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Review

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The functional and pathologic relevance of autophagy proteases

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Introduction

Originally identified as an adaptive response triggered by fasting and other bioenergetic demands in yeast, autophagy (derived from Greek, meaning "self-eating") was later described as an evolutionarily conserved pathway that is also essential for cellular homeostasis in higher eukaryotes. This catabolic route, in which cytoplasmic components are degraded by lysosomal hydrolases, plays an important role in the response to both extracellular and intracellular stress signals such as damaged organelles, misfolded proteins, or pathogenic infections (1, 2). Dysfunctions in the autophagic pathway have recently been described in several pathologic conditions including inflammation, cancer, and aging-associated diseases (3–8). Given the increasing relevance of autophagy in these processes, modulation of its activity has emerged as a potential therapeutic target, either through activation or inhibition of key components of the pathway.

There are at least three major autophagic pathways, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), which primarily differ in the method by which the cargo is transferred to the lysosome (9). In microautophagy the lysosomal membrane directly engulfs cytoplasmic components (10), whereas in CMA, cytoplasmic proteins are selectively delivered to the lysosome in a process dependent on the recognition of a sequence motif in the target protein by specific lysosomal receptors (11). Macroautophagy (herein referred to as autophagy) involves the formation and elongation of a membrane sac called the phagophore that develops into a double-membrane vesicle, the autophagosome, in which the cytoplasmic cargo is sequestered. Autophagosomes eventually fuse with lysosomes, allowing for the degradation of the cargo and the autophagosome's inner membrane. Once degraded, the contents of the autophagosome is returned to the cytoplasm as basic building blocks (mainly amino acids, lipids, sugars, and nucleotides) that can be reused by the cell to synthesize new biomolecules (12). Autophagy execution requires the activation of complex molecular machinery that includes regulatory signaling cascades, the formation and completion of autophagosomes, the transport of autophagosomes, and final fusion of autophagosomes with lysosomes (13). Although the intricate molecular machinery of this multistep pathway is not yet fully understood, some of its components have been identified as essential effectors required for a correct autophagic response. This is the case of proteins such as Atg5, Atg7, or the ubiquitin-like protein Atg8 and its conjugation system, which is indispensable for the correct expansion and closure of the preautophagosomal double membrane.

Proteases have recently been described as major signaling pathway initiators due to their ability to perform highly regulated proteolytic processing reactions for a variety of protein substrates (14, 15). In this regard, some proteolytic enzymes are known to indirectly modulate the autophagic response. Cleavage of the autophagy regulator beclin 1 (the mammalian ortholog of yeast Atg6) by different caspases abrogates its proautophagic activity, while the fragment resulting from this cleavage acquires proapoptotic activity (16, 17). Likewise, calpain-mediated processing of ATG5 switches autophagy to apoptosis in different cell types (18). Several caspases and calpains also have the ability to cleave in vitro a wide range of autophagic proteins that could be implicated in the molecular crosstalk between apoptosis and autophagy (19). Among these cell death proteases, caspase-3 targets the Crohn's disease T300A variant of ATG16L1 and causes defective autophagy, which in turn contributes to disease progression by sustaining cellular stress and facilitating pathogen expansion (20). Reversible ubiquitylation, a process involving different proteases such as proteasome components and

