Ubiquitin-like proteins and autophagy at a glance

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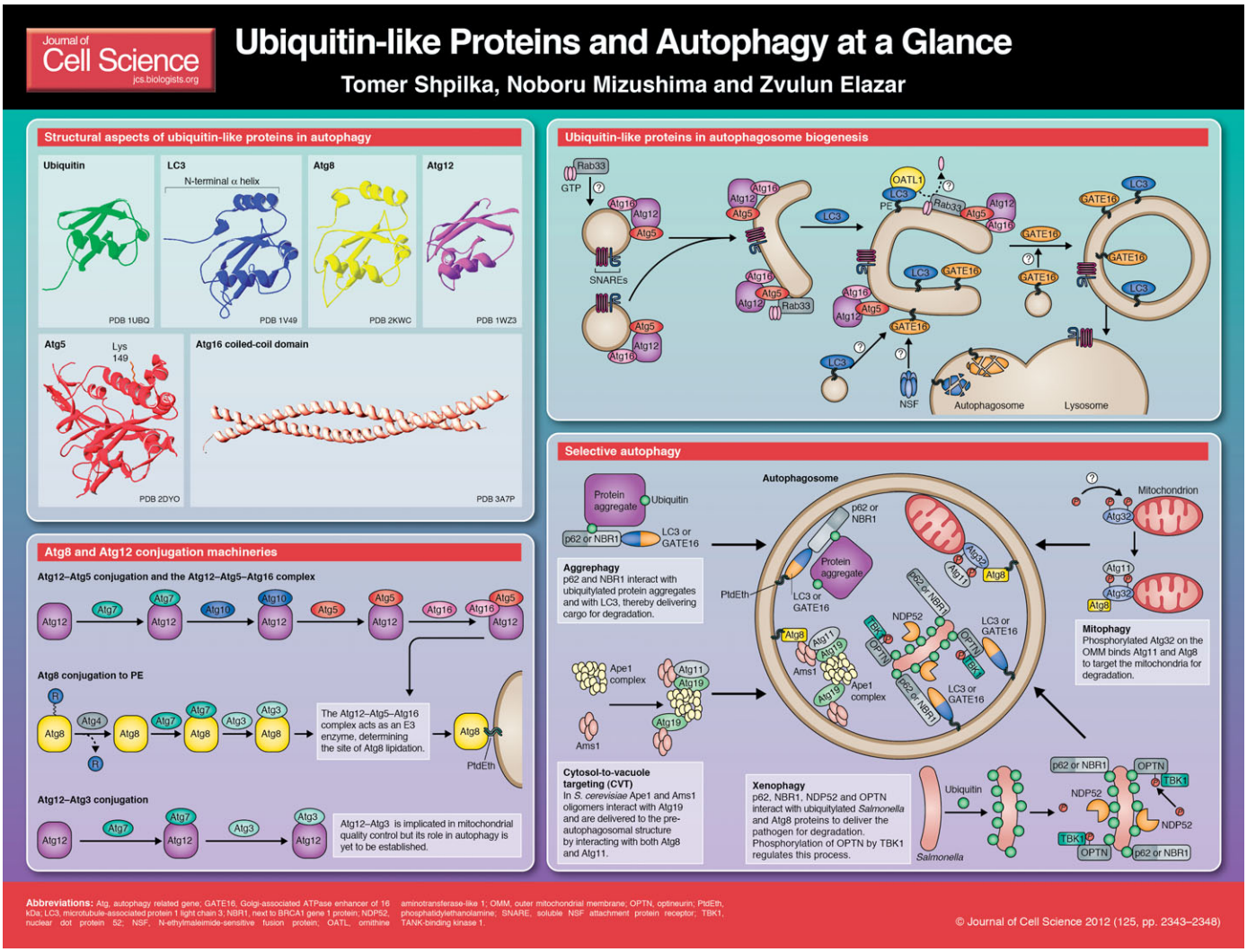
Autophagy is a major catabolic process that is predominantly controlled by amino acids and hormones, and delivers cytoplasmic components to lysosomes or the vacuole for degradation and recycling (Weidberg et al., 2011a). This process is essential for maintaining cell homeostasis by providing energy and clearing damaged or unnecessary substrates from the cytoplasm through lysosomes and the vacuole. Autophagy also has important roles in numerous physiological and pathophysiological processes, such as cell development, programmed cell death, cancer, defense mechanisms against pathogen infection, neurodegenerative disorders and myopathies (Ravikumar et al., 2010a). Several forms of autophagy have been described, including macroautophagy, microautophagy and chaperone-mediated autophagy (Mijaljica et al., 2011; Orenstein and Cuervo, 2010; Weidberg et al., 2011a).

