degradation of annexins by chaperone-mediated autophagy. *J Biol Chem.* (2000) 275:33329–35. doi: 10.1074/jbc.M005655200
22. Chiang HL, Terlecky SR, Plant CP, Dice JF. A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science* (1989) 246:382–5. doi: 10.1126/science.2799391
23. Stricher F, Macri C, Ruff M, Muller S. HSPA8/HSC70 chaperone protein: structure, function, and chemical targeting. *Autophagy* (2013) 9:1937–54. doi: 10.4161/auto.26448
24. Ferreira JV, Soares AR, Ramalho JS, Pereira P, Girao H. K63 linked ubiquitin chain formation is a signal for HIF1A degradation by Chaperone-Mediated Autophagy. *Sci Rep.* (2015) 5:10210. doi: 10.1038/srep10210
25. Kaushik S, Cuervo AM. AMPK-dependent phosphorylation of lipid droplet protein PLIN2 triggers its degradation by CMA. *Autophagy* (2016) 12:432–8. doi: 10.1080/15548627.2015.1124226
26. Quintavalle C, Di Costanzo S, Zanca C, Tasset I, Fraldi A, Incoronato M, et al. Phosphorylation-regulated degradation of the tumor-suppressor form of PED by chaperone-mediated autophagy in lung cancer cells. *J Cell Physiol.* (2014) 229:1359–68. doi: 10.1002/jcp.24569
27. Bonhoure A, Vallentin A, Martin M, Senff-Ribeiro A, Amson R, Telerman A, et al. Acetylation of translationally controlled tumor protein promotes its degradation through chaperone-mediated autophagy. *Eur J Cell Biol* (2017) 96:83–98. doi: 10.1016/j.ejcb.2016.12.002
28. Lv L, Li D, Zhao D, Lin R, Chu Y, Zhang H, et al. Acetylation targets the M2 isoform of pyruvate kinase for degradation through chaperone-mediated autophagy and promotes tumor growth. *Mol Cell* (2011) 42:719–30. doi: 10.1016/j.molcel.2011.04.025
29. Thompson LM, Aiken CT, Kaltenbach LS, Agrawal N, Illes K, Khoshnan A, et al. IKK phosphorylates Huntingtin and targets it for degradation by the proteasome and lysosome. *J Cell Biol.* (2009) 187:1083–99. doi: 10.1083/jcb.200909067
30. Cuervo AM, Dice JF. A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* (1996) 273:501–3. doi: 10.1126/science.273.5274.501
31. Cuervo AM, Dice JF. Unique properties of lamp2a compared to other lamp2 isoforms. *J Cell Sci.* (2000) 113(Pt 24):4441–50.
32. Bandyopadhyay U, Kaushik S, Varticovski L, Cuervo AM. The chaperone-mediated autophagy receptor organizes in dynamic protein

complexes at the lysosomal membrane. *Mol Cell Biol.* (2008) 28:5747–63. doi: 10.1128/MCB.02070-07
33. Bandyopadhyay U, Sridhar S, Kaushik S, Kiffin R, Cuervo AM. Identification of regulators of chaperone-mediated autophagy. *Mol Cell* (2010) 39:535–47. doi: 10.1016/j.molcel.2010.08.004
34. Arias E, Koga H, Diaz A, Mocholi E, Patel B, Cuervo AM. Lysosomal mTORC2/PHLPP1/Akt Regulate Chaperone-Mediated Autophagy. *Mol Cell* (2015) 59:270–84. doi: 10.1016/j.molcel.2015.05.030
35. Agarraberes FA, Terlecky SR, Dice JF. An intralysosomal hsp70 is required for a selective pathway of lysosomal protein degradation. *J Cell Biol.* (1997) 137:825–34. doi: 10.1083/jcb.137.4.825
36. Koga H, Martinez-Vicente M, Macian F, Verkhusha VV, Cuervo AM. A photoconvertible fluorescent reporter to track chaperone-mediated autophagy. *Nat Commun.* (2011) 2:386. doi: 10.1038/ncomms1393
37. Finn PF, Dice JF. Ketone bodies stimulate chaperone-mediated autophagy. *J Biol Chem.* (2005) 280:25864–70. doi: 10.1074/jbc.M502456200
38. Park C, Suh Y, Cuervo AM. Regulated degradation of Chk1 by chaperone-mediated autophagy in response to DNA damage. *Nat Commun.* (2015) 6:6823. doi: 10.1038/ncomms7823
39. Wu JH, Guo JP, Shi J, Wang H, Li LL, Guo B, et al. CMA down-regulates p53 expression through degradation of HMGB1 protein to inhibit irradiation-triggered apoptosis in hepatocellular carcinoma. *World J Gastroenterol.* (2017) 23:2308–17. doi: 10.3748/wjg.v23.i13.2308
40. Kiffin R, Christian C, Knecht E, Cuervo AM. Activation of chaperone-mediated autophagy during oxidative stress. *Mol Biol Cell* (2004) 15:4829–40. doi: 10.1091/mbc.e04-06-0477
41. Zhang L, Sun Y, Fei M, Tan C, Wu J, Zheng J, et al. Disruption of chaperone-mediated autophagy-dependent degradation of MEF2A by oxidative stress-induced lysosome destabilization. *Autophagy* (2014) 10:1015–35. doi: 10.4161/auto.28477
42. Wang Y, Singh R, Xiang Y, Czaja MJ. Macroautophagy and chaperone-mediated autophagy are required for hepatocyte resistance to oxidant stress. *Hepatology* (2010) 52:266–77. doi: 10.1002/hep.23645
43. Pajares M, Rojo AI, Arias E, Diaz-Carretero A, Cuervo AM, Cuadrado A. Transcription factor NFE2L2/NRF2 modulates chaperone-mediated autophagy through the regulation of LAMP2A. *Autophagy* (2018) 14:1310–22. doi: 10.1080/15548627.2018.1474992
44. Dohi E, Tanaka S, Seki T, Miyagi T, Hide I, Takahashi T, et al. Hypoxic stress activates chaperone-mediated autophagy and modulates neuronal cell survival. *Neurochem Int.* (2012) 60:431–42. doi: 10.1016/j.neuint.2012.01.020
45. Hubbi ME, Hu H, Kshitiz, Ahmed I, Levchenko A, Semenza GL. Chaperone-mediated autophagy targets hypoxia-inducible factor-1alpha (HIF-1alpha) for lysosomal degradation. *J Biol Chem.* (2013) 288:10703–14. doi: 10.1074/jbc.M112.414771
46. Koga H, Martinez-Vicente M, Arias E, Kaushik S, Sulzer D, Cuervo AM. Constitutive upregulation of chaperone-mediated autophagy in Huntington's disease. *J Neurosci.* (2011) 31:18492–505. doi: 10.1523/JNEUROSCI.3219-11.2011
47. Li W, Yang Q, Mao Z. Signaling and induction of chaperone-mediated autophagy by the endoplasmic reticulum under stress conditions. *Autophagy* (2018) 14:1094–96. doi: 10.1080/15548627.2018.1444314
48. Li W, Zhu J, Dou J, She H, Tao K, Xu H, et al. Phosphorylation of LAMP2A by p38 MAPK couples ER stress to chaperone-mediated autophagy. *Nat Commun.* (2017) 8:1763. doi: 10.1038/s41467-017-01609-x
49. Mak SK, McCormack AL, Manning-Bog AB, Cuervo AM, Di Monte DA. Lysosomal degradation of alpha-synuclein *in vivo*. *J Biol Chem.* (2010) 285:13621–9. doi: 10.1074/jbc.M109.074617
50. Wolfson RL, Sabatini DM. The dawn of the age of amino acid sensors for the mTORC1 pathway. *Cell Metab.* (2017) 26:301–9. doi: 10.1016/j.cmet.2017.07.001
51. Anthony TG. Homeostatic responses to amino acid insufficiency. *Anim Nutr.* (2015) 1:135–7. doi: 10.1016/j.aninu.2015.10.001
52. Castilho BA, Shanmugam R, Silva RC, Ramesh R, Himme BM, Sattlegger E. Keeping the eIF2 alpha kinase Gcn2 in check. *Biochim Biophys Acta* (2014) 1843:1948–68. doi: 10.1016/j.bbamcr.2014.04.006
53. Dikic I. Proteasomal and autophagic degradation systems. *Annu Rev Biochem.* (2017) 86:193–224. doi: 10.1146/annurev-biochem-061516-044908
54. Sorokin AV, Kim ER, Ovchinnikov LP. Proteasome system of protein degradation and processing. *Biochemistry* (2009) 74:1411–42. doi: 10.1134/S000629790913001X
55. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. *Cell Res.* (2014) 24:24–41. doi: 10.1038/cr.2013.168
56. Gatica D, Lahiri V, Klionsky DJ. Cargo recognition and degradation by selective autophagy. *Nat Cell Biol.* (2018) 20:233–242. doi: 10.1038/s41556-018-0037-z
57. Massey A, Kiffin R, Cuervo AM. Pathophysiology of chaperone-mediated autophagy. *Int J Biochem Cell Biol.* (2004) 36:2420–34. doi: 10.1016/j.biocel.2004.04.010
58. Lai MC, Chang CM, Sun HS. Hypoxia induces autophagy through translational up-regulation of lysosomal proteins in human colon cancer cells. *PLoS ONE* (2016) 11:e0153627. doi: 10.1371/journal.pone.0153627
59. Puertollano R, Ferguson SM, Brugarolas J, Ballabio A. The complex relationship between TFEB transcription factor phosphorylation and subcellular localization. *EMBO J.* (2018) 37:e98804. doi: 10.15252/embj.201798804
60. Martina JA, Puertollano R. TFEB and TFE3: the art of multi-tasking under stress conditions. *Transcription* (2017) 8:48–54. doi: 10.1080/21541264.2016.1264353
61. Le Y, Zhang S, Ni J, You Y, Luo K, Yu Y, et al. Sorting nexin (2018) 10 controls mTOR activation through regulating amino-acid metabolism in colorectal cancer. *Cell Death Dis.* 9:666. doi: 10.1038/s41419-018-0719-2
62. Han Q, Deng Y, Chen S, Chen R, Yang M, Zhang Z, et al. Downregulation of ATG5-dependent macroautophagy by chaperone-mediated autophagy promotes breast cancer cell metastasis. *Sci Rep.* (2017) 7:4759. doi: 10.1038/s41598-017-04994-x
63. Cuervo AM, Terlecky SR, Dice JF, Knecht E. Selective binding and uptake of ribonuclease A and glyceraldehyde-3-phosphate dehydrogenase by isolated rat liver lysosomes. *J Biol Chem.* (1994) 269:26374–80.
64. Zechner R, Madeo F, Kratky D. Cytosolic lipolysis and lipophagy: two sides of the same coin. *Nat Rev Mol Cell Biol.* (2017) 18:671–84. doi: 10.1038/nrm.2017.76
65. Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, et al. Autophagy regulates lipid metabolism. *Nature* (2009) 458:1131–5. doi: 10.1038/nature07976
66. Guo Y, Cordes KR, Farese RV Jr, Walther TC. Lipid droplets at a glance. *J Cell Sci.* (2009) 122:749–52. doi: 10.1242/jcs.037630
67. Sztalryd C, Brasaemle DL. The perilipin family of lipid droplet proteins: gatekeepers of intracellular lipolysis. *Biochim Biophys Acta* (2017) 1862:1221–32. doi: 10.1016/j.bbalip.2017.07.009
68. Cuervo AM, Mann L, Bonten EJ, A. d'Azzo, Dice JF. Cathepsin A regulates chaperone-mediated autophagy through cleavage of the lysosomal receptor. *EMBO J.* (2003) 22:47–59. doi: 10.1093/emboj/cdg002
69. Wang H, Sreenivasan U, Hu H, Saladino A, Polster BM, Lund LM, et al. Perilipin 5, a lipid droplet-associated protein, provides physical and metabolic linkage to mitochondria. *J Lipid Res.* (2011) 52:2159–68. doi: 10.1194/jlr.M017939
70. Ramos SV, Turnbull PC, MacPherson RE, LeBlanc PJ, Ward WE, Peters SJ. Changes in mitochondrial perilipin 3 and perilipin 5 protein content in rat skeletal muscle following endurance training and acute stimulated contraction. *Exp Physiol.* (2015) 100:450–62. doi: 10.1113/expphysiol.2014.084434
71. Bell M, Wang H, Chen H, McLenithan JC, Gong DW, Yang RZ, et al. Consequences of lipid droplet coat protein downregulation in liver cells: abnormal lipid droplet metabolism and induction of insulin resistance. *Diabetes* (2008) 57:2037–45. doi: 10.2337/db07-1383
72. Chen W, Chang B, Wu X, Li L, Sleeman M, Chan L. Inactivation of Plin4 downregulates Plin5 and reduces cardiac lipid accumulation in mice. *Am J Physiol Endocrinol Metab.* (2013) 304:E770–9. doi: 10.1152/ajpendo.00523.2012
73. Li Z, Schulze RJ, Weller SG, Krueger EW, Schott MB, Zhang X, et al. A novel Rab10-EHBP1-EHD2 complex essential for the autophagic engulfment of lipid droplets. *Sci Adv.* (2016) 2:e1601470. doi: 10.1126/sciadv.1601470
74. Wang P, Liu H, Wang Y, Liu O, Zhang J, Gleason A, et al. RAB-10 Promotes EHBP-1 bridging of filamentous actin and tubular recycling endosomes. *PLoS Genet.* (2016) 12:e1006093. doi: 10.1371/journal.pgen.1006093

75. Cai B, Giridharan SS, Zhang J, Saxena S, Bahl K, Schmidt JA, et al. Differential roles of C-terminal Eps15 homology domain proteins as vesiculators and tubulators of recycling endosomes. *J Biol Chem*. (2013) 288:30172-80. doi: 10.1074/jbc.M113.488627
76. Martinez-Lopez N, Garcia-Macia M, Sahu S, Athonvarangkul D, Liebling E, Merlo P, et al. Autophagy in the CNS and periphery coordinate lipophagy and lipolysis in the brown adipose tissue and liver. *Cell Metab*. (2016) 23:113–27. doi: 10.1016/j.cmet.2015.10.008
77. Schneider JL, Suh Y, Cuervo AM. Deficient chaperone-mediated autophagy in liver leads to metabolic dysregulation. *Cell Metab*. (2014) 20:417–32. doi: 10.1016/j.cmet.2014.06.009
78. Kaushik S, Cuervo AM. Degradation of lipid droplet-associated proteins by chaperone-mediated autophagy facilitates lipolysis. *Nat Cell Biol*. (2015) 17:759–70. doi: 10.1038/ncb3166
79. Mony VK, Benjamin S, O'Rourke EJ. A lysosome-centered view of nutrient homeostasis. *Autophagy* (2016) 12:619–31. doi: 10.1080/15548627.2016.1147671
80. Aniento F, Roche E, Cuervo AM, Knecht E. Uptake and degradation of glyceraldehyde-3-phosphate dehydrogenase by rat liver lysosomes. *J Biol Chem*. (1993) 268:10463–70.
81. Xia HG, Najafov A, Geng J, Galan-Acosta L, Han X, Guo Y, et al. Degradation of HK2 by chaperone-mediated autophagy promotes metabolic catastrophe and cell death. *J Cell Biol*. (2015) 210:705–16. doi: 10.1083/jcb.201503044
82. Bockus LB, Matsuzaki S, Vadvalkar SS, Young ZT, Giorgione JR, Newhardt MF, et al. Cardiac insulin signaling regulates glycolysis through phosphofructokinase 2 Content and Activity. *J Am Heart Assoc*. (2017) 6:e007159. doi: 10.1161/JAHA.117.007159
83. Susan PP, Dunn WA Jr. Starvation-induced lysosomal degradation of aldolase B requires glutamine 111 in a signal sequence for chaperone-mediated transport. *J Cell Physiol*. (2001) 187:48–58. doi: 10.1002/1097-4652(2001)9999:9999<00::AID-JCP1050>3.0.CO;2-I
84. McGuire CM, Forgac M. Glucose starvation increases V-ATPase assembly and activity in mammalian cells through AMP kinase and phosphatidylinositide 3-kinase/Akt signaling. *J Biol Chem*. (2018) 293:9113–23. doi: 10.1074/jbc.RA117.001327
85. Nwadike C, Williamson LE, Gallagher LE, Guan JL, Chan EYW. AMPK inhibits ULK1-dependent autophagosome formation and lysosomal acidification via distinct mechanisms. *Mol Cell Biol*. (2018) 38:e00023-18. doi: 10.1128/MCB.00023-18
86. Li L, Friedrichsen HJ, Andrews S, Picaud S, Volpon L, Ngeow K, et al. A TFEB nuclear export signal integrates amino acid supply and glucose availability. *Nat Commun*. (2018) 9:2685. doi: 10.1038/s41467-018-04849-7
87. Vogiatzi T, Xilouri M, Vekrellis K, Stefanis L. Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells. *J Biol Chem*. (2008) 283:23542–56. doi: 10.1074/jbc.M801992200
88. Martinez-Vicente M, Talloczy Z, Kaushik S, Massey AC, Mazzulli J, Mosharov EV, et al. Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy. *J Clin Invest*. (2008) 118:777–88. doi: 10.1172/JCI32806
89. Xilouri M, Vogiatzi T, Vekrellis K, Park D, Stefanis L. Abberant alpha-synuclein confers toxicity to neurons in part through inhibition of chaperone-mediated autophagy. *PLoS ONE* (2009) 4:e5515. doi: 10.1371/journal.pone.0005515
90. Xilouri M, Brekk OR, Polissidis A, Chrysanthou-Piterou M, Kloukina I, Stefanis L. Impairment of chaperone-mediated autophagy induces dopaminergic neurodegeneration in rats. *Autophagy* (2016) 12:2230–47. doi: 10.1080/15548627.2016.1214777
91. Issa AR, Sun J, Petitgas C, Mesquita A, Dulac A, Robin M, et al. The lysosomal membrane protein LAMP2A promotes autophagic flux and prevents SNCA-induced Parkinson disease-like symptoms in the Drosophila brain. *Autophagy* (2018) 14:1898–910. doi: 10.1080/15548627.2018.1491489
92. Wang B, Cai Z, Tao K, Zeng W, Lu F, Yang R, et al. Essential control of mitochondrial morphology and function by chaperone-mediated autophagy through degradation of PARK7. *Autophagy* (2016) 12:1215–28. doi: 10.1080/15548627.2016.1179401
93. Yang Q, She H, Gearing M, Colla E, Lee M, Shacka JJ, et al. Regulation of neuronal survival factor MEF2D by chaperone-mediated autophagy. *Science* (2009) 323:124–7. doi: 10.1126/science.1166088
94. Murphy KE, Gysbers AM, Abbott SK, Spiro AS, Furuta A, Cooper A, et al. Lysosomal-associated membrane protein 2 isoforms are differentially affected in early Parkinson's disease. *Move Disord*. (2015) 30:1639–47. doi: 10.1002/mds.26141
95. Sala G, Stefanoni G, Arosio A, Riva C, Melchionda L, Saracchi E, et al. Reduced expression of the chaperone-mediated autophagy carrier hsc70 protein in lymphomonocytes of patients with Parkinson's disease. *Brain Res*. (2014) 1546:46–52. doi: 10.1016/j.brainres.2013.12.017
96. Pang S, Chen D, Zhang A, Qin X, Yan B. Genetic analysis of the LAMP-2 gene promoter in patients with sporadic Parkinson's disease. *Neurosci Lett*. (2012) 526:63–7. doi: 10.1016/j.neulet.2012.07.044
97. Alvarez-Erviti L, Seow Y, Schapira AH, Rodriguez-Oroz MC, Obeso JA, Cooper JM. Influence of microRNA deregulation on chaperone-mediated autophagy and alpha-synuclein pathology in Parkinson's disease. *Cell Death Dis*. (2013) 4:e545. doi: 10.1038/cddis.2013.73
98. Liu H, Wang P, Song W, Sun X. Degradation of regulator of calcineurin 1 (RCAN1) is mediated by both chaperone-mediated autophagy and ubiquitin proteasome pathways. *FASEB J*. (2009) 23:3383–92. doi: 10.1096/fj.09-134296
99. Valdor R, Mocholi E, Botbol Y, Guerrero-Ros I, Chandra D, Koga H,., et al. Chaperone-mediated autophagy regulates T cell responses through targeted degradation of negative regulators of T cell activation. *Nat Immunol*. (2014) 15:1046–54. doi: 10.1038/ni.3003
100. Wang Y, Martinez-Vicente M, Kruger U, Kaushik S, Cuervo M, Wong E, et al. Tau fragmentation, aggregation and clearance: the dual role of lysosomal processing. *Hum Mol Genet*. (2009) 18:4153–70. doi: 10.1093/hmg/ddp367
101. Wang Y, Martinez-Vicente M, Kruger U, Kaushik S, Wong E, Mandelkow EM, et al. Synergy and antagonism of macroautophagy and chaperone-mediated autophagy in a cell model of pathological tau aggregation. *Autophagy* (2010) 6:182–3. doi: 10.4161/auto.6.1.10815
102. Park JS, Kim DH, Yoon SY. Regulation of amyloid precursor protein processing by its KFERQ motif. *BMB Rep*. (2016) 49:337–42. doi: 10.5483/BMBRep.2016.49.6.212
103. Qi L, Zhang XD, Wu JC, Lin F, Wang J, DiFiglia M, et al. The role of chaperone-mediated autophagy in huntingtin degradation. *PLoS ONE* (2012) 7:e46834. doi: 10.1371/journal.pone.0046834
104. Bauer PO, Goswami A, Wong HK, Okuno M, Kurosawa M, Yamada M, et al. Harnessing chaperone-mediated autophagy for the selective degradation of mutant huntingtin protein. *Nat Biotechnol*. (2010) 28:256–63. doi: 10.1038/nbt.1608
105. Huang CC, Bose JK, Majumder P, Lee KH, Huang JT, Huang JK, et al. Metabolism and mis-metabolism of the neuropathological signature protein TDP-43. *J Cell Sci*. (2014) 127:3024–38. doi: 10.1242/jcs.136150
106. Tamaki Y, Shodai A, Morimura T, Hikiami R, Minamiyama S, Ayaki T, et al. Elimination of TDP-43 inclusions linked to amyotrophic lateral sclerosis by a misfolding-specific intrabody with dual proteolytic signals. *Sci Rep*. (2018) 8:6030. doi: 10.1038/s41598-018-24463-3
107. Marino G, Fernandez AF, Lopez-Otin C. Autophagy and aging: lessons from progeria models. *Adv Exp Med Biol*. (2010) 694:61–8. doi: 10.1007/978-1-4419-7002-2_6
108. Cuervo AM, Dice JF. Age-related decline in chaperone-mediated autophagy. *J Biol Chem*. (2000) 275:31505–13. doi: 10.1074/jbc.M002102200
109. Kiffin R, Kaushik S, Zeng M, Bandyopadhyay U, Zhang C, Massey AC, et al. Altered dynamics of the lysosomal receptor for chaperone-mediated autophagy with age. *J Cell Sci*. (2007) 120:782–91. doi: 10.1242/jcs.001073
110. Schneider JL, Villarroya J, Diaz-Carretero A, Patel B, Urbanska AM, Thi M. M, et al. Loss of hepatic chaperone-mediated autophagy accelerates proteostasis failure in aging. *Aging Cell* (2015) 14:249–64. doi: 10.1111/acel.12310
111. Papagiannakis N, Xilouri M, Koros C, Stamelou M, Antonelou R, Maniati M, et al. Lysosomal alterations in peripheral blood mononuclear cells of Parkinson's disease patients. *Mov Disord*. (2015) 30:1830–4. doi: 10.1002/mds.26433
112. Klaver AC, Coffey MP, Aasly JO, Loeffler DA. CSF lamp2 concentrations are decreased in female Parkinson's disease patients with LRRK2 mutations. *Brain Res*. (2018) 1683:12–16. doi: 10.1016/j.brainres.2018.01.016

113. Loeffler DA, Klaver AC, Coffey MP, Aasly JO. Cerebrospinal fluid concentration of key autophagy protein lamp2 changes little during normal aging. *Front Aging Neurosci*. (2018) 10:130. doi: 10.3389/fnagi.2018.00130
114. Rodriguez-Navarro JA, Kaushik S, Koga H, Dall'Armi C, Shui G, Wenk MR, et al. Inhibitory effect of dietary lipids on chaperone-mediated autophagy. *Proc Natl Acad Sci USA* (2012) 109:E705–14. doi: 10.1073/pnas.1113036109
115. Khang R, Park C, Shin JH. Dysregulation of parkin in the substantia nigra of db/db and high-fat diet mice. *Neuroscience* (2015) 294:182–92. doi: 10.1016/j.neuroscience.2015.03.017
116. Rotermund C, Truckenmuller FM, Schell H, Kahle PJ. Diet-induced obesity accelerates the onset of terminal phenotypes in alpha-synuclein transgenic mice. *J Neurochem*. (2014) 131:848–58. doi: 10.1111/jnc.12813
117. Kim D, Cho J, Lee I, Jin Y, Kang H. Exercise attenuates high-fat diet-induced disease progression in 3xTg-AD Mice. *Med Sci Sports Exerc*. (2017) 49:676–86. doi: 10.1249/MSS.0000000000001166
118. Julien C, Tremblay C, Phivilay A, Berthiaume L, Emond V, Julien P, et al. High-fat diet aggravates amyloid-beta and tau pathologies in the (2010) 3xTg-AD mouse model. *Neurobiol Aging* 31:1516–31. doi: 10.1016/j.neurobiolaging.2008.08.022
119. Pennetta G, Welte MA. Emerging links between lipid droplets and motor neuron diseases. *Dev Cell* (2018) 45:427–32. doi: 10.1016/j.devcel.2018.05.002
120. Cole NB, Murphy DD, Grider T, Rueter S, Brasaemle D, Nussbaum RL. Lipid droplet binding and oligomerization properties of the Parkinson's disease protein alpha-synuclein. *J Biol Chem*. (2002) 277:6344–52. doi: 10.1074/jbc.M108414200
121. Sun X, Ueda M, Yamashita T, Nakamura M, Bergstrom J, Zeledon Ramirez ME, et al. Lipid droplets are present in amyloid deposits in familial amyloidotic polyneuropathy and dialysis related amyloidosis. *Amyloid* (2006) 13:20–3. doi: 10.1080/13506120500537137
122. Taylor MK, Sullivan DK, Swerdlow RH, Vidoni ED, Morris JK, Mahnken JD, et al. A high-glycemic diet is associated with cerebral amyloid burden in cognitively normal older adults. *Am J Clin Nutr*. (2017) 106:1463–70. doi: 10.3945/ajcn.117.162263
123. Vicente Miranda H, Outeiro TF. The sour side of neurodegenerative disorders: the effects of protein glycation. *J Pathol*. (2010) 221:13–25. doi: 10.1002/path.2682
124. Liu F, Zaidi T, Iqbal K, Grundke-Iqbal I, Gong CX. Aberrant glycosylation modulates phosphorylation of tau by protein kinase A and dephosphorylation of tau by protein phosphatase 2A and 5. *Neuroscience* (2002) 115:829–37. doi: 10.1016/S0306-4522(02)00510-9
125. Valente T, Gella A, Fernandez-Busquets X, Unzeta M, Durany N. Immunohistochemical analysis of human brain suggests pathological synergism of Alzheimer's disease and diabetes mellitus. *Neurobiol Dis*. (2010) 37:67–76. doi: 10.1016/j.nbd.2009.09.008
126. Seneff S, Wainwright G, Mascitelli L. Nutrition and Alzheimer's disease: the detrimental role of a high carbohydrate diet. *Eur J Intern Med*. (2011) 22:134–40. doi: 10.1016/j.ejim.2010.12.017
127. Dunn L, Allen GF, Mamais A, Ling H, Li A, Duberley KE, et al. Dysregulation of glucose metabolism is an early event in sporadic Parkinson's disease. *Neurobiol Aging* (2014) 35:1111–5. doi: 10.1016/j.neurobiolaging.2013.11.001
128. Bigl M, Bruckner MK, Arendt T, Bigl V, Eschrich K. Activities of key glycolytic enzymes in the brains of patients with Alzheimer's disease. *J Neural Transm (Vienna)* (1999) 106:499–511. doi: 10.1007/s007020050174
129. Barinova K, Khomyakova E, Semenyuk P, Schmalhausen E, Muronetz V. Binding of alpha-synuclein to partially oxidized glyceraldehyde-3-phosphate dehydrogenase induces subsequent inactivation of the enzyme. *Arch Biochem Biophys*. (2018) 642:10–22. doi: 10.1016/j.abb.2018.02.002
130. Gouarne C, Tardif G, Tracz J, Latyszenok V, Michaud M, Clemens LE, et al. Early deficits in glycolysis are specific to striatal neurons from a rat model of huntington disease. *PLoS ONE* (2013) 8:e81528. doi: 10.1371/journal.pone.0081528
131. Estrada-Sanchez AM, Montiel T, Massieu L. Glycolysis inhibition decreases the levels of glutamate transporters and enhances glutamate neurotoxicity in the R6/2 Huntington's disease mice. *Neurochem Res*. (2010) 35:1156–63. doi: 10.1007/s11064-010-0168-5
132. Rodriguez-Navarro JA, Cuervo AM. Dietary lipids and aging compromise chaperone-mediated autophagy by similar mechanisms. *Autophagy* (2012) 8:1152–4. doi: 10.4161/auto.20649
133. Kaushik S, Massey AC, Cuervo AM. Lysosome membrane lipid microdomains: novel regulators of chaperone-mediated autophagy. *EMBO J*. (2006) 25:3921–33. doi: 10.1038/sj.emboj.7601283

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Chaperone-Mediated Autophagy Upregulation Rescues Megalin Expression and Localization in Cystinotic Proximal Tubule Cells

Jinzhong Zhang[1], *Jing He*[1], *Jennifer L. Johnson*[1], *Farhana Rahman*[1], *Evripidis Gavathiotis*[2], *Ana Maria Cuervo*[3] *and Sergio D. Catz*[1]*

[1] *Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA, United States,* [2] *Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY, United States,* [3] *Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY, United States*

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***Correspondence:**
Sergio D. Catz
scatz@scripps.edu

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Cystinosis is a lysosomal storage disorder caused by defects in *CTNS*, the gene that encodes the lysosomal cystine transporter cystinosin. Patients with nephropathic cystinosis are characterized by endocrine defects, defective proximal tubule cell (PTC) function, the development of Fanconi syndrome and, eventually, end-stage renal disease. Kidney disease is developed despite the use of cysteamine, a drug that decreases lysosomal cystine overload but fails to correct overload-independent defects. Chaperone-mediated autophagy (CMA), a selective form of autophagy, is defective in cystinotic mouse fibroblasts, and treatment with cysteamine is unable to correct CMA defects *in vivo,* but whether the vesicular trafficking mechanisms that lead to defective CMA in cystinosis are manifested in human PTCs is not currently known and whether PTC-specific mechanisms are corrected upon CMA upregulation remains to be elucidated. Here, using CRISPR-Cas9 technology, we develop a new human PTC line with defective cystinosin expression (*CTNS*-KO PTCs). We show that the expression and localization of the CMA receptor, LAMP2A, is defective in *CTNS*-KO PTCs. The expression of the lipidated form of LC3B, a marker for another form of autophagy (macroautophagy), is decreased in *CTNS*-KO PTCs indicating decreased autophagosome numbers under basal conditions. However, the autophagic flux is functional, as measured by induction by starvation or by blockage using the v-ATPase inhibitor bafilomycin A, and by degradation of the macroautophagy substrate SQSTM1 under starvation and proteasome-inhibited conditions. Previous studies showed that LAMP2A accumulates in Rab11-positive vesicles in cystinotic cells. Here, we show defective Rab11 expression, localization and trafficking in *CTNS*-KO PTCs as determined by confocal microscopy, immunoblotting and TIRFM. We also show that both Rab11 expression and trafficking in cystinotic PTCs are rescued by the upregulation of CMA using small-molecule CMA activators. Cystinotic PTCs are characterized by PTC

de-differentiation accompanied by loss of the endocytic receptor megalin, and megalin recycling is regulated by Rab11. Here we show that megalin plasma membrane localization is defective in *CTNS*-KO PTCs and its expression is rescued by treatment with CMA activators. Altogether, our data support that CMA upregulation has the potential to improve PTC function in cystinosis.

Keywords: lysosomal storage disorder (LSD), fanconi syndrome, megalin, chaperone-mediated autophagy (CMA), cystinosis, vesicular trafficking, Rab 11 GTPase, LAMP2A

INTRODUCTION

Cystinosis is a lysosomal storage disorder (LSD) caused by genetic defects in *CTNS* (*Ctns* in mouse), the gene that codes for the cystine transporter cystinosin. Increased levels of intra-lysosomal cystine (1) lead to cell malfunction in cystinosis. Tissue deterioration manifests in kidneys and eyes but also affects other organs including the liver, brain, and muscle (2). Kidney proximal tubule cells (PTCs) are the first cell type to be affected in nephropathic cystinosis, causing, in the long term, end-stage kidney disease. Patients with severe cystinosis require kidney transplants. Endocrine disorders are also common in cystinosis such as hypothyroidism, growth retardation, and hypogonadism (3). Hypothyroidism is the most frequently reported endocrine manifestation of the disease (4). Altered thyroglobulin biosynthesis associated with endoplasmic reticulum stress is the cause of this manifestation. Cystinotic patients also suffer from insulin-dependent diabetes (5), which contributes other complications including muscle (6) and bone (7) alterations that are pathognomonic of the disease.

The current treatment for patients with cystinosis is cysteamine which reduces intra-lysosomal cystine, conjugates, and transports cysteine out of the lysosome through the exporter PQLC2 (8). Despite the efficiency of cysteamine in retarding the rate of renal deterioration and improving linear growth in children with cystinosis (9), cell malfunction, tissue failure, progressive renal disease, endocrine complications, and muscle abnormalities still occur (10), suggesting that cystine accumulation is not the only cause for all the defects observed in cystinosis (10, 11). Thus, to improve treatment of this LSD, it is crucial to understand the defective molecular mechanisms that lead to the various tissues dysfunction and injury. In order to understand these mechanisms, it is essential to develop and characterize models of the disease. To this end, the establishment of new cellular models of cystinotic proximal tubule cells, with defined genotypic and phenotypic characteristics, is essential to study disease-relevant mechanisms, to develop knowledge and to implement novel strategies for treating renal disease progression in this devastating disease.

Chaperone mediated autophagy (CMA) is a selective form of autophagy that contributes to proteostasis in several physiological and pathological conditions (12). CMA consists of the internalization of selected cytosolic substrates into the lysosome by a mechanism that includes: Recognition of a pentapeptide-like KFERQ in the substrate by the chaperone hsc70; substrate presentation by the chaperone to the receptor LAMP2A; receptor multimerization and protein internalization for degradation in the lysosome, assisted by a lumenal form of hsc70 (13). LAMP2A the only known lysosomal receptor for CMA, shows defective localization and impaired function in cystinosis (14, 15). Defects in CMA in cystinosis lead to the cytosolic accumulation of CMA substrates and are proposed to contribute to the pathological processes of the disease that are cysteamine treatment-insensitive (14). However, the specific CMA mechanism(s) that are defective in cystinotic proximal tubule cells are currently unknown and the impact of CMA upregulation on PTC function requires further analysis. Under oxidative stress CMA is activated. This activation correlates with increased expression levels of the lysosomal lumenal chaperone protein hsc70 (required for substrate uptake), and also correlates with a selective increase of the expression of the CMA receptor LAMP2A at the lysosomal membrane, leading to higher rates of CMA (16). However, despite the observations that cystinosis is associated with increased oxidative stress and that cystinotic patients have high serum levels of oxidative stress markers (11), cystinotic cells are actually susceptible to oxidative stress, most likely caused by downregulation of CMA. Remarkably, CMA induction by pharmacological enhancers protects cystinotic cells from the increased susceptibility to oxidative stress and reconstitutes the resistant levels observed in wild type cells, an effect dependent on LAMP2A expression and its lysosomal membrane localization (15). It then becomes clear that the correct lysosomal localization of LAMP2A is necessary to maintain cellular homeostasis in cystinosis. However, the mechanisms that mediate lysosomal localization of LAMP2A are not well-understood and the possible consequences of downregulated CMA in cystinotic PTCs is unknown.

In cystinosis, cystine accumulation induces apical PTC dedifferentiation (17). PTCs, which play a central role in maintaining homeostasis by mediating reabsorption of electrolytes and nutrients in the renal tube, rely on specialized apical receptors that control the internalization of specific substrates. In particular, megalin (gp330, LRP-2), a member of the low-density lipoprotein receptor family, is expressed in proximal tubule epithelial cells, and together with cubilin, mediates the endocytosis of an extensive number of diverse ligands including lipoproteins, vitamin-binding proteins, hormones, and enzymes (18). Based on their function as non-specific protein reabsorption molecules, megalin and cubilin are considered to operate as scavenger receptors (19). Megalin's apical localization in PTCs is mediated by fast-recycling

mechanisms from apical recycling endosomes, a mechanism regulated by the small GTPase Rab11 (18). Fast megalin recycling allows for its reutilization and delays its degradation (18). A possible role for CMA in the regulation of megalin expression and localization is currently unknown. However, the role of CMA in the control of trafficking mechanisms that are affected in cystinotic fibroblasts, as previously described by our group (14), suggests that autophagy and, in particular CMA, may be an important determinant in the control of megalin functions through the regulation of vesicular transport.

In this work, we developed and characterized human cystinotic proximal tubule cells (*CTNS*-KO PTCs) and demonstrated that these cells are characterized by CMA defects that affect vesicular trafficking mechanisms regulating megalin localization.

MATERIALS AND METHODS

Generation of Cystinosin Knockout Human Proximal Tubule Cell Line Using CRISPR/Cas9

To generate *CTNS* knockout human proximal tubule cells, Cas9 nickase was used along with a pair of gRNAs targeting exon 4 of the human *CTNS* gene. gRNAs were designed using the online tool (http://crispr.mit.edu/). Each gRNA was sub-cloned into pSpCas9n(BB)-2A-GFP(PX461) (Addgene), two pSpCas9n(BB)-2A-GFP(PX461)-*CTNS* gRNA constructs were then transfected into a model of human proximal tubule cell (HK-2 cells) at a 1:1 ratio using Lipofectamine LTX (Thermo Fisher Scientific). After 48 h, GFP-positive cells were sorted by FACS, seeded into plates and grown into single colonies. Single colonies were then expanded, the junction sequence was amplified by PCR, sub-cloned into pBluescript and sequenced to identify the variety of insertions and deletions (indels) at targeting sites. As a result, we obtained different indels in two alleles. One of the modifications has a 37 bp insertion, which abolishes the splicing site of the exon 4 at its 3′ end. The other allele has a 17 bp deletion, causing an amino acid shift, which led to a 52-amino acid product.

Constructs and Transfections

The GFP-Rab11 construct was obtained from Addgene. Cells were transfected with this construct using Lipofectamine® 2000 (Thermo Fisher Scientific) following the manufacturer's instructions.

Gel Electrophoresis, Immunoblotting, and Antibodies

Cells were lysed in RIPA lysis buffer in the presence of protease-inhibitors (Roche). Following electrophoresis using NuPAGE 4–12% gels (Thermo Fisher Scientific), proteins were transferred onto 0.45 μm nitrocellulose membranes and the membranes were incubated overnight at 4°C with the indicated primary antibodies, followed by incubation with HRP-conjugated secondary antibodies. The following antibodies were used in this study: mouse anti-LAMP1 (Santa Cruz, sc-20011), rabbit anti-LAMP2A (Abcam, ab18528), rabbit anti-actin (Sigma, A2066), mouse anti-Rab11 (Thermo Fisher Scientific, MA1-24919), rabbit anti-SQSTM1/p62 (Cell Signaling, 5114), rabbit anti-LC3B (Cell Signaling, 2775), and goat anti-Megalin (Santa Cruz, sc-16476).

Immunofluorescence and Confocal Microscopy Analysis

Cells were seeded on a 4-chamber 35 mm-glass bottom dish (in vitro Scientific). Where indicated, cells were treated with the indicated treatments, then fixed with 3.7% paraformaldehyde for 15 min and blocked with 1% BSA in PBS for 1 h. Samples were labeled with the indicated primary antibodies overnight at 4°C in the presence of 0.01% saponin and 1% BSA. Samples were washed 3 times and subsequently incubated with the appropriate combinations of Alexa Fluor (488 or 594)-conjugated anti-rabbit, anti-rat, or anti-mouse secondary antibodies (Thermo Fisher scientific). Samples were analyzed with a Zeiss LSM 710 laser scanning confocal microscope (LSCM) attached to a Zeiss Observer Z1 microscope, using a 63 × oil Plan Apo, 1.4 NA objective. Images were collected using ZEN-LSM software keeping the laser power and gain constant during all acquisitions for comparative analysis of wild type and knockout cells or treated with vehicle or various treatments. Images were then processed using Image*J* (National Institutes of Health, Bethesda, MD) and Photoshop CS4 (Adobe). The fluorescence intensities were quantified using the Image*J* software.

Total Internal Reflection Fluorescence (TIRF) Microscopy

Pseudo-TIRF microscopy analyses were performed using a 100X 1.45 numerical aperture TIRF objective (Nikon) on a Nikon TE2000U microscope custom modified with a TIRF illumination module as described (20). Images were acquired on a 16-bit, cooled charge-coupled device camera (Hamamatsu) controlled through NIS-Elements software. For live experiments, the images were recorded using 300–500 ms exposures depending on the fluorescence intensity of the sample.

Cystine Accumulation

Cystine levels were measured by mass spectrometry at the Biochemical Genetics Laboratory, University of California, San Diego as described previously (20).

Starvation Protocols

For studies of macroautophagy, cells were briefly washed in serum-free DMEM (containing 1x amino acids), media was aspirated, and fresh serum-free DMEM was added followed by 5 h incubation at 37°C, in the presence or absence of lysosomal inhibitor Bafilomycin A (LC laboratories, 100 nM) or proteasome inhibitor Clasto-Lactacystin β-lactone (Cayman Chemical, 1 uM), as described previously (14).

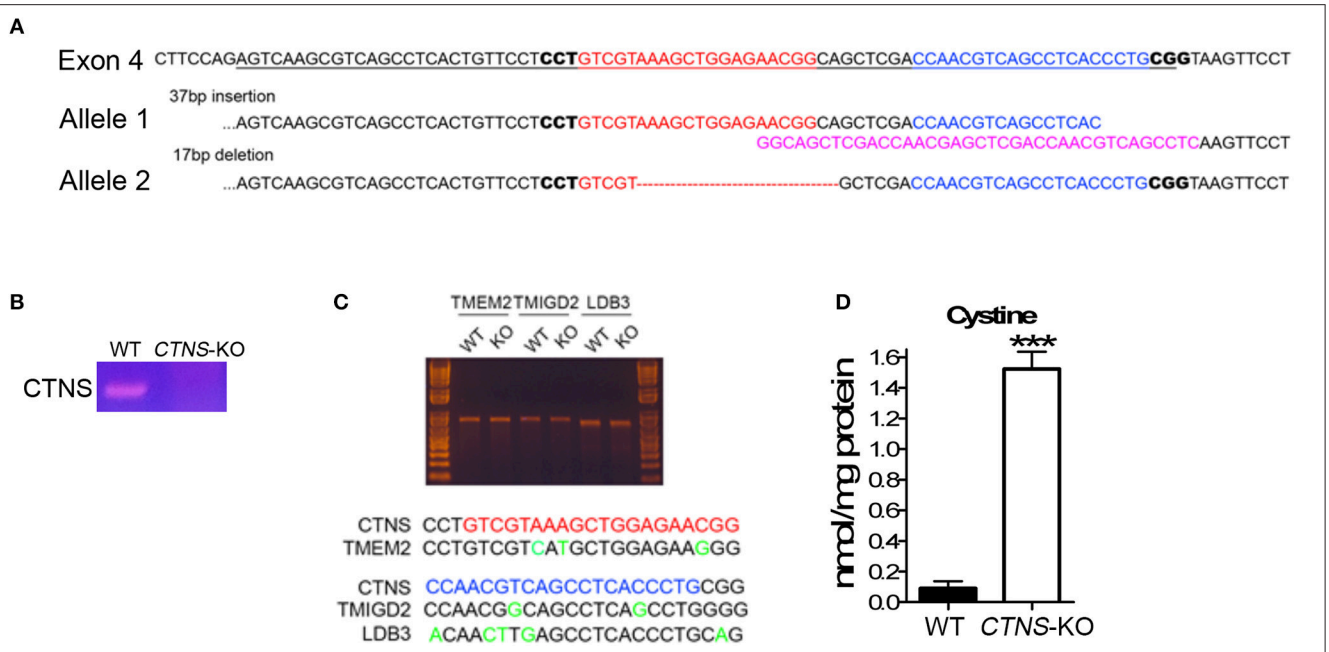

FIGURE 1 | Generation of cystinosin knockout human proximal tubule cell line using CRISPR/Cas9. **(A)** Human *CTNS* genomic sequence; underlined sequences indicate exon 4, gRNA sequences were marked as red and blue, and PAM sequences are in bold. The allele 1 has 37bp insertion which was marked in pink; allele 2 has 17 bp deletion which was marked as a red dashed line. **(B)** PCR analysis of *CTNS* gene expression in WT and *CTNS*-KO HK-2 cells. **(C)** Surveyor assay of three potential off-target sites that localize in TMEM2, TMIGD2, and LDB3 genes. **(D)** Cystine levels in WT and *CTNS*-KO HK-2 cells were determined by mass spectrometry. The data are represented as mean ± SEM from three independent experiments. ***$p < 0.001$.

PCR and RT-PCR Analysis

For RT-PCR analysis, RNA was isolated from wild-type or *Ctns*$^{-/-}$ mouse fibroblasts using the RNeasy mini-kit for RNA purification (Qiagen). A total of 100 ng of RNA for each cell line was reverse-transcribed (RT) using iScript cDNA synthesis kit (Bio-Rad). PCR analysis was then performed using Taq DNA Polymerase(Thermo Fisher Scientific, 18038042), with the following primer mixes: human *CTNS* forward, TCCTCCTGTCGTAAAGCTGGA, and human *CTNS* reverse, GCCGGTCTGATTGGAGTGAT.

Statistical Analysis

Data are presented as mean, and error bars correspond to standard errors of the means (SEMs) unless otherwise indicated. Statistical significance was determined using the pair or unpaired Student's *t*-test or one-way ANOVA, Tukey's multiple comparisons test for multiple groups using either Excel software or GraphPad Prism (version 4) software.

RESULTS

Generation and Characterization of Cystinotic Human Proximal Tubule Cells (PTCs)

Proximal tubule cells (PTCs) are the most affected cells in cystinosis and are characterized by the accumulation of cystine crystals, as well as by PTC de-differentiation accompanied by loss of the endocytic receptors megalin and cubilin (17). In cystinotic fibroblasts, the cystinosin deficiency leads to defects in autophagic pathways caused by defective intracellular trafficking of the CMA receptor LAMP2A (14). In cystinotic kidneys, the accumulation of substrates suggests defective CMA but whether LAMP2A function is defective in *CTNS*-KO PTCs and whether CMA upregulation improves PTC function are both currently unknown. To study the mechanisms of vesicular trafficking and CMA regulation in *CTNS*-KO PTCs, we developed human PTCs deficient in cystinosin using CRISPR-Cas9 technology. To this end, we used the human PTC cell line HK-2, which has been largely demonstrated to be an authentic PTC model (21). The strategy to design and generate *CTNS*-KO PTCs is described in **Figure 1**. Two gRNAs targeting Human *CTNS* exon 4 were designed and are shown in **Figure 1A**. The gRNA1 targeted one of the alleles, generated a 37 bp insertion, destroyed the splicing site, and was predicted to generate a 149aa product. The gRNA2, targeted the second allele to generate a 17 bp deletion, causing a shift leading to a theoretical 52aa product (**Figure 1A**). Next, the *CTNS*-KO PTCs were analyzed for the expression of *CTNS* mRNA by PCR (**Figure 1B**). Using a 5′ primer that sits just upstream of the mutation site and a downstream reverse primer, we show that wild type *CTNS* mRNA is not expressed in *CTNS*-targeted (*CTNS*-KO) cells. Although the mutations deplete expression of *CTNS*, aberrant *CTNS* mRNA could still be detected, at least partially (not shown). Possible off-target effects of CRISPR/Cas9 were also analyzed. Thus, three potential off-target loci were amplified by PCR followed with Surveyor assay and Sanger sequencing. Our data show that these three

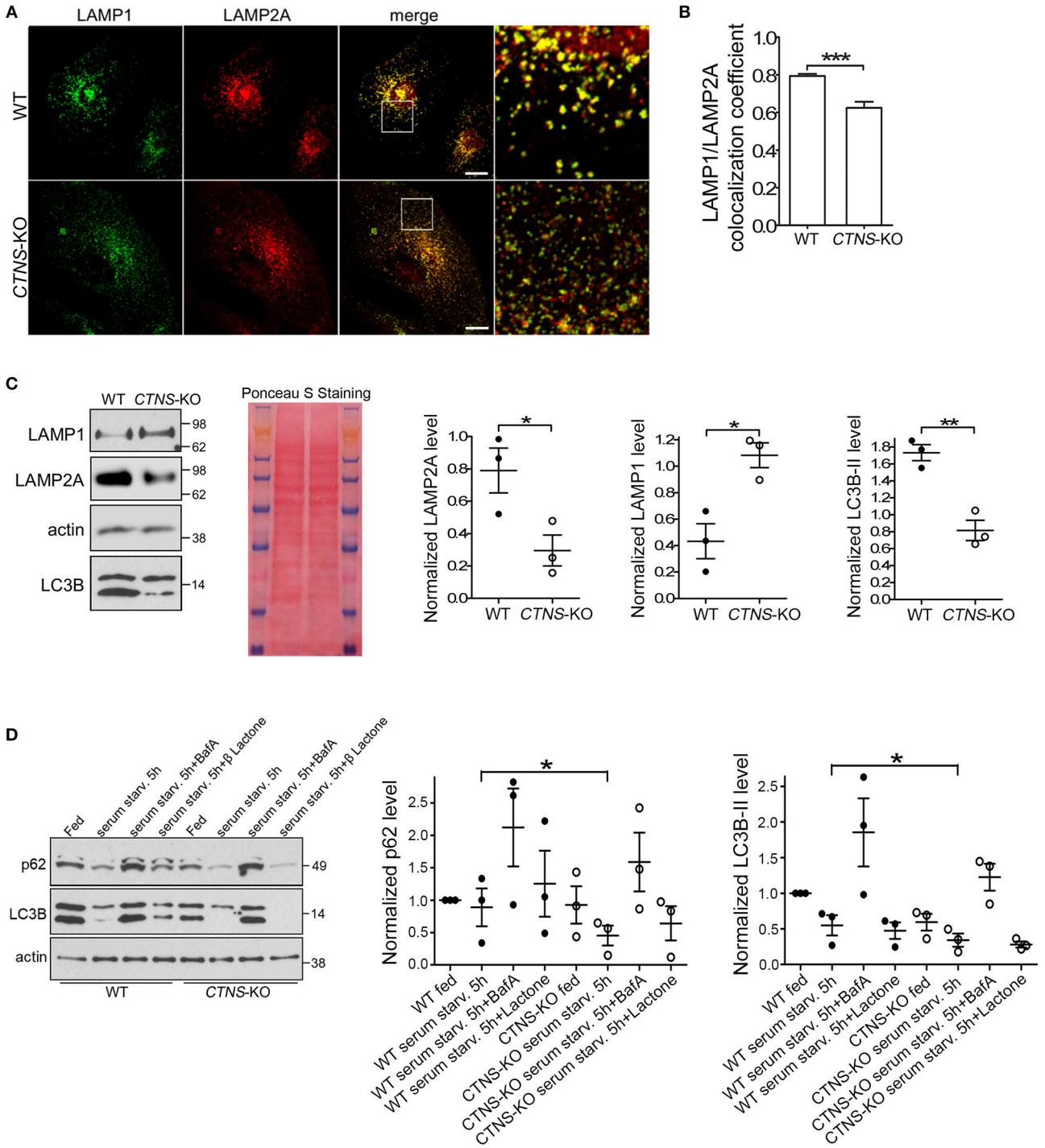

FIGURE 2 | Mislocalization and decreased expression of LAMP2A in *CTNS*-KO PTCs. **(A,B)** Confocal microscopy analysis and quantification of the distribution of endogenous LAMP1 and LAMP2A in WT and *CTNS*-KO PTCs. Scale bar, 10 μm. mean ± SEM. ***$p < 0.001$, Student's *t*-test. **(C)** Western blot analysis of LAMP1, LAMP2A, and LC3B expression levels in WT and *CTNS*-KO PTCs. Left panel, Representative immunoblots. Ponceaus S staining is shown for equal loading. Middle and right panels, Quantification of 3 independent experiments. The individual symbols correspond to independent biological replicates from 3 independent experiments. The bars represent the mean ± SEM. *$p < 0.05$; **$p < 0.01$, Student's *t*-test. **(D)** SQSTM1/p62 and LC3B-II levels in WT and *CTNS*-KO PTCs were analyzed by Western blot under fed and serum starvation conditions, in the absence or presence of 100 nM bafilomycin A (BafA) or 1 μM Clasto-Lactacystin β-lactone (proteasome inhibitor) for 5 h. Left panel, Representative immunoblots. Middle and right panel, Quantification of the expression levels of SQSTM1 (p62) and LC3B-II, respectively. Each value has been normalized to the actin expression level in the same sample. Results are expressed relatively to the wild type fed condition. The individual symbols correspond to independent biological replicates from 3 independent experiments. The bars represent the mean ± SEM. *$p < 0.05$.

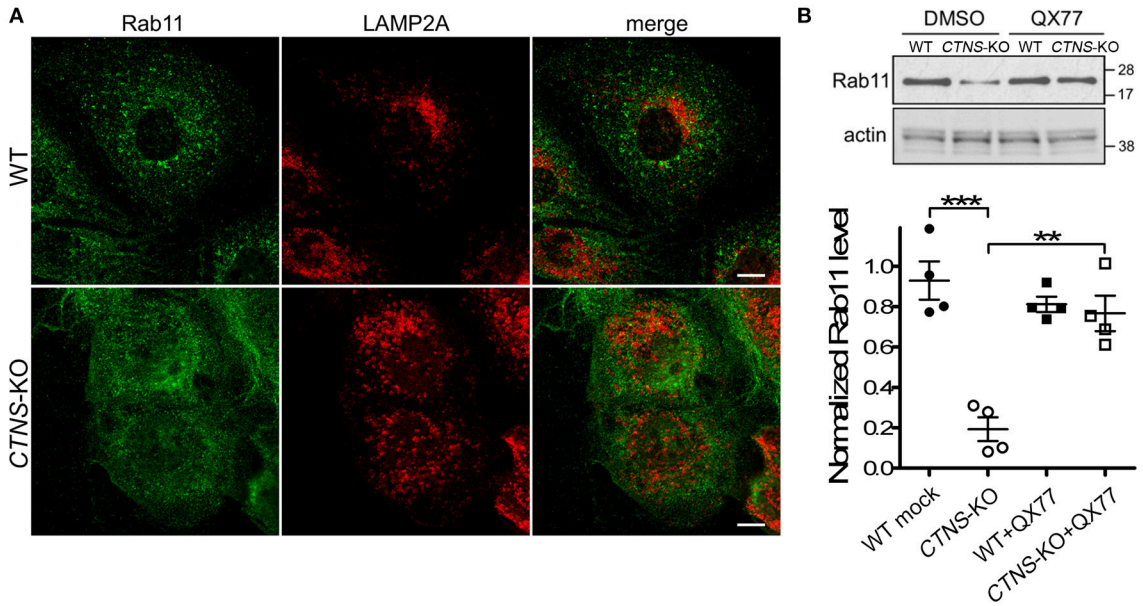

FIGURE 3 | Rab11 is down-regulated and mislocalized in *CTNS*-KO PTCs. **(A)** Confocal microscopy analysis of the distribution of endogenous Rab11 and LAMP2A in WT and *CTNS*-KO PTCs. Scale bar, 10 μm. **(B)** WT and *CTNS*-KO PTCs were treated with DMSO or 20 μM QX77 for 48 h, and Rab11 expression levels were analyzed by Western blot. Quantitative analysis of Rab11 expression levels. The individual symbols correspond to independent biological replicates from 4 independent experiments. The expression level of Rab11 was normalized to actin in each sample. The bars represent the mean ± SEM. $^{**}p < 0.01$, and $^{***}p < 0.001$, Student's *t*-test.

loci are intact (**Figure 1C**). For confirmation of cystinosin activity deficiency, we measured cystine accumulation using mass spectrometry analysis of cystine content. In **Figure 1D**, we show that cystine levels are barely detectable in wild type cells but cystine is significantly increased in *CTNS*-KO cells. Thus, *CTNS*-KO PTCs accumulate cystine at levels that are similar to those observed in cystinotic cells from the $Ctns^{-/-}$ mouse (14).

Decreased Expression and Mislocalization of LAMP2A in *CTNS*-KO PTCs

To study possible CMA defective mechanisms in *CTNS*-KO PTCs, and because previous studies from our laboratory suggested that the CMA receptor LAMP2A is mislocalized in cystinosis, we first analyzed the subcellular distribution of LAMP2A in wild type and *CTNS*-KO PTCs. In **Figures 2A,B**, we show that while LAMP2A shows high colocalization with LAMP1 in wild type cells, *CTNS*-KO PTCs have a subpopulation of LAMP2A-positive vesicles that does not overlap with the distribution of LAMP1. This is in agreement with previous studies from our laboratory showing that intermediate vesicles containing LAMP2A are mislocalized and have defective trafficking in cystinotic fibroblasts (15). Further, to better understand whether the mislocalization of LAMP2A affects protein stability, we determined the levels of expression of LAMP2A in *CTNS*-KO PTCs. Interestingly, LAMP2A expression was decreased in *CTNS*-KO PTCs, suggesting either downregulation at the transcriptional level or increased degradation (**Figure 2C**). LAMP2A-decreased expression was specific since LAMP1 expression is elevated in *CTNS*-KO PTCs (**Figure 2C**). These data recapitulate the defective phenotypes observed in $Ctns^{-/-}$ fibroblasts (14, 20) and thus confirmed that *CTNS*-KO PTCs constitute a valid cystinotic model.

Next, to determine whether changes in CMA are accompanied by defects in macroautophagy, we analyzed the expression levels of the lipidated form of LC3B (LC3B-II) by Western blot. In **Figure 2C**, we show that LC3B-II expression is decreased in *CTNS*-KO PTCs.

The autophagic flux in *CTNS*-KO PTCs was analyzed using well-established biochemical methods. Wild type and *CTNS*-KO PTCs were exposed to fed, serum starvation and autophagy-blocking conditions, using bafilomycin to inhibit the fusion of autophagosomes with lysosomes. In **Figure 2D**, we show that despite having decreased number of autophagosomes, *CTNS*-KO PTCs respond to starvation by degrading LC3B, indicating that macroautophagy is inducible by starvation in these cells. This was also confirmed by the decrease in protein levels of the autophagy substrate SQSTM1/p62 in these cells after starvation. Treatment with the proteasome inhibitor β-lactone did not affect protein degradation (**Figure 2D**), indicating that SQSTM1 is degraded mainly through macroautophagy in these cells. Increased LC3B levels after treatment with bafilomycin confirmed that autophagosome formation is functional in *CTNS*-KO PTCs and that the reduced LC3B levels together with reduced SQSTM1 levels are indicative of accelerated clearance rather than reduced autophagosome

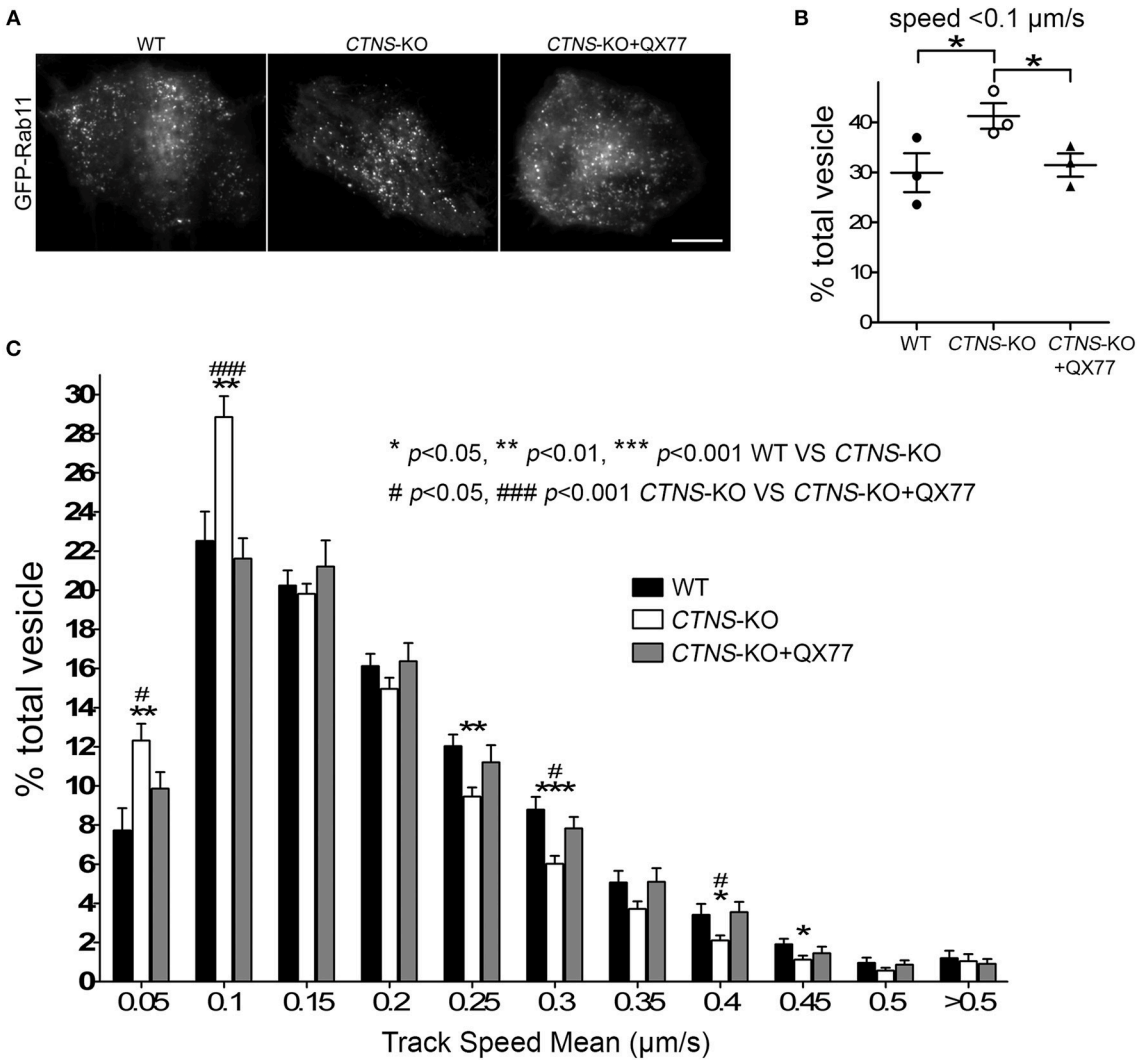

FIGURE 4 | Rab11 trafficking is impaired in *CTNS*-KO PTCs and is enhanced by CMA activation. **(A)** Representative images of GFP-Rab11 in WT, *CTNS*-KO PTCs, and *CTNS*-KO PTCs treated with QX77. Scale bar, 10 μm. **(B,C)** Quantitative analysis of the trafficking of GFP-Rab11 in WT, *CTNS*-KO PTCs, and *CTNS*-KO PTCs treated with 20 μM QX77 for 72 h. **(B)** Quantitative analysis of the numbers of vesicles with decreased motility (speed < 0.1 μm/s) in wild type, *CTNS*-KO PTCs and *CTNS*-KO PTCs treated with QX77. The individual symbols correspond to independent biological replicates from 3 independent experiments. The bars indicate the mean ± SEM. *$p < 0.05$. **(C)** Histograms represent the speeds of GFP-Rab11-containing organelles. The speeds for the independent vesicles were binned in 0.05 μm/s increments and plotted as a percentage of total vesicles for a given cell. Results are represented as mean ± SEM from at least 22 cells from 3 independent experiments. The statistically significant differences between the groups are indicated in the figure. Student's *t*-test.

biogenesis. This is in agreement with previous studies showing increased autophagic flux in cystinotic fibroblasts, albeit with accumulation of autophagosomes (14), and goes hand-in-hand with previous studies showing upregulation of macroautophagy as a compensatory mechanism in response to reduced CMA. This suggests that *CTNS*-KO PTCs have a dysregulated macroautophagy phenotype, similar to other cystinotic cellular systems.

We have previously shown that LAMP2A localizes at Rab11-positive vesicles in cystinotic cells (14), suggesting that Rab11-positive carrier vesicles may be responsible for trafficking of LAMP2A to the lysosome. Here, we next analyzed the distribution of endogenous Rab11 in *CTNS*-KO PTCs and found that Rab11 is mislocalized in these cells. In particular, we observed that the distribution of Rab11-positive vesicles is defective in *CTNS*-KO cells, showing a homogeneous distribution throughout the cystinotic cells as opposed to the more perinuclear distribution of Rab11 puncta observed in wild type cells (**Figure 3A**). This suggests defective trafficking of Rab11 vesicles in *CTNS*-KO cells. Next, we analyzed the expression levels of endogenous Rab11 and found that Rab11 is significantly downregulated in *CTNS*-KO PTCs (**Figure 3B**). Because LAMP2A mislocalization suggests defective CMA and as CMA upregulation may have a direct effect on the

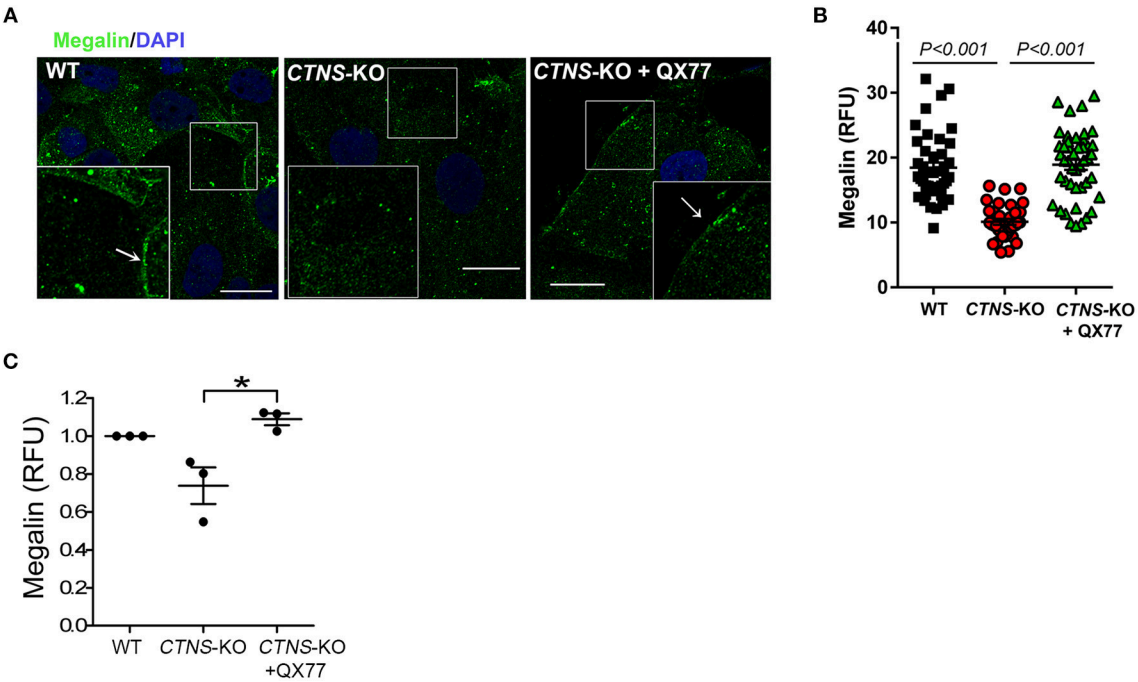

FIGURE 5 | Decreased membrane localization and expression of megalin in *CTNS*-KO PTCs is rescued by CMA activation. **(A)** Confocal microscopy analysis of the distribution of endogenous megalin. WT, *CTNS*-KO PTCs, and *CTNS*-KO PTCs treated with 20 μM QX77 or vehicle for 72 h were fixed and endogenous megalin was detected by immunofluorescence (green) and nuclei stained with DAPI (blue). The arrows indicate plasma membrane localization of megalin. Scale bar, 10 μm. **(B)** Megalin expression was quantified by analysis of the mean fluorescence intensity of megalin in each cell using ImageJ. Each symbol represents a cell. The data are from one experiment, and are representative of three independent experiments with the same result. **(C)** Megalin expression was analyzed as in B. The individual symbols correspond to independent biological replicates from 3 independent experiments. The bars indicate the mean ± SEM. The data are normalized to the values observed in wild type cells. Significant differences between *CTNS*-KO untreated and QX77-treated *CTNS*-KO PTCs were calculated using the Student's *t*-test. *$p < 0.05$.

expression levels of trafficking associated proteins (15), we next studied the effect of CMA enhancers on Rab11 expression in *CTNS*-KO cells. To this end, we treated wild type and *CTNS*-KO PTCs with the CMA enhancer QX77, a small molecule known to dysregulate the repressive function of retinoic acid receptor-alpha over the CMA machinery (15, 22). We found that treatment with this CMA enhancer significantly increased Rab11 expression in *CTNS*-KO PTCs, bringing the expression of this small GTPase to wild type levels (**Figure 3B**).

