

REVIEW

The machinery of macroautophagy

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Autophagy is a primarily degradative pathway that takes place in all eukaryotic cells. It is used for recycling cytoplasm to generate macromolecular building blocks and energy under stress conditions, to remove superfluous and damaged organelles to adapt to changing nutrient conditions and to maintain cellular homeostasis. In addition, autophagy plays a critical role in cytoprotection by preventing the accumulation of toxic proteins and through its action in various aspects of immunity including the elimination of invasive microbes and its participation in antigen presentation. The most prevalent form of autophagy is macroautophagy, and during this process, the cell forms a double-membrane sequestering compartment termed the phagophore, which matures into an autophagosome. Following delivery to the vacuole or lysosome, the cargo is degraded and the resulting macromolecules are released back into the cytosol for reuse. The past two decades have resulted in a tremendous increase with regard to the molecular studies of autophagy being carried out in yeast and other eukaryotes. Part of the surge in interest in this topic is due to the connection of autophagy with a wide range of human pathophysiologies including cancer, myopathies, diabetes and neurodegenerative disease. However, there are still many aspects of autophagy that remain unclear, including the process of phagophore formation, the regulatory mechanisms that control its induction and the function of most of the autophagy-related proteins. In this review, we focus on macroautophagy, briefly describing the discovery of this process in mammalian cells, discussing the current views concerning the donor membrane that forms the phagophore, and characterizing the autophagy machinery including the available structural information.

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Introduction

The definition of autophagy

Autophagy is a cellular process in which cytoplasmic contents are degraded within the lysosome/vacuole, and the resulting macromolecular constituents are recycled [1]. Macroautophagy is one type of autophagic process in which the substrates are sequestered within cytosolic double-membrane vesicles termed autophagosomes. The substrates of macroautophagy include superfluous and damaged organelles, cytosolic proteins and invasive microbes. Following degradation, the breakdown products are released back into the cytosol in order to recycle the macromolecular constituents and to generate energy to maintain cell viability under unfavorable conditions,

and to protect the cell during various conditions of stress [2, 3]. Autophagy is highly conserved from yeast to mammals, both morphologically and with regard to the protein constituents that make up the core autophagy machinery.

Terms and structures

The most readily identifiable feature of macroautophagy is the sequestration of cargo within cytosolic double-membrane vesicles, and many researchers still consider the analysis of macroautophagy by electron microscopy to be the “gold standard” for verifying macroautophagy activity. Below, we present some of the most common terms describing the principle structures of macroautophagy (Figure 1).

Autophagic body: The inner-membrane-bound structure released into the vacuole lumen after the outer membrane of the autophagosome fuses with vacuolar membrane [4]. Note that autophagic bodies only appear in yeast and plants due to the large size of the vacuole,

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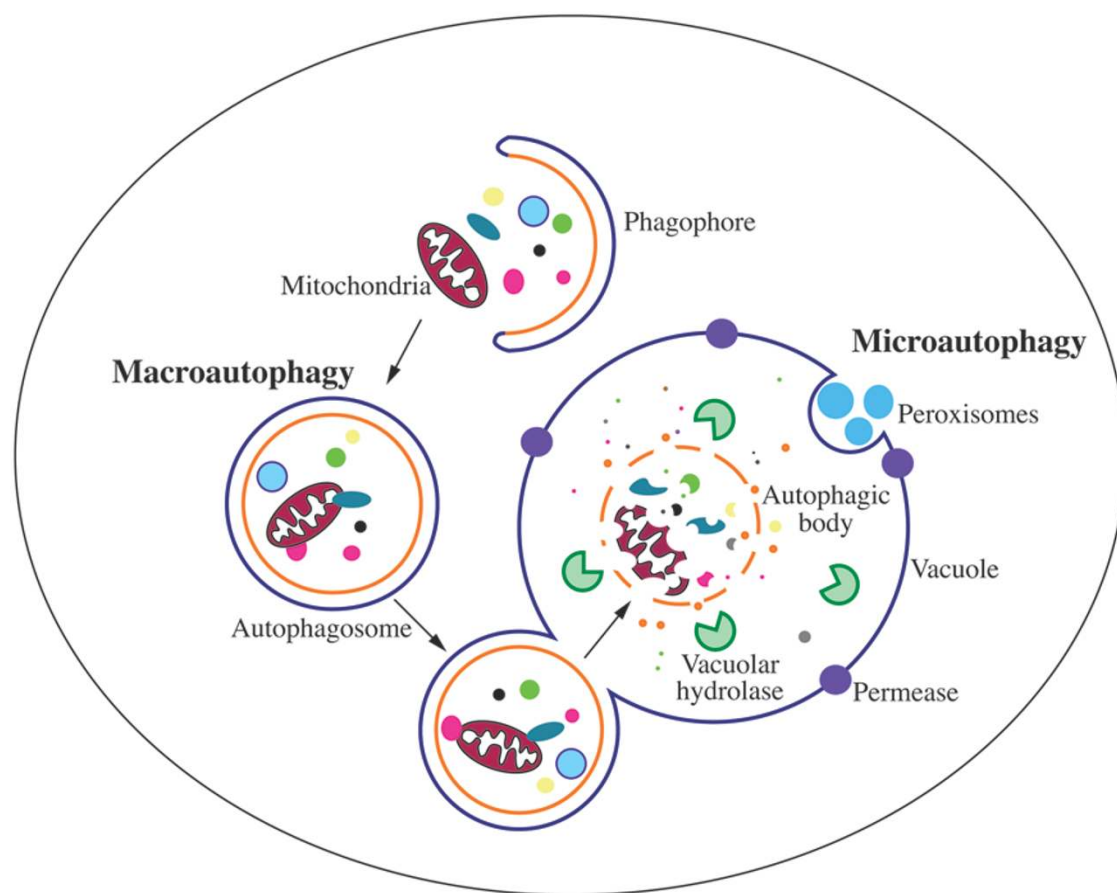


Figure 1 Schematic depiction of the two main types of yeast autophagy. In macroautophagy, random cytoplasm and dysfunctional organelles are sequestered by the expanding phagophore, leading to the formation of the autophagosome. The autophagosome subsequently fuses with the vacuole membrane, releasing the autophagic body into the vacuole lumen. Eventually, the sequestered cargos are degraded or processed by vacuolar hydrolases. In microautophagy, cargos are directly taken up by the invagination of the vacuole membrane, followed by scission, and subsequent lysis, exposing the cargo to vacuolar hydrolases for degradation.

and are not found in mammalian lysosomes.

Autophagosome: The completed double-membrane-bound compartment, the product of phagophore expansion and closure, which sequesters cytoplasmic cargos during macroautophagy [5, 6].

PAS: The phagophore assembly site; a peri-vacuolar location or compartment where the nucleation of the phagophore initiates. Most components of the macroautophagy core machinery locate at least transiently at the PAS in yeast; however, a mammalian equivalent of the PAS has not been identified [7-9].

Phagophore: The double-membrane structure that functions in the initial sequestering of cargo, and thus the active compartment of macroautophagy. The phagophore further elongates/expands and ultimately closes, generating a completed autophagosome [10].