Macroautophagy (hereafter referred to as autophagy) – the best characterized form of autophagy – refers to the formation of autophagic vesicles through the nucleation, assembly and elongation of membrane structures (Rubinsztein et al., 2012). It is initiated by the formation of a cup-shaped membrane called the phagophore, which engulfs parts of the cytoplasm. The phagophore subsequently elongates and becomes sealed, forming a unique double-membrane vesicle termed the autophagosome, which fuses with the lysosome and thereby leads to degradation of the inner membrane and the cargo of the autophagosome. In *Saccharomyces cerevisiae*, autophagosome formation takes



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place in a unique site termed the pre-autophagosomal structure (PAS) (Suzuki et al., 2001).

Genetic screens in yeast have led to the identification of 35 autophagy-related (*ATG*) genes, many of which are evolutionarily conserved in other species, and the genes identified in these screens have paved the way for elucidating the molecular machinery that drives autophagosome formation at the PAS (Thumm et al., 1994; Tsukada and Ohsumi, 1993). Atg proteins consist of several functional units: the Atg1–unc-51-like kinase (ULK) complex, the class III phosphoinositide 3-kinase (PI3K) complex, the Atg12 conjugation machinery, the Atg8 conjugation machinery, as well as a number of other proteins that are essential for autophagosome formation (Mizushima et al., 2011). The order in which these proteins are recruited to the PAS and participate in its organization has been established (Suzuki et al., 2007); however, the precise roles of each of the different Atgs is not yet fully understood.

Two of the proteins involved in the autophagic process, Atg12 and Atg8, are structurally related to ubiquitin (Paz et al., 2000; Suzuki et al., 2005). These molecules participate in the early stages of autophagosome biogenesis, where Atg12 is covalently attached (conjugated) to Atg5 and Atg8 becomes conjugated to phosphatidylethanolamine (PtdEth) (Ichimura et al., 2000; Mizushima et al., 1998a). In this Cell Science at a Glance article, we focus on the roles of these two proteins and their conjugation machineries in both yeast and mammals.

The Atg12–Atg5–Atg16 conjugation system

Atg12 was the first ubiquitin-like protein (Ubl) identified as an essential component for autophagy. Although Atg12 does not have a similar sequence to ubiquitin, its crystal structure reveals a ubiquitin fold (Suzuki et al., 2005). Atg12 is covalently conjugated to Atg5 through the Ubl-conjugation machinery. First, the Atg12 C-terminal Gly186 is activated by the E1-like enzyme Atg7 through the formation of a thioester bond with Cys507 of Atg7 (Tanida et al., 1999). Subsequently, Atg12 is transferred to the E2-like enzyme Atg10 (Shintani et al., 1999), which is followed by Atg12 conjugation to Lys149 on its target protein Atg5 (Mizushima et al., 1998b). The Atg12–Atg5 conjugate can further interact with the coiled-coil protein Atg16 (following its oligomerization) to form a dimeric complex (Fujioka et al., 2010; Kuma et al., 2002).

No deconjugation or E3-like enzyme that associates with the Atg12–Atg5 complex has been detected so far, and it seems that formation of the Atg12–Atg5 conjugate occurs constitutively. Atg5, which also has no substantial sequence similarity with ubiquitin, comprises two ubiquitin-like domains flanking a helix-rich domain (Matsushita et al., 2007). Binding of Atg5 to Atg16 and to Atg12 occurs on opposite sides of the protein. Atg16 in *S. cerevisiae* comprises a coiled-coil domain through which it forms homo-oligomers, and an N-terminal Atg5-binding domain (Fujioka et al., 2010). In addition, the mammalian Atg16 (ATG16L) has seven WD repeats in its C-terminus and forms a larger ATG12–ATG5–ATG16L complex (Mizushima et al., 2003). This complex is essential for the formation and elongation of the autophagosome (Mizushima et al., 2001) and orthologs of this system, with the respective functionality, are found in yeast and mammals (Ohsumi, 2001).