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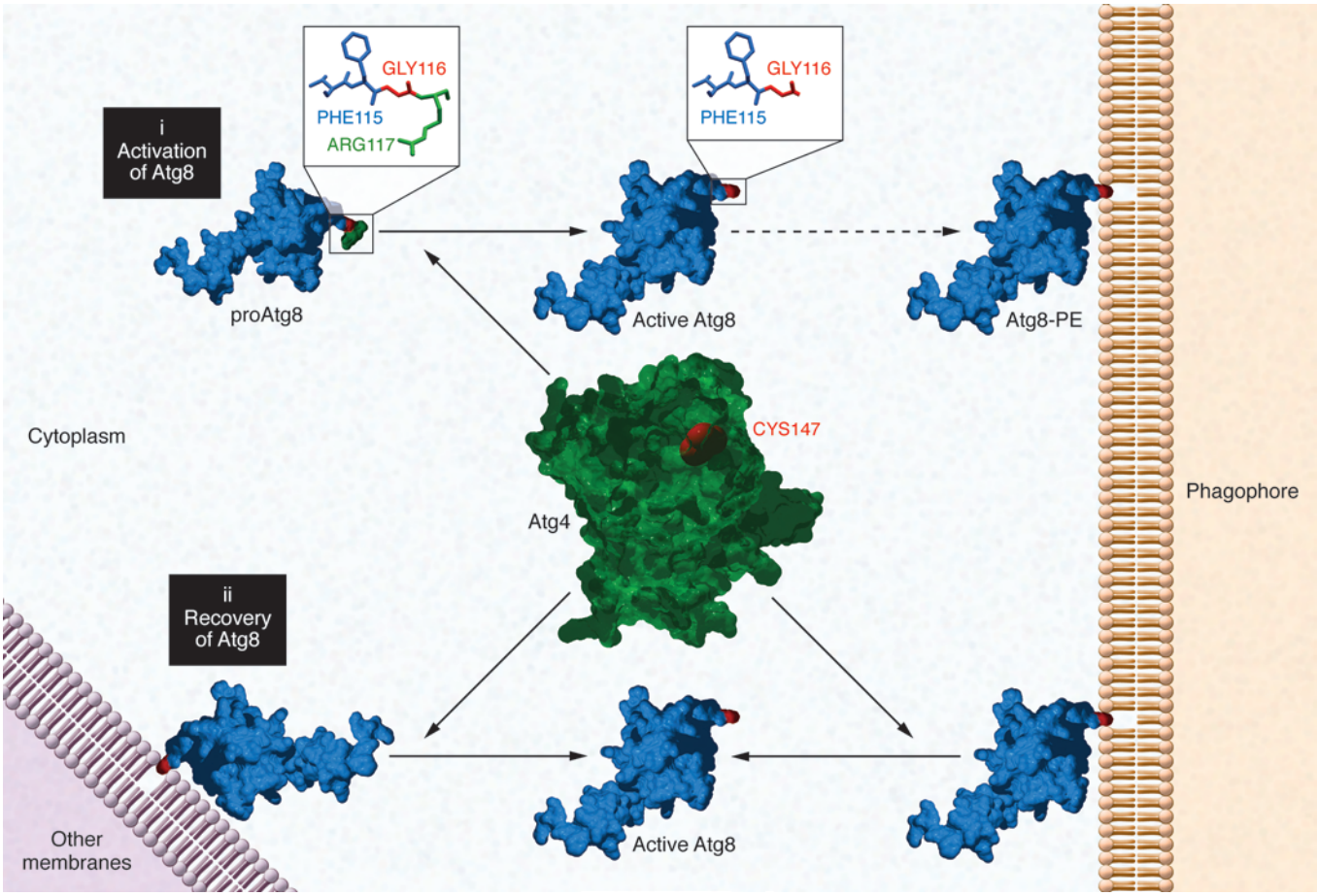

Figure 1. Atg4 functions. (i) Yeast Atg4 is a cysteine protease required for the activation of immature Atg8 protein, cleaving its C-terminal region and exposing a glycine residue (GLY116) that is essential for its attachment to the phagophore. (ii) Atg4 is also responsible for the recovery of Atg8 via delipidation of this protein from mature autophagosomes or organelle membranes, where it can be erroneously attached.

deubiquitinating enzymes (DUBs) (21), also contributes to selective autophagy of proteins and organelles (22–25). In fact, DUBs have been shown to control selective autophagy levels in basal conditions (26–28), and their activity could be crucial in different pathologies such as Parkinson's disease and pathogen infections associated with autophagy dysfunction (29, 30). Additionally, a large number of lysosomal proteases are implicated in the final degradative stages of the autophagic process (31). However, there is only one protease among the numerous Atg proteins originally identified in yeast and directly involved in autophagy: the cysteine protease Atg4, whose function is essential for the Atg8 conjugation system (32).

In this Review we focus on this Atg4 protease and its four mammalian orthologs, termed "autophagins," which have been identified in multiple species for which genome sequence data are available (33). We also address the functional relevance of this protein family in physiologic and pathologic processes and discuss the emerging importance of ATG4 proteins as potential targets of new therapeutic strategies in human diseases.

The yeast cysteine protease Atg4

The Atg8 ubiquitin-like conjugation system holds a pivotal role in the proper expansion of the phagophore (Figure 1). In yeast, Atg8 is synthesized as an inactive soluble protein and needs to be posttranslationally processed in order to be covalently attached to a molecule of phosphatidylethanolamine (PE) from the preautophagosomal membrane (13). This activation process starts with the cleavage of its C-terminal region by the cysteine protease Atg4 (34), exposing a glycine residue that is essential for subsequent interactions, including the final conjugation with the amino group of PE (Figure 1). Once Atg8 is activated, it undergoes several reactions similar to those involved in protein ubiquitylation, requiring the participation of additional autophagic proteins such as Atg7 (E1-ubiquitin ligase–like enzyme), Atg3 (E2-ubiquitin ligase–like enzyme), and the Atg12-Atg5-Atg16 complex (E3-like enzyme), which finally promotes the formation of the Atg8-PE amide bond. Atg8-PE recruits the Atg12-Atg5-Atg16 complex to the membrane, assembling a scaffold that is critical for phagophore biogenesis (35). This conjugation reaction is essential to retain Atg8 on the expanding structure, making membrane tethering and hemifusion possible (36).