The history of discovery

The term “autophagy” was coined by Christian de Duve at the CIBA Foundation Symposium on Lysosomes in 1963. This was based on his discovery of lysosomes in 1955, which won him the Nobel Prize in Physiology or Medicine in 1974 [11]. Autophagy was named morphologically by the observations from electron microscopy of rat hepatic cell lysosomes, where single membrane vesicles, containing cytoplasm or organelles such as mitochondria and endoplasmic reticulum (ER) were observed [12-15]. Based on Thomas Ashford’s and Keith Porter’s early findings in 1962 that revealed the presence of sequestered organelles in rat hepatocytes following their exposure to glucagon [12], de Duve and his colleagues confirmed that this hormone can induce autophagy [16]. Subsequently, other researchers further examined the hormonal and enzymatic regulation of autophagy.

For instance, Per Seglen and Paul Gordon discovered the inhibitory role of 3-methyladenine [17]. As de Duve mentioned in 1966 that autophagy could be selective [14], the specific sequestration of organelles including ER [18], mitochondria [19] and peroxisomes [20] by autophagy was demonstrated in the following decade. Although autophagy was initially revealed in mammalian systems, the molecular understanding of it was largely expanded and facilitated through genetic studies in yeast. Genetic screens of macroautophagy-defective mutants in *Saccharomyces cerevisiae* were carried out by the Ohsumi and Thumm laboratories [21, 22]. Shortly thereafter, the first autophagy-specific gene, *APG1* (now *ATG1* [23]), was identified, and the corresponding gene product, Apg1/Atg1, was characterized as a Ser/Thr protein kinase [24]. Multiple laboratories working on autophagy primarily in *S. cerevisiae*, *Pichia pastoris* and *Hansenula polymorpha* subsequently discovered more than thirty autophagy-related (*ATG*) genes. The molecular basis of autophagy was first connected to human diseases by Beth Levine's laboratory through the identification of *BECN1/VPS30/ATG6* as a tumor suppressor gene [25]. Subsequently, a series of studies uncovered the connections between

autophagy and pathophysiological conditions, such as pathogen infection [26-29] and neurodegeneration [30], and its dual role in cell growth and death [31, 32].

Nonselective and selective autophagy

There are three primary types of autophagy: microautophagy, macroautophagy and a mechanistically unrelated process, chaperone-mediated autophagy that only occurs in mammalian cells. Both micro and macroautophagy can be selective or nonselective and these processes have been best characterized in yeast [33] (Table 1). As noted above, the most distinguishing feature of macroautophagy is the formation of the double-membrane bound phagophore and autophagosome (Figure 1). In contrast, during microautophagy the cargos are sequestered by direct invagination or protrusion/septation of the yeast vacuole membrane [34]. Nonselective autophagy is used for the turnover of bulk cytoplasm under starvation conditions, whereas selective autophagy specifically targets damaged or superfluous organelles, including mitochondria and peroxisomes, as well as invasive microbes; each process involves a core set of machinery, as well as specific components, and accordingly is identified with

Table 1 The main types of autophagy in yeast

Name		Cargo	Characteristics
Macroautophagy			
	Nonselective		Random cytoplasm
		Cvt pathway	Vacuolar hydrolases
			Autophagosome or Cvt vesicle formation; Atg19 and Atg34 are receptors, Atg11 is a scaffold
	Selective		
		Mitophagy	Damaged or superfluous mitochondria
		Pexophagy	Superfluous peroxisomes
Microautophagy			
	Nonselective		Random cytoplasm, vacuole membrane
		Mitochondria	Vacuole invagination or protrusion
		Peroxisomes	Vacuole invagination or protrusion
		Nuclear membrane	Vacuole invagination, requires Nvj1 and Vac8

Adapted from Table 1 of ref [171].

a unique name — mitophagy for selective mitochondria degradation by autophagy, pexophagy for peroxisomes, xenophagy for microbes, *etc.* [35, 36]. In this review, we will mainly focus on macroautophagy, since the molecular or physiological studies of nonselective microautophagy are limited, and selective microautophagy shares most of the same machinery with macroautophagy (hereafter autophagy).

Identification of the PAS and omegasome, and the origin of the phagophore membrane

Identification of the PAS

Atg8 was the first Atg protein characterized to mark the phagophore and autophagosome [37, 38]. Immunofluorescence microscopy revealed that in rich conditions, Atg8 is primarily dispersed in the cytoplasm as small puncta, while it forms larger puncta in the vicinity of the vacuole when yeast cells are switched to starvation conditions; the nature of the smaller puncta is not known, whereas the larger puncta correspond to nascent autophagosomes. Subsequent studies with green fluorescent protein (GFP)-tagged chimeras demonstrated that most of the Atg proteins reside at this location, at least transiently [8]. Accordingly, this site is proposed to be where the phagophore assembles, and is thus termed the phagophore assembly site, or PAS [8, 9]. The precise nature of the PAS with regard to its protein and membrane composition, or the reason for its perivacuolar localization, is not known, but assembly of the Atg proteins at the PAS occurs in a hierarchical manner [39]. The PAS forms constitutively, and in vegetative conditions a key component that marks this site is Atg11; upon autophagy induction, the structure transitions into an autophagy-specific PAS and the function of Atg11 is replaced by a ternary complex of Atg17-Atg31-Atg29 that assembles at the PAS along with Atg1 and Atg13 [40, 41]. Atg9, which shuttles between the PAS and peripheral sites, plays an important role in directing membrane to the PAS that is needed for autophagosome formation, and it localizes to the PAS at a similar time as the Atg1 kinase complex. Subsequently, the PtdIns3K and Atg12-Atg5-Atg16 complexes are recruited to the PAS; the latter acts in part as an E3 ligase to facilitate the formation of Atg8-PE, one of the last proteins that are recruited to the PAS. In mammalian cells, the colocalization of ATG proteins is observed at multiple sites [42-44], instead of a single PAS as in yeast.

Origin of the phagophore

Until recently, the dogma was that the phagophore and autophagosome formed *de novo*, meaning that the se-

questering membrane does not form in “one step” from a pre-existing organelle already containing its cargo as occurs throughout the secretory pathway [45, 46]. Regardless of the specific mechanism, autophagosome formation is thought to occur by the expansion of the phagophore, that is, a sheet of membrane that would correspond to the size of a complete autophagosome does not separate from the endomembrane system and simply curl up to form an autophagosome. Rather, after nucleation the phagophore grows through the addition of membrane from one or more donor sources [1, 47]; however, the origin of the phagophore membrane is still under debate. Various lines of data suggest a role for almost every membrane compartment in contributing to formation of the autophagosome, including the ER, mitochondria, the Golgi apparatus and the plasma membrane [48-52]. As discussed above, there is no absolute equivalent of the yeast PAS in mammalian cells. Instead, there is evidence that there may be at least two separate mechanisms for generating autophagosomes. One mechanism would involve the delivery of membrane from various organelles, as is thought to occur in yeast, and the second utilizes an omega-shaped membrane structure or cradle, derived from phosphatidylinositol-3-phosphate (PtdIns3P)-enriched ER subdomains, termed an omegasome [53].