Next, to determine whether the mislocalization of Rab11 was caused by defective trafficking, we transfected wild type and *CTNS*-KO cells with the recycling endosome trafficking reporter, GFP-Rab11 (**Figure 4A**), and measured vesicular transport using Total Internal Reflection Fluorescence microscopic analysis. In **Figure 4B**, we show that cystinotic PTCs have defective Rab11 trafficking characterized by significant decrease in Rab11-positive vesicle movement. Thus, *CTNS*-KO cells showed increased numbers of vesicles moving at very low speed or not moving at all (**Figure 4B**), with a concomitant decrease of faster-moving Rab11-positive vesicles (**Figure 4C**). Interestingly, treatment with the CMA enhancer QX77 rescued the defective trafficking phenotype in *CTNS*-KO PTCs thus favoring a net and significant increment in Rab11 transport (**Figures 4B,C** and **Movies 1–3**).

Upregulation of Chaperone-Mediated Autophagy Increases Megalin Localization at the Plasma Membrane in Cystinotic PTCs

Megalin, an endocytosis receptor located apically in PTCs and other epithelial cells including endocrine glands, the lung and the brain, plays a fundamental role in PTC function by facilitating the uptake of plasma solutes for ultrafiltration. Cystinotic PTCs have progressive loss of megalin expression and other apical receptors (17), and megalin-KO mice are characterized by urinary loss of ultra-filtrated plasma proteins (23). The apical distribution of megalin in PTCs is proposed to be mediated by the trafficking of apical recycling endosomes, regulated by the small GTPase Rab11 (18). Because Rab11 trafficking is defective in *CTNS*-KO PTCs, we next investigated the expression and localization of endogenous megalin in *CTNS*-KO PTCs. In **Figure 5A**, we show that *CTNS*-KO PTCs are characterized by decreased plasma membrane localization of megalin. Further, because treatment with CMA enhancers has a direct impact on Rab11 trafficking in cystinotic cells, we studied whether the upregulation of CMA has a beneficial effect on megalin localization. To this end, we treated *CTNS*-KO PTCs with the CMA activator QX77 (20 μM) for 72 h and determined

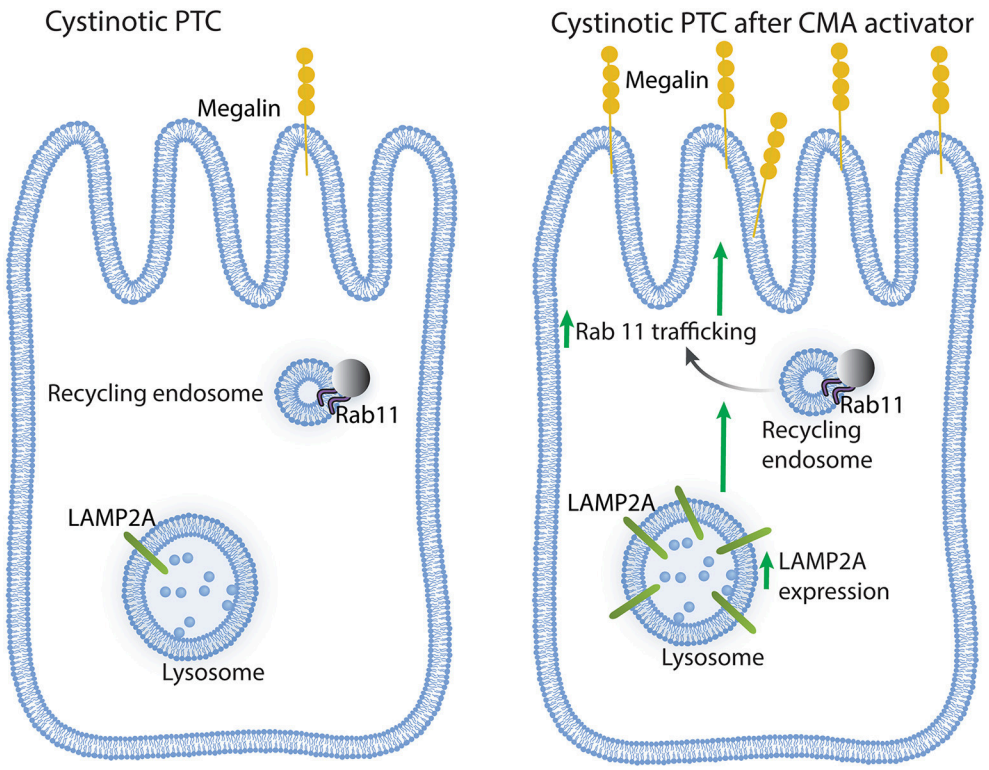

FIGURE 6 | Schematic representation of the effect of chaperone-mediated autophagy (CMA) enhancers on cystinotic proximal tubule cells (PTCs). Left panel, Cystinotic PTCs are characterized by decreased localization of the CMA receptor LAMP2A at the lysosomal membrane, reduced Rab11 expression, impaired Rab11 trafficking and decreased plasma membrane localization and expression of the apical receptor megalin, a phenotype that is associated with the development of Fanconi syndrome in cystinosis. Right panel, upon treatment with CMA enhancers, cystinotic PTCs show increased LAMP2A at the lysosomal membrane, enhanced Rab11 trafficking and increased apical distribution of megalin along with increased megalin expression, suggesting that CMA upregulation has direct positive implications for PTC function.

the plasma membrane distribution of megalin in these cells. In **Figure 5A**, we show that CMA upregulation significantly increases megalin localization at the plasma membrane in *CTNS*-KO PTCs, indicating that upregulation of CMA has a direct positive implication on megalin distribution, likely affecting PTC function. Finally, because recycling through the plasma membrane is known to stabilize megalin and prevent its degradation (18), we next analyzed the effect of CMA enhancers on megalin expression. In **Figures 5B,C**, we show that megalin expression is decreased in *CTNS*-KO PTCs. We also show that treatment with the CMA enhancer, QX77, induces the significant upregulation of megalin expression in cystinotic PTCs.

DISCUSSION

Proximal tubule cell dedifferentiation is a hallmark of nephropathic cystinosis (17). One of the most important changes underwent by PTCs in cystinosis is the loss of megalin apical distribution which is accompanied by a net increase of protein and electrolyte loss (17). This progressive loss of the expression of megalin and other endocytic receptors, including the glucose and sodium transporter SGC2 and phosphate transporters, is proposed to account for apical dedifferentiation leading to the development of Fanconi syndrome, which is not corrected by cysteamine treatment in cystinotic patients. Megalin is a fast recycling receptor and has been proposed to define the apical recycling pathway of epithelial cells. Its localization and recycling at the apical membrane depends on the function of Rab11 (18). In this work, using newly generated cystinotic PTCs, we show that Rab11 expression and trafficking is defective in *CTNS*-KO cells. We also demonstrate that both the impaired Rab11 trafficking and the defective localization of the CMA receptor LAMP2A at lysosomes are corrected by treatment with CMA enhancers, thus linking CMA defects to PTC disfunction and de-differentiation in cystinosis.

Rab GTPases and their effectors are determinants of membrane identity and master regulators of vesicular function. Decreased expression or defective functions of these small GTPases are associated with specific defects in vesicular transport. Cystinotic cells are characterized by defective endolysosomal transport, while the induction of vesicular trafficking mechanisms through the upregulation of Rab27a or Rab7 improves vesicular trafficking in cystinosis (20). Furthermore, a Rab11-specific defect in cystinotic cells was

recently described (15). In particular, the CMA receptor LAMP2A shows an anomalous distribution in mouse cystinotic fibroblasts, where it localizes to Rab11-positive carrier vesicles instead of reaching the lysosomes (14). LAMP2A mislocalization was directly associated with defects in CMA, observed both in isolated cystinotic fibroblasts and, *in vivo*, in cystinotic mice (14, 15). Constitutively active Rab11 partially rescues the localization of LAMP2A at the lysosomal membrane (15). It is current knowledge that not all defects in cystinosis are corrected by cysteamine, that Fanconi syndrome is developed before the apparition of cystine crystals and independently of early cysteamine treatment, suggesting that CMA defects occur independently of lysosomal overload. Because the defects in CMA function are not repaired by treatment with cysteamine, the only lysosomal depletion therapy currently available for cystinotic patients, CMA function acquires important clinical significance. In this context, the observations that CMA upregulation, through treatment of proximal tubule cells with novel CMA enhancers, upregulates Rab11 expression, corrects Rab11 trafficking and corrects LAMP2A localization in PTCs is highly significant. Of note, although LAMP2A is cleaved by lysosomal cathepsin A (24) and lysosomal protease inhibitors increase the amount of lysosomal membrane-localized LAMP2A in cystinosis (14), it is not yet clear whether this mechanism of LAMP2A upregulation may have direct implications in proximal tubule cell function.

How defective CMA affects PTC function in cystinosis is currently unknown but since dysfunction of CMA has been implicated in PTC hypertrophy (25), it is expected that CMA upregulation would be beneficial in cystinotic patients. Kidney proximal epithelial cells are characterized by low levels of macroautophagy, but high basal levels of CMA activity. In PTCs, CMA has been shown to regulate the protein levels of important transcription factors that control PTC differentiation, including Pax2 (25). In mouse cystinotic PTCs *in vivo,* the accumulation of CMA substrates (including GAPDH) is caused by mislocalization of LAMP2A (15), suggesting that CMA may directly affect PTC function in this lysosomal disorder and that LAMP2A trafficking may be affected in these cells. Despite this knowledge, a direct impact of CMA deficiency on PTC function in cystinosis has not been demonstrated thus far and the mechanisms underlying PTC defects caused by defective CMA in cystinosis remain unknown. Here, the observation that CMA enhancers upregulate Rab11 trafficking in cystinotic PTCs is highly significant, not only because Rab11 is able to re-localize LAMP2A at the lysosomal membrane (15) but also because Rab11 upregulation has direct consequences on plasma membrane expression of the endocytic receptor megalin, which as mentioned above, is essential for substrate resorption from the ultrafiltrate in the proximal tubule. Thus, it is expected that CMA enhancers would have a direct impact on cystinotic PTC function and potentially on the reduction of some of the symptoms associated with Fanconi syndrome. Whether CMA enhancers increase upregulation of other apical receptors in cystinosis is currently unknown, but because of their effect on Rab11 trafficking and the function of Rab11 as a recycling endosome regulator, it is likely that the effects of CMA enhancers go beyond the benefits mediated by megalin upregulation.

In conclusion, CMA upregulation in cystinotic PTCs has a direct positive impact on Rab11 trafficking and megalin expression (**Figure 6**), whose reduced expression in cystinosis is associated with Fanconi syndrome. Thus, CMA constitutes a potential new therapeutic target, independent of lysosomal overload-reducing therapies such as cysteamine.

AUTHOR CONTRIBUTIONS

JZ and JH: performed experiments and organized data; JJ: supervised experiments, analyzed data, and edited manuscript; FR: performed experiments; EG and AC: contributed important reagents and edited manuscript; SC: conceived the idea, analyzed data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00021/full#supplementary-material

Movie 1 | Pseudo-TIRF microscopy analysis of GFP-Rab11 in WT HK-2 cells. Cells were transfected with GFP-Rab11 plasmid. After 48 h, cells were analyzed by TIRFM. The video was recorded for 60 s, 61 frames and 200 ms exposure. Playback is 7 fps.

Movie 2 | Pseudo-TIRF microscopy analysis of GFP-Rab11 in CTNS-KO HK-2 cells. Cells were transfected with GFP-Rab11 plasmid. After 48 h, cells were analyzed by TIRFM. The video was recorded for 60 s, 61 frames and 200 ms exposure. Playback is 7 fps.

Movie 3 | Pseudo-TIRF microscopy analysis of GFP-Rab11 in CTNS-KO HK-2 cells treated with QX77. Cells were transfected with GFP-Rab11 plasmid and incubated with QX77. After 48 h, cells were analyzed by TIRFM. The video was recorded for 60 s, 61 frames and 200 ms exposure. Playback is 7 fps.

REFERENCES

1. Town M, Jean G, Cherqui S, Attard M, Forestier L, Whitmore SA, et al. A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis. *Nat Genet.* (1998) 18:319–324. doi: 10.1038/ng0498-319
2. Gahl WA, Thoene JG, Schneider JA. Cystinosis. *N Eng J Med.* (2002) 347:111–21. doi: 10.1056/NEJMra020552

3. Gultekingil Keser A, Topaloglu R, Bilginer Y, Besbas N. Long-term endocrinologic complications of cystinosis. *Minerva Pediatr* (2014) 66:123–30.
4. Besouw MT, Van Dyck M, Cassiman D, Claes KJ, Levtchenko EN. Management dilemmas in pediatric nephrology: Cystinosis. *Pediatric Nephrol.* (2015) 30:1349–60. doi: 10.1007/s00467-015-3117-3
5. Robert JJ, Tete MJ, Guest G, Gagnadoux MF, Niaudet P, Broyer M. Diabetes mellitus in patients with infantile cystinosis after renal transplantation. *Pediatric nephrology* (1999) 13:524–9. doi: 10.1007/s004670050651
6. Cheung WW, Cherqui S, Ding W, Esparza M, Zhou P, Shao J, et al. Muscle wasting and adipose tissue browning in infantile nephropathic cystinosis. *J Cachexia Sarcopenia Muscle* (2016) 7:152–64. doi: 10.1002/jcsm.12056
7. Klusmann M, Van't Hoff W, Monsell F, Offiah AC. Progressive destructive bone changes in patients with cystinosis. *Skeletal Radiol.* (2013) doi: 10.1007/s00256-013-1735-z
8. Cherqui S, Courtoy PJ. The renal Fanconi syndrome in cystinosis: pathogenic insights and therapeutic perspectives. *Nat Rev Nephrol.* (2017) 13:115–31. doi: 10.1038/nrneph.2016.182
9. da Silva V, Zurbrügg R, Lavanchy P, Blumberg A, Suter H, Wyss S, et al. Long-term treatment of infantile nephropathic cystinosis with cysteamine. *N Eng J Med.* (1985) 313:1460–3.
10. Cherqui S. Cysteamine therapy: a treatment for cystinosis, not a cure. *Kidney Int.* (2012) 81:127–9. doi: 10.1038/ki.2011.301
11. Vaisbich M, Pache de Faria Guimaraes L, Shimizu M, Seguro A. Oxidative stress in cystinosis patients. *Nephron Extra* (2011) 1:73–7. doi: 10.1159/000331445
12. Kaushik S, Cuervo AM. The coming of age of chaperone-mediated autophagy. *Nat Rev Mol Cell Biol.* (2018) 19:365–81. doi: 10.1038/s41580-018-0001-6
13. Kaushik S, Bandyopadhyay U, Sridhar S, Kiffin R, Martinez-Vicente M, Kon M, et al. Chaperone-mediated autophagy at a glance. *J Cell Sci.* (2011) 124:495–9. doi: 10.1242/jcs.073874
14. Napolitano G, Johnson JL, He J, Rocca CJ, Monfregola J, Pestonjamasp K, et al. Impairment of chaperone-mediated autophagy leads to selective lysosomal degradation defects in the lysosomal storage disease cystinosis. *EMBO Mol Med.* (2015) 7:158–74. doi: 10.15252/emmm.201404223
15. Zhang J, Johnson JL, He J, Napolitano G, Ramadass M, Rocca C, et al. Cystinosin, the small GTPase Rab11, and the Rab7 effector RILP regulate intracellular trafficking of the chaperone-mediated autophagy receptor LAMP2A. *J Biol Chem.* (2017) 292:10328–46. doi: 10.1074/jbc.M116.764076
16. Kiffin R, Christian C, Knecht E, Cuervo AM. Activation of chaperone-mediated autophagy during oxidative stress. *Mol Biol Cell* (2004) 15:4829–40. doi: 10.1091/mbc.e04-06-0477
17. Gaide Chevronnay HP, Janssens V, Van Der Smissen P, N'Kuli F, Nevo N, Guiot Y, et al. Time course of pathogenic and adaptation mechanisms in cystinotic mouse kidneys. *J Am Soc Nephrol.* (2014) 25:1256–69. doi: 10.1681/ASN.2013060598
18. Perez Bay AE, Schreiner R, Benedicto I, Paz Marzolo M, Banfelder J, Weinstein AM, et al. The fast-recycling receptor Megalin defines the apical recycling pathway of epithelial cells. *Nat Commun.* (2016) 7:11550. doi: 10.1038/ncomms11550
19. Christensen EI, Birn H. Megalin and cubilin: multifunctional endocytic receptors. *Nat Rev Mol Cell Biol.* (2002) 3:256–66. doi: 10.1038/nrm778
20. Johnson J, Napolitano G, Monfregola J, Rocca C, Cherqui S, Catz S. Upregulation of the Rab27a-dependent trafficking and secretory mechanisms improves lysosomal transport, alleviates endoplasmic reticulum stress, and reduces lysosome overload in cystinosis. *Mol Cell Biol.* (2013) 33:2950–62. doi: 10.1128/MCB.00417-13
21. Ryan MJ, Johnson G, Kirk J, Fuerstenberg SM, Zager RA, Torok-Storb B. HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney Int.* (1994) 45:48–57. doi: 10.1038/ki.1994.6
22. Anguiano J, Garner TP, Mahalingam M, Das BC, Gavathiotis E, Cuervo AM. Chemical modulation of chaperone-mediated autophagy by retinoic acid derivatives. *Nat Chem Biol.* (2013) 9:374–82. doi: 10.1038/nchembio.1230
23. Leheste JR, Rolinski B, Vorum H, Hilpert J, Nykjaer A, Jacobsen C, et al. Megalin knockout mice as an animal model of low molecular weight proteinuria. *Am J Pathol.* (1999) 155:1361–70. doi: 10.1016/S0002-9440(10)65238-8
24. Cuervo A, Mann L, Bonten E, d'Azzo A, Dice J. Cathepsin A regulates chaperone-mediated autophagy through cleavage of the lysosomal receptor. *EMBO J.* (2003) 22:47–59. doi: 10.1093/emboj/cdg002
25. Franch HA. Chaperone-mediated autophagy in the kidney: the road more traveled. *Semin Nephrol.* (2014) 34:72–83. doi: 10.1016/j.semnephrol.2013.11.010

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Modulation of Autophagy Influences the Function and Survival of Human Pancreatic Beta Cells Under Endoplasmic Reticulum Stress Conditions and in Type 2 Diabetes

M. Bugliani[1], S. Mossuto[1], F. Grano[1], M. Suleiman[1], L. Marselli[1], U. Boggi[2], P. De Simone[3], D. L. Eizirik[4], M. Cnop[4], P. Marchetti[1†] and V. De Tata[3†]*

[1] Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy, [2] Department of Surgical Pathology, Medicine, Molecular and Critical Area, University of Pisa, Pisa, Italy, [3] Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy, [4] Medical Faculty, ULB Center for Diabetes Research, Université Libre de Bruxelles, Brussels, Belgium

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***Correspondence:**
V. De Tata
vincenzo.detata@med.unipi.it

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

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Autophagy is the major mechanism involved in degradation and recycling of intracellular components, and its alterations have been proposed to cause beta cell dysfunction. In this study, we explored the effects of autophagy modulation in human islets under conditions associated to endoplasmic reticulum (ER) stress. Human pancreatic islets were isolated by enzymatic digestion and density gradient purification from pancreatic samples of non-diabetic (ND; $n = 17$; age 65 ± 21 years; gender: 5 M/12 F; BMI 23.4 ± 3.3 kg/m^2) and T2D ($n = 9$; age 76 ± 6 years; 4 M/5 F; gender: BMI 25.4 ± 3.7 kg/m^2) organ donors. Nine ND organ donors were treated for hypertension and 1 for both hypertension and hypercholesterolemia. T2D organ donors were treated with metformin (1), oral hypoglycemic agents (2), diet + oral hypoglycemic agents (3), insulin (3) or insulin plus metformin (3) as for antidiabetic therapy and, of these, 3 were treated also for hypertension and 6 for both hypertension and hypercholesterolemia. Two days after isolation, they were cultured for 1–5 days with 10 ng/ml rapamycin (autophagy inducer), 5 mM 3-methyladenine or 1.0 nM concanamycin-A (autophagy blockers), either in the presence or not of metabolic (0.5 mM palmitate) or chemical (0.1 ng/ml brefeldin A) ER stressors. In ND islets palmitate exposure induced a 4 to 5-fold increase of beta cell apoptosis, which was significantly prevented by rapamycin and exacerbated by 3-MA. Similar results were observed with brefeldin treatment. Glucose-stimulated insulin secretion from ND islets was reduced by palmitate (−40 to 50%) and brefeldin (−60 to 70%), and rapamycin counteracted palmitate, but not brefeldin, cytotoxic actions. Both palmitate and brefeldin induced PERK, CHOP and BiP gene expression, which was partially, but significantly prevented by rapamycin. With T2D islets, rapamycin alone reduced the amount of p62, an autophagy receptor that accumulates in cells when macroautophagy is inhibited. Compared to untreated T2D cells, rapamycin-exposed diabetic islets showed improved insulin secretion, reduced proportion of beta cells showing signs of apoptosis and better preserved insulin granules, mitochondria and

ER ultrastructure; this was associated with significant reduction of PERK, CHOP and BiP gene expression. This study emphasizes the importance of autophagy modulation in human beta cell function and survival, particularly in situations of ER stress. Tuning autophagy could be a tool for beta cell protection.

Keywords: autophagy, human islets, type 2 diabetes, ER stress, insulin secretion, beta cell dysfunction

INTRODUCTION

Autophagy represents a highly conserved intracellular recycling pathway by which cellular components are degraded through the lysosomal machinery (3). Classically considered as a mechanism to promote cell survival during starvation (2), autophagy can also be induced by several physiological and pathological conditions, such as growth factors deprivation, hypoxia, oxidative stress, and physical exercise (4). Furthermore, autophagy seems to be constitutively activated at low levels to remove misfolded proteins and damaged and/or senescent organelles (1). Hence, autophagy can be viewed as a mechanism to protect cells against several stressors as well as a cellular response to wear-and-tear processes (5). On the other hand, it has been demonstrated that dysregulated activation of autophagy can also induce different types of cell death (6). Thus, it seems that autophagy can either protect or promote cell death in relation to the cellular and environmental context (7). Accordingly, altered autophagy could play a key pathogenic role in several disease processes, especially where the accumulation of damaged molecules and organelles might elicit a condition of increased cellular stress (8).

Pancreatic beta cells are specialized to secrete insulin in response to variations in blood glucose concentration. In order to maintain glucose homeostasis, beta cells are able to raise several fold their insulin synthesis and secretion in response to increased plasma glucose levels. Therefore, they must continually deal with a high protein burden, as proinsulin biosynthesis has been calculated to reach 10^6 molecules/min (9). This represents a major challenge for their ER, where protein translation and quality control takes place, and therefore beta cells are particularly susceptible to ER stress (10–12). When faced with ER stress, beta cells respond to activating the unfolded protein response (UPR) (13, 14), whose signaling is mediated via three main transmembrane sensors: IRE1α (endoribonuclease inositol requiring protein), PERK (protein kinase RNA-like endoplasmic reticulum kinase), and ATF6 (activating transcription factor 6) (10–15). In basal conditions, the chaperone immunoglobulin heavy chain binding protein (BiP), a key member of the Hsp70 family, is constitutively bound to the luminal domain of these three sensors and prevents their activation. When misfolded protein accumulates in the ER, BiP dissociates from the UPR sensors leading to their consequent activation. While a moderate ER stress-induced UPR represents a compensatory mechanism, a chronic or overwhelming ER stress impairs cellular functions and can induce apoptosis to remove irreversibly damaged cells (11–15).

Autophagy has been shown to have a protective role against ER stress (16) and facilitate mitochondrial turnover (17). Indeed, it has been demonstrated that beta-cell Atg7$^{-/-}$ mice are characterized by islet degeneration, impaired insulin secretion, and glucose intolerance (18, 19). In addition, we have previously shown that a proportion of beta cells of type 2 diabetic (T2D) subjects presents a major increase of autophagic vacuoles and autophagosomes, associated with cell damage, which further suggests that altered autophagy might contribute to the loss of beta cell functional mass (20).

To shed further light on these issues, we presently explored the effects of autophagy modulation in isolated human islets under conditions of metabolically (palmitic acid) or chemically (brefeldin A) induced ER stress (21, 22). In addition, activators and inhibitors of autophagy were tested with pancreatic islets from T2D organ donors.

MATERIALS AND METHODS

Human Islet Isolation and Culture

Human islet collection and handling were approved by the local Ethics Committee. Human pancreatic islets were isolated by enzymatic digestion and density gradient purification from pancreatic samples of non-diabetic (ND; $n = 17$; age 65 ± 21 years; gender: 5 M/12 F; BMI 23.4 ± 3.3 kg/m^2) and T2D ($n = 9$; age 76 ± 6 years; 4 M/5 F; gender: BMI 25.4 ± 3.7 kg/m^2) organ donors as detailed elsewhere (23, 24). For the experiments with palmitate, ND islets were cultured for 1–5 days in M199 medium containing 1% BSA with 10 ng/ml rapamycin (autophagy inducer) (25), 5.0 mmol/l 3-methyladenine or 1.0 nmol/l concanamycin-A (autophagy blockers) (25), either in the presence or absence of 0.5 mmol/l palmitate, prepared as previously reported (24, 26). For the experiments with brefeldin A, ND islets were exposed to the autophagy modulators either in the presence or absence of 0.1 ng/ml of this chemical ER stress inducer. The islets prepared from T2D donors were studied after 24 h incubation with M199 medium containing or not 10 ng/ml rapamycin.

Electron Microscopy Evaluation

Electron microscopy studies were performed on isolated islets as previously described (27). Islets were fixed with 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.4 for 1 h at 4°C. After rinsing in cacodylate buffer, the sample was postfixed in 1% cacodylate buffered osmium tetroxide for 2 h at room temperature, then dehydrated in a graded series of ethanol, briefly transferred to propylene oxide and embedded in Epon-Araldite. Ultrathin sections (60–80 nm thick) were cut with a diamond knife, placed on formvar-coated copper grids (200 mesh), and stained with uranyl acetate and lead citrate. The presence of

marked chromatin condensation and/or blebs was considered as signs of apoptosis (28). Morphometric analyses were performed by stereological techniques (19, 24, 27). In particular, volume density of insulin granules, mitochondria and rough endoplasmic reticulum (RER) was estimated. Micrographs, obtained at 10,000× were analyzed by overlay with a graticule (11 × 11 cm) composed of 169 points. Volume density was calculated according to the formula: VD = Pi/Pt, where Pi is the number of points within the subcellular component and Pt is the total number of points, and expressed in ml/100 ml of tissue (ml%) (19, 24, 27).

Insulin Secretion

Insulin secretion studies were performed by the batch incubation technique as previously described (29–31). Groups of approximately 15 islets of comparable size were incubated at 37°C for 45 min in Krebs-Ringer bicarbonate solution (KRB), 0.5% albumin, pH 7.4, containing 3.3 mmol/l glucose. Then, the medium was removed and replaced with KRB containing 16.7 mmol/l glucose. After additional 45 min incubation, medium was collected. Insulin levels were measured by a commercially available immunoradiometric assay (Pantec Forniture Biomediche, Turin, Italy). Insulin secretion was expressed as stimulation index (SI), i.e., the ratio of stimulated (16.7 mmol/l glucose) over basal (3.3 mmol/l glucose) insulin secretion (29–31).

Quantitative RT-PCR Experiments in Isolated Islets

Gene expression studies were performed as previously detailed (30). Total RNA was extracted using the PureLink™ RNA Mini kit (Life technologies, Carlsbad, CA, USA) according to manufacturer recommendations and quantified by absorbance at A260/A280 nm (ratio >1.95) in a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). After it was reverse-transcribed with SuperScript VILO Master Mix (Life technologies), the levels of the genes of interest were normalized for the housekeeping gene beta actin and quantified by the $2^{-\Delta\Delta Ct}$ method in a VIIA7 instrument (Life technologies). The primers/ probe for the analyzed genes were purchased from Taqman® Assay on-demand library (Life technologies).

p62 Evaluation in Isolated Human Islets

The levels of p62 were assessed in T2D islets by the *p62 Elisa kit* (Enzo Life Sciences, Lausen, Switzerland) following the manufacturer protocol. In brief, after 24 h exposure to rapamycin or 3-MA, the islets were collected, protein were extracted and aliquoted on a plate pre-coated with a p62 specific antibody. After having incubated the samples in presence of a second anti-p62 antibody (rabbit polyclonal), the amount of p62 was revealed by adding a secondary donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase and a mix composed by TMB and hydrogen peroxide. The plate was read in a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany) and the amount of p62 normalized for the total amount of proteins.

Statistical Analysis

Data are presented as mean ± SD. Differences between groups were assessed by the two-tailed Student's *t*-test or one-way ANOVA followed by the Bonferroni correction, as appropriate. A $P < 0.05$ was considered statistically significant.

RESULTS

Beta Cell Apoptosis and Insulin Secretion

Exposure of ND human islets to 0.5 mmol/l palmitate for 5 days or 0.1 ng/ml brefeldin-A for 24 h induced a significant increase of beta cell apoptosis compared to control islets (**Figures 1A,B**), confirming previously reported results (32, 33). In agreement with previous reports (20, 24) apoptosis was identified on the basis of internationally acknowledged criteria based on the appearance of marked chromatin condensation and blebs (28). Electron microscopy can be considered one of the best methods to identify apoptotic cells, because it enables not only the detection of apoptosis but also enables to identify which type of cell is undergoing apoptosis (see also **Supplementary Figure 1** ESM). The deleterious effects of both ER stressors were prevented by rapamycin (autophagy activator), whereas 3-MA, but not concanamycin (both are autophagy inhibitors), enhanced the rate of beta cell apoptosis in presence of palmitate (**Figures 1A,B**). Insulin secretion was significantly decreased by islet exposure to either palmitate or brefeldin A, and the presence of rapamycin protected beta cells from palmitate-induced insulin secretion alterations (**Figure 1C**).

Electron Microscopy Studies

We then investigated the ultrastructural changes induced by the exposure of human islets to palmitate or brefeldin by quantitative morphometry. In particular, we assayed the volume density of insulin granules, mitochondria, and ER in beta cells exposed to 0.5 mmol/l palmitate for 5 days and 0.1 ng/ml brefeldin for 24 h. In addition, we evaluated the effects of the stimulation of autophagy by the concomitant exposure to 10 ng/ml rapamycin. Five days of palmitate exposure significantly decreased the volume density of insulin granules, whereas volume density of both mitochondria and ER was significantly increased (**Figures 2A–C**). In all cases, co-incubation with rapamycin was able to prevent the changes caused by palmitate exposure (**Figures 2A–C**). Brefeldin A also induced a significant reduction of insulin granule volume density, although quantitatively less compared to palmitate, and both mitochondria and ER volume density was significantly increased (**Figures 2A–C**). Rapamycin had no effects on the changes in insulin granules and mitochondria induced by brefeldin, whereas it was able to partially prevent brefeldin A-induced increase of ER volume density (**Figures 2A–C**).

Gene Expression in Isolated Human Islets

The expression of selected ER markers was then studied in isolated ND human islets exposed for 24 h to 0.5 mmol/l palmitate or to 0.1 ng/ml brefeldin; the modulating effect of the concomitant exposure to 10 ng/ml rapamycin was also evaluated. Palmitate exposure significantly increased the

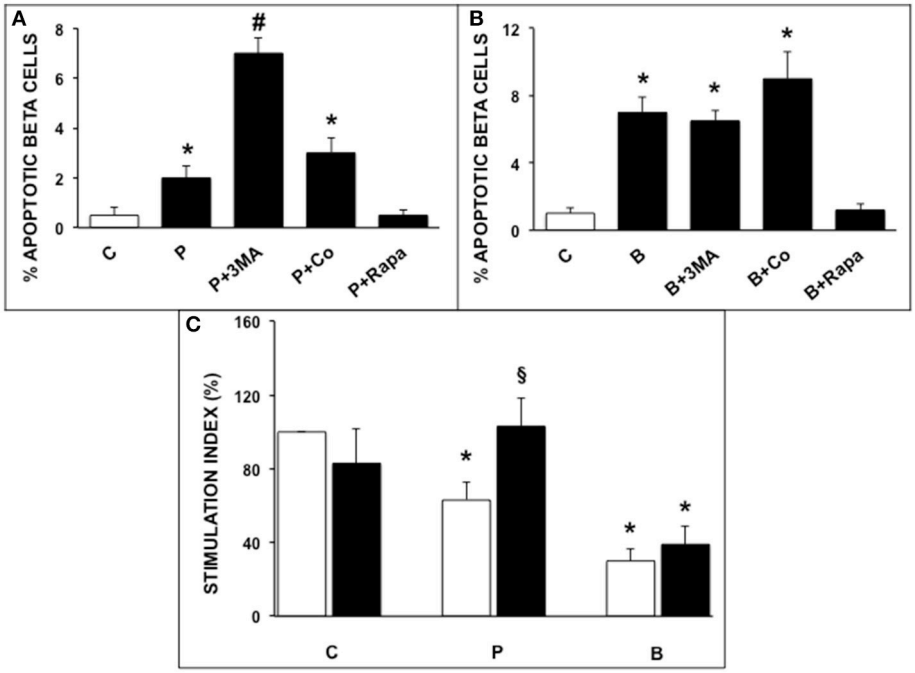

FIGURE 1 | (A,B) Ultrastructural morphometric analysis of beta-cell apoptosis in human islets after 5 days exposure to palmitate **(A)** or 1 day exposure to brefeldin **(B)**. C, control; P = 0.5 mmol/l palmitate; B = 0.1 ng/ml brefeldin; P + 3MA = palmitate + 5 mmol/l 3-methyladenine; P + Co = palmitate+ 1 nmol/l concanamycinA; P + Rapa = palmitate + 10 ng/ml rapamycin; B + Rapa = brefeldin + 10 ng/ml rapamycin. For palmitate experiments around 200–300 cells were considered, corresponding to 6 islets and two islet preparations. For brefeldin around 200 cells were analyzed corresponding to 3 islets and 1 islet preparation. Statistical analysis: $^{*}p < 0.05$ vs. C and P + Rapa or B + Rapa, $^{\#}p < 0.05$ vs. all groups, after Bonferroni correction. **(C)** Glucose-stimulated insulin secretion of control (C), palmitate-treated (P), and brefeldin-treated (B) human islets in the presence (black bars) or absence (white bars) of 10 ng/ml rapamycin ($N = 4$–7). Statistical analysis: $^{*}p < 0.05$ vs. C; $^{\S}p < 0.05$ vs. P, after Bonferroni correction.

expression of PERK, CHOP, and BiP with respect to control human islets, and this effect was significantly prevented by the concomitant presence of rapamycin (**Figure 3**, panels on the left). Brefeldin-A exposure markedly increased the expression of all the assayed ER stress markers in human islets with respect to controls, and the concomitant stimulation of autophagy by rapamycin in part counteracted this effect (**Figure 3**, panels on the right).

Rapamycin and T2D Human Islets

Finally, we explored the effects of rapamycin in human islets isolated from T2D donors. In T2D islets beta cell apoptosis was significantly higher than in ND islets, in agreement with previous results (31, 34), and exposure to rapamycin significantly decreased the number of apoptotic beta cells (**Figure 4A**). Rapamycin also caused a significant increase of glucose-stimulated insulin secretion T2D human islets (**Figure 4B**).

Quantitative morphometry confirmed the ultrastructural alterations previously observed in human islets isolated from T2D patients (31, 34): volume density of insulin granules was significantly decreased whereas volume density of both mitochondria and ER was significantly increased compared to ND islets (**Figure 4C**). Exposure of diabetic islets to rapamycin was able to significantly counteract these alterations (**Figure 4C**). These favorable changes induced by rapamycin were associated with decreased expression of PERK, CHOP, and BiP similar to those observed in ND islets exposed to palmitate (**Figure 4D**).

p62 in T2D Islets

In order to confirm whether rapamycin and 3-MA could modulate autophagy flux, we evaluated the amount of p62, an autophagy receptor that accumulates in cells when macroautophagy is inhibited (21). In ND islets, we found 1.15 ± 0.36 ng/ml of p62/μg total proteins. In T2D islets, p62 levels (3.62 ± 0.90 ng/ml of p62/μg total proteins) were higher with respect to non-diabetic islets ($p < 0.05$). Exposure to rapamycin significantly reduced the amount of p62 (**Figure 5**), indicating that the drug could increase the autophagy flux in T2D islets. Conversely, the use of 3-MA, an autophagy inhibitor, significantly increased the levels of the receptor (**Figure 5**).

DISCUSSION

The synthesis, modification and delivery of proteins to their target sites occur in the ER. Several physiological and pathological conditions are able to impair these processes leading to ER stress, and in the last years a large body of evidences has

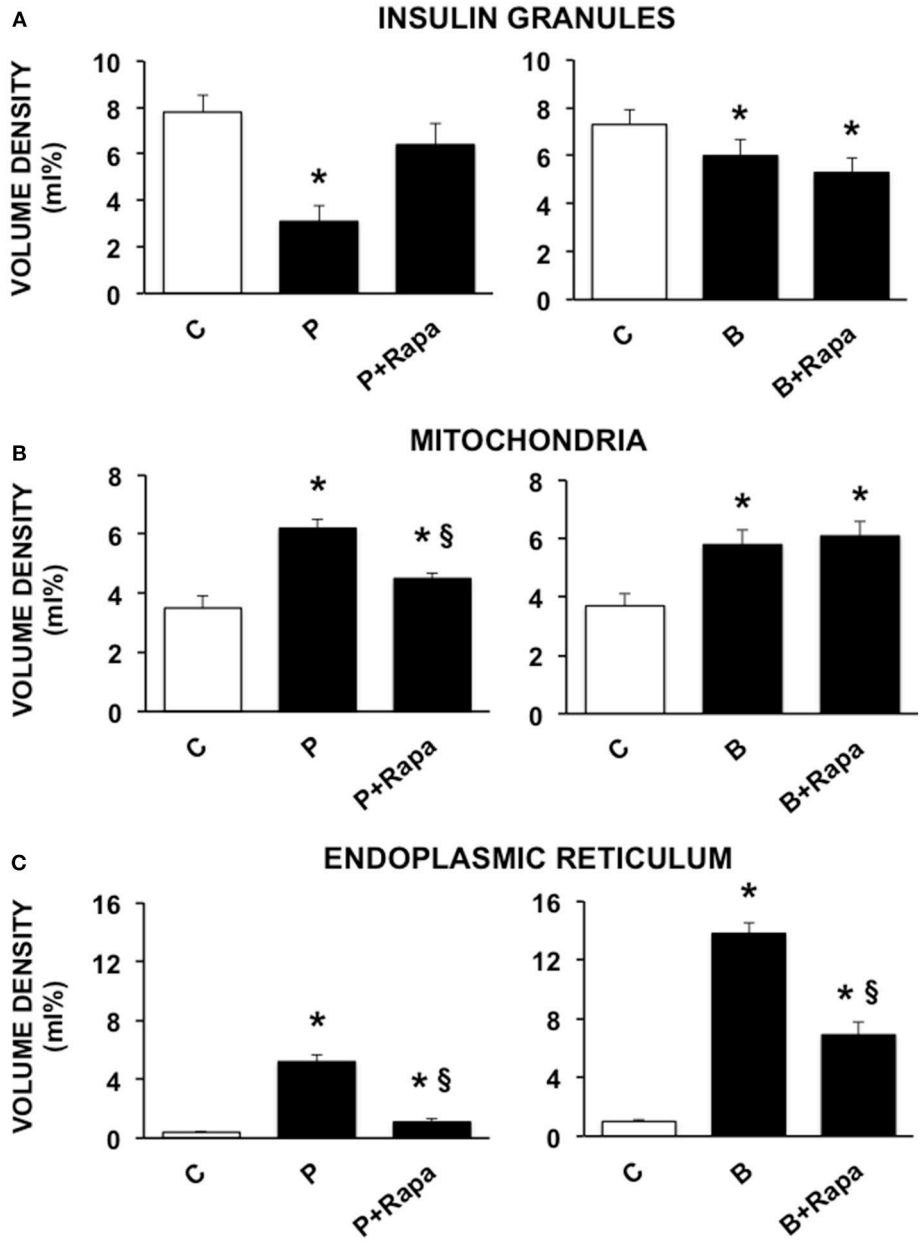

FIGURE 2 | Quantitative morphometric analysis of volume density of insulin granules **(A)**, mitochondria **(B)**, and endoplasmic reticulum **(C)** in beta cells of isolated human islets after 5 days exposure to palmitate (left panels) or 1 day exposure to brefeldin (right panels). C, control; P = 0.5 mmol/l palmitate; B = 0.1 ng/ml brefeldin; P + Rapa = palmitate + 10 ng/ml rapamycin; B + Rapa = brefeldin + 10 ng/ml rapamycin. For palmitate experiments around 200–300 cells were considered, corresponding to 6 islets and two islet preparations. For brefeldin around 200 cells were analyzed corresponding to 3 islets and 1 islet preparation. Statistical analysis: $^{*}p < 0.05$ vs. C; $^{\S}p < 0.05$ vs. P, after Bonferroni correction.

been provided indicating that ER stress may be implicated in beta cell dysfunction and death in diabetes (22, 23, 35–42).

Recently, autophagy has emerged as a crucial protective mechanism during ER stress (43, 44). An early study reported that cells undergoing ER stress, as indicated by ER expansion, showed a concomitant increase in autophagosome abundance (44). This activation of autophagy was interpreted as a survival mechanism to prevent ER stress-induced toxicity (43, 44). In mammals, ER stress inducers generally act as on-off switches for mTOR-regulated cell growth, survival and energy balance, through the downregulation of AKT1, which induces the activation of autophagy by decreasing mTOR activity (45). Autophagy is a degradation mechanism that can be induced by starvation or other form of nutrient deprivation to supply substrates for cellular energy generation (46). Autophagy also serves as a catabolic pathway to recycle excessive or damaged

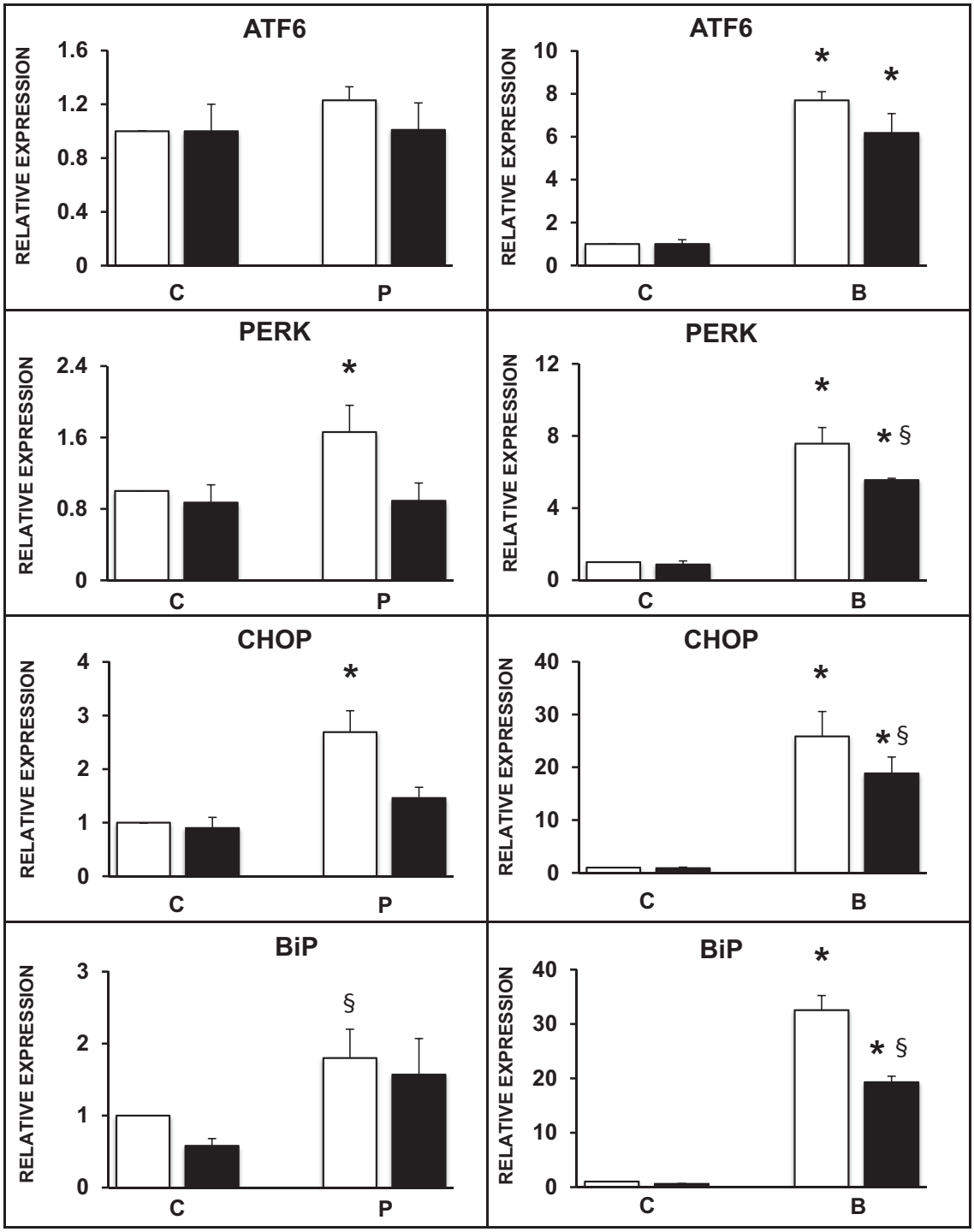

FIGURE 3 | Expression of several ER stress markers in isolated human islets after 5 days exposure to palmitate (left panels) or 1 day exposure to brefeldin (right panels) in the presence (black bars) or absence (white bars) of 10 ng/ml rapamycin. $N = 4–5$ for both treatments. C, control; P = 0.5 mmol/l palmitate; B = 0.1 ng/ml brefeldin; Statistical analysis: $^{*}p < 0.05$ vs. all the other groups; $^{\S}p < 0.05$ vs. C, after Bonferroni correction.

intracellular organelles such as mitochondria (47). Therefore, it can act as a housekeeping mechanism in the absence of stress, while under stress conditions it exerts a crucial protective role (48).

The aim of this study was to explore the effects of the modulation of autophagy on the ER stress-induced beta-cell dysfunction in isolated human pancreatic islets. We induced ER stress in cultured human islets by their

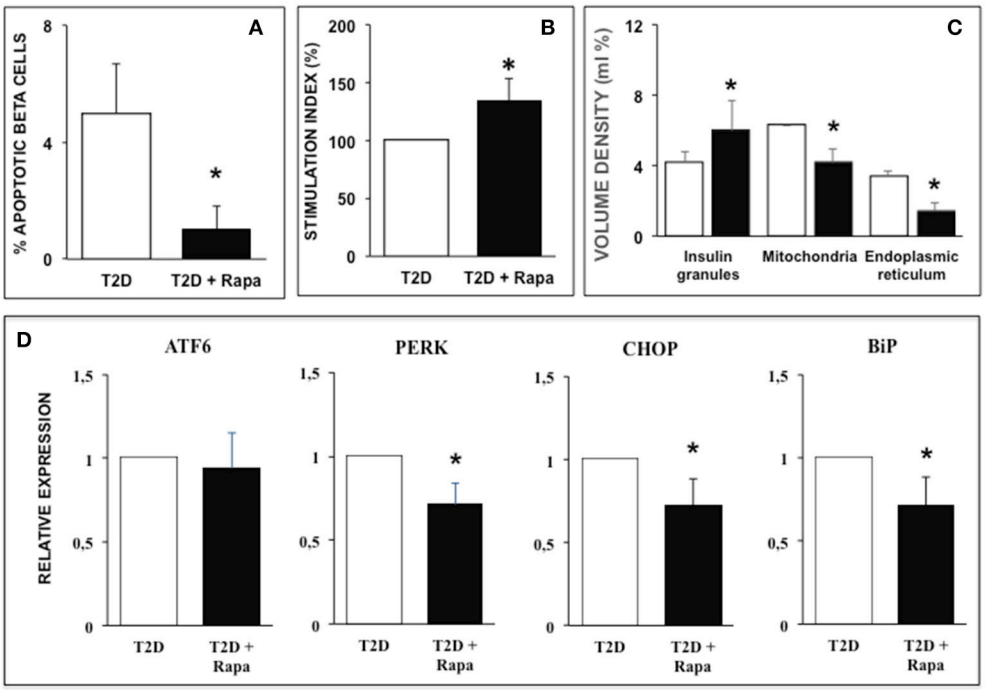

FIGURE 4 | (A) Ultrastructural morphometric analysis of beta-cell death in T2D human islets in the presence (black bar) or absence (white bar) of 10 ng/ml rapamycin. $N = 9$ (300–400 cells, two islet preparations analyzed). Statistical analysis: $^*p < 0.05$ vs. T2D. **(B)** Glucose-stimulated insulin secretion of type 2 diabetes human islets in the presence (black bar) or absence (white bar) of 10 ng/ml rapamycin. $N = 5$. Statistical analysis: $^*p < 0.05$ vs. T2D. **(C)** Quantitative morphometric analysis of volume density of insulin granules, mitochondria, and endoplasmic reticulum in beta cells of type 2 diabetes human islets in the presence (black bar) or absence (white bar) of 10 ng/ml rapamycin. $N = 9$ (300–400 cells, two islet preparations analyzed). Statistical analysis: $^*p < 0.05$ vs. T2D. **(D)** Expression of several ER stress markers in type 2 diabetes human islets in the presence (black bars) or absence (white bar) of 10 ng/ml rapamycin. $N = 5$. Statistical analysis: $^*p < 0.05$ vs. T2D.

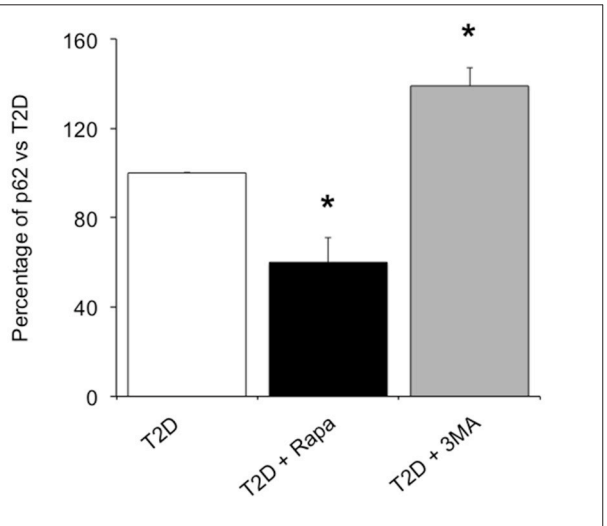

FIGURE 5 | Levels of p62 in T2D human islets in presence of 10 ng/ml of the autophagy inducer, rapamycin, or 5.0 mmol/l of the autophagy inhibitor, 3-MA. $N = 3$–4. Statistical analysis: $^*p < 0.05$ vs. all groups.

exposure to a metabolic (0.5 mmol/l palmitic acid for 5 days) or a chemical (0.1 ng/ml brefeldin for 1 day) stressor. The increased levels of fatty acids, commonly associated with obesity, can induce insulin resistance and beta-cell dysfunction, making them good candidates to explain the link between obesity and the development of T2D (49–51). It has been proposed that palmitate-induced ER stress may arise from the disruption of protein processing and trafficking (49), or from incorrect Ca^{++} regulation (52). Brefeldin A is a macrocyclic lactone antibiotic which is synthetized from palmitate by several fungi and has been shown to induce ER stress through the inhibition of ADP ribosylation factor (ARF) and the consequent disruption of the ER-Golgi vesicular transport (53, 54).

Here, we confirm that lipotoxic conditions (such as palmitate exposure) and chemically-induced (brefeldin) ER stress are associated with alterations of beta cell survival and function, and show that the modulation of autophagy influences these effects. In particular, rapamycin, an inducer of autophagy through inhibition of mTORC1 complex (25), was able to prevent beta cell apoptosis induced by palmitate or brefeldin, and restore a proper insulin secretion in response to glucose which was altered by the presence of the fatty acid. These data are in line with those previously published on the effect of rapamycin in *Akita* mice (55), where the drug attenuated cellular stress and apoptosis that, conversely, were exacerbated by autophagy inhibitors in conditions of accumulation of

misfolded proinsulin. Conversely, 3-MA and concanamycin-A were used as autophagy inhibitors. The first blocks an early stage of autophagy by inhibiting the class III PI3K (21), while concanamycin causes an increase in lysosomal/vacuolar pH, and, ultimately, blocks fusion of autophagosomes with the vacuole by inhibiting V-ATPase (25). In our study, the presence of 3-MA caused a significant increase of beta-cell apoptosis in human islets exposed to palmitate, whereas concanamycin A did not show any effect on the cytotoxicity induced by palmitate or brefeldin. Our data are in agreement with previously published results in other cell types (56), where it was observed that inhibition of autophagy at an early stage, but not at a late stage, potentiated chemosensitivity, increasing caspase 3/7 activation, especially in conditions of high levels of autophagy. More in detail, 3-MA could exert this pro-apoptotic action making available beclin-1 (a master component of the PI3K-III complex inhibited by the drug, playing a role also in apoptosis) for caspase 8 cleavage in order to elicit cell death (57–60). However, other studies exist reporting a more deleterious effect of late autophagy blockade on cell survival (61).

At the ultrastructural level, we found that in both palmitate and brefeldin-treated human islets a significant decrease in the volume density of insulin granules, and a significant increase in volume density of mitochondria and ER was present. Co-exposure with rapamycin was able to prevent these alterations mainly in the islets treated with palmitate. These beneficial effects on ultrastructure were associated with a reduction in the expression of some ER stress-related genes. The ultrastructural alterations found in our experiments were similar to those previously observed in other studies. In particular, they look like those observed in beta cells isolated from $Atg^{\Delta\beta-cell}$ mice with beta cell-specific deletion of Atg7 (autophagy-related 7) (19) indicating that autophagy could be necessary to maintain beta-cell homeostasis (62, 63) and in human islets isolated from T2D patients (20, 23, 27) confirming a pathogenetic role played by ER stress (50, 64). However, the role of autophagy in diabetes pathophysiology has not been fully elucidated. Recently, it was suggested that impaired autophagy could lead to accumulation of dysfunctional organelles such as mitochondria (65) and that in type 2 diabetic pancreatic beta cells, a massive overload of autophagic vacuoles and autophagosomes might contribute to the loss of beta-cell mass (20). In addition, some authors reported that rapamycin improved insulin resistance and hepatic steatosis in T2D rats via activation of autophagy (66). Our results in T2D islets showed that the promotion of autophagic process by rapamycin, as evaluated by the clearance of the p62 protein, is associated with amelioration of function, survival and ultrastructure possibly due to reduction of ER stress. Recently, it was hypothesized that mTORC1 (one of the major signaling complex in beta cells, where it is responsible for nutrient sensing and beta cell growth) (67), if short term and transiently activated, regulates beta cell replication, anabolic growth and insulin secretion under physiological conditions (67). Conversely, its sustained activation (as in presence of chronic excess of nutrients) is associated with impaired insulin release, ER stress and reduced beta cell survival. In this regards, several authors showed that chronic exposure to high glucose and/or high free fatty acid concentrations could activate mTORC1 in beta cells and that its inhibition could prevent gluco- lipotoxic-induced beta cell derangement (68). In particular, Yuan and colleagues (69) showed that islets isolated from T2D organ donors had increased mTORC1 activity in comparison with non-diabetic islets and that this augmentation was present in beta but not alpha cells. Moreover, mTORC1 genetic or chemical inhibition was associated with restoration of insulin release in T2D islets. Differently from them, in our study, 10 ng/ml rapamycin (corresponding to around 11 nmol/l) were able to promote beta cell function, survival and ultrastructure in T2D islets. Similarly, it has been observed that the defective autophagic flux associated with a lysosomal dysfunction observed in T2D islets can be restored also by a GLP-1 receptor agonist (70). It should be underlined that the beneficial effects observed with rapamycin in presence of ER stress modulators, especially those related to insulin release, are exerted in a short-term setting. Additional experiments should be performed to assess the role of a chronic exposure to the drug. In this regards, some authors have observed how some pharmacological agents, currently used in diabetes treatment, show deleterious effects in beta cells when chronically administered in presence of rapamycin (71).

In conclusion, this study provides information on how conditions of metabolically or chemically induced stress on human beta cells associate with reduced beta cell survival, impaired beta cell function, and ultrastructural alterations, which are also mediated by ER stress. More importantly, our data suggest that promotion of autophagy at the beta cell level, in some context, might be helpful to protect beta cell health.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the University of Pisa.

AUTHOR CONTRIBUTIONS

MB, PM, and VD conceived the study and wrote the manuscript. MB, SM, FG, MS, LM, UB, PD, DE, and MC researched data. All the authors contributed to discussion and reviewed the manuscript. VD is the guarantor of this study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00052/full#supplementary-material

REFERENCES

1. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell* (2011) 147:728–41. doi: 10.1016/j.cell.2011.10.026
2. Efeyan A, Comb WC, Sabatini DM. Nutrient-sensing mechanisms and pathways. *Nature* (2015) 517:302–10. doi: 10.1038/nature 14190
3. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science* (2000) 290:1717–21. doi: 10.1126/science.290.5497.1717
4. Rocchi A, He C. Emerging roles of autophagy in metabolism and metabolic disorders. *Front Biol.* (2015) 10:154–64. doi: 10.1007/s11515-015-1354-2
5. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature* (2008) 451:1069–75. doi: 10.1038/nature06639
6. Fitzwalter BE, Thorburn A. Recent insights into cell death and autophagy. *FEBS J.* (2015) 282:4279–88. doi: 10.1111/febs.13515
7. Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A. Life and death partners: apoptosis, autophagy and the cross-talk between them. *Cell Death Differ.* (2009) 16:966–75. doi: 10.1038/cdd.2009.33
8. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell* (2008) 132:27–42. doi: 10.1016/j.cell.2007.12.018
9. Schuit FC, In't Veld PA, Pipeleers DG. Glucose stimulates proinsulin biosynthesis by a dose-dependent recruitment of pancreatic beta cells. *Proc Natl Acad Sci USA.* (1998) 85:3865–9. doi: 10.1073/pnas.85.11.3865
10. Huang CJ, Lin CY, Haataja L, Gurlo T, Butler AE, Rizza RA, et al. High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress mediated beta-cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes. *Diabetes* (2007) 56:2016–27. doi: 10.2337/db07-0197
11. Fonseca SG, Gromada J, Urano F. Endoplasmic reticulum stress and pancreatic β-cell death. *Trends Endocrinol Metab.* (2011) 22:266–74. doi: 10.1016/j.tem.2011.02.008
12. Eizirik DL, Cardozo AK, Cnop M. The role for endoplasmic reticulum stress in diabetes mellitus. *Endocr Rev.* (2008) 29:42–61. doi: 10.1210/er.2007-0015
13. Senft D, Ronai ZA. UPR, autophagy, and mitochondria crosstalk underlies the ER stress response. *Trends Biochem Sci.* (2015) 40:141–8. doi: 10.1016/j.tibs.2015.01.002
14. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science* (2011) 334:1081–6. doi: 10.1126/science.1209038
15. Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep.* (2006) 7:880–5. doi: 10.1038/sj.embor.7400779
16. Høyer-Hansen M, Jäättelä M. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. *Cell Death Differ.* (2007) 14:1576–82. doi: 10.1038/sj.cdd.4402200
17. Chen H, Chan DC. Mitochondrial dynamics–fusion, fission, movement, and mitophagy–in neurodegenerative diseases. *Hum Mol Genet.* (2009) 18:R169–76. doi: 10.1093/hmg/ddp326
18. Ebato C, Uchida T, Arakawa M, Komatsu M, Ueno T, Komiya K, et al. Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. *Cell Metab.* (2008) 8:325–32. doi: 10.1016/j.cmet.2008.08.009
19. Jung HS, Chung KW, Won Kim J, Kim J, Komatsu M, Tanaka K, et al. Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell Metab.* (2008) 8:318–24. doi: 10.1016/j.cmet.2008.08.013
20. Masini M, Bugliani M, Lupi R, del Guerra S, Boggi U, Filipponi F, et al. Autophagy in human type 2 diabetes pancreatic beta cells. *Diabetologia* (2009) 52:1083–6. doi: 10.1007/s00125-009-1347-2
21. Arunagiri A, Haataja L, Cunningham CN, Shrestha N, Tsai B, Qi L, et al. Misfolded proinsulin in the endoplasmic reticulum during development of beta cell failure in diabetes. *Ann N Y Acad Sci.* (2018) 1418:5–19. doi: 10.1111/nyas.13531
22. Preston AM, Gurisik E, Bartley C, Laybutt DR, Biden TJ. Reduced endoplasmic reticulum (ER)-to-Golgi protein trafficking contributes to ER stress in lipotoxic mouse beta cells by promoting protein overload. *Diabetologia* (2009) 52:2369–73. doi: 10.1007/s00125-009-1506-5
23. Marchetti P, Bugliani M, Lupi R, Marselli L, Masini M, Boggi U, et al. The endoplasmic reticulum in pancreatic beta cells of type 2 diabetes patients. *Diabetologia* (2007) 50:2486–94. doi: 10.1007/s00125-007-0816-8
24. Martino L, Masini M, Novelli M, Beffy P, Bugliani M, Marselli L, et al. Palmitate activates autophagy in INS-1E β-cells and in isolated rat and human pancreatic islets. *PLoS ONE* (2012) 7:e36188. doi: 10.1371/journal.pone.0036188
25. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* (2016) 12:1–222. doi: 10.1080/15548627.2015.1100356
26. Ciregia F, Bugliani M, Ronci M, Giusti L, Boldrini C, Mazzoni MR, et al. Palmitate-induced lipotoxicity alters acetylation of multiple proteins in clonal β cells and human pancreatic islets. *Sci Rep.* (2017) 7:13445. doi: 10.1038/s41598-017-13908-w
27. Masini M, Marselli L, Bugliani M, Martino L, Masiello P, Marchetti P, et al. Ultrastructural morphometric analysis of insulin secretory granules in human type 2 diabetes. *Acta Diabetol.* (2012) 49(Suppl. 1):S247–52. doi: 10.1007/s00592-012-0446-6
28. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ.* (2012) 19:107–20. doi: 10.1038/cdd.2011.96
29. Masini M, Anello M, Bugliani M, Marselli L, Filipponi F, Boggi U, et al. Prevention by metformin of alterations induced by chronic exposure to high glucose in human islet beta cells is associated with preserved ATP/ADP ratio. *Diabetes Res Clin Pract.* (2014) 104:163–70. doi: 10.1016/j.diabres.2013.12.031
30. Bugliani M, Syed F, Paula FMM, Omar BA, Suleiman M, Mossuto S, et al. DPP-4 is expressed in human pancreatic beta cells and its direct inhibition improves beta cell function and survival in type 2 diabetes. *Mol Cell Endocrinol.* (2018) 73:186–93. doi: 10.1016/j.mce.2018.01.019
31. Marchetti P, Del Guerra S, Marselli L, Lupi R, Masini M, Pollera M, et al. Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin. *J Clin Endocrinol Metab.* (2004) 89:5535–41. doi: 10.1210/jc.2004-0150
32. Cnop M, Abdulkarim B, Bottu G, Cunha DA, Igoillo-Esteve M, Masini M, et al. RNA sequencing identifies dysregulation of the human pancreatic islet transcriptome by the saturated fatty acid palmitate. *Diabetes* (2014) 63:1978–93. doi: 10.2337/db13-1383
33. Ladrière L, Igoillo-Esteve M, Cunha DA, Brion JP, Bugliani M, Marchetti P, et al. Enhanced signaling downstream of ribonucleic Acid-activated protein kinase-like endoplasmic reticulum kinase potentiates lipotoxic endoplasmic reticulum stress in human islets. *J Clin Endocrinol Metab.* (2010) 95:1442–9. doi: 10.1210/jc.2009-2322
34. Masini M, Martino L, Marselli L, Bugliani M, Boggi U, Filipponi F, et al. Ultrastructural alterations of pancreatic beta cells in human diabetes mellitus. *Diabetes Metab Res Rev.* (2017) 33:e2894. doi: 10.1002/dmrr.2894
35. Cunha DA, Igoillo-Esteve M, Gurzov EN, Germano CM, Naamane N, Marhfour I, et al. Death protein 5 and p53-upregulated modulator of apoptosis mediate the endoplasmic reticulum stress-mitochondrial dialog triggering lipotoxic rodent and human β-cell apoptosis. *Diabetes* (2012) 61:2763–75. doi: 10.2337/db12-0123
36. Dandekar A, Mendez R, Zhang K. Cross talk between ER stress, oxidative stress, and inflammation in health and disease. *Methods Mol Biol.* (2015) 1292:205–14. doi: 10.1007/978-1-4939-2522-3_15
37. Cnop M, Welsh N, Jonas JC, Jörns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes* (2005) 54 (Suppl. 2):S97–107. doi: 10.2337/diabetes.54.suppl_2.S97
38. Oyadomari S, Araki E, Mori M. Endoplasmic reticulum stress-mediated apoptosis in pancreatic beta-cells. *Apoptosis* (2002) 7:335–45. doi: 10.1023/A:1016175429877
39. Cardozo AK, Ortis F, Storling J, Feng YM, Rasschaert J, Tonnesen M, et al. Cytokines downregulate the sarcoendoplasmic reticulum pump Ca^{2+} ATPase 2b and deplete endoplasmic reticulum Ca^{2+}, leading to induction of endoplasmic reticulum stress in pancreatic beta-cells. *Diabetes* (2005) 54:452–61. doi: 10.2337/diabetes.54.2.452

40. Kharroubi I, Ladriere L, Cardozo AK, Dogusan Z, Cnop M, Eizirick DL. Free fatty acids and cytokines induce pancreatic β-cell apoptosis by different mechanisms: role of the nuclear factor-kB and endoplasmic reticulum stress. *Endocrinology* (2004) 145:5087–96. doi: 10.1210/en.2004-0478
41. Araki E, Oyadomari S, Mori M. Impact of endoplasmic reticulum stress pathway on pancreatic beta-cells and diabetes mellitus. *Exp Biol Med.* (2003) 228:1213–7. doi: 10.1177/153537020322801018
42. Laybutt DR, Preston AM, Akerfeldt MC, Kench JG, Busch AK, Biankin AV, et al. Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. *Diabetologia* (2007) 50:752–63. doi: 10.1007/s00125-006-0590-z
43. Rashid HO, Yadav RK, Kim HR, Chae HJ. ER stress: autophagy induction, inhibition and selection. *Autophagy* (2015) 11:1956–77. doi: 10.1080/15548627.2015.1091141
44. Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol Cell Biol.* (2006) 26:9220–31. doi: 10.1128/MCB.01453-06
45. Qin L, Wang Z, Tao L, Wang Y. ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. *Autophagy* (2010) 6:239–47. doi: 10.4161/auto.6.2.11062
46. Kadowaki M, Karim MR, Carpi A, Miotto G. Nutrient control of macroautophagy in mammalian cells. *Mol Aspects Med.* (2006) 27:426–43. doi: 10.1016/j.mam.2006.08.010
47. Mizushima N, Hara T. Intracellular quality control by autophagy: how does autophagy prevent neurodegeneration? *Autophagy* (2006) 2:302–4. doi: 10.4161/auto.2945
48. Kaushik S, Singh R, Cuervo AM. Autophagic pathways and metabolic stress. *Diabetes Obes Metab.* (2010) 12 (Suppl. 2):4–14. doi: 10.1111/j.1463-1326.2010.01263.x
49. Ly LD, Xu S, Choi SK, Ha CM, Thoudam T, Cha SK, et al. Oxidative stress and calcium dysregulation by palmitate in type 2 diabetes. *Exp Mol Med.* (2017) 49:e291. doi: 10.1038/emm.2016.157
50. Biden TJ, Boslem E, Chu KY, Sue N. Lipotoxic endoplasmic reticulum stress, β cell failure, and type 2 diabetes mellitus. *Trends Endocrinol Metab.* (2014) 25:389–98. doi: 10.1016/j.tem.2014.02.003
51. Maris M, Robert S, Waelkens E, Derua R, Hernangomez MH, D'Hertog W, et al. Role of the saturated nonesterified fatty acid palmitate in beta cell dysfunction. *J Proteome Res.* (2013) 12:347–62. doi: 10.1021/pr300596g
52. Hara T, Mahadevan J, Kanekura K, Hara M, Lu S, Urano F. Calcium efflux from the endoplasmic reticulum leads to β-cell death. *Endocrinology* (2014) 155:758–68. doi: 10.1210/en.2013-1519
53. Klausner RD, Donaldson JG, Lippincott-Schwartz J. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J Cell Biol.* (1992) 116:1071–80. doi: 10.1083/jcb.116.5.1071
54. Choi SE, Lee SM, Lee YJ, Li LJ, Lee SJ, Lee JH, et al. Protective role of autophagy in palmitate-induced INS-1 beta-cell death. *Endocrinology* (2008) 150:126–34. doi: 10.1210/en.2008-0483
55. Li C, Liu Y, Liu H, Zhang W, Shen C, Cho K, et al. Impact of autophagy inhibition at different stages on cytotoxic effect of autophagy inducer in glioblastoma cells. *Cell Physiol Biochem.* (2015) 35:1303–16. doi: 10.1159/000373952
56. Bachar-Wikstrom E, Wikstrom JD, Ariav Y, Tirosh B, Kaiser N, Cerasi E, et al. Stimulation of autophagy improves endoplasmic reticulum stress-induced diabetes *Diabetes* (2013) 62:1227–37. doi: 10.2337/db12-1474
57. Follo C, Cheng Y, Richards WG, Bueno R, Broaddus VC. Inhibition of autophagy initiation potentiates chemosensitivity in mesothelioma. *Mol Carcinog.* (2018) 57:319–32. doi: 10.1002/mc.22757
58. McKnight NC, Zhenyu Y. Beclin 1, an essential component and master regulator of PI3K-III in health and disease. *Curr Pathobiol Rep.* (2013) 1:231–8. doi: 10.1007/s40139-013-0028-5
59. Li H, Wang P, Sun Q, Ding WX, Yin XM, Sobol RW, et al. Following cytochrome c release, autophagy is inhibited during chemotherapy-induced apoptosis by caspase 8-mediated cleavage of Beclin 1. *Cancer Res.* (2011) 71:3625–34. doi: 10.1158/0008-5472.CAN-10-4475
60. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ.* (2011) 18:571–80. doi: 10.1038/cdd.2010.191
61. Song S, Tan J, Miao Y, Li M, Zhang Q. Crosstalk of autophagy and apoptosis: involvement of the dual role of autophagy under ER stress. *J Cell Physiol.* (2017) 232:2977–84. doi: 10.1002/jcp.25785
62. Quan W, Lim YM, Lee MS. Role of autophagy in diabetes and endoplasmic reticulum stress of pancreatic β-cells. *Exp Mol Med.* (2012) 44:81–8. doi: 10.3858/emm.2012.44.2.030
63. Riahi Y, Wikstrom JD, Bachar-Wikstrom E, Polin N, Zucker H, Lee MS, et al. Autophagy is a major regulator of beta cell insulin homeostasis. *Diabetologia* (2016) 59:1480–91. doi: 10.1007/s00125-016-3868-9
64. Eizirik DL, Cnop M. ER stress in pancreatic beta cells: the thin red line between adaptation and failure. *Sci Signal.* (2010) 3:pe7. doi: 10.1126/scisignal.3110pe7
65. Gonzalez CD, Lee MS, Marchetti P, Pietropaolo M, Towns R, Vaccaro MI, et al. The emerging role of autophagy in the pathophysiology of diabetes mellitus. *Autophagy* (2011) 7:2–11. doi: 10.4161/auto.7.1.13044
66. Zhou W, Ye S. Rapamycin improves insulin resistance and hepatic steatosis in type 2 diabetes rats through activation of autophagy. *Cell Biol Int.* (2018) 42:1282–91. doi: 10.1002/cbin.11015
67. Dibble CC, Manning BD. Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nat Cell Biol.* (2013) 15:555–64. doi: 10.1038/ncb2763
68. Ardestani A, Lupse B, Kido Y, Leibowitz G, Maedler K. mTORC1 signaling: a double-edged sword in diabetic β cells. *Cell Metab.* (2018) 27:314–31. doi: 10.1016/j.cmet.2017.11.004
69. Yuan T, Rafizadeh S, Gorrepati KD, Lupse B, Oberholzer J, Maedler K, et al. Reciprocal regulation of mTOR complexes in pancreatic islets from humans with type 2 diabetes. *Diabetologia* (2017) 60:668–78. doi: 10.1007/s00125-016-4188-9
70. Zummo FP, Cullen KS, Honkanen-Scott M, Shaw JAM, Lovat PE, Arden C. Glucagon-like peptide 1 protects pancreatic β-cells from death by increasing autophagic flux and restoring lysosomal function. *Diabetes* (2017) 66:1272–85. doi: 10.2337/db16-1009
71. Wang Q, Heimberg H, Pipeleers D, Ling Z. Glibenclamide activates translation in rat pancreatic beta cells through calcium-dependent mTOR, PKA and MEK signalling pathways. *Diabetologia* (2008) 51:1202–12. doi: 10.1007/s00125-008-1026-8

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Palmitic Acid Reduces the Autophagic Flux and Insulin Sensitivity Through the Activation of the Free Fatty Acid Receptor 1 (FFAR1) in the Hypothalamic Neuronal Cell Line N43/5

*María Paz Hernández-Cáceres[1,2‡], Lilian Toledo-Valenzuela[1,2‡], Francisco Díaz-Castro[2,3], Yenniffer Ávalos[1,2†], Paulina Burgos[1,2], Carla Narro[1,2], Daniel Peña-Oyarzun[2,4,5], Jasson Espinoza-Caicedo[1,2], Flavia Cifuentes-Araneda[1,2], Fernanda Navarro-Aguad[6], Cecilia Riquelme[6], Rodrigo Troncoso[2,3,4], Alfredo Criollo[2,4,5] and Eugenia Morselli[1,2]**

[1] Laboratory of Autophagy and Metabolism, Department of Physiology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Santiago, Chile, [2] Autophagy Research Center, Santiago, Chile, [3] Research Laboratory of Nutrition and Physical Activity, Institute of Nutrition and Food Technology, Universidad de Chile, Santiago, Chile, [4] Advanced Center for Chronic Diseases and Center for Molecular Studies of the Cell, Universidad de Chile, Santiago, Chile, [5] Facultad de Odontología, Instituto de Investigación en Ciencias Odontológicas, Universidad de Chile, Santiago, Chile, [6] Laboratory of Differentiation and Pathology, Department of Cell and Molecular Biology, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Santiago, Chile

Chronic consumption of high fat diets (HFDs), rich in saturated fatty acids (SatFAs) like palmitic acid (PA), is associated with the development of obesity and obesity-related metabolic diseases such as type II diabetes mellitus (T2DM). Previous studies indicate that PA accumulates in the hypothalamus following consumption of HFDs; in addition, HFDs consumption inhibits autophagy and reduces insulin sensitivity. Whether malfunction of autophagy specifically in hypothalamic neurons decreases insulin sensitivity remains unknown. PA does activate the Free Fatty Acid Receptor 1 (FFAR1), also known as G protein-coupled receptor 40 (GPR40); however, whether FFAR1 mediates the effects of PA on hypothalamic autophagy and insulin sensitivity has not been shown. Here, we demonstrate that exposure to PA inhibits the autophagic flux and reduces insulin sensitivity in a cellular model of hypothalamic neurons (N43/5 cells). Furthermore, we show that inhibition of autophagy and the autophagic flux reduces insulin sensitivity in hypothalamic neuronal cells. Interestingly, the inhibition of the autophagic flux, and the reduction in insulin sensitivity are prevented by pharmacological inhibition of FFAR1. Our findings show that dysregulation of autophagy reduces insulin sensitivity in hypothalamic neuronal cells. In addition, our data suggest FFAR1 mediates the ability of PA to inhibit autophagic flux and reduce insulin sensitivity in hypothalamic neuronal cells. These results reveal a novel cellular mechanism linking PA-rich diets to decreased insulin sensitivity in the hypothalamus and suggest that hypothalamic autophagy might represent a target for future T2DM therapies.