The lipidation of Atg8 is a reversible process because the protein can be deconjugated and released back to the cytosol (34). Interestingly, the recovery of Atg8 is also Atg4 dependent, as this protease is able to cleave the amide bond with PE, disassembling the scaffold (ref. 35 and Figure 1). This additional role of Atg4 has been described as an important process for the dynamism of autophagy, as it provides a new source of active Atg8 to maintain a

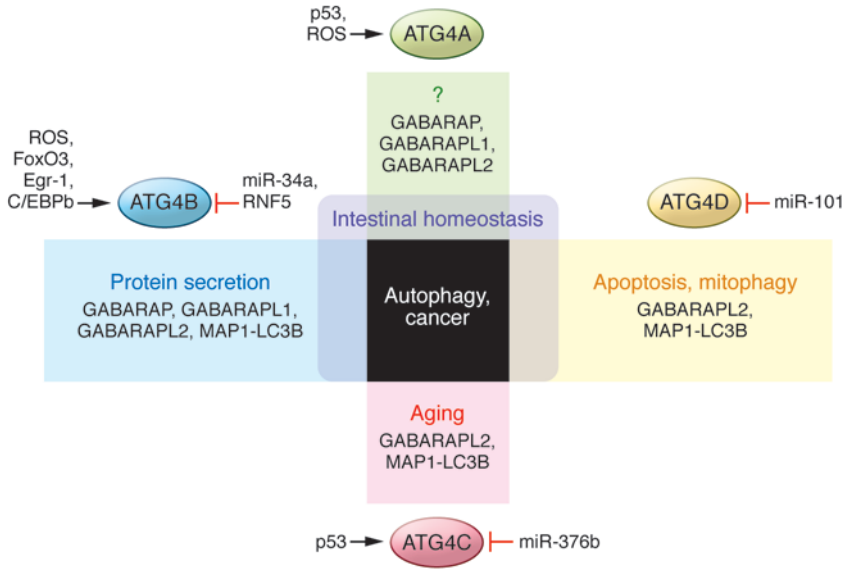

Figure 2. Roles and regulation of the ATG4 protease family. The complexity of the mammalian ATG8 protein system is reflected in the substrate specificity of ATG4 proteases and their particular roles. Besides the autophagic response, all ATG4 proteases have been involved in cancer, and defects in ATG4A, ATG4B, and ATG4D are also linked to intestinal disorders. Variants of ATG4C have been identified in aging free of major diseases, and ATG4D might be essential in the crosstalk between autophagy and apoptosis and is required for correct mitophagy during erythropoiesis. ATG4B has been widely described as necessary for the proper function of secretory cells. These proteases must be finely controlled by different regulators to coordinate this wide range of functions. Thus transcription factors such as p53, FOXO3, EGR11, or C/EBPb and several microRNAs such as miR-101, miR-376b, and miR-34a are responsible for the modulation of *Atg4* genes. Additionally, ATG4 proteases can also be posttranslationally regulated by ROS-dependent oxidation or by E3 ubiquitin ligases such as RNF5.

longer autophagic response (37). Moreover, it allows the recycling of Atg8 molecules that have been attached to inappropriate membranes (38). Finally, the release of Atg8 from mature autophagosomes facilitates the fusion of these structures with lysosomes, allowing the completion of the autophagic process (39).

The mammalian Atg4 family

Autophagy is evolutionarily well conserved, and the Atg8 ubiquitin–like conjugation system has been described in higher eukaryotes (40–42). Accordingly, four mammalian orthologs of yeast protease Atg4 were identified and cloned in our laboratory, which led us to define the autophagin protease family (ref. 42 and Figure 2). These four proteases were called autophagin-1/ATG4B, autophagin-2/ATG4A, autophagin-3/ATG4C, and autophagin-4/ATG4D and contained all the residues required for the catalytic activity of cysteine proteases, including the conserved cysteine residue within the catalytic site. Intriguingly, the mammalian ATG8 protein family of putative ATG4 substrates is more complex; it is divided into two main subfamilies that differ in both structural features and functional role in the formation of the autophagosome (43). Thus, the microtubule-associated protein 1–light chain 3 (MAP1-LC3) subfamily (including MAP1-LC3A, MAP1-LC3B, and MAP1-LC3C) is involved in the elongation of the initial phagophore, while the GATE-16/GABARAP subfamily (including GABARAP, GABARAPL1/ATG8L, and GABARAPL2/GATE-16) is required during the final maturation of the double-membrane vesicle. Notably, the latter subfamily is preferred by the Unc-51–like autophagy-activating kinase (ULK) complex as the scaffold necessary for its recruitment in the phagophore, an essential step for the efficient formation and maturation of the autophagosome (44).