Core molecular machinery of autophagosome formation

The transition of autophagy studies from morphology to molecular machinery relied on the identification of the *ATG* genes [23]. As mentioned above, genetic screens for autophagy-defective mutants in yeast have led to the identification of over 30 *ATG* genes [21-23, 54], many of which have known orthologs in higher eukaryotes.

Among these *ATG* genes, one subgroup, consisting of approximately 18 genes (Table 2), is shared among the various types of autophagy including nonselective macroautophagy, the cytoplasm-to-vacuole-targeting (Cvt) pathway (a biosynthetic autophagy-like pathway), mitophagy and pexophagy. More specifically, the corresponding gene products of this subgroup are required for autophagosome formation, and thus are termed the core autophagy machinery. The core Atg proteins can be divided into different functional subgroups: (A) the Atg1/ULK complex (Atg1, Atg11, Atg13, Atg17, Atg29 and Atg31) is the initial complex that regulates the induction of autophagosome formation; (B) Atg9 and its cycling system (Atg2, Atg9 and Atg18) play a role in membrane delivery to the expanding phagophore after the assembly of the Atg1 complex at the PAS; (C) the PtdIns 3-kinase (PtdIns3K) complex (Vps34, Vps15, Vps30/Atg6, and Atg14) acts at the stage of vesicle nucleation, and is in-

Table 2 Atg/ATG proteins in the core machinery of autophagosome formation

	Yeast	Mammals	Characteristics and functions
Atg1/ULK complex	Atg1	ULK1/2	Ser/Thr protein kinase; phosphorylated by M/TORC1; recruitment of Atg proteins to the PAS
	Atg13	ATG13	Regulatory subunit through phosphorylation by M/TORC1 and/or PKA, linker between Atg1 and Atg17
	Atg17	RB1CC1/FIP200 (functional homolog)	Scaffold protein, ternary complex with Atg29 and Atg31. Phosphorylated by ULK1; scaffold for ULK1/2 and ATG13
	Atg29		Ternary complex with Atg17 and Atg31
	Atg31		Ternary complex with Atg17 and Atg29
	Atg11		Scaffold protein in selective autophagy for PAS organization
		C12orf44/Atg101	Component of the complex with ATG13 and RB1CC1
Atg9 and its cycling system	Atg2	ATG2	Interacts with Atg18
	Atg9	ATG9A/B	Transmembrane protein, directs membrane to the phagophore
	Atg18	WIP1/2	PtdIns3P-binding protein
PtdIns3K complex	Vps34	PIK3C3/VPS34	PtdIns 3-kinase
	Vps15	PIK3R4/VPS15	Ser/Thr protein kinase
	Vps30/Atg6	BECN1	Component of PtdIns3K complex I and II
	Atg14	ATG14	Component of PtdIns3K complex I
Atg8 Ubl conjugation system	Atg8	LC3A/B/C, GABARAP, GABARAPL1/2	Ubl, conjugated to PE
	Atg7	ATG7	E1-like enzyme
	Atg3	ATG3	E2-like enzyme
	Atg4	ATG4A/B/C/D	Deconjugating enzyme, cysteine proteinase
Atg12 Ubl conjugation system	Atg12	ATG12	Ubl
	Atg7	ATG7	E1-like enzyme
	Atg10	ATG10	E2-like enzyme
	Atg16	ATG16L1	Interacts with Atg5 and Atg12
	Atg5	ATG5	Conjugated by Atg12

Adapted from Table 1 of ref [56].

volved in the recruitment of PtdIns3P-binding proteins to the PAS; (D) two ubiquitin-like (Ubl) conjugation systems: the Atg12 (Atg5, Atg7, Atg10, Atg12 and Atg16) and Atg8 (Atg3, Atg4, Atg7 and Atg8) conjugation systems play roles in vesicle expansion [39, 55-57].

Characterization and function of the Atg proteins

The autophagy-related proteins have been described in great detail in numerous reviews. Here, we highlight some of the key information.

Yeast Atg1 kinase complex

The Atg1 kinase complex regulates the magnitude

of autophagy [58-62]. This complex also plays a role in mediating the retrieval of Atg9 from the PAS [63]. The Atg1 kinase complex is extensively regulated, receiving input from several signaling pathways. In yeast, much of the regulation centers around nutrient signaling and involves kinases that sense the nutritional status of the cell including the target of rapamycin (TOR), protein kinase A (PKA), Gcn2 and Snf1. The yeast Atg1 kinase complex includes Atg1, a Ser/Thr protein kinase, Atg13, a regulatory subunit, and the Atg17-Atg31-Atg29 complex, which may function in part as a scaffold (Figure 2). Atg1 is the only kinase of the core autophagy machinery; however, despite the fact that it was the first Atg protein to be identified, the key physiological substrate of

yeast Atg1 is not known. Atg1 kinase activity is required for autophagosome formation and the Cvt pathway, although there are conflicting data concerning the changes in kinase activity when yeast cells shift from vegetative growth to autophagy-inducing conditions [24, 64, 65]. Atg1 recruitment to the PAS may be regulated by PKA-dependent phosphorylation [66]. TOR also phosphorylates Atg1, and autophosphorylation is important in regulating Atg1 kinase activity [67, 68].

Atg13 is required for Atg1 kinase activity. Atg13 is hyperphosphorylated under nutrient-rich conditions, and its phosphorylation is regulated by TOR complex 1 (TORC1) [69] and/or PKA [70]. Atg13 is rapidly, but only partially, dephosphorylated upon autophagy induction [71]. These data, coupled with affinity isolation studies, led to a model suggesting that the interaction between Atg1 and Atg13 is controlled by Atg13 phosphorylation — dephosphorylation would lead to the formation of an Atg1-Atg13 complex, and subsequent activation of Atg1 kinase activity [61]. Recent data, however, suggest that Atg1 and Atg13 interact in a constitutive manner [72], which is similar to the situation in other organisms.

Atg17-Atg31-Atg29 exists as a stable complex under both vegetative and starvation conditions, suggesting that formation of the complex is not involved in autophagy induction. Atg31 bridges Atg17 and Atg29, Atg17 binds Atg13, and Atg29 binds Atg11; the latter is a scaffold protein that may also be considered part of the Atg1 kinase complex because it binds Atg1 and is involved in the transition of the PAS that occurs during the switch from vegetative to starvation conditions [40, 41, 58, 73–75]. Both Atg29 and Atg31 are phosphoproteins. The phosphorylation of Atg29 appears to be involved in regulation and is proposed to alter the conformation of an inhibitory C-terminal peptide to allow the protein to become active [41].

