Review

Autophagy: molecular mechanisms, physiological functions and relevance in human pathology

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Abstract. Autophagy is a degradative mechanism mainly involved in the recycling and turnover of cytoplasmic constituents from eukaryotic cells. Over the last years, yeast genetic screens have considerably increased our knowledge about the molecular mechanisms of autophagy, and a number of genes involved in fundamental steps of the autophagic pathway have been identified. Most of these autophagy genes are present in higher eukaryotes indicating that this process has been evolutionarily conserved. In yeast, autophagy is mainly involved in adaptation to starvation, but in multicellular organisms this route has emerged as a multifunctional pathway involved in a variety of additional processes such as programmed cell death, removal of damaged organelles and development of different tissue-specific functions. Furthermore, autophagy is associated with a growing number of pathological conditions, including cancer, myopathies and neurodegenerative disorders. The physiological and pathological roles of autophagy, as well as the molecular mechanisms underlying this multifunctional pathway, are discussed in this review.

Key words. Autophagy; lysosome; proteasome; protease; cancer; myopathy; Alzheimer; Parkinson; apoptosis; cell death.

Introduction

Cell homeostasis is maintained by a precisely regulated balance between synthesis and degradation of cellular components. Cells are able to detect environmental changes and transduce them into a variety of anabolic or catabolic responses, facilitating cell adaptation to different adverse conditions such as starvation. Adaptation to nutrient deprivation is favoured by the recycling of cellular components necessary for cell maintenance. The constitutive degradation of proteins by the proteasome maintains a continuous protein turnover [1]. However, this process is limited to cytoplasmic short-lived proteins, and the majority of cellular components are long lived. Therefore, there must be another route for large-scale macro-molecular recycling which helps cell survival under adverse conditions. This alternative degradative route is the lysosomal pathway.

The lysosome/vacuole is a separate organelle that possesses the proper environment for the catalytic activity of a large variety of hydrolytic enzymes [2]. This property makes it capable of degrading almost any kind of cellular constituent, including entire organelles. The mechanisms responsible for delivering cytoplasmic cargo to the lysosome/vacuole are known collectively as autophagy and play an important role in the maintenance of homeostasis [3]. This process has been observed in all eukaryotic cells analyzed and shows an essentially identical morphology in plants, yeast and animal cells, indicating the widespread occurrence of this evolutionarily conserved pathway. Although autophagy has been studied at the cellular level for more than 4 decades, its molecular mechanisms have only...
started to be elucidated in the last 10 years, mainly due to the application of yeast genetics. Autophagy pathways can be classified into at least three different types: macroautophagy, microautophagy and chaperone-mediated autophagy [3–7]. In microautophagy, portions of the cytoplasm are sequestered by invagination of the lysosomal membrane and subsequently degraded. Chaperone-mediated autophagy involves the selective delivery of cytoplasmic proteins to the lysosome/vacuole in a process which depends upon the recognition by lysosomal receptors of a sequence motif present in cytosolic proteins. Macroautophagy is the major lysosomal route for the turnover of cytoplasmic components and will hereafter be referred as autophagy. This process begins with a sequestration event consisting of an engulfment of cytoplasmic constituents by a membrane sac, called the isolation membrane. This structure results in a double-membrane vesicle called the autophagosome, containing bulk portions of cytoplasm, which eventually fuses with the lysosome/vacuole (fig. 1). The inner membrane of the autophagosome and its protein and organelle contents are degraded by lysosomal/vacuolar hydrolases and recycled [8, 9].

In the last few years, autophagy emerged not just as a simply degradative process but also as a cellular mechanism necessary for several tissue-specific functions [10–12], and for essential processes such as programmed cell death during development [13]. In addition, autophagic dysfunction is associated with several pathologies, including neurodegenerative disorders and cancer [14–16]. In this review, we present an analysis of autophagy at the molecular level, with a special emphasis aimed at describing the diversity of components involved in the process and their functional roles during autophagy. We will also describe the physiological functions of this degradative pathway and the growing relevance of autophagic dysfunctions in the development of a variety of pathological conditions.

**Autophagy at the molecular level**

Under certain stress situations, eukaryotic cells are able to generate autophagic responses. This process requires the activity of multiple cellular factors which are involved

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**Figure 1.** The process of autophagy. When autophagy is induced, double membrane vesicles called autophagosomes are formed, enclosing bulk cytoplasm which may contain nonfunctional proteins and damaged organelles. Once formed, autophagosomes fuse with the lysosome/vacuole, and their cytoplasmic content is degraded by acid lysosomal/vacuolar hydrolases together with the autophagosome inner membrane.
in distinct steps of this degradative route. We will focus on the discussion of the molecular mechanisms underlying autophagy in the yeast *Saccharomyces cerevisiae* because of the better understanding of these processes in this organism. Nevertheless, we will also point out the differences between this route in mammals and yeast, remarking those parts of the process which remain to be elucidated in higher eukaryotic cells.