Additionally, these Atg8 orthologs are differentially cleaved by the different Atg4 family members, with ATG4B representing the most potent and promiscuous of all of them in terms of substrate specificity (ref. 45 and Figure 2). In fact, ATG4B is able to process a wide range of Atg8 orthologs (46), and the analysis of their structures has been very useful to understand the interaction between both enzyme and substrate (47–50). ATG4A has also been described as an effective protease with the ability to target GABARAPL2 (51), although it is unable to cleave MAP1-LC3B, which is one of the most important orthologs of yeast Atg8. Finally, ATG4C and ATG4D efficiently bind these substrates but show reduced catalytic activity against them, as they can only process MAP1-LC3B and GABARAPL2. In fact, human ATG4D must be cleaved by caspase-3 to increase its activity against GABARAPL1 (52). ATG4C also presents the canonical cleavage site DEVD, which allows its caspase-mediated activation in vitro (19). However, ATG4A and ATG4B lack this motif and do not seem to require any caspase-mediated cleavage for their activation, which could explain their high proteolytic activity compared with that of other ATG4 family members. Notably, the cleavage of ATG4D exposes a BH3-like domain that contributes to the recruitment of the protein to the mitochondria, where it induces apoptosis. Thus, ATG4D could be a crucial component in the crosstalk between autophagy and apoptosis, while it has also been proposed to be involved in the mitochondrial clearance by mitophagy during erythropoiesis (53, 54).

The complex ATG8 conjugation system observed in mammals, formed by several orthologs of both Atg4 and Atg8 proteins, points to the existence of a complex network of protease-substrate interactions with specific spatiotemporal modulation at different