Small fractions of the cytosolic ATG12–ATG5–ATG16L complex associate with the outer membrane of the phagophore and dissociate from it on or near completion of the double-membrane autophagosome (Mizushima et al., 2001; Mizushima et al., 2003). Atg5 and Atg16 depend on each other for their membrane targeting, whereas Atg12 is dispensable for Atg5–Atg16 membrane association (Mizushima et al., 2003; Suzuki et al., 2001). The targeting of this complex to the membranes also depends on the formation of phosphatidylinositol 3-phosphate [PtdIns(3)*P*], but no known PtdIns(3)*P*-binding domain is found within the Atg12–Atg5–Atg16 complex. However, during the process of xenophagy (the degradation of pathogens by autophagy; for further details, see below), recruitment of the ATG12–ATG5–ATG16L complex appears to be independent of PtdIns(3)*P* and other upstream ATG proteins (Kageyama et al., 2011).

The Atg8 conjugation system

The second ubiquitin-like conjugation system involves the conjugation of Atg8 proteins to the lipid PtdEth (Ichimura et al., 2000). Yeast have one *ATG8* gene, whereas mammalians possess several genes encoding Atg8-like proteins that are divided into three subfamilies: microtubule-associated protein 1 light chain 3 (LC3), γ-aminobutyric acid (GABA) receptor-associated protein (GABARAP) and Golgi-associated ATPase enhancer of 16 kDa (GATE16, also known as GABARAPL2) (Shpilka et al., 2011). Mammalian ATG8 proteins were originally characterized as factors that participate in membrane trafficking processes (Kuznetsov and Gelfand, 1987; Mann and Hammarback, 1994), and it was only discovered later that they are indispensable for autophagy (Lang et al., 1998; Scott et al., 1996).

Atg8 proteins are synthesized as precursors that are immediately cleaved in their C-terminus by the specific cysteine protease Atg4, which leads to the exposure of a glycine residue (Kabeya et al., 2000; Kirisako et al., 2000). The carboxy group of the exposed glycine is then activated by Atg7, the same E1-like enzyme that acts on Atg12, by forming a thioester bond between Atg8 and Atg7. The glycine residue of Atg8 is subsequently transferred to the specific E2-like enzyme Atg3, and this interaction leads to formation of an amide bond between PtdEth and the exposed glycine residue of Atg8 (Ichimura et al., 2000). The conjugation of Atg8 to PtdEth is reversible, as Atg4 can also function as a deconjugating enzyme (Kirisako et al., 2000), and Atg8 proteins that are conjugated to PtdEth reside on both sides of the phagophore membrane as well as on the inner and outer membranes of complete autophagosomes.

Three-dimensional structure analysis has revealed that all Atg8 proteins are structurally similar to ubiquitin (Coyle et al., 2002; Kouno et al., 2005; Kumeta et al., 2010; Paz et al., 2000). Atg8s comprise a C-terminal ubiquitin core that is decorated with two additional N-terminal α-helices. The three-dimensional structure of GABARAP has been solved and has revealed two different conformations of the protein, in which the α-helices are projected either towards the ubiquitin core (closed conformation) or away from it (open conformation) (Coyle et al., 2002). It has been suggested that Atg8 is found in an open conformation following conjugation to PtdEth, implying that there is a function for the Atg8 N-terminus in autophagy (Ichimura et al., 2004). Furthermore, this unique region differs in the different mammalian ATG8 subfamilies: the first α-helix of the mammalian LC3 subfamily is basic, whereas in the GABARAP and GATE16 subfamilies this region is acidic (Sugawara et al., 2004).

Functional roles of ubiquitin-like conjugation systems

Atg12–Atg5–Atg16 as an E3-like enzyme

The Atg12–Atg5–Atg16 complex has an important regulatory role in Atg8 conjugation. Thus, Atg5 deficiency or

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Box 1. Additional cellular roles for Atg12–Atg5–Atg16

The ATG12–ATG3 conjugate and mitochondrial quality control

Disruption of the ATG12–ATG3 conjugate by mutagenesis does not seem to affect nonselective autophagy. Instead, it leads to an increase in mitochondrial mass, fragmentation of the mitochondrial network and resistance to mitochondrial cell death. The mechanism by which the ATG12–ATG3 conjugate regulates mitochondrial homeostasis is not yet understood, but it is known that following disruption of the conjugate, the basal levels of the anti-apoptotic protein Bcl-XL increase, which is correlated to the resistance of these cells to mitochondria-mediated apoptosis (Radoshevich et al., 2010).