**Genes associated with autophagy**

The characterization of the molecular mechanisms of autophagy has been mainly carried out by mutagenesis-based genetic screens in *S. cerevisiae*. The subsequent discovery of a number of mammalian genes involved in autophagy has been possible because of their homology with the corresponding yeast counterparts. Interestingly, the autophagic route in *S. cerevisiae* overlaps with a biosynthetic process termed the cytoplasm-to-vacuole targeting (Cvt) pathway, which consists in the transport of inactive precursors of hydrolases, as aminopeptidase I or α-mannosidase, from the cytoplasm to the vacuole, where they become active due to the acid vacuolar environment [17, 18]. There are also several genes involved in other degradative routes, such as peroxyphagy, which overlap with those functioning in the autophagic pathway. Several groups have made independent genetic screens to identify mutants defective in these pathways, which has led to a situation in which a given autophagy-related gene may have different names, owing to its involvement in distinct overlapping processes. Accordingly, a unified gene and protein nomenclature was adopted recently [19]. The new names, also used in this review, are ATG, which stands for ‘autophagy-related’, followed by a number to identify the corresponding gene or protein.

**The molecular machinery of autophagy**

At least 25 yeast genes have been found to be specifically involved in autophagy, whereas there are more than 40 additional yeast genes required for autophagy but also involved in other pathways [19]. Nevertheless, the physiological functions of many of these genes remain to be elucidated. Among the characterized components of this complex molecular machinery, we can identify several functional subgroups which are involved in fundamental steps of the autophagic route, such as signalling, autophagosome formation, transport and fusion with the vacuole/lysosome.

**Signalling complexes**

There are several signalling complexes and pathways involved in the development of an autophagic response (fig. 2). These include the Tor signalling pathway, the ATG1 complex and the Vps34/class III PI3K complex. The first of these signalling pathways is mediated by Tor (Target of rapamycin), a serine/threonine kinase involved in the control of multiple cell processes in response to changes in nutrient conditions. Treatment of cells with rapamycin – an immunosuppressant drug which inhibits Tor activity – blocks cell cycle progression and triggers autophagy [20]. In yeast, Tor phosphorylates the protein Tap42, causing its association with protein phosphatase 2A (PP2A). This interaction significantly reduces the enzymatic activity of PP2A [21]. Inhibition of Tor by nutrient stress or rapamycin treatment results in the dephosphorylation of Tap42, which dissociates from PP2A. PP2A may then dephosphorylate its targets, which eventually leads to a variety of antiproliferative responses and induction of autophagy. In mammalian cells, there is a Tor orthologue – mTor – which appears to modulate autophagy in a manner similar to that observed in yeast. Likewise, the activity of the mammalian PP2A orthologue strongly correlates with autophagy induction [22].

The second autophagy signalling pathway involves the participation of ATG1, a protein kinase which forms part of a dynamic protein complex involved in triggering Cvt and autophagy pathways [23]. The composition of this complex may vary depending on nutrient conditions. Under nutrient-rich conditions, Tor activation causes hyperphosphorylation of ATG13, preventing its association with ATG1. This event promotes interaction between ATG1 and ATG11, which possibly determines the use of the autophagic machinery in the Cvt pathway. On the other hand, under nutrient starvation conditions or after treatment with rapamycin, ATG13 becomes partially dephosphorylated, leading to an ATG1-ATG13 interaction which subsequently triggers autophagy and the generation of autophagosomes instead of Cvt vesicles. It is not clear whether the kinase activity of ATG1 is required for this function. In fact, ATG1 kinase activity against myelin basic protein in vitro is enhanced when interacting with ATG13 [23]. Recently, it was proposed that ATG1 kinase activity is required for the Cvt pathway but not for autophagy in vivo [24]. This model suggests that ATG1 may have a non-kinase structural role in autophagy induction. This autophagy-specific ATG1 activity may be caused by a conformational change induced by variations in upstream factors such as ATG13. This conformational change may allow nucleation of autophagosomes instead of nucleation of Cvt vesicles. Therefore, it seems that ATG1, ATG11 and ATG13 are involved in the signalling of a switch from the Cvt pathway to autophagy under starvation conditions, but the molecular mechanisms underlying this event remain unclear. Other autophagy- or Cvt-related proteins, such as ATG17, Vae8 ATG24 and ATG20 [23, 25, 26], are associated with the ATG1 complex, but their physiological functions are not yet fully understood. In mammalian cells, a putative ATG1 orthologue called ULK1 has been described [27]. The finding of an inter-
action between ULK1 and GATE-16 and GABARAP [28] suggests that these two mammalian orthologues of ATG8 (a yeast protein essential for autophagosome formation) may be functionally related to the ATG1 protein complex.