Keywords: saturated fatty acids, G-protein coupled receptor 40, central nervous system, AKT, insulin resistance, autophagy, glucose uptake

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***Correspondence:**
Eugenia Morselli
emorselli@bio.puc.cl

†Present Address:
Yenniffer Ávalos, Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile

‡*These authors have contributed equally to this work*

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INTRODUCTION

Obesity is currently considered a global epidemic both in developed and developing countries. Excessive accumulation of body fat promotes obesity-associated metabolic dysfunctions, such as insulin resistance, type II diabetes mellitus (T2DM), cardiovascular diseases, neurodegeneration, and certain cancers (1).

Consumption of high fat diets (HFDs), and especially of diets high in saturated fatty acids (SatFAs) such as the palmitic acid (PA), increases body weight and is associated with the onset of several metabolic diseases, such as insulin resistance and T2DM (2, 3). Previous studies from our and other groups show that SatFAs accumulate in the central nervous system (CNS), specifically in the hypothalamus (4–7). Interestingly, fatty acid (FA) sensing neurons reside in the hypothalamus and play important roles in feeding behavior, as well as lipid and glucose metabolism. Due to their critical role in the regulation of energy homeostasis, these neuronal populations have been implicated in development of obesity and T2DM (8, 9).

Macroautophagy, hereafter referred to as "autophagy," is a conserved adaptive mechanism that maintains the balance between synthesis, degradation, and recycling of cellular components (10). Autophagy supports cellular homeostasis and studies in multiple cell types have demonstrated autophagic dysregulation following chronic consumption of HFDs (11). Previous studies from our and other groups have shown that hypothalamic autophagic flux is defective in rodents chronically fed with HFDs (12, 13). Consistently, previous work demonstrates that the inhibition of autophagy in proopiomelanocortin (POMC) neurons leads to metabolic disorders, including obesity and insulin resistance (14–16); however the mechanism by which this occurs is still largely unknown.

Evidence exists of crosstalk between autophagy and insulin signaling pathways. For example, hyperinsulinemia reduces the autophagic response in different peripheral tissues and cell types (17–20), whereas inhibition of autophagy (by autophagy related gene 7 -ATG7- tissue-specific knockout) promotes insulin resistance in liver tissue (21) and reduces insulin secretion in pancreatic β-cells (22, 23). However, whether inhibition of autophagy directly reduces neuronal insulin sensitivity is currently unknown.

The Free Fatty Acid Receptor 1 (FFAR1), also known as G protein-coupled receptor 40 (GPR40), is a seven-transmembrane domain receptor activated by medium and long-chain fatty acids, including PA (24, 25). Previous studies in GPR40$^{-/-}$ mice show that this receptor plays a significant role in the chain of events linking obesity and metabolic disorders, as GPR40$^{-/-}$ mice are protected from obesity-induced hyperinsulinemia, hyperglycemia, and glucose intolerance (26). Moreover, FFAR1 is required to mediate the insulin response to FA in the pancreas (27–29). However, its function in the hypothalamus, specifically in the context of autophagy and insulin sensitivity, is not known.

Here, we demonstrate that exposure to the SatFA PA inhibits the autophagic flux and reduces insulin sensitivity in N43/5 cells, a hypothalamic neuronal cell line. Furthermore, our findings show that autophagy malfunction promotes insulin resistance in hypothalamic neuronal cells. In addition, our data suggest FFAR1 may link PA with reduced insulin sensitivity and impaired autophagic flux in hypothalamic neuronal cells. Altogether, these results suggest activation of autophagy should be considered as treatment for insulin resistance.

MATERIALS AND METHODS

Cell Culture and Treatments

N43/5 cells (Cellutions Biosystems) were cultured in Dulbecco's modified eagle medium (DMEM) high glucose (11995-040, Gibco, USA) supplemented with 10% of fetal bovine serum (FBS) (10437028, Gibco), 100 U/ml penicillin streptomycin (15140122, Gibco) and maintained at 37 °C with 5% CO2. To evaluate the changes in the autophagic flux in response to PA exposure, cells were incubated with DMEM high glucose supplemented with 2% of FBS 24 h before treatments and then exposed to 100 μM PA (P0500, Sigma-Aldrich, St. Louis, MO, USA) conjugated to fatty acid-free bovine serum albumin (BSA) (152401, MP Biomedicals, Santa Ana, CA, USA). BSA treatment was used as control. To assess the autophagic flux and insulin response, cells were incubated at the time points indicated in each experiment with the autophagic flux inhibitor Bafilomycin A1 (BafA1, 100 nM) (B1793, Sigma-Aldrich) or with its vehicle DMSO (BM-0660, Winkler). To determine the effect of 6-h treatment with PA or BafA1 on insulin signaling, cells were serum starved in medium DMEM/F-12 (11330-32, Gibco) overnight prior to treatments. Then, cells were co-treated with insulin (1 nM) (I0516, Sigma-Aldrich) or phosphate-buffered saline (PBS). To evaluate the involvement of FFAR1 in PA-mediated inhibition of the autophagic flux or reduction in insulin signaling, cells were pre-incubated with the FFAR1 antagonist GW1100 (1 μM; CAS 306974-70-9, Calbiochem, San Diego, CA, USA) for 20 min or with its vehicle (DMSO), followed by co-incubation with PA or BSA at the indicated time point, depending on the readouts.

Animals

Animal care and procedures were approved by the Ethical Committee of the Pontificia Universidad Católica de Chile. Male C57BL/6 mice were housed in a temperature-controlled environment in groups of two to four at 22–24°C using a 12 h light/dark cycle. Mice were fed a standard chow (Prolab® RMH3000).

Animals Gavage

Mice (n = 3–5/group) received an intragastric gavage of nutritionally complete diet as described by Benoit et al. (30). Briefly, the PA diet contains 20 g fat/100 g diet (19 g of ethyl palmitate dissolved in medium-chain triglyceride and 1 g of soybean oil to provide essential fatty acids), while the control diet contains 3 g ethyl palmitate and 1 g soybean oil/100 g diet. This liquid diet was injected into the stomach of the mice using a gavage-feeding needle. Mice were given 3 equally sized feedings daily at 7:00 AM, 12:00 PM, and 5:00 PM for 3 days. At the end of day 3, mice were sacrificed 3 h after the final gavage. Brains were removed, hypothalami dissected and stored in RNAlater

(Ambion, Life Technologies) at 4 °C. 24 h later, tissues were homogenized in 1 ml of TRIzol (Ambion, Life Technologies) and RNA was extracted using the RNeasy Kit (Qiagen Sciences, Inc., Germantown, MD, USA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions and cDNA was used for RT-PCR assays.

siRNA Transfections

Cells were cultured in six-well plates and transfected at 50% confluence with siRNAs targeting murine Beclin 1 (BECN1) (SASI_Mm01_00048143, Sigma-Aldrich) or murine autophagy related gene 7 (ATG7) (SASI_Mm01_00044616, Sigma-Aldrich). Transfection was performed using Lipofectamine RNAiMAX® Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. As negative control, cells were incubated with Lipofectamine RNAiMAX® Transfection Reagent only. 48 h after siRNA transfection, cells were treated as indicated or directly lysed for protein or RNA extraction.

Western Blot Analysis

Cells were lysed in RIPA buffer and 30–40 μg of denatured proteins from each sample were resolved in 8–12% SDS-PAGE. Gels were transferred to nitrocellulose membranes and incubated with 5% BSA (BM-0150, Winkler, RM, Chile)-tris-buffered saline-0.1% Tween-20 (TBS-T) to block nonspecific binding. Membranes were incubated with the primary antibodies anti LC3A/B (4108, Cell Signaling Technology, Danvers, MA, USA), SQSTM1 (H00008878-M01, Abnova, Jhouzih St., Taipei, Taiwan), p-Insulin Receptor β (Tyr1150/1151) (3024, Cell Signaling Technology), IR (ab131238, Abcam), p-AKT (Ser473) (9271, Cell Signaling Technology), AKT (9272, Cell Signaling Technology), BECN1 (H-300; sc-11427, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), at dilution of 1:1,000 in 5% BSA-TBS-T overnight on a rocking platform at 4 °C. Then, membranes were washed 3 times for 10 min in TBS-T and revealed with the appropriate horseradish peroxidase-labeled secondary antibodies (Goat Anti-Mouse IgG (H + L)-HRP Conjugate, 1706516; Goat Anti-Rabbit IgG (H + L)-HRP Conjugate, 1706515; Bio-Rad, CA, USA) and the chemiluminescent substrate. GAPDH (1:1000; sc-365062, Santa Cruz Biotechnology, Inc.) and β-actin (1:10,000; A1978, Sigma-Aldrich) were used as loading control. To evaluate insulin signaling the same samples were run on parallel gels, one for p-AKT and the other for AKT. Analysis of data was performed by comparing p-AKT vs. β-actin and AKT vs. β-actin. The obtained ratios were analyzed.

Immunofluorescence and Fluorescence Microscopy

For fluorescence microscopy determinations in N43/5 cells, cells cultured on coverslips were fixed with cold methanol (−20 °C) for 10 min. Cells were blocked in 3% BSA in PBS for 1 h and then incubated with the following primary antibodies overnight at 4 °C. The primary antibodies used are LC3A/B (1:250; Cell Signaling Technology), p62/SQSTM1 (1:300; Abnova). Primary antibodies staining was followed by conjugation with its respective secondary antibody (1:300; Alexa Fluor®, Life Technologies) for 1 h at room temperature. Nuclei were counterstained with Hoechst 33342 (10 mg/ml) (Molecular Probes, Eugene, OR, USA) or with ProLong® Gold Antifade Mountant (P36931, Molecular Probes, Eugene, OR, USA). Images were taken in an inverted fluorescence microscopy (Nikon Eclipse Ti, Tokio, Japon).

For fluorescence microscopy determinations on brain sections, mice were anesthetized and perfused with 10% formalin. Brains were dissected and post-fixed in 10% formalin for 24 h followed by treatment with 30% sucrose in PBS. Brain sections were cut at 30 μm using a Thermo Scientific HM 450 sliding microtome (Thermo Scientific). The sections were permeabilized in 0.01% Triton and blocked in 3% bovine BSA (BM-0150 Winkler) for 1 h. Brain sections were incubated overnight at 4 °C with the primary antibody against GPR40 (Y17; sc-28416, Santa Cruz Biotechnology, Inc.) in combination with either a marker of neurons (NeuN; MAB377, Millipore, Burlington, MA, USA), astrocytes (GFAP; G9269, Sigma-Aldrich) or microglia (IBA-1; 019-19741, Wako, USA), followed by conjugation with the respective secondary antibodies (Alexa Fluor, Life Technologies) for 1 h at room temperature. Sections were placed on gelatinized slides, mounted with VECTASHIELD anti-fading medium with DAPI (Vector Laboratories), and coverslipped. Pictures containing the arcuate nucleus (ARC) region of the hypothalamus were taken in a confocal fluorescence microscopy (Carl Zeiss, LSM700, Oberkochen, Germany).

Real Time PCR (RT-PCR)

For analysis of gene expression, mice were anesthetized and decapitated. Tissues were stored in RNA*later* (Ambion, Life Technologies) at 4° C, and 24 h later, hypothalami dissected and then homogenized in 1 ml of TRIzol (Ambion, Life Technologies). RNA was extracted using the RNeasy Kit (Qiagen Sciences, Inc., Germantown, MD, USA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions.

For analysis of *Atg7* gene expression in N43/5 cells, RNA was extracted using the E.Z.N.A.® Total RNA Kit (OMEGA Bio-Tek, Norcross, GA, USA) according to the manufacture's indications. cDNA was synthesized using iScriptTM cDNA kit (Bio-Rad) from 1 μg of total RNA.

Quantitative PCR reactions were carried out on a Step One System Real Time PCR (Applied Biosystems) using Fast SYBR Green Master Mix (4385370, Life Technologies). Specific forward and reverse primers sequences used to evaluate gene expression are indicated in **Supplemental Table 1**. *Hprt1* was used as housekeeping gene. The $\Delta\Delta C_T$ method was used for relative quantification analysis.

Intracellular Calcium Measurement

Intracellular calcium (Ca^{2+}) was determined using a spectrofluorometric technique described by Darling R.A. and collaborators (31). N43/5 cells were loaded with 1.5 μM Fura 2-AM (Molecular Probes) for 45 min at 37 °C. Cells were

excited at a wavelength of 340 and 380 nm in a Nikon Diaphot microscope equipped with a Photon Technology International spectrofluorometer (Lawrenceville, NJ). To confirm the presence of a functional FFAR1 in our cellular model, cells were pre-incubated with its antagonist GW1100 (1 μM) for 20 min. After 10 min of stabilization, the intensity ratio was continuously recorded and cultures were co-stimulated for 210 seconds with 100 μM PA or BSA. Fluorescence emission intensity was detected with a photomultiplier and analyzed using a fluorescence analysis program (FELIX version 1.1).

2-NBDG Uptake

To assess the insulin-dependent glucose uptake, N43/5 cells were stimulated with insulin 1 nM for 30 min and incubated with the fluorescent analog of glucose (2-NBDG, 300 mM) for 15 min at 37 °C as previously described (32). To evaluate the involvement of FFAR1 in PA-mediated inhibition of insulin-dependent glucose uptake, cells were pre-incubated with the FFAR1 antagonist GW1100. Cells were transferred to an inverted Nikon Ti Eclipse microscope equipped with 40X oil objective [numerical aperture, N.A. 1.3]. A Xenon lamp was coupled to the monochromator device (Cairn Research Ltd, Faversham, UK). Digital images were acquired by means of a cooled CCD camera (Hamamatsu ORCA 03, Japan). Images were quantified by ImageJ software (NIH, Bethesda, MD).

Results and Statistical Analysis

Results are shown as mean ± SEM from at least 3 independent experiments. Two groups were compared using two-tailed Student's *t* tests. For more than two groups, one- or two-way ANOVA was used, as appropriate, followed by *post hoc* adjustment. All analyses were performed with GraphPad software (San Diego, CA, USA). *P* value of < 0.05 was considered statistically significant.

RESULTS

Palmitic Acid Inhibits the Autophagic Flux in N43/5 Cells

We and others have previously shown that autophagy is dysregulated *in vivo* in the hypothalamus of male mice chronically fed with HFDs (12, 13, 33). Interestingly, we also demonstrated that in the brain of mice fed with the same diet for 16 weeks, SatFAs are significantly increased (4, 6, 7). Among those PA represents the most abundant SatFA, thus, we decided to evaluate whether its increase affects basal autophagy in hypothalamic neurons. We exposed N43/5 cells, a model of POMC neurons (34), to 100 μM PA, a concentration of PA similar to the one identified in the brain of obese mice chronically exposed to HFDs (7). As indicated by western blot, the levels of the autophagy receptor Sequestosome 1/p62 (SQSTM1), which is degraded during the autophagic process, increased compared to control (BSA vs. PA: $^{**}p < 0.01$; **Figures 1A,D**), suggesting PA inhibited autophagic flux. This result was supported by measuring levels of microtubule-associated protein light chain 3 II (LC3II) in presence and absence of Bafilomycin A1 (BafA1), a classic autophagic flux inhibitor which prevents fusion of autophagosomes and lysosomes (**Figures 1A–C**). PA exposure increased the levels of LC3II; however, addition of BafA1 had not effect (BSA vs. PA: $^{**}p < 0.01$; BafA1 vs. BafA1 + PA: non-significant; **Figures 1A–C**). Consistent with this, immunofluorescence studies in N43/5 cells stained against LC3 showed that treatment with PA stimulated the formation of autophagic puncta *per se* already 1 h following PA exposure (**Figures 1E,F** and **Supplemental Figures 1A,B**). However, exposure to BafA1 did not further enhance the PA-triggered induction of LC3 puncta (**Figures 1E,F** and **Supplemental Figures 1A,B**). Finally, in addition to LC3 puncta, PA treatment also increased the number of SQSTM1 puncta, already 1 h following PA treatment (**Figures 1E,G** and **Supplemental Figures 1A,C**). Importantly, the levels of *Atg5*, *Atg7, Atg16, Beclin 1, Fip200, and Gabarap* are not increased by PA exposure, and *Lc3* and *Sqstm1* levels, even if affected, are significantly increased only 6 and 4 h, respectively, following PA exposure (**Supplemental Figures 1D–K**), thus suggesting PA treatment specifically affects the autophagic flux.

Importantly, as PA is not the only SatFA increased in the brain of male mice chronically fed with HFDs, to determine if the inhibition of the autophagic flux is specific to PA or not, we evaluated the effect of stearic acid (SA) on autophagy on N43/5 cells. The results we obtained show also SA inhibits the autophagic flux (**Supplemental Figure 2**). Indeed, as indicated by western blot, the levels of SQSTM1 are significantly increased when compared to BSA (BSA vs. SA: $^{*}p < 0.05$; **Supplemental Figures 2C,D**). Consistent with this, the levels of LC3II, which are increased by SA *per se*, were not affected by BafA1 (BSA vs. SA: $^{**}p < 0.01$, BafA1 + BSA vs. BafA1 + SA: non-significant; **Supplemental Figures 2A,B**). Accordingly, SA stimulated the formation of autophagic puncta, as assessed by immunofluorescence (**Supplemental Figures 2E,F**) and exposure to BafA1 did not further enhance the SA-triggered induction of LC3 puncta (**Supplemental Figures 2E,F**). Finally, in addition to LC3 puncta, SA treatment also increased the number of SQSTM1 puncta (**Supplemental Figures 2E,G**). Altogether, these data indicate that SatFAs inhibit the basal rate of autophagy in N43/5 hypothalamic neurons.

Palmitic Acid Reduces Insulin Sensitivity in N43/5 Cells

Consumption of HFDs causes hypothalamic insulin resistance in rodents (35). Importantly, insulin activates neurons located in the arcuate nucleus of the hypothalamus (such as POMC neurons), thereby reducing food intake. In conditions of chronic caloric excess (as occurs in HFDs feeding), insulin resistance develops and contributes to pathological weight gain (36). Moreover, consumption of HFDs increases PA levels in the hypothalamus (4, 5), and intracerebroventricular injection of PA blunts hypothalamic insulin signaling (30). Here, we evaluated the sensitivity of our cellular model to insulin, and whether PA exposure could reduce insulin sensitivity in the same cell type. Following exposure to insulin, levels of AKT phosphorylation (p-AKT) increased (**Figures 2A,B**), showing that N43/5 hypothalamic neuronal cells are, indeed, sensitive

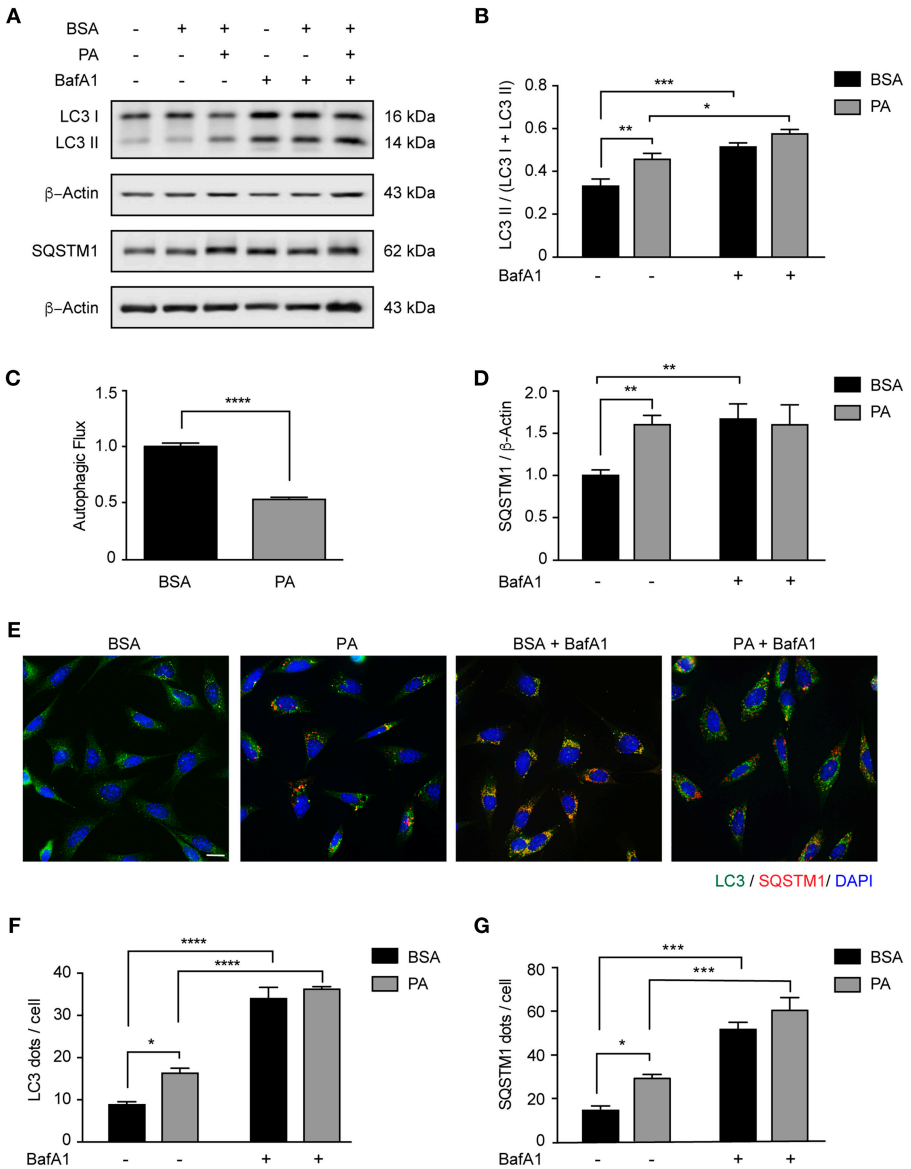

FIGURE 1 | Palmitic acid inhibits the autophagic flux in N43/5 cells. **(A)** Representative western blot of LC3 and SQSTM1 levels in N43/5 cell lysates, incubated with vehicle (BSA) or PA (100 μM) for 6 h, in presence or absence of BafA1 (100 nM), with their respective quantifications **(B,D)**. **(C)** Autophagic flux calculated as the difference in LC3II levels in presence and absence of BafA1 (100 nM). **(E)** Representative images of N43/5 cells treated with BSA or PA (100 μM) for 6 h in presence or absence of BafA1 (100 nM) and stained against LC3 (green) and SQSTM1 (red). Nuclei are stained with DAPI (blue). Quantification of LC3 **(F)** and SQSTM1 **(G)** dots per cell. Size bar: 10 μm. Data are presented as mean ± SEM. Two ways ANOVA followed by Sidak's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. $n = 3$.

to insulin. Critically, PA treatment reduced phosphorylation levels (BSA + Ins vs. PA + Ins: $^{**}p < 0.01$; **Figures 2A,B**) and consistently, PA exposure inhibited insulin-induced glucose uptake (BSA + Ins vs. PA + Ins: $^{**}p < 0.01$; **Figures 2C,D**). Importantly, these effects seem not be specific of PA, as also SA exposure reduces insulin sensitivity, as indicated by p-AKT levels in presence and absence of insulin (BSA + Ins vs. SA + Ins: $^{****}p < 0.0001$; **Supplemental Figure 3**). Altogether these results indicate SatFAs reduce insulin sensitivity in N43/5 cells.

Inhibition of Autophagy Reduces Insulin Sensitivity in N43/5 Cells

Our data indicate PA inhibits the autophagic flux and reduces insulin sensitivity. In the next series of experiments, we determined if this response is specific to PA or if other compounds that inhibit the autophagic flux also affect the insulin response. First, we exposed N43/5 cells to BafA1 or vehicle for 6 h and added insulin during the last 3 or 15 min. While insulin treatment increased p-Insulin Receptor (IR) and p-AKT

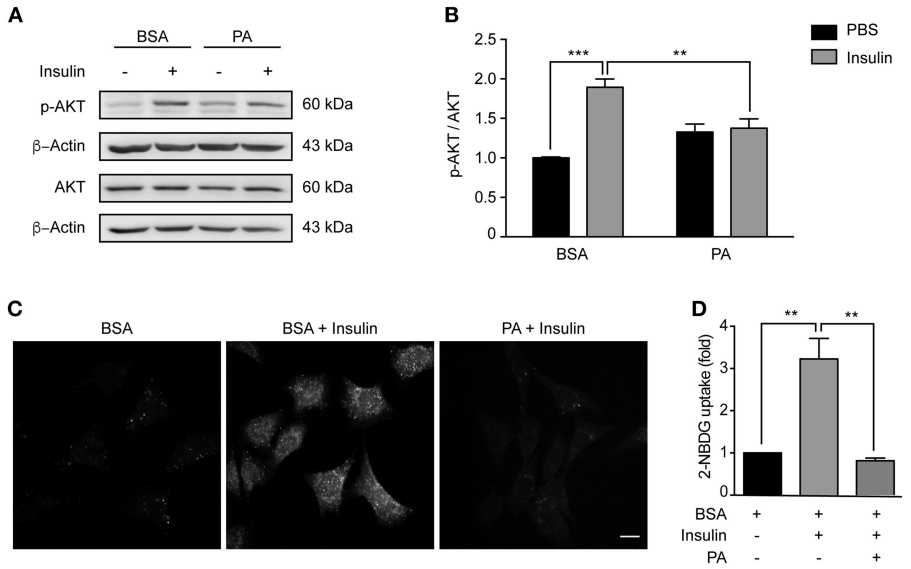

FIGURE 2 | Palmitic acid reduces insulin sensitivity in N43/5 cells. **(A)** Representative western blot showing relative levels of AKT phosphorylation (Ser473) induced by 15 min of insulin or PBS treatment in N43/5 cells pre-incubated with PA (100 μM) or BSA for 6 h, with its respective quantification **(B)**. **(B)** Data are presented as mean ± SEM. Two ways ANOVA followed by Sidak's multiple comparisons test. $^{**}p < 0.01$, $^{***}p < 0.001$. $n = 3$. **(C)** Representative images of 2-NBDG uptake in N43/5 cells incubated with vehicle (BSA) or PA for 6 h and then stimulated with insulin 1 nM for 30 min, with its representative quantification **(D)**. Size bar: 10 μm. **(D)** Data are presented as mean ± SEM. One-way ANOVA followed by Holm-Sidak's multiple comparisons test. $^{**}p < 0.01$. $n = 3$.

levels (3 and 15 min following insulin treatment, respectively), again suggesting N43/5 cells are sensitive to insulin, pretreatment with BafA1 prevented the increase in their phosphorylation (Ins vs. BafA1 + Ins: $^{**}p < 0.01$ for p-IR and $^{*}p < 0.05$ for p-AKT; **Figures 3A–C**). Importantly, the reduction in insulin sensitivity is confirmed by the reduction in glucose uptake, which is blunted by BafA1 treatment (Ins vs. BafA1 + Ins: $^{*}p < 0.05$; **Figures 3D,E**). No differences were seen in *Glut4* expression, suggesting the effect of BafA1 does not occur at the transcriptional level (**Supplemental Figure 4**). In addition, BafA1 exposure increased the levels of LC3II and SQSTM1 (**Figure 3A**), suggesting that inhibition of the autophagic flux reduces the insulin response in hypothalamic neuronal cells.

Then, we assessed if inhibition of autophagy, and not only the inhibition of the autophagic flux, could affect the response to insulin in our cellular model. To do this, we downregulated the expression of two autophagy essential genes *Beclin1* and *Atg7* (**Figures 3F,J**) and evaluated the levels of IR and p-AKT in response to insulin treatment. Interestingly, downregulation of both autophagy essential genes significantly reduced the levels of p-IR and p-AKT following insulin treatment (Ins vs. siBeclin + Ins: $^{*}p < 0.05$ for p-IR and $^{**}p < 0.01$ for p-AKT. Ins vs. siAtg7 + Ins: $^{**}p < 0.01$ for p-IR and p-AKT; **Figures 3F–L**). *Glut4* expression levels were increased following *Atg7* downregulation, while no differences were identified following BafA1 exposure or *Beclin1* downregulation (**Supplemental Figure 4**). Altogether these results indicate inhibition of autophagy, by downregulation of autophagy essential genes, reduces insulin sensitivity of N43/5 cells *in vitro*.

Palmitic Acid Activates FFAR1 in N43/5 Cells

Given the increased PA in the hypothalamus of HFDs animals (5), we addressed whether PA affects the levels of FFAR1. Consistent with previous work (37, 38), we identified FFAR1 in hypothalamic neurons but not astrocytes or microglia (**Figures 4A,B**). To determine the effect of PA on FFAR1 levels we gavaged male mice with PA. Importantly, this experiment was performed only in male mice as the increase in PA concentration in the brain, and more specifically in the hypothalamus, was identified only in male mice (4–7). Our data showed *Ffar1* mRNA levels increased following PA gavage (BSA vs. PA $^{*}p < 0.05$; **Figure 4C**). Furthermore, consistently with the results we obtained in N43/5 cells, *Sqstm1* levels increased, while *Atg* genes were not affected (**Supplemental Figure 5**).

The activation of FFAR1 by FAs triggers the phospholipase C (PLC)/ Inositol trisphosphate (IP_3) signaling pathway leading to Ca^{2+} release from the endoplasmic reticulum (24, 39). Thus, to determine if PA exposure activates FFAR1 in our cell model, we assessed intracellular Ca^{2+} levels following PA treatment. Intracellular Ca^{2+} levels were significantly increased in N43/5 cells after exposure to PA relative to vehicle (BSA vs. PA $^{***}p < 0.001$; **Figures 4D,E**). Importantly, this increase was significantly reduced in cultures exposed to GW1100, a specific FFAR1 antagonist, prior to PA treatment (PA vs. GW1100 + PA $^{*}p < 0.05$; **Figures 4D,E**). These data suggest that PA, among other factors, activates FFAR1 in N43/5 cells.

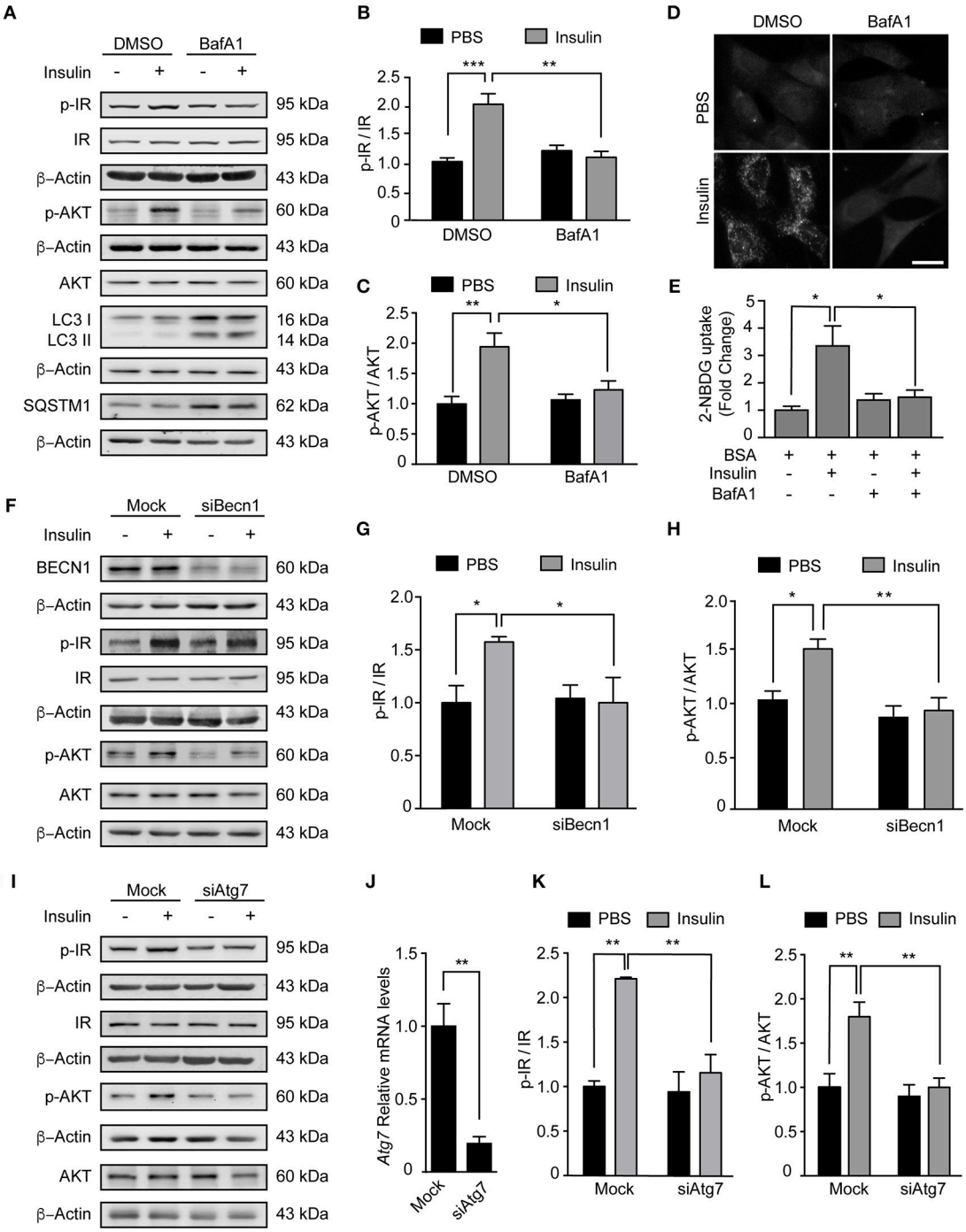

FIGURE 3 | Inhibition of autophagy reduces insulin sensitivity in N43/5 cells. **(A)** Representative western blots of the indicated proteins of N43/5 cells pre-incubated with BafA1 (100 nM) or its vehicle (DMSO) during 6 h and then stimulated with insulin for 3 min to evaluate IR phosphorylation (Tyr1150/1115) or 15 min to evaluate AKT phosphorylation. **(B,C)** Quantification of IR (Tyr1150/115) and AKT (Ser473) phosphorylation in cells exposed to the treatments as indicated in **(A)**. **(B,C)** Data are presented as mean ± SEM. Two ways ANOVA followed by Sidak's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. $n = 3$. **(D)** Representative images and quantification **(E)** of 2-NBDG uptake in N43/5 cells pre-incubated with BafA1 (100 nM) or its vehicle (DMSO) during 6 h and then stimulated with insulin for 30 min. Size bar: 10 μm. **(E)** Data are presented as mean ± SEM. One-way ANOVA followed by Holm-Sidak's multiple comparisons test. $^{*}p < 0.05$. $n = 3$. Representative blot of the indicated proteins of N43/5 cells transfected with siRNA against BECN1 **(F)** or ATG7 **(I)** followed by insulin or PBS treatment for 3 min to evaluate IR phosphorylation or 15 min to evaluate AKT phosphorylation, with its respective quantifications **(G,H,K,L)**. **(G,H,K,L)** Data are presented as mean ± SEM. Two ways ANOVA followed by Sidak's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$. $n = 3$. **(J)** mRNA levels of *Atg7* in N43/5 cells transfected with a siRNA to downregulate ATG7. As control condition, cells were incubated with Lipofectamine RNAiMAX reagent only (Mock). Data are presented as mean ± SEM. Unpaired *t* test. $^{**}p < 0.01$. $n = 3$.

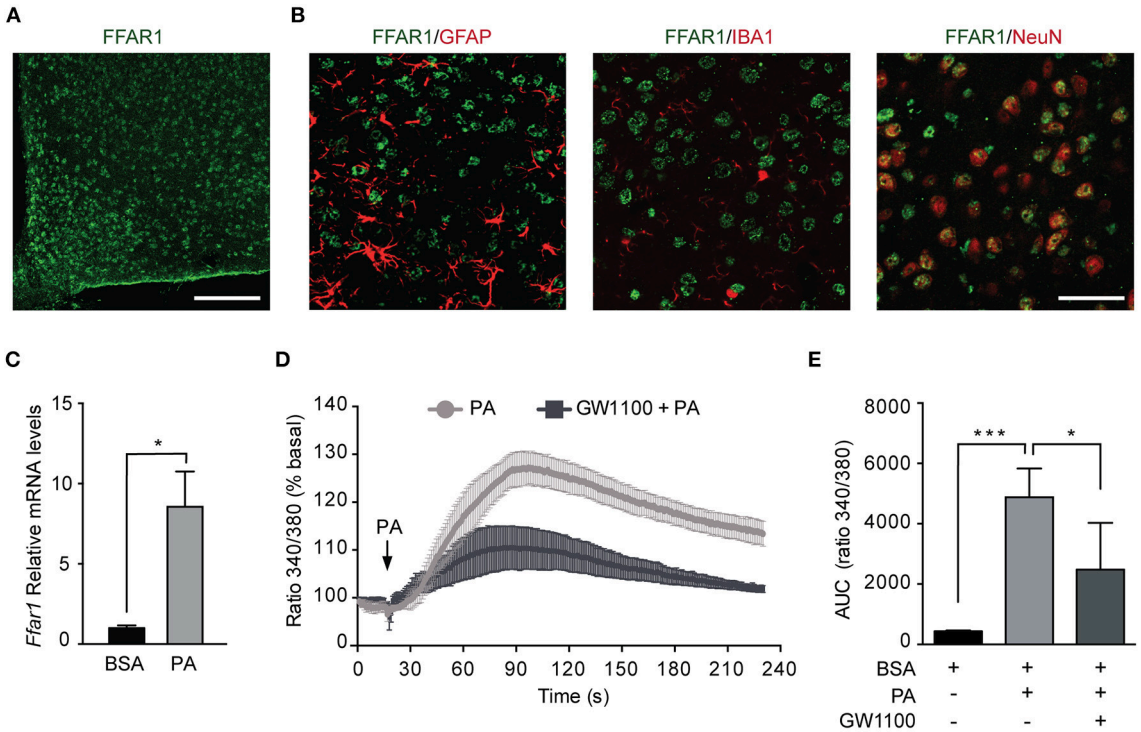

FIGURE 4 | Palmitic acid activates FFAR1 in N43/5 cells. **(A)** Representative confocal images showing FFAR1 immunoreactivity in the arcuate (ARC) nucleus of the hypothalamus of male mice. Scale bar: 125 μM. **(B)** Representative confocal images showing colocalization between FFAR1 and glial fibrillary acidic protein (GFAP), which stains astrocytes, or ionized calcium-binding adapter molecule 1 (IBA-1), which stains microglia or neuronal nuclei (NeuN), which stains neurons, in the hypothalamus. Scale bar: 25 μM. **(C)** mRNA levels of *Ffar1* in hypothalamic tissue in mice gavaged with BSA or PA for 3 days. Data are presented as mean ± SEM. Unpaired *t* test. BSA, $n = 3$; PA, $n = 4$. **(D)** Representative plot showing the percent increase in intracellular Ca^{2+} levels induced by PA (100 μM), in the presence or absence of FFAR1 antagonist GW1100 (1 μM). **(E)** Area under the curves (AUC) represented in **(D)**. Data are shown as mean ± SEM. One-way ANOVA followed by Holm-Sidak's multiple comparisons test. $^{*}p < 0.05$, $^{***}p < 0.001$. $n = 4$.

FFAR1 Pharmacological Inhibition Restores Autophagy and Insulin Sensitivity

Our data indicate that PA inhibits the autophagic flux and activates FFAR1 (**Figures 1**, **4**); thus, we determined if the activation of FFAR1 by PA is mechanistically involved in the inhibition of the autophagic flux. We exposed N43/5 cells to the FFAR1 antagonist GW1100 prior to PA treatment. GW1100 pretreatment reduced the levels of LC3II in cells pre-exposed to PA (PA vs. GW1100+PA $^{*}p <$ 0.05; **Figures 5A,B**). As we previously showed that PA inhibits the autophagic flux (**Figure 1**), these results suggest FFAR1 inhibition restores the autophagic flux in cells treated with PA (**Figure 5C**).

Interestingly, pharmacological inhibition of FFAR1 activity in cells exposed to PA also restored insulin sensitivity in N43/5 cells. Indeed, PA exposure reduced p-AKT levels when cells were stimulated with insulin (**Figures 2**, **5D,E**). However, p-AKT levels in cells treated with insulin were similar between control cells (treated with BSA) and cells pre-treated with GW1100, prior to PA exposure (PA + Ins vs. GW1100 + PA + Ins $^{*}p < 0.05$; **Figures 5D,E**). Consistently, glucose uptake, significantly reduced by PA treatment, is restored by GW1100 pretreatment (**Figures 5F,G**). Again, no significant differences were seen in *Glut4* mRNA levels following treatments (**Supplemental Figure 6**). These data indicate FFAR1 pharmacological inhibition promotes insulin sensitivity in N43/5 cells exposed to PA.

DISCUSSION

Here, we demonstrate that exposure to the SatFA PA inhibits the autophagic flux and reduces insulin sensitivity in N43/5 hypothalamic neurons (**Figures 1**, **2**). Furthermore, we show that inhibition of autophagy and the autophagic flux reduces insulin sensitivity in the same cellular model (**Figure 3**). Lastly, our data indicate that PA activates FFAR1 in hypothalamic neuronal cells (**Figure 4**) and that the inhibition of the autophagic flux and the reduction in insulin sensitivity are prevented by pharmacological inhibition of FFAR1 (**Figure 5**).

Chronic consumption of HFDs promotes the accumulation of PA in the hypothalamus of male mice (4–7); thus, we decided to determine whether this increase in PA might affect autophagy, which also appears dysfunctional in the hypothalamus following chronic consumption of HFDs. Importantly, previous studies indicate autophagy malfunction in hypothalamic POMC neurons contributes to obesity-associated metabolic dysfunctions, including increased plasma insulin

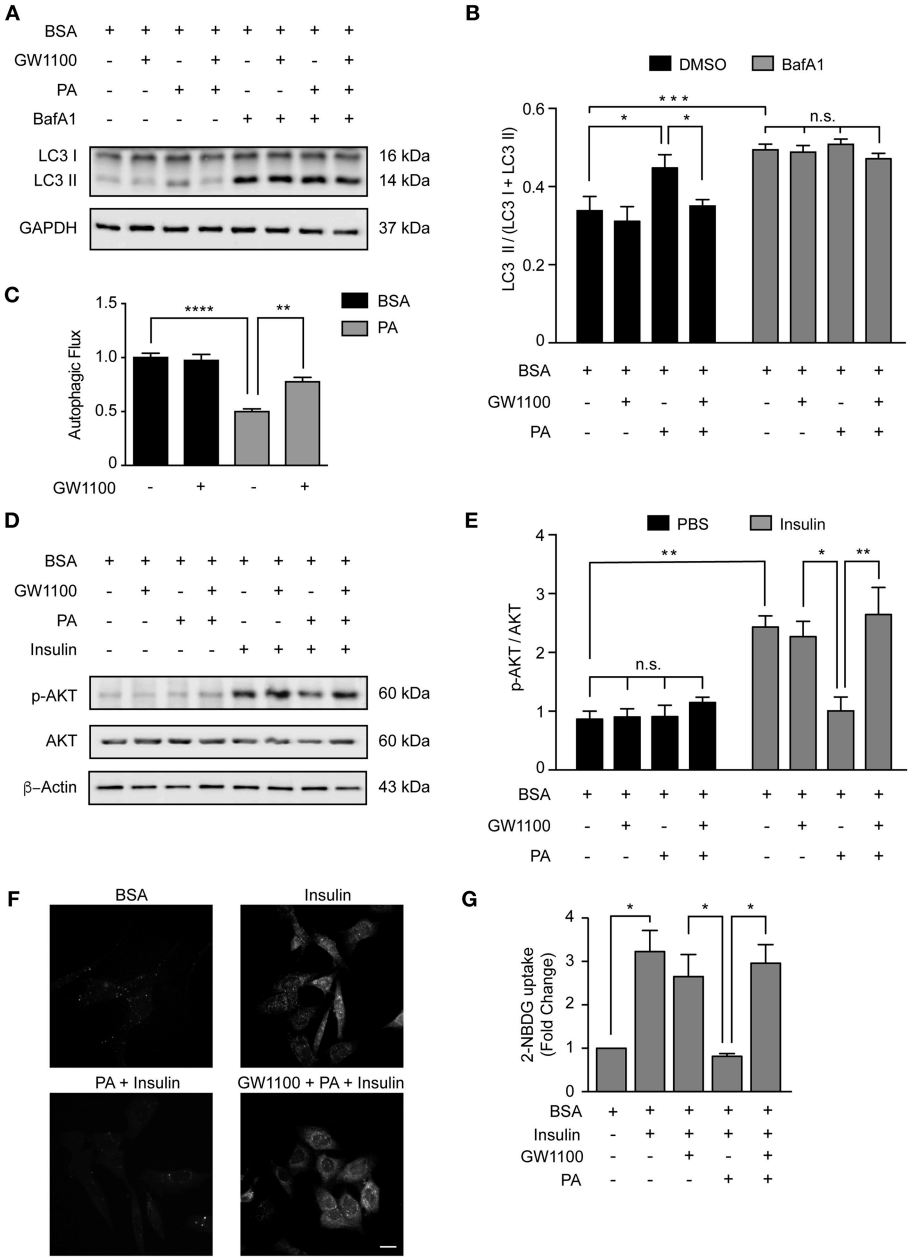

FIGURE 5 | FFAR1 pharmacological inhibition restores autophagy and insulin sensitivity. **(A)** Representative western blot of LC3 levels in N43/5 cell lysates, incubated with vehicle (BSA), GW1100 (1 μM), or PA (100 μM) for 1 h as indicated, in presence or absence of BafA1 (100 nM), with its respective quantification **(B)**. **(B)** Data are presented as mean ± SEM. Two ways ANOVA followed by Sidak's multiple comparisons test. $^{*}p < 0.05$, $^{***}p < 0.001$. $n = 3$. In case of GW1100 and PA treatment, cells were pre-exposed to GW1100 for 20 min, followed by GW1100 and PA exposure for 1 h. **(C)** Autophagic flux calculated as the difference in LC3II levels in presence and absence of BafA1 (100 nM). Data are shown as mean ± SEM. One-way ANOVA followed by Holm-Sidak's multiple comparisons test. $^{**}p < 0.01$, $^{****}p < 0.0001$. **(D)** Representative western blot of N43/5 cell lysates, incubated with vehicle (BSA), GW1100 (1 μM) or PA (100 μM) for 15 min in presence or absence of insulin (1 nM) for additional 15 min, as indicated, with its respective quantification **(E)**. In case of GW1100 and PA treatment, cells were pre-treated with GW1100 for 20 min, followed by GW1100 and PA exposure for 15 min. Data are presented as mean ± SEM. Two ways ANOVA followed by Sidak's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$. $n = 3$. **(F)** Representative images of 2-NBDG uptake in N43/5 cells incubated with vehicle (BSA), GW1100, PA and then stimulated with insulin 1 nM for 30 min, with its representative quantification **(G)**. Size bar: 10 μm. Data are shown as mean ± SEM. One-way ANOVA followed by Holm-Sidak's multiple comparisons test. $^{*}p < 0.05$. $n = 3$.

levels, hyperglycemia, glucose intolerance, impaired lipolysis, and leptin resistance (14–16). Taken together, these data suggest that HFDs-induced SatFAs accumulation in the CNS in male mice promotes a vicious cycle where autophagy inhibition might further enhance obesity-associated diseases. In this context, our data indicate that, at least in our cellular model, exposure to PA

inhibits the autophagic flux, a condition that also affects insulin sensitivity. Future research, should confirm these data in *in vivo* models. Special attention should be paid to assess the possible sexual dimorphism is this response, especially considering previous studies indicating the sex-dependent function of POMC neurons in the regulation of energy homeostasis (40, 41).

The existence of a crosstalk between autophagy and insulin sensitivity has been previously suggested and identified in peripheral metabolic tissues (22, 23, 42). Interestingly, it was recently demonstrated that autophagy degrades insulin-containing vesicles in β-cells of autophagy-hyperactive mice, whereas in insulin-sensitive cells, autophagy enhances insulin response (42). Indeed, induction of autophagy, by different means, in skeletal muscle, hepatocytes, podocytes and adipocytes (43–45), stimulates insulin sensitivity; suggesting increased autophagy might be a general mechanism to boost insulin response. Despite this, there are no reports that determine if this crosstalk also occurs in the CNS, or specifically, in the hypothalamus where insulin sensitive neurons key in the regulation of food intake and peripheral glucose homeostasis reside. In the present study, we evaluated if inhibition of autophagy or autophagic flux blockade in a hypothalamic neuronal cell line affects insulin response. Our data indicate that this might be the case, as downregulation of different autophagy essential genes (*Atg7* and *Beclin1*), as well as inhibition of the autophagic flux using a classic autophagy inhibitor (Bafilomycin A1), reduced the ability of the neuron to respond to insulin, as indicated by IR and p-AKT level and the reduction in glucose uptake following Bafilomycin A1 exposure. How this might be occurring has not been elucidated; however, a possibility is that, by inhibiting autophagy we prevent the degradation of negative regulators of the insulin signaling pathway, such as the protein

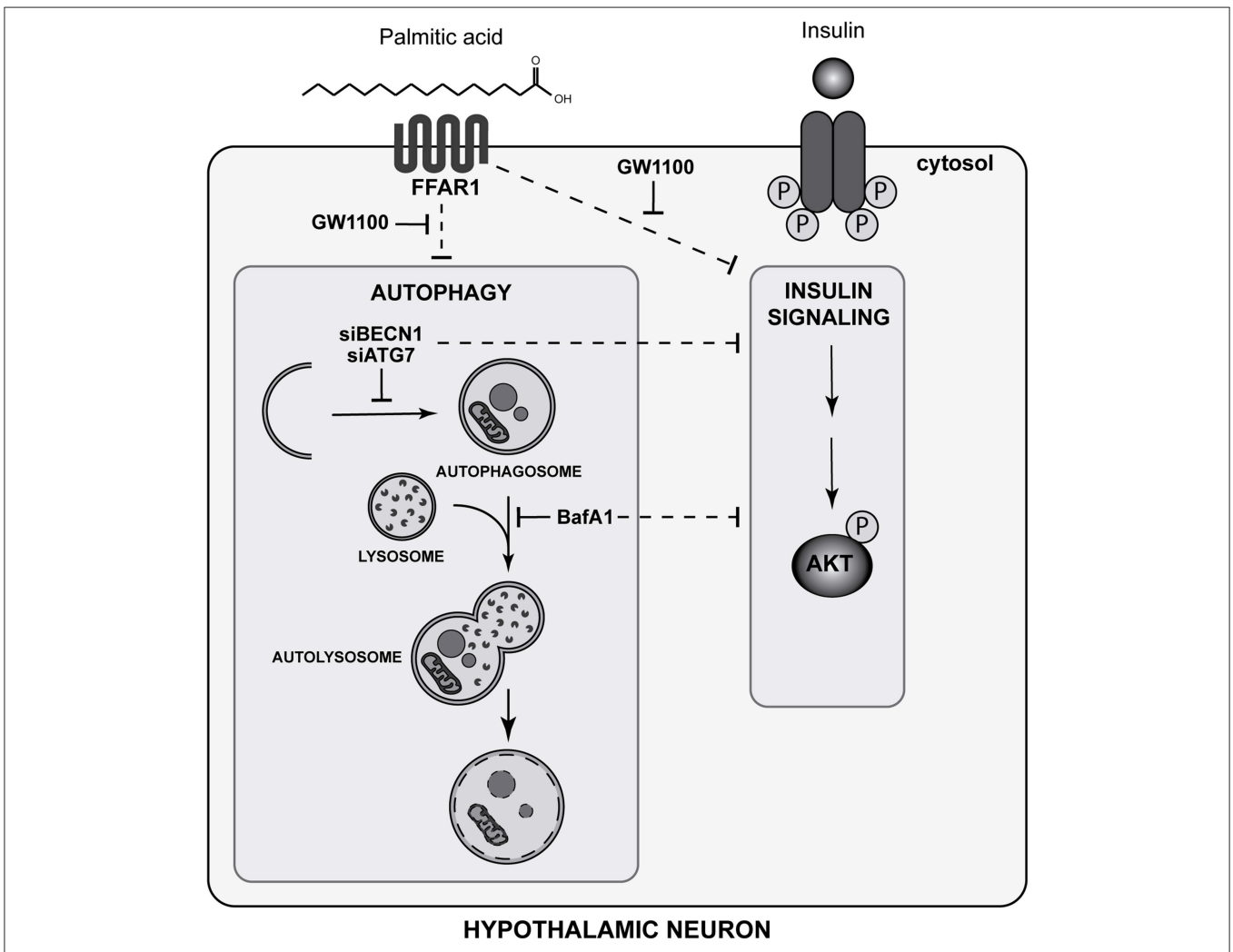

FIGURE 6 | Palmitic acid-mediated FFAR1 activation decreases autophagic flux and insulin sensitivity in hypothalamic neuronal cells (Graphical abstract). The FFAR1 antagonist GW1100 prevents autophagic flux inhibition and restores insulin sensitivity, as indicated by AKT phosphorylation levels, in cells exposed to PA. Downregulation of autophagy essential genes (ATG7 and Beclin 1 -BECN1-), as well as pharmacological inhibition of the autophagic flux by BafA1, reduces the increase in AKT and IR phosphorylation levels stimulated by insulin.

phosphatase and tensin homolog (PTEN), which can be degraded by the autophagic/lysosomal pathway (46). In addition, based on these results, it is tempting to speculate that the maintenance of the autophagic balance in hypothalamic neurons might be key in the regulation of peripheral glucose homeostasis, as well as food intake. Future studies in neuron-specific transgenic mouse models need to be performed to confirm this hypothesis *in vivo*.

Sensing lipids is a mechanism through which the hypothalamus controls energy balance. Brain lipid sensing is important to control feeding behavior, hepatic glucose production, and insulin secretion (8). Fatty acids sensitive neurons can increase/decrease their activity in response to FAs (8). Different types of proteins can act as receptors of fatty acids, such as fatty acid transporters, fatty acid binding proteins, and fatty acid receptors. Among those, we focused our attention on FFAR1, the activation of which in pancreatic β-cells, leads to insulin secretion (25, 47), suggesting that this receptor might be involved in the chain of events that regulate the insulin response. Importantly, Steneberg et al. demonstrated that FFAR1 overexpression impairs β-cells function, promoting hypoinsulinemia and diabetes following HFDs feeding (26). In addition, in immortalized and primary pancreatic β-cells, FFAR1 activation induces lipotoxicity (48). Altogether these studies suggest that, in pancreatic β-cells, the effects of FFAR1 activation appear to be dual, since it is involved in both homeostatic and pathologic effects. In addition, FFAR1 overexpression causes hypoinsulinemia and autophagy hyperactivation reduces insulin release in β-cells, suggesting that an additional crosstalk might be occurring between FFAR1 and autophagy, specifically in pancreatic β-cells. Considerably less information is available regarding FFAR1 function in the CNS, where its role is still elusive. Our data, supported by previously published studies (37), show that, within the hypothalamus, FFAR1 is only expressed by neurons and not by astrocytes and microglial cells. Dragano et al. also confirmed FFAR1 is expressed in hypothalamic neurons (both in neuropeptide Y (NPY) and POMC neurons) and acts to maintain whole body energy homeostasis by decreasing energy efficiency and reducing hypothalamic inflammation when FFAR1 is chemically activated by receptor specific agonists (38). Additional studies demonstrate that polyunsaturated fatty acids (PUFAs)-mediate FFAR1 activation in the hypothalamus to produce an anti-inflammatory and neuroprotective effect (37, 49–52). This apparent discrepancy in the effects of FFAR1 is most likely attributable to a ligand-dependent activation of distinct allosteric sites on the receptor by the different molecular structures (53). Furthermore, differences between SatFAs-mediated activation of the receptor in hypothalamic neurons *in vitro* vs. *in vivo* have not been investigated. In this study, we show PA-mediated FFAR1 activation in N43/5 hypothalamic neuronal cells reduces insulin sensitivity and the autophagic flux. Future research, using *in vivo* models, should evaluate if hypothalamic stimulation of this receptor may lead to the development of metabolic diseases, by affecting whole-body insulin sensitivity.

In summary, our study identifies a new role of autophagy in N43/5 hypothalamic neuronal cells in the regulation of insulin sensitivity. In addition, we identified PA as negative modulator of the autophagic flux in the same cell type. Finally, our data suggest FFAR1 mediates the ability of PA to reduce insulin sensitivity promoted by autophagic flux inhibition in hypothalamic neuronal cells (**Figure 6**).

These results may help understanding the cellular mechanisms that drive to insulin resistance induced by PA in hypothalamic neurons.