ATG16L and Crohn's disease

Genetic studies in humans have shown that a single nucleotide polymorphism in the C-terminal WD repeats of the ATG16L1 protein – which results in substitution of alanine for threonine at amino acid 300 – leads to an increased susceptibility to Crohn's disease (Cadwell et al., 2008). The pathogenesis of this inflammatory disease of the intestine is poorly understood (Cadwell et al., 2009). Dendritic cells taken from patients with the ATG16L1 (T300A) variant are defective in the induction of autophagy, bacterial trafficking and antigen presentation (Cooney et al., 2010). This polymorphism has been further shown to impair autophagy and clearance of several pathogens (Kuballa et al., 2008; Lapaquette et al., 2010). Additionally, macrophages derived from mice with a deletion of the coiled-coil domain of ATG16L1 show an enhanced inflammatory response to Toll-like receptor ligands (Saitoh et al., 2008). Paneth cells of mice that are hypomorphic for ATG16L1 protein expression show defects in handling antimicrobial granules and exhibit the same defects as Paneth cells taken from Crohn's disease patients that are homozygous for the ATG16L1 risk allele (Cadwell et al., 2008). Taken together, these studies suggest that ATG16L1 and autophagy influence the immune response and the biology of Paneth cells. The role of the WD repeats in ATG16L1 is unknown, but this region is known to participate in protein–protein interactions. Revealing the proteins that interact with the WD region of ATG16L1 might therefore prove useful in understanding the pathogenesis of Crohn's disease.

mutations that hamper the formation of the Atg12–Atg5 complex almost completely abolish the conjugation of Atg8 to PtdEth (Hanada and Ohsumi, 2005; Mizushima et al., 2001). Furthermore, in vitro experiments have demonstrated that the Atg12–Atg5 conjugate strongly enhances the formation of Atg8–PtdEth by interacting with and stimulating the activity of Atg3 (Hanada et al., 2007). Atg16 is required for the localization of Atg8 to the pre-autophagosomal structure in yeast (Suzuki et al., 2001), and mammalian ATG16L determines the site of LC3 lipidation (Fujita et al., 2008). Taken together, these findings suggest that the Atg12–Atg5–Atg16 complex has an E3-like enzyme activity, which determines the site of Atg8 conjugation to PtdEth. A recent study has shown that ATG12 is covalently conjugated to ATG3 through a reaction that is mediated by ATG7 and the autocatalytic activity of ATG3. The ATG12–ATG3 conjugate participates in mitochondrial quality control (Box 1), and blocking this interaction does not affect the conjugation of mammalian ATG8 proteins to PtdEth. Evidently, the interaction between ATG12–ATG5 and ATG3, and their effect on ATG8 lipidation, requires further study (Radoshevich et al., 2010).

The role of Atg8 in cargo recruitment

The autophagic pathway was initially considered to be nonselective, but it is now clear that both yeast and mammalian ATG8 proteins participate in selective cargo recruitment to the autophagosome (Weidberg et al., 2011a). In yeast, Atg8 participates in the selective recruitment of prApe1 (for precursor Ape1) to the vacuole by interacting with Atg19 (Lynch-Day and Klionsky, 2010), whereas in mammals the two scaffold proteins p62 (also known as sequestosome-1, SQSTM1) and neighbour of BRCA1 gene 1 (NBR1) are recruited to the autophagosome by mammalian ATG8 proteins through their Atg8-family interacting motif (AIM) [also known as LC3-interacting region (LIR)]. The AIM motif is composed of acidic residues followed by the sequence W/YxxL/I/V (Ichimura et al., 2008; Noda et al., 2010). This motif is found in numerous proteins and enables them to interact directly with Atg8 family members (Noda et al., 2010). Importantly, different mammalian ATG8 proteins appear to differ in their selectivity towards cargo molecules. Thus, p62 is recruited to the autophagosomes by LC3 but not by GATE16, although it is able to interact with both proteins in their soluble form (Shvets et al., 2011).

Autophagy also participates in the selective delivery of protein aggregates (aggrephagy), organelles, such as peroxisomes (pexophagy) and mitochondria (mitophagy), bacteria and viruses (xenophagy) and ribosomes (ribophagy) to the lysosome and/or vacuole (Johansen and Lamark, 2011). In the following sections we exemplify the role of Atg8 proteins in two of these processes, mitophagy and xenophagy.