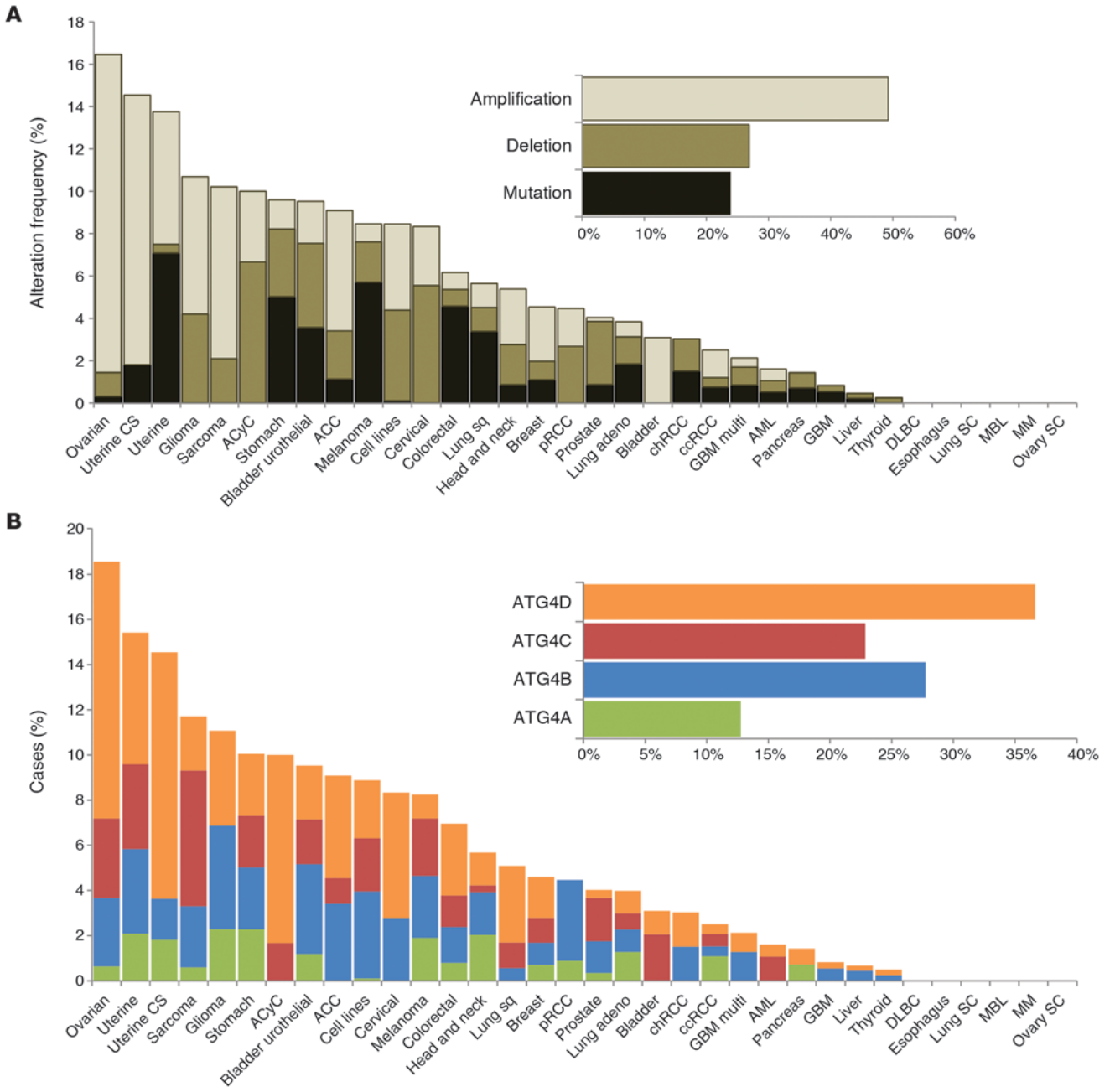

Figure 3. ATG4 proteases in human cancer. (**A**) Structural alterations of *ATG4* genes in several malignancies. (**B**) Percentage of genetic alterations in the different *ATG4* genes found in human tumors. ACC, adrenocortical carcinoma; ACyC, adenoid cystic carcinoma; adeno, adenocarcinoma; AML, acute myeloid leukemia; ccRCC, kidney renal clear cell carcinoma; chRCC, kidney chromophobe; CS, carcinosarcoma; DLBC, lymphoid neoplasm diffuse large B cell lymphoma; GBM, glioblastoma; GBM multi, GBM multiforme; MBL, medulloblastoma; MM, multiple myeloma; pRCC, kidney renal papillary cell carcinoma; SC; small cell carcinoma; Sq, squamous cell carcinoma. Data were obtained from cBioPortal database (133, 134), and those human tumors with structural alterations in *ATG4* genes were compiled and used to construct both graphs.

levels, although the specific regulatory mechanisms are not yet fully understood (55). Interestingly, the binding between an ATG4 autophagic protease and its corresponding substrate seems to be the main regulator of its activity. It has been shown that ATG4B is autoinhibited in its free form by two domains that hide the catalytic cysteine: a regulatory loop that prevents the entry of the substrate, and the N-terminal tail that blocks its exit (49). However, the interaction of the ATG4 enzyme with MAP1-LC3 induces conformational changes that displace the loop and open the tail, uncovering the active site and permitting the cleavage of the substrate (48). Moreover, this open conformation of ATG4B can be stabilized by the interaction of the N-terminal tail with adjacent non-substrate MAP1-LC3, facilitating the membrane targeting of this protease required for MAP1-LC3 delipidation. This additional binding is mediated by the LC3-interacting region present in the N-terminal tail of ATG4B (56).

Transcriptionally, ATG4 proteases are part of intricate signal transduction pathways, complicating the identification of transcription factors with the ability to induce their expression (Figure 2). In this regard, it has been shown that p53 can bind and regulate *Atg4a* and *Atg4c* (among other several *Atg* genes such as *Atg7*, *Atg10*, *Ulk1*, and *Ulk2*) in response to DNA damage, inducing an autophagic response that could contribute to tumor suppression by enhancing p53-dependent apoptosis (57). Moreover, FoxO3 has been described to upregulate *Atg4b* and other autophagic genes in mouse skeletal muscle (58, 59). FOXO3a can also regulate *ATG4* genes in ovarian cancer cells, although it must be dephosphorylated and retained in the nucleus after inhibition of PI3K and Ras/MAPK signaling pathways by the tumor suppressor AHRI (DIRAS3) (60, 61). Expression of human *ATG4B* also depends on tissue-specific transcription factors, including EGR1 in lung tissue (62) and C/EBPb in differentiating murine 3T3-L1 adipocytes (63). Notably, the activity of the different ATG4 family members can be fine-tuned by microRNAs after transcription. The tumor suppressor miR-101 inhibits autophagy by targeting *ATG4D* (64), miR-376b modulates human autophagy by regulating intracellular levels of *ATG4C* (65), and miR-34a targets *ATG4B* (66).