The third autophagy signalling pathway is that involving the Vps34/class III phosphatidylinositol 3-kinase (PI3K) complex. The PI3Ks represent a family of enzymes implicated in diverse cellular processes, such as intracellular trafficking, proliferation and assembly of cytoskeletal elements [29]. The discovery of the autophagy inhibitory properties of 3-methyladenine, a PI3K inhibitor, was the first evidence for the implication of this family of protein kinases in autophagy [30]. Subsequently, other PI3K inhibitors, such as wortmannin and LY294002, have also been shown to inhibit this process. S. cerevisiae has only one PI3K, Vps34, which is mainly involved in vacuolar protein targeting through the endosomal/prevacuolar compartment, although recent data indicate that it is also required for autophagy [31]. Yeast Vps34 is associated with the membrane-anchored Vps15 protein which tethers the kinase to cytoplasmic membranes. Vps34/Vps15 can be present in two complexes which are involved in a variety of membrane transport events. Complex I, which is composed of Vps34/Vps15, ATG6 and ATG14, is essential for autophagy, whereas complex II, composed of Vps34/Vps15, ATG6 and Vps38, controls the vesicular transport to the vacuole. There are different possibilities to explain how Vps34 complex I controls autophagy. First, PI3- P, the reaction product of these PI3K enzymes, which would recruit proteins necessary for autophagosome formation to preautophagosomal membranes.
for autophagy regulation, but class III PI3K plays a crucial role at an early step of autophagosome formation in mammalian cells, as occurs with yeast Vps34, its functional orthologue. In fact, 3-methyladenine and wortmannin also inhibit the formation of autophagosome precursors in mammalian cells. In addition, an increase in the class III PI3K product – PI3-P – stimulates autophagy [34], whereas activation of the autophagic pathway in response to amino acid depletion is associated with an increase of class III PI3K activity in murine myotubes [33].

Class III PI3K is associated with beclin-1 and with p150, the orthologue of Vps15, forming a complex similar to yeast Vps34 autophagy-related complex. Because class III PI3K also controls distinct processes involving membrane transport [35], the understanding of the basis for the selective regulation of autophagy awaits the identification of new partners of the class III PI3K complex. Beclin-1, the mammalian orthologue of yeast ATG6, is the first autophagy-related tumour suppressor gene reported so far [36], and its role in tumorigenesis will be further discussed in this review.

**Origin of autophagosomal membrane**

Formation of the autophagosomal membrane starts with enwrapping of the bulk cytoplasm or selected organelles by a double membrane, the isolation sac, which eventually becomes a double-membrane vesicle called an autophagosome and containing cytoplasmic constituents. Over the past few decades, there has been a controversy concerning the origin of autophagosome membranes. In mammalian cells, the ribosome-free region of the rough endoplasmic reticulum (ER) has been proposed as the source of these membranes [37, 38]. However, an alternative model suggests that the phagophore – a poorly characterized organelle – may be the origin of the autophagosomal membrane and related structures [39]. In yeast, a novel perivacuolar structure called PAS (preautophagosomal structure) has been proposed as the precursor of autophagosomes [40]. Several autophagy-related proteins such as ATG1, ATG2, ATG5, ATG8, ATG9, ATG11, ATG12, ATG14 and ATG16, colocalize, at least transiently, in this perivacuolar structure. In addition, no Golgi, ER or late-endosomal markers have been found at the PAS, suggesting that this is a novel structure which had not been described so far in the context of the endomembrane system [41]. However, little is known about the existence of a PAS-like structure in mammalian cells, and the models proposed for autophagosome origin have to be confirmed at the experimental level.