Atg8 proteins and mitophagy

The term 'mitophagy' refers to the selective clearance of mitochondria by autophagy. In yeast, mitophagy involves Atg32, an outer mitochondrial membrane (OMM)-spanning protein, which interacts with the cargo protein Atg11 (Kanki et al., 2009; Okamoto et al., 2009). The interaction is mediated by Atg32 phosphorylation and is thought to be the initial step in mitophagy (Aoki et al., 2011). In addition, Atg32 contains AIMs in its cytosolic domain and is thus able to interact with Atg8. Mutations in the Atg32 AIM result in partial defects in mitophagy and hamper the Atg32–Atg8 interaction in yeast two-hybrid assays (Okamoto et al., 2009). In mammals, NIX (also known as BNIP3-like protein, BNIP3L), an OMM protein, contains the AIM in its cytosolic domain. As in yeast, this AIM mediates direct binding to mammalian ATG8 proteins, and NIX can recruit mitochondria to the autophagosomes. Ablation of the interaction between NIX and mammalian ATG8 proteins hinders clearance of mitochondria (Novak et al., 2010).

In addition, mitophagy can also occur in a NIX-independent manner (reviewed by Youle and Narendra, 2011). For example, the E3 ubiquitin ligase Parkin has been shown to mediate mitophagy. Although p62 has been reported to function as an adaptor protein for Parkin-mediated mitophagy, this idea remains controversial (Narendra et al., 2010; Okatsu et al., 2010).

Mammalian ATG8 proteins and xenophagy

As described above, xenophagy refers to the selective autophagy of pathogens. In this process, adaptor proteins such as p62, NBR1, nuclear dot protein 52 (NDP52) and optineurin (OPTN), which all contain AIMs, bind both ubiquitylated pathogens and mammalian ATG8 proteins, thereby targeting the pathogens to autophagosomes. In addition, LC3, through a pathway involving p62, targets cytosolic proteins to the lysosome, where they are processed into anti-microbial peptides (Ponpuak et al.,

2010). A recent study has shown that TANK-binding kinase 1 (TBK1) phosphorylates the autophagic adaptor OPTN, which is a ubiquitin-binding protein. This phosphorylation, which occurs at a site within the OPTN AIM motif, increases the affinity of OPTN binding to LC3 and thereby promotes selective autophagy of ubiquitylated *Salmonella enterica* (Wild et al., 2011). The regulation of cargo recruitment to mammalian ATG8 proteins by phosphorylation is of great importance in the elucidation of signaling events that target cargo proteins for degradation.

Atg proteins in autophagosome biogenesis

A key question in autophagy focuses on the mechanism of autophagosome biogenesis. Atg8 proteins have certain characteristics that could be important for this process, and they are thought to have a role in autophagosome biogenesis. However, their mode of action is not fully understood. Early studies have shown that Atg8 proteins promote elongation of the autophagosomal membrane and determine the size of the autophagosome (Abeliovich et al., 2000; Xie et al., 2008). It has been suggested that the yeast Atg8, following lipidation, mediates membrane tethering and hemifusion, which drive the growth of autophagosomal membranes (Nakatogawa et al., 2007). A recent in vitro study has indeed shown that mammalian ATG8 proteins, through their N-terminus, mediate fusion processes that are required for autophagosome biogenesis (Weidberg et al., 2011b). Furthermore, preventing the lipidation of all mammalian ATG8 proteins blocks autophagosome biogenesis (Fujita et al., 2008; Sou et al., 2008), and the mammalian ATG8 subfamilies LC3, GABARAP and GATE16 are essential for autophagosome biogenesis and have been suggested to act at different stages of this process (Weidberg et al., 2010).

The role of yeast Atg8 in autophagosome biogenesis is expanded by its ability to recruit essential factors that participate in the process. For example, it has been intriguing to find that Atg8 interacts with the cell division control protein 48 (Cdc48) cofactor Shp1 (for suppressor of high-copy PP1 protein 1). Cdc48 belongs to the AAA ATPase superfamily and participates in numerous cellular functions, including homotypic membrane fusion, endoplasmic reticulum (ER)-associated protein degradation (ERAD) and protein degradation through the ubiquitin–proteasome system (Dargemont and Ossareh-Nazari, 2011). Cdc48 and Shp1 mediate Golgi reassembly by extracting a monoubiquitylated protein regulator (Wang et al., 2004). A recent study has revealed that Cdc48 and its cofactor Shp1 have a role in autophagosome biogenesis (Krick et al., 2010), and the authors of this study suggest that this role resembles their function in Golgi reassembly, where the ubiquitin-like protein Atg8 acts as the essential monoubiquitylated protein that is extracted by Cdc48 and Shp1 (Krick et al., 2010).