AUTHOR CONTRIBUTIONS

EM and MH-C conceived and planned the experiments. MH-C, LT-V, YÁ, FD-C, CN, JE-C, and DP-O carried out the experiments. EM took the lead in writing the manuscript. All the authors contributed to the interpretation of the results and provided critical feedback and helped shape the research, analysis, and manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00176/full#supplementary-material

REFERENCES

1. Hotamisligil GS. Inflammation, metaflammation and immunometabolic disorders. *Nature.* (2017) 542:177–85. doi: 10.1038/nature21363

2. Milanski M, Degasperi G, Coope A, Morari J, Denis R, Cintra DE, et al. Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. *J Neurosci.* (2009) 29:359–70. doi: 10.1523/JNEUROSCI.2760-08.2009

3. Dorfman MD, Thaler JP. Hypothalamic inflammation and gliosis in obesity. *Curr Opin Endocrinol Diabetes Obes.* (2015) 22:325–30. doi: 10.1097/MED.0000000000000182
4. Morselli E, Fuente-Martin E, Finan B, Kim M, Frank A, Garcia-Caceres C, et al. Hypothalamic PGC-1alpha protects against high-fat diet exposure by regulating ERalpha. *Cell Rep.* (2014) 9:633–45. doi: 10.1016/j.celrep.2014.09.025
5. Valdearcos M, Robblee MM, Benjamin DI, Nomura DK, Xu AW, Koliwad SK. Microglia dictate the impact of saturated fat consumption on hypothalamic inflammation and neuronal function. *Cell Rep.* (2014) 9:2124–38. doi: 10.1016/j.celrep.2014.11.018
6. Morselli E, Frank AP, Palmer BF, Rodriguez-Navas C, Criollo A, Clegg DJ. A sexually dimorphic hypothalamic response to chronic high-fat diet consumption. *Int J Obes.* (2016) 40:206–9. doi: 10.1038/ijo.2015.114
7. Rodriguez-Navas C, Morselli E, Clegg DJ. Sexually dimorphic brain fatty acid composition in low and high fat diet-fed mice. *Mol Metab.* (2016) 5:680–9. doi: 10.1016/j.molmet.2016.06.014
8. Migrenne S, Le Foll C, Levin BE, Magnan C. Brain lipid sensing and nervous control of energy balance. *Diabetes Metab.* (2011) 37:83–8. doi: 10.1016/j.diabet.2010.11.001
9. Moulle VS, Picard A, Le Foll C, Levin BE, Magnan C. Lipid sensing in the brain and regulation of energy balance. *Diabetes Metab.* (2014) 40:29–33. doi: 10.1016/j.diabet.2013.10.001
10. Singh R, Cuervo AM. Autophagy in the cellular energetic balance. *Cell Metab.* (2011) 13:495–504. doi: 10.1016/j.cmet.2011.04.004
11. Zhang Y, Sowers JR, Ren J. Targeting autophagy in obesity: from pathophysiology to management. *Nat Rev Endocrinol.* (2018) 14:356–76. doi: 10.1038/s41574-018-0009-1
12. Morselli E, Criollo A, Rodriguez-Navas C, Clegg DJ. Chronic high fat diet consumption impairs metabolic health of male mice. *Inflamm Cell Signal.* (2014) 1:e561. doi: 10.14800/ics.561
13. Portovedo M, Ignacio-Souza LM, Bombassaro B, Coope A, Reginato A, Razolli DS, et al. Saturated fatty acids modulate autophagy's proteins in the hypothalamus. *PLoS ONE.* (2015) 10:e0119850. doi: 10.1371/journal.pone.0119850
14. Coupe B, Ishii Y, Dietrich MO, Komatsu M, Horvath TL, Bouret SG. Loss of autophagy in pro-opiomelanocortin neurons perturbs axon growth and causes metabolic dysregulation. *Cell Metab.* (2012) 15:247–55. doi: 10.1016/j.cmet.2011.12.016
15. Kaushik S, Arias E, Kwon H, Lopez NM, Athonvarangkul D, Sahu S, et al. Loss of autophagy in hypothalamic POMC neurons impairs lipolysis. *EMBO Rep.* (2012) 13:258–65. doi: 10.1038/embor.2011.260
16. Quan W, Kim HK, Moon EY, Kim SS, Choi CS, Komatsu M, et al. Role of hypothalamic proopiomelanocortin neuron autophagy in the control of appetite and leptin response. *Endocrinology.* (2012) 153:1817–26. doi: 10.1210/en.2011-1882
17. Lopez-Vicario C, Alcaraz-Quiles J, Garcia-Alonso V, Rius B, Hwang SH, Titos E, et al. Inhibition of soluble epoxide hydrolase modulates inflammation and autophagy in obese adipose tissue and liver: role for omega-3 epoxides. *Proc Natl Acad Sci USA.* (2015) 112:536–41. doi: 10.1073/pnas.1422590112
18. Jia G, DeMarco VG, Sowers JR. Insulin resistance and hyperinsulinaemia in diabetic cardiomyopathy. *Nat Rev Endocrinol.* (2016) 12:144–53. doi: 10.1038/nrendo.2015.216
19. Liu H, Javaheri A, Godar RJ, Murphy J, Ma X, Rohatgi N, et al. Intermittent fasting preserves beta-cell mass in obesity-induced diabetes via the autophagy-lysosome pathway. *Autophagy.* (2017) 13:1952–68. doi: 10.1080/15548627.2017.1368596
20. Qian Q, Zhang Z, Orwig A, Chen S, Ding WX, Xu Y, et al. S-Nitrosoglutathione reductase dysfunction contributes to obesity-associated hepatic insulin resistance via regulating autophagy. *Diabetes.* (2018) 67:193–207. doi: 10.2337/db17-0223
21. Yang L, Li P, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. *Cell Metab.* (2010) 11:467–78. doi: 10.1016/j.cmet.2010.04.005
22. Ebato C, Uchida T, Arakawa M, Komatsu M, Ueno T, Komiya K, et al. Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. *Cell Metab.* (2008) 8:325–32. doi: 10.1016/j.cmet.2008.08.009
23. Jung HS, Chung KW, Won Kim J, Kim J, Komatsu M, Tanaka K, et al. Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell Metab.* (2008) 8:318–24. doi: 10.1016/j.cmet.2008.08.013
24. Briscoe CP, Peat AJ, McKeown SC, Corbett DF, Goetz AS, Littleton TR, et al. Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. *Br J Pharmacol.* (2006) 148:619–28. doi: 10.1038/sj.bjp.0706770
25. Covington DK, Briscoe CA, Brown AJ, Jayawickreme CK. The G-protein-coupled receptor 40 family (GPR40-GPR43) and its role in nutrient sensing. *Biochem Soc Trans.* (2006) 34(Pt 5):770–3. doi: 10.1042/BST0340770
26. Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, Edlund H. The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. *Cell Metab.* (2005) 1:245–58. doi: 10.1016/j.cmet.2005.03.007
27. Kebede M, Ferdaoussi M, Mancini A, Alquier T, Kulkarni RN, Walker MD, et al. Glucose activates free fatty acid receptor 1 gene transcription via phosphatidylinositol-3-kinase-dependent O-GlcNAcylation of pancreas-duodenum homeobox-1. *Proc Natl Acad Sci USA.* (2012) 109:2376–81. doi: 10.1073/pnas.1114350109
28. Kristinsson H, Smith DM, Bergsten P, Sargsyan E. FFAR1 is involved in both the acute and chronic effects of palmitate on insulin secretion. *Endocrinology.* (2013) 154:4078–88. doi: 10.1210/en.2013-1352
29. Verma MK, Sadasivuni MK, Yateesh AN, Neelima K, Mrudula S, Reddy M, et al. Activation of GPR40 attenuates chronic inflammation induced impact on pancreatic beta-cells health and function. *BMC Cell Biol.* (2014) 15:24. doi: 10.1186/1471-2121-15-24
30. Benoit SC, Kemp CJ, Elias CF, Abplanalp W, Herman JP, Migrenne S, et al. Palmitic acid mediates hypothalamic insulin resistance by altering PKC-theta subcellular localization in rodents. *J Clin Invest.* (2009) 119:2577–89. doi: 10.1172/JCI36714
31. Darling RA, Zhao H, Kinch D, Li AJ, Simasko SM, Ritter S. Mercaptoacetate and fatty acids exert direct and antagonistic effects on nodose neurons via GPR40 fatty acid receptors. *Am J Physiol Regul Integr Comp Physiol.* (2014) 307:R35–43. doi: 10.1152/ajpregu.00536.2013
32. Bernal-Sore I, Navarro-Marquez M, Osorio-Fuentealba C, Diaz-Castro F, Del Campo A, Donoso-Barraza C, et al. Mifepristone enhances insulin-stimulated Akt phosphorylation and glucose uptake in skeletal muscle cells. *Mol Cell Endocrinol.* (2018) 461:277–83. doi: 10.1016/j.mce.2017.09.028
33. Meng Q, Cai D. Defective hypothalamic autophagy directs the central pathogenesis of obesity via the IkappaB kinase beta (IKKbeta)/NF-kappaB pathway. *J Biol Chem.* (2011) 286:32324–32. doi: 10.1074/jbc.M111.254417
34. Oh TS, Cho H, Cho JH, Yu SW, Kim EK. Hypothalamic AMPK-induced autophagy increases food intake by regulating NPY and POMC expression. *Autophagy.* (2016) 12:2009–25. doi: 10.1080/15548627.2016.1215382
35. Posey KA, Clegg DJ, Printz RL, Byun J, Morton GJ, Vivekanandan-Giri A, et al. Hypothalamic proinflammatory lipid accumulation, inflammation, and insulin resistance in rats fed a high-fat diet. *Am J Physiol Endocrinol Metab.* (2009) 296:E1003–1012. doi: 10.1152/ajpendo.90377.2008
36. De Souza CT, Araujo EP, Bordin S, Ashimine R, Zollner RL, Boschero AC, et al. Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. *Endocrinology.* (2005) 146:4192–9. doi: 10.1210/en.2004-1520
37. Nakamoto K, Nishinaka T, Sato N, Mankura M, Koyama Y, Kasuya F, et al. Hypothalamic GPR40 signaling activated by free long chain fatty acids suppresses CFA-induced inflammatory chronic pain. *PLoS ONE.* (2013) 8:e81563. doi: 10.1371/journal.pone.0081563
38. Dragano NRV, Solon C, Ramalho AF, de Moura RF, Razolli DS, Christiansen E, et al. Polyunsaturated fatty acid receptors, GPR40 and GPR120, are expressed in the hypothalamus and control energy homeostasis and inflammation. *J Neuroinflamm.* (2017) 14:91. doi: 10.1186/s12974-017-0869-7
39. Fujiwara K, Maekawa F, Yada T. Oleic acid interacts with GPR40 to induce Ca2+ signaling in rat islet beta-cells: mediation by PLC and L-type Ca2+ channel and link to insulin release. *Am J Physiol Endocrinol Metab.* (2005) 289:E670–677. doi: 10.1152/ajpendo.00035.2005
40. Xu Y, Nedungadi TP, Zhu L, Sobhani N, Irani BG, Davis KE, et al. Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. *Cell Metab.* (2011) 14:453–65. doi: 10.1016/j.cmet.2011.08.009

41. Wang C, He Y, Xu, P, Yang, Y, Saito K, Xia Y, et al. TAp63 contributes to sexual dimorphism in POMC neuron functions and energy homeostasis. *Nat Commun.* (2018) 9:1544. doi: 10.1038/s41467-018-03796-7
42. Yamamoto S, Kuramoto K, Wang N, Situ X, Priyadarshini M, Zhang W, et al. Autophagy differentially regulates insulin production and insulin sensitivity. *Cell Rep.* (2018) 23:3286–99. doi: 10.1016/j.celrep.2018.05.032
43. Xin W, Li Z, Xu Y, Yu Y, Zhou Q, Chen L, et al. Autophagy protects human podocytes from high glucose-induced injury by preventing insulin resistance. *Metab Clin Exp.* (2016) 65:1307–15. doi: 10.1016/j.metabol.2016.05.015
44. Ahlstrom P, Rai E, Chakma S, Cho HH, Rengasamy P, Sweeney G. Adiponectin improves insulin sensitivity via activation of autophagic flux. *J Mol Endocrinol.* (2017) 59:339–50. doi: 10.1530/JME-17-0096
45. Zhou W, Ye S. Rapamycin improves insulin resistance and hepatic steatosis in type 2 diabetes rats through activation of autophagy. *Cell Biol Int.* (2018) 42:1282–91. doi: 10.1002/cbin.11015
46. Wang M, Wu H, Li S, Xu Z, Li X, Yang Y, et al. SYNJ2BP promotes the degradation of PTEN through the lysosome-pathway and enhances breast tumor metastasis via PI3K/AKT/SNAI1 signaling. *Oncotarget.* (2017) 8:89692–706. doi: 10.18632/oncotarget.21058
47. Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, et al. Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. *Nature.* (2003) 422:173–6. doi: 10.1038/nature01478
48. Yamashima T. Dual effects of the non-esterified fatty acid receptor 'GPR40' for human health. *Prog Lipid Res.* (2015) 58:40–50. doi: 10.1016/j.plipres.2015.01.002
49. Nakamoto K, Nishinaka T, Matsumoto K, Kasuya F, Mankura M, Koyama Y, et al. Involvement of the long-chain fatty acid receptor GPR40 as a novel pain regulatory system. *Brain Res.* (2012) 1432:74–83. doi: 10.1016/j.brainres.2011.11.012
50. Nakamoto K, Aizawa F, Nishinaka T, Tokuyama S. Regulation of prohormone convertase 2 protein expression via GPR40/FFA1 in the hypothalamus. *Eur J Pharmacol.* (2015) 762:459–63. doi: 10.1016/j.ejphar.2015.06.013
51. Khan MZ, He L. The role of polyunsaturated fatty acids and GPR40 receptor in brain. *Neuropharmacology.* (2017) 113(Pt B):639–51. doi: 10.1016/j.neuropharm.2015.05.013
52. Nakamoto K, Aizawa F, Miyagi K, Yamashita T, Mankura M, Koyama Y, et al. Dysfunctional GPR40/FFAR1 signaling exacerbates pain behavior in mice. *PLoS ONE.* (2017) 12:e0180610. doi: 10.1371/journal.pone.0180610
53. Lin DC, Guo Q, Luo J, Zhang J, Nguyen K, Chen M, et al. Identification and pharmacological characterization of multiple allosteric binding sites on the free fatty acid 1 receptor. *Mol Pharmacol.* (2012) 82:843–59. doi: 10.1124/mol.112.079640

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Polycystin-2 Is Required for Starvation- and Rapamycin-Induced Atrophy in Myotubes

Catalina Kretschmar[1,2†], Daniel Peña-Oyarzun[1,2†], Cecilia Hernando[1,2], Nadia Hernández-Moya[1,2], Alfredo Molina-Berríos[1], María Paz Hernández-Cáceres[3], Sergio Lavandero[2,4,5], Mauricio Budini[1,6], Eugenia Morselli[3,6], Valentina Parra[2,4,6], Rodrigo Troncoso[2,6,7] and Alfredo Criollo[1,2,6]**

[1] Facultad de Odontología, Instituto de Investigación en Ciencias Odontológicas, Universidad de Chile, Santiago, Chile, [2] Facultad Ciencias Químicas y Farmacéuticas and Facultad Medicina, Advanced Center for Chronic Diseases, Universidad de Chile, Santiago, Chile, [3] Departamento de Fisiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile, [4] Facultad de Medicina, Centro de Estudios en Ejercicio, Metabolismo y Cáncer, Universidad de Chile, Santiago, Chile, [5] Cardiology Division, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, United States, [6] Autophagy Research Center, Santiago, Chile, [7] Laboratorio de Investigación en Nutrición y Actividad Física, Instituto de Nutrición y Tecnología de los Alimentos, Universidad de Chile, Santiago, Chile*

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***Correspondence:**
Rodrigo Troncoso
rtroncoso@inta.uchile.cl
Alfredo Criollo
alcriollo@u.uchile.cl

[†]These authors have contributed equally to this work

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Muscle atrophy involves a massive catabolism of intracellular components leading to a significant reduction in cellular and tissue volume. In this regard, autophagy, an intracellular mechanism that degrades proteins and organelles, has been implicated with muscle breakdown. Recently, it has shown that polycystin-2 (PC2), a membrane protein that belongs to the transient receptor potential (TRP) family, is required for the maintenance of cellular proteostasis, by regulating autophagy in several cell types. The role of PC2 in the control of atrophy and autophagy in skeletal muscle remains unknown. Here, we show that PC2 is required for the induction of atrophy in C2C12 myotubes caused by nutrient deprivation or rapamycin exposure. Consistently, overexpression of PC2 induces atrophy in C2C12 myotubes as indicated by decreasing of the myogenic proteins myogenin and caveolin-3. In addition, we show that inhibition of mTORC1, by starvation or rapamycin is inhibited in cells when PC2 is silenced. Importantly, even if PC2 regulates mTORC1, our results show that the regulation of atrophy by PC2 is independent of autophagy. This study provides novel evidence regarding the role of PC2 in skeletal muscle cell atrophy.

Keywords: atrophy, polycystin-2, myotubes, mTOR, starvation, rapamycin

INTRODUCTION

Atrophy defined as a decrease in the mass and size of tissues or cells, is caused by a massive loss of proteins, cytoplasm, and organelles. Muscle cells respond to different pathophysiological stimuli by activating pathways involved in protein degradation. Stimuli such as cancer pharmacological treatments, AIDS, sepsis, heart failure, burn injury, and multiple sclerosis among others can induce severe muscle atrophy (1–4). The preservation of the homeostasis in muscle cells is crucial, not only because of the maintenance of an optimal muscle performance, but also because muscle represents an important source of amino acids and nutrients, which can be metabolized by

different organs such as brain, heart and liver (5). A severe or aggressive episode of atrophy can aggravate other co-lateral diseases, and seriously increase morbidity and mortality. Importantly, massive macroautophagy, hereafter referred as autophagy, is one of the mechanisms involved in muscle cell atrophy (6). Autophagy is a fundamental intra cellular process for degrading and recycling components such as proteins, organelles, and cytoplasm. Autophagy is characterized by the formation of autophagosomes, which fuse with the lysosome to form the autolysosome where the intravacuolar material is degraded. Studies *in vitro* and *in vivo* have shown that there is a strong relationship between autophagy and skeletal muscle atrophy (7–9). Studies in C2C12 myotubes and a murine cancer model showed that activation of autophagy contributes to muscle wasting in cancer cachexia (10). Consistently, other studies have shown that the mechanistic target of rapamycin complex 1, mTOR, a constitutive kinase protein that inhibits autophagy, blocks atrophy in muscle and other cell types (11). In this regard, muscle-specific mTOR knockout mice present a severe muscle atrophy phenotype. Consistently, the insulin- or IGF-1-induced overactivation of mTOR blunts atrophy through the inhibition of autophagy in cardiac muscle (12, 13). Furthermore, inhibition of mTOR, induced by nutrient restriction or treatment with rapamycin (which inhibits mTORC1), causes autophagy and atrophy in skeletal muscle (11, 12, 14, 15). These evidences indicate the existence of a cross-talk between mTOR, autophagy and atrophy in the control of metabolism and cell and tissue size. Although different signaling pathways have been elucidated during the process of atrophy, the molecular mechanisms by which they can be modulated are still unknown. Recently, we have showed that the protein polycystin-2, PC2, a member of the transient receptor potential family which acts as a non-selective cation channel, is required for nutrient deprivation-induced autophagy *in vivo* and by hypertonicity, rapamycin and starvation *in vitro* in different types of cells (16, 17). Others studies also supported the role of PC2 in the regulation of autophagy. Indeed in renal epithelial cells and human embryonic stem cell-derived cardiomyocytes, PC2 is required for fluid flow- and glucose starvation-induced autophagy, respectively (18, 19).

PC2 regulates autophagy by activating different pathways; among those are listed classic (mTOR-dependent) and mTOR independent pathways, such as Ca^{2+}-dependent modulation of autophagy. Interestingly, as PC2 is not an ubiquitous protein and since PC2 can regulate autophagy thanks to its Ca^{2+} channel function, the modulation of autophagy by PC2 can be cell, tissue and stimuli dependent (16–19).

Here, we show that PC2 is required for starvation- and rapamycin- induced atrophy and inactivation of mTOR in C2C12 myotubes, without affecting the modulation of autophagy in the same cell type.

Abbreviations: AU, arbitrary units; Baf A1, Bafilomycin A1; mTOR, mechanistic target of rapamycin; PC2, polycystin-2; PKD, polycystic kidney disease; VPS34, vacuolar protein sorting 34; 4EBP1, 4E-binding protein 1; ULK1, 51-like kinase 1 protein; Cav3, Caveolin-3; Myo, Myogenin; LC3, light chain.

RESULTS

Classical Autophagic Inducers Causes Atrophy in C2C12 Myotubes

C2C12 myotubes were exposed to classical autophagy inducers such as nutrient deprivation, by treatment with Earle's Balanced Salt Solution (EBSS) or by pharmacological inhibition of mTORC1 with rapamycin, 0.1 μM, at different time points (0–6 h) (**Figure 1**). Protein levels of myogenic markers Myogenin (MYO) and Caveolin-3 (CAV3), which are down-regulated in different *in vitro* and *in vivo* models of atrophy, decrease in C2C12 myotubes following EBSS or rapamycin exposure (**Figures 1A–J**). In addition, as expected, both EBSS and rapamycin induce autophagy in C2C12 myotubes as assessed by the analysis of LC3 I to LC3 II conversion by western blotting, reaching the highest level at 0.5 and 1 h post-treatments (**Figures 1A,D,F,I**). We also analyzed the levels of p62/SQSTM1, a protein that binds specifically to LC3 and thus is degraded in the autolysosome (20). Consistently, the level of p62/SQSTM1 decreases when cells are exposed to EBSS or treated with rapamycin (**Figures 1A,E,F,J**), confirming that autophagy is up-regulated in C2C12 myotubes following the aforementioned treatments. Altogether these data show that nutrient deprivation and rapamicyn not only induce autophagy but also atrophy in C2C12 myotubes.

PC2 Deficiency Prevents Atrophy in an Autophagy-Independent Manner in C2C12 Myotubes

PC2 is a member of the TRP channels protein family and studies have shown that PC2 is involved in mechanisms of mechanotransduction mostly in renal epithelial cells (21, 22). In addition, recently we and others have shown that PC2 regulates autophagy in different cell types (16, 17, 19). Indeed, we showed PC2 is required for hyperosmotic stress-induced autophagy in human cervical and colon cancer cell lines, HeLa and HCT116, respectively (16). Furthermore, it has been shown that PC2 is required for autophagy induction also in primary rat neonatal cardiomyocytes submitted to starvation or exposed to rapamycin (17), as well as in kidney mouse epithelial cells exposed to changes in fluid flow (18). In addition, animals knocked-out for PC2 in cardiomyocytes are resistant to starvation-induced autophagy, specifically in the heart (17). Given that PC2 regulates autophagy, and that autophagy is enhanced by action of different atrophy inducers in skeletal muscle cells, we evaluated if PC2 is required for atrophy in C2C12 myotubes exposed to nutrient deprivation or rapamycin. Our results showed that down regulation of PC2, by the use of specific siRNAs, prevented atrophy as indicated by the myotube diameter in C2C12 cells submitted to starvation (**Figures 2A,B**). In addition, western blot assays revealed that the decrease in the levels of MYO and CAV3 in cells exposed to nutrient deprivation or rapamycin for 4 and 6 h, respectively, is inhibited in C2C12 myotube cultures transfected with a specific siRNA against PC2 (siPC2) (**Figures 2C–H**). Despite the effects of PC2 in the regulation of atrophy, we did not observe inhibition of starvation- and rapamycin-induced autophagy,

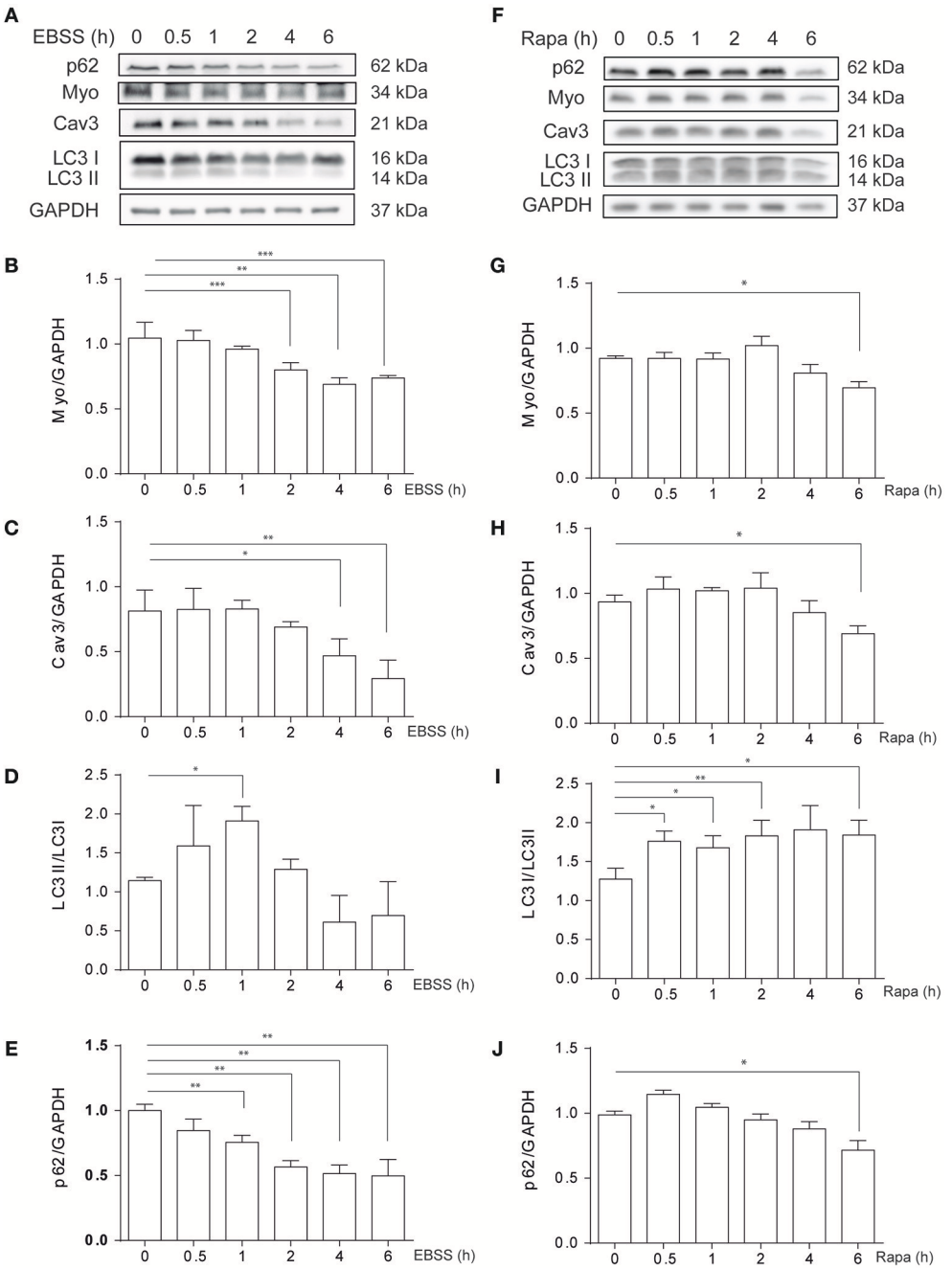

FIGURE 1 | Starvation- and rapamycin-induced autophagy and atrophy in C2C12 myotubes. C2C12 myotube cultures were submitted to starvation with EBSS **(A–E)** or treated with rapamycin 0.1 μM **(F–J)** by 0, 0.5, 1, 2, 4, and 6 h. Subsequently, starvation- and rapamycin- induced atrophy were evaluated by western blot, by evaluating the levels of myo **(A,B,F,G)** and Cav3 **(A,C,F,H)**. Autophagy was evaluated by western blot by assessing the levels of LC3 I and II **(A,D,F,I)**. GAPDH was used as loading control. Representative gels are showed in **(A)** and **(F)** and gels quantifications are depicted in **(B–E)** and **(G–J)**, respectively (mean± S.E.M., $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{**}p < 0.001$).

evaluated by the conversion of LC3 I to LC3 II, in cells down-regulated for PC2 (**Supplementary Figure 1**). Altogether these results show that PC2 is required for starvation- and rapamycin-induced atrophy in an autophagy-independent manner in C2C12 myotubes.

PC2 Overexpression Induces Atrophy in C2C12 Myotubes

Our results showed that PC2 is required for starvation- and rapamycin-induced atrophy in C2C12 myotubes (**Figure 2**). However, if PC2 overexpression is sufficient to induce atrophy in

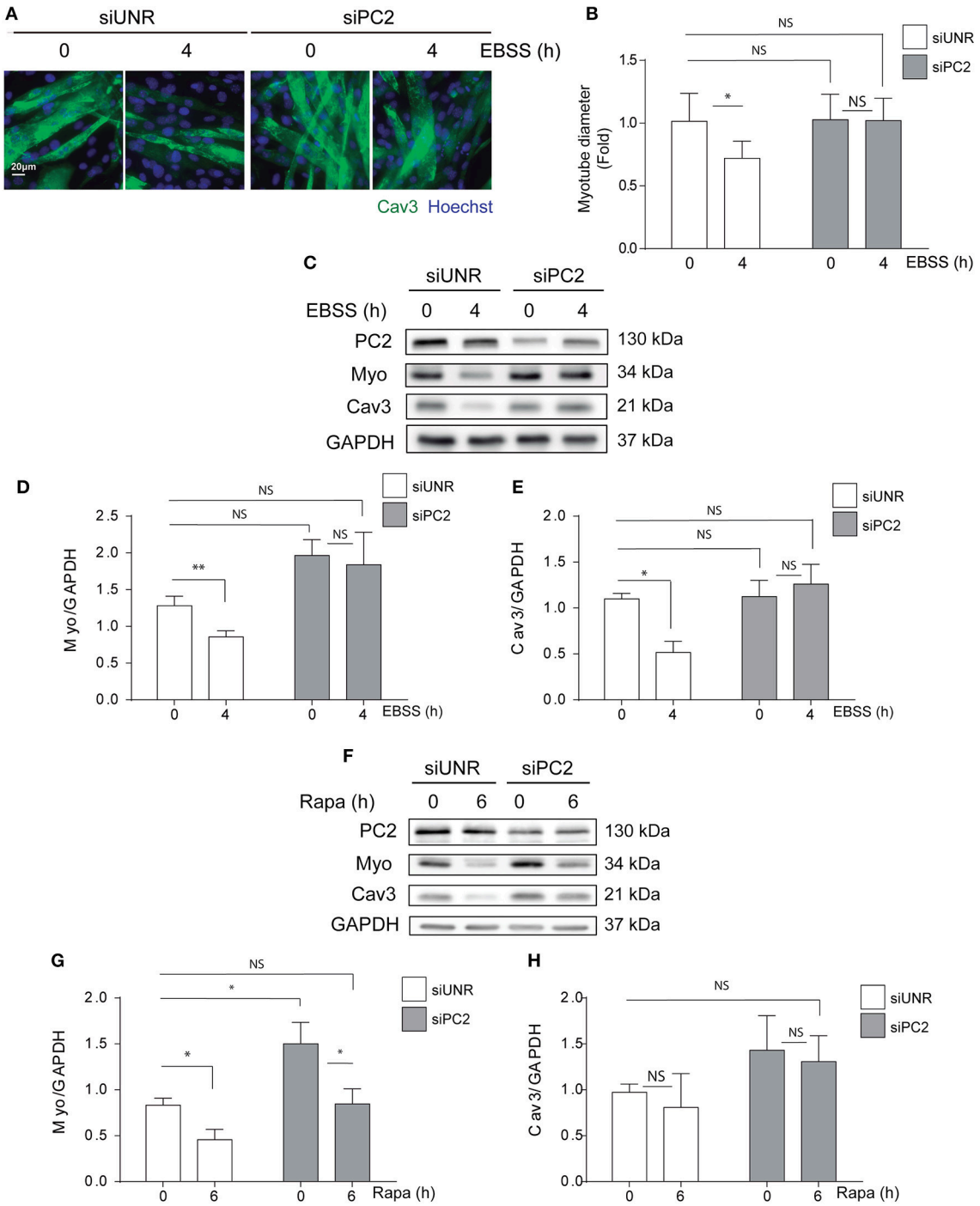

FIGURE 2 | PC2 is required for atrophy but not autophagy in C2C12 myotubes. PC2 was downregulated in C2C12 myotube cultures using a specific siRNA against PC2 (siPC2). An unrelated siRNA (siUNR) was used as control. Subsequently, 48 h post-transfection cells were subjected to nutrient deprivation by incubation in EBSS for 0 and 4 h **(A–D)** or treated with rapamycin 0.1 μM for 0 and 6 h **(F–H)**. **(A,B)** Cells were fixed and immunostaining against caveolin-3 (Cav3) was performed to evaluate myotube diameter by fluorescent microscopy. Nuclei were stained with 1 μg/mL Hoechst 33342. Representative pictures are showed in **(A)** and myotube diameter quantification is represented in **(B)** (mean± S.E.M., $n = 3$, $^{*}p < 0.05$). **(C,D)** Whole lysates were resolved by western blot and Myogenin (Myo), Cav-3, LC3 I, and LC3 II levels were evaluated by the use of specific antibodies. GAPDH was used as a loading control. Representative gels are showed in **(C,F)** and gels quantifications are depicted in **(E,D,H,G)** (mean± S.E.M., $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$).

C2C12 myotubes remains elusive. To this aim, we overexpressed PC2 by the use of the adenovirus, Ad PC2, or control adenovirus, Ad Co, and we evaluated atrophy in C2C12 myotubes. Our data showed that over expression of PC2 induced a reduction in myotubes diameter (**Figures 3A,B**), which correlated with a decrease in the levels of MYO and CAV3 (**Figures 3C–E**). Furthermore, PC2 overexpression did not induce autophagy, as evaluated by LC3 I to LC3 II turnover, indicating that regulation of autophagy is not involved as mechanism by which PC2 regulates atrophy in C2C12 myotubes (**Supplementary Figures 2A,B**). Given that C2C12 myotubes are a syncytium formed by the fusion of multiple cells, it is possible that they might be more resistant to autophagy. Thus, we also evaluated if PC2 induces autophagy in myoblasts. Our results showed that, similar to the results obtained in myotubes, overexpression of PC2 did not induce autophagy in myoblasts (**Supplementary Figures 2C,D**). These data, together with the results depicted in **Figure 2**, indicate that PC2 induces atrophy in C2C12 skeletal muscle cells by an autophagy-independent mechanism.

PC2 Modulates mTOR Pathway in C2C12 Myotubes

It is known that the mTOR and AKT axis not only regulates hypertrophy, but also atrophy in skeletal muscle *in vivo* and *in vitro* (11, 15, 23, 24). Given that it is well-known that starvation affects mTOR (14) and that our results indicate PC2 is required for starvation-induced atrophy, we evaluated if PC2 modulates mTOR signaling in C2C12 myotubes. To this aim, myotubes were submitted to siRNA-mediated down regulation of PC2 or transfected with an unrelated siRNA, siUNR. Then, cells were subjected to starvation with EBSS medium at different time points. Our results show that down regulation of PC2 prevents the decrease in the phosphorylation of the downstream-mTOR proteins, S6, and 4EBP1, when atrophy is induced by starvation (**Figures 4A–C**). Another signaling pathway implicated in the induction of atrophy is the inactivation of AKT with the subsequent translocation from the cytoplasm to the nucleus of the forkhead box O (FoXO) proteins (25, 26). In this regard, our data showed that downregulation of PC2 does not regulate starvation-induced dephosphorylation of AKT on Ser473 (**Supplementary Figures 3A,B**). Altogether, these results suggest that PC2 regulates starvation-induced atrophy via mTORC1 in C2C12 myotubes, independently of AKT (**Figure 4A–C** and **Supplementary Figures 3A,B**).

DISCUSSION

Severe and progressive muscle atrophy is observed in different human pathological conditions and several of the molecular mechanism that control atrophy remains elusive (1–4). Previous studies of our group demonstrated that PC2 positively regulates autophagy and that depending on the stimuli, cell type and tissue the modulation of mTOR is implicated in the process (16, 17). Furthermore, downregulation of PC2 inhibits autophagy induced by nutrient deprivation, rapamycin, and hypertonicity in different types of cells, while its overexpression triggers autophagy (16, 17). The requirement of PC2 for autophagy induction has also been demonstrated *in vivo*, in mouse heart tissues, where starvation-induced autophagy was inhibited in the heart mouse knocked-out for PC2 in cardiomyocytes (17). Given that massive autophagy has been observed in different models of atrophy and that there are no evidences about the role of PC2 in skeletal muscle cell models, we evaluated here if PC2 is regulating atrophy in C2C12 myotubes induced by classical stimuli of autophagy, such as nutrient deprivation or treatment with rapamycin. We observed that starvation and rapamycin induced atrophy in C2C12 myotubes and that PC2 is necessary in this process (**Figures 1**, **2**). Although, other studies have showed that PC2 has a role in the control of autophagy, specifically in human embryonic stem cell-derived cardiomyocytes, neonatal cardiomyocytes, and renal epithelial cells (16, 18, 19) we did not observe that PC2 regulates autophagy in C2C12 myotubes, as assessed by the conversion of LC3 I to LC3 II. Importantly, previous studies that identified a role for PC2 in the regulation of autophagy in renal epithelial cells showed that it is related with pathways activated by fluid flow-induced mechanical stress, where the role of primary cilium has been shown to be key. In this regard, we can speculate that the intracellular location of PC2 as well as the type of stimuli or condition that affects autophagy might differentially affect PC2 function and/or autophagy. Here, our data showed that modulation of atrophy by PC2 is autophagy-independent given that PC2 did not modulate rapamycin-induced LC3 I to LC3 II conversion (**Supplementary Figure 1**) and consistently, autophagy was not altered following PC2 over expression-induced atrophy conditions (**Figure 3** and **Supplementary Figure 2**).

mTOR has a pro-myogenic role given that it positively regulates protein synthesis (14). Indeed, skeletal muscle-specific ablation of Raptor, an adapter protein of the mTORC1, or deletion of the mTORC1 substrate S6 kinase, causes muscle dystrophy, and suppresses muscle growth adaptations to nutrient availability, respectively (27). Given the pro-myogenic relevance of mTOR, we evaluated if PC2 regulates atrophy by modulation of the mTOR pathway. Our results showed that downregulation of PC2 inhibited starvation-induced dephosphorylation of the protein S6, a key downstream target of mTOR, suggesting that regulation of atrophy by PC2 can be mediated by the modulation of mTOR signaling (**Figure 4**). Studies have shown that the role of mTOR not only has been related with the modulation of the size of the muscle fibers but its function has been also observed in other types of cells. Indeed, MDCK cells show a dramatically increase in cell size when the mTOR pathway is activated (28). Interestingly, our results showed that although the down regulation of PC2 attenuates the effects of starvation on the mTOR pathway, however, despite this it was unable to inhibit autophagy induced by rapamycin (**Supplementary Figure 1**), indicating that PC2, by modulating mTOR, and not autophagy, regulates atrophy in C2C12 myotubes.

To elucidate the mechanism by which PC2 regulates atrophy, we evaluated the phosphorylation status of AKT, which controls

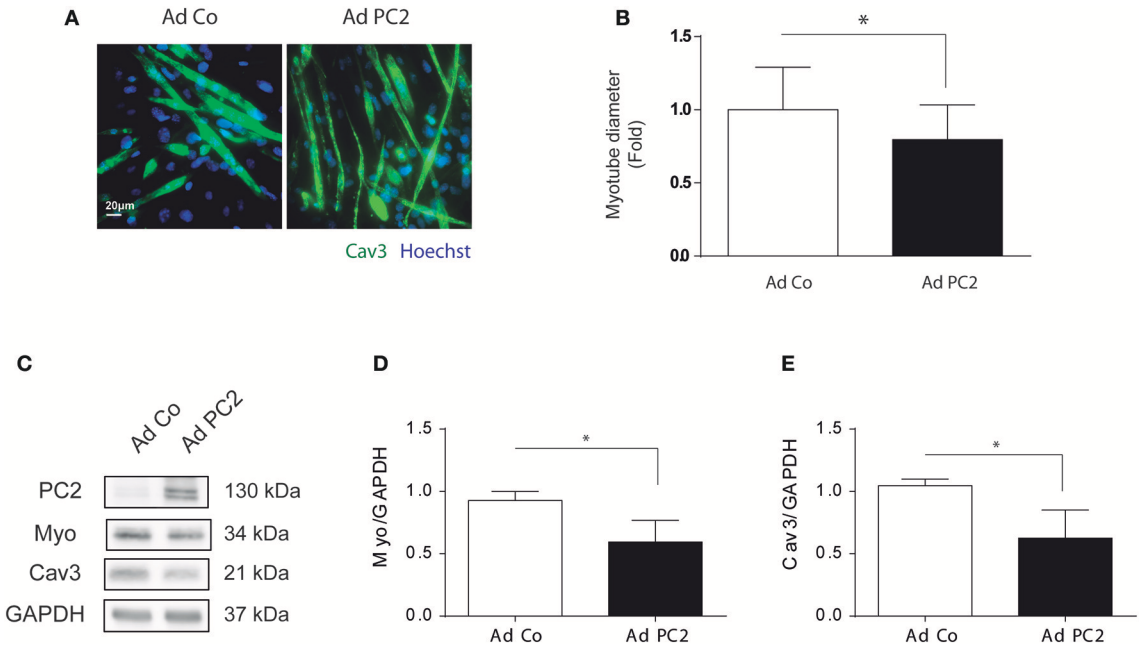

FIGURE 3 | Overexpression of PC2 induces atrophy and autophagy in C2C12 myotubes. PC2 was overexpressed in C2C12 myotubes by adenoviral transduction with Ad PC2 and an empty adenovirus was used as control (Ad Co) **(A–E)**. Twenty-four hours post transduction cells were fixed and immunostained against Caveolin-3 (Cav3), subsequently myotube diameter was evaluated by fluorescent microscopy. Nuclei were stained with 1 μg/mL Hoechst 33342. Representative pictures are showed in **(A)** and myotube diameter quantification is showed in **(B)** (mean ± S.E.M., $n = 3$, $^*p < 0.05$). **(C–E)** Whole lysates were resolved by western blot and Polycystin-2 (PC2), Myogenin (Myo), and Cav3 levels were evaluated by the use of specific antibodies. GAPDH was used as a loading control. Representative gels are showed in **(C)** and gels quantifications are depicted in **(D,E)** (mean ± S.E.M., $n = 3$, $^*p < 0.05$).

the activation of the transcription factor FoXO1, a known regulator of atrophy-related genes expression. Even if the status of phosphorylation of AKT was sensitive to starvation, it was unaffected by PC2 downregulation, indicating that AKT pathway does not participate in the regulation of atrophy by PC2. In this regard, other studies have also shown events of atrophy independent of AKT/FOXO1 pathway, especially in glucocorticoid-induced muscle atrophy (29).

Regarding the function of PC2, it is a Ca^{2+}-permeable ion channels with a relevant role in the maintenance of the cytosolic Ca^{2+} (30). Indeed, mutations in PC2 lead to impaired calcium homeostasis in cardiac muscle which predispose cardiomyopathies *in vivo* (31). Thus, given that PC2 was required for starvation- and rapamycin-induced atrophy and that autophagy was not implicated in the mechanism by which PC2 regulates atrophy, it is possible that calcium microdomains controlled by PC2 may be also required to induce atrophy in C2C12. In fact, agents such as angiotensin II, tumor necrosis factor-α (TNFα) and lipopolysaccharide not only induce muscle atrophy but also a rise in Ca^{2+} which is necessary for both proteolysis and decreasing in protein synthesis in muscle cells (29, 32–34). This work did not evaluate the role of Ca^{2+}. However, it could be addressed in future studies.

In conclusion, all these findings reveal a novel role of PC2 on the regulation of atrophy, which is mediated by the modulation of mTOR in C2C12 myotubes.

MATERIALS AND METHODS

Cell Culture and Treatments

C2C12 myoblasts were purchased in Sigma-Aldrich (Cat N° 91031101-1VL). Cells were grown in DMEM (glucose 4.5 g/L) containing L-glutamine, 110 mg/L sodium pyruvate, 10% FBS, and 10 mM HEPES. Differentiation of C2C12 myoblast to myotubes was performed by culturing cells in DMEM (glucose 4.5 g/L) containing L-glutamine, 110mg/L sodium pyruvate, 10 mM HEPES and 2% horse serum by 1 week. Media, supplements and reagents for cell culture were purchased from Gibco-Invitrogen (Carlsbad, USA). Cells were submitted to nutrient deprivation by culture of cells in Earle's Balanced Salt Solution medium, EBSS (Sigma-Aldrich, St. Louis, USA). Rapamycin and Bafilomycin A1 were purchased in Sigma-Aldrich. All experiments were independently repeated at least three times.

siRNA Transfection and Adenovirus Infection

siRNAs were purchased by Sigma-Aldrich Corporation. An unrelated-siRNA sequence was used as negative control. Lipofectamine iMax (Invitrogen) and Optimem culture medium were used for siRNA transfections. Thirty-six hours after transfection cells were stimulated. Protein quantification of the targeted protein was used to evaluate the efficiency of the different siRNAs. For adenovirus-mediated protein

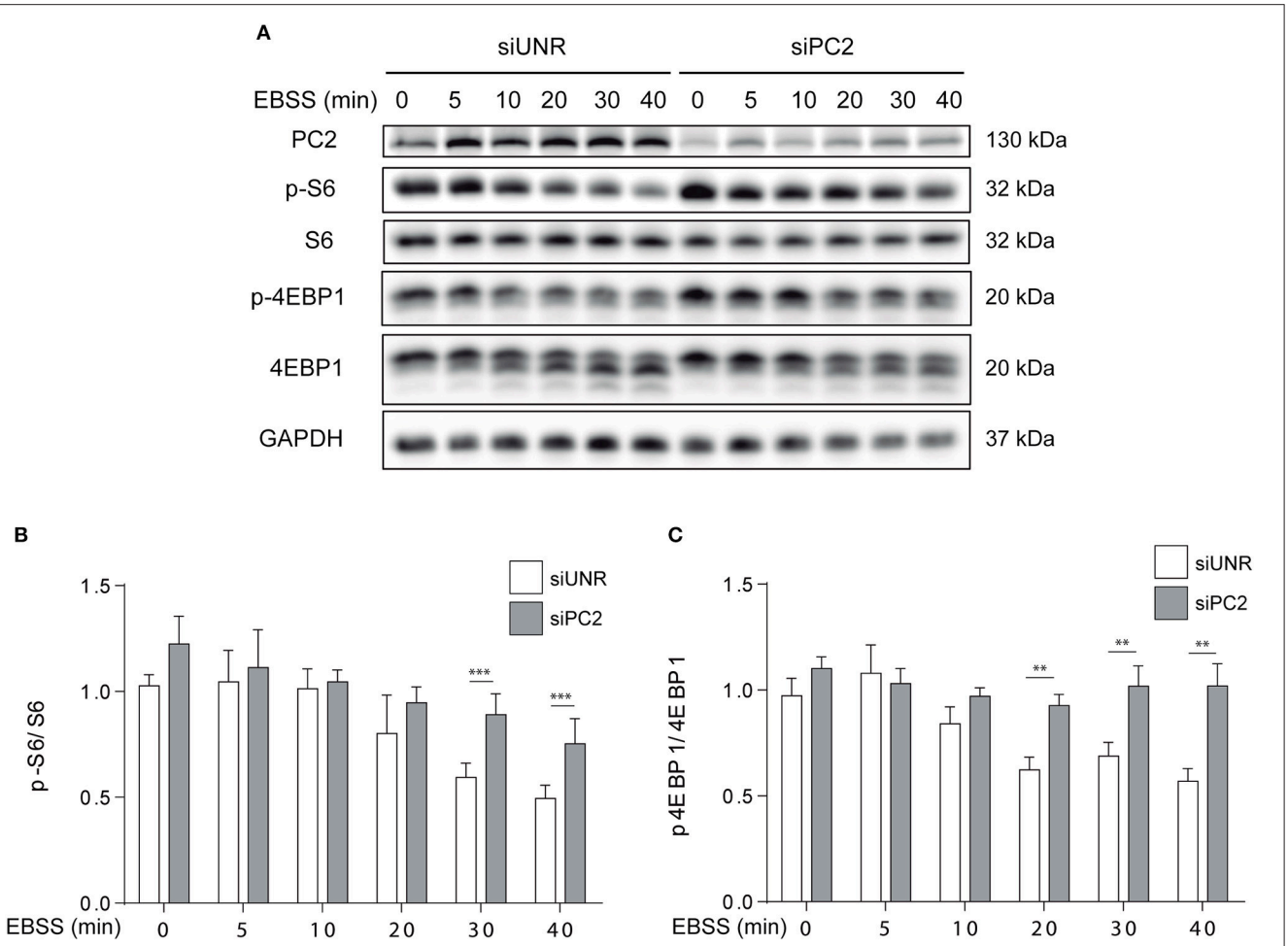

FIGURE 4 | PC2 modulates the mTOR pathway. PC2 was downregulated in C2C12 myotubes using a specific siRNA against PC2 (siPC2) and an unrelated siRNA (siUNR) was used as control **(A–C)**. C2C12 myotubes downregulated for PC2 were submitted to starvation with EBSS for 0, 5, 10, 20, 30, and 40 min **(A–C)**. Then, whole lysates were resolved by western blot and Polycystin-2 (PC2), S6, P-S6 Ser235/236, 4EBP1, and P-4EBP1 Thr37/46 levels were evaluated. GAPDH was used as loading control. Representative gels are showed in **(A)** and relative levels of P-S6/S6 and P-4EBP1/4EBP1 are depicted in the graphs in **(B,C)** (mean ± S.E.M., $n = 3$, $^{**}p < 0.01$, $^{***}p < 0.001$).

overexpression, cells were incubated for 12 h with the AdPC2 adenovirus.

Western Blot Analysis

Protein samples of C2C12 myoblasts cells were prepared in M-PER lysis buffer (Thermo Scientific) supplemented with protease and phosphatase inhibitors (ROCHE). Aliquots of the extracted proteins (∼30 μg/lane) were resolved in 10 or 12% SDS-PAGE gels and then subjected to immunoblotting using antibodies specific for Myogenin (mouse monoclonal IgG clone F5D, cat. n° sc-12732; Santa Cruz Biotechnology), Caveolin-3 (Mouse monoclonal IgG clone 26, cat. n° 610420; BD Bioscience Laboratories™), GAPDH (mouse monoclonal IgG, cat n° MAB274; Chemicon International), 4EBP1(rabbit polyclonal IgG cat n° 9452; Cell Signaling Technology), P-4EBP1 Thr37/46 (rabbit polyclonal IgG clone 236B4, cat n° 2855; Cell Signaling Technology), LC3 I and II (rabbit polyclonal IgG, cat n° 9748; Cell Signaling Technology), p62/SQSTM1 (rabbit polyclonal IgG, cat n°, NBP1-42822; Novus Biologicals), PC2 (rabbit polyclonal IgG clone H-280, cat. n° sc-25749; Santa Cruz Biotechnology), AKT/PKB (mouse monoclonal IgG cat n°2966; Cell Signaling Technology), P-AKT/PKB Ser473 (rabbit polyclonal IgG cat n°4060; Cell Signaling Technology), S6 (mouse monoclonal IgG clone 54D2, cat n° 2317; Cell Signaling Technology), P-S6 Ser235/236 (rabbit polyclonal IgG clone 236B4, cat n° 2211; Cell Signaling Technology). Then, membranes were incubated with secondary goat anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (SouthernBiotech, Birmingham, USA) prior to revelation by means of ECL Detection Kit (Amersham Pharmacia, Pittsburgh, USA). Gels were analyzed and quantified with the software ImageJ (http://rsb.info.nih.gov/ij/).

Confocal and Fluorescence Microscopy

Following treatments cells were washed twice with ice-cold PBS, fixed in paraformaldehyde (4% w/v) for 15 min, permeabilized with Triton 0.1%, PBS for 10 min and blocked in 3% BSA-PBS for 1 h. Nuclei were counterstained with Hoechst 33342 (1 μg/mL) (Molecular Probes). Fluorescence and confocal fluorescence

images were captured using an IRE2 microscope equipped with a DC300F camera (both from Leica Microsystems GmbH, Wetzlar, Germany) and an LSM 510 microscope (Carl Zeiss, Jena, Germany). Images were analyzed with the software ImageJ (http://rsb.info.nih.gov/ij/).

Myotube Diameter Measurement

Myoblasts were differentiated into myotubes by culturing cells in DMEM containing 2% horse serum for 1 week. Then, myotubes were used to evaluate the cell diameter. Briefly, images were obtained with an epifluorescent microscope (Nikon Eclipse TI) and analyzed by a transverse line across the myotube. Myotubes with more than three nuclei were used for diameter measurements. We draw the line of distance across the myotube, which represents the myotube diameter (μm). At least three diameters per myotube were measured and at least 100 myotubes per well were analyzed using ImageJ Software. Data is presented as fold changes relative to control levels.

RESULTS AND STATISTICAL ANALYSIS

Results are shown as mean ± S.E.M. from at least three independent experiments. Statistical analyses were performed using Student's *t*–test when analyzing two independent groups, one-way ANOVA for more than two independent groups and two-way ANOVA for two independent variables followed by a Sidak *post-hoc* test (GraphPad Software Inc.). $P < 0.05$ was considered to be statistically significant.

AUTHOR CONTRIBUTIONS

CK, DP-O, CH, NH-M, and AM-B performed the experiments. MH-C performed experiments and statistical analysis in the second round of revisions. SL and MB contributed to the experimental design and manuscript preparation. EM contributed to the experimental design and image analysis. VP performed mitochondrial morphology studies in the second round of revisions. RT and AC conceived the project and contributed to manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00280/full#supplementary-material

Supplementary Figure 1 | PC2 is not required for rapamycin-induced autophagy. PC2 was downregulated in C2C12 myotubes using a specific siRNA against PC2 (siPC2) **(A,B)**. Unrelated siRNA (siUNR) was used as control. C2C12 myotubes downregulated for PC2 were treated with rapamycin 0.1 μM **(A–B)** for 0 and 6 h. Whole lysates were resolved by western blot and Polycystin-2 (PC2), LC3 I and LC3II were evaluated. GAPDH was used as loading control. Representative gel is showed in **(A)** and the relative levels of LC3 II/LC3 I are depicted in the graph in **(B)** (mean ± S.E.M., $n = 3$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Supplementary Figure 2 | Overexpression of PC2 does not regulate autophagy both in C2C12 myotubes and myoblasts. PC2 was overexpressed in C2C12 myotubes **(A,B)** or myoblast **(C,D)** for 24 h by using an adenovirus for PC2 (Ad PC2). Empty adenovirus was used as control (Ad Co). Whole lysates were resolved by western blot and Polycystin-2 (PC2), LC3 I, and LC3II were evaluated. GAPDH was used as loading control. Representative gels for myotubes and myoblasts are showed in **(A)** and **(C)**, respectively. Relative levels of LC3 I to LC3 II turnover in myotubes and myoblasts are depicted in the graph in **(C)** and **(D)**, respectively (mean ± S.E.M., $n = 3$).

Supplementary Figure 3 | PC2 does not regulate AKT/PBK pathway. PC2 was downregulated in C2C12 myotubes by transfection with a specific siRNA against PC2 (siPC2). Unrelated siRNA (siUNR) was used as control. Then, cultures were submitted to starvation with EBSS **(A,B)** for 0, 5, 10, 20, 30, and 40 min. C2C12 myotubes whole lysates were resolved by western blot and total AKT/PKB and P-AKT/PKB Ser473 levels were evaluated by the use of specific antibodies. GAPDH was used as loading control. Representative gels are showed in **(A)** and relative levels of P-AKT/AKT are depicted in the graphs in **(B)** (mean ± S.E.M., $n = 3$).

REFERENCES

1. Bonaldo P, Sandri M. Cellular and molecular mechanisms of muscle atrophy. *Dis Model Mech.* (2013) 6:25–39. doi: 10.1242/dmm.010389
2. Erlandson KM, Allshouse AA, Jankowski CM, MaWhinney S, Kohrt WM, Campbell TB. Functional impairment is associated with low bone and muscle mass among persons aging with HIV infection. *J Acquir Immune Defic Syndr.* (2013) 63:209–15. doi: 10.1097/QAI.0b013e318289bb7e
3. Wasserman P, Segal-Maurer S, Rubin DS. High prevalence of low skeletal muscle mass associated with male gender in midlife and older HIV-infected persons despite CD4 cell reconstitution and viral suppression. *J Int Assoc Provid AIDS Care.* (2014) 13:145–52. doi: 10.1177/2325957413495919
4. Sandri M. Protein breakdown in cancer cachexia. *Semin Cell Dev Biol.* (2016) 54:11–9. doi: 10.1016/j.semcdb.2015.11.002
5. Porporato PE. Understanding cachexia as a cancer metabolism syndrome. *Oncogenesis.* (2016) 5:e200. doi: 10.1038/oncsis.2016.3

6. Milan G, Romanello V, Pescatore F, Armani A, Paik JH, Frasson L, et al. Regulation of autophagy and the ubiquitin-proteasome system by the FoxO transcriptional network during muscle atrophy. *Nat Commun.* (2015) 6:6670. doi: 10.1038/ncomms7670
7. Masiero E, Agatea L, Mammucari C, Blaauw B, Loro E, Komatsu M, et al. Autophagy is required to maintain muscle mass. *Cell Metab.* (2009) 10:507–15. doi: 10.1016/j.cmet.2009.10.008
8. Troncoso R, Paredes F, Parra V, Gatica D, Vasquez-Trincado C, Quiroga C, et al. Dexamethasone-induced autophagy mediates muscle atrophy through mitochondrial clearance. *Cell Cycle.* (2014) 13:2281–95. doi: 10.4161/cc.29272
9. Fan J, Kou X, Jia S, Yang X, Yang Y, Chen N. Autophagy as a potential target for sarcopenia. *J Cell Physiol.* (2016) 231:1450–9. doi: 10.1002/jcp.25260
10. Penna F, Costamagna D, Pin F, Camperi A, Fanzani A, Chiarpotto EM, et al. Autophagic degradation contributes to muscle wasting in cancer cachexia. *Am J Pathol.* (2013) 182:1367–78. doi: 10.1016/j.ajpath.2012.12.023
11. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, et al. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy *in vivo*. *Nat Cell Biol.* (2001) 3:1014–9. doi: 10.1038/ncb1101-1014
12. Risson V, Mazelin L, Roceri M, Sanchez H, Moncollin V, Corneloup C, et al. Muscle inactivation of mTOR causes metabolic and dystrophin defects leading to severe myopathy. *J Cell Biol.* (2009) 187:859–74. doi: 10.1083/jcb.200903131
13. Troncoso R, Vicencio JM, Parra V, Nemchenko A, Kawashima Y, Del Campo A, et al. Energy-preserving effects of IGF-1 antagonize starvation-induced cardiac autophagy. *Cardiovasc Res.* (2012) 93:320–9. doi: 10.1093/cvr/cvr321
14. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell.* (2017) 169:361–71. doi: 10.1016/j.cell.2017.03.035
15. Yoon MS. mTOR as a key regulator in maintaining skeletal muscle mass. *Front Physiol.* (2017) 8:788. doi: 10.3389/fphys.2017.00788
16. Pena-Oyarzun D, Troncoso R, Kretschmar C, Hernando C, Budini M, Morselli E, et al. Hyperosmotic stress stimulates autophagy via polycystin-2. *Oncotarget.* (2017) 8:55984–97. doi: 10.18632/oncotarget.18995
17. Criollo A, Altamirano F, Pedrozo Z, Schiattarella GG, Li DL, Rivera-Mejias P, et al. Polycystin-2-dependent control of cardiomyocyte autophagy. *J Mol Cell Cardiol.* (2018) 118:110–21. doi: 10.1016/j.yjmcc.2018.03.002
18. Orhon I, Dupont N, Zaidan M, Boitez V, Burtin M, Schmitt A, et al. Primary-cilium-dependent autophagy controls epithelial cell volume in response to fluid flow. *Nat Cell Biol.* (2016) 18:657–67. doi: 10.1038/ncb3360
19. Lu J, Boheler KR, Jiang L, Chan CW, Tse WW, Keung W, et al. Polycystin-2 plays an essential role in glucose starvation-induced autophagy in human embryonic stem cell-derived cardiomyocytes. *Stem Cells.* (2018) 36:501–13. doi: 10.1002/stem.2764
20. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy.* (2016) 12:1–222. doi: 10.1080/15548627.2015.1100356
21. Dai XQ, Perez PL, Soria G, Scarinci N, Smoler M, Morsucci DC, et al. External Ca(2+) regulates polycystin-2 (TRPP2) cation currents in LLC-PK1 renal epithelial cells. *Exp Cell Res.* (2017) 350:50–61. doi: 10.1016/j.yexcr.2016.11.004
22. Liu X, Vien T, Duan J, Sheu SH, DeCaen PG, Clapham DE. Polycystin-2 is an essential ion channel subunit in the primary cilium of the renal collecting duct epithelium. *Elife.* (2018) 7:e33183. doi: 10.7554/eLife.33183
23. Pallafacchina G, Calabria E, Serrano AL, Kalhovde JM, Schiaffino S. A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification. *Proc Natl Acad Sci USA.* (2002) 99:9213–8. doi: 10.1073/pnas.142166599
24. Schiaffino S, Dyar KA, Ciciliot S, Blaauw B, Sandri M. Mechanisms regulating skeletal muscle growth and atrophy. *FEBS J.* (2013) 280:4294–314. doi: 10.1111/febs.12253
25. Cohen S, Lee D, Zhai B, Gygi SP, Goldberg AL. Trim32 reduces PI3K-Akt-FoxO signaling in muscle atrophy by promoting plakoglobin-PI3K dissociation. *J Cell Biol.* (2014) 204:747–58. doi: 10.1083/jcb.201304167
26. Kang SH, Lee HA, Kim M, Lee E, Sohn UD, Kim I. Forkhead box O3 plays a role in skeletal muscle atrophy through expression of E3 ubiquitin ligases MuRF-1 and atrogin-1 in Cushing's syndrome. *Am J Physiol Endocrinol Metab.* (2017) 312:E495–507. doi: 10.1152/ajpendo.00389.2016
27. Bentzinger CF, Romanino K, Cloetta D, Lin S, Mascarenhas JB, Oliveri F, et al. Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy. *Cell Metab.* (2008) 8:411–24. doi: 10.1016/j.cmet.2008.10.002
28. Boehlke C, Kotsis F, Patel V, Braeg S, Voelker H, Bredt S, et al. Primary cilia regulate mTORC1 activity and cell size through Lkb1. *Nat Cell Biol.* (2010) 12:1115–22. doi: 10.1038/ncb2117
29. Wang XJ, Xiao JJ, Liu L, Jiao HC, Lin H. Excessive glucocorticoid-induced muscle MuRF1 overexpression is independent of Akt/FoXO1 pathway. *Biosci Rep.* (2017) 37:BSR20171056. doi: 10.1042/BSR20171056
30. Lemos FO, Ehrlich BE. Polycystin and calcium signaling in cell death and survival. *Cell Calcium.* (2018) 69:37–45. doi: 10.1016/j.ceca.2017.05.011
31. Paavola J, Schliffke S, Rossetti S, Kuo IY, Yuan S, Sun Z, et al. Polycystin-2 mutations lead to impaired calcium cycling in the heart and predispose to dilated cardiomyopathy. *J Mol Cell Cardiol.* (2013) 58:199–208. doi: 10.1016/j.yjmcc.2013.01.015
32. Doyle A, Zhang G, Abdel Fattah EA, Eissa NT, Li YP. Toll-like receptor 4 mediates lipopolysaccharide-induced muscle catabolism via coordinate activation of ubiquitin-proteasome and autophagy-lysosome pathways. *FASEB J.* (2011) 25:99–110. doi: 10.1096/fj.10-164152
33. De Larichaudy J, Zufferli A, Serra F, Isidori AM, Naro F, Dessalle K, et al. TNF-alpha- and tumor-induced skeletal muscle atrophy involves sphingolipid metabolism. *Skelet Muscle.* (2012) 2:2. doi: 10.1186/2044-5040-2-2.
34. Mirza KA, Tisdale MJ. Role of Ca2+ in proteolysis-inducing factor (PIF)-induced atrophy of skeletal muscle. *Cell Signal.* (2012) 24:2118–22. doi: 10.1016/j.cellsig.2012.07.016

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Autophagic and Proteasomal Mediated Removal of Mutant Androgen Receptor in Muscle Models of Spinal and Bulbar Muscular Atrophy

*Maria Elena Cicardi[1†], Riccardo Cristofani[1†], Valeria Crippa[1], Veronica Ferrari[1], Barbara Tedesco[1], Elena Casarotto[1], Marta Chierichetti[1], Mariarita Galbiati[1], Margherita Piccolella[1], Elio Messi[1], Serena Carra[2], Maria Pennuto[3,4,5], Paola Rusmini[1] and Angelo Poletti[1,6]**

[1] Dipartimento di Scienze Farmacologiche e Biomolecolari (DiSFeB), Dipartimento di Eccellenza 2018-2022, Centro di Eccellenza sulle Malattie Neurodegenerative, Università degli Studi di Milano, Milan, Italy, [2] Dipartimento di Scienze Biomediche, Metaboliche e Neuroscienze, Centro Interdipartimentale di Neuroscienze e Neurotecnologie (CfNN), Università degli Studi di Modena e Reggio Emilia, Modena, Italy, [3] Department of Neurosciences, Neuromuscular Center, University of Padova, Padova, Italy, [4] Department of Biomedical Sciences, University of Padova, Padova, Italy, [5] Dulbecco Telethon Institute, Centre for Integrative Biology (CIBIO), University of Trento, Povo, Italy, [6] Centro InterUniversitario sulle Malattie Neurodegenerative, Università degli Studi di Firenze, Milan, Italy

Spinal and bulbar muscular atrophy (SBMA) is an X-linked motoneuron disease (MND) caused by a mutant androgen receptor (AR) containing an elongated polyglutamine (polyQ) tract. ARpolyQ toxicity is triggered by androgenic AR ligands, which induce aberrant conformations (misfolding) of the ARpolyQ protein that aggregates. Misfolded proteins perturb the protein quality control (PQC) system leading to cell dysfunction and death. Spinal cord motoneurons, dorsal root ganglia neurons and skeletal muscle cells are affected by ARpolyQ toxicity. Here, we found that, in stabilized skeletal myoblasts (s-myoblasts), ARpolyQ formed testosterone-inducible aggregates resistant to NP-40 solubilization; these aggregates did not affect s-myoblasts survival or viability. Both wild type AR and ARpolyQ were processed via proteasome, but ARpolyQ triggered (and it was also cleared via) autophagy. ARpolyQ reduced two pro-autophagic proteins expression (BAG3 and VCP), leading to decreased autophagic response in ARpolyQ s-myoblasts. Overexpression of two components of the chaperone assisted selective autophagy (CASA) complex (BAG3 and HSPB8), enhanced ARpolyQ clearance, while the treatment with the mTOR independent autophagy activator trehalose induced complete ARpolyQ degradation. Thus, trehalose has beneficial effects in SBMA skeletal muscle models even when autophagy is impaired, possibly by stimulating CASA to assist the removal of ARpolyQ misfolded species/aggregates.

Keywords: autophagy, chaperones, protein aggregation, androgen receptor, motoneuron disease

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Heather Lynn Montie, Philadelphia College of Osteopathic Medicine, United States

***Correspondence:**
Angelo Poletti
angelo.poletti@unimi.it

†These authors have contributed equally to this work

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INTRODUCTION

Spinal and bulbar muscular atrophy (SBMA) is an inherited X-linked motoneuron disease (MND) linked to a CAG triplet repeat expansion present in the exon 1 of the gene coding for the androgen receptor (AR) (1). Because of that, the AR protein carries an elongated polyglutamine (polyQ) tract in its N-terminus. In normal individuals, the polyQ tract is comprised between 9 and 37 Qs with an average value of 22, but SBMA patients have a polyQ tract longer than 38 Qs (ARpolyQ) with a maximum of 68 Qs observed so far in some patients affected by a pathology characterized by an unusual early onset (2, 3). The physiological role of the polyQ tract is still largely debated, but the region could act as a transcriptional regulatory domain (4–6). Other eight totally unrelated proteins presenting expanded CAG/polyQ repeats have been involved in neurodegenerative diseases (CAG/polyQ diseases) (7). Thus, the polyQ expansion is likely to confer a gain of neurotoxic function(s) to these mutant proteins. Indeed, the polyQ tract induces the acquisition of aberrant protein conformation (misfolding) to the host proteins making them prone to aggregate. Misfolded proteins affect the protein quality control (PQC) system functioning and, in SBMA, this event occurs in the cells expressing high levels of ARpolyQ. In fact, AR is abundantly expressed in motoneurons located in the anterior horns of the spinal cord and in the brain stem, as well as in sensory neurons of the dorsal root ganglia. These neurons degenerate in SBMA leading to atrophy of bulbar, facial and limb muscles, and in sensory function alterations (8–14). Also, non-neuronal cells, like the motoneuron-controlled skeletal muscle cells, are directly affected by mutant protein toxicity. Indeed, even if originally classified as a typical MND, due to the relevant involvement of muscle tissue, SBMA has been reclassified as neuromuscular disease (15–26). The involvement of the muscle cells is rather complex. The atrophy of muscle cells may result from the loss of innervation arising from affected motoneurons and/or may be a direct consequence of ARpolyQ proteotoxicity on skeletal muscle cells. In fact, like spinal cord motoneurons and dorsal root ganglia neurons, also skeletal muscle cells are post-mitotic cells highly sensitive to the presence of misfolded species of ARpolyQ (27). Several evidences obtained initially in SBMA animal models support the direct involvement of muscle in the pathogenesis of SBMA, since the inhibition of AR production selectively in muscle correlates with an amelioration of the phenotype in mice; this notion has been proposed to be valid also in SBMA patients (24, 28, 29). Even human wild type AR (wtAR) overexpressed in mouse skeletal muscle induces several alterations normally observed in SBMA (16, 18, 30, 31). In addition, the downregulation of ARpolyQ levels specifically in skeletal muscle, by mean of antisense oligonucleotides (ASOs), results in prolonged survival in different SBMA mouse models, proving ARpolyQ direct action on muscle (25, 26, 32, 33); also, the restricted overexpression of ARpolyQ in muscle cells determines a delay of the SBMA onset in mouse models. Moreover, muscle samples from SBMA patients show dysregulation of several important pathways such as mitochondrial turnover, or the neuromuscular transmission at birth with an increased expression of the neonatal isoform of acetylcholine receptor (34). A major aspect of SBMA is that castration completely rescues SBMA phenotype in male mice, ascribing SBMA onset to testosterone activation of ARpolyQ (35–38), even if some early symptoms could appear in an androgen-independent manner (39). Muscle is the typical direct target of the anabolic androgenic activity of the AR (40, 41), and, thus, testosterone-triggered ARpolyQ toxicity may sensitize skeletal muscle cells to "toxic" ARpolyQ conformations, which cause ARpolyQ aggregation. In addition, testosterone induces the translocation of misfolded ARpolyQ into the nucleus where the protein exerts most of its toxicity (42, 43). These aggregates may not be toxic *per se* (44), but their presence in cell environment can lead to many cellular dysfunctions. However, misfolded ARpolyQ are likely to be formed soon after the release from HSPs which occurs in the cell cytoplasm, and there is the possibility to clear them as soon as they are formed prior to their migration into the cell nuclei. A typical cytoplasmic degradative process which may prevent misfolded ARpolyQ accumulation, or aberrant nuclear migration, is autophagy. Unfortunately, in the cytoplasm the ARpolyQ protein may block the autophagic flux due to misfolded proteins overload (45–51). Autophagy is considered one of the most important degradative system in cells, since its impairment in neurons leads to their death (52, 53). Autophagy is based on the formation of autophagosomes that entrap the waste material which will be then degraded when autophagosomes fuse with lysosomes (54). Indeed, by using trehalose, a well-known activator of the autophagy master regulator transcription factor EB (TFEB) (55, 56), to restore a normal autophagic flux in SBMA neuronal models, we found an improved clearance of misfolded ARpolyQ and the prevention of its aggregation (49, 51, 56), particularly in motoneuron (57–59).