Mammalian ULK1/2 Complex

The mammalian ULK1/2 complex includes ULK1/2 (mammalian homologs of Atg1), ATG13 (a homolog of yeast Atg13), RB1CC1/FIP200 (a putative Atg17 homolog) and C12orf44/ATG101 (the latter component is not conserved in *S. cerevisiae*) [76–80]. ULK1/2 interact with ATG13, which directly binds RB1CC1 and mediates the latter's interaction with the ULKs [76, 81]. In mammalian cells, ULK1 kinase can be activated by both AMP-activated protein kinase (AMPK)-dependent (glucose starvation) and -independent (amino acid starvation) processes [82]. The ULK1/2-ATG13-RB1CC1 interaction is nutrient independent, forming a complex even in nutrient-rich conditions [80]. In this situation, MTORC1 phosphorylates and inhibits ULK1/2 and ATG13, disrupt-

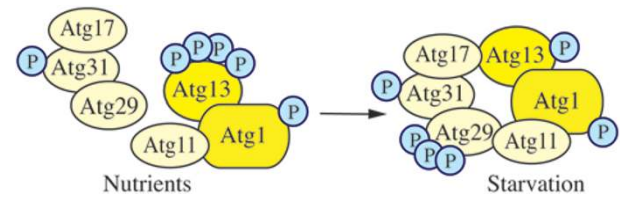


Figure 2 Yeast Atg1 kinase complex. The yeast Atg1 kinase complex includes Atg1, a Ser/Thr protein kinase, Atg13, a regulatory subunit required for Atg1 kinase activity, and the Atg17-Atg31-Atg29 complex, which may function in part as a scaffold. Atg31 bridges Atg17 and Atg29, Atg17 binds Atg13, and Atg29 binds Atg11.

ing the interaction between ULK1 and AMPK [82]. In contrast, under autophagy-inducing conditions, MTOR is released from the complex, resulting in activation of ULK1/2, which phosphorylates, and presumably activates, ATG13 and RB1CC1. AMBRA1 and BECN1, components of the autophagy-promoting PtdIns3K complexes, are also phosphorylated by ULK1; these modifications result in localization of the lipid kinase complex to the ER and its activation [83, 84].

Yeast Atg9

In yeast cells, Atg9 is a transmembrane protein that transits between the PAS and peripheral sites (Figure 3); the latter have been termed Atg9 reservoirs or tubulovesicular clusters, and are proposed to represent sites from which membrane is delivered to the forming phagophore [49, 63, 85], although the exact role of Atg9 in this process is not clear. Atg9 is proposed to consist of six transmembrane domains, with both the amino and carboxyl termini exposed in the cytosol. Atg9 self-interacts and may exist in a complex [85, 86].

Atg9 localization to the PAS from the peripheral sites (referred to as anterograde transport) depends on Atg11, Atg23 and Atg27 [63, 87–89]. Return to the peripheral sites (retrograde movement) requires Atg1-Atg13, Atg2 and Atg18; PAS recruitment of the latter involves binding to PtdIns3P and thus necessitates the function of Atg14 and the class III PtdIns3K complex [63]. Atg27 is a type I integral transmembrane protein, while the other components involved in Atg9 movement are soluble and/or peripherally associated with membranes.

Mammalian ATG9

The mammalian Atg9 homolog ATG9A localizes to the *trans*-Golgi network and late endosomes in nutrient-rich conditions [44]; another Atg9 ortholog, ATG9B, displays a similar localization, but is expressed only in the placenta and pituitary gland, whereas ATG9A is expressed ubiqu-

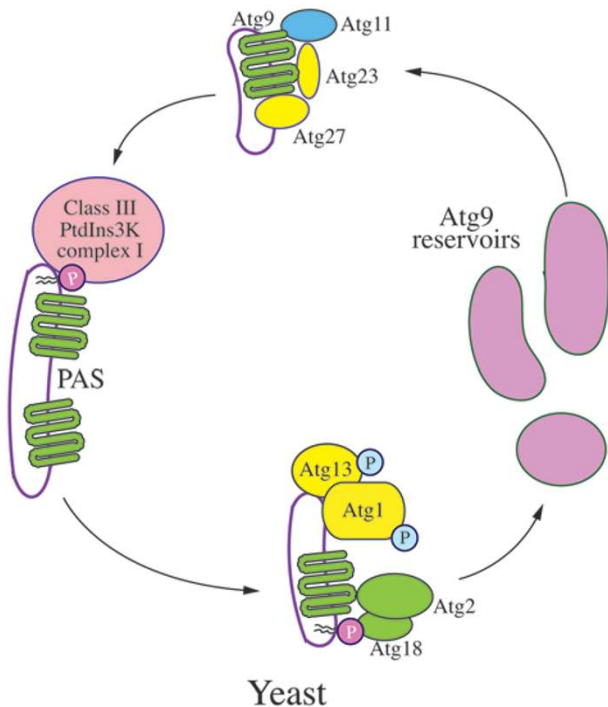


Figure 3 Trafficking of yeast Atg9. In yeast cells, Atg9 is a transmembrane protein that transits between the PAS and peripheral sites (Atg9 reservoirs). Atg9 localization to the PAS from the peripheral sites depends on Atg11, Atg23 and Atg27. Return to the peripheral sites requires Atg1-Atg13, Atg2 and Atg18; PAS recruitment of the latter involves binding to PtdIns3P, the product of the class III PtdIns3K complex.

uitously [43]. ATG9 appears to have a conserved role in coordinating membrane transport from donor sources to the phagophore and displays a cycling pattern similar to that seen in yeast [44, 90]. ATG9 and ATG16L1 also appear to traffic at least in part on vesicles derived from the plasma membrane, which are delivered to the recycling endosome; the two proteins are initially present in separate vesicular populations that subsequently fuse as part of the process of autophagosome formation [91]. ATG9 movement is dependent on the activity of the ULK1 and PIK3C3/VPS34 kinases, as well as WIPI2 (a homolog of yeast Atg18).

Yeast PtdIns3K complexes I and II

In yeast, Vps15 (a putative regulatory protein kinase that is required for Vps34 membrane association), Vps30/Atg6, Vps34 (the PtdIns3K), Atg14 and Atg38, form the autophagy-specific class III PtdIns3K complex I (Figure 4), which localizes at the PAS [92-94]. One key role of the PtdIns3K complex, the generation of PtdIns-3P, is presumably to recruit PtdIns3P-binding proteins such as Atg18 to the PAS. Vps15, Vps34 and Vps30 are

present in a second complex that includes Vps38 instead of Atg14. Thus, the latter protein appears to determine the specificity of the PtdIns3K complex I for macroautophagy.

Mammalian class III PtdIns3K

Mammalian cells have both a class I phosphoinositide 3-kinase and a class III PtdIns3K; the class III enzyme complexes consist of homologs of Vps34 (PIK3C3), Vps15 (PIK3R4) and Vps30 (BECN1) [95]. These core components are part of at least three different complexes [96, 97]. The ATG14 complex contains ATG14 and is regulated by the binding of BECN1 to AMBRA1 and BCL2, which stimulate and inhibit the complex, respectively. This complex functions similarly to the yeast PtdIns3K complex I. The UVRAG complex replaces ATG14 and AMBRA1 with UVRAG and its positive regulator SH3GLB1, and participates in both endocytosis and macroautophagy. In the third complex, SH3GLB1 is replaced with KIAA0226/Rubicon, which inhibits UVRAG; this complex acts to negatively regulate macroautophagy.