**Autophagosome formation machinery**

The variety of proteins involved in autophagosome formation can be classified into three major functional subgroups, including two novel ubiquitin-like (UBL) conjugation systems and a putative membrane complex containing the ATG9 protein. The two UBL systems associated with autophagy are those involving the ATG12-ATG5 conjugate and the ATG8 protein. Both systems are similar to those catalyzing the ubiquitylation of cytoplasmic proteins for their subsequent degradation by the proteasome. They are composed of three different enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein ligase enzyme (E3) [5]. The first of these UBL conjugation systems requires the participation of ATG12-ATG5, a protein conjugate necessary for autophagosome formation (fig. 3). In this system, ATG12 is transferred to a unique target protein, ATG5, instead of being a nonspecific conjugate for misfolded or nonfunctional proteins, as occurs in the ubiquitin system. ATG12 is first activated by the E1-like enzyme ATG7, leading to the formation of a thioester bond between the C-terminal glycine of ATG12 and a cysteine residue of ATG7 [42–45]. Afterwards, ATG12 is transferred to ATG10 (which functions as an E2 enzyme), forming a new thioester bond between the C-terminal glycine of ATG12 and a cysteine of ATG10 [46, 47]. The last step in this UBL conjugation system is the covalent linkage of ATG12 to a lysine residue of ATG5 [42, 43, 48]. To date, no E3 enzyme has been identified for this conjugation system, and it is likely that ATG10 directly catalyzes the ATG12-ATG5 conjugate formation. It seems that this UBL system is constitutively active, as the formation of the ATG12-ATG5 conjugate is not dependent upon starvation or other autophagy induction conditions. Once formed, this ATG12-ATG5 conjugate interacts with ATG16, a protein containing a coiled-coil region which may facilitate its homo-oligomerization [49]. The new complex formed by ATG12-ATG5 and ATG16 is then able to oligomerize forming an ~350-kDa structure apparently composed of four subunits of ATG12-ATG5-ATG16 conjugates. This complex is necessary for the elongation of the isolation membranes, but not for the generation of the precursor structures. When autophagosomes are forming, the ATG12-ATG5-ATG16 conjugates show an asymmetric localization, and most of them are associated with the outer side of the isolation membranes. Before autophagosome completion, these conjugates start to dissociate from the membranes and are not detected in mature autophagosomes [50].

Mammalian orthologues for ATG5, ATG7, ATG10 and ATG12 have been identified, suggesting that this UBL system is well conserved among eukaryotes. Mammalian ATG5 and ATG12 are conjugated to each other as in yeast, but the complex interacts with a larger protein called ATG16L, forming an ~800-kDa structure instead of the yeast ~350-kDa complex [51]. Although the ATG12-ATG5-ATG16 complex is fundamental for autophagosome formation, little is known about its function. Recent studies indicate that the presence of the com-
plex on nascent isolation membranes is necessary for the formation of a lipid-protein conjugate essential for the completion of autophagosomes. The formation of this unusual conjugate is dependent upon the ATG8 system, the second UBL system involved in autophagy.

ATG8 is a soluble cytoplasmic protein which undergoes a series of reactions similar to those involving protein ubiquitylation, and becomes membrane attached after being covalently conjugated with a molecule of phosphatidylethanolamine (PE) (fig. 4). As in the first autophagy-related UBL system, the E1 enzyme which catalyzes the activation of ATG8 is ATG7. However, ATG8 must be first processed by the yeast cysteine proteinase ATG4 [52, 53]. This proteolytic modification results in the loss of the C-terminal region, leaving a glycine at the C-terminus. Once processed, ATG8 is activated by ATG7, leading to the formation of a thioester bond between the terminal glycine of ATG8 and a cysteine residue of ATG7 [45, 52, 54]. ATG8 is then transferred to ATG3, an E2 enzyme, forming a new thioester bond between both proteins [53–55]. In the final step, ATG8 is covalently conjugated to PE through an amide bond between the C-terminal glycine of ATG8 and the amino group of PE. The presence of this rare lipid-protein conjugate in both sides of the isolation sac is fundamental for autophagosome completion, but little is known about its function. Some authors have proposed this conjugation event as a mechanism for lipid or protein recruitment to the preautophagosomal structures [56], whereas other authors consider the function of the ATG8-PE conjugate as merely structural [18]. The enzyme or enzymes that catalyze this lipid-protein linkage remain to be characterized, but it has been suggested that the ATG12-ATG5 complex may act as an E3 enzyme for ATG8-PE conjugation [57]. This hypothesis would connect in an elegant way the two UBL systems involved in autophagy. Recently, it has been reported that mammalian ATG12-ATG5 colocalizes with LC3 (a mammalian orthologue of ATG8) at the nascent isolation membranes or at membranous punctate structures present in the cytoplasm [58]. Considering that ATG8-PE conjugation is blocked in the absence of ATG12-ATG5 conjugate [53] or in the presence of mutated forms of ATG5 unable to bind to ATG12 [57], it is reasonable to consider a possible connection between ATG12-ATG5 conjugate and ATG8/LC3 processing. On the other hand, it has been reported that mammalian ATG12, but not the ATG12-ATG5 conjugate, facilitates LC3 processing, suggesting a complex but interesting relationship between ATG12, ATG12-ATG5 conjugate and ATG8/LC3 [59]. Once autophagosome formation is completed, the ATG8 molecules attached to the cytoplasmic side of the autophagosome membrane are recycled. This
Figure 4. The ATG8 ubiquitin-like system. Once formed, nascent ATG8 is processed by the cysteine protease ATG4, leaving a glycine at the C-terminus. Apg8 is then activated by ATG7 (E1-like) and transferred to ATG3 (E2-like) through the formation of thioester intermediates, in a chain of reactions similar to ubiquitylation. Finally, ATG8 conjugates covalently with phosphatidylethanolamine (PE) and becomes membrane attached. No enzymes have been found to catalyze this lipid-protein conjugation, but ATG5-ATG12-ATG16 complex has been suggested to play a crucial role in this step. When autophagosome is formed, membrane attached ATG8 is deconjugated from PE by ATG4, providing a new source of cytoplasmic ATG8.
Autophagosome fusion with the vacuole/lysosome