It has been proposed that ROS may also regulate ATG4 proteins (Figure 2). In fact, hydrogen peroxide can directly inhibit the delipidating activity of ATG4A and ATG4B by oxidation of a non-catalytic residue near the active site (67). Accumulation of H_2O_2 after amino acid starvation has been described to promote ATG8 lipidation while preventing its release from the autophagosome. ROS can also be generated in autophagosomes and lysosomes, reinforcing the hypothesis of a ROS-mediated protection of ATG8-PE in the autophagosome (68), while recycling of additional ATG8 molecules from other membranes is still possible in the reducing environment of the cytosol. Thus, the oxidative regulation of ATG4 proteases might limit their activities to specific areas, allowing for the proper initiation and completion of autophagy. In vascular smooth muscle, for example, 7-ketocholesterol induces the expression of NADPH oxidase 4 and increases H_2O_2 levels that inhibit ATG4B delipidating activity, maintaining the autophagic response triggered by ER stress or atherosclerosis (69). The modification of catalytic or non-catalytic residues (including those responsible for the interaction between protease and substrate that, once oxidized, can alter the structural conformation of the enzyme) controls the ability of these proteins to cleave substrates at specific sites under different conditions.

Additional effectors might also contribute to the spatiotemporal regulation of ATG4 proteins (Figure 2). The E3 ligase RNF5 spatially controls ATG4B stability by inducing its ubiquitination and proteasome-mediated degradation at membrane domains like the phagophore. This mechanism thus represents a new regulatory layer of basal autophagy, blocking LC3 priming at the beginning of the process (70). In yeast, an Atg18-Atg21 complex is a key regulator during the phagophore formation (71), recruiting and protecting Atg8-PE. This new scaffold could also be present in higher organisms, acting as a barrier to ATG4 that prevents the access of the enzyme to the substrate until the autophagosome is mature, when the complex disassociates and allows the recycling of ATG8.

Given the wide diversity of orthologs and activities described in the mammalian Atg8 system, it has been hypothesized that Atg4 autophagic proteases may be involved in different physiologic processes. Historically, knockout mice have been research tools with important limitations for global in vivo studies on autophagy, as proteins like ATG5 or ATG7 are essential for the viability of these animals (72, 73). However, the apparent functional redundancy of the ATG4 family has made possible the generation of *Atg4*-deficient mice, which exhibit impaired autophagic flux but are perfectly viable. Accordingly, these animals represent excellent models to assess the in vivo roles of autophagy. By using *Atg4c*-deficient mice, we have found that under prolonged periods of starvation, ATG4C is necessary for maximal autophagy activation in tissues dependent on continuous energy consumption, such as the diaphragm (74). We have also found that mutant mice lacking ATG4B show altered secretion and assembly of otoconial components in the inner ear vestibular regions, ultimately leading to balance disorders (75, 76). Moreover, *Atg4b*-deficient mice show impaired release of lysozyme granules in Paneth cells during dextran sodium sulfate–induced (DSS-induced) experimental colitis, resulting in exacerbated inflammation that leads to the death of these animals (77). This surprising role of ATG4B in protein secretion has also been described in bone resorption by osteoclasts (78), pointing to a novel function of autophagy in secretory cells (79) and further supporting the idea that ATG proteins are involved in non-canonical autophagic processes (80–82). Similarly to the described intestinal phenotype, *Atg4b*-deficient mice subjected to an endotoxemia model show increased mortality and lung inflammation caused by sequestration of the antiinflammatory transcription factor ATF3 (83). Paradoxically, autophagy impairment in these mutant mice ameliorates the inflammatory response to mechanical ventilation and decreases lung injury by blockade of the NF-κB pathway (84), indicating that autophagic functions of ATG4 proteases in pathologic conditions may be dual and highly dependent on the cell and tissue context.