The importance of a functional autophagy flux in SBMA is also sustained by several studies performed in animal and cell models of SBMA (50, 60, 61). In particular, autophagy is dysregulated in muscles of AR113Q knock-in SBMA mice (19, 22, 26), and this dysregulation includes alteration of TFEB, and its physiological antagonist ZKSCAN3 (22), as well as TFEB-target genes (coding for LC3, VPS11, VPS18 and LAMP1), both in mice and in patients (22). Notably, the inhibition of BECN1/Beclin1-mediated autophagy activation in AR113Q knock-in SBMA mice reduces skeletal muscle atrophy, extends survival and improves the phenotype, while over-activation of autophagy worsens phenotype (19). Thus, a crucial point when considering autophagy is that its levels of activation must be finely tuned, and, thus, any autophagy stimulator must be able to prevent accumulation of harmful material preserving cell's functionality. In this scenario, autophagic clearance of ARpolyQ in skeletal muscle, and how this is related to alternative degradative systems could have a high relevance. However, autophagy works in conjunction with the ubiquitin-proteasome system (UPS) in the removal of misfolded ARpolyQ, and its aggregated forms.

Interestingly, skeletal muscles of SBMA mice also display a high activation of *Tgfb1*, *Ppargc1a*, *Pax7*, *Myog*, E2-ubiquitin ligase *Ube2q1*, but not of *Myod*, and of two E3-ligases (*Trim63/Murf*-1 and *Cul3*). We found that the skeletal muscle of SBMA mice are characterized by a dramatic perturbation of several components of the autophagic pathways (*Becn-1*,

Atg10, *Sqstm1/p62*, *Lc3*), particularly those involved in the peculiar autophagic process now recognized as chaperone-assisted selective autophagy (CASA) (56, 61–72), like the CASA complex components: the small heat shock protein (HSP) B8 and BAG3, which in cooperation with the co-chaperone BAG1 control the correct routing of misfolded proteins to clearance (61). The *Hspb8, Bag3* and *Bag1* gene are all iper-induced in skeletal muscle of SBMA mice, and the *Bag3:Bag1* ratio is increased in these muscles (73). Of note, the equilibrium between UPS and autophagy is critical to maintain the regular misfolded ARpolyQ clearance in SBMA (61). The molecular players regulating the equilibrium that re-routes substrates to UPS or autophagy are BAG1, which mediates UPS clearance of clients, and BAG3 which controls autophagic clearance of clients (46, 48, 49, 61, 68, 72, 74). BAG3 interacts (in a 2:1 ratio) with HSPB8, and the complex reduces ARpolyQ aggregation, by enhancing its solubility and clearance acting as an autophagy facilitator (49, 61). In this process HSPB8/BAG3 complex needs to interact with HSC70/CHIP dimer and the client misfolded protein, allowing its ubiquitination for SQSTM1/p62-mediated insertion into autophagosomes (63, 65). Only few studies aimed to unravel the involvement of the HSPB8-BAG3 and BAG1 systems in SBMA skeletal muscle (73), but the identification of specific autophagy related molecular target might represent a therapeutic valuable strategy for counteracting ARpolyQ toxicity (73). Notably, both *HSPB8* and *BAG3* mutations have been linked to neuromuscular disorders suggesting that they may be deeply involved in the regulation and in the control of the proteotoxic response of muscle cells (70, 71, 75–79).

For all these reasons, in this study, we have provided an extensive characterization of the autophagic activation, the role of the CASA complex and the HSPB8/BAG3 machinery as well as of the BAG1 co-chaperone in the PQC system response in a SBMA muscle cellular model.

MATERIALS AND METHODS

Chemicals

Testosterone; Z-Leu-Leu-Leu-al or MG132; Bafilomycin A1 from *Streptomyces griseus*; D-(+)-Trehalose dihydrate were all obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell Cultures, Treatments, and Transfection

Immortalized mouse myoblast C2C12, stably transfected, respectively, with cDNA encoding the human full length wt AR (with 24 Qs = ARQ24), or the mutant AR (with elongated polyQ of 100 Qs = ARQ100), were obtained by infection with the Lentiviral vector #945.PCCL.sin.cPPT.SV40ployA.eGFP.minCMV.hPGK.deltaLN-GFR containing the human cDNA encoding the ARQ24 or the ARQ100 (s-myoblasts) (80). After transfection, cells were sorted using the GFP fluorescence to identify positive cells. Sorted cells of both lines were cultured with DMEM high glucose medium (Euroclone, Pero, MI, Italy) supplemented with 1 mM glutamine (Euroclone), (30 μg/mL) penicillin [SERVA, Electrophoresis GmbH, Heidelberg, Germany (64μg/mL)] streptomycin (SERVA), and 10% charcoal-stripped fetal bovine serum (CS-FBS) (GIBCO, Thermo Scientific Life Sciences Research, Waltham, MA, USA), to deplete hormones contained in the serum. Basal C2C12 cells were grown in medium containing unstripped serum. Testosterone was added in presence of CS-FBS. Cells were regularly maintained at 37°C, with 5% CO_2, and propagated after trypsin (Euroclone) dissociation as previously described (81). Cells were treated with testosterone (10 nM) for 48 h (ethanol was used as control); MG132 (10 μM) for 16 h (DMSO was used as control); Bafilomycin A1 from *Streptomyces griseus* (100 μM) for 16 h (DMSO was used as control); D-(+)-Trehalose dihydrate (100 mM) for 48 h (diluted directly in the culture medium), as detailed in figure legends.

Lipofectamine® 2000 Transfection Reagent (Thermo Scientific Life Sciences Research) was used to transfect cells, using 2 μL for transfecting 1 μg of DNA. 12-well plates were transfected with 1 μg of DNA, while 24-well plate were transfected with 0.5 μg of DNA. After 5 h, medium was replaced.

The following plasmids were used: p5HBhARQ112 (kindly provided by Dr. A.P. Lieberman, University of Michigan, Ann Harbor) here referred as ARQ112; pARQ16ΔHA, pARQ112ΔHA (kindly provided by Dr. Diane Merry, Thomas Jefferson University, Philadelphia); pCI-HSPB8 encoding human HSPB8, pCI-neo-6xHisBAG3 encoding the full-length form of human BAG3 and pCDNA/HA-BAG1 encoding the human BAG1, were all kindly provided by Prof. H. H. Kampinga (Groeningen University, Groeningen, The Netherlands); pEGFP-N1 (Clontech-Takara Bio, Saint-Germain-en-Laye, France) was utilized to determine transfection efficiency.

PBS and NP-40 Protein Extraction

PBS extracts: cells were plated in 12-well plate at a density of 65,000 cells/well, and the day after plating, cells were transfected and/or treated. At the end of the experiment, cells were harvested, centrifuged (100 x g; 6 min; 4°C), and diluted in 60μL of PBS (Euroclone) added of protease inhibitor cocktail (Sigma-Aldrich), containing individual components including AEBSF at 104 mM, Aprotinin at 80 μM, Bestatin at 4 mM, E-64 at 1.4 mM, Leupeptin at 2 mM and Pepstatin A at 1.5 mM. After slight sonication using Bandelin Sonoplus Ultrasonic Homogenizers –HD 2070, protein content of each sample was quantified by bicinchoninic acid (BCA) assay (Euroclone).

NP-40 extracts: cells were plated in 6-well plate at a density of 130,000 cells/well. After treatments, cells were harvested, centrifuged (100 x g; 6 min; 4°C), and diluted in 65 μL in NP-40 extraction buffer (composition: 150 mM NaCl (Sigma-Aldrich); 20 mM TrisBase (Sigma-Aldrich); 0.5% Nonidet P-40 (NP-40) (Sigma-Aldrich); 1,5 mM MgCl2 (Sigma-Aldrich); 3% Glycerol (Sigma-Aldrich), pH 7.4), added of protease inhibitors [complete EDTA-free Tablet 25X (Sigma-Aldrich)], and 1 mM 1,4-Dithiotreitol (Sigma-Aldrich). Cells were lysed by passage in syringe (27 gauges). Samples were then centrifuged (16,000 x g; 15 min; 4°C). Supernatants were transferred in new tubes, and the pellets were rinsed in 65 μL of NP-40 extraction buffer. Protein content of the NP-40 soluble fraction was quantified by BCA assay (Euroclone). The insoluble fraction was sonicated following the same protocol described above.

Filter Retardation Assay

Filter retardation assay (FRA) was performed using Bio-Dot SF Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA). Six micrograms of both PBS and NP-40 soluble extracts were loaded on a cellulose acetate membrane with pores of 0.22 μm. For NP-40 insoluble extracts, the amount to be loaded was calculated as equal volume to NP-40 soluble extracts. After loading, the samples onto the cellulose acetate membrane, vacuum was applied at the apparatus and protein suspension was filtered. Proteins were fixed at the membrane using a 20% methanol solution, and the membrane was incubated for 1 h at RT in blocking solution [5% non-fat dried milk (Euroclone) in TBS-T 1X]. The membrane was then incubated with rabbit polyclonal anti-AR antibody (AR-H280, Santa-Cruz, sc-13162; dilution 1:1,000 in blocking solution) for at least 2 h at RT. After two washes with 1X TBS-T, the membrane was incubated for 1 h at RT with goat anti-rabbit HRP-conjugate secondary antibody (Santa Cruz Biotechnology, sc-2004; dilution 1:5,000 in 1X TBS-T). After three washes in 1X TBS-T signal was revealed with Clarity™ Western ECL Blotting Substrate (Bio-Rad) and optical density was acquired by ChemiDoc XRS System (Bio-Rad). Results were analyzed using Prism 5.0. Sample variations were related alternatively to ARQ24 (EtOH) or ARQ100 (EtOH). Statistical differences were obtained applying the two-way ANOVA test followed by Bonferroni *post-hoc* test. Each experiment was replicated three times, and each bar represents mean ± SEM of three independent biological replicates.

Western Blot Analysis

Western blot experiments were performed using 10% polyacrylamide gels. To visualize AR protein, 15 μg of each PBS extract or 30 μg of each NP-40 soluble and insoluble extracts were loaded on gels. After electrophoresis, proteins were transferred over night at 4°C on nitrocellulose membrane (Bio-Rad). Membrane was then incubated 1 h at RT in blocking solution, and then overnight at 4°C with primary antibody diluted in blocking solution (5% dried non-fat milk (Euroclone) in 1X T-BST). After two washes with 1X TBS-T, the membrane was incubated 1 h at RT with secondary antibody diluted in 1X TBS-T. Signal was revealed using Clarity™ Western ECL Blotting Substrate (Bio-Rad) and images were acquired by ChemiDoc XRS System (Bio-Rad) as described for FRA. The following primary antibody were used: rabbit polyclonal AR-H280 antibody (Santa-Cruz Biotechnology, sc-13162; dilution 1:1,000) rabbit polyclonal anti-LC3-B antibody (Sigma-Aldrich, L8918; dilution 1:1,000), rabbit polyclonal anti-p62/SQSTM1 antibody (Abcam, Cambridge, UK, ab91526; dilution 1:3,000), home-made rabbit polyclonal anti-HSPB8 (kindly provided by Dr. Landry, Centre of Recherche Cancerologie, University of Laval, Canada; dilution 1:2,000), rabbit polyclonal anti-GAPDH (Santa Cruz Biotechnology, sc-32233; dilution 1:1,000), goat polyclonal anti-ACTIN (Santa Cruz Biotechnology, sc1615; dilution 1:1,000), mouse monoclonal anti-α-TUBULIN (Sigma-Aldrich, T6199; dilution 1:3,000). The following secondary antibodies were used: goat anti-rabbit HRP-conjugate secondary antibody (Santa Cruz Biotechnology, sc-2004; 1:10,000), goat anti-mouse HRP-conjugate secondary antibody (Santa Cruz Biotechnology, sc-2005; 1:10,000), donkey anti-goat HRP-conjugate secondary antibody (Santa Cruz Biotechnology, sc-2020; 1:10,000).

Immunostaining and Confocal Microscope Analysis

Cells were seeded on coverslips at a density of 25,000 cells/well (in 24-well plate), and the day after plating were transfected and/or treated. After treatments, cells were fixed at 37°C for 25 min using a solution 1:1 of 4% paraformaldehyde (Sigma-Aldrich) in PB 0.2 M [a solution made of KH2PO4 (0.06M) and Na2HPO4 (0,26M)] and 4% sucrose (Sigma-Aldrich) in PB 0.2 M. Then, fixing solution was removed and iced methanol was added for 10 min to complete the fixation. Cell permeabilization was performed using a solution of 0.2% TRITON X100 (Sigma-Aldrich) followed by incubation for 1 h in blocking solution (5% dried non-fat milk in 1X T-BST). Incubation with the primary antibody was kept o/n at 4°C. Incubation with the fluorescent-tagged secondary antibody was preceded by three washes with PBS, to remove the excess of primary antibody. Nuclei were stained with DAPI (Sigma-Aldrich). The following primary antibodies were used: rabbit polyclonal AR-H280 antibody (Santa-Cruz Biotechnology, sc-13162; dilution 1:500), rabbit polyclonal anti-LC3 antibody (Sigma-Aldrich, L8918; dilution 1:500), rabbit polyclonal anti-p62/SQSTM1 antibody (Abcam, ab91526; dilution 1:500). The following secondary antibodies were used: goat anti-rabbit Alexa 594 (Life technologies, Thermo Scientific, A-11012; dilution 1:1,000). All the primary and secondary antibodies were diluted in blocking solution. Coverslips were mounted on a glass support using MOWIOL and images were acquired using an Axiovert 200 microscope (Zeiss Instr., Oberkochen, Germany) combined with a Photometric Cool-Snap CCD camera (Ropper Scientific, Trenton, NJ, USA) or using Eclipse T*i*2 (Nikon, Netherlands) confocal microscope equipped with A1 plus camera (Nikon) and processed with the NIS-Elements software (Nikon) or using LSM510 Meta system confocal microscope (Zeiss, Oberkochen, Germany) and processed with the Aim 4.2 software (Zeiss).

Real Time PCR

Cells were plated in 6-well plate at a density of 130,000 cells/well, and the day after plating were transfected and/or treated. At the end of the experiment, cells were harvested, centrifuged (100 x g; 6 min; 4°C) and lysed using TRI Reagent (Sigma-Aldrich). RNA was extracted following manufacturer instructions and quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific). After DNA removal using DNase I (Sigma-Aldrich), 0.5 μg of the total mRNA was reverse-transcribed using High-Capacity cDNA Archive Kit (Thermo Scientific). mRNA levels were assayed using iTaq SYBR Green Supermix (Bio-Rad) on CFX 96 Real-Time System (Bio-Rad). All results were normalized to *RplP0* used as control. All the primers used were obtained by Eurofins Genomics, sequences of primers have been previously reported (73). The following primers were newly designed: *Vcp* FW- 5′-TGCCATCCTAAAAGCCAATC-3′ RV-5′-TCAGCTCCAGAAAAGCCATT-3′.

Statistical Analysis

Statistical analysis has been performed by using Student's *t*-test to compare two groups and analysis of variance (ANOVA) to compare three or more groups. Two-Way ANOVA was used to compare the effect of two independent variables. Analyses were performed with the PRISM (version 5) software (GraphPad Software).

RESULTS

AR Aggregation in Muscle Cells

Here we used immortalized C2C12 myoblasts, that are widely used as model to mimic muscle cells in culture; this cell line has been infected with viral vectors expressing ARQ24 or ARQ100, subcloned and stabilized in culture (s-myoblast). We initially performed a characterization of the AR biochemical properties in s-myoblasts to assure that the viral expression of this protein was retained even after several passages in culture. Immunofluorescence (IF) analysis showed that ARQ24 and ARQ100 have similar fluorescence intensity, and are both localized in the cell cytoplasm in basal conditions; as expected, upon testosterone treatment they both translocated into the nucleus (**Figure 1A**) and signal intensity also increased upon testosterone treatment. No visible aggregates or inclusions were seen by IF in C2C12-ARQ100 cell line, even after testosterone treatment. Western blot (WB) correctly showed ARQ24 with a higher SDS-PAGE motility than ARQ100, because of the presence of the polyQ tract of different length which results in different molecular weights (MW) of the two AR proteins. Moreover, both ARQ24 and ARQ100 expression was stabilized by testosterone treatment which also induced a mild upshift of the band possibly linked to AR phosphorylation during activation process (27, 82) (**Figure 1B**, upper panel). No high MW (HMW) forms were observed in the stacking gel in all the tested conditions (not shown), suggesting that the AR does not form SDS-resistant insoluble species in s-myoblasts. A low intensity band, possibly related to ARpolyQ fragmentation (or to the endogenous mouse AR) appeared to be mildly increased in ARpolyQ testosterone-treated samples. Interestingly, filter retardation assay (FRA) showed that, after testosterone exposure, ARQ100 formed aggregated species that can be retained on cellulose acetate membrane (with size exclusion of 0.22 μm) (***$p < 0.001$ vs. testosterone-treated C2C12-ARQ24; **$p < 0.01$ vs. untreated C2C12-ARQ100) (**Figure 1B**, lower panel). In this analysis, ARQ24 immunoreactivity was very low even after its activation with testosterone. To better characterize the ARQ100 aggregated species identified in FRA (but not visible in IF), we performed a detergent fractionation assay using NP-40 extraction on cell lysates. In WB, we found that large amounts of ARQ24 and ARQ100 were present in the NP-40 soluble fraction of testosterone activated ARs samples, which were considerably higher than those found for the corresponding untreated ARs samples (**Figure 1C**, upper panel). This confirmed testosterone stabilization of AR protein (83). Of note, in the NP-40 insoluble extracts we found a much more abundant amounts of testosterone-treated ARQ100 compared to testosterone-treated ARQ24, and to untreated controls (**Figure 1D**, upper panel). Using FRA analysis, we found that testosterone treatment triggered the formation of NP-40 soluble, and NP-40 insoluble aggregates retained on the cellulose acetate membrane of ARQ100, while these species were not formed by ARQ24 (**$p < 0.01$ vs. ARQ24 cell line) (**Figures 1C,D**, lower panels). Despite these data, we found no differences in cell viability, or cell survival in cells expressing ARQ24 or ARQ100, even after testosterone treatment (data not shown), suggesting that s-myoblasts are not sensitive to ARpolyQ toxicity.

Collectively these data suggest that testosterone induces the formation of ARQ100 aggregates detectable in FRA. These species are present both in PBS extracts and in NP-40 soluble, and insoluble extracts. Surprisingly, no aggregates were observed in IF. It might be possible that their size is lower than the detection sensitivity of IF as in the case of small oligomeric species.

The Impact of the Modulation of the Protein Quality Control System on AR Aggregation in Muscle Cells

We next investigated which degradative pathway is specifically responsible for ARQ24 and ARQ100 degradation in s-myoblasts by inhibiting the UPS or autophagy, using MG132 or bafilomycin A1, respectively. Proteasome inhibition resulted in an increase of the accumulation of the total amounts of ARQ24 in FRA, which is normally processed via this degradative pathway (47) (**Figure 2A**, lower inset), showing that a high concentration of wtAR inside cells (associated to its impaired clearance) could lead to its accumulation in HMW species. In s-myoblasts, proteasome inhibition resulted in a dramatic increase of the accumulation of mutant ARQ100 in FRA independently from its activation, as we already reported for immortalized motoneurons (47). We performed detergent fractionation assay, and we found no difference in the levels of ARQ24 species after proteasome inhibition (**Figures 2B,C**), suggesting a variability in the response of normal (ARQ24) cells to UPS inhibition. Conversely, both NP-40 soluble and insoluble testosterone-induced ARQ100 aggregates, which are retained on cellulose acetate membrane, were increased after proteasome inhibition (**Figures 2B,C**, lower insets).

With regards to autophagy, we found no involvement of this pathway in the clearance of the wtAR (ARQ24) in s-myoblasts, while the perturbation of autophagosome and lysosome fusion with bafilomycin A1 resulted in a robust increase of PBS soluble form of ARQ100 in presence of testosterone (**Figure 2A**) in FRA. Bafilomycin A1-mediated inhibition of autophagy resulted also in a dramatic increase of both ARQ100 NP-40 soluble and insoluble species, independently from testosterone treatment (**Figures 2B,C**).

These data suggest that in s-myoblasts proteasome is the main mediator of the clearance of both wt and mutant AR, while autophagy appears to be predominantly involved in the clearance of the mutant ARpolyQ.

Next, we evaluated whether the presence and activation of mutant ARpolyQ have an impact on the expression of genes involved in the PQC system. We found no variation in the expression of *Tfeb*, *Becn1*, *Bag1*, *Hspb8*, *Sqstm1/p62*, *Lc3* in all

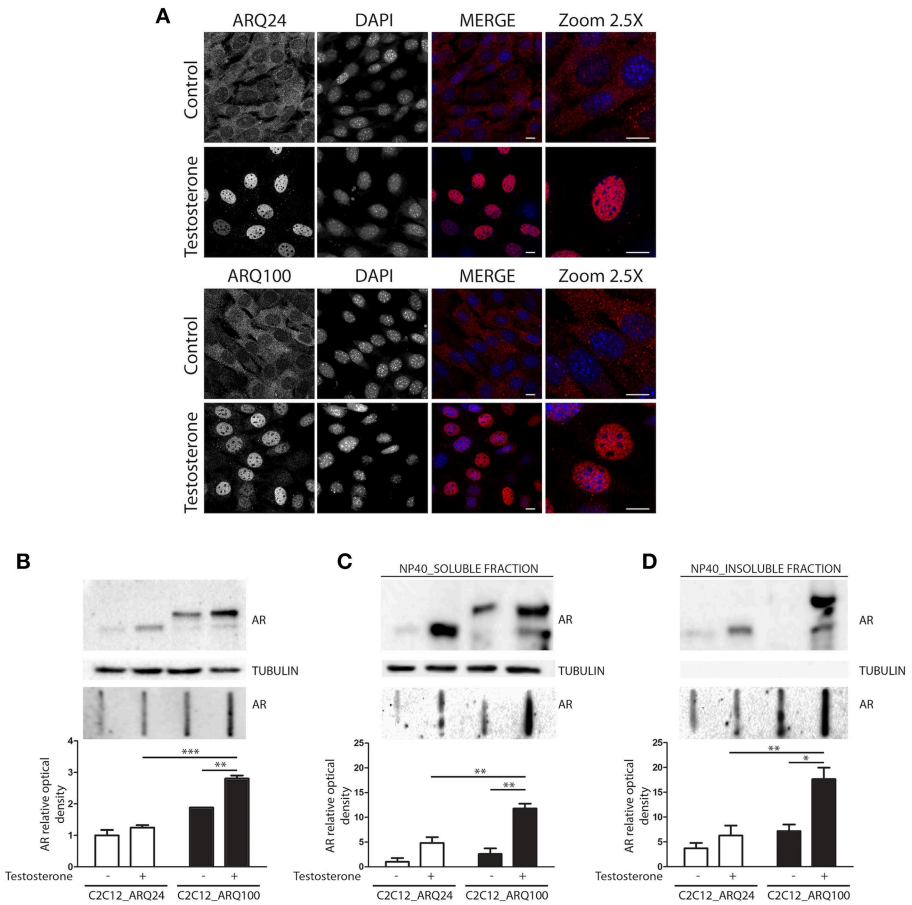

FIGURE 1 | Characterization of the cellular model. **(A)** Immunostaining for AR. Nuclei staining: DAPI. 60X magnification. Confocal microscope: Eclipse T*i*2 (Nikon). Scale bar = 10 μm **(B)** WB (upper inset) and FRA (middle inset) of PBS extracts. Optical densitometry quantification of FRA (lower inset). ***$p < 0.001$ vs. ARQ24+T; **$p > 0.01$ vs. ARQ100-T. **(C)** WB (upper inset) and FRA (middle inset) of NP-40 soluble extracts. Optical densitometry quantification of FRA (lower inset). (**$p < 0.01$ vs. ARQ24+T or vs. ARQ100-T). **(D)** WB (upper inset) and FRA (middle inset) of NP-40 insoluble extracts. Optical densitometry quantification of FRA (lower inset). (**$p < 0.01$ vs. ARQ24+T; *$p < 0.05$ vs. ARQ100-T). Two-way ANOVA followed by Bonferroni *post-hoc* test was used. Each experiment was independently replicated three times. Graphs show quantification of three independent biological samples ($n = 3$).

conditions tested (**Figure 3A**). We found that the expression of mutant ARpolyQ correlated with a reduction in the expression of *Bag3* and *Vcp* (another autophagy associated proteins found to be involved in motoneuron diseases), but these changes were not linked to the presence of testosterone (**Figure 3A**).

We then analyzed whether the levels of ARpolyQ species entrapped in FRA could be modulated by the overexpression of components required to route misfolded proteins to either UPS or autophagy. The data shown in **Figure 3B** indicate that the formation of testosterone-induced aggregated species of mutant ARpolyQ in transiently transfected C2C12 (ARQ112 HMW aggregates) can be counteracted by the overexpression of HSPB8 and BAG3. These two proteins are essential components of the CASA complex, which delivers misfolded proteins to the microtubule organization center where aggresomes are formed before their engulfment into nascent autophagosomes. Notably, both HSPB8 alone, and BAG3 alone preserve their pro-autophagic activity even if the CASA complex required both proteins in association with HSP70 and CHIP. This suggests that may be both considered limiting factor for the CASA complex activity. Interestingly, also the overexpression of BAG1 resulted in a great reduction of the accumulation of testosterone-induced aggregated species of mutant ARpolyQ measured in FRA (**Figure 3B**). It must be noted that BAG1 exerts its activity by preventing HSP70 and CHIP to become part of the CASA complex (61, 84–86), thus routing misfolded proteins to UPS degradation as an alternative to autophagy. Since, it has been demonstrated that testosterone induces the formation of ARpolyQ aggregates via the generation of a N-terminal caspase-3 cleaved fragment containing the polyQ stretch, which is highly prone to aggregate, we wanted to test whether the routing system may also be involved in the removal of this highly neurotoxic AR species. The results (**Figure 3C**) clearly demonstrated that both the overexpression of HSPB8 and BAG3, as well as that of BAG1, are capable to revert the accumulation in FRA of HMW aggregates of a highly

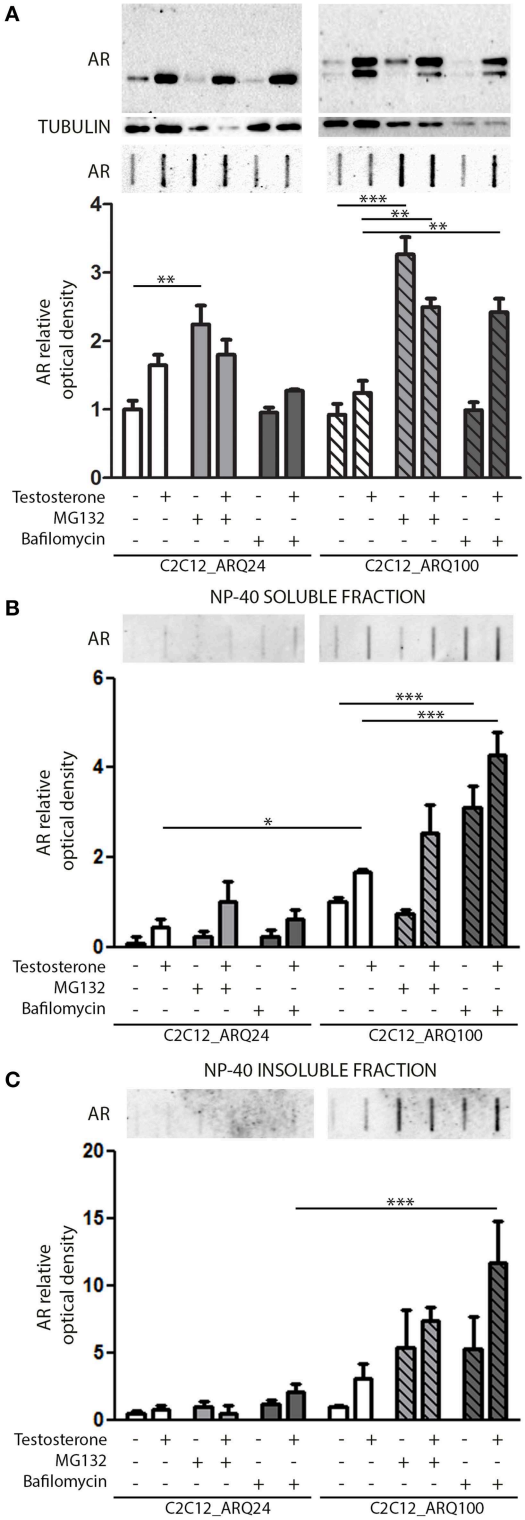

FIGURE 2 | Degradative systems involvement. **(A)** WB (upper inset) and FRA (middle inset) of PBS extract of cells treated with testosterone, MG132 and bafilomycin A1. Optical densitometry quantification of FRA (lower inset). (**$p <$ 0.01; ***$p < 0.001$ vs. relative control conditions –T/+T). **(B)** FRA (upper inset) of NP-40 soluble extracts of cells treated with testosterone, MG132 and bafilomycin A1. Optical densitometry quantification of FRA (lower inset) (***$p < 0.001$ vs. relative control conditions –T/+T; *$p < 0.05$ vs. ARQ24 -T). **(C)** FRA (upper inset) of NP-40 insoluble extracts of cells treated with testosterone, MG132 and bafilomycin A1. Optical densitometry quantification of FRA (lower inset) (***$p < 0.001$ vs. relative control conditions +T). For each panel, FRA images derive from the same membranes with identical exposure time to permit direct comparison of wtAR and ARpolyQ levels. Two-way ANOVA followed by Bonferroni *post-hoc* test was used. Each experiment was independently replicated three times. Graphs show quantification of three independent biological samples ($n = 3$).

neurotoxic caspase-3 released N-terminal fragment of ARpolyQ ARQ112ΔHA (87–90).

Collectively, these data suggest that, by modulating specific components of the PQC system, the ARpolyQ and its highly neurotoxic aggregate-prone caspase-3 released fragment can be eliminated from muscle cells using both the proteasome and the autophagy system, when they are still normal and functioning as in our cell line [ARQ100 does not greatly affect proteasome and autophagy machinery (**Figure 3A**)].

Pharmacological Induction of the Autophagic System Reduces ARpolyQ Accumulation and Aggregation

Based on these data, we hypothesized that compounds capable of activating autophagy may serve to enhance the ARpolyQ clearance from muscle cells. We use a well-known autophagy activator, trehalose, which acts in a mTOR-independent manner. We recently described that trehalose causes a transient lysosomal damage, which in turn activates TFEB and, consequently, promotes autophagosome and lysosome assembly and fusion (56). We found that in s-myoblasts, trehalose retained its capability to activate autophagy, as demonstrated by the conversion of LC3 from its LC3-I diffuse form to the LC3-II lipidated form associated to autophagosomes in its punctate status (**Figure 4A**, left insets) or by the relocalization of SQSTM1/p62 into p62 bodies (**Figure 4A**, right insets). These data were also corroborated by the mRNA expression analysis showing that trehalose induced the *de novo* expression of several pro-autophagic genes, including *Foxo3, Tfeb, Becn1, Bag3, Bag1, Hspb8, Lc3, Sqstm1/p62, Vcp*, and *AchR* (**Figure 4B**).

The effects of trehalose were then tested on the accumulation of ARQ100 in s-myoblasts and the data showed that this autophagy activator reduced the levels of monomeric soluble ARQ100, and fully counteracted the accumulation of testosterone-induced HMW aggregated species of ARQ100 (**Figure 4C**). Trehalose effect on mutant ARpolyQ was fully blocked by bafilomycin A1, proving that its pro-degradative activity is mediated by autophagy. The effects of trehalose on ARpolyQ clearance were also maintained when the mutant protein was transiently overexpressed in basal C2C12, since this autophagy activator significantly reduced both the monomeric soluble ARQ112 evaluated in WB (**Figure 4D**, upper inset), and testosterone-induced aggregated species of ARQ112 evaluated in FRA (**Figure 4D**, lower inset). Trehalose activation of autophagy was tested in basal C2C12 expressing ARQ112

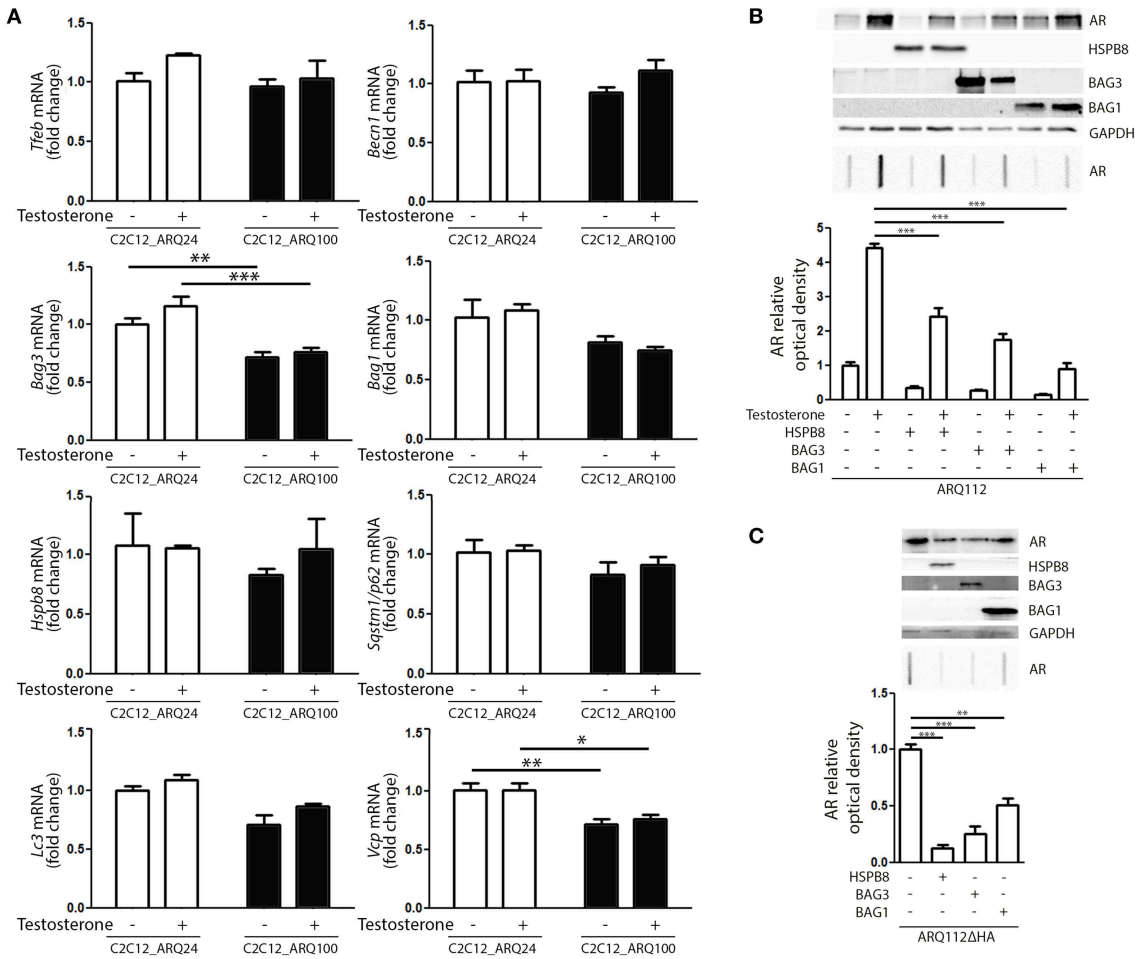

FIGURE 3 | PQC activation and role against AR accumulation. **(A)** RT-qPCR of PQC system related genes performed on C2C12_ARQ24 and C2C12_ARQ100. *$p <$ 0.05; **$p < 0.01$; ***$p < 0.001$ vs. ARQ24 in the same conditions. Graphs show quantification of four independent biological samples ($n = 4$). **(B)** WB (upper inset) and FRA (middle inset) of C2C12 transiently transfected with p5HBhARQ112 and co-transfected with plasmids coding for HSPB8, BAG3 and BAG1. Optical densitometry quantification of FRA (lower inset). (***$p < 0.001$ vs. relative control conditions +T). **(C)** WB (upper inset) and FRA (middle inset) of C2C12 transiently transfected with pARQ112ΔHA and co-transfected with plasmids coding for HSPB8, BAG3 and BAG1. Optical densitometry quantification of FRA (lower inset). (**$p < 0.01$; ***$p < 0.001$ vs. relative control conditions pcDNA3). Each experiment was independently replicated three times. Graphs show quantification of three independent biological samples ($n = 3$).

only by LC3 conversion in WB, assuming that the effects observed by RT-qPCR and IF in s-myoblasts expressing ARQ100 were recapitulated also in basal C2C12, and the data confirmed that trehalose acts as a potent autophagy inducer as previously shown (51, 56, 91, 92). Importantly, testosterone-dependent ARQ112 inclusions observed in IF (**Figure 4E**) were found to be fully degraded after trehalose treatment. Finally, we found that activation of autophagy with trehalose counteracted the accumulation also of the aggregated species of fragmented ARQ112ΔHA retained in FRA (**Figure 4F**), and the ARQ112ΔHA inclusions evaluated in IF (**Figure 4G**).

DISCUSSION

In this study, we characterized the biochemical behavior of the mutant ARpolyQ in s-myoblasts, and we compared it to the one of the wtAR. We found that, in these cells, the mutant ARpolyQ does not form inclusions visible by microscopy, or detectable by immunoblotting as SDS-insoluble aggregates in WB, even after its testosterone activation. Instead, we found that ARpolyQ generated testosterone-inducible aggregated species readily detectable in FRA, which were resistant to NP-40 solubilization. Notably wtAR insoluble species were detected only in the PBS resistant fraction, but not in NP-40 soluble or insoluble fractions, suggesting that even if formed they remain largely soluble, while those formed by mutant ARpolyQ becomes detergent-insoluble. Both the wtAR and the mutant ARpolyQ are processed via the proteasome, while only mutant ARpolyQ is cleared by autophagy, since autophagy inhibition resulted in a robust accumulation of ARpolyQ insoluble species in FRA. Despite this, the presence of ARpolyQ was insufficient to trigger an autophagic response, since no variation were found in the expression of classical autophagy related genes (e.g., *Tfeb, Becn1, Bag1, Hspb8, Sqstm1/p62, Lc3*) even after testosterone treatment.

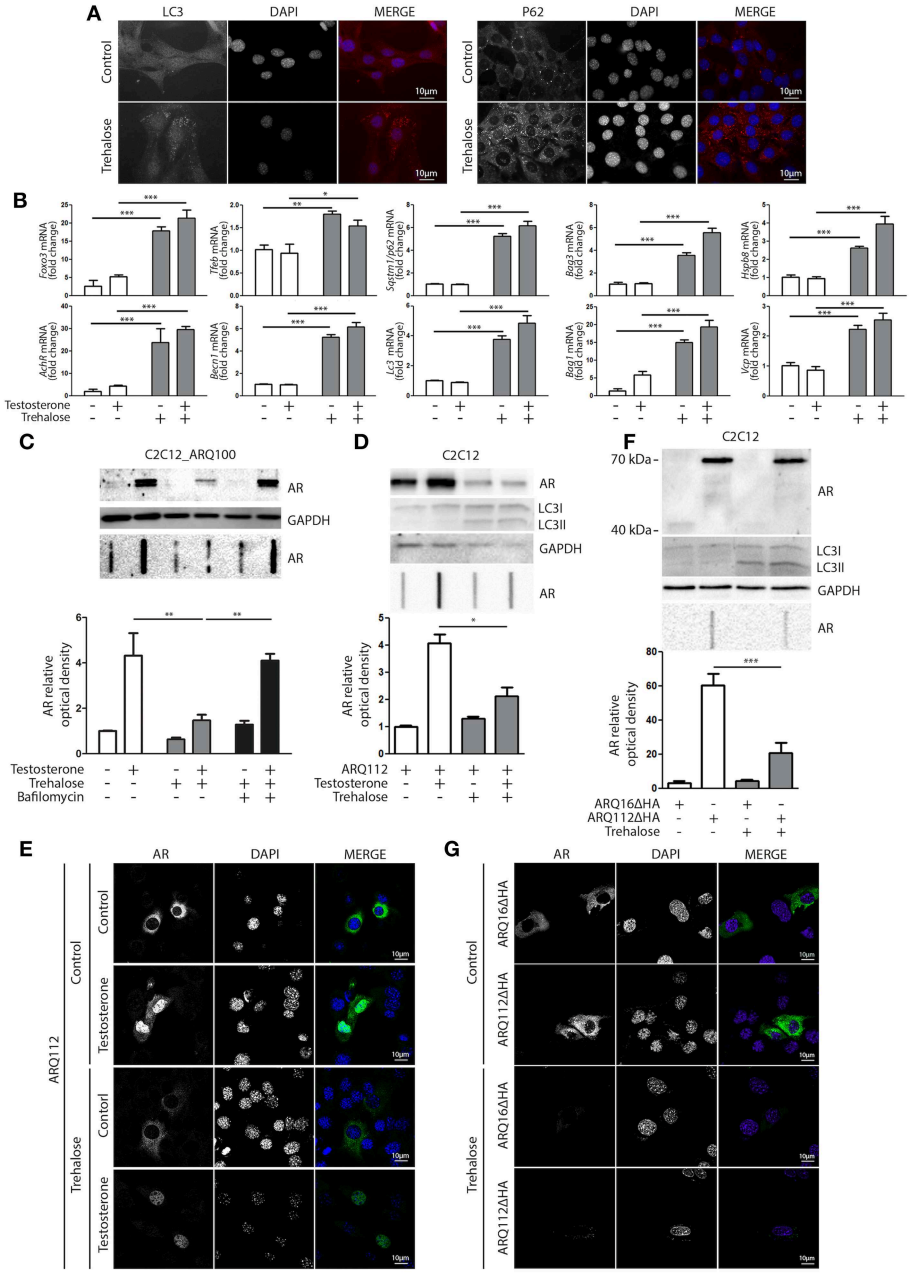

FIGURE 4 | Trehalose activates autophagy and reduces ARpolyQ accumulation **(A)** Immunostaining for LC3 (left inset) and p62 (right inset) of C2C12_ARQ100 in presence of trehalose. Nuclei staining: DAPI. Magnification: 63X. Microscope: Axiovert 2000 **(B)** C2C12_ARQ100 treated with trehalose. RTqPCR of PQC system related genes. (**$p < 0.01$; ***$p < 0.001$ vs. relative untreated control). **(C)** C2C12_ARQ100 treated with trehalose and bafilomycin A1. WB (upper inset) and FRA (middle inset) of PBS extracts of Optical densitometry quantification of FRA (lower panel) (**$p < 0.01$ vs. relative untreated control; ***$p < 0.001$ vs. trehalose +T). **(D,E)** C2C12 transiently transfected with p5HBhARQ112 and treated with testosterone and trehalose. **(D)** WB (upper inset) and FRA (middle inset). Optical densitometry quantification of FRA (lower inset). *$p < 0.05$ vs. relative control conditions +T. **(E)** Immunostaining for AR. Nuclei staining: DAPI. Magnification: 63X. Microscope: confocal LSM510 Zeiss **(F,G)** C2C12 transiently transfected with pARQ16ΔHA or pARQ112ΔHA and treated with trehalose. **(F)** WB (upper inset) and FRA (middle inset). Optical densitometry quantification of FRA (lower inset). **$p < 0.01$ vs. relative control conditions +T. **(G)** Immunostaining for AR. Nuclei staining: DAPI. Magnification: 63X. Microscope: confocal LSM510 Zeiss. Each experiment was independently replicated three times ($n = 3$). Graphs show quantification of three independent biological samples ($n = 3$).

Of note, we found that ARpolyQ activation in s-myoblasts correlated with a reduced expression of two pro-autophagic proteins such as BAG3 and VCP. Mutations in *BAG3* and *VCP* genes are responsible for late onset degenerative diseases affecting skeletal muscle (93, 94), suggesting that these proteins might play an important role in the maintenance of muscle

cell homeostasis. This phenomenon is not correlated to the presence of testosterone, but still suggestive of a decreased autophagic response in s-myoblasts in presence of ARpolyQ. Overall these results point to the fact that activated ARpolyQ does not greatly affect the functionality of the PQC system in our muscle cell model. Anyway, it might be possible that the mildly reduced autophagic potential causes ARpolyQ aggregation only after testosterone activation, slowing down ARpolyQ clearance via autophagy. To facilitate the degradation of ARpolyQ, we overexpressed BAG3 or its partner HSPB8, essential components of the CASA complex, showing that they are both able to enhance ARpolyQ clearance even in presence of testosterone. Also, the overexpression of BAG1, the co-chaperone which routes the HSP70/CHIP/misfolded protein complex to UPS (95–97), exerted a similar effect on ARpolyQ clearance. Overexpression of these chaperones was effective also against aggregates formed by the N-terminal ARpolyQ fragment, physiologically formed upon testosterone treatment. Thus, the modulation of the PQC could be viewed as a potential target to ameliorate the removal of toxic ARpolyQ from our muscle cell models. Indeed, by adopting a pharmacological treatment with trehalose, which is a mTOR independent autophagy activator, we have clearly shown that the insoluble species of ARpolyQ disappeared, both using the ARQ100 in stably infected cells as well as with ARQ112 or the caspase-3 released N-terminal fragment transiently transfected in s-myoblasts. The involvement of autophagy in mediating the pro-degradative activity of trehalose in s-myoblasts was proved by the fact that treatment with bafilomycin A1 fully reverted the protection exerted by trehalose against ARpolyQ accumulation.

In our view, these data acquire particular relevance keeping in mind that SBMA, regarded for years as a MND (3, 98), has now been defined as a neuromuscular disease (25). Muscle tissue is a primary site for SBMA toxicity as muscle atrophy often precedes motoneuron loss and the onset of SBMA is rescued by specific repression of ARpolyQ in muscle cells (33, 50). Even if these studies were carried out in murine models and findings remain to be confirmed in human cell lines, they support the notion of a direct muscle involvement in SBMA onset and progression. Here, we found that autophagy activation, or facilitation, prevents ARpolyQ accumulation in our muscle cell models, suggesting that autophagy could be a specific pathway for the degradation of testosterone activated ARpolyQ insoluble species. In addition, autophagy appears to be partially impaired, making it an important target to facilitate misfolded ARpolyQ clearance in SBMA. Studies performed in SBMA mouse models indicated that at later stage of disease, autophagy is altered in skeletal muscle (22, 32, 50, 73, 99), but its role is still largely debated. One of the major problem linked to these analysis is that the specific time window in which the mice are analyzed (pre-symptomatic, symptomatic or end stage of disease) may influence the relative involvement of autophagy in response to mutant ARpolyQ due to several compensatory mechanisms triggered during muscle atrophy progression. In addition, the mouse SBMA models utilized significantly differ in term of level of protein expression, and its tissue distribution. However, the analysis of TFEB activity, measured by evaluating its target genes, showed that autophagy is enhanced in presence of ARpolyQ in muscle tissue (22). Using the same SBMA model, we also confirmed and extended the activation of TFEB-mediated autophagy (73). In addition, in the same animals, we demonstrated that, at the symptomatic stage, also the expression of genes involved in CASA-complex (e.g., *Hspb8* and *Bag3*) resulted upregulated. Thus, in skeletal muscle autophagy is activated during disease progression, and its upregulation might be an attempt to respond to ARpolyQ toxicity, or to mediate the catabolic activity induced by muscle atrophy associated to the chronic exposure to ARpolyQ (100). This may suggest that the autophagy response observed in the skeletal muscle of the SBMA mice is an adaptive mechanism related to both the presence of the misfolded ARpolyQ, and the muscle atrophy. In any case, we cannot exclude that aberrant autophagic upregulation contribute to SBMA progression. Overall, these data suggest that restoration of physiological autophagic function might represent an important therapeutic target for SBMA. Our s-myoblast model may be particularly relevant for the screening of compounds that may modulate autophagy dysregulation in muscle cells. In addition, our s-myoblast model will permit to evaluate the acute response to ARpolyQ activation by testosterone. Indeed, acute ARpolyQ expression leads to a mild autophagy response. Misfolded ARpolyQ production is insufficient to induce a *de novo* expression of all gene tested, with the exception of *Bag3* and *Vcp*. Our s-myoblast SBMA model may thus contribute to understand the different events taking place in skeletal muscle cells exposed to misfolded ARpolyQ allowing to discriminate between early and/or adaptive response. A possible limitation of this model is the fact that s-myoblasts do not show modification in cell viability induced by ARpolyQ. Despite this, they are characterized by the specific accumulation of testosterone-induced ARpolyQ NP-40 insoluble species (not detectable in the case of wtAR); these species clearly represent a biochemical form of misfolded ARpolyQ which play a role in SBMA pathogenesis recapitulating the disease phenotype. As it has been published (80, 101, 102), the formation of the insoluble ARpolyQ species, might be a valuable biomarker to follow the progression of muscle degeneration.

Importantly, the systems here described, including some chaperones and autophagy/proteasome, are highly conserved and work in a similar manner in neuronal, muscular and non-neuronal cells. Therefore, boosting them could provide protection by enhancing the clearing capacities, and maintaining protein homeostasis in different cell types affected by the disease.

In this context, targeting autophagy could be an efficient strategy to reduce the accumulation of ARpolyQ. Trehalose not only activates the basal autophagy process (e.g., TFEB activation enhanced SQSTM1/p62 and LC3 expression), but also increased the expression of key factors of the CASA-complex, like HSPB8, that probably helps in the recognition of selected cargo avoiding the uncontrolled degradation of every intracellular element.

Even if C2C12 SBMA cell model does not show a reduction in cell viability induced by ARpolyQ NP-40 insoluble species, it might be helpful to understand molecular mechanisms responsible for muscle degeneration observed in SBMA patients as it has been published in other publications (80, 101, 102). Since in this model AR NP-40 insoluble species are polyQ and

testosterone-dependent, recapitulating the disease phenotype, these C2C12 cell lines could be used to co-culture skeletal muscle and motoneurons in order to study if ARpolyQ expression in myoblast can alter motoneuron functionality and viability.

Overall these results show that ARpolyQ aggregation may occur also in muscle cells, and that targeting aggregation of ARpolyQ could be beneficial in SBMA, since the permanence of inclusions in the cells could cause the damage of several pathways and the recruitment of other soluble proteins, impairing other pathways. Concluding, trehalose plays beneficial effects against ARpolyQ aggregation and autophagy appears as a valuable pathway for the degradation of insoluble ARpolyQ species. In parallel and supporting this study, there are several ongoing studies that are testing, in *in vivo* models, novel compounds that will address the PQC system, to reduce the presence of the misfolded toxic proteins.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study is not subjected to ethical committee approval, since no animal and human data have been collected.

AUTHOR CONTRIBUTIONS

MEC and RC performed most of the experiments. VC, VF, BT, EC, and MC contributed to perform experiments and critically revised the manuscript. MG, MPi, and EM contributed to design the experiments and critically discussed the data. SC provided expertise on BAG1-BAG3 functions and revised the manuscript. MPe provided the stably infected cells and assisted in experiment design with the expertise on SBMA. PR provided expertise on trehalose experiments and critically revised the manuscript. AP supervised the entire study and wrote the paper.

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REFERENCES

1. La Spada AR, Wilson EM, Lubahn DB, Harding AE, Fischbeck KH. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature*. (1991) 352:77–9. doi: 10.1038/352077a0
2. Fratta P, Collins T, Pemble S, Nethisinghe S, Devoy A, Giunti P, et al. (2014). Sequencing analysis of the spinal bulbar muscular atrophy CAG expansion reveals absence of repeat interruptions. *Neurobiol Aging*. 35:e441–443. doi: 10.1016/j.neurobiolaging.2013.07.015
3. Grunseich C, Kats IR, Bott LC, Rinaldi C, Kokkinis A, Fox D, et al. Early onset and novel features in a spinal and bulbar muscular atrophy patient with a 68 CAG repeat. *Neuromuscul Disord*. (2014) 24:978–81. doi: 10.1016/j.nmd.2014.06.441
4. Mhatre AN, Trifiro MA, Kaufman M, Kazemi-Esfarjani P, Figlewicz D, Rouleau G, et al. Reduced transcriptional regulatory competence of the androgen receptor in X-linked spinal and bulbar muscular atrophy. *Nat Genet*. (1993) 5:184–8. doi: 10.1038/ng 1093-184
5. Kazemi-Esfarjani P, Trifiro MA, Pinsky L. Evidence for a repressive function of the long polyglutamine tract in the human androgen receptor: possible pathogenetic relevance for the (CAG)n-expanded neuronopathies. *Hum Mol Genet*. (1995) 4:523–7. doi: 10.1093/hmg/4.4.523
6. Tut TG, Ghadessy FJ, Trifiro MA, Pinsky L, Yong EL. Long polyglutamine tracts in the androgen receptor are associated with reduced trans-activation, impaired sperm production, and male infertility. *J Clin Endocrinol Metab*. (1997) 82:3777–82. doi: 10.1210/jcem.82.11.4385
7. Orr HT, Zoghbi HY. Trinucleotide repeat disorders. *Annu Rev Neurosci*. (2007) 30:575–621. doi: 10.1146/annurev.neuro.29.051605.113042
8. Kennedy WR, Alter M, Sung JH. Progressive proximal spinal and bulbar muscular atrophy of late onset. A sex-linked recessive trait. *Neurology*. (1968) 18:671–80. doi: 10.1212/wnl.18.7.671
9. Sobue G, Hashizume Y, Mukai E, Hirayama M, Mitsuma T, Takahashi A. X-linked recessive bulbospinal neuronopathy. A clinicopathological study. *Brain*. (1989) 112 (Pt 1), 209–232. doi: 10.1093/brain/112.1.209
10. Polo A, Teatini F, D'Anna S, Manganotti P, Salviati A, Dallapiccola B, et al. Sensory involvement in X-linked spino-bulbar muscular atrophy

(Kennedy's syndrome): an electrophysiological study. *J Neurol.* (1996) 243:388–92.
11. Adachi H, Katsuno M, Minamiyama M, Waza M, Sang C, Nakagomi Y, et al. Widespread nuclear and cytoplasmic accumulation of mutant androgen receptor in SBMA patients. *Brain.* (2005) 128(Pt 3), 659–670. doi: 10.1093/brain/awh381
12. Atsuta N, Watanabe H, Ito M, Banno H, Suzuki K, Katsuno M, et al. Natural history of spinal and bulbar muscular atrophy (SBMA): a study of 223 Japanese patients. *Brain.* (2006) 129(Pt 6), 1446–1455. doi: 10.1093/brain/awl096
13. Suzuki K, Katsuno M, Banno H, Takeuchi Y, Atsuta N, Ito M, et al. CAG repeat size correlates to electrophysiological motor and sensory phenotypes in SBMA. *Brain.* (2008) 131(Pt 1), 229–239. doi: 10.1093/brain/awm289
14. Fratta P, Nirmalananthan N, Masset L, Skorupinska I, Collins T, Cortese A, et al. Correlation of clinical and molecular features in spinal bulbar muscular atrophy. *Neurology.* (2014) 82:2077–84. doi: 10.1212/WNL.0000000000000507
15. Jordan CL, Lieberman AP. Spinal and bulbar muscular atrophy: a motoneuron or muscle disease? *Curr Opin Pharmacol.* (2008) 8:752–8. doi: 10.1016/j.coph.2008.08.006
16. Johansen JA, Yu Z, Mo K, Monks DA, Lieberman AP, Breedlove SM, et al. Recovery of function in a myogenic mouse model of spinal bulbar muscular atrophy. *Neurobiol Dis.* (2009) 34:113–20. doi: 10.1016/j.nbd.2008.12.009
17. Yu Z, Wang AM, Robins DM, Lieberman AP. Altered RNA splicing contributes to skeletal muscle pathology in Kennedy disease knock-in mice. *Dis Model Mech.* (2009) 2:500–7. doi: 10.1242/dmm.003301
18. Mo K, Razak Z, Rao P, Yu Z, Adachi H, Katsuno M, et al. Microarray analysis of gene expression by skeletal muscle of three mouse models of Kennedy disease/spinal bulbar muscular atrophy. *PLoS ONE.* (2010) 5:e12922. doi: 10.1371/journal.pone.0012922
19. Yu Z, Wang AM, Adachi H, Katsuno M, Sobue G, Yue Z, et al. Macroautophagy is regulated by the UPR-mediator CHOP and accentuates the phenotype of SBMA mice. *PLoS Genet.* (2011) 7:e1002321. doi: 10.1371/journal.pgen.1002321
20. Chua JP, Lieberman AP. Pathogenic mechanisms and therapeutic strategies in spinobulbar muscular atrophy. *CNS Neurol Disord Drug Target.* (2013) 12:1146–56. doi: 10.2174/18715273113120124
21. Malena A, Pennuto M, Tezze C, Querin G, D'Ascenzo C, Silani V, et al. Androgen-dependent impairment of myogenesis in spinal and bulbar muscular atrophy. *Acta Neuropathol.* (2013) 126:109–21. doi: 10.1007/s00401-013-1122-9
22. Chua JP, Reddy SL, Merry DE, Adachi H, Katsuno M, Sobue G, et al. Transcriptional activation of TFEB/ZKSCAN3 target genes underlies enhanced autophagy in spinobulbar muscular atrophy. *Hum Mol Genet.* (2014) 23:1376–86. doi: 10.1093/hmg/ddt527
23. Orsucci D, Rocchi A, Caldarazzo Ienco E, Ali G, LoGerfo A, Petrozzi L, et al. Myopathic involvement and mitochondrial pathology in Kennedy disease and in other motor neuron diseases. *Curr Mol Med.* (2014) 14:598–602. doi: 10.2174/1566524014666140603100131
24. Ramzan F, McPhail M, Rao P, Mo K, Halievski K, Swift-Gallant A, et al. Distinct etiological roles for myocytes and motor neurons in a mouse model of Kennedy's disease/spinobulbar muscular atrophy. *J Neurosci.* (2015) 35:6444–51. doi: 10.1523/JNEUROSCI.3599-14.2015
25. Giorgetti E, Lieberman AP. Polyglutamine androgen receptor-mediated neuromuscular disease. *Cell Mol Life Sci.* (2016) 73:3991–9. doi: 10.1007/s00018-016-2275-1
26. Giorgetti E, Yu Z, Chua JP, Shimamura R, Zhao L, Zhu F, et al. Rescue of metabolic alterations in AR113Q skeletal muscle by peripheral androgen receptor gene silencing. *Cell Rep.* (2016) 17:125–36. doi: 10.1016/j.celrep.2016.08.084
27. Poletti A. The polyglutamine tract of androgen receptor: from functions to dysfunctions in motor neurons. *Front Neuroendocrinol.* (2004) 25:1–26. doi: 10.1016/j.yfrne.2004.03.001
28. Palazzolo I, Stack C, Kong L, Musaro A, Adachi H, Katsuno M, et al. Overexpression of IGF-1 in muscle attenuates disease in a mouse model of spinal and bulbar muscular atrophy. *Neuron.* (2009) 63:316–28. doi: 10.1016/j.neuron.2009.07.019
29. Jimenez Garduno AM, Juarez-Hernandez LJ, Polanco MJ, Tosatto L, Michelatti D, Arosio D, et al. Altered ionic currents and amelioration by IGF-1 and PACAP in motoneuron-derived cells modelling SBMA. *Biophys Chem.* (2017) 229:68–76. doi: 10.1016/j.bpc.2017.05.003
30. Monks DA, Johansen JA, Mo K, Rao P, Eagleson B, Yu Z, et al. Overexpression of wild-type androgen receptor in muscle recapitulates polyglutamine disease. *Proc Natl Acad Sci USA.* (2007) 104:18259–64. doi: 10.1073/pnas.0705501104
31. Huguenard AL, Fernando SM, Monks DA, Sengelaub DR. Overexpression of androgen receptors in target musculature confers androgen sensitivity to motoneuron dendrites. *Endocrinology.* (2011) 152:639–50. doi: 10.1210/en.2010-1197
32. Cortes CJ, Ling SC, Guo LT, Hung G, Tsunemi T, Ly L, et al. Muscle expression of mutant androgen receptor accounts for systemic and motor neuron disease phenotypes in spinal and bulbar muscular atrophy. *Neuron.* (2014) 82:295–307. doi: 10.1016/j.neuron.2014.03.001
33. Lieberman AP, Yu Z, Murray S, Peralta R, Low A, Guo S, et al. Peripheral androgen receptor gene suppression rescues disease in mouse models of spinal and bulbar muscular atrophy. *Cell Rep.* (2014) 7:774–84. doi: 10.1016/j.celrep.2014.02.008
34. Xu Y, Halievski K, Henley C, Atchison WD, Katsuno M, Adachi H, et al. Defects in neuromuscular transmission may underlie motor dysfunction in spinal and bulbar muscular atrophy. *J Neurosci.* (2016) 36:5094–106. doi: 10.1523/JNEUROSCI.3485-15.2016
35. Katsuno M, Adachi H, Kume A, Li M, Nakagomi Y, Niwa H, et al. Testosterone reduction prevents phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. *Neuron.* (2002) 35:843–54. doi: 10.1016/S0896-6273(02)00834-6
36. Katsuno M, Adachi H, Doyu M, Minamiyama M, Sang C, Kobayashi Y, et al. Leuprorelin rescues polyglutamine-dependent phenotypes in a transgenic mouse model of spinal and bulbar muscular atrophy. *Nat Med.* (2003) 9:768–73. doi: 10.1038/nm878
37. Katsuno M, Adachi H, Inukai A, Sobue G. Transgenic mouse models of spinal and bulbar muscular atrophy (SBMA). *Cytogenet Genome Res.* (2003) 100:243–51. doi: 10.1159/000072860
38. Katsuno M, Tanaka F, Adachi H, Banno H, Suzuki K, Watanabe H, et al. Pathogenesis and therapy of spinal and bulbar muscular atrophy (SBMA). *Prog Neurobiol.* (2012) 99:246–56. doi: 10.1016/j.pneurobio.2012.05.007
39. Xu Y, Halievski K, Katsuno M, Adachi H, Sobue G, Breedlove SM, et al. Pre-clinical symptoms of SBMA may not be androgen-dependent: implications from two SBMA mouse models. *Hum Mol Genet.* (2018) 27:2425–42. doi: 10.1093/hmg/ddy142
40. Galbiati M, Onesto E, Zito A, Crippa V, Rusmini P, Mariotti R, et al. The anabolic/androgenic steroid nandrolone exacerbates gene expression modifications induced by mutant SOD1 in muscles of mice models of amyotrophic lateral sclerosis. *Pharmacol Res.* (2012) 65:221–30. doi: 10.1016/j.phrs.2011.12.001
41. Aggarwal T, Polanco MJ, Scaramuzzino C, Rocchi A, Milioto C, Emionite L, et al. Androgens affect muscle, motor neuron, and survival in a mouse model of SOD1-related amyotrophic lateral sclerosis. *Neurobiol Aging.* (2014) 35:1929–38. doi: 10.1016/j.neurobiolaging.2014.02.004
42. Montie HL, Cho MS, Holder L, Liu Y, Tsvetkov AS, Finkbeiner S, et al. Cytoplasmic retention of polyglutamine-expanded androgen receptor ameliorates disease via autophagy in a mouse model of spinal and bulbar muscular atrophy. *Hum Mol Genet.* (2009) 18:1937–50. doi: 10.1093/hmg/ddp115
43. Montie HL, Pestell RG, Merry DE. SIRT1 modulates aggregation and toxicity through deacetylation of the androgen receptor in cell models of SBMA. *J Neurosci.* (2011) 31:17425–36. doi: 10.1523/JNEUROSCI.3958-11.2011
44. Simeoni S, Mancini MA, Stenoien DL, Marcelli M, Weigel NL, Zanisi M, et al. Motoneuronal cell death is not correlated with aggregate formation of androgen receptors containing an elongated polyglutamine tract. *Hum Mol Genet.* (2000) 9:133–44. doi: 10.1093/hmg/9.1.133
45. Rusmini P, Sau D, Crippa V, Palazzolo I, Simonini F, Onesto E, et al. Aggregation and proteasome: the case of elongated polyglutamine aggregation in spinal and bulbar muscular atrophy. *Neurobiol Aging.* (2007) 28:1099–111. doi: 10.1016/j.neurobiolaging.2006.05.015