Yeast Ubl conjugation systems

There are two Ubl protein conjugation systems that participate in macroautophagy [48]. These include two Ubl proteins, Atg8 and Atg12, which are used to generate the conjugation products Atg8-PE and Atg12-Atg5, respectively (Figure 5). The Ubl conjugation systems

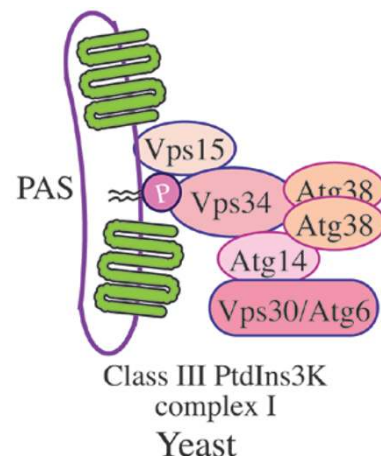


Figure 4 Yeast PtdIns3K complex I. In yeast, Vps15 (a putative regulatory protein kinase that is required for Vps34 membrane association), Vps30/Atg6, Vps34 (the PtdIns3K), Atg14 and Atg38 form the autophagy-specific class III PtdIns3K complex, which localizes at the PAS. Vps15, Vps34 and Vps30 are present in the second class III PtdIns3K complex that includes Vps38 instead of Atg14.

participate in phagophore expansion, although their functions are not fully understood. The Atg12–Atg5 conjugate, along with a third component, Atg16, may act in part as an E3 ligase to facilitate the conjugation of Atg8 to PE, and the amount of Atg8 can regulate the size of autophagosomes [98]; however, Atg8 also functions in cargo binding during selective autophagy [99].

Yeast Atg8 is initially synthesized with a C-terminal arginine residue that is removed by Atg4, a cysteine protease [38, 100]. During autophagy, Atg8 is released from Atg8–PE by a second Atg4-dependent cleavage referred to as deconjugation [101], whereas there is no similar cleavage that separates Atg12 from Atg5. Both of the Ubl protein conjugation systems share a single E1-like activating enzyme, Atg7 [100, 102–104]. The E2-like conjugating enzymes are Atg3 for Atg8, and Atg10 for Atg12 [102, 105].

Mammalian Ubl protein conjugation systems

The Ubl protein conjugation systems are highly conserved from yeast to mammals [106–109]. One of the primary differences is the existence of multiple homologs of yeast Atg8, which are divided into two subfamilies, LC3 and GABARAP. LC3 functions at the stage of phagophore elongation, whereas GABARAP proteins act at a later stage of maturation [110]. There are four mammalian ATG4 homologs, with ATG4B carrying out the primary role in macroautophagy [111]. As in yeast, this

enzyme cleaves after the C-terminal glycine of proLC3 to form cytosolic LC3-I, followed by its subsequent conjugation to PE to generate the membrane-associated LC3-II form [112]. The GABARAP proteins undergo a similar type of posttranslational modification.

Ligand-receptor-scaffold system

In addition to the core machinery of autophagosome formation, other proteins play significant roles in autophagy, in particular during selective types of macroautophagy. Thus, in *S. cerevisiae*, Atg19 and its paralog, Atg34, are receptors for the biosynthetic delivery of resident vacuolar hydrolases [113, 114], Atg32 is a receptor for mitophagy [115, 116] and Atg36 functions in a similar manner during pexophagy [117] (Figure 6). In the case of Atg19, Atg34 and Atg36, the receptor binds a ligand on the cargo, which corresponds to the propeptide of prApe1 for the Cvt pathway, and Pex3 during pexophagy; Atg32 is a mitochondrial membrane protein and does not appear to bind a separate ligand. Each of the receptors binds the scaffold Atg11 and subsequently interacts with Atg8 to link the cargo with the autophagic machinery [118].

Selective autophagy: Cvt pathway, pexophagy and mitophagy

In selective autophagy, the overall morphology is large-

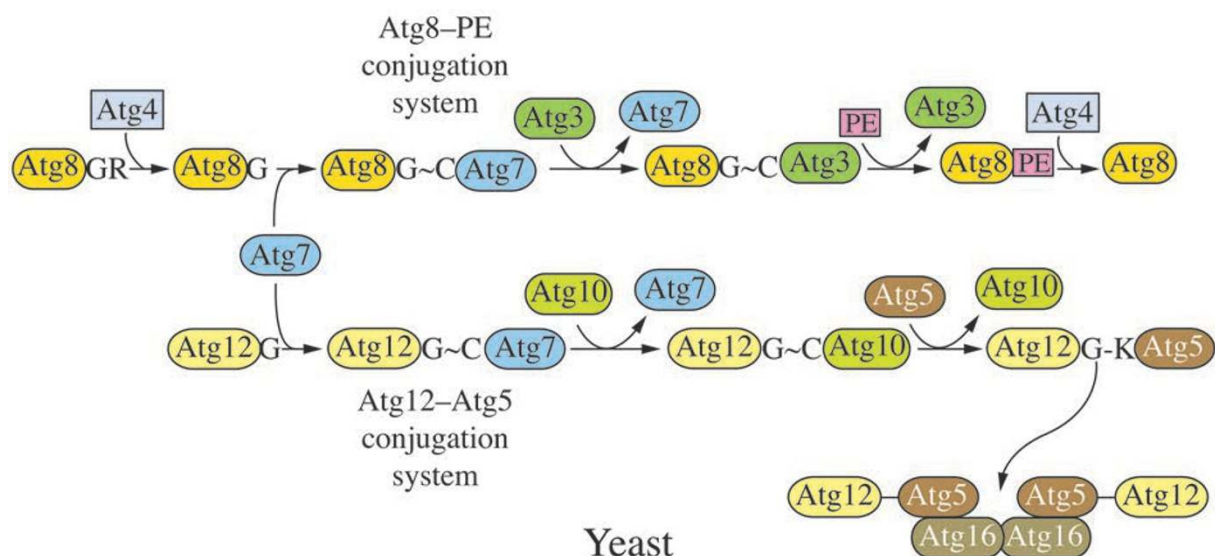


Figure 5 Yeast Ubl conjugation systems. There are two Ubl protein conjugations systems, involving Atg8 and Atg12, which are used to generate the conjugation products Atg8–PE and Atg12–Atg5, respectively. Atg8 is initially processed by Atg4, a cysteine protease, and during autophagy, Atg8 is released from Atg8–PE by a second Atg4-dependent cleavage. The Atg12–Atg5 conjugate, along with Atg16, facilitates the conjugation of Atg8 to PE. Both of the Ubl protein conjugation systems share a single activating enzyme, Atg7. The conjugating enzymes are Atg3 for Atg8, and Atg10 for Atg12.