Dysregulation of Atg4 proteases in disease

Dysfunctional autophagic responses have been reported in several pathologies, including cancer and pathogen infection (1–5). In some cases, this aberrant autophagy is associated with a dysregulation of members of the ATG4 family during the development or progression of these diseases. As for the relevance of ATG4 proteases in cancer, different structural alterations in the genes encoding these enzymes have been found in several malignancies, with copy number amplification representing the most common of these modifications (Figure 3A). Furthermore, a large proportion of these alterations have been detected in female reproductive tissue tumors, including ovarian serous cystadenocarcinoma and uterine cancers. Intriguingly, hypomethylation of *ATG4A* in ovarian tumor–initiating cells increases their stem properties and is linked to poor prognosis in ovarian cancer patients (85). *ATG4A* expression has also been reported to be essential for breast cancer stem cells, regulating their tumorigenicity in vivo (86). Nevertheless, *ATG4D* seems to be the most frequently altered *ATG4* gene in human cancer, followed by *ATG4B* and *ATG4C* (Figure 3B). *ATG4B* overexpression has been described in linearly patterned programmed cell necrosis, a special type of cell death observed during early tumor growth of aggressive melanomas (87). Even though there is substantial genetic evidence, little is known about the specific roles of these autophagic proteases in cancer progression. Loss of *Atg4c* in a chemically induced murine model of fibrosarcoma suggests a tumor suppressor role for

this protease (74). Nevertheless, further in vivo studies with other *Atg4*-deficient models are needed, especially in light of the dual role of autophagy in cancer, either promoting cancer cell survival or suppressing tumorigenesis by maintaining cellular homeostasis in a context-dependent manner (88).

ATG4 mutations are also linked to some inflammatory bowel diseases (IBDs), as genetic variants in *ATG4A* and *ATG4D* have been proposed to contribute to granuloma formation in Crohn's disease (89). Additionally, patients with IBD show low expression of *ATG4B* in colon (77). These observations of altered ATG4 function in IBD are consistent with the fact that disrupted autophagy results in impaired processing of bacterial components, triggering the exacerbated inflammatory responses that characterize these disorders (90–94).

Autophagic proteins, including several ATG4 proteases, have also been implicated in the pathogenesis of infectious diseases (3). This is the case for HIV infection, which induces the expression of *ATG4D* and other *ATG* genes during viral morphogenesis and propagation (95). Hepatitis C virus is another example of a pathogen that evades and exploits autophagy, using autophagic components such as ATG4B to initiate its replication (96). Infections by parasites including *Trypanosoma cruzi*, which causes Chagas disease, or *Leishmania major*, responsible for leishmaniasis, are different from those caused by other pathogens, as these eukaryotic organisms also utilize autophagy (97). In fact, the activity of their Atg4 orthologs is essential for their survival, differentiation, and virulence, and new therapeutic strategies targeting these proteases could block or slow the infection (98–101).

Consistent with the growing relevance of autophagy in the modulation of aging (6, 8, 102, 103), it is remarkable that *ATG4C* variants have been linked to both aging in the absence of major diseases and increased longevity in a GWAS (104).

Therapeutic options targeting Atg4 proteases

The increasing evidence that dysregulation of ATG4 proteins occurs in a number of different diseases has opened new possibilities for the development of therapies targeting these proteases. Moreover, the essential role of ATG4 proteases in autophagy suggests that their chemical regulation may help to control the autophagic response in some contexts. In fact, ATG4 levels are correlated with autophagic flux, and synthetic substrates have been developed to measure ATG4 activity and monitor autophagy both in cultured cells and in vitro (105, 106). Similar approaches have recently been used to screen for specific inhibitors of these enzymes, which could be useful for the treatment of pathologic conditions associated with excess autophagic protease activity (107, 108). Another strategy to inhibit autophagy, either for research or therapeutic purposes, is the utilization of inactive mutant forms of ATG4 proteases with the ability to sequester their corresponding substrates (109–111).

In recent years, attenuation of ATG4 protease activity in different contexts has produced promising results. Overexpression of an inactive mutant of *ATG4B* in hepatocellular carcinoma cells reduced their viability (112), and autophagy inhibition by miR-101, which targets *ATG4D*, enhanced cisplatin-induced apoptosis in these tumor cells (113). Disruption of the autophagic response has also been proposed as a therapeutic option in cisplatin-resistant patients with squamous cell carcinoma, in which *ATG4A* is upregulated (114), and chronic myeloid leukemia, in which *ATG4B* expression is involved in resistance of $CD34^+$ cells to imatinib mesylate (66). Apart from the interest in ATG4 inhibition in cancer, a treatment based on the blockade of these proteases has also been suggested for type 1 diabetes mellitus, as some autophagic proteins (including ATG4A) could be related to neural injury of young patients with early neuronal deficits and diabetic ketoacidosis (115).