46. Rusmini P, Bolzoni E, Crippa V, Onesto E, Sau D, Galbiati M, et al. Proteasomal and autophagic degradative activities in spinal and bulbar muscular atrophy. *Neurobiol Dis*. (2010) 40:361–9. doi: 10.1016/j.nbd.2010.06.016
47. Rusmini P, Simonini F, Crippa V, Bolzoni E, Onesto E, Cagnin M, et al. 17-AAG increases autophagic removal of mutant androgen receptor in spinal and bulbar muscular atrophy. *Neurobiol Dis*. (2011) 41:83–95. doi: 10.1016/j.nbd.2010.08.023
48. Carra S, Crippa V, Rusmini P, Boncoraglio A, Minoia M, Giorgetti E, et al. Alteration of protein folding and degradation in motor neuron diseases: implications and protective functions of small heat shock proteins. *Prog Neurobiol*. (2012) 97:83–100. doi: 10.1016/j.pneurobio.2011.09.009
49. Rusmini P, Crippa V, Giorgetti E, Boncoraglio A, Cristofani R, Carra S, et al. Clearance of the mutant androgen receptor in motoneuronal models of spinal and bulbar muscular atrophy. *Neurobiol Aging*. (2013) 34:2585–603. doi: 10.1016/j.neurobiolaging.2013.05.026
50. Cortes CJ, Miranda HC, Frankowski H, Batlevi Y, Young JE, Le A, et al. Polyglutamine-expanded androgen receptor interferes with TFEB to elicit autophagy defects in SBMA. *Nat Neurosci*. (2014) 17:1180–9. doi: 10.1038/nn.3787
51. Giorgetti E, Rusmini P, Crippa V, Cristofani R, Boncoraglio A, Cicardi ME, et al. Synergic prodegradative activity of Bicalutamide and trehalose on the mutant androgen receptor responsible for spinal and bulbar muscular atrophy. *Hum Mol Genet*. (2015) 24:64–75. doi: 10.1093/hmg/ddu419
52. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, et al. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature*. (2006) 441:880–4. doi: 10.1038/nature04723
53. Komatsu M, Wang QJ, Holstein GR, Friedrich VL Jr, Iwata J, Kominami E, et al. Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *Proc Natl Acad Sci USA*. (2007) 104:14489–94. doi: 10.1073/pnas.0701311104
54. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy*. (2016) 12:1–222. doi: 10.1080/15548627.2015.1100356
55. Dehay B, Bove J, Rodriguez-Muela N, Perier C, Recasens A, Boya P, et al. Pathogenic lysosomal depletion in Parkinson's disease. *J Neurosci*. (2010) 30:12535–44. doi: 10.1523/JNEUROSCI.1920-10.2010
56. Rusmini P, Cortese K, Crippa V, Cristofani R, Cicardi ME, Ferrari V, et al. Trehalose induces autophagy via lysosomal-mediated TFEB activation in models of motoneuron degeneration. *Autophagy*. (2019) 15:631–51. doi: 10.1080/15548627.2018.1535292
57. Zhang X, Chen S, Song L, Tang Y, Shen Y, Jia L, et al. MTOR-independent, autophagic enhancer trehalose prolongs motor neuron survival and ameliorates the autophagic flux defect in a mouse model of amyotrophic lateral sclerosis. *Autophagy*. (2014) 10:588–602. doi: 10.4161/auto.27710
58. Li Y, Guo Y, Wang X, Yu X, Duan W, Hong K, et al. Trehalose decreases mutant SOD1 expression and alleviates motor deficiency in early but not end-stage amyotrophic lateral sclerosis in a SOD1-G93A mouse model. *Neuroscience*. (2015) 298:12–25. doi: 10.1016/j.neuroscience.2015.03.061
59. Wang Y, Liu FT, Wang YX, Guan RY, Chen C, Li DK, et al. Autophagic Modulation by Trehalose Reduces Accumulation of TDP-43 in a Cell Model of Amyotrophic Lateral Sclerosis via TFEB Activation. *Neurotox Res*. (2018) 34:109–20. doi: 10.1007/s12640-018-9865-7
60. Settembre C, Ballabio A. TFEB regulates autophagy: an integrated coordination of cellular degradation and recycling processes. *Autophagy*. (2011) 7:1379–81. doi: 10.4161/auto.7.11.17166
61. Cristofani R, Crippa V, Rusmini P, Cicardi ME, Meroni M, Licata NV, et al. Inhibition of retrograde transport modulates misfolded protein accumulation and clearance in motoneuron diseases. *Autophagy*. (2017) 13:1280–303. doi: 10.1080/15548627.2017.1308985
62. Gamerdinger M, Hajieva P, Kaya AM, Wolfrum U, Hartl FU, Behl C. Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. *EMBO J*. (2009) 28:889–901. doi: 10.1038/emboj.2009.29
63. Arndt V, Dick N, Tawo R, Dreiseidler M, Wenzel D, Hesse M, et al. Chaperone-assisted selective autophagy is essential for muscle maintenance. *Curr Biol*. (2010) 20:143–8. doi: 10.1016/j.cub.2009.11.022
64. Crippa V, Carra S, Rusmini P, Sau D, Bolzoni E, Bendotti C, et al. A role of small heat shock protein B8 (HspB8) in the autophagic removal of misfolded proteins responsible for neurodegenerative diseases. *Autophagy*. (2010) 6:958–60. doi: 10.4161/auto.6.7.13042
65. Crippa V, Sau D, Rusmini P, Boncoraglio A, Onesto E, Bolzoni E, et al. The small heat shock protein B8 (HspB8) promotes autophagic removal of misfolded proteins involved in amyotrophic lateral sclerosis (ALS). *Hum Mol Genet*. (2010) 19:3440–56. doi: 10.1093/hmg/ddq257
66. Behl C. BAG3 and friends: co-chaperones in selective autophagy during aging and disease. *Autophagy*. (2011) 7:795–8. doi: 10.4161/auto.7.7.15844
67. Gamerdinger M, Carra S, Behl C. Emerging roles of molecular chaperones and co-chaperones in selective autophagy: focus on BAG proteins. *J Mol Med*. (2011) 89:1175–82. doi: 10.1007/s00109-011-0795-6
68. Gamerdinger M, Kaya AM, Wolfrum U, Clement AM, Behl C. BAG3 mediates chaperone-based aggresome-targeting and selective autophagy of misfolded proteins. *EMBO Rep*. (2011) 12:149–56. doi: 10.1038/embor.2010.203
69. Ulbricht A, Gehlert S, Leciejewski B, Schiffer T, Bloch W, Hohfeld J. Induction and adaptation of chaperone-assisted selective autophagy CASA in response to resistance exercise in human skeletal muscle. *Autophagy*. (2015) 11:538–46. doi: 10.1080/15548627.2015.1017186
70. Behl C. Breaking BAG: the co-chaperone BAG3 in health and disease. *Trends Pharmacol Sci*. (2016) 37:672–88. doi: 10.1016/j.tips.2016.04.007
71. Sturner E, Behl C. The role of the multifunctional BAG3 protein in cellular protein quality control and in disease. *Front Mol Neurosci*. (2017) 10:177. doi: 10.3389/fnmol.2017.00177
72. Cristofani R, Crippa V, Vezzoli G, Rusmini P, Galbiati M, Cicardi ME, et al. The small heat shock protein B8 (HSPB8) efficiently removes aggregating species of dipeptides produced in C9ORF72-related neurodegenerative diseases. *Cell Stress Chaperones*. (2018) 23:1–12. doi: 10.1007/s12192-017-0806-9
73. Rusmini P, Polanco MJ, Cristofani R, Cicardi ME, Meroni M, Galbiati M, et al. Aberrant autophagic response in the muscle of a knock-in mouse model of spinal and bulbar muscular Atrophy. *Sci Rep*. (2015) 5:15174. doi: 10.1038/srep15174
74. Carra S, Rusmini P, Crippa V, Giorgetti E, Boncoraglio A, Cristofani R, et al. Different anti-aggregation and pro-degradative functions of the members of the mammalian sHSP family in neurological disorders. *Philos Trans R Soc Lond B Biol Sci*. (2013) 368:20110409. doi: 10.1098/rstb.2011.0409
75. Vicario M, Skaper SD, Negro A. The small heat shock protein HspB8: role in nervous system physiology and pathology. *CNS Neurol Disord Drug Target*. (2014) 13:885–95. doi: 10.2174/1871527313666140711093344
76. D'Avila F, Meregalli M, Lupoli S, Barcella M, Orro A, De Santis F, et al. Exome sequencing identifies variants in two genes encoding the LIM-proteins NRAP and FHL1 in an Italian patient with BAG3 myofibrillar myopathy. *J Muscle Res Cell Motil*. (2016) 37:101–15. doi: 10.1007/s10974-016-9451-7
77. Ghaoui R, Palmio J, Brewer J, Lek M, Needham M, Evila A, et al. Mutations in HSPB8 causing a new phenotype of distal myopathy and motor neuropathy. *Neurology*. (2016) 86:391–8. doi: 10.1212/WNL.0000000000002324
78. Fang X, Bogomolovas J, Wu T, Zhang W, Liu C, Veevers J, et al. Loss-of-function mutations in co-chaperone BAG3 destabilize small HSPs and cause cardiomyopathy. *J Clin Invest*. (2017) 127:3189–200. doi: 10.1172/JCI94310
79. Bouhy D, Juneja M, Katona I, Holmgren A, Asselbergh B, De Winter V, et al. A knock-in/knock-out mouse model of HSPB8-associated distal hereditary motor neuropathy and myopathy reveals toxic gain-of-function of mutant Hspb8. *Acta Neuropathol*. (2018) 135:131–48. doi: 10.1007/s00401-017-1756-0
80. Milioto C, Malena A, Maino E, Polanco MJ, Marchioretti C, Borgia D, et al. Beta-agonist stimulation ameliorates the phenotype of spinal and bulbar muscular atrophy mice and patient-derived myotubes. *Sci Rep*. (2017) 7:41046. doi: 10.1038/srep41046
81. Cicardi ME, Cristofani R, Rusmini P, Meroni M, Ferrari V, Vezzoli G, et al. Tdp-25 routing to autophagy and proteasome ameliorates its aggregation in amyotrophic lateral sclerosis target cells. *Sci Rep*. (2018) 8:12390. doi: 10.1038/s41598-018-29658-2
82. Gioeli D, Ficarro SB, Kwiek JJ, Aaronson D, Hancock M, Catling AD, et al. Androgen receptor phosphorylation. Regulation and identification

of the phosphorylation sites. *J Biol Chem*. (2002) 277:29304–14. doi: 10.1074/jbc.M204131200
83. Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM. Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. *Mol Endocrinol*. (1995) 9:208–18. doi: 10.1210/mend.9.2.7776971
84. Nollen EA, Brunsting JF, Song J, Kampinga HH, Morimoto RI. Bag1 functions in vivo as a negative regulator of Hsp70 chaperone activity. *Mol Cell Biol*. (2000) 20:1083–8. doi: 10.1128/mcb.20.3.1083-1088.2000
85. Tsukahara F, Maru Y. Bag1 directly routes immature BCR-ABL for proteasomal degradation. *Blood*. (2010) 116:3582–92. doi: 10.1182/blood-2009-10-249623
86. Rusmini P, Cristofani R, Galbiati M, Cicardi ME, Meroni M, Ferrari V, et al. The role of the heat shock protein B8 (HSPB8) in motoneuron diseases. *Front Mol Neurosci*. (2017) 10:176. doi: 10.3389/fnmol.2017.00176
87. Abel A, Taye AA, Fischbeck KH, Merry DE. Truncated androgen receptor gene with an expanded CAG repeat causes phenotypic changes in transgenic mice. *Am J Hum Genet*. (1998) 63:A319
88. Merry DE, Kobayashi Y, Bailey CK, Taye AA, Fischbeck KH. Cleavage, aggregation and toxicity of the expanded androgen receptor in spinal and bulbar muscular atrophy. *Hum Mol Genet*. (1998) 7:693–701
89. Ellerby LM, Hackam AS, Propp SS, Ellerby HM, Rabizadeh S, Cashman NR, et al. Kennedy's disease: caspase cleavage of the androgen receptor is a crucial event in cytotoxicity. *J. Neurochem.* (1999) 72:185–95. doi: 10.1046/j.1471-4159.1999.0720185.x
90. Young JE, Garden GA, Martinez RA, Tanaka F, Sandoval CM, Smith AC, et al. Polyglutamine-expanded androgen receptor truncation fragments activate a Bax-dependent apoptotic cascade mediated by DP5/Hrk. *J Neurosci*. (2009) 29:1987–97. doi: 10.1523/JNEUROSCI.4072-08.2009
91. Tanaka M, Machida Y, Niu S, Ikeda T, Jana NR, Doi H, et al. Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. *Nat Med*. (2004) 10:148–54. doi: 10.1038/nm985
92. Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. *J Biol Chem*. (2007) 282:5641–52. doi: 10.1074/jbc.M609532200
93. Meister-Broekema M, Freilich R, Jagadeesan C, Rauch JN, Bengoechea R, Motley WW, et al. Myopathy associated BAG3 mutations lead to protein aggregation by stalling Hsp70 networks. *Nat Commun*. (2018) 9:5342. doi: 10.1038/s41467-018-07718-5
94. Falcao de Campos C, de Carvalho M. Distal myopathy and rapidly progressive dementia associated with a novel mutation in the VCP gene: Expanding inclusion body myopathy with early-onset Paget disease and frontotemporal dementia spectrum. *J Clin Neurosci*. (2019) 64:8–10. doi: 10.1016/j.jocn.2019.03.063
95. Luders J, Demand J, Hohfeld J. The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. *J Biol Chem*. (2000) 275:4613–7.
96. Demand J, Alberti S, Patterson C, Hohfeld J. Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. *Curr Biol*. (2001) 11:1569–77.
97. Alberti S, Demand J, Esser C, Emmerich N, Schild H, Hohfeld J. Ubiquitylation of BAG-1 suggests a novel regulatory mechanism during the sorting of chaperone substrates to the proteasome. *J Biol Chem*. (2002) 277:45920–7. doi: 10.1074/jbc.M204196200
98. Grunseich C, Rinaldi C, Fischbeck KH. Spinal and bulbar muscular atrophy: pathogenesis and clinical management. *Oral Dis*. (2014) 20:6–9. doi: 10.1111/odi.12121
99. Rocchi A, Milioto C, Parodi S, Armirotti A, Borgia D, Pellegrini M, et al. Glycolytic-to-oxidative fiber-type switch and mTOR signaling activation are early-onset features of SBMA muscle modified by high-fat diet. *Acta Neuropathol*. (2016) 132:127–44. doi: 10.1007/s00401-016-1550-4
100. Sandri M. Protein breakdown in muscle wasting: role of autophagy-lysosome and ubiquitin-proteasome. *Int J Biochem Cell Biol*. (2013) 45:2121–9. doi: 10.1016/j.biocel.2013.04.023
101. Iida M, Katsuno M, Nakatsuji H, Adachi H, Kondo N, Miyazaki Y, et al. Pioglitazone suppresses neuronal and muscular degeneration caused by polyglutamine-expanded androgen receptors. *Hum Mol Genet*. (2015) 24:314–29. doi: 10.1093/hmg/ddu445
102. Hijikata Y, Katsuno M, Suzuki K, Hashizume A, Araki A, Yamada S, et al. Impaired muscle uptake of creatine in spinal and bulbar muscular atrophy. *Ann Clin Transl Neurol*. (2016) 3:537–46. doi: 10.1002/acn3.324

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Secretory Autophagy and Its Relevance in Metabolic and Degenerative Disease

Claudio Daniel Gonzalez[1,2], *Roxana Resnik*[1,2] *and Maria Ines Vaccaro*[1,2]*

[1] Department of Pathophysiology, Institute of Biochemistry and Molecular Medicine (UBA-CONICET), School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina, [2] CEMIC University Institute, Buenos Aires, Argentina

Proteins to be secreted through so-called "conventional mechanisms" are characterized by the presence of an N-terminal peptide that is a leader or signal peptide, needed for access to the endoplasmic reticulum and the Golgi apparatus for further secretion. However, some relevant cytosolic proteins lack of this signal peptides and should be secreted by different unconventional or "non-canonical" processes. One form of this unconventional secretion was named secretory autophagy (SA) because it is specifically associated with the autophagy pathway. It is defined by ATG proteins that regulate the biogenesis of the autophagosome, its representative organelle. The canonical macroautophagy involves the fusion of the autophagosomes with lysosomes for content degradation, whereas the SA pathway bypasses this degradative process to allow the secretion. ATG5, as well as other factors involved in autophagy such as BCN1, are also activated as part of the secretory pathway. SA has been recognized as a new mechanism that is becoming of increasing relevance to explain the unconventional secretion of a series of cytosolic proteins that have critical biological importance. Also, SA may play a role in the release of aggregation-prone protein since it has been related to the autophagosome biogenesis machinery. SA requires the autophagic pathway and both, secretory autophagy and canonical degradative autophagy are at the same time, integrated and highly regulated processes that interact in ultimate cross-talking molecular mechanisms. The potential implications of alterations in SA, its cargos, pathways, and regulation in human diseases such as metabolic/aging pathological processes are predictable. Further research of SA as potential target of therapeutic intervention is deserved.

Keywords: unconventional protein secretion, IL-1β, aggregate-prone proteins, macroautophagy, ATG (autophagy-related) proteins

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***Correspondence:**
Maria Ines Vaccaro
mvaccaro@ffyb.uba.ar;
maria.vaccaro@gmail.com

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AUTOPHAGY

Autophagy is an evolutionarily conserved cellular process induced by nutrient starvation or lack of growth factors that sequester and delivers cytoplasmic components to the lysosome for degradation (1). The classical functions of autophagy are nutrient recycling functions by bulk sequestration from the cytoplasm (2). It is also involved in the cytoplasmic component quality control by removing

specifically damaged or aging organelles, such as depolarized mitochondria (3). Autophagy is also important in proteostasis, sequestering and degrading long-life proteins and invading microbes (4). However, recent reports found that autophagy also presents non-canonical functions (5), especially regulating unconventional secretory processes. Thus, a novel non-degradative role of autophagy has emerged, raising the concept of Secretory Autophagy (SA) (6–8).

In the classical view, according to the pathway that cargo follows to reach the lysosomal compartment, there are three mayor types of canonical degradative autophagy. These types are: microautophagy/endosomal microautophagy (9, 10), chaperone-mediated autophagy (CMA) (11, 12), and macroautophagy. The last one is characterized by the engulfment of cytoplasmic contents by a double membrane vesicle, named autophagosome. Therefore, macroautophagy (hereafter mentioned as autophagy) is distinguished by the formation of the autophagosome as its characteristic and representative intracellular organelle (13).

The autophagic process involves the fusion of the outer membrane of the autophagosomes with lysosomes to deliver the inner vesicle with its cargo to the degradation compartment forming the autolysosome. In the autolysosome, the inner vesicle is degraded, and its products recycled. So far, more than 100 molecules have been related to autophagy regulation and were named ATG molecules (14). Two signaling pathways are associated with autophagy induction: those involve mTOR and AMPK activation. These signaling pathways can sense the environmental, nutritional and energetic status of the cell and promote autophagy through the ULK1-complex, which is the first member of the core molecular machinery in the autophagosome biogenesis [reviewed in (15, 16)]. In brief: Following ULK1 complex activation, the transmembrane protein VMP1 (17) recruits on the ER surface contact site (18) where the first structure in the autophagosome biogenesis, called omegasome, is formed. VMP1 also interacts with the BH3 domain of BECN1 recruiting the kinase complex PI3KC3-C1 to the autophagosome membrane (19). The events that lead to the initial structures (isolation membrane) are followed by the BECN1-PI3K complex activity that phosphorylates the autophagosome membrane and two ubiquitin-like systems ATG12 and LC3 that promote the proper recognition of PI3P (20). In this way, cytoplasmic ATG12 is covalently attached to a C-terminal glycine of ATG5. Furthermore, the ATG5-ATG12 complex promotes LC3 conjugation to phosphatidylethanolamine (PE) on the autophagosomal membrane and this process is mediated by ATG16L, which interacts with ATG5 to eventually form the ATG12-ATG5-ATG16L complex [(21); **Figure 1**].

It is well-known that LC3 plays a central role in autophagy being involved in vesicle elongation, maturation, fusion of autophagosome-lysosome, and even as an adaptor to cargo recognition (22, 23). The lipidated LC3, (LC3B) is present at the isolation membrane and in the autophagosome, in both sides of the membrane. The arrival of autophagosome to the lysosome, is a fusion dependent mechanism of the HOPS complex, through STX17 (24), and RAB7 (25). Thus, LC3 from the inner membrane of the autophagosome is degraded with the cargo. LC3 localized in the external membrane is cleaved from the PE by ATG4B and then recycled (26–28).

Apart from its physiological/homeostatic function, autophagy is also considered as a cell adaptation-to-stress process, which frequently starts as a consequence of organelle damage caused by oxidative species and other stress conditions. Any specific sequestration of a selected type of cargo by autophagy for its delivery to the lysosome is called *selective autophagy* (29). Selective autophagy has a role in intracellular homeostasis, mediating the specific degradation of cytoplasmic material such as aggregated proteins or damaged mitochondria (30). Interactions between autophagy receptors and ubiquitin-like proteins constitute the molecular basis of selective autophagy. In selective autophagy, a cargo-receptor-protein, such as p62, makes the connection between the selected cargo and LC3 in the autophagosomal membrane (31). Importantly, selective degradative autophagy is involved in the cellular response to complex diseases, such as metabolic/aging pathological processes, by the specific degradation of aggregation-prone or aggregated proteins (30, 32) and organelles. These well-studied aspects of degradative autophagy are widely considered an attractive target for therapeutic strategies (33).

SECRETORY AUTOPHAGY

In most cases, especially in exocrine glands and neurons, proteins are secreted by exocytosis (34). The amino-terminal signal peptide (leader sequence) leads eukaryotic secretory proteins into the endoplasmic reticulum (ER), following a well-defined secretory pathway via the Golgi apparatus and eventually progress to the cell surface through vesicular flow. However, some relevant cytosolic proteins lack of this signal peptides and are not able to enter the endoplasmic reticulum (ER). Therefore, they should be secreted by different unconventional or "non-canonical" processes that differ from the classical ER-Golgi pathway (35–37). The autophagy machinery participates in at least one of these pathways. Thus, as mentioned above, this autophagy-dependent secretion pathway is also referred to as SA (6–8).

SA is becoming of increasing relevance to explain the secretion of a series of peptides that have critical biological importance. Interestingly, SA has been shown to play a role in the release of aggregation-prone proteins. This highlights the pathophysiological relevance of this novel, but still not fully elucidated autophagy mediated secretory pathway (38, 39). Autophagy has been also involved in extracellular export of cytosolic organelles, such as mitochondria that can also be released by secretory autophagy (40). Furthermore, different types of non-canonical autophagy have been involved in pathogen released from infected cells (41) and associated with the unconventionally trafficking of proteins to the plasma membrane (42).

Interleukin-1β (IL-1β) secretion is mediated by SA. LC3B-positive carrier sequesters IL1β from the cytosol and fuses with

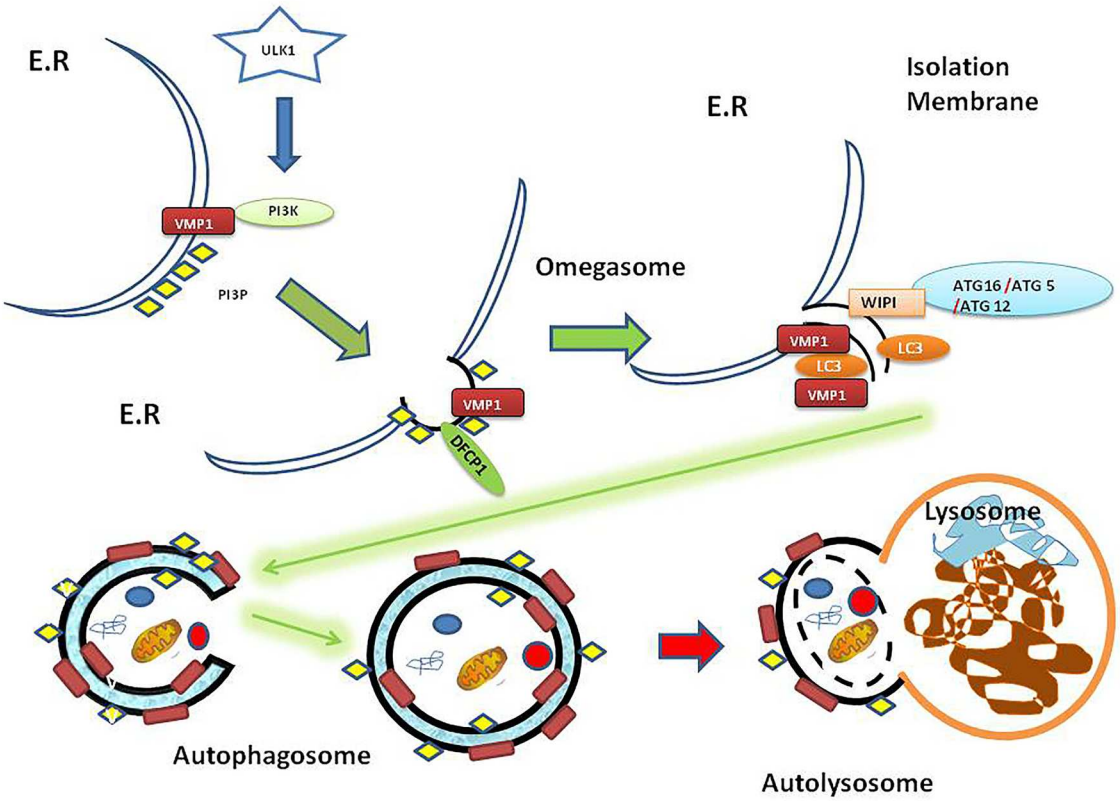

FIGURE 1 | Autophagy overview diagram flow. Autophagosome biogenesis is mediated by ULK1 activation. Here is shown that VMP1, a transmembrane protein, recruits PI3K complex on the ER surface. Then DFCP1 recognizes the PI3P subdomain on the omegasome structure. Besides, WIPI proteins recruit the ATG16-ATG5-ATG12 protein complex on the isolation membrane. In turn, the ATG16-ATG5-ATG12 complex mentioned above mediates LC3 lipidation on the membrane. The genesis of the autophagosome as a double membrane vesicle allows carrying its cargo to the lysosome where the cargo is eventually degraded in the resulting autolysosome as a final structure [reviewed in (15)]. ER, endoplasmic reticulum; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol (3,4,5) triphosphate (PI3P); ULK1, Unc-51-like kinase 1; VMP1, Vacuole Membrane Protein 1; DFCP1, Double FYVE-containing protein 1 (omegasome marker); WIPI, WD40-repeat phosphoinositide-interacting protein (isolation membrane marker); LC3, Microtubule-associated proteins 1A/1B light chain 3B (vesicle maturation/cargo recognition); ATG12, Autophagy-related protein 12 (member of ATG12-ATG5-ATG16L involved in LC3 conjugation to autophagosome membrane); ATG5, autophagy-related protein 5; ATG16, autophagy-related protein 16.

the plasma membrane to release this cytokine through a SA process (6–8, 43). IL-1β release seems to request the participation of the TRIM family proteins as receptors for cargo to be secreted. It has been reported that the TRIM family interacting with SEC22B, as well as some Qa-SNARE (syntaxins 3 and 4), and Qbc SNARE (SNAP 23 and 29) are needed to promote the secretory release of IL-1β. Also, these molecules are needed for other unconventional secretion processes, such as those involving Lysozymes, Cathepsin A, B, C, S, Z, and other dipeptidyl-peptidases and Tubulin (7, 44). Other SA cargos that do not contain a signal peptide are IL-18 and HMGB1 (45, 46). SA is not restricted to inflammasome substrates and autophagic mediated secretion of other cytosolic proteins lacking leader peptide have been reported, such as Galectin-3, Ferritin, and Annexin-I (47).

It has been observed that SA is involved in α-Synuclein aggregates associated with Parkinson's disease (48–50). SA has been linked to the release of aggregates of amyloid-beta (Aβ) peptide associated with Alzheimer's disease (46). A decrease in Aβ secretion and extracellular Aβ plaque formation and an increase of intracellular Aβ aggregate in the perinuclear zone of neurons were reported in neuron-specific ATG7-deficient mice. Also, ATG7 was able to rescue the ability to release Aβ, whereas the induction of autophagy with rapamycin decreased the secretion of Aβ from wild-type primary neurons (46). Moreover, the secretion of Parkinson's disease- and cancer-associated protein Park7/DJ-1 is mediated by SA. Park7 secretion is induced by autophagy through AMPK and ULK1 activation and it is suppressed in Atg5, Atg9, or Atg16lL deficiency animals (51). On the other hand, the inhibition of degradative autophagy was reported to induce the unconventional secretion of α-Synuclein and Huntingtin protein (50). Therefore, growing evidence accumulates to point at a relevant role of SA in pathological protein aggregate secretion and their intracellular accumulation as mechanisms of cell response to degenerative diseases.

SA has a role in intestinal defense mechanisms being involved in the secretion of Lysozyme in Paneth cells, mediated by ATG161L (52). SA is triggered by bacteria-induced ER stress and it is disrupted in Paneth cells of mice harboring ATG16L1, which is a variant of ATG16L related to high risk for Crohn's disease. Another example is the secretion of CFTR (cystic fibrosis transmembrane conductance regulator): knockdown of ATG5 and ATG7, and treatment with autophagy inhibitors, such as wortmannin and 3-methyladenine, abolished the unconventional secretion of CFTR stimulated by ER stress and an ER-to-Golgi blockade (53).

Several ATG-proteins involved in the biogenesis of the autophagosome have been directly related to the molecular mechanisms in SA (**Table 1**). However, the participation of the autophagosomes in the membrane trafficking pathways of SA is not completely elucidated (37, 58). The analysis of the mechanisms underlying unconventional protein secretion includes a wide range of pathways that goes from different forms of plasma membrane translocation to the generation of extracellular vesicles (EVs).

EVs are a heterogeneous group of cell-derived membranous structures that can be originated from the endosomal system and micro-vesicles that are shed from the plasma membrane (59). A type of EVs with a diameter range between 40 and 120 nm were called exosomes. It is interesting to highlight that the secretion of exosomes was also related to the SA pathway (60). This finding suggests that exosomes can be another cargo for SA. Moreover, some cytoplasmic components, from single molecules to organelles, could be involved in the SA mechanism and may also work as SA cargos (61). However, the intracellular membrane trafficking involved in the exosome secretion pathway largely remains poorly understood. In the classic view, exosome biogenesis would start when early endosomes mature into late endosomes or MVBs (Multi Vesicle Bodies) that fuse with the plasma membrane to release the extracellular vesicles to the environment (62). Hence, during this process, the endosomal membrane invaginates to generate intraluminal vesicles (ILVs). These ILVs are finally released into the extracellular space as exosomes, after the fusion of MVBs with the plasma membrane. On the other hand, ATG proteins such as ATG5, participate in exosome production (63). The location of LC3B on the lumen side of the ILVs suggests that a lipidation event takes place at the MVB membrane, or membrane invaginations. Besides, the fact that intact LC3B-positive EVs are eventually released, strongly suggests a secretory fate of selected autophagosomes by its fusion with MVBs in another vesicle called amphisomes (62). A recent article provided solid evidence regarding the involvement of SA in the EVs secretion. In this model, LC3-conjugation controlled the formation and secretion of EVs containing RNA-binding proteins (64). Nevertheless, the biological function of ATG5 and other ATG proteins in exosome production and release remains unclear (58). Finally, as it was recently reviewed, autophagy also participates in the control of conventional secretion, including selective types of autophagy such as such as ribophagy (65) and zymophagy (66, 67). It acts on the secretory apparatus at different steps, with selectivity between different secretory cell types (68).

TABLE 1 | Examples of autophagy related molecules and modulators affecting unconventional protein secretion.

Autophagy-related molecules and modulators	References	Protein secretion	Potentially affected disease
ULK1-complex	(51)	Park7	Parkinson's disease
ATG5	(8, 51, 53–55)	IL-1β; Park7; CFTR; IDE	Parkinson's disease; Cystic Fibrosis; Alzheimer's disease; several chronic inflammatory diseases*
ATG16L1	(51, 52)	Lysozyme; Park7	Parkinson's disease; Crohn's disease
ATG7	(50, 53, 55–57)	α-synuclein; CFTR; Amyloid beta; IDE	Parkinson's disease; type 2 diabetes; cystic fibrosis; Alzheimer's disease
LC3	(8)	IL-1β	Several chronic inflammatory diseases*; carcinogenesis
BECN1	(43, 55)	IL-1β; IDE	Alzheimer's disease
SEC22B	(7)	IL-1β	Several chronic inflammatory diseases*; carcinogenesis
TRIM16	(7)	IL-1β	Several chronic inflammatory diseases*; carcinogenesis
3-MA	(8, 53)	IL-1β; CFTR; IDE	Cystic fibrosis; Alzheimer's disease
Bafilomycin A	(45, 55)	IDE; IL-1β	Alzheimer's disease
Rapamycin	(46)	Amyloid beta	Alzheimer's disease
Spautin 1	(57)	Amyloid beta	Alzheimer's disease
Starvation	(8, 54)	IL-1β	Several chronic inflammatory diseases*; carcinogenesis

Including: rheumatoid arthritis, inflammatory bowel disease; autoimmune thyroiditis; type 2 diabetes.

THE RELEVANCE OF SECRETORY AUTOPHAGY IN DEGENERATIVE, ENDOCRINE, AND METABOLIC DISEASE

Disease Linked With Aggregation-Prone Proteins Deposition and Accumulation May Be Associated With Disturbances in Secretory Autophagy

The potential implication of alterations in autophagy mechanisms in human health is the subject of strong research interest. Parkinson's disease (PD), Alzheimer's disease (AD), and another severe neurological diseases might be at least partially associated with alterations in degradative autophagy and/or in the autophagy-based secretion of certain peptides. This may be also the case for endocrine diseases. In neurodegenerative diseases such as PD and AD, as well as in endocrine and metabolic disease such as Diabetes mellitus, the SA mechanism may be altered in a way that toxic products are secreted and accumulated outside the cell as detritus that in time can cause cell death. In AD, Amyloid beta (Aβ) aggregates and accumulates outside the cell causing neuron functional impairment, structural changes and eventually cell death. The complete process reflects a severe imbalance between production, secretion, aggregation, and clearance of Aβ which progresses until the most advanced stages of the disease. Some studies have shown that autophagy plays a critical role in Aβ secretion (57, 69). Degradative autophagy blockade is followed by Aβ intracellular accumulation which has been demonstrated to be toxic to neurons as well as degradative autophagy in microglia is deteriorated by long exposure to Aβ (69). Moreover, the secretion of Aβ depends on autophagy, since Aβ secretion and plaque formation are reduced in mice lacking ATG7 in the excitatory forebrain neurons (57). It has been observed that ATG7 deficient mice tend to accumulate Aβ in the Golgi (57). Interestingly, it seems that autophagy machinery mediates the transport of certain peptides from the Golgi to endosomes (57). Alterations in these processes may affect Aβ secretion and promote the intracellular accumulation of Aβ, which in turn, results in toxic effects for cells. On the other hand, the administration of rapamycin, an autophagy activator, is followed by a reduction in the intracellular content of Aβ and an improvement in cognitive function in mice (57). Some alterations in SA have also been proposed as playing a role in the pathophysiology of the disease. Dysfunctional insulin-degrading enzyme (IDE), secretion may serve as an example of these SA alterations affecting Aβ aggregation (55). IDE -a ubiquitous enzyme of the inverzincin family of peptidases, is involved in the clearance of insulin, insulin-like growth factors, glucagon, amylin, and other peptides. IDE also degrades some neurotransmitters/neurohormones, including transforming growth factor-α, somatostatin, and endorphins. IDE inactivates calcitonin gene-related peptide and seems to modulate inflammatory responses, and the production of some tumor-associated antigens (70). Cellular and oxidative stress, insulin concentrations, free-fatty acid, and starvation seem to modulate IDE expression. IDE dysfunction may be associated with some forms of type 2 diabetes (T2DM), in humans and several single nucleotide polymorphisms in non-coding regions of the IDE gene, are associated with the disease (71–75). However, many IDE inhibitors have resulted in non-conclusive effects in terms of their potential as glucose tolerance improvers (76). As this enzyme is part of the catabolic pathways of insulin and insulin-like growth factors, high renal and hepatic expression of IDE is not a surprise under normal conditions. However, other cells such as astrocytes and some neurons also display relatively high levels of IDE expression (55). Predominantly located in the cytosol, IDE is also found in other organelles including endosomes, peroxisomes and mitochondria, and it is also found at the cell surface. A close to C terminus Sly sequence motif prevents IDE from enzymatic degradation when present at its lysosomal location (70). Usually <10% of the enzyme is secreted, following a non-conventional pathway. IDE secretion in microglia is enhanced by HMG-CoA reductase inhibitors (statins) (77). Having no signal sequence, IDE secretory pathway is mediated by autophagy. Son et al. (55) found that simvastatin is able to induce the degradation of extracellular Aβ40, which depended on IDE secretion from primary astrocytes. Simvastatin increased IDE secretion is mediated by the activation of autophagy through the LKB1-AMPK-mTOR signaling pathway in astrocytes. Importantly, IDE acts as a key protease for Aβ in the central nervous system. Also, Aβ induces IDE secretion involving the contribution of ATG genes. On the other hand, IDE secretion is associated with GORASP (Golgi reassembly and stacking protein) physiology (36). The dose dependent Aβ induced secretion of IDE is also mediated by GORASP and RAB8A. Moreover, the integrity of the autophagy flow is needed for this process. It also has been shown that Aβ injection in mice with alterations in ATG7 results in reduced expression and activity of IDE in the cerebrospinal fluid potentially associated with alterations in SA (55). Even when Aβ induced IDE secretion by astrocytes, it seems to fulfill many of the critical characteristics required to be considered an autophagy-mediated process. It is worth to be mentioned that some steps in this pathway are not completely understood. Interestingly, some epidemiological studies suggest that patients with T2DM are at a higher risk of developing AD. However, it is difficult to be certain about the nature of the link of both diseases with a dysfunctional SA. A standard oral agent for the treatment of type 2 diabetes (metformin), has shown some positive effects in experimental models of AD. Metformin decreases Beta-secretase 1 (BACE 1) activity which results in reduced production of Aβ (78). The inhibition of acetylcholinesterase (AChE) in the central nervous system, may explain at least in part, some beneficial effects on cognitive function, learning, and memory. Furthermore, Metformin reduces oxidative stress and exhibits anti-inflammatory properties (78). However, metformin is also an autophagy regulatory agent that may modulate both, degradative and secretory autophagy, and enhance autophagic clearance of intracellular neurofibrillary tangles formed by hyperphosphorylated tau protein (79). The real impact of these potential beneficial effects in clinical practice remains to be elucidated.

Amyloid Polypeptide and other aggregation-prone proteins accumulation surrounding the Langerhans Islet cells is a

common finding in type 2 diabetes in humans. Islet Amyloid Polypeptide (IAPP) accumulation is long recognized as a phenomenon occurring in human type 2 diabetes. IAPP is co-synthetized in beta cells and secreted along with insulin, this is a 37-amino-acid residue polypeptide. Human IAPP forms oligomeric structures and fibrous extracellular precipitates that accumulate in the islets. The genetic background seems to be associated with the degree of IAPP deposition (80) and some nutritional factors (such as fat intake), would facilitate IAPP deposition (81). The implications of the IAPP accumulation in the evolution of human forms of diabetes is poorly understood. Furthermore, extracellular human IAPP can promote autophagy in beta cells and the reactive Oxygen Species (ROS), mediates part of the IAPP induced degradative autophagy by involving the AMPK pathway (82). On the other hand, it has been suggested that intracellular IAPP oligomer formation would be toxic for beta cells. In this way, IAPP oligomers may damage mitochondrial membranes as well as the endoplasmic reticulum (ER) (83). In humans, the augmented expression of IAPP in beta cells is followed by an increase of the autophagy flux (84). Moreover, lack of autophagy in hIAPP-expressing animals resulted in hIAPP oligomer and amyloid accumulation in pancreatic islets, leading to β cell death (83, 85–87). It has been suggested that IAPP oligomers may cross plasma membranes inducing damage by a "prion-like" effect. In this way, the intraperitoneal injection of IAPP aggregates from the pancreas homogenate to the transgenic mouse that expresses hIAPP, dramatically accelerates IAPP amyloid deposition, which was accompanied by abnormalities resembling T2DM (88, 89). In this process, degradative autophagy integrity seems to be of relevance. In general, increased degradative autophagy would protect against IAPP induced damage on beta cells. However, evidence on the potential role of SA remains less conclusive. Alterations in IDE secretion has been suggested as part of the pathophysiology of IAPP deposition. IAPP is a substrate of IDE. However, IDE inhibition does not seem to increase amyloid deposition of endogenous IAPP *in vivo* (90). The recently described expression of another aggregable protein, α-synuclein, in beta cells adds complexity to the landscape of aggregation-prone proteins induced damage in type 2 diabetes. Alpha-synuclein expresses in murine pancreatic islets and exogenous overexpression of α-synuclein reduces insulin secretion by INS1 cells (91). It has been proposed that α-synuclein interacts with the Kir6.2 subunit at the KATP channel located at the beta-cell membrane suppressing insulin secretion (91). In normal conditions, this mechanism may protect beta-cell from ER stress by down-regulating exaggerated insulin secretion. In the presence of type 2 diabetes, α-synuclein may play a negative role in insulin release contributing to hyperglycemia, oxidative stress, and glycosylation of protein (91). Steneberg et al. (56) showed that autophagic flux is reduced by increased levels of α-synuclein present in β-cells from IDE KO mice and in T2D patients. On the other hand, Sharma et al. (92), proposed that α-synuclein is able to activate IDE, while IDE inhibits amyloid formation by α-synuclein. However, it is unclear if these factors may result in increased IAPP deposition in T2DM in humans. Moreover, several reported changes may represent indirect evidence of the IAPP induced damage and cell adaptation. Also, these alterations might precede or follow the intracellular accumulation of oligomers. Finally, direct or indirect alterations in SA may result in an increased concentration of proinflammatory mediators such as IL-1β increasing IAPP deposition, functional alteration, oxidative stress, and cell death.

In PD's Disease (PD), the accumulation of α-synuclein, a protein with high binding affinity for smaller vesicles (as synaptic vesicles, for instance), was reported. Alpha-synuclein mutation or over-expression impairs membrane trafficking including exocytosis and ER-to-Golgi transport (93, 94). These alterations are associated with ER stress, increasing oxidative stress affecting cell homeostasis. Moreover, lysosome impairment seems to play a critical role in the progression of the disease (95). It has been proposed that the Secretory Carrier Membrane Protein 5 (SCAMP5), promotes the secretion of α-synuclein and other neurotoxic proteins via exosomes (50). It has been demonstrated that SCAMP5 has some effects as an inhibitor of degradative autophagy, playing role in balancing several processes involved in cell homeostasis, including vesicle trafficking as well as constitutive, degradative and secretory autophagy (50). Also, several other alterations linked to autophagy have been identified in different models of PD. Alterations in the TMEM230 gene (Transmembrane Protein 230), have been recently associated with the pathophysiology of familial forms of the disease. TMEM230 regulates the autophagy-mediated clearance of α-synuclein and mediates Rab8a-associated SA. As a result, this protein regulates Golgi-derived vesicle secretion (96). Lewy-bodies formation, which is typically associated with cognitive dysfunction and dementia seen in some patients with advanced PD, may result from these alterations (96). PARK7/DJ-1 protein has been associated with PD and certain forms of cancer. PARK7/DJ-1 participates in the regulation of several cell processes, including anti-oxidative protection. Furthermore, an increased pro-oxidative environment is associated with degenerative damage along with the disease evolution. The autophagy pathway involving ATG molecules was associated with PARK7/DJ-1 secretion (51). Thus, impairment in autophagy might result in the defective secretion of this protein with potential implications in terms of anti-oxidative stress protection, playing a role in PD progression.

Amyotrophic lateral sclerosis (ALS), is a degenerative disorder that affects cortical, bulbar and spinal motor neurons and is mainly characterized by a progressive adult-onset. Like other neurodegenerative diseases, ALS is categorized as a "proteinopathy" since SOD1, TDP-43, and FUS are pathological proteins that accumulate, interfere, and impair neuronal functions leading to cell death. Sproviero et al. suggest that these toxic proteins are transported mainly by EVs that might play a role in prion-like propagation of ALS disease (97).

Impairment in SA May Affect the Effect and the Concentration of Secreted Peptides and Other Co-secreted Substances

Disruptions in SA may result in relevant changes in the concentrations of secreted peptides and other co-secreted molecules, as well as in their paracrine and endocrine effects.

Altered secretion of lysozyme is related to some forms of inflammatory bowel disease and relevant changes in host defense mechanism. Paneth cells are specialized secretory cells in the small intestine that have been related to Crohn disease through a mutation of ATG16L1 (98). It has been observed, that lysozyme and other critical secreted antibacterial proteins are rerouted by Paneth cells through SA as a reaction to bacterial invasion. Moreover, activation of innate lymphoid cells type 3 (ILC3) which secrete IL22 licenses Paneth cells to secrete lysozyme through SA (52, 99). These data suggest that Crohn disease may be associated, at least in part, to some genetically mediated alterations in SA.

Impaired secretion of other inflammation regulatory molecules by a disrupted SA has been reported. IL-1β is a major pro-inflammatory cytokine, that also doesn't follow the classical endoplasmic reticulum-to-Golgi route. IL-1β release is mediated by SA requiring the participation of the microtubule-associated protein EB1 (100). This autophagy-dependent, unconventional secretion pathway is of special interest (45). IL-1β transcription is induced by different stimuli ranging from microbiological agents to other cytokines and also growth factors (101). Moreover, neutrophils, macrophages, and microglia are relevant sources of IL-1β under infectious and other conditions and it was demonstrated that autophagy is responsible for IL-1β exocytosis under these challenges. As expected for autophagy-based secretory mechanisms, it was shown that siRNA-mediated knockdown of ATG5 reduces IL-1β secretion in neutrophils (54). In contrast, cell starvation increases the colocalization of IL-1β and LC3B promoting IL-1β secretion. Extending data including all data mentioned above strongly suggests that SA is involved in IL-1β secretion by neutrophils and other secreting cells.

IL-1β derives from an inactive precursor, pro- IL-1β, that requires cleavage by caspase-1 for activation. Activation of caspase 1 is mediated by the protein complex called 'inflammasome'. Release of mature IL-1β relays on SA and it has been suggested that GRASP (a Golgi apparatus associated factor) participates in IL-1β secretion (45). After release, a series of inhibitory molecules including IL-1Ra, sIL-1RI, sIL-1RII, and sIL-1RAcP regulates IL-1β mediated inflammation. IL-1β secretion has been demonstrated to be increased in several endocrine, metabolic and degenerative conditions, as well as some acute entities and infections. In fact, a key role in the defense against microbial pathogens and in tissue injury repair, is performed by IL-1β. Local and systemic responses to this cytokine are responsible for homeostatic effects. Exaggerated responses to IL-1β, however, are associated with potentially deleterious effects; and excessive IL-1β activity is associated to vasculitis and thrombosis. IL-1β also plays a role in rheumatoid arthritis and other inflammatory diseases. Part of the structural damage associated with PD, AD, and other degenerative entities seems to be linked to IL-1β effects. For instance, β cells exposure to increased concentrations of IL-1β is associated with functional deterioration and cell death. Moreover, IL-1β is linked to inflammation in obesity, type 2 diabetes, insulin-resistance associated entities (e.g., polycystic ovary syndrome), atherosclerosis and other conditions. In addition, IL-1β seems to play a role in the pathogenesis of Autoimmune Thyroid Diseases (102). Regarding type 2 diabetes, only when basal levels of IL-1β mRNA are low, hyperglycemia induced IL-1β production in β cells can be observed (103). IL-1β is also produced by infiltrating immune cells in the pancreas (104). Taking all this information into account, it seems that IL-1β acts as a "metabolic sensor" aside of its well-recognized role as pro-inflammatory mediator (105). IL-1β physiology is a highly illustrative example of the complexities of degradative and secretory autophagic processes. Hence, the induction of inflammasomes triggers autophagosome formation in macrophages (106). In some tissues, this effect may be part of a negative mechanism to control and limit the inflammatory response confronting challenges of infectious origin. It seems to be also linked to an increased cytokine-mediated anti-microbial defense.

In some cases, IL-1β was found to be increased in degradative autophagy and may be linked to tissue damage. In pancreatic acinar cells, IL-1β hyperactivity seems to be associated with increased endoplasmic reticulum stress-inducing autophagy. This mechanism may be related to an impaired autophagic flux leading to trypsin activation and pancreatic injury. It has been observed that Atg 5 alterations are followed by an increment in IL-1β plasma concentration though pro-IL-1β caspase-mediated cleavage (107). As mentioned before, autophagy is critical to IL-1β release through the SA mechanism that involves the AIM2 inflammasome. The interplay between different cytokines (many of them affecting autophagy in several manners), adds complexity to this homeostatic network. This effect may result in a chain of autophagic process modifications affecting the SA of regulatory peptides. Although precise mechanisms articulating the balance between the complex processes enumerated above remain unclear, many of them seem to be of major relevance for a better understanding of the immune-inflammatory components in degenerative and metabolic conditions.

Autoimmune thyroiditis (AIT) is among the main causes of hypothyroidism in human. An increased expression of some inflammasome components as well as IL-1β was observed in thyroid gland tissues from AIT patients (108). This phenomenon suggests an upregulated SA activity in these subjects and may play a role in the pathophysiology of the disease. Other autoimmune endocrinopathies may also reflect a certain degree of dysfunctional SA with dysregulated secretion of IL-1β and other cytokines. Elevated levels of pro-osteoporotic cytokines including IL-1β have been found in patients with different forms of hyperthyroidism (109). IL-1β secreted by peripheral monocytes induces IL-6 secretion by stromal cells and osteoblast, IL-6 increments osteoclast proliferation and differentiation inducing increased bone resorption (110) A role of IL-1β hypersecretion cannot be excluded as a potential mediator of hyperthyroidism associated altered bone resorption (111).

Emerging role of secretory autophagy in carcinogenesis and endocrine tumor progression is a topic of significant clinical relevance. Secretory autophagy may be involved in the secretion of tumor-promoting proteins. It has been suggested, that low expression of Rasal2 gene was associated with the recurrence of

luminal B breast cancer (112). Recently has been shown that the Rasal2 gene knock out induces secretory autophagy. Although the potential mediators are not elucidated, the increase in SA seems to be associated with luminal breast cancer proliferation (113). Altered secretory patterns of IL-1β, IL-6, IL-8, bFGF, and other growth factors have been demonstrated for certain tumor types (114). Interestingly, IDE is expressed in some human undifferentiated breast and ovarian types of cancer, as well as in retinoblastoma. A tumor-suppressing activity was suggested for this enzyme (115, 116). Dysfunctional SA of IDE might play a role in very aggressive tumors. Paracrine secretion between cancer-cell interactions would facilitate tumor initiation, growth, and spreading. It has been suggested that paracrine secretion of IL6, IL8, and bFGF induces autophagy in head and neck cancer-associated fibroblasts (114). Therefore, SA and degradative autophagy modulates critical interactions between tumor cells and the surrounding microenvironment and may result in significant metabolic modifications, with a strong impact in cancer cell adaptations by Warburg and reverse Warburg effects (117).

CONCLUSIONS

Macroautophagy is a complex cellular pathway characterized by the formation of the double-membrane vesicle called autophagosome that sequesters cytoplasmic contents to be delivered to the lysosomal compartment for degradation. However, increased experimental evidences strongly support that another fate of the autophagosome biogenesis molecular machinery exists, leading to a novel pathway of unconventional secretion. This last process was named SA to differentiate it from the canonical degradative autophagy (6, 61).

SA has been recognized as a novel mechanism to explain the secretion of a series of peptides which have critical biological importance. Although the molecular pathways and vesicular trafficking in SA are not fully elucidated, canonical autophagy machinery is mechanistically involved (7). Thus, the autophagy-related proteins, such as ATG5 and several other components of the autophagosome biogenesis (13) play a central role in the secretion of critical disease-related proteins. Indeed, autophagosome formation seems to be involved in the eventual release of these relevant proteins (8). Interestingly, the fact that cargo recognition molecules, such as the TRIM family are mechanistically involved in this process (7) strongly suggests that SA could be considered as a non-canonical type of selective autophagy. In SA the process does not end in lysosomal degradation but in the secretion of the selected cargo. A wide range of proteins secreted by mammalian cells following different pathways are related to autophagy and associated with human diseases. This includes proteins related to some endocrine and aging associated diseases.

Degradative and secretory autophagy are two integrated multistep processes highly regulated by several physiological and disease-related factors. Starvation, oxidative stress, and hypoxia are well recognized stimuli for degradative autophagy. SA efficiency depends at least in part, on the integrity of the molecular machinery involved in the degradative processes. Changes in degradative and secretory autophagy may result in impairment of the release of different peptides that, in turn, may affect autophagy in other cells by paracrine or even endocrine ways. Implications of this interplay in the prevention, prognosis and treatment of several diseases are still to be elucidated.

Multiple neurodegenerative disorders have in common abnormal protein accumulation and aggregation (32). Autophagosomes or MVBs would be able to sequester and degrade cytosolic-protein aggregates through lysosomes. Externalization of protein aggregates may also be mediated by secretory autophagy. On the other hand, changes in the secretion of protein aggregates might decrease the proteotoxic stress in the releasing cells or reduce the spreading of protein aggregates to neighboring cells.

There is substantial experimental data that allow us to consider that canonical degradative autophagy and its related factors are mechanistically associated with secretion. It has been demonstrated that SA requires the autophagic pathway and both, secretory and degradative autophagy are integrated and highly regulated processes that interact in ultimate cross-talking molecular mechanisms. Impaired secretory autophagy may result in the aggregation-prone proteins deposition and accumulation, or severe alterations in the release and concentration of other secreted proteins. The relevance and the mechanisms involved in these interactions seem to be very important in metabolic and degenerative diseases. The pharmacological modulation of SA and its regulatory pathways might also be a clear target for drug research. Although there is available evidence that outline the potential relevance of the pharmacological control of SA, important gaps in the evidence remain to be filled. Metformin, for instance, may modulate SA by its demonstrated effects on degradative autophagy. It has been observed that in patients with type 2 diabetes, metformin upregulates mitophagy, and improves mitochondrial function in a glucose-lowering independent manner (118). By inhibiting macrophage activation and activating autophagic flux, metformin reduces pro-inflammatory cytokines release (including IL-1β) (119). However, a direct effect on SA mechanisms cannot be excluded. Statins (HMGCoA reductase inhibitors) increases IDE release by astrocytes in a dose-dependent manner. These agents directly modulate SA at least in some tissues. However, the potential relevance of this mechanism on the beneficial and/or adverse events associated with statin use in practice remains obscure. Iron enhances Aβ stimulated IL-1β secretion in microglia (120). The impact of this finding in humans is still unknown. Many other agents seem to modulate IL-1β secretion by regulating SA processes in different ways, and in different cell types (121). Translation of these findings to drug development and application into clinical practice will take a while. However, the potential implication of impairment in secretory autophagy, its cargos, pathways, and regulation in human diseases such as metabolic/aging pathological processes is a clear focus of biomedical investigation. Further research on

secretory autophagy pathways as a potential target of therapeutic intervention is deserved.

AUTHOR CONTRIBUTIONS

MV selected the subject, wrote the 1st and 2nd sections, and the conclusions, selected part of the bibliography, reviewed the whole manuscript, and made the table. CG wrote the abstract and the third part of the manuscript and selected part of the bibliography and reviewed the manuscript. RR collaborated in the writing of the whole manuscript and made the figures.

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REFERENCES

1. Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat Rev Mol Cell Biol.* (2007) 8:931–7. doi: 10.1038/nrm2245
2. Galluzzi L, Pietrocola F, Levine B, Kroemer G. Metabolic control of autophagy. *Cell*. (2014) 159:1263–76. doi: 10.1016/j.cell.2014.11.006
3. Yamano K, Fogel A, Wang C, Van der Bliek A, Youle R. Mitochondrial Rab GAPs govern autophagosome biogenesis during mitophagy. *Elife.* (2014) 3:e01612. doi: 10.7554/eLife.01612
4. Deretic V, Kimura T, Timmins G, Moseley P, Chauhan S, Mandell M, et al. Immunologic manifestations of autophagy. *J Clin Invest.* (2015) 125:75–84. doi: 10.1172/JCI73945
5. Subramani S, Malhotra V. Non-autophagic roles of autophagy-related proteins. *EMBO Rep.* (2013) 14:143–51. doi: 10.1038/embor.2012.220
6. Ponpuak M, Mandell MA, Kimura T, Chauhan S, Cleyrat C, Deretic V. Secretory autophagy. *Curr Opin Cell Biol.* (2015) 35:106–16. doi: 10.1016/j.ceb.2015.04.016
7. Kimura T, Jia J, Kumar S, Choi SW, Gu Y, Mudd M, et al. Dedicated SNAREs and specialized TRIM cargo receptors mediate secretory autophagy. *EMBO J.* (2017) 36:42–60. doi: 10.15252/embj.201695081
8. Zhang M, Kenny S, Ge L, Xu K, Schekman R. Translocation of interleukin-1β into a vesicle intermediate in autophagy-mediated secretion. *Elife.* (2015) 4:e11205. doi: 10.7554/eLife.11205.023
9. Li WW, Li J, Bao JK. Microautophagy: lesser-known self-eating. *Cell Mol Life Sci.* (2012) 69:1125–36. doi: 10.1007/s00018-011-0865-5
10. Galluzzi L, Baehrecke EH, Ballabio A, Boya P, Bravo-San Pedro JM, Cecconi F. Molecular definitions of autophagy and related processes. *EMBO J.* (2017) 36:1811–36. doi: 10.15252/embj.201796697
11. Cuervo AM, Wong E. Chaperone-mediated autophagy: roles in disease and aging. *Cell Res.* (2014) 24:92–104. doi: 10.1038/cr.2013.153
12. Kaushik S, Cuervo AM. The coming of age of chaperone-mediated autophagy. *Nat Rev Mol Cell Biol.* (2018) 19:365–81. doi: 10.1038/s41580-018-0001-6
13. Mizushima N, Yoshimori T, Ohsumi Y. The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol.* (2011) 27:107–32. doi: 10.1146/annurev-cellbio-092910-154005
14. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd Edition). *Autophagy.* (2016) 12:1–222. doi: 10.1080/15548627.2015.1100356
15. Grasso D, Renna FJ, Vaccaro MI. Initial steps in mammalian autophagosome biogenesis. *Front Cell Dev Biol.* (2018) 6:146. doi: 10.3389/fcell.2018.00146
16. Mercer TJ, Gubas A, Tooze SA. A molecular perspective of mammalian autophagosome biogenesis. *J Biol Chem.* (2018) 293:5386–95. doi: 10.1074/jbc.R117.810366
17. Ropolo A, Grasso D, Pardo R, Sacchetti ML, Archange C, Lo Re A, et al. The pancreatitis-induced vacuole membrane protein 1 triggers autophagy in mammalian cells. *J Biol Chem.* (2007) 282:37124–33. doi: 10.1074/jbc.M706956200
18. Nascimbeni AC, Giordano F, Dupont N, Grasso D, Vaccaro MI, Codogno E, et al. ER-plasma membrane contact sites contribute to autophagosome biogenesis by regulation of local PI3P synthesis. *EMBO J.* (2017) 36:2018–33. doi: 10.15252/embj.201797006
19. Molejon MI, Ropolo A, Re AL, Boggio V, Vaccaro MI. The VMP1-beclin 1 interaction regulates autophagy induction. *Sci Rep.* (2013) 3:1055. doi: 10.1038/srep01055
20. Mizushima N. The ATG conjugation systems in autophagy. *Curr Opin Cell Biol.* (2019) 63:1–10. doi: 10.1016/j.ceb.2019.12.001
21. Wilson MI, Dooley HC, Tooze SA. WIPI2b and Atg16L1: setting the stage for autophagosome formation. *Biochem Soc Trans.* (2014) 42:1327–34. doi: 10.1042/BST20140177
22. Nakatogawa H, Ichimura Y, Ohsumi Y. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell.* (2007) 130:165–78. doi: 10.1016/j.cell.2007.05.021
23. Lee YK, Lee JA. Role of the mammalian ATG8/LC3 family in autophagy: differential and compensatory roles in the spatiotemporal regulation of autophagy. *BMB Rep.* (2016) 49:424–30. doi: 10.5483/BMBRep.2016.49.8.081
24. Jiang P, Nishimura T, Sakamaki Y, Itakura E, Hatta T, Natsume T, et al. The HOPS complex mediates autophagosome-lysosome fusion through interaction with syntaxin 17. *Mol Biol Cell.* (2014) 25:1327–37. doi: 10.1091/mbc.e13-08-0447
25. Gutierrez MG, Munafo DB, Beron W, Colombo MI. Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. *J Cell Sci.* (2004) 117(Pt 13):2687–97. doi: 10.1242/jcs.01114
26. Noda NN, Fujioka Y, Hanada T, Ohsumi Y, Inagaki F. Structure of the Atg12-Atg5 conjugate reveals a platform for stimulating Atg8-PE conjugation. *EMBO Rep.* (2013) 14:206–11. doi: 10.1038/embor.2012.208
27. Otomo C, Metlagel Z, Takaesu G, Otomo T. Structure of the human ATG12∼ATG5 conjugate required for LC3 lipidation in autophagy. *Nat Struct Mol Biol.* (2013) 20:59–66. doi: 10.1038/nsmb.2431
28. Dooley HC, Wilson MI, Tooze SA. WIPI2B links PtdIns3P to LC3 lipidation through binding ATG16L1. *Autophagy.* (2015) 11:190–1. doi: 10.1080/15548627.2014.996029
29. Kraft C, Reggiori F, Peter M. Selective types of autophagy in yeast. *Biochim Biophys Acta*. (2009) 1793:1404–12. doi: 10.1016/j.bbamcr.2009.02.006
30. Rogov V, Dötsch V, Johansen T, Kirkin V. Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. *Mol Cell.* (2014) 53:167–78. doi: 10.1016/j.molcel.2013.12.014
31. Khaminets A, Behl C, Dikic I. Ubiquitin-dependent and independent signals in selective autophagy. *Trends Cell Biol.* (2015) 26:6–16. doi: 10.1016/j.tcb.2015.08.010
32. Frake RA, Ricketts T, Menzies FM, Rubinsztein DC. Autophagy and neurodegeneration. *J Clin Invest*. (2015) 125:65–74. doi: 10.1172/JCI73944
33. Levine B, Packer M, Codogno PL. Development of autophagy inducers in clinical medicine. *J Clin Invest.* (2015) 125:14–24. doi: 10.1172/JCI73938
34. Südhof TC, Rizo J. Synaptic vesicle exocytosis. *Cold Spring Harb Perspect Biol.* (2011) 3:a005637. doi: 10.1101/cshperspect.a005637
35. Rabouille C, Malhotra V, Nickel W. Diversity in unconventional protein secretion. *J Cell Sci.* (2012) 125:5251–5. doi: 10.1242/jcs.103630
36. Jiang S, Dupont N, Castillo EF, Deretic V. Secretory versus degradative autophagy: unconventional secretion of inflammatory mediators. *J Innate Immun.* (2013) 5:471–9. doi: 10.1159/000346707

37. New J, Thomas SM. Autophagy-dependent secretion: mechanism, factors secreted, and disease implications. *Autophagy*. (2019) 15:1682–93. doi: 10.1080/15548627.2019.1596479
38. Lee JG, Takahama S, Zhang G, Tomarev S, Ye Y. Unconventional secretion of misfolded proteins promotes adaptation to proteasome dysfunction in mammalian cells. *Nat Cell Biol.* (2016) 18:765–76. doi: 10.1038/ncb3372
39. Xu J, Camfield R, Gorski SM. The interplay between exosomes and autophagy - partners in crime. *J Cell Sci.* (2018) 131:jcs215210. doi: 10.1242/jcs.215210
40. Schweers RL, Zhang J, Randall MS, Loyd MR, Li W, Dorsey FC, et al. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. *Proc Natl Acad Sci USA*. (2007) 104:19500–5. doi: 10.1073/pnas.0708818104
41. Gerstenmaier L, Pilla R, Herrmann L, Herrmann H, Prado M, Villafano GJ, et al. The autophagic machinery ensures nonlytic transmission of mycobacteria. *Proc Natl Acad Sci USA*. (2015) 112:E687–92. doi: 10.1073/pnas.1423318112
42. Gee HY, Noh SH, Tang BL, Kim KH, Lee MG. Rescue of DeltaF508-CFTR trafficking via a GRASP-dependent unconventional secretion pathway. *Cell*. (2011) 146:746–60. doi: 10.1016/j.cell.2011.07.021
43. Kraya AA, Piao S, Xu X, Zhang G, Herlyn M, Gimotty P, et al. Identification of secreted proteins that reflect autophagy dynamics within tumor cells. *Autophagy*. (2015) 11:60–74. doi: 10.4161/15548627.2014.984273
44. Deretic V. Autophagy: an emerging immunological paradigm. *J Immunol*. (2012) 189:15–20. doi: 10.4049/jimmunol.1102108
45. Dupont N, Jia S, Pilli M, Ornatowsking W, Bhattacharya D, Deretic V. Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta. *EMBO J.* (2011) 30:4701–11. doi: 10.1038/emboj.2011.398
46. Thorburn J, Horita H, Redzic J, Hansen K, Frankel AE, Thorburn A. Autophagy regulates selective HMGB1 release in tumor cells that are destined to die. *Cell Death Differ*. (2009) 16:175–83. doi: 10.1038/cdd.2008.143
47. Ohman T, Teirilä L, Lahesmaa-Korpinen AM, Cypryk W, Veckman V, Shinobu Saijo S, et al. Dectin-1 pathway activates robust autophagy-dependent unconventional protein secretion in human macrophages. *J Immunol*. (2014) 192:5952–62. doi: 10.4049/jimmunol.1303213
48. Ejlerskov P, Rasmussen I, Nielsen TT, Bergström AL, Tohyama Y, Jensen PH, et al. Tubulin polymerization-promoting protein (TPPP/p25alpha) promotes unconventional secretion of alpha-synuclein through exophagy by impairing autophagosome- lysosome fusion. *J Biol Chem*. (2013) 288:17313–35. doi: 10.1074/jbc.M112.401174
49. Iguchi Y, Eid L, Parent M, Soucy G, Bareil C, Riku Y, et al. Exosome secretion is a key pathway for clearance of pathological TDP-43. *Brain*. (2016) 139(Pt 12):3187–201. doi: 10.1093/brain/aww237
50. Yang Y, Qin M, Bao P, Xu W, Xu J. Secretory carrier membrane protein 5 is an autophagy inhibitor that promotes the secretion of α-synuclein via exosome. *PLoS ONE*. (2017) 12:e0180892. doi: 10.1371/journal.pone.0180892
51. Urano Y, Mori C, Fuji A, Konno K, Yamamoto T, Yashirogi S, et al. 6-Hydroxydopamine induces secretion of PARK7/DJ-1 via autophagy-based unconventional secretory pathway. *Autophagy*. (2018) 14:1943–58. doi: 10.1080/15548627.2018.1493043
52. Bel S, Pendse M, Wang Y, Li Y, Kelly A, Ruhn KA, et al. Paneth cells secrete lysozyme via secretory autophagy during bacterial infection of the intestine. *Science*. (2017) 357:1047–52 doi: 10.1126/science.aal4677
53. Noh SH, Kim Y, Piao H, Kim J, Kang CM, et al. Specific autophagy and ESCRT components participate in the unconventional secretion of CFTR. *Autophagy*. (2018) 14:1761–78. doi: 10.1080/15548627.2018.1489479
54. Iula L, Keitelman IA, Sabbione F, Fuentes F, Guzman M, Galletti JG, et al. Autophagy Mediates interleukin-1β secretion in human neutrophils. *Front Immunol*. (2018) 9:269. doi: 10.3389/fimmu.2018.00269
55. Son SM, Cha MY, Choi H, Kang S, Choi H, Lee MS, et al. Insulin-degrading enzyme secretion from astrocytes is mediated by an autophagy-based unconventional secretory pathway in Alzheimer disease. *Autophagy*. (2016) 12:784–800. doi: 10.1080/15548627.2016.1159375
56. Steneberg P, Bernardo L, Edfalk S, Lundberg L, Backlund F, Östenson CG, et al. The type 2 diabetes-associated gene ide is required for insulin secretion and suppression of α-synuclein levels in β-cells. *Diabetes*. (2013) 62:2004–14. doi: 10.2337/db12-1045
57. Nilsson P, Loganathan K, Sekiguchi M, Matsuba Y, Hui K, Tsubuki S, et al. A β secretion and plaque formation depend on autophagy. *Cell Rep*. (2013) 5:61–9. doi: 10.1016/j.celrep.2013.08.042
58. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol*. (2018) 19:213–28. doi: 10.1038/nrm.2017.125
59. Théry C, Witwer K, Aikawa E, Alcaraz MJ, Anderson D, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles*. (2018) 7:1535750. doi: 10.1080/20013078.2018.1535750
60. Cheng Q, Li X, Wang Y, Dong M, Zhan FH, Liu J. (2018). The ceramide pathway is involved in the survival, apoptosis and exosome functions of human multiple myeloma cells in vitro. *Acta Pharmacol Sin*. (2018) 39:561–568. doi: 10.1038/aps.2017.118
61. Claude-Taupin A, Bissa B, Jia J, Gu Y, Deretic V. Role of autophagy in IL-1β export and release from cells. *Semin Cell Dev Biol*. (2018) 83:36–41. doi: 10.1016/j.semcdb.2018.03.012
62. Xu Y, Cui L, Dibello A, Wang L, Lee J, Saidi L, et al. DNAJC5 facilitates USP19-dependent unconventional secretion of misfolded cytosolic proteins. *Cell Discov*. (2018) 4:11. doi: 10.1038/s41421-018-0012-7
63. Guo H, Chitiprolu M, Roncevic L, Javalet C, Hemming FJ, Trung MT. Atg5 disassociates the V 1 V 0 -ATPase to promote exosome production and tumor metastasis independent of canonical macroautophagy. *Dev Cell*. (2017) 43:716–730.e7. doi: 10.1016/j.devcel.2017.11.018
64. Leidal M, Huang H, Marsh T, Solvik T, Zhang D, Ye J, et al. The LC3-conjugation machinery specifies the loading of RNA-binding proteins into extracellular vesicles. *Nat Cell Biol*. (2020) 22:187–99. doi: 10.1038/s41556-019-0450-y
65. An H, Harper JW. Systematic analysis of ribophagy in human cells reveals bystander flux during selective autophagy. *Nat Cell Biol*. (2018) 20:135–43. doi: 10.1038/s41556-017-0007-x
66. Grasso D, Ropolo A, Lo Ré A, Boggio V, Molejón MI, Iovanna JL. Zymophagy, a novel selective autophagy pathway mediated by VMP1-USP9x-p62, prevents pancreatic cell death. *J Biol Chem*. (2011) 286:8308–24. doi: 10.1074/jbc.M110.197301
67. Vaccaro MI. Zymophagy: selective autophagy of secretory granules. *Int J Cell Biol*. (2012) 2012:396705. doi: 10.1155/2012/396705
68. Cavalli G, Cenci S. Autophagy and protein secretion. *J Mol Biol*. (2020). doi: 10.1016/j.jmb.2020.01.015. [Epub ahead of print].
69. Pomilio C, Gorojod RM, Riudavets M, Vinuesa A, Presa J, Gregosa A, et al. Microglial autophagy is impaired by prolonged exposure to β-amyloid peptides: evidence from experimental models and Alzheimer's disease patients. *Geroscience*. (2020). doi: 10.1007/s11357-020-00161-9. [Epub ahead of print].
70. Glebov K, Schütze S, Walter J. Functional relevance of a novel SlyX motif in non-conventional secretion of insulin-degrading enzyme. *J Biol Chem*. (2011) 286:22711–5. doi: 10.1074/jbc.C110.217893
71. Ghosh S, Watanabe RM, Valle TT, Hauser ER, Magnuson VL, Langefeld CD, et al. The Finland-United States investigation of non-insulin-dependent diabetes mellitus genetics (FUSION) study. I. An autosomal genome scan for genes that predispose to type 2 diabetes. *Am J Hum Genet*. (2000) 67, 1174–85. doi: 10.1016/S0002-9297(07)62948-6
72. Kim M, Hersh LB, Leissring MA, Ingelsson M, Matsui T, Farris W, et al. Decreased catalytic activity of the insulin-degrading enzyme in chromosome 10-linked Alzheimer's disease families. *J Biol Chem*. (2007) 282:7825–32. doi: 10.1074/jbc.M609168200
73. Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D, et al. A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature*. (2007) 445:881–5. doi: 10.1038/nature05616
74. Zeggini E, Weedon MN, Lindgren CM, Frayling TM, Elliott KS, Lango H, et al. Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. *Science*. (2007) 316:1336–41. doi: 10.1126/science.1142364