Autophagosome fusion with lysosomes/vacuoles is an essential process for completion of the autophagic pathway. In fact, several human muscular disorders are caused by a defect in autophagosome-lysosome fusion. The molecular mechanisms underlying the transport and fusion of autophagosomes are beginning to be understood, and several major events involved in the process have recently been clarified.

In yeast, the fusion of autophagosomes with the vacuole requires several factors which are also involved in other types of vesicular transport. The SNARE machinery – which functions in vacuolar-targeted vesicular traffic and in homotypic vacuole membrane-fusion events – is required for correct autophagosome maturation. Proteins such as the vacuolar syntaxin homologue Vam3 [71], the SNAP-25 homologue Vam7 [72], the Rab family GTP-binding protein Ypt7 [73] or the orthologue of the N-ethylmaleimide-sensitive fusion protein (NSF), Sec18 [74], are required for autophagosome-vacuole fusion in yeast.

In mammalian cells, autophagosome fusion with lysosomes is a complex process in which the autophagosome requires a series of maturation steps prior to its fusion with the lysosome. Similar to yeast, the activity of monomeric GTPases such as Rab22 and Rab24 is needed for correct autophagosome maturation [75, 76]. Mammalian orthologues of SNARE protein family members and the NSF protein may also be involved in the maturation of autophagic vesicles. Prior to their fusion with lysosomes, autophagosomes have to fuse with endosomes or endosome-derived vesicles [39, 77, 78]. These structures are called amphisomes or intermediate autophagic vacuoles and contain endosome markers, but few lysosome proteins [77]. Specific autophagosome-endosome fusion factors remain to be identified. However, overexpression of a mutated form of the regulator of endosome sorting, SKD1 ATPase, which is unable to hydrolyze ATP, hampers endosome function, causing abnormal endosome morphology and defects in recycling of plasma membrane receptors [79]. A massive accumulation of nascent autophagosomes is also seen in cells expressing this mutated form of SKD1, suggesting that fusion with endosomes provides autophagosomes with the machinery required for fusion with lysosomes. Finally, it is remarkable that cytoskeletal elements are also involved in either autophagosome maturation or autophagosome-lysosome fusion. Microtubules are important for this event, because treatment of cells with microtubule-destabilizing drugs blocks autophagosome maturation [80, 81]. Likewise, taxol-mediated microtubule stabilization increases the fusion of amphisomes with lysosomes [82], whereas cells treated with cytochalasin D, an agent that disrupts actin filaments, display a significant reduction in autophagosome formation [81, 83].

Roles of autophagy

In yeast, the autophagy pathway mainly acts as a survival mechanism induced under starvation conditions. However, in multicellular organisms and especially in mammals, the roles of autophagy are more diverse than in lower eukaryotes. Thus, beyond classical function of autophagy during starvation, this degradative route is involved in programmed cell death and in different tissue-specific functions.

Autophagy in cell death

In 1973, three types of cell death were described based on ultrastructural morphological differences in dying cells [84]. Type 1 cell death had already been characterized as apoptosis [85], whereas type 3 would correspond to necro-
tic death, in which plasma and nuclear membrane lose their integrity after swelling of cellular organelles [86]. Type 2 cell death or autophagic cell death involves accumulation of autophagic vacuoles in the cytoplasm of dying cells as well as mitochondria dilatation and enlargement of the ER and the Golgi apparatus. There is some controversy regarding the precise role that autophagy might play in programmed cell death. There are many reports describing situations in which autophagy activation accompanies apoptosis, but there are also cases in which autophagy enhancement leads to cell death in the absence of caspase activation [87–90]. Thus, the autophagic pathway may play different roles in distinct types of cell death.