Numerous Rab proteins, Sec proteins and soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptors (SNAREs) have been implicated in autophagosome biogenesis and some have been shown to interact with Atg proteins (Chua et al., 2011; Longatti and Tooze, 2009; Moreau et al., 2011; Nair et al., 2011; Ohashi and Munro, 2010). RAB33A and RAB33B interact directly and in a GTP-dependent manner with the coiled-coil domain of ATG16L (Itoh et al., 2008), and it has been suggested that interaction modulates autophagosome formation (Itoh et al., 2008). Ornithine aminotransferase-like 1 (OATL1, also known as TBC1D25), a putative Rab GTP-activating protein (GAP), has recently been shown to bind mammalian ATG8 proteins (Itoh et al., 2011). This protein, through its interaction with mammalian ATG8 proteins, is recruited to isolation membranes and to autophagosomes, where it participates in autophagosome maturation. Furthermore, RAB33B is a target of the OATL1 GAP activity (Itoh et al., 2011). Thus, the interaction between Atg8 and the Atg12–Atg5–Atg16 complex might serve as a scaffold that brings RAB33 and OATL1 into close proximity. In summary, the participation of Atg8 proteins in autophagosome biogenesis might occur through their ability to promote vesicle fusion, as well as by their ability to recruit essential factors to the phagophore and/or to work together with these factors.

The NSF protein Sec18, which mediates the disassembly of SNARE proteins, has been implicated as an essential component of autophagosome biogenesis in yeast (Nair et al., 2011). Interestingly, mammalian NSF associates with both the GATE16 and the GABARAP subfamilies (Kittler et al., 2001; Sagiv et al., 2000). However, a role for this interaction in autophagosome biogenesis has not yet been established. It has been suggested GATE16 that acts as an intermediary between NSF activity and SNARE activation (Sagiv et al., 2000). If so, GATE16 could act in concert with NSF and SNARE proteins to mediate autophagosome biogenesis. Recent studies have revealed that vesicles containing the ATG5–ATG12–ATG16L complex, derived from the plasma membrane, undergo SNARE-mediated homotypic fusion, and that this process is important for autophagosome formation (Moreau et al., 2011; Ravikumar et al., 2010b).

To sum up these recent findings, both the Atg12 and Atg8 conjugation systems act at early stages of autophagosome formation by recruiting the protein machinery involved in this process and by participating in membrane remodeling.

Perspectives

Numerous studies have recently established that the Atg12 and Atg8 conjugation systems have a dual role in early stages of autophagosome formation. These systems are needed for the elongation and, possibly, sealing of the autophagosomal membrane and, at the same time, act as part of the machinery for selective cargo recruitment into autophagosomes. Nevertheless, there are still many questions regarding these systems that need to be addressed.

Until recently, Atg5 was thought to be the sole target of Atg12. New research has revealed, however, that Atg12 is also conjugated to Atg3. This conjugation, and its surprising role in mitochondrial quality control, raises the exciting possibility that both Atg12 and Atg3 have additional interacting partners with important regulatory functions, and this is a fertile area for future research. Another major question in the autophagy field has to do with the biogenesis of autophagosomes. This process requires numerous factors, including Atg proteins and SNAREs. However, the details of the interplay between these proteins and their contribution to autophagosome biogenesis are still vague. Studying autophagosome biogenesis is extremely difficult because of the large number of factors and organelles that participate in this process. Isolation of phagophores might be a crucial step towards understanding the machinery involved and the necessary steps in autophagosome biogenesis. Moreover, the finding that the Atg12–Atg5–Atg16 complex possesses E3-like activity for Atg8 lipidation but does not reside on the phagophore inner membrane raises an

important question: does the Atg12–Atg5–Atg16 complex direct Atg8 conjugation on both sides of the phagophore or only on its outer membrane? It is unclear, moreover, how the Atg12–Atg5–Atg16 complex associates with the phagophore and dissociates from it. Moreover, it will be essential to determine the exact localization of Atg8 proteins to understand their role in autophagosome biogenesis. Furthermore, the crosstalk between Atg8 proteins and other factors, such as NSF and CDC48, is in need of elucidation. These are among the questions to be addressed in future studies.

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