75. Flannick J, Thorleifsson G, Beer NL, Jacobs SB, Grarup N, Burtt NP, et al. Loss-of-function mutations in SLC30A8 protect against type 2 diabetes. *Nat Genet*. (2014) 46:357–63. doi: 10.1038/ng.2915
76. Tang J. Targeting insulin-degrading enzyme to treat type 2 diabetes. *Trends Endocrinol Metab*. (2015) 27:24–34. doi: 10.1016/j.tem.2015.11.003
77. Tamboli IY, Barth E, Christian L, Siepmann M, Kumar S, Singh S, et al. Statins promote the degradation of extracellular amyloid b-peptide by microglia via stimulation of exosome-associated insulin-degrading enzyme (IDE) secretion. *J Biol Chem.* (2010) 285:37405–14. doi: 10.1074/jbc.M110.149468
78. Markowicz-Piasecka M, Sikora J, Szydłowska A, Skupien A, Mikiciuk-Olasik E, Huttunen KM. Metformin - a future therapy for neurodegenerative diseases: theme: drug discovery, development and delivery in Alzheimer disease. *Pharm Res*. (2017) 34:2614–27. doi: 10.1007/s11095-017-2199-y
79. Chen JL, Luo C, Pu D, Zhang GQ, Zhao YX, Sun Y, et al. Metformin attenuates diabetes-induced tau hyperphosphorylation *in vitro* and *in vivo* by enhancing autophagic clearance. *Exp Neurol*. (2019) 311:44–56. doi: 10.1016/j.expneurol.2018.09.008
80. Hull RL, Shen Z, Watts MR, Kodama K, Carr DB, Utzschneider KM, et al. Long term treatment with rosiglitazone and metformin reduce the extent of, but do not prevent, islet amyloid deposition in mice expressing the gene for human islet amyloid polypeptide. *Diabetes.* (2005) 54:2235–44. doi: 10.2337/diabetes.54.7.2235
81. Hull RL, Andrikopoulos S, Verchere CB, Vidal J, Wang F, Cnop M. Increased dietary fat promotes islet amyloid formation and ß-cell secretory dysfunction n in a transgenic mouse model of islet amyloid. *Diabetes.* (2003) 52:372–9. doi: 10.2337/diabetes.52.2.372
82. Xia J, Lu L, Jin C, Wang S, Zhou J, Ni Y, et al. Effects of short term lead exposure on gut microbiota and hepatic metabolism in adult zebrafish. *Comp Biochem Physiol C Toxicol Pharmacol*. (2018) 209:1–8. doi: 10.1016/j.cbpc.2018.03.007
83. Kim J, Cheon H, Jeong YT, Quan W, Kim KH, Cho JM, et al. Amyloidogenic peptide oligomer accumulation in autophagy-deficient β cells induces diabetes. *J Clin Invest*. (2014) 124:3311–24. doi: 10.1172/JCI69625
84. Rivera JF, Gurlo T, Daval M, Huang CJ, Matveyenko AV, Butler PC, et al. IAPP disrupts the autophagy/lysosomal pathway in pancreatic β-cells: protective role of p62-positive cytoplasmic inclusions. *Cell Death Differ*. (2010) 18:415–26. doi: 10.1038/cdd.2010.111
85. Rivera JF, Costes S, Gurlo T, Glabe CG, Butler PC. Autophagy defends pancreatic β cells from human islet amyloid polypeptide-induced toxicity. *J Clin Invest*. (2014) 124:3489–500. doi: 10.1172/JCI71981
86. Shigihara N, Fukunaka A, Hara A, Komiya K, Honda A, et al. Human IAPP–induced pancreatic β cell toxicity and its regulation by autophagy. *J Clin Invest*. (2014) 124:3634–44. doi: 10.1172/JCI69866
87. Cortes CJ, La Spada AR. The many faces of autophagy dysfunction in Huntington's disease: from mechanism to therapy. *Drug Discov Today*. (2014) 19:963–71. doi: 10.1016/j.drudis.2014.02.014
88. Mukherjee A, Soto C. Prion-like protein aggregates and type 2 diabetes. *Cold Spring Harb Perspect Med.* (2017) 7:a024315. doi: 10.1101/cshperspect.a024315
89. Mukherjee A, Morales-Scheihing D, Salvadores N, Moreno-Gonzalez I, Gonzalez C, Taylor-Presse K. Induction of IAPP amyloid deposition and associated diabetic abnormalities by a prion-like mechanism. *J Exp Med*. (2017) 214:2591–610. doi: 10.1084/jem.20161134
90. Hogan MF, Meier DT, Zraika S, Templin AT, Mellati M, Hull RL, et al. Inhibition of insulin-degrading enzyme does not increase islet amyloid deposition *in vitro*. *Endocrinology*. (2016) 157:3462–8. doi: 10.1210/en.2016-1410
91. Geng X, Haiyan L, Wang J, Li L, Swanson AL, Sun M, et al. α-Synuclein binds the KATP channel at insulin-secretory granules and inhibits insulin secretion. *Am J Physiol Endocrinol Metab.* (2011) 300:E276–86. doi: 10.1152/ajpendo.00262.2010
92. Sharma SK, Chorell E, Steneberg P, Vernersson-Lindahl E, Edlund H, Wittung-Stafshede P. Insulin-degrading enzyme is activated b the C-terminus of α-synuclein. *Biochem Biophys Res Commun*. (2015) 466:192–5. doi: 10.1016/j.bbrc.2015.09.002
93. Burré J, Sharma M, Tsetsenis T, Buchman V, Etherton MR, Südhof TC. Alpha-synuclein promotes SNARE-complex assembly *in vivo* and *in vitro*. *Science*. (2010) 329:1663–7. doi: 10.1126/science.1195227
94. Diao J, Burré J, Vivona S, Cipriano DJ, Sharma M, Kyoung M, et al. Native α-synuclein induces clustering of synaptic-vesicle mimics via binding to phospholipids and synaptobrevin-2/VAMP2. *Elife.* (2013) 2:e00592. doi: 10.7554/eLife.00592
95. Dehay B, Decressac M, Bourdenx M, Guadagnino I, Fernagut PO, Tamburrino A, et al. Targeting α-synuclein: therapeutic options. *Mov Disord*. (2016) 31:882–8. doi: 10.1002/mds.26568
96. Kim MJ, Deng HX, Wong YC, Siddique T, Krainc D. The Parkinson's disease-linked protein TMEM230 is required for Rab8a-mediated secretory vesicle trafficking and retromer trafficking. *Hum Mol Genet*. (2017) 26:729–41. doi: 10.1093/hmg/ddw413
97. Sproviero D, La Salvia S, Gianinni M, Crippa V, Gagliardi S, Bernuzzi S, et al. Pathological proteins are transported by extracellular vesicles of sporadic amyotrophic lateral sclerosis patients. *Front Neurosci*. (2018) 12:487. doi: 10.3389/fnins.2018.00487
98. Murthy A, Li Y, Peng I, Reichelt M, Katakam AK, Noubade R, et al. A Crohn's disease variant in Atg16l1 enhances its degradation by caspase 3. *Nature*. (2014) 506:456–62. doi: 10.1038/nature13044
99. Bel S, Hooper LV. Secretory autophagy of lysozyme in Paneth cells. *Autophagy*. (2018) 14:719–21. doi: 10.1080/15548627.2018.1430462
100. Wang LJ, Huang HY, Huang MP, Liou W, Chang YT, Wu CC, et al. The microtubule-associated protein, EB1, Links AIM2 inflammasomes with autophagy-dependent secretion. *J Biol Chem*. (2014) 289:29322–33. doi: 10.1074/jbc.M114.559153
101. Yoshimura A, Hara W, Kaneko T, Kato I. Secretion of IL-1β, TNF-α, IL-8 and IL-1ra by human polymorphonuclear leukocytes in response to lipopolysaccharides from periodontopathic bacteria. *J Periodont Res.* (1997) 32:279–86. doi: 10.1111/j.1600-0765.1997.tb00535.x
102. Zaaber I, Mestiri S, Hammedi H, Marmouch H, Mahjoub S, Tensaout BB, et al. Association of interleukin-1β and interleukin-4 gene variants with autoimmune thyroid diseases in tunisian population. *Immunol Invest*. (2016) 45:284–97. doi: 10.3109/08820139.2016.1153650
103. Zhao G, Dharmadhikari G, Maedler K, Meyer-Hermann M. Possible role of interleukin-1β in type 2 diabetes onset and implications for antiinflammatory therapy strategies. *PLoS Comput Biol.* (2014) 10:e1003798. doi: 10.1371/journal.pcbi.1003798
104. Reddy S, Krogvold L, Martin C, Holand R, Choi J, Woo H, et al. Distribution of IL-1β immunoreactive cells in pancreatic biopsies from living volunteers with new-onset type 1 diabetes: comparison with donors without diabetes and with longer duration of disease. *Diabetologia.* (2018) 61:1362–73. doi: 10.1007/s00125-018-4600-8
105. Böni-Schnetzler M, Thorne J, Parnaud G, Marselli L, Ehses JA, Kerr-Conte J, et al. Increased interleukin (IL)-1beta messenger ribonucleic acid expression in beta-cells of individuals with type 2 diabetes and regulation of IL-1beta in human islets by glucose and autostimulation. *J Clin Endocrinol Metab.* (2008) 93:4065–74. doi: 10.1210/jc.2008-0396
106. Shi C, Shenderov K, Huang N, Kabat J, Abu-Asab M, Fitzgerals KA, et al. Activation of autophagy by inflammatory signals limits IL-1β production by targeting ubiquitinated inflammasomes for destruction. *Nat Immunol.* (2012) 13:255–63. doi: 10.1038/ni.2215
107. Mann SM, Kanneganti TD. Regulation of lysosomal dynamics and autophagy by CTSB/cathepsin B. *Autophagy*. (2016) 12:2504–5. doi: 10.1080/15548627.2016.1239679
108. Guo Q, Wu Y, Hou Y, Liu Y, Liu T, Zhang H, et al. Cytokine secretion and pyroptosis of thyroid follicular cells mediated by enhanced NLRP3, NLRP1, NLRC4, and AIM2 inflammasomes are associated with autoimmune thyroiditis. *Front Immunol*. (2018) 9:1197. doi: 10.3389/fimmu.2018.01197
109. Lakatos P, Foldes J, Horvath C, Kiss L, Tatrai A, Takacs I, et al. Serum interleukin-6 and bone metabolism in patients with thyroid function disorders. *J Clin Endocrinol Metab*. (1997) 82:78–81. doi: 10.1210/jc.82.1.78
110. Kurihara N, Bertolini D, Sida T, Akiyama Y, Roodman GD, et al. IL-6 stimulates osteoclast-like multinucleated cell formation in long term human marrow cultures by inducing IL-1 release. *J Immunol*. (1990) 144:4226–30.
111. Simsek G, Karter Y, Aydin S, Uzun H. Osteoporotic cytokines and bone metabolism on rats with induced hyperthyroidism; changes as a result of reversal to euthyroidism. *Chin J Physiol.* (2003) 4:181–6.

112. McLaughlin SK, Olsen SN, Dake B, et al. The RasGAP gene, RASAL2, is a tumor and metastasis suppressor. *Cancer Cell.* (2013) 24:365–78. doi: 10.1016/j.ccr.2013.08.004
113. Wang X, Yin X, Yang Y. Rasal2 suppresses breast cancer cell proliferation modulated by secretory autophagy. *Mol Cell Biochem.* (2019) 462:115–22. doi: 10.1007/s11010-019-03615-7
114. New J, Arnold L, Ananth M, Alvi S, Thornton M, Werner L, et al. Secretory autophagy in cancer-associated fibroblasts promotes head and neck cancer progression and offers a novel therapeutic target. *Cancer Res.* (2017) 77:6679–91. doi: 10.1158/0008-5472.CAN-17-1077
115. Radulescu RT, Hufnagel C, Luppa P, Hellebrand H, Kuo WL, Rosner MR, et al. Immunohistochemical demonstration of the zinc metalloprotease insulin-degrading enzyme in normal and malignant human breast: correlation with tissue insulin levels. *Int J Oncol.* (2007) 30:73–80. doi: 10.3892/ijo.30.1.73
116. Radulescu RT, Duckworth WC, Levy JL, Fawcett J. Retinoblastoma protein co-purifies with proteasomal insulin-degrading enzyme: implications for cell proliferation control. *Biochem Biophys Res Commun.* (2010) 395:196–9. doi: 10.1016/j.bbrc.2010.03.157
117. Gonzalez CD, Alvarez S, Ropolo A, Rosenzvit C, Gonzalez Bagnes MF, Vaccaro MI. Autophagy, Warburg, and Warburg reverse effects in human cancer. *Bio Med Res Int.* (2014) 2014:926729. doi: 10.1155/2014/926729
118. Bhansali S, Bhansali A, Dutta P, Walia R, Dhawan V. Metformin upregulates mitophagy in patients with T2DM: a randomized placebo-controlled study. *J Cell Mol Med.* (2020) 24:2832–46. doi: 10.1111/jcmm.14834
119. Wang M, Qu S, Ma J, Wang X, Yang Y. Metformin suppresses LPS-induced inflammatory responses in macrophage and ameliorates allergic contact dermatitis in mice via autophagy. *Biol Pharm Bull.* (2020) 43:129–37. doi: 10.1248/bpb.b19-00689
120. Nnah IC, Lee CH, Wessling-Resnick M. Iron potentiates microglial interleukin-1β secretion induced by amyloid-β. *J Neurochem.* (2018) 11:129–143. doi: 10.3390/ph11040129
121. Terabe K, Ohashi Y, Tsuchiya S, Ishizuka S, Knudson CB, Knudson W. Chondroprotective effects of 4-methylumbelliferone and hyaluronan synthase-2 overexpression involve changes in chondrocyte energy metabolism. *J Biol Chem.* (2019) 294:17799–817. doi: 10.1074/jbc.RA119.009556

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A Novel E2F1-EP300-VMP1 Pathway Mediates Gemcitabine-Induced Autophagy in Pancreatic Cancer Cells Carrying Oncogenic KRAS

Alejandro Ropolo[1], Cintia Catrinacio[1], Felipe Javier Renna[1], Veronica Boggio[1], Tamara Orquera[1], Claudio D. Gonzalez[1,2] and Maria I. Vaccaro[1]*

[1] Department of Pathophysiology, Institute of Biochemistry and Molecular Medicine (UBA-CONICET), School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina, [2] CEMIC University Institute, Buenos Aires, Argentina

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Giorgio Stassi, University of Palermo, Italy

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***Correspondence:**
Alejandro Ropolo
aropolo@ffyb.uba.ar

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Autophagy is an evolutionarily preserved degradation process of cytoplasmic cellular constituents, which participates in cell response to disease. We previously characterized VMP1 (Vacuole Membrane Protein 1) as an essential autophagy related protein that mediates autophagy in pancreatic diseases. We also demonstrated that VMP1-mediated autophagy is induced by HIF-1A (hypoxia inducible factor 1 subunit alpha) in colon-cancer tumor cell lines, conferring resistance to photodynamic treatment. Here we identify a new molecular pathway, mediated by VMP1, by which gemcitabine is able to trigger autophagy in human pancreatic tumor cell lines. We demonstrated that gemcitabine requires the VMP1 expression to induce autophagy in the highly resistant pancreatic cancer cells PANC-1 and MIAPaCa-2 that carry activated *KRAS*. E2F1 is a transcription factor that is regulated by the retinoblastoma pathway. We found that E2F1 is an effector of gemcitabine-induced autophagy and regulates the expression and promoter activity of VMP1. Chromatin immunoprecipitation assays demonstrated that E2F1 binds to the *VMP1* promoter in PANC-1 cells. We have also identified the histone acetyltransferase EP300 as a modulator of VMP1 promoter activity. Our data showed that the E2F1-EP300 activator/co-activator complex is part of the regulatory pathway controlling the expression and promoter activity of VMP1 triggered by gemcitabine in PANC-1 cells. Finally, we found that neither VMP1 nor E2F1 are induced by gemcitabine treatment in BxPC-3 cells, which do not carry oncogenic KRAS and are sensitive to chemotherapy. In conclusion, we have identified the E2F1-EP300-VMP1 pathway that mediates gemcitabine-induced autophagy in pancreatic cancer cells. These results strongly support that VMP1-mediated autophagy may integrate the complex network of events involved in pancreatic ductal adenocarcinoma chemo-resistance. Our experimental findings point at E2F1 and VMP1 as novel potential therapeutic targets in precise treatment strategies for pancreatic cancer.

Keywords: pancreatic cancer, gemcitabine, autophagy, VMP1, E2F1

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human malignancies with 8–9% 5-year survival rate (1). Despite the progress in the knowledge of the disease, it remains a calamitous neoplasia. Up to 60% of patients have advanced pancreatic cancer at the time of diagnosis, and their median survival time is 3–6 months (2). Its poor prognosis has been attributed to a tendency regarding early vascular dissemination and spreading to regional lymph nodes, and to the incapacity to make a diagnosis while the tumor is still surgically removable (2). This is caused both by the aggressive nature of the disease, the lack of specific symptoms and early detection tools, and the refractory response to traditional cytotoxic agents and radiotherapy (3, 4). Furthermore, pancreatic cancer cells get more malignant and survive with an extremely low blood supply (2, 3). Up to now, contradictory data are available concerning autophagy activity and its regulation by specific autophagy related (ATG) proteins in pancreatic cancer cells. Experimental evidence places autophagy as a mechanism for survival of tumor cell under adverse environmental conditions, or as a defective mechanism of programmed cell death that promotes pancreatic cancer cell resistance to treatment (5–7).

At present, the first option for resectable tumors in pancreatic cancer is adjuvant chemotherapy before surgical resection (8, 9). However, most patients are in an advanced stage at the time of diagnosis and in these cases, chemotherapy is used as the first option. Regarding chemotherapy, gemcitabine used alone or in combination with nabpaclitaxel represent one of the most effective therapy (9, 10), despite its poor efficacy in terms of overall patient survival (11). Gemcitabine works by causing apoptosis of malignant cells in pancreatic cancer (12, 13). Intrinsic and acquired factors are involved in gemcitabine resistance. Several of them related to the transport and metabolism of gemcitabine (14) and associated with the tumor microenvironment, among others (15, 16).

Interestingly, recent studies highlight the importance of autophagic flux in acquiring resistance to gemcitabine in pancreatic cancer tumor cells (17–19). Macroautophagy (hereafter autophagy), is an evolutionarily conserved process that involves the sequestration and delivery of cytoplasmic components into the lysosome, where they are degraded and recycled (20). Autophagy is involved in the turnover of long-lived proteins and other cellular macromolecules. It has also been involved in the physiological responses to exercise and aging and is implicated in different pathophysiological processes such as neurodegenerative disorders, cardiovascular, pulmonary diseases, and cancer (21–24). Autophagy correlates with poor patient outcome in pancreatic cancers (25), and it has been suggested that autophagy is required for tumor growth (6, 7). For instance, in mice with pancreas containing an activated oncogenic allele of *KRAS* proto-oncogene, GTPase (KRAS), the most frequent mutation in PDAC (26), a small number of pre-cancerous lesions are developed that become PDAC randomly over time (27). KRAS activates the expression of the Vacuole Membrane Protein 1 (VMP1) to induce and maintain autophagy levels in pancreatic tumor cells (28). Accordingly, mice lacking the essential autophagy genes ATG5 or ATG7 acquire pre-invasive low-grade pancreatic intraepithelial neoplasia lesions, but progression to high-grade pancreatic intraepithelial neoplasia lesions and PDAC is blocked (27). This evidence highlight the relevance of KRAS-induced autophagy in the malignant transformation of pancreatic tumor cells.

Autophagy involves the formation of double-membrane structure, autophagosomes, around the cellular components targeted for degradation, which include large structures such as organelles and protein aggregates (29). Autophagy is mediated by a set of evolutionarily conserved gene products (termed the ATG proteins) originally discovered in yeast (30). In mammalian cells, the sequential association of at least a subset of the ATG proteins, referred to as the core molecular machinery (29), leads to the autophagosome formation. VMP1 belongs to these essential ATG proteins. We have demonstrated that VMP1 expression triggers autophagy in mammalian cells even under nutrient-rich conditions (31, 32). By contrast, autophagy is completely blocked in the absence of VMP1 expression (31). VMP1 autophagy-related function requires its hydrophilic C-terminal domain of 20 amino acids (VMP1-ATGD) (32). This domain binds directly to the Bcl-2 binding domain (BH3) motif of beclin 1 (BECN1) leading to the formation of a VMP1-BECN1-PI3KC3 (phosphatidylinositol 3-kinase catalytic subunit type 3) complex at the site where autophagosomes are generated (33, 34).

VMP1 is not expressed in normal pancreas, however its expression is early activated in pancreas suffering experimental diabetes mellitus, experimental and human pancreatitis, and in human pancreatic cancer cells (35–39). Interestingly, VMP1 prevents pancreatic cell death induced by acute pancreatitis (35). In previous studies, we found that VMP1 expression is induced by mutated KRAS in pancreatic tumor cells (28). KRAS is a member of the Ras family of GTP-binding proteins that mediate a wide variety of cellular functions including proliferation, differentiation, and survival. KRAS mutation is one of the earliest genetic events in human PDAC (40). Besides, it has been demonstrated that VMP1 down-regulation reduces cell resistance of pancreatic cells to chemotherapeutic drugs as Imatinib, Cisplatin, Adriamycin, Staurosporin, and Rapamycin (41). In colon cancer cells, we have recently shown that the HIF-1A-VMP1 autophagic pathway is involved in the resistance to photodynamic therapy in colon cancer cells (42). Therefore, we hypothesized that VMP1 is involved in the tumor cell response to chemotherapy in pancreatic cancer cells.

Here, we study the role of autophagy and its molecular mechanism involved in the pancreatic tumor cell response to chemotherapy. We identified a new regulatory pathway,

Abbreviations: ATG, related to autophagy; ChIP, chromatin immunoprecipitation; E2F1, E2F transcription factor 1; EGFP, enhanced green fluorescent protein; EP300, E1A binding protein p300; HIF1A, hypoxia inducible factor 1 subunit alpha; KRAS, Kirsten rat sarcoma viral oncogene homolog; LC3, microtubule-associated protein 1 light chain 3; PDAC, pancreatic ductal adenocarcinoma; PBS, phosphate-buffered saline; RLU, Relative light units; RFP, red fluorescent protein; shControl, short hairpin RNA with a scrambled sequence; shE2F1, short hairpin RNA targeting E2F1; shEP300, short hairpin RNA targeting EP300; shVMP1, short hairpin RNA targeting VMP1; TBS, TRIS-buffered saline; TTS, transcriptional starting site; VMP1, Vacuole Membrane Protein 1.

which is activated in high resistant pancreatic tumor cells, carrying oncogenic KRAS, under gemcitabine treatment but not in sensitive cells to chemotherapy. This molecular mechanism includes the activation of E2F transcription factor 1 (E2F1) that binds to VMP1 promoter to enhance VMP1-mediated autophagy. We also identified the histone acetyltransferase EP300 (E1A binding protein p300), as a modulator of this promoter activity. Our data show that the E2F1-EP300 activator/co-activator complex is part of the regulatory pathway controlling VMP1 expression triggered by gemcitabine. Together these data point at E2F1 as a regulatory factor modulating VMP1-mediated autophagy in human pancreatic cancer cells and integrate this degradative cellular process into the complex network of events involved in PDAC chemoresistance.

MATERIALS AND METHODS

Mammalian Cell Lines, Transfections, and Treatments

Human pancreatic cancer cell lines with mutated KRAS, PANC-1 (KRASG12D), and MIAPaCa-2 (KRASG12C), and human pancreatic cancer cell line with wild type *KRAS*, BxPC-3 (43), and also a human HeLa cell line were obtained from American Type Culture Collection. PANC-1, MIAPaCa-2, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Biological Industries) containing 10% fetal bovine serum (Natocor). BxPC-3 cells were cultured in RPMI 1640 medium (Biological Industries) containing 10% fetal bovine serum (Natocor). All cell culture mediums were supplemented with 100 U μl^{-1} penicillin, and 100 μg μl^{-1} streptomycin (Biological Industries). All cell lines were maintained at 37°C under a humidified atmosphere with 5% CO_2. Mycoplasma contamination is periodically checked by PCR, each time a cell line enters the laboratory, and then monthly for each cell line currently in use. Cells were seeded 24 h before transfection and treatments to reach a 60% confluence. Cells were transfected using FuGENE-6 Transfection Reagent (Promega) as indicated by the manufacturer. Gemcitabine (Elli Lilly) and chloroquine (Sigma-Aldrich) were prepared according to the manufacturer's instructions. Cells were treated with 20 μM gemcitabine (Elli Lilly) and/or 10 μM chloroquine (Sigma) for different times when appropriate.

Expression Vectors

Plasmid pRFP-LC3 (microtubule-associated protein 1 light chain 3 fused to red fluorescent protein) was kindly provided by Dr. Maria I. Colombo [Universidad Nacional de Cuyo, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina]. The vector pEGFP-VMP1 (enhanced green fluorescent protein fused to VMP1) was designed and constructed as previously reported (31). Expression vector for E2F1 was kindly provided by Dr. Cánepa (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina). The expression vector Flag-EP300 was kindly provided by Dr. Donald Tindall (Mayo Clinic, Rochester, MN, USA). The shRNA targeting EP300 (shEP300) was obtained from Sigma-Aldrich (St. Louis, MO). VMP1 and E2F1 down-regulation was made using the plasmid pCMS3-1p-EGFP, which was kindly provided by Dr D. Billadeau (Department of Immunology, College of Medicine, Mayo Clinic, USA), and contains a separate transcriptional cassette for EGFP to identify transfected cells. A plasmid contains an VMP1 short hairpin RNA (shVMP1) construction (sense 5′-GGCAGAAUAUUGUCCUGUG-3′, and antisense 5′-CACA GGACAAUAUUCUCUGCC-3′) for VMP1 down-regulation. Another plasmid contains an E2F1 short hairpin RNA (shE2F1) construction (sense 5′-GACGTGTCAGGACCTTCGT-3′, and antisense 5′-CTGCACAGTCCTGGAAGCA-3′) for E2F1 down-regulation. A vector with a scramble sequence was used as a control short hairpin RNA (shControl).

Cell Viability Assay by Trypan Blue Method

Cells were seeded into 6-well-plates at 1×10^5 cells per well with 3 ml growth medium. The following day, PANC-1, MIAPaCA-2, and BxPC-3 cells were treated with 20, 200, or 2,000 μM gemcitabine for 24, 48, and 72 h. The number of viable cells was then determined by the Trypan blue method. Cells were raised by gentle pipetting into the growth medium, and 0.5 ml of the cell suspension was taken and mixed with 0.5 ml of a Trypan Blue 0.2% w/v in phosphate-buffered saline (PBS) solution and incubated for 3 min at room temperature. Colored (dead) and non-colored (live) cells were counted in a Neubauer chamber. The percentage of viable cells under gemcitabine treatment regarding to control was determined according to the following formula: % Viable cells = NT/NC × 100, where NC is the number of viable cells in the control and NT is the number of viable cells under treatment at the same time of incubation.

RNA Extraction and Quantitative Real-Time PCR (qPCR)

Cells lines were grown in 6-well-plates at 3.5×10^5 cells per well with 3 ml growth medium. After the corresponding transfections and treatments, RNA extraction from cell cultures was performed with the TRIzol reagent (Invitrogen). The concentration of RNA was determined by Vision Life Science Spectrophotometer (Hoefer) and RNA was running in an agarose gel to check its quality. RNA purified (2 μg) were used and treated with 1 μl of RNase-free DNase I (Invitrogen) in a final volume of 10 μl containing 1X DNAse (deoxyribonuclease) buffer. The 10 μl of DNAse treatment was incubated with 100 nM random primers (N6) at 70°C for 5 min. Then, 1X MMLV buffer, dNTPs (deoxynucleoside triphosphates) to 1 mM and 1 μl of the enzyme MMLV reverse transcriptase (Promega) were added in a final volume of 20 μl, incubated at 25°C for 5 min and then at 37°C for 1 h (RT). The RT-PCR reaction was performed on a Techne cycler. For the qPCR, the primers described below were used with 0.5 μl of RT per reaction in a final volume of 25 μl using the Master Mix Real Mix (Biodynamics). The qPCR was performed on RG 6000 cycler (Corvette). The cycling conditions were as follows: 95°C for 2 min followed by 40 cycles of 95°C for 20 s, 58°C for 20 s, and 72°C for 30 s. Transcript level of VMP1 mRNA was normalized by comparison with mRNA of β-actin and was calculated using the $2^{-\Delta\Delta CT}$ method (44, 45). Specific primers were used for VMP1: forward 5′-GGTGCTGAACCA

GATGATGA-3′ and reverse, 5′-GCACCAAAGAAGGTCCAAA-3′; and for β-actin: forward 5′-GACTTCGAGCAAGAGATGG-3′ and reverse, 5′-GCACTGTGTTGGCGTACAG-3′.

Western Blot Analysis

Cells were seeded into 60 mm culture dishes at 8×10^5 cells per dish with 5 ml growth medium. After different treatments and transfections, cells were lysed in ice-old RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM Tris-HCl pH 7.2, 5 mM EDTA) containing Phosphatase and Protease Inhibitor Cocktail (Sigma–Aldrich). Protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce). Equal amount of protein was analyzed on SDS-PAGE and transferred to polyvinylidene fluoride PVDF membranes (0.22 μm pore size, Millipore). The membranes were blocked with Odyssey Blocking Buffer (LI-COR) at room temperature for 1 h and incubated with the corresponding primary antibodies overnight at 4°C. The primary antibodies used were anti-VMP1 (1:1,000; rabbit mAb #12929, Cell Signaling Technology), anti-E2F1 (1:500; mouse mAb CS204394; Millipore), anti-Actin (1:4,000; rabbit polyAb A2066; Sigma–Aldrich). After incubation, the membrane was washed four times with PBS containing 0.1% Tween-20 (PBST) and twice with PBS, and then incubated with the corresponding IRDye secondary antibody (1:15,000, IRDye® 680LT Goat anti-Rabbit IgG or 1:10,000, IRDye® 800CW Goat anti-Mouse IgG, LI-COR) in Odyssey Blocking Buffer (LI-COR) for 2 h at room temperature. After, the membrane was washed four times with PBST, twice with PBS, and scanned with Odyssey® SA (LI-COR). For LC3 western blotting, the membranes were blocked with 5% (w/v) non-fat dry milk in TRIS-buffered saline (TBS) containing 0.1% Tween-20 (TBST) at room temperature for 1 h and incubated with the primary antibody anti-LC3B (1:1,000, Rabbit mAb #3868, Cell Signaling Technology) overnight at 4°C. After the incubation, the membrane was washed four times with TBST and twice with TBS, then incubated with anti-rabbit HRP-conjugated (1:3,000, Amersham NA934, GE Healthcare) secondary antibody in TBST with 5% (w/v) non-fat milk for 2 h at room temperature. Next, the membrane was washed four times with TBST and twice with TBS and incubated with PIERCE ELC Plus Western blotting Substrate (Cat# 32134, Thermo Scientific) according to manufacturer's instructions. Finally, the membrane was scanned with cDigit Blot Scanner (LI-COR). We used ImageJ software to determine protein bands density. Relative densitometry normalized to actin is expressed as the mean ± SD of three different experiments.

Fluorescence Microscopy

To determine autophagy, cells were growing on glass slides into 24-well-plates. They were seeded at 5×10^4 cells per well with 1 ml growth medium. Twenty-four hours later, cells were co-transfected with a red fluorescent protein fused to LC3 (RFP-LC3) expression vector and the indicated plasmid, and then treated with gemcitabine. Next, cells were fixed with 4% p-formaldehyde in PBS for 15 min, and immediately washed several times with PBS. Samples were mounted in DABCO (Sigma–Aldrich) and observed using a fluorescence microscope Nikon Eclipse 200 (Plan100), or an inverted LSM Olympus FV1000 using an UPLSAPO 60X O NA: 1.35 objective. We consider a cell positive for autophagy when RFP-LC3 has a punctate staining and not diffused protein remains. The number of fluorescent cells with punctate staining per 100 fluorescent RFP-LC3 transfected cells was determined in three independent experiments. To quantify, the number of fluorescent cells with punctate staining was counted in six random fields representing 100 fluorescent cells and expressed as the mean ± SD of combined results.

In silico Analysis

Genomic details and characteristics from human VMP1 gene were collected from the Ensembl Genome database. VMP1 promoter prediction was done using the Gene2Promoter utility; transcription factors binding sites and additional information was obtained using RegionMiner, MatBase, and MatInspector tools (Genomatix Software). Supporting evidence was found using the Neural Network Promoter Prediction program (BDGP version 2.2) and FPROM (Softberry). Alibaba 2.1 and PROMO 3.0 were also used for transcription factors' consensus sequences search.

Cloning of *VMP1* Promoter Into Reporter Vector

Genomic DNA from HeLa cells was extracted using TRIzol reagent (Invitrogen). PCR was performed to amplify a fragment of 3,005 bp and then three progressively shorter ones of 1,977, 1,469, and 883 bp from the 5′ upstream region of *VMP1* using specific primers and HeLa DNA as template. PCR was done in a final volume of 50 ml as follows: 1× Pfu DNA Polymerase Buffer (with MgSO4), 300 mM dNTPs (deoxynucleosyde triphosphates), 0.4 mM Primers sense/anti-sense, 50 ng genomic DNA, 1.5U Pfu DNA Polymerase (Promega). Amplification was performed according to an initial denaturation step of 1.5 min at 94°C, followed by 30 cycles at 94°C for 30 s, 59°C for 30 s, 73°C for 8 min, and a final step at 73°C for 5 min. PCR products were run in agarose gels and amplicons were recovered with a Gel Band Purification Kit (GE Healthcare). Primers had restriction sites for SalI (Forward) and HindIII (Reverse) enzymes for subsequent cloning of the fragments in pGemT easy vector (Promega). Amplicons integrity was confirmed by sequence analysis (MacroGen). All constructs were then subcloned in the luciferase reporter pGL3 Basic Vector (Promega), with SalI and HindIII enzymes. As a result, the constructs pGL3.vmp1-883, pGL3.vmp1-1469, pGL3.vmp1-1977, and pGL3.vmp1-3005 were obtained.

Luciferase Reporter Assays

For luciferase assays cells were plated 24 h before transfection in 12-well-plates at 1.4×10^5 cells per well. Cells were used at 60% confluence for pGL3.vmp1 promoter constructs transfection with FuGENE6 Transfection Reagent (Promega). Ratio used in each case was 1.5 ml FuGENE6 per 1 mg DNA. When two plasmids were transfected, we used 0.4 mg of pGL3 reporter and 0.6 mg of expression vector. In shRNA assays we used 1.5 mg of shRNA and 0.5 mg pGL3 reporter vectors. Treatments were done 24 h after transfection. In co-transfection experiments with

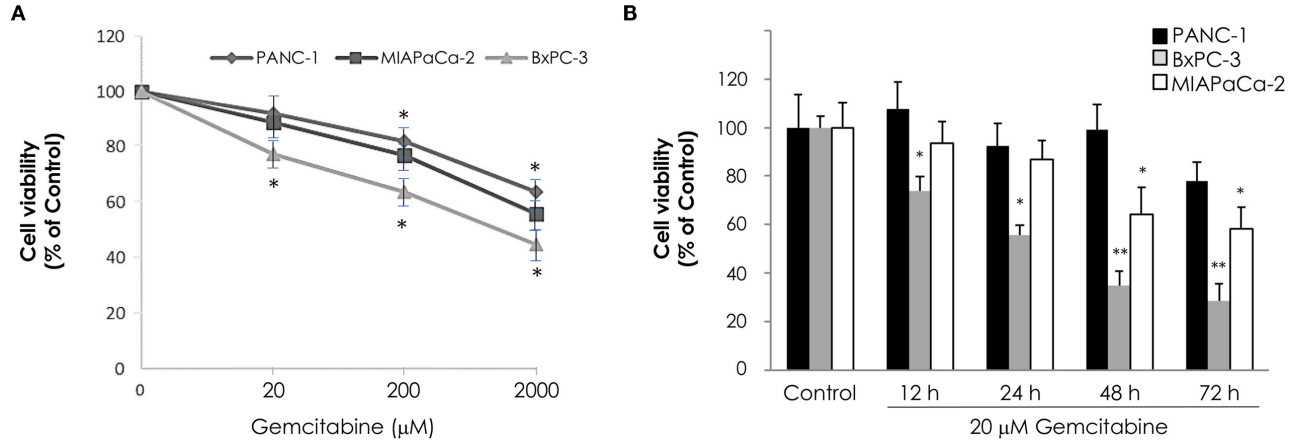

FIGURE 1 | Relative sensitivity of pancreatic cancer cell lines PANC-1, MIAPaCa-2 and BxPC-3 to gemcitabine treatment. The number of viable cells was determined by the trypan blue dye exclusion test. **(A)** Cells were treated with increasing concentrations of gemcitabine (from 20 to 2,000 μM) for 24 h. BxPC-3 cells are sensitive to all gemcitabine concentrations tested, and PANC-1 and MIAPaCa-2 cells are sensitive from 200 μM gemcitabine. **(B)** Cells were treated with 20 μM gemcitabine and cell viability was determined at 0, 12, 24, 48, and 72 h. There were no significant differences within 72 h of treatment in PANC-1 cells, and within 48 h of treatment in MIAPaCa-2 cells. However, a significant reduction in cell viability was observed in BxPC-3 cells from 12 h of treatment. Results are presented as percentage of viable cells compared to the untreated control (mean ± SE; $n = 3$). *$p < 0.05$ vs. untreated cells, **$p < 0.01$ vs. untreated cells.

expression vectors, cells were processed 48 h after transfection. Each condition was tested in triplicate. Cells were washed with cold PBS and lysed with 100 ml of 1× Cell Culture Lysis Buffer (Promega). 96-well-plates were used for activity assays. In each well 40 μl of luciferase substrate was added to 20 μl of lysate and a 5-s reading was done for luminescence measurement in a Victor3 1420 multilabel counter (PerkinElmer). Results were normalized to protein concentration. Relative light units (RLU) value was calculated as luciferase activity/protein concentration.

Chromatin Immunoprecipitation (ChIP) Assay

Chromatin immunoprecipitation was conducted following the Pierce Agarose Chip kit (Thermo Scientific). Briefly, PANC-1 cells were culture into 100 mm culture dishes at 3×10^6 cells per dish with 13 ml growth medium. The next day, cells were treated with 20 μM gemcitabine and 24 h were cross-linked with 1% formaldehyde directly into the media for 10 min at room temperature. The cells were then washed and scraped with PBS and collected by centrifugation at 800 × g for 5 min at 4°C, resuspended in cell lysis buffer and incubated on ice for 15 min. The pellet was then resuspended in nuclear lysis buffer and sheared to fragment DNA to about 700 bp. Samples were then immunoprecipitated using a E2F1 antibody or normal rabbit IgG (Millipore) overnight at 4°C on a rotating wheel. Following immunoprecipitation, samples were washed and eluted using the chromatin immunoprecipitation kit in accordance with the manufacturer's instructions. Cross-links were removed at 62°C for 2 h followed by 10 min at 95°C and immunoprecipitated DNA was purified and subsequently amplified by PCR. PCR was performed using seven primer sets for the seven areas containing potential E2F1 binding sites in the VMP1 promoter sequence: forward, 5′-GCAT CTCACTTTGTCACCCAG-3′ and reverse, 5′-ACTTGAGG TCAGGAGTTCGAGAC-3′; (2) forward, 5′-CAGGCTGTTCTC AAACTCCTGG-3′ and reverse, 5′-GCACCATACTAGACTC TGGGA; (3) forward, 5′-TCCCAGAGTCTAGTATGGTGC-3′ and reverse, 5′-CGATATCGCTCCATTGCTCTCCA-3′; (4) forward, 5′-GAGTAGCTGGGATTACAGGC-3 and reverse, 5′-ACCTGAGGTCAGAAGTTCGAGAC-3′; (5) forward, 5′-GTCT CGAACTTCTGACCTCAGGT-3′ and reverse, 5′-CAGCTGGG CACTTATGAATATCCC-3′; (6) forward, 5′-GATATTGGTCTC CTTCGCCCTGT-3′ and reverse, 5′-GCAAGAGGAAGAATGA CTGCTC-3′; and (7) forward, 5′-GAGCCTAACTGAAATCCCG CGA-3′ and reverse, 5′-CAAGCTCTGAGGACAGCCTCA-3. PCR products were visualized on a 2% agarose gel.

Statistical Analysis

Data are expressed as mean ± SD. We performed a minimum of three independent experiments, where individual data points were based on at least technical duplicates each. Student's *t*-test was used for comparisons between two groups and ANOVA test to assess more than two groups. $P < 0.05$ were considered statistically significant. Statistical analysis of data was performed using GraphPad Prism 6.

RESULTS

Gemcitabine Requires VMP1 Expression to Induce Autophagy in Pancreatic Cancer Cells Carrying Oncogenic *KRAS*

In order to analyze the time course effect of gemcitabine treatment on VMP1 expression we used PANC-1 and MIAPaCa-2 pancreatic tumor cells harboring a *KRAS* activating mutation,

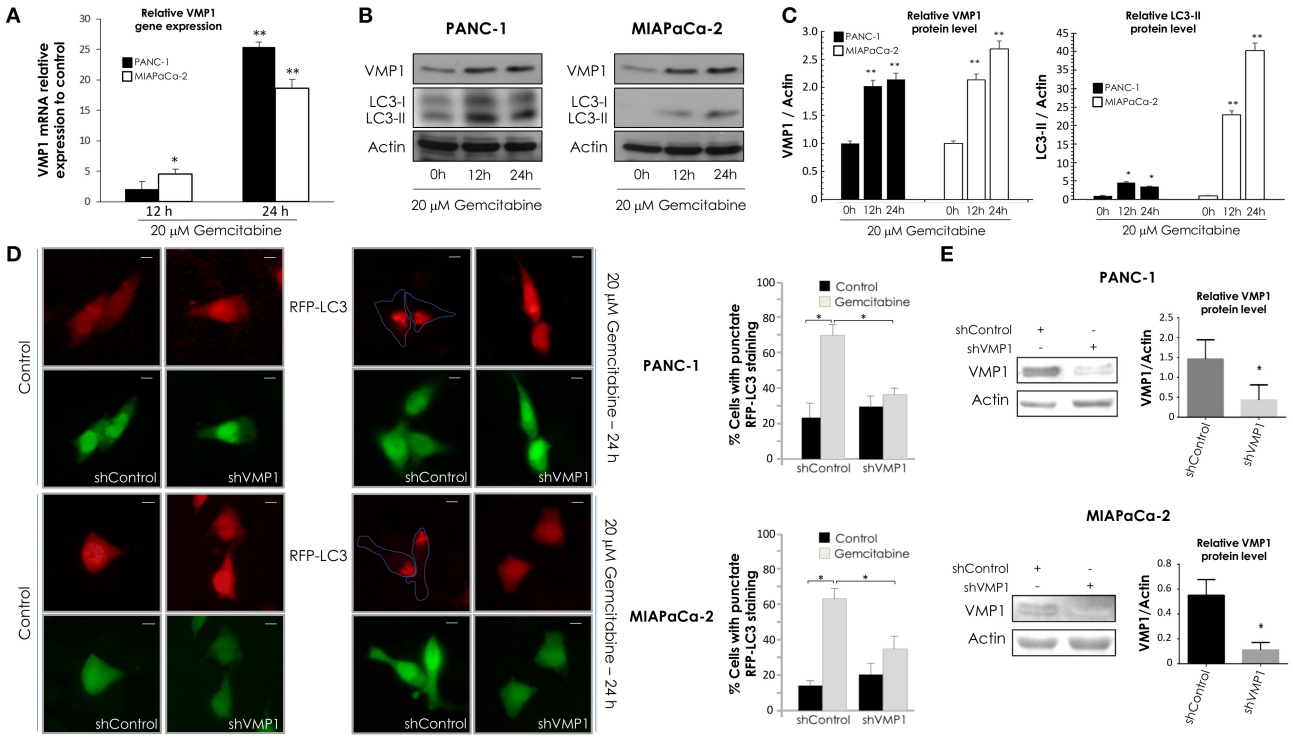

FIGURE 2 | Gemcitabine requires VMP1 expression to induce autophagy in PANC-1 and MIAPaCa-2 cells. PANC-1 and MIAPaCa-2 cells were treated with 20 μM gemcitabine for 12 and 24 h. **(A)** qPCR and western blot analysis showed an increase of VMP1 mRNA **(A)** and protein expression **(B)** in PANC-1 and MIAPaCa-2 cells under treatment ($n = 3$). **(B)** Western blot analysis shows an increase of in PANC-1 and MIAPaCa-2 cells submitted to gemcitabine treatment. **(C)** Relative densitometry (VMP1/Actin and LC3-II/Actin) of western blots are shown in panel **(B)** ($n = 3$). **(D)** PANC-1 and MIAPaCa-2 cells were transiently co-transfected with RFP-LC3 and shVMP1-EGFP or shControl-EGFP expression vectors, and they were treated with 20 μM gemcitabine for 24 h. RFP-LC3 distribution was detected using fluorescence microscopy. Representative images show the punctate RFP-LC3 in cells expressing EGFP (bar scale = 10 μm). Quantitation represents the percentage of RFP-LC3 cells with punctate staining. LC3 redistribution was significantly reduced in shVMP1 expressing cells under gemcitabine treatment in both cell lines (*$p < 0.05$ vs. gemcitabine-treated shControl transfected cells). **(E)** PANC-1 and MIAPaCa-2 cells were transfected with the shControl or shVMP1 expression vectors. VMP1 expression was reduced by shVMP1 in PANC-1 and MIAPaCa-2 cells as was demonstrated by western blotting. Relative densitometry (VMP1/Actin) is shown ($n = 3$). *$p < 0.05$, **$p < 0.01$.

that are highly resistant to chemotherapy, and BxPC-3 pancreatic tumor cells that do not carry *KRAS* mutation. The relative sensitivity of pancreatic cancer cells to gemcitabine treatment was analyzed. PANC-1, MIAPaCa-2, and BxPC-3 cells were treated with 20, 200, and 2,000 μM gemcitabine. The relative number of viable cells was determined by the trypan blue dye exclusion test 24 h later. **Figure 1A** shows cell viability as a percentage relative to control according to the gemcitabine dose. BxPC-3 cells were more sensitive in all doses of gemcitabine analyzed (**Figure 1A**). However, cell viability was significantly reduced from 200 μM gemcitabine in PANC-1 and MIAPaCa-2 cells (**Figure 1A**). Thus, gemcitabine 20 μM is the lowest tested dose in which BxPC-3 cells are sensitive, and PANC-1 and MIAPaCa-2 are resistant, at least 24 h after treatment. In view of this, we analyzed cell viability of PANC-1, MIAPaCa-2, and BxPC-3 cells under 20 μM gemcitabine treatment during 72 h. There were no significant differences during the 72 h of treatment for PANC-1 cells and up to 48 h of treatment for MIAPaCa-2 cells. On the other hand, BxPC-3 cells showed a significant reduction in cell viability from 12 h of treatment (**Figure 1B**). These results indicate that at 12 and 24 h with 20 μM gemcitabine, PANC-1 and MIAPaCa-2 cells are resistant, and by comparison, BxPC-3 cells are sensitive to treatment. Consequently, from now on we use this dose of gemcitabine for the following experiments.

Next, PANC-1 and MIAPaCa-2 cells were incubated with 20 μM gemcitabine for 24 h and we evaluated VMP1 mRNA expression by qPCR assays. A significant induction of VMP1 mRNA was found after 12 and 24 h of gemcitabine treatment in PANC-1 and MIAPaCa-2 cells (**Figure 2A**). LC3 is currently used as a specific marker of autophagy (46). During the autophagic process, the cytosolic form of LC3 (LC3-I) undergoes C-terminal proteolytic and lipid modifications (LC3-II) and translocates from the cytosol to the autophagosomal membrane (47, 48). Then, we analyzed VMP1 expression and LC3 lipidation to determinate autophagy by western blot. **Figure 2B** shows that gemcitabine induced VMP1 protein expression and

LC3-II formation, and therefore autophagy, in PANC-1 and MIAPaCa-2 cells. The quantification of western blots is shown in **Figure 2C**.

Afterward, PANC-1 and MIAPaCa-2 cells were co-transfected with RFP-LC3 and shVMP1 expression vectors and treated with 20 μM gemcitabine for 24 h. A diffuse pattern of LC3-RPF expression in the cytoplasm indicates that autophagy is not occurring, and when autophagy is induced RFP-LC3 is relocated as dots indicating the formation of autophagosomes. We counted cells with punctate RFP-LC3 staining to determinate autophagy (46). **Figure 2D** shows that gemcitabine induced RFP-LC3 redistribution to autophagosomes was significantly reduced when VMP1 expression was down-regulated in both cell lines. **Figure 2E** shows downregulation of VMP1 by shVMP1 expression in PANC-1 and MIAPaCa-2 cell lines. Therefore, gemcitabine requires VMP1 expression to induce autophagy in pancreatic tumor cells harboring a *KRAS* activating mutation.

Gemcitabine Induces E2F1 Activation of VMP1-Mediated Autophagy Only in Pancreatic Tumor Cells That Carry Oncogenic *KRAS*

Following, in order to analyze VMP1 expression and autophagy in cells that carry wild type *KRAS*, BxPC-3 cells were incubated with 20 μM gemcitabine for 24 h. Interestingly, gemcitabine treatment did not increase VMP1 mRNA and protein levels compared to basal conditions in BxPC-3 cells (**Figures 3A,B**). Then, we analyzed LC3 lipidation to determinate autophagy, and western blot analyses shows that gemcitabine treatment did not induce autophagy evidenced by LC3-II formation in BxPC-3 cells (**Figure 3B**). In addition, choloroquine treatment was used to evaluate autophagic process by inhibiting autophagy flux (46). The use of chloroquine alone or in combination with gemcitabine treatment induced LC3-II accumulation compared to control cells or cells only treated with gemcitabine in the same proportion, respectively, indicating that gemcitabine did not interrupt autophagy flux in these cells. The quantification of western blots is shown in **Figure 3C**. These results suggest BxPC-3 cells have a basal VMP1 expression and autophagy that are not up-regulated by gemcitabine.

E2F transcription factors are involved in cell proliferation and DNA repair (49). As gemcitabine incorporation into DNA is critical for its toxicity (50), we evaluated E2F1 expression in response to gemcitabine treatment. To characterize this response of E2F1 to gemcitabine, we chose the sensitive cell line BxPC-3 and the most resistant to treatment by comparison, PANC-1 cells. Western blot assay shows E2F1 protein levels were significantly increased after 12 and 24 h of gemcitabine treatment in PANC-1 cells, and they were similar with respect to control in treated BxPC-3 cells (**Figure 3D**). In consequence, E2F1 is a candidate to mediate increased VMP1 expression and autophagy in response to gemcitabine in PANC-1 but not in BxPC-3 cells.

Considering that VMP1 expression induces autophagosome formation (31), we tested whether E2F1 expression is capable of inducing VMP1 expression and autophagy in PANC-1 cells. We performed qPCR on samples from cells transfected with an expression vector for E2F1. As seen in **Figure 3E**, E2F1 expression induced VMP1 mRNA expression in PANC-1 tumor cells. Then, PANC-1 cells were concomitantly transfected with an expression plasmid encoding for the RFP-LC3 fusion protein and E2F1 expression vector. **Figure 3F** shows the recruitment of LC3 fusion protein in punctate structures in E2F1-transfected cells in contrast to the diffuse RFP-LC3 fusion protein signal observed in control cells. Quantitation showed that recruitment of LC3 was significantly increased in cells expressing E2F1 compared to control cells (**Figure 3F**). These results demonstrate that E2F1 is capable of inducing VMP1 expression and autophagy in pancreatic tumor cells resistant to gemcitabine treatment.

VMP1 Promoter Is Activated by Gemcitabine

We have previously demonstrated that starving conditions and rapamycin treatment induce VMP1 expression in HeLa cells (31). On the other hand, VMP1 expression is activated in PANC-1 human tumor cells carrying mutated (G12D) KRAS (28). In order to study the molecular mechanism that regulates VMP1 expression in the context of gemcitabine induced-autophagy in pancreatic tumor cells, a 3,005 bp sequence of the 5′ upstream region of the human gene *VMP1* was amplified and cloned in the pGL3 reporter vector (pGL3.vmp1-3005) (**Figure 4A**). Following, we analyzed if this sequence cloned has a promoter activity. In these experiments, we used HeLa cells under starving conditions and rapamycin treatment as a positive control of autophagy induction, and the pGL3.vmp1-3005 construct was used to perform luciferase reporter assays. As a result, we found increased VMP1 mRNA expression and *VMP1* promoter activity in response to starving conditions and to rapamycin treatment in PANC-1 and HeLa cells (**Figures 4B,C**). Next, we analyzed if gemcitabine was able to increase VMP1 promoter activity. **Figure 4D** shows that the activity of the 3,005 bp sequence of *VMP1* promoter was significantly increased when PANC-1 and HeLa cells were treated with 20 μM gemcitabine for 24 h.

In order to localize the essential promoter sequence in the 3,005 bp sequence of the 5′ upstream region of the human gene *VMP1*, we amplified and subcloned consecutive shorter fragments of this region. Three more constructs were created and named as follows: pGL3.vmp1-1977, pGL3.vmp1-1469, and pGL3.vmp1-883 (**Figure 4E**). Luciferase activity assays were performed in HeLa cells transfected with each construct and submitted to starvation, rapamycin, or gemcitabine treatment. Relative promoter activity was analyzed for each sequence (**Figure 4F**). Results showed a decreased activation for the pGL3.vmp1-1977 and pGL3.vmp1-1469 constructs comparing to the initial 3,005 bp sequence. On the other hand, the activity was increased when the shorter pGL3.vmp1-883bp construct was used. The same results were observed in all the conditions analyzed. These data suggest that essential regulation motifs involved in VMP1 expression are contained into this *VMP1* shorter promoter region of 883 bp.

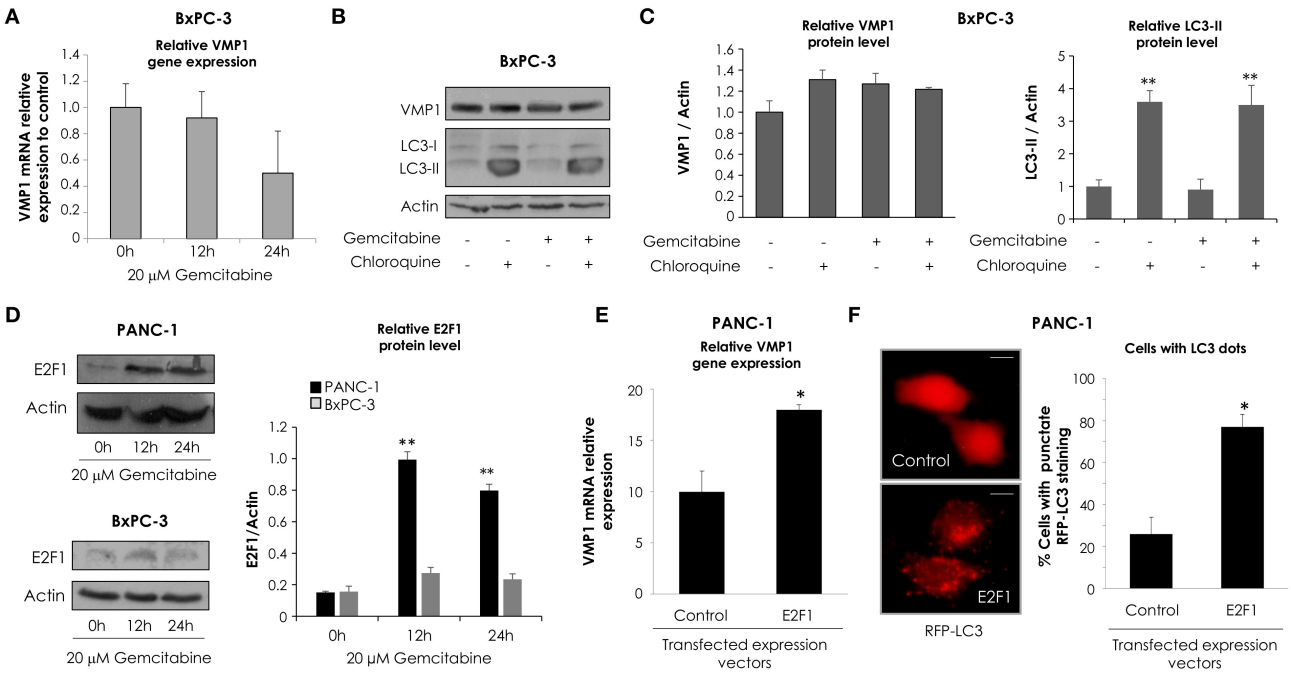

FIGURE 3 | Gemcitabine induces E2F1 activation of VMP1-mediated autophagy only in pancreatic tumor cells that carry oncogenic KRAS. qPCR and western blot analysis showed no significant differences of VMP1 mRNA **(A)** and protein expression **(B)** in BxPC-3 treated with 20 μM gemcitabine for 12 and 24 h ($n = 3$). **(B)** Western blot analysis shows no difference of VMP1 expression and LC3 lipidation in BxPC-3 cells submitted to gemcitabine treatment. The use of chloroquine alone or in combination with gemcitabine treatment induced LC3-II accumulation compared to control cells or cells only treated with gemcitabine in the same proportion, respectively, indicating that gemcitabine does not interrupt autophagy flux in these cells. **(C)** Relative densitometry (VMP1/Actin and LC3-II/Actin) of western blots shown in B ($n = 3$). **(D)** Western blot analysis shows that E2F1 expression was increased in PANC-1 cells submitted to gemcitabine treatment for 12 and 24 h, and there were no significant differences in the expression of E2F1 in BxPC-3 cells. Relative densitometry (E2F1/Actin) of western blots is shown ($n = 3$). PANC-1 cells were transiently transfected with E2F1 expression vector or empty vector as control. **(E)** qPCR assay demonstrated an increase of VMP1 mRNA expression in PANC-1 cells expressing E2F1 compared to control cells ($n = 3$). **(F)** E2F1 expression induced autophagy as was determined by counting cells with punctate RFP-LC3 staining. Representative images of RFP-LC3 distribution using confocal microscopy are shown (bar scale = 10 μm). The graph presents the percentage of RFP-LC3 cells with punctate staining ($n = 3$). $^*p < 0.05$, $^{**}p < 0.01$.

E2F1 Directly Activates the *VMP1* Promoter and Regulates VMP1 Expression Under Gemcitabine Treatment

Considering that maximal promoter activity in luciferase reporter assays was observed for the pGL3.vmp1-883 construct, we analyzed this sequence using bioinformatics tools. This *in silico* analysis data showed a putative promoter in this sequence containing a TATA. In addition, we identified several putative binding sites for relevant transcription factors related to the cellular stress response including E2F1 (**Figure 5A**).

In order to know if *VMP1* gene is a direct target of E2F1, we used a combination of transcriptional and chromatin assays to determine a possible involvement of E2F1 transcription factor in *VMP1* promoter activation. First, we performed luciferase reporter assays using the shorter sequence of VMP1 promoter, pGL3.vmp1-883bp construct. Reporter studies demonstrate that expression of E2F1 led to an increase in *VMP1* promoter activity (**Figure 5B**). Moreover, endogenous E2F1 can bind to *VMP1* promoter in PANC-1 cells treated with gemcitabine as demonstrated by ChIP assay (**Figure 5C**). Also, gemcitabine increased VMP1 promoter activity (see shControl transfected PANC-1 cells) (**Figure 5D**). Moreover, down-regulation of E2F1 expression in PANC-1 cells treated with gemcitabine significantly reduced VMP1 promoter activity (**Figure 5D**) and VMP1 expression (**Figure 5E**). These results demonstrate that VMP1 is a novel direct target of the E2F1 transcription factor under gemcitabine treatment.

E2F1 and EP300 Cooperate in *VMP1* Promoter Activation

Transcription factors regulate gene expression through their inherent activation or repression properties, and through functional interactions with co-regulatory molecules. Here, we tested whether activation by E2F1 involves the histone acetyltransferases EP300. First, we analyzed if EP300 could induce VMP1 mRNA expression in PANC-1 cells. **Figure 6A**, shows that VMP1 mRNA expression was up-regulated in PANC-1 cells expressing EP300. Additionally, EP300 expression significantly activated the *VMP1* promoter compared to control cells (**Figure 6B**). Then, we evaluated if EP300 participates

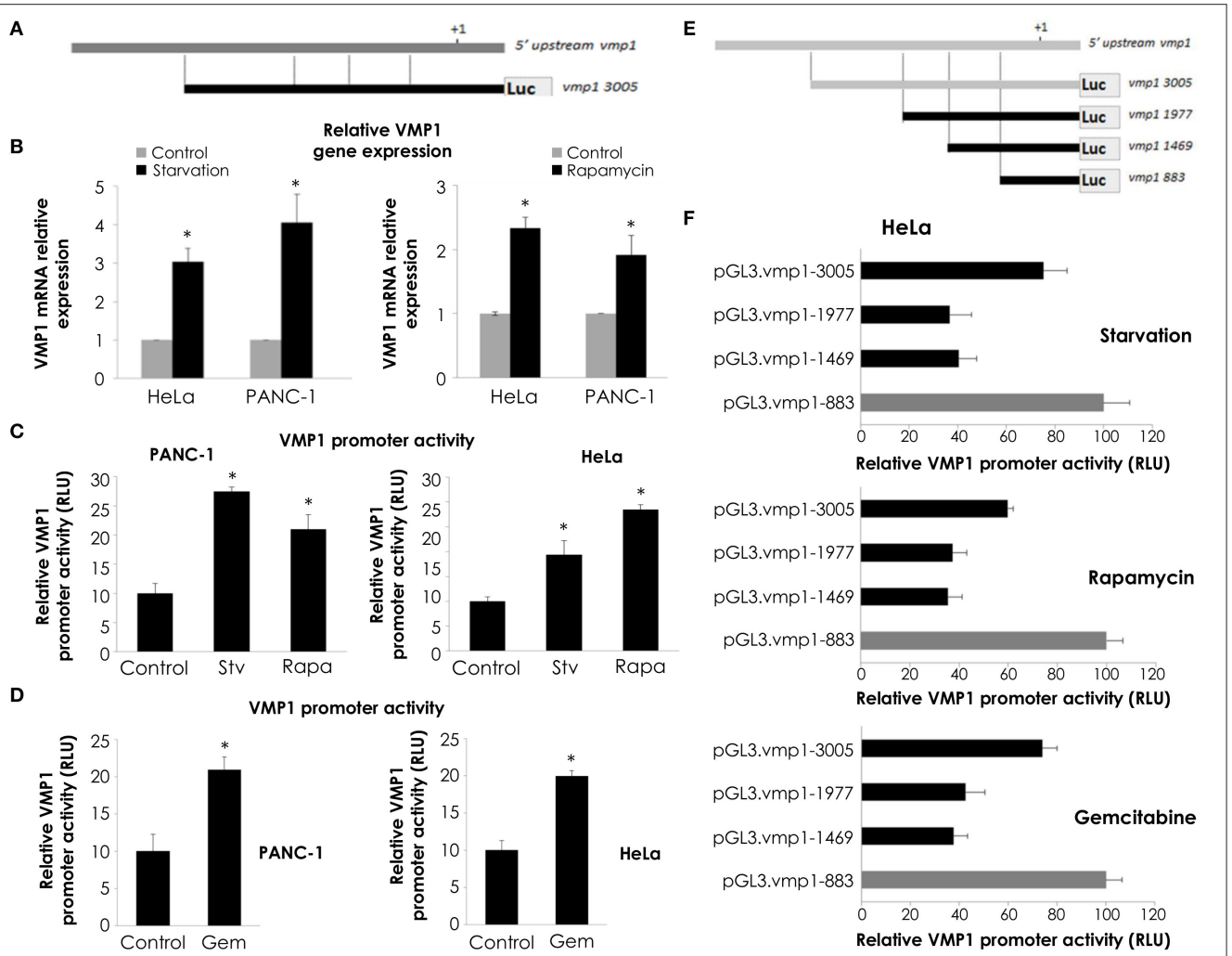

FIGURE 4 | *VMP1* promoter analysis. **(A)** To assay *VMP1* promoter activity, a 3,005 bp sequence of the 5′ upstream region of the *VMP1* gene was amplified and cloned into the pGL3 reporter vector (pGL3.vmp1-3005) driving Luciferase (Luc) expression. **(B)** qPCR assay showed an increase of VMP1 mRNA expression in HeLa and PANC-1 cells submitted to starving conditions and rapamycin treatment for 4 h ($n = 3$). Next, HeLa and PANC-1 cells were transfected with pGL3.vmp1-3005. **(C)** Relative Luciferase activity shows an increase of *VMP1* promoter activation in HeLa and PANC-1 cells submitted to starving (Stv) conditions and rapamycin (Rapa) treatment. ($n = 3$). **(D)** Relative Luciferase activity shows an increase of *VMP1* promoter activation in HeLa and PANC-1 cells submitted to gemcitabine treatment for 24 h ($n = 3$). **(E)** Consecutive shorter fragments of 3005 bp sequence of *VMP1* promoter were amplified and subcloned in the pGL3 reporter vector (pGL3.vmp1-1977, pGL3.vmp1-1469, pGL3.vmp1-883). **(F)** HeLa cells were submitted to starvation conditions, rapamycin and gemcitabine treatment for 24 h. Relative Luciferase activity showed higher levels of *VMP1* promoter activation in the shorter construct (pGL3.vmp1-883) compared to 3,005 bp sequence of *VMP1* promoter in all treatments. $^*p < 0.05$.

with E2F1 in *VMP1* promoter activation. Interestingly, down-regulation of EP300 impaired E2F1-mediated activation of the *VMP1* promoter in PANC-1 cells (**Figure 6C**). Furthermore, co-expression of E2F1 and EP300 led to a synergistic activation of the VMP1 promoter (**Figure 6D**). These findings demonstrate that E2F1 and EP300 cooperates in *VMP1* promoter regulation.