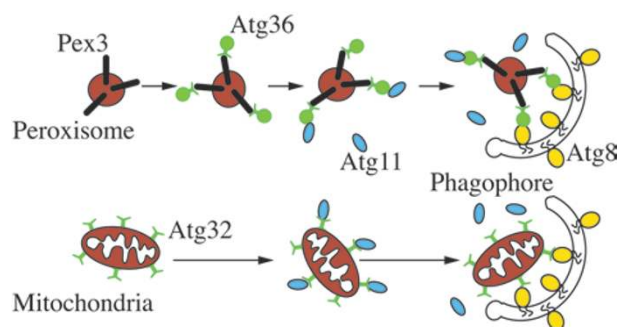


Figure 6 Schematic depiction of the mechanism of selective autophagy. Using mitophagy and pexophagy as examples, the model of ligand-receptor-scaffold complex is shown, with Pex3 functioning as a ligand, Atg36 and Atg32 as receptors and Atg11 as the scaffold protein; in the case of mitophagy there does not appear to be a separate ligand on the cargo since Atg32 is an integral membrane protein of the mitochondrial outer membrane.

ly the same as during nonselective autophagy with one primary distinguishing feature; in selective autophagy, the membrane-bound vesicle targets specific cargos, instead of random cytoplasm. In addition to the core machinery, a cargo ligand (a molecular entity on the surface of the cargo that is recognized by a receptor), receptor and scaffold are involved; the latter act to specifically bind the cargo and provide a bridge between the cargo and the core machinery [118, 119]. Accordingly, during selective autophagy the phagophore is closely apposed to the cargo, and the completed autophagosome excludes bulk cytoplasm. As noted above, there are different types of selective autophagy according to the cargo types. Here we will focus on the Cvt pathway, mitophagy and pexophagy as examples (Table 1).

Cvt pathway

With regard to the details of cargo recognition, the Cvt pathway was the first well-studied type of selective autophagy in yeast; it is also the primary example of a biosynthetic rather than degradative use of autophagy [119-122]. The double-membrane sequestering vesicle is termed a Cvt vesicle rather than an autophagosome; as noted above, this vesicle contains selective cargos of the Cvt pathway, but excludes bulk cytoplasm, in accordance with the biosynthetic and constitutive nature of the Cvt pathway compared to starvation-induced autophagy. The Cvt pathway delivers precursor forms of hydrolases, such as Ape1 (aminopeptidase I), Ape4 and Ams1 (α -mannosidase), from the cytoplasm to the vacuole, where they carry out their functions. The formation of the Cvt vesicle at the PAS is driven by the formation

of a cargo complex, composed primarily of oligomerized precursor (prApe1) along with a few other resident vacuolar hydrolases [123-125]. Atg19 acts as a cargo receptor that binds the ligand (the prApe1 propeptide) and is thus recruited to the complex [113]. Subsequently, the interaction between Atg19 and Atg11, a key scaffold that functions in most or all types of selective autophagy in yeast, mediates the delivery of the cargo complex to the PAS [99, 126]. Atg19 also interacts with Atg8, which might play a role in tethering the Cvt complex to the phagophore [127] and in directing the selective sequestration of the cargo during vesicle formation [99].

Mitophagy

Mitophagy, the selective degradation of mitochondria by autophagy, occurs through both macro- and microautophagic processes. When yeast cells are shifted from a nonfermentable carbon source such as glycerol to a fermentable/preferred carbon source such as glucose, a part of the mitochondria population that is now superfluous is subjected to degradation through mitophagy, and this is particularly enhanced when cells are simultaneously starved for nitrogen. The selective recognition of the cargo is mediated through the mitochondria outer-membrane protein Atg32, which functions as a receptor; this protein is not required for the Cvt pathway or nonselective autophagy [128]. Similar to the situation with Atg19, Atg32 interacts with Atg11 to recruit mitochondria to the PAS, and also binds to Atg8 to complete vesicle formation [116, 128] (Figure 6). Atg33 is another protein that is specifically involved in mitophagy [115, 129]. Mitophagy can also be induced by growth in a nonfermentable carbon source when yeast cells grow past the logarithmic phase. Though post-logarithmic-induced mitophagy does not completely share the same mechanism with starvation-induced mitophagy, they are both controlled by two mitogen-activated protein kinases (MAPKs), Slt2 and Hog1 [129, 130]. Slt2 affects the recruitment of mitochondria to the PAS, and is also required in pexophagy, whereas Hog1 is mitophagy-specific [130]. In addition, the absence of the mitochondrial outer membrane protein Uth1 impairs the induction of autophagy by rapamycin treatment or starvation [131], suggesting its potential role as a ligand [118]. Ptc6/Aup1, a yeast mitochondrial protein phosphatase homolog, is required for post-logarithmic phase mitophagy [132], and the function of Ptc6 in mitophagy may be based on its regulation of Rtg3-dependent transcription [133].

Specific degradation of obsolete mitochondria is also observed in mammalian cells, although Atg32 is not conserved in higher eukaryotes [134-136]. Instead, FUNDC1 and BNIP3, BNIP3L/NIX and SQSTM1/p62 function

as receptors in mammalian mitophagy, depending on the inducing conditions, during hypoxia, erythrocyte maturation or damage-induced mitophagy, respectively [136–138]. Moreover, mutations of PARK2/Parkin and PINK1 are linked to Parkinson [136, 139, 140] and Gaucher diseases [141]. An elegant model for PINK1 stabilization on, and PINK1-dependent recruitment of PARK2 to, the mitochondrial outer membrane, that relies on the E3 ubiquitin ligase activity of PARK2 to induce mitophagy, has been proposed in mammalian cells [136].

Pexophagy

Similar to mitophagy, pexophagy happens when the medium of fungi is switched from oleic acid or methanol, where peroxisomes (the only organelle in yeast that can carry out β -oxidation) proliferate, to glucose or nitrogen-starvation medium [142]. Atg36 and PpAtg30 are receptors for pexophagy in *S. cerevisiae* and *P. pastoris*, respectively [143, 144]. In both yeast and mammalian cells Pex3 and Pex14 function as cargo ligands [145, 146]. As with mitophagy, pexophagy is regulated by the Slt2 pathway [130].

Structural studies of the Atg proteins

The tremendous amount of molecular genetic data on the yeast Atg proteins has provided considerable information regarding protein-protein interactions; however, detailed structural information at the atomic level has lagged behind. Structural data for the Atg proteins have started to provide direct evidence for protein interactions and are also generating insights about protein function. An increasing amount of crystallography data for the Atg proteins has been reported in recent years. While these data have contributed to the elucidation of autophagy, structural information for many of the Atg proteins is still unavailable. Here, we review the current data for those proteins with available structural information.

Atg1 kinase complex

Atg1, together with the regulatory subunit Atg13, forms the core machinery of the Atg1 kinase complex. Structural data of the entire putative holo-complex (i.e., containing at least Atg1-Atg11-Atg13-Atg17-Atg31-Atg29, Figure 2) are still unavailable. Crystallization of the Atg13 N-terminal domain (NTD) shows a HORMA (Hop1, Rev1 and Mad2) fold [147]. This domain is important for Atg14 recruitment but not for Atg13 localization or its interaction with Atg1.