Insect metamorphosis is an excellent example of apoptotic cell death involving autophagic events. The destruction of useless larval tissues such as salivary glands in Drosophila is associated with apoptotic features such as caspase activation and DNA fragmentation, but also with massive accumulation of lysosomal vesicles [13]. In a classical apoptotic event, the apoptotic bodies resulting from cell death are degraded by the lysosomes of the phagocytic cells. In contrast, in cells undergoing programmed cell death with autophagy activation, degradation is carried out by the lysosomal machinery of the dying cells. This type of cell death may be necessary when degradation of a whole organ or tissue is needed and the activity of phagocytic cells is not sufficient to eliminate all of the death cell bodies. In these cases, autophagy would represent a mechanism of cell clearance rather than a real cell death pathway. Nevertheless, there is multiple evidence of apoptosis-independent autophagic cell death. For example, MCF-7 breast cancer cells treated with tamoxifen undergo a type of cell death in which autophagic vacuoles accumulate in the cytoplasm, in the absence of significant apoptotic features [87]. Likewise, Jurkat T-cells undergoing apoptosis due to Bax overexpression develop a process of cell death which involves autophagosome accumulation in response to caspase inhibition [88]. Furthermore, in HL-60 leukemia cells, bcl-2 downregulation leads to a cell death event independent of mitochondrial signalling or caspase activity, also characterized by the presence of multiple autophagic vacuoles in the cytoplasm [91]. It has also been reported that overexpression of RasV12 in glioblastoma or gastric cancer cell lines results in autophagic caspase-independent cell death [89]. The fact that Ras mutations are rarely observed in these tumours suggests that autophagy may act as an alternative death program when apoptosis is defective.

**Other roles of autophagy**

The autophagic pathway is also involved in a variety of processes different from programmed cell death which are relevant for the maintenance of cellular homeostasis. Similar to the situation originally described in yeast, nutrient starvation induces autophagy in cultured mammalian cells from diverse sources. Analysis of this aspect has been facilitated by the recent generation of transgenic mice expressing a fusion protein between LC3 and GFP (green fluorescent protein) which allows in vivo detection of autophagosomes [92]. By using these mice, it has been observed that autophagy can be induced in most organs by nutrient deprivation, although there are differences in the response generated in different tissues [92]. In addition to this role in the starvation response, autophagy has also been implicated in other physiological processes. Thus, the autophagy machinery may be used to recycle damaged organelles, thereby contributing to maintain cellular integrity. For example, damaged mitochondria resulting from an incomplete apoptotic event are sequestered by autophagosomes to prevent cellular damage caused by the uncontrolled release of reactive molecules, such as free radicals [93]. Likewise, mitochondria with an active production of oxygen reactive species or with alteration in their membrane potential are preferentially sequestered by autophagic vacuoles [94, 95]. Similarly, autophagic degradation of peroxisomal vesicles in fibroblasts from patients with Zellweger syndrome, a peroxisomal disorder, has also been observed [96]. Consistent with this, autophagy is constitutively activated in transgenic mice expressing a mutant form of α1-antitrypsin which accumulates in the ER, causing hepatocarcinoma and severe liver injury [97]. Finally, autophagy is involved in several tissue-specific functions. For example, erythroid maturation requires autophagy, which contributes to eliminate ribosomes or mitochondria after the expulsion of the nucleus. In addition, the biconcave shape of erythrocytes is acquired after the expulsion of the autophagic vacuole [12]. Lastly, neuromelanin biosynthesis in dopaminergic neurons has been reported to be caused by the sequestration of cytoplasmic dopamine-quinone into autophagosomes [11].

**Autophagy in human pathology**

The variety of functions that can be assigned to autophagy in mammalian cells explains, at least in part, the number of diseases in which autophagy deficiencies have been observed. A number of neuronal or myodegenerative diseases, infectious diseases and cancer have been related to autophagy dysfunction.

**Autophagy in neuronal diseases**

Neuronal cell death is a normal physiological process which occurs during development or can be a consequence of a pathological process such as those associated with neurodegenerative diseases. Apoptosis is the best-understood mechanism of neuronal cell death and has
been exhaustively studied, but autophagic cell death has also been reported in neurons [15]. This form of neuronal death involving autophagic features has been observed during vertebrate development. For example, during chick isthmo-optic nucleus formation, the neurons that make inappropriate connections undergo a type of cell death which involves formation of autophagic vacuoles [98]. Furthermore, death of Purkinje cells in heterozygous Lurcher mice is associated with the presence of an excess of autophagosomes [99, 100]. In fact, Lurcher cell death is caused by the expression of a constitutively activated form of the glutamate receptor GRID2, which interacts with a protein complex that contains beclin-1. Likewise, cell death of sympathetic neurons induced by deprivation of GNGF exhibits autophagy activation [101]. Regarding neurodegenerative pathologies, there are reports of autophagic cell death in neurons from patients with Huntington, Parkinson and Alzheimer diseases [102–105]. All these in vivo observations suggest a functional role for autophagy in neuronal cell death. It is likely that uncontrolled activation of autophagy may cause cell death due to degradation of intracellular organelles, but there is not enough experimental evidence to conclude whether autophagy itself is an independent pathway of programmed cell death in neurons or simply a consequence of a much more complex process.