Strategies based on the induction of ATG4 autophagic proteases have also provided relevant results in several cellular models. In fact, treatment of human breast carcinoma MCF-7 cells with BBP (N-benzoyl-O-[N′-(1-benzyloxycarbonyl-4-piperidiylcarbonyl)-D-phenylalanyl]- D-phenylalaninol), a novel asperphenamate derivative, requires the upregulation of ATG4B activity (116). This modulation is JNK dependent and involves the regulation of ATG4 proteins by ROS. Overexpression of these proteases is also a potential therapeutic strategy in lung infections such as chronic obstructive pulmonary disease (62), ischemia and reperfusion injury in liver and heart (117, 118), and neurodegenerative diseases such as Huntington's disease (119), in which in vitro stimulation of the autophagic response ameliorates the effects of this pathologic condition. However, the role of ATG4 proteases in most diseases is context dependent, and the consequences of their modulation could differ greatly. For example, it has been suggested that changes in the expression of *ATG4B* in prostate cancer could either amplify the action of both chemotherapy and radiotherapy or contribute to the development of treatment resistance (120). Furthermore, inhibition of ATG4 proteases in radiation therapies sensitizes resistant carcinoma cells in most cases, although it also promotes resistance in some conditions (121), demonstrating the importance of a more comprehensive understanding of ATG4 function.

Nevertheless, the development of new therapies based on the modulation of ATG4 proteases is still at a very preliminary stage. To date, no modulators of these enzymes have been successfully tested in clinical trials, and specific inhibitors for them are still being characterized (122). This could be partially due to the fact that autophagins are cysteine proteases, which have been historically difficult to target when compared with other proteases, due to the metabolic instability and lack of specificity of small-molecule drugs (123). Moreover, our current knowledge of the activity and regulation of ATG4 proteases is still limited, and specific modulators should be able to discriminate between distinct ATG4 enzymes in order to avoid unexpected effects. Ideally, priming and delipidation steps should also be specifically targeted, although we still do not fully understand these processes. Consequently, new genetic and proteomic approaches will be required for the development of ATG4-based treatments.

Conclusions and perspectives

Autophagy is a well-conserved pathway that has gained functional complexity throughout evolution. The identification of four mammalian orthologs of yeast protease Atg4 has helped to dissect the complex ATG8 conjugation system present in mammals, which consists of a large number of substrate orthologs of these proteolytic enzymes. This complexity contrasts with other key autophagic components such as Atg3, Atg5, or Atg7, for which unique genes have been described in mammals. The reasons behind this

genetic redundancy in mammalian orthologs of yeast Atg4 and Atg8 remain elusive, but it may reflect the existence of specific functions for these proteins that are not limited to the canonical autophagic response observed in yeast. In fact, members of the GATE-16/GABARAP subfamily were first described in membrane trafficking processes (124–126), and MAP1-LC3B was initially characterized because of its interaction with microtubule-associated proteins MAP1A and MAP1B (127, 128). Accordingly, mammalian ATG4 autophagic proteases are also involved in several physiologic processes distinct from macromolecular recycling, including protein secretion and apoptosis.

Dysregulation of the activity of these cysteine proteases has been associated with several diseases such as cancer, inflammatory disorders, and vertigo, supporting their importance as emerging therapeutic targets. However, many questions remain concerning the specific functions of ATG4 proteases, including their potential dual roles in cancer, which can complicate future strategies aimed at targeting these enzymes in human malignancies. Moreover, expression of the different ATG4 family members depends on intricate signaling pathways, and their specific spatiotemporal regulation remains largely unknown. Further understanding of this protein family is still necessary to develop efficient treatments while avoiding undesired side effects. In this regard, the generation and characterization of additional gain- or loss-of-function animal models would be a valuable tool for the study of these proteases. Mutant mice lacking some of these enzymes such as ATG4B and ATG4C are viable due to the functional redundancy among members of this protease family, thereby resulting in very useful models to analyze the role of autophagy in vivo (129, 130). *Atg4b*-deficient mice, for example, show minor basal autophagy defects but impaired induced autophagic response after diverse stress signals. This alteration is comparable to that described in several pathologies in which the pathway is not completely disrupted but attenuated (131), which reinforces the value of these mutant mice as models of human diseases with autophagy deficiency. Furthermore, the generation of specific ATG4 inhibitors is currently ongoing and may contribute to the development of valuable tools to clarify the roles of these proteolytic enzymes in health and disease (132). Thus, ATG4 proteases are emerging as potential pharmaceutical targets for the treatment of dysfunctional autophagy or specific alterations involving these enzymes, but additional efforts will be needed to elucidate their role in physiologic and pathologic processes and to develop new therapies for human diseases associated with dysregulation of ATG4 proteolytic enzymes.

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