In addition to the core machinery, a stable ternary complex composed of Atg17-Atg31-Atg29 (written in the order that reflects the known protein interactions) is

also important for the induction of nonselective autophagy. Structural studies [41, 75, 148] reveal that Atg17 is a coiled-coil protein that is made up of four helices. The Atg17 monomer exhibits a crescent shape, which suggests the possibility that it binds curved membranes. The N terminus of Atg17 is involved in binding Atg13. The Atg31-Atg29 proteins are located toward the ends of the Atg17 crescent, where Atg31 serves as a connector bridging Atg17 and Atg29. Atg31 binds to Atg17 through its C-terminal α -helix and interacts with Atg29 via its N-terminal β sandwich. No direct interaction is detected between Atg17 and Atg29. The position of Atg31-Atg29 indicates that this subcomplex may function in regulating Atg17 membrane binding. The ternary complex needs dimerization to be functional, and this is essential for the initiation of autophagy. The dimerization is only mediated by Atg17 helix α_4 , which is located in the C-terminal region; thus, it does not involve Atg31-Atg29 and may be independent of ternary complex formation. Atg17 dimerization stabilizes the two crescents where they oppose each other, making a “wave” or “S” shape. This two-crescent conformation suggests that the dimer may be involved in both vesicle binding and tethering. Previous studies suggest that the C-terminal domain of Atg1 can also bind vesicles and contains the binding site for Atg13 [149]. The relationship between this domain and Atg17-Atg31-Atg29 has not been determined. Both Atg29 and Atg31 are phosphoproteins, and phosphorylation of Atg29 appears to alter the conformation of the protein to alleviate inhibition by the extreme C terminus as discussed above [41]. The structure of the C-terminal EAT (“early autophagy targeting/tethering”) domain of Atg1 has not been solved, but based on its predicted helical structure and the observation that it can bind highly curved vesicles, it may sense membrane curvature, dimerize and tether liposomes [75].

Atg9 complex

Atg9 was the first identified transmembrane protein in the Atg protein family, but detailed structural information is not available. Similarly, no structural information has been published for the Atg11, Atg23 and Atg27 proteins, which are involved in Atg9 localization to the PAS [63, 87–89] (Figure 3). Atg18, together with Atg2, helps Atg9 cycle from the PAS to peripheral sites [63]. Although the structure of Atg18 has not been solved, a paralog, Hsv2, which shares high sequence similarity with Atg18, has been studied in different yeast strains [150–152]. Both proteins belong to the “PROPPINs” family, which are defined by β propeller structures and the existence of phosphoinositide binding sites [152, 153]. Hsv2 displays the typical propeller fold that consists of seven β blades.

In addition, Hsv2 (and Atg18 along with a second paralog, Atg21) harbor an FRRG motif, which is required for phosphoinositide binding. Detailed analysis indicates that Hsv2 possesses two PtdIns3P binding sites, located in blade 5 and blade 6. Mutations in the corresponding sites in Atg18 result in loss of Atg18 localization. Moreover, the blade 6 $\beta 3$ - $\beta 4$ loop of Hsv2 proves to be important for protein docking to the membrane. When several hydrophobic residues in this region, or the corresponding residues in Atg18, are mutated, the binding of the protein to liposomes is eliminated [152]. These results suggest a potential Atg18-docking model: the disk-like Atg18 touches the membrane with its edge; two binding sites interact with PtdIns3P on the membrane surface and the hydrophobic loop penetrates the membrane to stabilize the interaction.

The Atg18 $\beta 1$ - $\beta 2$ loop and $\beta 2$ - $\beta 3$ loop in blade 2 are important for PAS targeting [151]. Blade 2 is located at the opposite position to blade 5 and blade 6, and thus is not involved in membrane docking. Additional studies indicate that the loops in blade 2 are important for Atg18-Atg2 interaction [151, 154], allowing a refinement of the model for Atg18 membrane binding. While Atg18 binds to the membrane through blades 5 and 6, blade 2, which is located on the opposite side of the protein, interacts with Atg2. The structural data further suggest that the Atg18-Atg2 interaction is independent of membrane docking. Detailed information about the Atg18-Atg2 interaction awaits progress on the crystallization of Atg2, which may also provide insight into their interdependent localization to the PAS.

Two Ubl protein conjugation systems

By far, the most structural information for the Atg proteins concerns those involved in the two Ubl protein conjugation systems (Figure 5). Atg8 is the first identified Ubl Atg protein, and there are several mammalian homologs, comprising the LC3 and GABARAP subfamilies. The crystal structure reveals that the major part of LC3 resembles a ubiquitin core, which consists of a five-stranded β sheet and two α -helices [155]. Besides the ubiquitin fold, LC3 also contains two N-terminal α -helices and an extended C-terminal tail. As noted above, Atg4 is a cysteine protease that is responsible for removal of the C-terminal residues that follow Gly of Atg8/LC3. The structural analysis of human ATG4B, the homolog of yeast Atg4 that appears to be the most relevant isoform for autophagy, shows that the catalytic residue Cys74 is located in the center of the unique papain-like domain [156, 157]. The catalytic site is masked by a regulatory loop of ATG4B when it is not interacting with its substrate. When LC3 binds ATG4B through its

ubiquitin core, both proteins undergo large conformational changes. Trp142 of ATG4B is spatially replaced by Phe119 of LC3, which generates an open conformation of the ATG4B regulatory loop, resulting in exposure of its catalytic site. The opening of the active site is so limited that only the small Gly120 of LC3 can gain access to Cys74, which then attacks the covalent bond between Gly120 and the neighboring residue. At this moment, the open regulatory loop, together with Trp142 of ATG4B, serves as a clamp to stabilize the conformation of LC3-ATG4B [157]. After the C-terminal amino acid residues are removed, processed LC3 with an exposed Gly120 is released and ATG4B goes back to its auto-inhibited conformation.

LC3/Atg8 is then sent to a non-canonical E1-like enzyme, ATG7/Atg7. The Atg7 monomer is composed of three parts: NTD, adenylation domain (AD) and C-terminal Atg7-specific domain (ECTD) [158]. The NTD of Atg7 is highly basic, and Atg3, the cognate E2 enzyme, recognizes this positively charged domain. The AD is the key functional domain in Atg7. This domain possesses a long loop region containing the catalytic residue Cys507. ATP, which is important for activation of Atg8, also binds to this domain. The C-terminal tail of the ECTD shows no secondary structure and can interact with Atg8. Biochemical data further show that this interaction is important for recognition of Atg8 by Atg7 [158, 159]. Based on the structural and biochemical data, Atg8 is first recognized by the ECTD C-terminal tail of Atg7. Next, the long loop in the Atg7 AD undergoes a large conformational change, which allows Atg8 binding to the AD through its ubiquitin core. At the same time, ATP is also bound to the Atg7 AD. The C-terminal Gly of Atg8 then becomes adenylated. Subsequently, the catalytic Cys507 of Atg7 forms a thioester bond with Atg8. The crystal structure data reveal that Atg7 functions as a homodimer, in which the monomers interact with each other through their AD [158]. Considering that the NTD of Atg7 is responsible for Atg3 binding, this suggests that Atg8 is activated by one Atg7 and then transferred to Atg3 that is bound to another Atg7 [160]. This *trans*-transfer model displays higher efficiency than a *cis*-model and may explain why Atg7 forms a homodimer.

The crystal structure of Atg3 shows a hammer-like conformation with an N-terminal head moiety and a C-terminal handle moiety [161]. The head moiety shares some similarities to canonical E2 enzymes, while the handle moiety, which is suggested to bind Atg7, is unique to Atg3. Cys234 in Atg3 forms the thioester bond with Atg8, generating the Atg3-Atg8 intermediate. The function of an E3 enzyme in the Atg8 Ubl conjugation system is not fully elucidated. One hypothesis is that the Atg12–

Atg5-Atg16 complex, which is the product of the Atg12-Ubl conjugation system in autophagy, acts as the E3 enzyme [162]. Biochemical studies indicate that the unique catalytic site of Atg3 involves a Thr residue, instead of the more typical Arg, for the transfer of Atg8 to PE [163]. Furthermore, interaction with the Atg12-Atg5 conjugate results in a change in the orientation of the catalytic cysteine, such that it moves closer to the Thr residue, facilitating the conjugation activity of Atg3.