Autophagy has also been shown to contribute to the clearance of aggresomes, intracellular protein aggregates which are a common feature of numerous neurodegenerative disorders. In fact, huntingtin and α-synuclein aggresomes, caused by overexpression of mutant forms of these proteins, are effectively cleared from cells treated with rapamycin, an autophagy inducer, whereas treatment with 3-methyladenine, a potent autophagy inhibitor, reduces their clearance [104, 106]. Accordingly, autophagy induction can reduce the formation of insoluble polyglutamine inclusion bodies under certain cell culture conditions [107]. These findings suggest that autophagy may be activated as a compensatory mechanism due to an insufficiency in the proteasome pathway, which eventually facilitates the accumulation of these protein aggregates [108].

Autophagy in cancer

Although a few studies have focused on the analysis of putative variations in the ability of cancer cells for developing autophagy, it is widely believed that the autophagic pathway is downregulated in tumour cells. Cell lines derived from hepatic, pancreatic and breast carcinomas exhibit low autophagic activity, as compared with normal cells from the same origin [36, 109, 110]. It has also been suggested that the differentiation status of a tumour is related to its autophagic activity [111], whereas the loss of autophagic potential usually occurs during the transition from adenoma to carcinoma [109, 112]. Nevertheless, several tumour cell lines retain a high autophagic capacity. Starvation induced autophagy has been observed in cervical cancer HeLa cells, and in lymphocytes isolated from patients with chronic lymphocytic leukemia [113, 114]. These findings strongly suggest that autophagy may play dual roles in cancer progression.

The characterization of the tumour suppressor activity of the autophagy-related gene beclin-1 [36, 115, 116] established an important relationship between cancer and the autophagic pathway. Beclin-1 was originally isolated as a bel-2-interacting protein [117], but it is still unclear whether this interaction is instrumental in autophagy. Nevertheless, it is remarkable that downregulation of bel-2 stimulates autophagy [91]. Beclin-1 expression can induce autophagy in MCF7 cells, which is associated with inhibition of MCF7 cellular proliferation, in vitro clonogenicity and tumorigenesis in nude mice [36]. Consistently, this gene is mono-allelically deleted in 40–75% of human sporadic breast, prostate and ovarian cancers, whereas beclin-1-haploinsufficient mice show a pronounced increase in epithelial and hematopoietic tumours and reduced autophagy [115, 116]. The tumour suppressor activity of beclin-1 and the involvement of PTEN (a tumour suppressor which dephosphorylates class I PI3K products and prevents autophagy inhibition) in autophagy regulation has led to a better understanding of the variability of autophagy potential in cancer cells. Thus, tumour cells that retain high autophagic activity also show normal expression levels of these two genes [111, 118, 119]. In contrast, tumour cell lines with a low rate of autophagy, such as MCF-7 cells, show defective expression of beclin-1 and PTEN [36, 120]. The observation of a similar correlation in a large number of breast tumours [36, 121], suggests the possibility of establishing a link between the autophagic potential of cancer cells and the expression levels of different autophagy-related genes, oncogenes and tumour suppressor genes.

Infectious diseases and autophagy

Some bacteria and viruses use the autophagic machinery to invade host tissues [122]. For example, Porphyromonas gingivalis and Brucella abortus localize in autophagosomes, and their virulence or replicative capacity are likely associated with this event. During infection, these pathogens are sequestered into phagosomes and induce autophagy, thereby promoting phagosome fusion with autophagic vacuoles instead of endosomes. This fact prevents pathogen degradation by lysosomal hydrolases, because autophagosome maturation is impaired in cells infected with virulent P. gingivalis and B. abortus. Autophagic vacuoles also provide these pathogens with nutrients that facilitate their growth into the autophagosome compartment, as they are able to use autophagy-recycled
proteins as substrates for their metabolic pathways. Consistent with these observations, autophagy inhibition reduces *P. gingivalis* persistence in infected cells. Likewise, cells infected with virulent *B. abortus* show inhibition of autophagosome-lysosome fusion [123]. *Legionella pneumophila* and *Coxiella burnetii*, which are able to grow into lysosomes, can use autophagic vacuoles to invade new vesicles [124]. More recently, *Salmonella* has been reported to induce macrophage cell death by promoting autophagy activation. This enteric bacteria induces the formation of multimembranous autophagosome-like structures which contain both mitochondria and ER markers by expressing a protein with membrane fusion activity [125]. The autophagic machinery is used by some viruses during their replication inside cells, thereby facilitating viral dissemination [126, 127]. However, autophagy induction may also serve cells to prevent virus infection. In fact, expression of beclin-1 protects murine cells against infection by Sindbis virus [117], whereas infection with *Herpes simplex* prevents autophagosome formation [128].