Altogether, these data show that the E2F1-EP300 activator/co-activator complex is part of the novel signaling pathway controlling the promoter activity and, consequently, the expression of the autophagy-related gene VMP1 in pancreatic tumor cells carrying oncogenic *KRAS*.

DISCUSSION

Tumor cells with a high prevalence of *KRAS* activating mutations, like pancreatic cancer, have the distinction of a poor prognosis (4). Previously, it has been demonstrated that many human cancer cell lines with *KRAS* activating mutations have basal levels of autophagy (51, 52). Yang et al. (6), have showed that pancreatic cancer cells exhibit constitutive autophagy under basal conditions, and it is increased in the advanced stages of PDAC being required for malignant transformation. In a previous work, we have identified VMP1 as a transcriptional target of oncogenic KRAS signaling

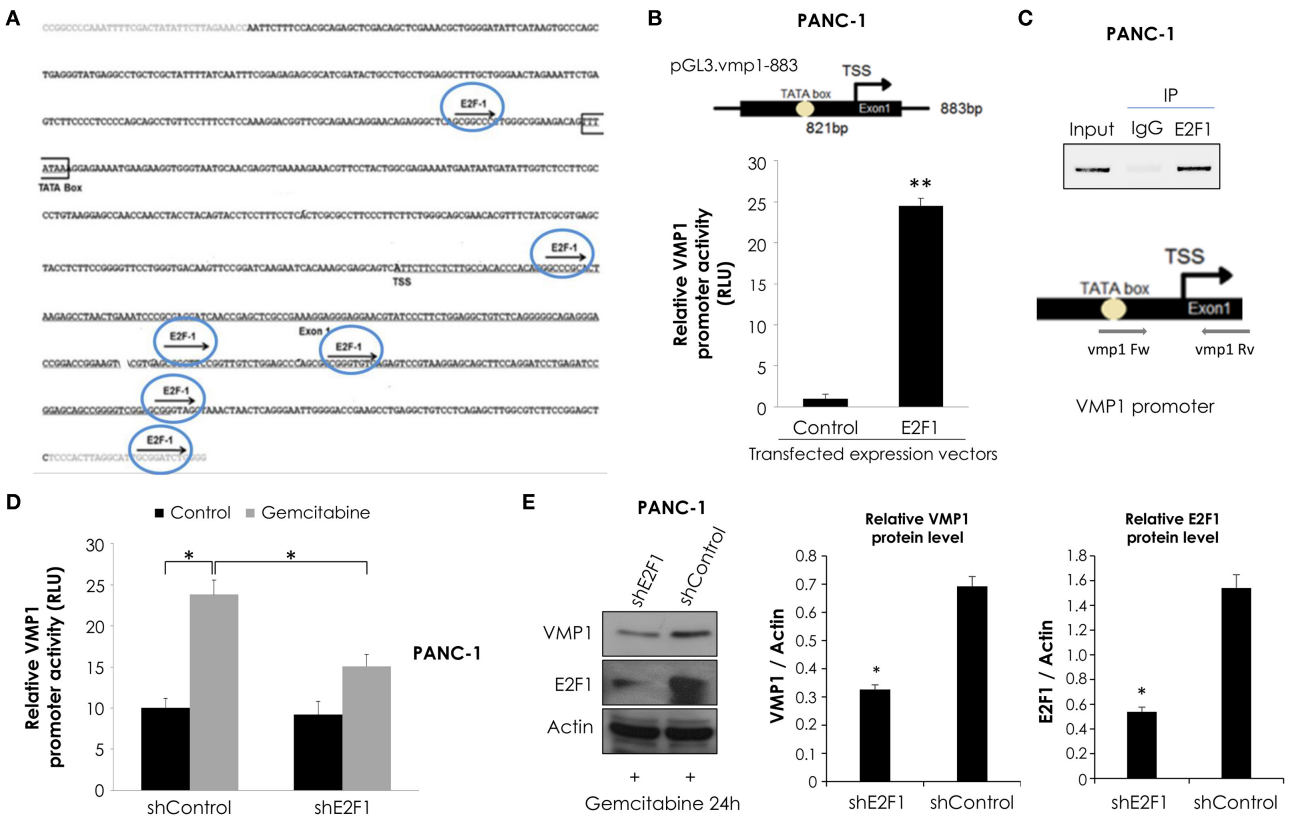

FIGURE 5 | E2F1 binds to *VMP1* promoter and regulates VMP1 expression induced by gemcitabine. **(A)** Bioinformatics analysis of the *VMP1* promoter identified candidate E2F1 binding sites. *TSS*, transcription starting site. **(B)** PANC-1 cells were transiently transfected with pGL3.vmp1-883, E2F1 expression vector or empty vector as control. Relative luciferase activity showed an increase of *VMP1* promoter activation in PANC-1 cells expressing E2F1 compared to control ($n = 3$). **(C)** ChIP assay using chromatin extracts precipitated with an antibody against E2F1 or IgG as a negative control in PANC-1 cells treated with 20 μM gemcitabine for 24 h. Through PCR we amplified the region of *VMP1* promoter in anti-E2F1 precipitated products using a specific set of primers (vmp1 Fw and vmp1 Rv) as described in Experimental Procedures, indicating that E2F1 binds directly to the *VMP1* promoter. **(D)** PANC-1 cells were transiently transfected with shControl or shE2F1 expression vectors and treated with 20 μM gemcitabine for 24 h. Relative Luciferase activity showed a significant reduction of *VMP1* promoter activation in PANC-1 cells expressing shE2F1 and treated with 20 μM gemcitabine for 24 h ($n = 3$). **(E)** Western blot analysis showed reduction of VMP1 expression in PANC-1 cells transfected with shE2F1 targeting vector and treated with 20 μM gemcitabine for 24 h. Relative densitometries (VMP1/Actin and E2F1/Actin) of western blots are shown ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

pathway and demonstrated that KRAS requires VMP1 to induce and maintain basal autophagy in pancreatic tumor cells (28). On the other hand, VMP1-mediated autophagy is early induced above basal conditions by gemcitabine treatment in MIAPaCa-2 cells (36). Moreover, VMP1 is highly expressed in poorly differentiated human pancreatic cancer (41). In this study, we identified a regulatory pathway, which is activated by gemcitabine treatment, in pancreatic tumor cells carrying a KRAS mutation at amino acid position 12. This molecular mechanism involves a novel E2F1-EP300-VMP1 pathway controlling VMP1 expression triggered by gemcitabine.

According to the *Ensembl Genome database*, *VMP1* gene is localized in Chromosome 17 of the human genome. Using informatics tools, we found out four putative promoter regions given for *VMP1* gene; among them we particularly focus on one of 821 bp localized in the positive strand at the following position 57784363–57785183 of chromosome 17. In the analysis, it was also found the proposed transcription-starting site (TSS) (**Figure 5A**) at position 501 of the promoter area. This prediction would regulate the expression of a 12 exons transcript and we consider this transcript to be the one coding for the 406 aa protein VMP1. The TSS is located at the start of the first exon sequence, while the starting codon (ATG) is in the second exon. Considering these data, we amplified and cloned a 3,005 bp sequence of the 5′ upstream regions of the *VMP1* gene sequence which includes the promoter area analyzed above. Using luciferase reporter assays we demonstrated that this region is activated by previously reported VMP1 stimuli, such as rapamycin, starvation, and gemcitabine (**Figure 4**). We found that regulatory motifs involved in VMP1 expression are contained into an 883 bp fragment in the 5′ upstream region of the *VMP1* gene. This essential promoter sequence showed the highest activation

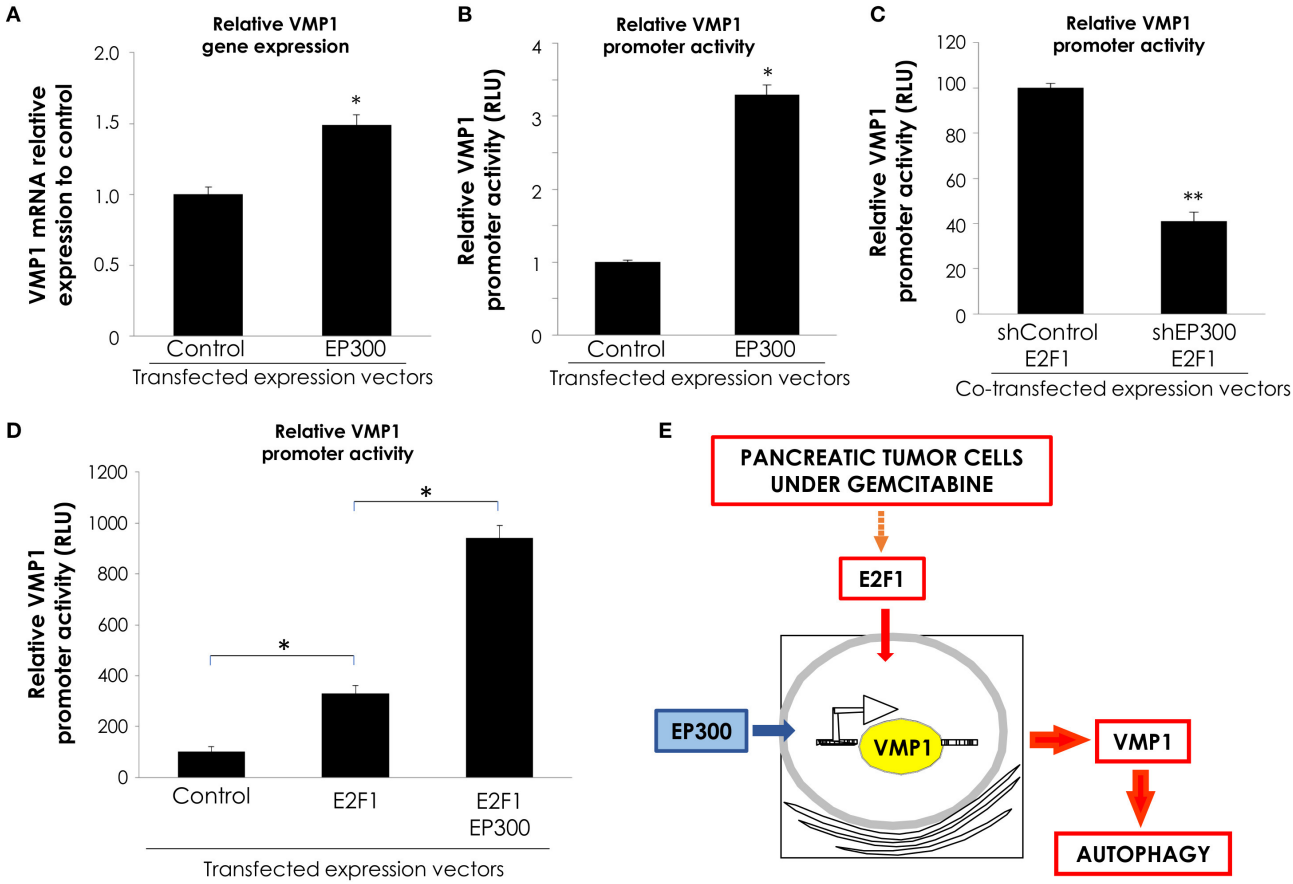

FIGURE 6 | E2F1 and EP300 cooperates in *VMP1* promoter regulation. PANC-1 cells were transiently transfected with an EP300 expression vector or with and empty expression vector. **(A)** VMP1 mRNA increased expression was observed by qPCR. In PANC-1 transfected with an EP300 expression vector compared to control cells ($n = 3$). **(B)** PANC-1 cells were transiently transfected with pGL3.vmp1-883. Relative Luciferase activity showed a significant increase of *VMP1* promoter activation in PANC-1 cells co-transfected with EP300 expression vector compared to cells transfected with a control vector ($n = 3$). **(C)** PANC-1 cells were transiently co-transfected with pGL3.vmp1-883, an E2F1 expression vector and shEP300 targeting vector or shControl targeting vector. Relative Luciferase activity showed a reduction of *VMP1* promoter activity in PANC-1 cells co-transfected with E2F1 and shp300 targeting vector compared to cells transfected with a E2F1 and shControl targeting vector ($n = 3$). **(D)** PANC-1 cells were transiently co-transfected with pGL3.vmp1-883, E2F1, and EP300 expression vectors. Relative Luciferase activity showed an increase of *VMP1* promoter activation in PANC-1 cells co-transfected with E2F1 and EP300 expression vectors compared to cells transfected with a E2F1 expression vector alone ($n = 3$). **(E)** Schematic model. E2F1-p300-VMP1 pathway that mediates gemcitabine-induced autophagy in pancreatic cancer cells. $^{*}p < 0.05$, $^{**}p < 0.01$.

by rapamycin, starvation, and gemcitabine treatment using luciferase reporter assays.

The *in silico* analysis of VMP1 essential promoter sequence revealed the presence of binding sites for several transcription factors related to cellular stress. We focus our attention on E2F1 because it has been correlated with high-grade tumors and unfavorable patient survival in PDAC (53, 54). E2F1 was the first identified member of the E2F family of transcription factors. E2F activity is linked to retinoblastoma tumor suppressor (RB)–dependent cell-cycle control. E2F transcription factors are found downstream of growth factor signaling cascades, acting as transcriptional activators or repressors of genes necessary for cell cycle progression (55). Most human tumors harbor the functionally inactivated retinoblastoma protein, resulting in deregulated E2F1 and its target genes are highly up-regulated in these transformed cells (56). This up-regulation leads to the activation of cytoplasmic (PIK3CA/AKT and RAS/MAPK/ERK) and nuclear signaling cascades related to invasion and metastasis (54). Also, activation of E2F1 transcription factor has been shown to induce autophagy (57) by up-regulating the expression of the autophagy genes LC3, ULK1 (unc-51 like autophagy activating kinase 1), ATG5 and DRAM1 (DNA damage regulated autophagy modulator 1). The E2F1-mediated induction of LC3, ULK1, and DRAM1 is direct (through interaction with the promoter), whereas the up-regulation of the expression of ATG5 is indirect (58). In this work, we provide evidence of another gene related to autophagy that is up-regulated by E2F1. We demonstrated that

E2F1 is able to induce autophagy in pancreatic tumor cells and regulates VMP1-mediated autophagy by a direct binding to VMP1 promoter.

Gemcitabine inhibits DNA synthesis via a process called masked chain termination where gemcitabine is incorporated into DNA via DNA polymerase α, leading to the inhibition of DNA repair and synthesis (59). It has been shown that E2F1 induces genes involved in DNA repair in normal cells and in tumor cells undergoing chemotherapy through complex formation on the promoters of these genes (54). In this sense, BxPC-3 cells that are sensitive to the dose of 20 μM gemcitabine did not increase the expression of E2F1 or VMP1 during treatment. In contrast, PANC-1 cells resistant to treatment increased the expression of E2F1, result consistent with data of previous works (60, 61). Lai et al. (60) have demonstrated that PANC-1 cells respond to gemcitabine by increasing the expression of ribonucleotide reductase M2 catalytic subunit (RRM2) through E2F1-mediated transcriptional activation, as a DNA damage response to enhance DNA repair capacity in these cells. Here, we demonstrated E2F1 and VMP1 expressions are both increased in PANC-1 cells treated with gemcitabine. Besides, pancreatic tumor cells transfected with an expression vector for E2F1 induced VMP1 expression and activated autophagy. Therefore, in this study we demonstrated another mechanism activated by E2F1 in response to chemotherapy, in which E2F1 activates VMP1 expression and autophagy as a resistance response to gemcitabine.

Autophagy is constitutively activated in oncogenic KRAS-driven tumors and is necessary for the development of these tumors (6). Previously, we identified the PI3KCA (phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha)-AKT1 (AKT serine/threonine kinase 1) pathway as the signaling pathway mediating the expression and promoter activity of VMP1 in KRAS driven tumors (28). PANC-1 cells harbor a *KRAS* mutated allele (KRAS G12D) and these cells present basal expression of VMP1 and autophagy. In this work, we show that gemcitabine treatment increased that basal VMP1 expression and autophagy in PANC-1 cells. As well as GLI3 (GLI family zinc finger 3) is the transcription factor implicated in *VMP1* promoter activation in basal conditions in PANC-1 cells (28), E2F1 plays that function under gemcitabine treatment. Here, we show that down-regulation of E2F1 reduced VMP1 expression and *VMP1* promoter activity induced by gemcitabine. Besides, we demonstrated a direct binding of E2F1 on *VMP1* promoter under gemcitabine treatment in pancreatic tumor cells. Therefore, these data suggest VMP1 expression is regulated by different transcription factors depending on the cellular context that autophagy is induced. Further research will be necessary to clarify if function or mechanisms involved in basal VMP1-induced autophagy differs from VMP1-induced autophagy under gemcitabine treatment.

The regulation of gene expression depends on the characteristic activation/repression properties of each transcription factor, but also on functional interactions with co-regulatory molecules. EP300 belongs to the type 3 family of lysine acetyltransferases (KAT3) (62), and this enzyme is involved in the regulation of important physiological processes such as proliferation, differentiation, and apoptosis, due to its ability to function as transcriptional coactivator interacting and regulating more than 400 transcription factors (63, 64). However, the role of EP300 in gene regulation is not only restricted to its property of allowing the binding of transcription factors to large protein complexes in the transcription machinery, but also implies the required KAT activity for the acetylation of transcription factors and histones that allow access to chromatin (65). Thus, EP300 contributes to DNA repair through histone acetylation, facilitating the recruitment of DNA repair factors to the site of damage (66). We demonstrated that EP300 potentiates the *VMP1* promoter activation by E2F1. Even, down-regulation of EP300 impaired E2F1-mediated activation of the VMP1 promoter in pancreatic tumor cells. These findings demonstrate that E2F1 and p300 cooperate in VMP1 promoter regulation.

Our results agree with Hashimoto et al. (67), who have shown that autophagy has a cytoprotective effect against 5-fluorouracil and gemcitabine in pancreatic cancer cells. They demonstrated that inhibition of autophagy potentiates the inhibition of PANC-1 cell proliferation by 5-fluorouracil and gemcitabine. Here, we showed an induction of VMP1 expression and autophagy in PANC-1 and MIAPaCa-2 cells under gemcitabine treatment and down-regulation of VMP1 expression significantly reduced autophagy induced by gemcitabine. These data strongly suggest that VMP1 expression is involved in PDAC chemoresistance to gemcitabine.

In conclusion, we have identified the E2F1-EP300-VMP1 pathway that mediates gemcitabine-induced autophagy in pancreatic cancer cells (**Figure 6E**). This pathway would be activated by gemcitabine like a resistance mechanism. Our results point at E2F1 as a regulatory factor modulating gemcitabine induced VMP1-mediated autophagy in human pancreatic cancer cells and mechanistically integrate the autophagic degradative process into the complex network of events involved in PDAC chemoresistance.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AR designed and performed experiments, analyzed and interpreted data, prepared figures, and wrote the manuscript. CC performed experiments, contributed to interpret data, and prepared figures. FR performed experiments. VB developed analytical tools. TO developed expression vectors. CG contributed to interpret data. MV developed the

hypothesis, designed experiments, analyzed, and interpreted data, and wrote the manuscript. All authors reviewed the manuscript.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics. *CA Cancer J Clin.* (2019) 69:7–34. doi: 10.3322/caac.21551
2. Jaffee EM, Hruban RH, Canto M, Kern SE. Focus on pancreas cancer. *Cancer Cell.* (2002) 2:25–8. doi: 10.1016/S1535-61080200093-4
3. Warshaw AL, Fernandez-del Castillo C. Pancreatic carcinoma. *N Engl J Med.* (1992) 326:455–65. doi: 10.1056/NEJM199202133260706
4. Hidalgo M, Von Hoff DD. Translational therapeutic opportunities in ductal adenocarcinoma of the pancreas. *Clin Cancer Res.* (2012) 18:4249–56. doi: 10.1158/1078-0432.CCR-12-1327
5. Kimmelman AC. The dynamic nature of autophagy in cancer. *Genes Dev.* (2011) 25:1999–2010. doi: 10.1101/gad.17558811
6. Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, et al. Pancreatic cancers require autophagy for tumor growth. *Genes Dev.* (2011) 25:717–29. doi: 10.1101/gad.2016111
7. Yang A, Rajeshkumar NV, Wang X, Yabuuchi S, Alexander BM, Chu GC, et al. Autophagy is critical for pancreatic tumor growth and progression in tumors with p53 alterations. *Cancer Discov.* (2014) 4:905–13. doi: 10.1158/2159-8290.CD-14-0362
8. Chawla A, Ferrone CR. Neoadjuvant therapy for resectable pancreatic cancer: an evolving paradigm shift. *Front Oncol.* (2019) 9:1085. doi: 10.3389/fonc.2019.01085
9. Ducreux M, Seufferlein T, Van Laethem JL, Laurent-Puig P, Smolenschi C, Malka D, et al. Systemic treatment of pancreatic cancer revisited. *Semin Oncol.* (2019) 46:28–38. doi: 10.1053/j.seminoncol.2018.12.003
10. Raufi AG, Manji GA, Chabot JA, Bates SE. Neoadjuvant treatment for pancreatic cancer. *Semin Oncol.* (2019) 46:19–27. doi: 10.1053/j.seminoncol.2018.12.002
11. Cao H, Le D, Yang LX. Current status in chemotherapy for advanced pancreatic adenocarcinoma. *Anticancer Res.* (2013) 33:1785–91.
12. Huang P, Plunkett W. Induction of apoptosis by gemcitabine. *Semin Oncol.* (1995) 22:19–25.
13. Bold RJ, Chandra J, McConkey DJ. Gemcitabine-induced programmed cell death (apoptosis) of human pancreatic carcinoma is determined by Bcl-2 content. *Ann Surg Oncol.* (1999) 6:279–85. doi: 10.1007/s10434-999-0279-x
14. Bergman AM, Pinedo HM, Peters GJ. Determinants of resistance to 2′,2′-difluorodeoxycytidine (gemcitabine). *Drug Resist Updat.* (2002) 5:19–33. doi: 10.1016/S1368-76460200002-X
15. Sarvepalli D, Rashid MU, Rahman AU, Ullah W, Hussain I, Hasan B, et al. Gemcitabine: a review of chemoresistance in pancreatic cancer. *Crit Rev Oncog.* (2019) 24:199–212. doi: 10.1615/CritRevOncog.2019031641
16. Zeng S, Pottler M, Lan B, Grutzmann R, Pilarsky C, Yang H. Chemoresistance in pancreatic cancer. *Int J Mol Sci.* (2019) 20:E4504. doi: 10.3390/ijms20184504
17. Adiseshaiah PP, Crist RM, Hook SS, McNeil SE. Nanomedicine strategies to overcome the pathophysiological barriers of pancreatic cancer. *Nat Rev Clin Oncol.* (2016) 13:750–65. doi: 10.1038/nrclinonc.2016.119
18. Ma T, Chen W, Zhi X, Liu H, Zhou Y, Chen BW, et al. USP9X inhibition improves gemcitabine sensitivity in pancreatic cancer by inhibiting autophagy. *Cancer Lett.* (2018) 436:129–38. doi: 10.1016/j.canlet.2018.08.010
19. Zeh H, Bahary N, Boone BA, Singhi AD, Miller-Ocuin JL, Normolle DP, et al. A randomized phase II preoperative study of autophagy inhibition with high-dose hydroxychloroquine and gemcitabine/nab-paclitaxel in pancreatic cancer patients. *Clin Cancer Res.* (2020). doi: 10.1158/1078-0432.CCR-19-4042. [Epub ahead of print].
20. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell.* (2004) 6:463–77. doi: 10.1016/S1534-58070400099-1
21. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, et al. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature.* (2006) 441:885–9. doi: 10.1038/nature04724
22. Qu X, Zou Z, Sun Q, Luby-Phelps K, Cheng P, Hogan RN, et al. Autophagy gene-dependent clearance of apoptotic cells during embryonic development. *Cell.* (2007) 128:931–46. doi: 10.1016/j.cell.2006.12.044
23. Choi AM, Ryter SW, Levine B. Autophagy in human health and disease. *N Engl J Med.* (2013) 368:1845–46. doi: 10.1056/NEJMc1303158
24. Wilde L, Tanson K, Curry J, Martinez-Outschoorn U. Autophagy in cancer: a complex relationship. *Biochem J.* (2018) 475:1939–54. doi: 10.1042/BCJ20170847
25. Fujii S, Mitsunaga S, Yamazaki M, Hasebe T, Ishii G, Kojima M, et al. Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. *Cancer Sci.* (2008) 99:1813–9. doi: 10.1111/j.1349-7006.2008.00893.x
26. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev.* (2006) 20:1218–49. doi: 10.1101/gad.1415606
27. Rosenfeldt MT, O'Prey J, Morton JP, Nixon C, MacKay G, Mrowinska A, et al. p53 status determines the role of autophagy in pancreatic tumour development. *Nature.* (2013) 504:296–300. doi: 10.1038/nature12865
28. Lo Re AE, Fernandez-Barrena MG, Almada LL, Mills LD, Elsawa SF, Lund G, et al. Novel AKT1-GLI3-VMP1 pathway mediates KRAS oncogene-induced autophagy in cancer cells. *J Biol Chem.* (2012) 287:25325–34. doi: 10.1074/jbc.M112.370809
29. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol.* (2010) 22:124–31. doi: 10.1016/j.ceb.2009.11.014
30. Klionsky DJ, Cregg JM, Dunn WA Jr, Emr SD, Sakai Y, Sandoval IV, et al. A unified nomenclature for yeast autophagy-related genes. *Dev Cell.* (2003) 5:539–45. doi: 10.1016/S1534-5807(03)00296-X
31. Ropolo A, Grasso D, Pardo R, Sacchetti ML, Archange C, Lo Re A, et al. The pancreatitis-induced vacuole membrane protein 1 triggers autophagy in mammalian cells. *J Biol Chem.* (2007) 282:37124–33. doi: 10.1074/jbc.M706956200
32. Vaccaro MI, Ropolo A, Grasso D, Iovanna JL. A novel mammalian transmembrane protein reveals an alternative initiation pathway for autophagy. *Autophagy.* (2008) 4:388–90. doi: 10.4161/auto.5656

33. Molejon MI, Ropolo A, Re AL, Boggio V, Vaccaro MI. The VMP1-Beclin 1 interaction regulates autophagy induction. *Sci Rep.* (2013) 3:1055. doi: 10.1038/srep01055
34. Molejon MI, Ropolo A, Vaccaro MI. VMP1 is a new player in the regulation of the autophagy-specific phosphatidylinositol 3-kinase complex activation. *Autophagy.* (2013) 9:933–5. doi: 10.4161/auto.24390
35. Grasso D, Ropolo A, Lo Re A, Boggio V, Molejon MI, Iovanna JL, et al. Zymophagy, a novel selective autophagy pathway mediated by VMP1-USP9x-p62, prevents pancreatic cell death. *J Biol Chem.* (2011) 286:8308–24. doi: 10.1074/jbc.M110.197301
36. Pardo R, Lo Ré A, Archange C, Ropolo A, Papademetrio DL, Gonzalez CD, et al. Gemcitabine induces the VMP1-mediated autophagy pathway to promote apoptotic death in human pancreatic cancer cells. *Pancreatology.* (2010) 10:19–26. doi: 10.1159/000264680
37. Grasso D, Sacchetti ML, Bruno L, Lo Ré A, Iovanna JL, Gonzalez CD, et al. Autophagy and VMP1 expression are early cellular events in experimental diabetes. *Pancreatology.* (2009) 9:81–8. doi: 10.1159/000178878
38. Jiang PH, Motoo Y, Vaccaro MI, Iovanna JL, Okada G, Sawabu N. Expression of vacuole membrane protein 1 (VMP1) in spontaneous chronic pancreatitis in the WBN/Kob rat. *Pancreas.* (2004) 29:225–30. doi: 10.1097/00006676-200410000-00008
39. Vaccaro MI, Grasso D, Ropolo A, Iovanna JL, Cerquetti MC. VMP1 expression correlates with acinar cell cytoplasmic vacuolization in arginine-induced acute pancreatitis. *Pancreatology.* (2003) 3:69–74. doi: 10.1159/000069150
40. Bryant KL, Mancias JD, Kimmelman AC, Der CJ. KRAS: feeding pancreatic cancer proliferation. *Trends Biochem Sci.* (2014) 39:91–100. doi: 10.1016/j.tibs.2013.12.004
41. Gilabert M, Vaccaro MI, Fernandez-Zapico ME, Calvo EL, Turrini O, Secq V, et al. Novel role of VMP1 as modifier of the pancreatic tumor cell response to chemotherapeutic drugs. *J Cell Physiol.* (2013) 228:1834–43. doi: 10.1002/jcp.24343
42. Rodriguez ME, Catrinacio C, Ropolo A, Rivarola VA, Vaccaro MI. A novel HIF-1alpha/VMP1-autophagic pathway induces resistance to photodynamic therapy in colon cancer cells. *Photochem Photobiol Sci.* (2017) 16:1631–42. doi: 10.1039/C7PP00161D
43. Lyons JF, Wilhelm S, Hibner B, Bollag G. Discovery of a novel Raf kinase inhibitor. *Endocr Relat Cancer.* (2001) 8:219–25. doi: 10.1677/erc.0.0080219
44. Wei L, Ye H, Li G, Lu Y, Zhou Q, Zheng S, et al. Cancer-associated fibroblasts promote progression and gemcitabine resistance via the SDF-1/SATB-1 pathway in pancreatic cancer. *Cell Death Dis.* (2018) 9:1065. doi: 10.1038/s41419-018-1104-x
45. Wang X, Yin L, Yang L, Zheng Y, Liu S, Yang J, et al. Silencing ubiquitin-conjugating enzyme 2C inhibits proliferation and epithelial-mesenchymal transition in pancreatic ductal adenocarcinoma. *FEBS J.* (2019) 286:4889–909. doi: 10.1111/febs.15134
46. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy.* (2016) 12:1–222. doi: 10.1080/15548627.2015.1100356
47. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* (2000) 19:5720–8. doi: 10.1093/emboj/19.21.5720
48. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. *Cell Res.* (2014) 24:24–41. doi: 10.1038/cr.2013.168
49. DeGregori J, Johnson DG. Distinct and overlapping roles for E2f family members in transcription, proliferation and apoptosis. *Curr Mol Med.* (2006) 6:739–48. doi: 10.2174/156652401060607073 9
50. Liao WC, Chien KL, Lin YL, Wu MS, Lin JT, Wang HP, et al. Adjuvant treatments for resected pancreatic adenocarcinoma: a systematic review and network meta-analysis. *Lancet Oncol.* (2013) 14:1095–103. doi: 10.1016/S1470-20451370388-7
51. Elgendy M, Sheridan C, Brumatti G, Martin SJ. Oncogenic Ras-induced expression of Noxa and Beclin-1 promotes autophagic cell death and limits clonogenic survival. *Mol Cell.* (2011) 42:23–35. doi: 10.1016/j.molcel.2011.02.009
52. Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev.* (2011) 25:460–70. doi: 10.1101/gad.2016311
53. Schild C, Wirth M, Reichert M, Schmid RM, Saur D, Schneider G. PI3K signaling maintains c-myc expression to regulate transcription of E2F1 in pancreatic cancer cells. *Mol Carcinog.* (2009) 48:1149–58. doi: 10.1002/mc.20569
54. Engelmann D, Putzer BM. The dark side of E2F1: in transit beyond apoptosis. *Cancer Res.* (2012) 72:571–5. doi: 10.1158/0008-5472.CAN-11-2575
55. Putzer BM, Engelmann D. E2F1 apoptosis counterattacked: evil strikes back. *Trends Mol Med.* (2013) 19:89–98. doi: 10.1016/j.molmed.2012.10.009
56. Uchida C. The retinoblastoma protein: functions beyond the G1-S regulator. *Curr Drug Targets.* (2012) 13:1622–32. doi: 10.2174/138945012803529938
57. Korah J, Canaff L, Lebrun JJ. The retinoblastoma tumor suppressor protein (pRb)/E2 promoter binding factor 1 (E2F1) pathway as a novel mediator of TGFβ-induced autophagy. *J Biol Chem.* (2016) 291:2043–54. doi: 10.1074/jbc.M115.678557
58. Polager S, Ofir M, Ginsberg D. E2F1 regulates autophagy and the transcription of autophagy genes. *Oncogene.* (2008) 27:4860–4. doi: 10.1038/onc.2008.117
59. Soo RA, Yong WP, Innocenti F. Systemic therapies for pancreatic cancer–the role of pharmacogenetics. *Curr Drug Targets.* (2012) 13:811–28. doi: 10.2174/138945012800564068
60. Lai IL, Chou CC, Lai PT, Fang CS, Shirley LA, Yan R, et al. Targeting the Warburg effect with a novel glucose transporter inhibitor to overcome gemcitabine resistance in pancreatic cancer cells. *Carcinogenesis.* (2014) 35:2203–13. doi: 10.1093/carcin/bgu124
61. Zhu X, Shi C, Peng Y, Yin L, Tu M, Chen Q, et al. Thymidine kinase 1 silencing retards proliferative activity of pancreatic cancer cell via E2F1-TK1-P21 axis. *Cell Prolif.* (2018) 51:e12428. doi: 10.1111/cpr.12428
62. Yuan LW, Giordano A. Acetyltransferase machinery conserved in p300/CBP-family proteins. *Oncogene.* (2002) 21:2253–60. doi: 10.1038/sj.onc.1205283
63. Chan HM, La Thangue NB. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J Cell Sci.* (2001) 114:2363–73.
64. Kalkhoven E. CBP and p300: HATs for different occasions. *Biochem Pharmacol.* (2004) 68:1145–55. doi: 10.1016/j.bcp.2004.03.045
65. Dancy BM, Cole PA. Correction to protein lysine acetylation by p300/CBP. *Chem Rev.* (2016) 116:8314. doi: 10.1021/acs.chemrev.6b00351
66. Ogiwara H, Kohno T. CBP and p300 histone acetyltransferases contribute to homologous recombination by transcriptionally activating the BRCA1 and RAD51 genes. *PLoS One.* (2012) 7:e52810. doi: 10.1371/journal.pone.0052810
67. Hashimoto D, Blauer M, Hirota M, Ikonen NH, Sand J, Laukkarinen J. Autophagy is needed for the growth of pancreatic adenocarcinoma and has a cytoprotective effect against anticancer drugs. *Eur J Cancer.* (2014) 50:1382–90. doi: 10.1016/j.ejca.2014.01.011
68. Vaccaro MI, Ropolo A, Giovenco M, Catrinacio C, Renna F, Grasso D, et al. Autophagy mediates resistance of pancreatic cancer cells to chemotherapy through a novel E2F1-p300-VMP1 pathway. *Gastroenterology.* (2015) 148(Suppl 1):S-341. doi: 10.1016/S0016-50851531135-5

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Increased Expression of Autophagy Protein LC3 in Two Patients With Progressing Chronic Lymphocytic Leukemia

Daniela S. Arroyo[1,2†], *Cecilia M. Rodriguez*[1,2†], *Claudio Bussi*[3], *Clarisa Manzone-Rodriguez*[2,4], *Darío Sastre*[1], *Viviana Heller*[1], *Carmen Stanganelli*[5], *Irma Slavutsky*[6] *and Pablo Iribarren*[2,4*]

[1] Facultad de Ciencias Médicas, Hospital Nacional de Clínicas, Universidad Nacional de Córdoba, Córdoba, Argentina, [2] Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina, [3] The Francis Crick Institute, London, United Kingdom, [4] Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI-CONICET), Córdoba, Argentina, [5] Patología Molecular, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Buenos Aires, Argentina, [6] Laboratorio de Genética de Neoplasias Linfoides, Instituto de Medicina Experimental, CONICET-Academia Nacional de Medicina, Buenos Aires, Argentina

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***Correspondence:**
Pablo Iribarren
piribarren@unc.edu.ar

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

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Chronic lymphocytic leukemia (CLL) is the most common type of adult leukemia in the western hemisphere. It is characterized by a clonal proliferation of a population of CD5+ B lymphocytes that accumulate in the secondary lymphoid tissues, bone marrow, and blood. Some CLL patients remain free of symptoms for decades, whereas others rapidly become symptomatic or develop high-risk disease. Studying autophagy, which may modulate key protein expression and cell survival, may be important to the search for novel prognostic factors and molecules. Here, we applied flow cytometry technology to simultaneously detect autophagy protein LC3B with classical phenotypical markers used for the identification of tumoral CLL B cell clones. We found that two patients with progressing CLL showed increased expression of the autophagy protein LC3B, in addition to positive expression of CD38 and ZAP70 and unmutated status of IGHV. Our data suggest that activation of autophagy flux may correlate with CLL progression even before Ibrutinib treatment.

Keywords: chronic lymphocytic leukemia, autophagy, LC3, progressing, cancer

INTRODUCTION

Chronic lymphocytic leukemia (CLL) represents 25% of all leukemias and 1.3% of all cancers in the western hemisphere, but it has lower prevalence in Asia (1, 2). It is characterized by the expansion of a population of monoclonal CD5+ B lymphocytes that accumulate in blood, bone marrow, and secondary lymphoid tissues (3, 4). Chronic lymphocytic leukemia frequently presents with adverse prognostic features in older patients, significantly affecting their survival (1).

The average age at the time of diagnosis is around 70 years old, though, rarely, it is seen in people under age 40. Patients with CLL may be asymptomatic and may initially be diagnosed by the detection of lymphocytosis on a routine complete blood cell count (1). Sometimes the patients develop anemia, thrombocytopenia, lymphadenopathy, and/or hepatosplenomegaly (5). Other cases may present fever, fatigue, night sweats, and weight loss (1). As we describe in a

previous report (4), chronic lymphocytic leukemia can present two clinical forms, aggressive and indolent. The worst prognosis is associated with CD38 expression, high expression levels of ZAP-70, and the absence of mutations in the immunoglobulin heavy chain variable (IGHV) genes (4, 6, 7). Discrimination of patients with different outcomes can be done by finding chromosomal alterations, which are normally detected in >80% of cases (4, 8). The most frequent genomic aberration (deletions at chromosome 13q14) can be detected by FISH analysis. Most of the CLL patients show constitutively elevated expression of Bcl-2, indicating a role for resistance to apoptosis in the disease pathogenesis.

The particular ability of autophagy to promote cell survival during metabolic stress or cell death as a result of an imbalance in cell metabolism, where autophagic cellular consumption exceeds the cellular capacity for synthesis, is a promising avenue for cancer therapy (9). As described by Bologna et al. (3), autophagy is activated in leukemia cells upon treatment with different chemotherapeutic agents, inducing cell death. In particular, many currently used drugs for CLL, including fludarabine, dexamethasone, idelalisib, and Bcl-2 antagonists, have been suggested to have an autophagy-mediated effect (3).

It was proposed that conventional CLL prognostic markers like genetic mutations, the mutation status of the IGHV, and expression of ZAP-70 and CD38, have predictive value for the responses to first-line therapy in CLL (4, 10). Many initiatives have aimed at integrating all of the prognostic factors defined previously into a single prognostic score (11, 12). As stated by Strati et al. (1), it has recently been suggested that "published evidence is sufficient to recommend that FISH and IGHV analysis be performed as standard clinical tests for all patients with newly diagnosed CLL in those countries with the resources to do so" (1, 13). Recently, there was a report of differential expression of at least 20 miRs in B cells from progressive CLL patients compared to non-progressive CLL controls (14). Several of these miRs promote resistance to apoptosis and/or progression of neoplastic B cell clones. Therefore, studying cell responses able to modulate miR expression and cell survival, such as autophagy, may be important for the search for novel prognostic factors and molecules (14).

BACKGROUND

We present two cases of progressing CLL; the characteristics of the patients are summarized in **Table 1**. Two additional non-progressing CLL patients are included for comparison purposes. These patients were included in our previous study (4). Here, we describe the patients accordingly to onset, diagnosis, prognostic markers, and evolution.

Patient #1

A 58-year-old man was diagnosed with CLL in February 2008. At that time, routine analysis showed a total white blood cell count of 34 $\times$ 10^9/L (normal range: 4.5–10 $\times$ 10^9/L), 82% of lymphocytes (normal range: 20–45%), with splenomegaly and no anemia or thrombocytopenia. Lactate dehydrogenase (LDH) and B2-microglobulin (B2M) values were 312 UI/L and 4.85 mg/L, respectively (reference values: LDH: 180–450 UI/L; B2M: 0.8–2.20 mg/L). Flow cytometry analysis revealed a clonal B cell population with a typical CLL phenotype. Prognostic marker analysis showed 58% of ZAP-70- and 80% of CD38-positive cells (15). Thus, a diagnostic of CLL Rai stage II was done, and the patient was treated with bendamustine from 2009 to 2016 because progressive systemic symptoms were present. At that moment, genetic markers were: 17p deleted 4.7 %, 11q deleted 62.5%, 13q14 14.4%, and the IGHV gene was unmutated. A stringent follow-up was adopted until July 2016, when he arrived at our Center because of progression with lymphadenopathy, hepatosplenomegaly, and systemic symptoms. At this time, he received targeted drug treatment with Ibrutinib for 16 months, achieving partial response. Finally, he progressed to Richter Syndrome, and the patient died 1 month later.

Patient #2

A 51-year-old man was diagnosed with CLL in February 2010. At that time, routine analysis showed a total white blood cell count of 51.7 $\times$ 10^9/L (normal range: 4.5–10 $\times$ 10^9/L), 91% of lymphocytes (normal range: 20–45%), with hepatosplenomegaly and no anemia or thrombocytopenia. Lactate dehydrogenase (LDH) and B2-microglobulin (B2M) values were 257 UI/L and 3.6 mg/L, respectively (reference values: LDH: 180–450 UI/L; B2M: 0.8–2.20 mg/L). Flow cytometry analysis revealed a clonal B cell population with a typical CLL phenotype, expressing prognostic markers ZAP-70 and CD38 (15). Thus, a diagnostic of CLL Rai stage II was done, and progression was detected in October 2010. In March 2011, first-line treatment of six cycles with Fludarabine, Cyclophosphamide, and Rituximab was administered, followed by two additional cycles in May 2015. Genetic markers were: 17p deleted 20%, 13q 53.9%, 11q normal, and the IGHV gene was unmutated. Relapse was observed in 2014 with profuse sweating and progression to stage IIB. In 2016, further progression to stage IV was observed with weight loss, hepatosplenomegaly, lymphadenopathy, asthenia, sweating, and low platelet count. Ibrutinib treatment was initiated in May 2016, and the patient died in March 2018.

Patients #3 and #4

Patients 3 and 4 presented non-progressing CLL, RAI stages I and 0, respectively, mutated IGHV gene, and negative for CD38 and ZAP70. They were diagnosed in 2006 and 2009, respectively. They subsequently maintained the typical non-progressive phenotype, and both are still alive (**Table 1**).

LC3 Expression in Cells From CLL Patients

At the moments of progression of patients 1 and 2, we evaluated autophagy protein LC3B expression in CLL cells by flow cytometry (FlowCellect^{TM}, MilliporeSigma, Darmstadt, Germany). Peripheral blood mononuclear cells (PBMC) from patients with CLL were cultured in the presence or the absence of bafilomycin and then stained using antibodies specific for CD5, CD19, and LC3B. Chronic lymphocytic leukemia B cells were identified and gated using the combination of CD5 plus CD19 staining, for the evaluation of LC3B expression. Lipidated membrane-located LC3B fraction (LC3BII) was accumulated in

TABLE 1 | Characterization of the CLL patients.

Sample	Age at diagnosis	Date of diagnosis	Rai stage at diagnosis	Gender (male/female)	CD38/ZAP70	IgVH mutation	del 17p	del 11q	Progression
CLL#1	58	2008	II	M	+/+	UM	Yes	Yes	Yes
CLL#2	51	2010	IIB	M	+/+	UM	Yes	No	Yes
CLL#3	43	2006	I	F	–/–	MM	No	Yes	No
CLL#4	55	2009	0	M	–/–	MM	Yes	Yes	No

Chronic lymphocytic leukemia was diagnosed according to standard clinical and laboratory criteria. At the time of analysis, all patients were free from clinically relevant infectious complications. For all in vitro studies, written and informed consent was obtained from patients in accordance with the Declaration of Helsinki. CLL#1 and CLL#2 represent a subgroup of patients with adverse prognostic factors, and CLL#3 and CLL#4 represent patients with more favorable prognostic factors.
M, male; F, female; UM, unmutated; MM, mutated.

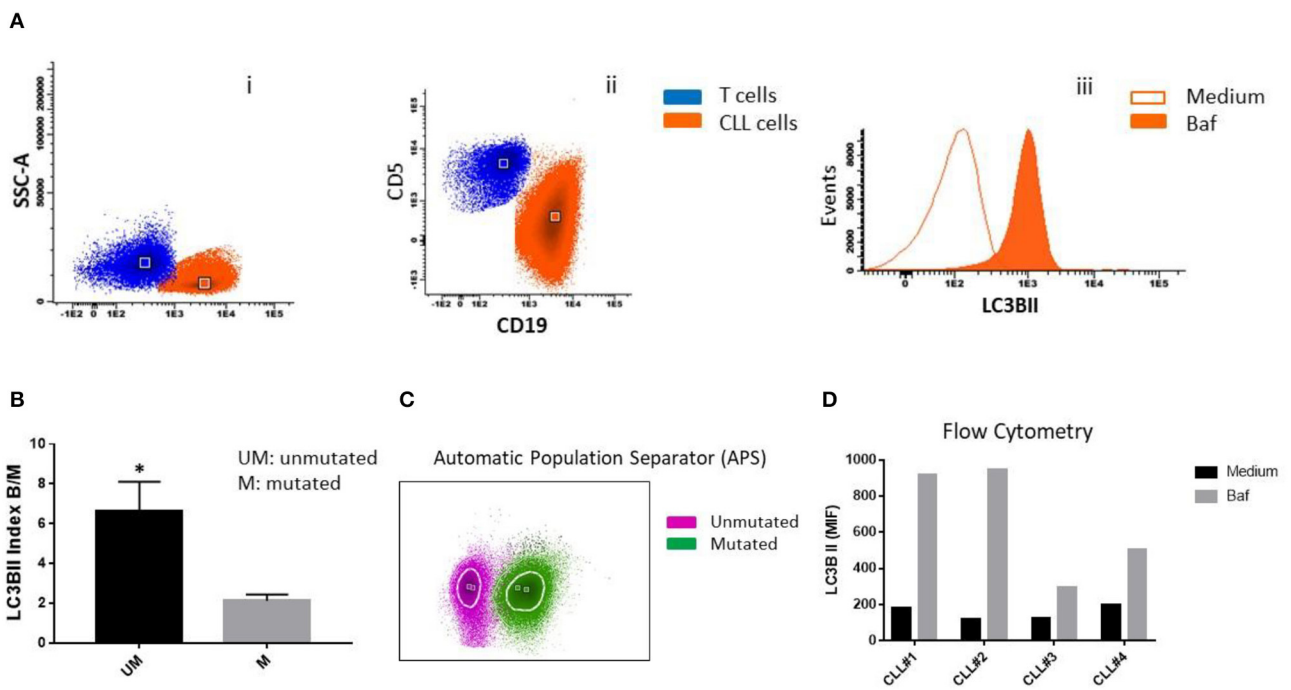

FIGURE 1 | LC3B expression in CLL cells. **(A)** PBMC cells from patients with CLL were treated with 25 nM bafilomycin (Baf) or medium (untreated control) for 24 h. Flow cytometric analysis of LC3B expression in CLL cells is shown. (i) Dot plot of CD19 vs. side scatter (SSC) of B lymphocytes from CLL patients. (ii) Dot plot of CD19 vs. CD5 shows clonal CLL cells and residual T cells. (iii) Histogram showing LC3B expression (MFI) in clonal B cells treated with Baf and untreated cells. **(B)** LC3B index B/M was calculated by dividing MFI of LC3B of CD19+CD5+ (clonal B cells) treated with bafilomycin by MFI of LC3B of untreated clonal B cells (*$p < 0.05$, unpaired *t*-test). **(C)** Automatic population separator (APS) shows two populations: LC3B+ CLL cells from two patients with unmutated IGHV (Purple) and LC3B+ CLL cells from two patients with mutated IGHV (Green). **(D)** PBMC cells from all four patients (CLL#1, Patient #1; CLL#2, Patient #2; CLL#3, Patient #3; CLL#4, Patient #4) were treated with bafilomycin 25 nM for 2 h. After that, MFI of LC3B was determined by flow cytometry, as described in **(A)**.

the presence of bafilomycin (**Figure 1A**). Samples from non-progressing (mutated IGHV) patients 3 and 4 were also included as controls.

We observed higher expression of LC3BII in cells from unmutated progressing CLL patients compared to the cells from mutated non-progressing CLL patients ($p < 0.05$) (**Figure 1B**). In addition, using Infinicyt^{TM} software, we merged files of all the patients into one file and then analyzed immunophenotype-based automatic separation of cell clusters (automatic population separation, APS) (Infinicyt^{TM} software, Cytognos S.L., Salamanca, Spain) based on LC3BII expression (**Figure 1C**). The APS algorithm clearly discriminated two groups of data corresponding to samples from progressing and non-progressing CLL patients, respectively (**Figure 1C**). This suggests that LC3BII expression may contribute to the discrimination between progressing and non-progressing patients. In addition, similar results were obtained when LC3BII expression was studied in cells from CLL patients by flow cytometry (**Figure 1D**) and the classical Western immunoblotting detection of LC3B (**Figure 2A**). In the presence of bafilomycin, increased LC3BII levels were observed in samples from unmutated progressing CLL patients (**Figure 1D**). Minimal

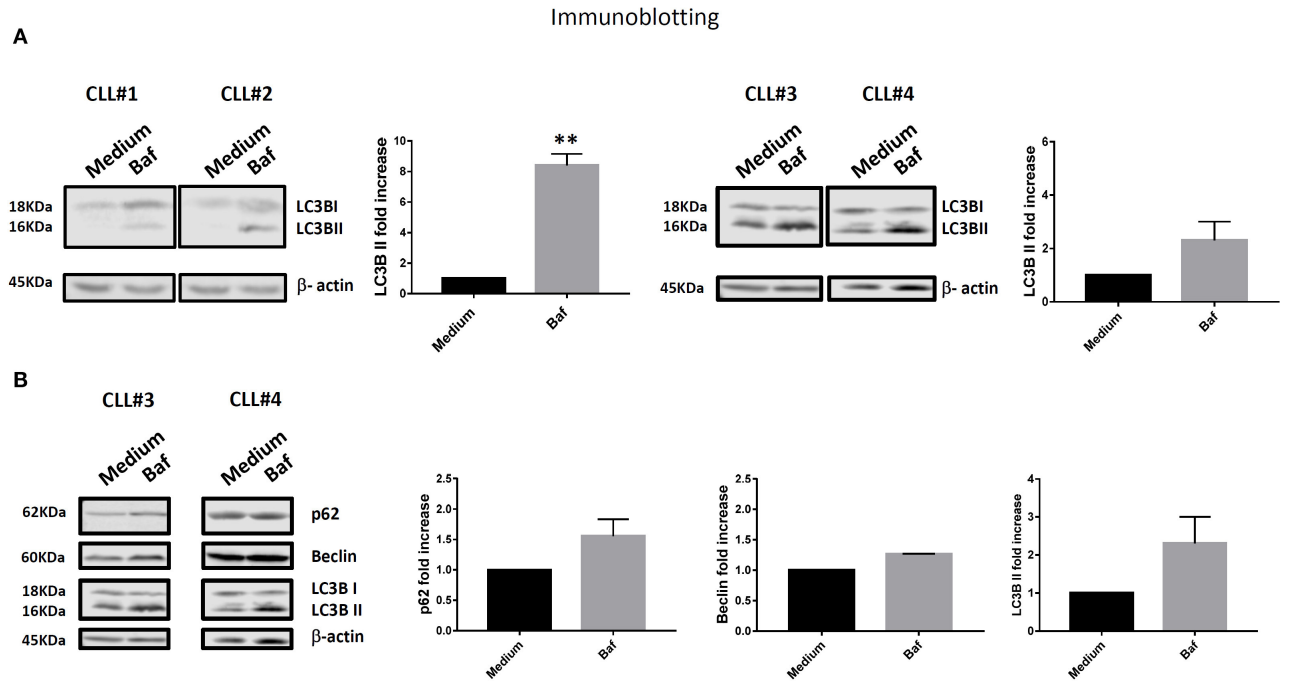

FIGURE 2 | (A) PBMC cells from all four patients (CLL#1, Patient #1; CLL#2, Patient #2; CLL#3, Patient #3; CLL#4, Patient #4) were treated as described in **Figure 1D**, then LC3B and β-actin protein levels were examined by immunoblotting. The figure shows image and relative band intensity quantification. The results were analyzed by *t*-test. Error bars represent SEM (**$p < 0.01$). **(B)** In addition to LC3B and β-actin, p62 and Beclin protein levels were examined by immunoblotting in new samples from survivor Patients #3 and #4 (CLL#3 and CLL#4, respectively). The results were analyzed by *t*-test. No significant differences were observed.

LC3BII detection was observed in samples from mutated patients (CLL#3 and CLL#4) (**Figure 1D**). In additional experiments, we analyzed p62/SQSTM1 and Beclin expression in new samples from survivor (mutated IGHV) patients 3 and 4 by Western immunoblotting. We did not find significant differences in the expression levels of autophagy proteins between untreated and bafilomycin-treated samples (**Figure 2B**).

It is important to notice that increased LC3BII expression was detected by Western immunoblotting (**Figure 2A**) when the patients progressed but before Ibrutinib treatment. Similar results were obtained by flow cytometry (**Figure 1**) after Ibrutinib treatment.

These results, taken together, suggest that autophagy activation may correlate with CLL progression beyond Ibrutinib treatment.

DISCUSSION

Here we found that two patients with progressing CLL showed increased expression of the autophagy protein LC3B in addition to positive CD38 and ZAP70 expression and unmutated status of IGHV.

Autophagy, and autophagy-related proteins (ATG), play a central role in integrating many stress signals to determine the fate of cells (16). There are multiple reports in the literature of autophagy providing resistance to anticancer treatments *in vitro* (17–20), but the resistance mechanisms have yet to be completely determined (16). Autophagy is considered a fundamental survival mechanism that allows cells to adapt to a hostile microenvironment through the recycling of cytosolic molecules in double-membrane vesicles named autophagosomes (21). This mechanism can be induced by several stressors blocking both extrinsic and intrinsic apoptotic pathways (21). It has been described that neoplastic cells can exploit autophagy to survive under hypoxia and low-nutrient conditions (22, 23). Recently, it has become evident that combinatory drug therapy can benefit from the cross-sensitization induced in tumoral cells by cross-modulation of the molecular pathways targeted by each drug. For instance, we recently observed that rapamycin, a mTOR inhibitor, enhanced Fludarabine-induced cytotoxicity in CLL B cells (4). It was reported that pre-treatment of CLL cells with Bruton's tyrosine kinase inhibitor Ibrutinib, whether *ex vivo* or *in vivo* in patients, enhances mitochondrial Bcl-2 dependence, increasing the killing of CLL cells by Venetoclax (24). Similarly, we observed that cells from patients with progressing CLL treated *in vivo* with Ibrutinib were more sensitive to *in vitro* treatment with Venetoclax than cells from patients with non-progressing CLL (data not shown).

Kipps et al. highlighted that the "clinical course of newly diagnosed CLL is very variable; some patients remain free of symptoms and are fully active for decades, whereas others rapidly become symptomatic or develop high-risk disease, which requires treatment soon after diagnosis and might result in death due to therapy-related and/or disease-related complications (2).

However, most patients have a clinical course that is in between these two extremes.

More robust prognostic markers are provided by newer techniques, such as flow cytometry, cytogenetics, and molecular biology" (2). Here, we applied flow cytometry technology to simultaneously detect autophagy protein LC3B together with classical phenotypical markers that identify tumoral CLL B cell clones. In addition, we exploited immunophenotype-based (including LC3B expression) automatic separation of cell clusters (APS) to discriminate different groups of data that correlated with the disease progression and IGHV mutational status of the patients. Our results suggest that activation of autophagy flux may correlate with CLL progression even before Ibrutinib treatment. Kong et al. (25) found increased levels of ATG5 and Beclin mRNA in a Chinese cohort of CLL patients compared to healthy controls. However, these results were inconsistent with the findings by Kristensen et al. (26) showing high expression of Beclin being associated with more aggressive disease. The detailed study of the dynamics of autophagosome formation and disappearance during the autophagic flux may solve discrepancies in the interpretation of the role of autophagy in pathogenesis, progression, and therapeutic outcome in CLL.

Autophagy is a very complex response that involves the expression, modification, association, and degradation of ATG proteins. Alterations in the expression of autophagy genes contribute to the tumorigenesis process in numerous types of cancer during tumor initiation, progression, and development and to the maintenance of the malignant state (27). The activation of autophagy flux plays key roles in controlling the tumor microenvironment and in the therapeutic response (28). However, in different subtypes of hematopoietic malignancies, the role of autophagy in cell transformation and the impact of autophagy on the response to different treatment strategies remains ambiguous. In this regard, it has been reported that decreased Bcl-2 levels and an increase in Beclin-1 expression correlate with a favorable clinical outcome in high-grade B-cell lymphomas (29, 30). In line with these observations, it was reported that high expression of LC3 or BECN1 is associated with a favorable outcome in multiple myeloma (31). Nevertheless, other studies have demonstrated an opposite role for the autophagy machinery in disease malignancy development, showing that autophagy activation is required to induce multiple myeloma cell survival (32, 33). Moreover, autophagy machinery activation has been shown to be involved in the progression of other hematopoietic cancers. Indeed, Giatromanolaki et al. reported that patients with follicular and diffuse large B-cell lymphomas express high LC3 levels compared to normal B-cells (34). Considering all of this information, the role of autophagy in lymphoid malignancies is still debated and might be subtype-specific. While several studies have addressed the impact of autophagy in treatment response in several subtypes of hematopoietic malignancies, further studies are needed to better understand the effect of changes in the autophagy flux during disease progression and therapy responses.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Nacional de Clínicas, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Argentina. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DA and CR designed and carried out all the experiments and wrote the manuscript. CB helped with experiments and project discussion. CM-R revised the manuscript. DS helped with sample preparation. VH helped in the organization of the clinical data. CS and IS carried out and helped discuss the results of molecular biology studies. PI conceived and designed the study, supervised all of the experiments, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Strati P, Jain N, O'Brien S. Chronic lymphocytic leukemia: diagnosis and treatment. *Mayo Clin Proc.* (2018) 93:651–64. doi: 10.1016/j.mayocp.2018.03.002
2. Kipps TJ, Stevenson FK, Wu CJ, Croce CM, Packham G, Wierda WG, et al. Chronic lymphocytic leukaemia. *Nat Rev Dis Primers.* (2017) 3:16096. doi: 10.1038/nrdp.2016.96
3. Bologna C, Buonincontri R, Serra S, Vaisitti T, Audrito V, Brusa D, et al. SLAMF1 regulation of chemotaxis and autophagy determines CLL patient response. *J Clin Invest.* (2016) 126:181–94. doi: 10.1172/JCI83013
4. Rodriguez CM, Bussi C, Arroyo DS, Sastre D, Heller V, Stanganelli C, et al. Effects of rapamycin in combination with fludarabine on primary chronic lymphocytic leukemia cells. *Leuk Lymphoma.* (2019) 60:1299–303. doi: 10.1080/10428194.2018.1529309

5. Strati P, Shanafelt TD. Monoclonal B-cell lymphocytosis and early-stage chronic lymphocytic leukemia: diagnosis, natural history, and risk stratification. *Blood.* (2015) 126:454–62. doi: 10.1182/blood-2015-02-585059
6. Burger JA, Chiorazzi N. B cell receptor signaling in chronic lymphocytic leukemia. *Trends Immunol.* (2013) 34:592–601. doi: 10.1016/j.it.2013.07.002
7. Malavasi F, Deaglio S, Damle R, Cutrona G, Ferrarini M, Chiorazzi N. CD38 and chronic lymphocytic leukemia: a decade later. *Blood.* (2011) 118:3470–8. doi: 10.1182/blood-2011-06-275610
8. Van Dyke DL, Werner L, Rassenti LZ, Neuberg D, Ghia E, Heerema NA, et al. The Dohner fluorescence *in situ* hybridization prognostic classification of chronic lymphocytic leukaemia (CLL): the CLL Research Consortium experience. *Br J Haematol.* (2016) 173:105–13. doi: 10.1111/bjh.13933
9. Arroyo DS, Gaviglio EA, Ramos JM, Bussi C, Rodriguez-Galan MC, Iribarren P, et al. Autophagy in inflammation, infection, neurodegeneration and cancer. *Int Immunopharmacol.* (2014) 18:55–65. doi: 10.1016/j.intimp.2013.11.001
10. White E. Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer.* (2012) 12:401–10. doi: 10.1038/nrc3262
11. Wierda WG, O'Brien S, Wang X, Faderl S, Ferrajoli A, Do KA, et al. Prognostic nomogram and index for overall survival in previously untreated patients with chronic lymphocytic leukemia. *Blood.* (2007) 109:4679–85. doi: 10.1182/blood-2005-12-051458
12. Pflug N, Bahlo J, Shanafelt TD, Eichhorst BF, Bergmann MA, Elter T, et al. Development of a comprehensive prognostic index for patients with chronic lymphocytic leukemia. *Blood.* (2014) 124:49–62. doi: 10.1182/blood-2014-02-556399
13. Parikh SA, Strati P, Tsang M, West CP, Shanafelt TD. Should IGHV status and FISH testing be performed in all CLL patients at diagnosis? A systematic review and meta-analysis. *Blood.* (2016) 127:1752–60. doi: 10.1182/blood-2015-10-620864
14. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *Lancet.* (2010) 376:1164–74. doi: 10.1016/S0140-6736(10)61381-5
15. Rodríguez CM, Stanganelli C, Bussi C, Arroyo D, Sastre D, Heller V, et al. Intracytoplasmic filamentous inclusions and IGHV rearrangements in a patient with chronic lymphocytic leukemia. *Leuk Lymphoma.* (2018) 59:1239–43. doi: 10.1080/10428194.2017.1370549
16. Macintosh RL, Ryan KM. Autophagy in tumour cell death. *Semin Cancer Biol.* (2013) 23:344–51. doi: 10.1016/j.semcancer.2013.05.006
17. Bellodi C, Lidonnici MR, Hamilton A, Helgason GV, Soliera AR, Ronchetti M, et al. Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. *J Clin Invest.* (2009) 119:1109–23. doi: 10.1172/JCI35660
18. Firat E, Weyerbrock A, Gaedicke S, Grosu AL, Niedermann G. Chloroquine or chloroquine-PI3K/Akt pathway inhibitor combinations strongly promote gamma-irradiation-induced cell death in primary stem-like glioma cells. *PLoS ONE.* (2012) 7:e47357. doi: 10.1371/journal.pone.0047357
19. Han J, Hou W, Goldstein LA, Lu C, Stolz DB, Yin XM, et al. Involvement of protective autophagy in TRAIL resistance of apoptosis-defective tumor cells. *J Biol Chem.* (2008) 283:19665–77. doi: 10.1074/jbc.M710169200
20. Lamoureux F, Thomas C, Crafter C, Kumano M, Zhang F, Davies BR, et al. Blocked autophagy using lysosomotropic agents sensitizes resistant prostate tumor cells to the novel Akt inhibitor AZD5363. *Clin Cancer Res.* (2013) 19:833–44. doi: 10.1158/1078-0432.CCR-12-3114
21. Kroemer G, Mariño G, Levine B. Autophagy and the integrated stress response. *Mol Cell.* (2010) 40:280–93. doi: 10.1016/j.molcel.2010.09.023
22. Dikic I, Johansen T, Kirkin V. Selective autophagy in cancer development and therapy. *Cancer Res.* (2010) 70:3431–4. doi: 10.1158/0008-5472.CAN-09-4027
23. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. *Nat Rev Cancer.* (2007) 7:961–7. doi: 10.1038/nrc2254
24. Deng J, Isik E, Fernandes SM, Brown JR, Letai A, Davids MS. Bruton's tyrosine kinase inhibition increases BCL-2 dependence and enhances sensitivity to venetoclax in chronic lymphocytic leukemia. *Leukemia.* (2017) 31:2075–84. doi: 10.1038/leu.2017.32
25. Kong YL, Huang Y, Wu JZ, Cao X, Liang JH, Xia Y, et al. Expression of autophagy related genes in chronic lymphocytic leukemia is associated with disease course. *Leuk Res.* (2018) 66:8–14. doi: 10.1016/j.leukres.2017.12.007
26. Kristensen L, Kristensen T, Abildgaard N, Thomassen M, Frederiksen M, Mourits-Andersen T, et al. High expression of PI3K core complex genes is associated with poor prognosis in chronic lymphocytic leukemia. *Leuk Res.* (2015) 39:555–60. doi: 10.1016/j.leukres.2015.02.008
27. Levy JM, Towers CG, Thorburn A. Targeting autophagy in cancer. *Nat Rev Cancer.* (2017) 17:528–42. doi: 10.1038/nrc.2017.53
28. White E. The role for autophagy in cancer. *J Clin Invest.* (2015) 125:42–6. doi: 10.1172/JCI73941
29. Nicotra G, Mercalli F, Peracchio C, Castino R, Follo C, Valente G, et al. Autophagy-active beclin-1 correlates with favourable clinical outcome in non-Hodgkin lymphomas. *Mod Pathol.* (2010) 23:937–50. doi: 10.1038/modpathol.2010.80
30. Huang JJ, Zhu YJ, Lin TY, Jiang WQ, Huang HQ, Li ZM. Beclin 1 expression predicts favorable clinical outcome in patients with diffuse large B-cell lymphoma treated with R-CHOP. *Hum Pathol.* (2011) 42:1459–66. doi: 10.1016/j.humpath.2010.12.014
31. Jung G, Roh J, Lee H, Gil M, Yoon DH, Suh C, et al. Autophagic markers BECLIN 1 and LC3 are associated with prognosis of multiple myeloma. *Acta Haematol.* (2015) 134:17–24. doi: 10.1159/000368848
32. De Veirman K, Menu E, Maes K, De Beule N, De Smedt E, Maes A, et al. Myeloid-derived suppressor cells induce multiple myeloma cell survival by activating the AMPK pathway. *Cancer Lett.* (2019) 442:233–41. doi: 10.1016/j.canlet.2018.11.002
33. Gao D, Lv AE, Li HP, Han DH, Zhang YP. LncRNA MALAT-1 elevates HMGB1 to promote autophagy resulting in inhibition of tumor cell apoptosis in multiple myeloma. *J Cell Biochem.* (2017) 118:3341–8. doi: 10.1002/jcb.25987
34. Giatromanolaki A, Koukourakis MI, Pouliliou S, Gatter KC, Pezzella F, Harris AL, et al. Overexpression of LC3A autophagy protein in follicular and diffuse large B-cell lymphomas. *Hematol Oncol Stem Cell Ther.* (2013) 6:20–5. doi: 10.1016/j.hemonc.2013.02.001

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