ATG12 was first crystallized from plant [164]. The topology of *Arabidopsis thaliana* ATG12 is similar to other ubiquitin-fold proteins, and ATG12 shares a high degree of structural similarity with LC3/Atg8 except for a unique hydrophobic patch. Similar to Atg8, Atg12 is activated by Atg7. Next, it is sent to another E2 enzyme, Atg10, forming an Atg12-Atg10 intermediate. It is unclear whether Atg3 will compete with Atg10 for interaction with Atg7. Previous studies show that Atg10 bound to Atg7 could be easily replaced by Atg3, but not the reverse, indicating that Atg3 has a higher affinity for binding Atg7 [159]. Atg10 possesses a conserved E2 core that is comprised of four β sheets and two α -helices. However, Atg10 contains two additional accessory α -helices and three additional β sheets, which are important for its normal function [165, 166]. Atg12 is finally conjugated to Lys149 of Atg5, and both structural and biochemical evidence indicate that Atg10 directly interacts with Atg5 through its accessory β 5 and β 6 sheets.

Atg5 has two Ubl domains, an NTD UblA and C-terminal domain UblB, with a helix-rich domain connecting them, and an extreme N-terminal α -helix [165, 167]. Analysis suggests that Atg5 β 7 located in UblB is responsible for interacting with Atg10 [165]. After Atg12 is conjugated to Atg5, no major conformational change is detected [168]. Atg12-Atg5 prefers to interact with Atg16, forming an Atg12-Atg5-Atg16 complex. Atg16 possesses a coiled-coil C-terminal region, which is responsible for Atg16 homodimer formation [169]. The NTD of Atg16 is comprised of an α -helix and a loop moiety, and binds Atg5. Structural data reveal that the helix binds to the groove formed by UblA, UblB and the N-terminal α -helix of Atg5, while the loop moiety mainly binds to UblA [167].

As mentioned above, previous studies indicate that the Atg12-Atg5-Atg16 complex functions as an E3 ligase in Atg8 lipidation. The structural data of the ATG12-ATG5-ATG16L1N (containing the N-terminal part of ATG16L1) complex reveal that ATG16L1N binds to ATG5 at the opposite side to where ATG12 binds; no interaction is detected between ATG16L1N and ATG12 [170]. Human ATG12 can interact with ATG3 through its Phe108 residue [162]. Phe154 in plant ATG12 is

equivalent to the human ATG12 Phe108, and the former is masked in the ATG12-ATG5 complex [168]. This observation raises a possible explanation for the function of Atg12-Atg5-Atg16: The complex has two states; in the absence of Atg3-Atg8, the complex is in the closed state where Atg5 buries the Atg3-binding surface of Atg12. When Atg3-Atg8 forms, a conformational change is triggered that exposes the Atg3-binding surface in Atg12. Atg12 then interacts with Atg3 and brings the Atg3-Atg8 intermediate to the membrane, where the Atg12-Atg5-Atg16 complex facilitates Atg8-PE conjugation. Further studies are needed to support and/or refine this model.

The PtdIns3K Complexes

The PtdIns3K complex plays a critical role in autophagy through the generation of PtdIns3P, which allows the recruitment of several of the Atg proteins to the PAS. Vps34, the lipid kinase, is the only PtdIns3K in yeast. As described above, Vps34 together with Vps15, Vps30/Atg6, Atg14 and Atg38, form the class III PtdIns3K complex I, which specifically functions in autophagy [171].

The structure of Vps34 contains a C2 domain at the N terminus, a helical domain in the middle and a C-terminal catalytic domain. The C2 domain does not affect the catalytic activity of the kinase and is thought to be involved in protein-protein interactions. The crystal structure of Vps34 lacking the C2 domain has been determined [172]. Vps34 has a highly ordered phosphatidylinositol-binding loop located in its catalytic domain, while the corresponding activation loop that is critical in substrate recognition in phosphoinositide 3-kinases is largely disordered. Vps34 also contains a catalytic loop in this domain. The conserved DRH motif is found in the loop and the His residue in the motif could be the catalytic base. The C-terminal tail that is important for kinase activity is helical. Structural information also reveals that the loop between the last helices (α 11 and α 12) is flexible, which enables the helix to cover/uncover the reaction center. Thus, the final helices help to regulate the activity of Vps34 by covering the catalytic site and preventing it from touching the membrane [172].

Vps30/Atg6 forms a complex with Atg14, which, along with Atg38, links it to Vps34. The structures of the separate domains of BECN1, the mammalian ortholog of Vps30, have been solved [173-175]. BECN1 contains three domains, a BH3 domain located at the N terminus, a coiled-coil domain in the middle and a C-terminal evolutionarily conserved domain (ECD). The crystal structure information for the BECN1 ECD indicates that it consists of four α -helices, six β -strands and six loops. One significant feature of the BECN1 ECD concerns loop 4; the main ECD displays an ellipsoid conforma-

tion except for the protrusion of loop 4. At the tip of the protrusion, three aromatic amino acids form an “aromatic finger,” which is important for membrane association [173]. The coiled-coil domain is involved in forming a metastable homodimer that plays a critical role in the interactions of BECN1 with some of its many binding partners [175].

Vps15 plays a regulatory role in the complex and its kinase activity is important for Vps34 function. Vps15 contains an N-terminal kinase domain, HEAT repeats in the middle, and a C-terminal WD domain. The structure of the WD domain reveals that it is a seven-bladed propeller [176], but the function behind this structure needs to be elucidated with further analysis.

Future directions

Our knowledge of autophagy has expanded significantly during recent years. However, many challenges remain with regard to understanding the detailed mechanism of this process. Perhaps the most complex step of autophagy is that of autophagosome biogenesis. We still do not understand the details of the nucleation process, including the origin of the phagophore membrane or the function of the PAS. For example, how are lipids directed away from their normal destination and into the autophagy pathway? Similarly, there are many fundamental questions concerning phagophore and autophagosome maturation. For example, how is the curvature and size of the phagophore regulated, and what controls the timing of fusion of the completed autophagosome with the lysosome or vacuole? Although the SNARE STX17 was recently shown to bind only to completed autophagosomes [177], it is not known what controls the interaction of this protein with the sequestering compartment.

Another challenge in the field relates to the structural studies. Although the structures of some of the Atg/ATG proteins are solved, the crystal structures for many are still unavailable. In addition, x-ray crystallography provides only a static image of a protein and cannot offer any information about disordered regions that may play critical roles in function [41]. Knowing detailed structural information for the autophagy-related proteins will not only help us better understand the process of autophagy, but can also contribute to rational drug design [178]. The studies of autophagy regulation are also promising in terms of their potential health applications, and continued studies in this field will provide insight into human diseases and enable the discovery of new therapeutics aimed at their treatment [179].

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