Taken together, these observations suggest that in some cases, the autophagic machinery is used by several viruses or bacteria to infect and grow inside host cells, but in other cases autophagy may function as a protective mechanism against infection. The elucidation of the molecular strategies used by viruses and bacteria to control the autophagic machinery may help us to understand the complex molecular mechanisms regulating autophagy in mammalian cells, and could also contribute to develop autophagy-based strategies for the treatment and prevention of infectious diseases.

**Autophagy and myopathies**

Several hereditary muscular disorders are associated with the accumulation of autophagic or lysosomal vacuoles. These pathologies are collectively known as vacuolar myopathies, and are characterized by the accumulation of multiple vacuoles from lysosomal and autophagic origin. According to morphological, biochemical and genetic criteria, vacuolar myopathies have been classified into several groups, including rimmed vacuolar myopathies, Danon disease and X-linked vacuolar myopathies [129].

The rimmed vacuolar myopathies are the most common vacuolar myopathies, and are characterized by the presence of apparent small holes in the muscle fiber lined by ‘rims’, which are red granules observed by modified Gomori trichrome staining. These disorders are caused by mutations in different genes which eventually lead to the accumulation of misfolded protein aggregates. Then, autophagy is activated to degrade these inclusion bodies, whose accumulation may lead to cell death [130–132]. Danon disease is characterized by myopathy, hypertrophic cardiomyopathy and mild mental retardation [133]. This disease is caused by mutations in the LAMP-2 gene, which encodes a protein that covers the inner side of the lysosomal membrane, preventing its degradation by lysosomal hydrolytic enzymes [134]. Muscle biopsies of Danon disease patients show an accumulation of small autophagic vacuoles with acetylcholinesterase activity and sarcosomal features. Interestingly, it has been demonstrated that fusion of autophagic vacuoles with lysosomes is not affected in Danon disease [135], and it is likely that vacuole accumulation is due to the impaired capacity of lysosomes for degradation. X-linked myopathy with excessive autophagy (XMEA) is similar to Danon disease and is characterized by progressive muscle atrophy, involving cardiac and respiratory muscle degeneration [136–138]. Muscle biopsy shows similar features to those observed in Danon disease, but no LAMP-2 mutations have been found in patients of XMEA. Other forms of autophagic vacuolar myopathies, such as infantile autophagic vacuolar myopathy [139] or a late-onset multigenic vacuolar myopathy [140], have been reported. These disorders show some of the abnormalities characteristic of XMEA and Danon disease but also exhibit distinctive features and lack mutations in the LAMP-2 gene.

**Conclusions and perspectives**

Over the last years, our understanding of autophagy has drastically increased, mainly due to the application of genetic approaches. In yeast, many questions concerning molecular mechanisms of autophagy have already been addressed, but there are fundamental tasks ahead, such as clarification of the putative links between the different signalling complexes involved in the process and elucidation of the specific mechanisms mediating autophagosome biogenesis, transport and fusion. In mammalian cells, our knowledge regarding the autophagic pathway is more limited, although recent advances have shed light on the major cellular and molecular events underlying this degradative route. The molecular machinery of the autophagic pathway is well conserved during evolution, but the high number of autophagy-related genes in multicellular organisms, especially in mammals, indicates that this process is much more complex in these organisms when compared with yeast. The creation and analysis of transgenic and knockout animal models will be essential to understand the evolutionary-acquired complexity of autophagy mediated processes in mammals [92].

Multiple studies analyzing autophagy in pathological conditions have concomitantly expanded our knowledge regarding the physiological roles of autophagy in multicellular organisms. However, a better understanding of the connections between autophagy and processes such as programmed cell death, malignant transformation and
tumour growth would be of particular interest. Autophagy is also deregulated in a variety of muscular and neurological diseases. Recent analysis of murine models of both types of diseases have contributed to clarify some aspects of the role of autophagy pathway in neurodegeneration and myopathies [99, 134]. It will also be very interesting to analyze the connections between autophagy and aging. It has been proposed that accumulation of aberrant or functionally disabled mitochondria and other oxidatively damaged structures is a consequence of an age-related decline in autophagic and lysosomal activity [6, 141–144]. Furthermore, it has been reported that the age decline of autophagy is caused by the alteration in glucose metabolism and hormone levels which is inherent to aging [142]. These observations, together with the finding that autophagy genes are essential for life-span extension in Caenorhabditis elegans [145] and that inhibition of Tor kinase (whose activity inhibits autophagy) doubles the life-span of this nematode [146], support the idea that dysfunction of the autophagic pathway may promote aging.

In summary, a better understanding of the mechanisms underlying autophagy will be very helpful to clarify the multiple functions of this degradative pathway in biological processes as well as to develop pharmacological strategies designed to modulate the altered autophagic responses occurring in a number of pathological situations ranging from cancer to neurological diseases.

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