論文 / 著書情報
Article / Book Information

Title	Dynamics and diversity in autophagy mechanisms: lessons from yeast
Author	Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y.
Journal/Book name	NATURE REVIEWS MOLECULAR CELL BIOLOGY, , , pp. 458-467
発行日 / Issue date	2009, 6
DOI	http://dx.doi.org/10.1038/nrm2708
URL	http://www.nature.com/nrm/index.html
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Dynamics and diversity in molecular mechanisms of autophagy: lessons from yeast studies

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Autophagy is one of the fundamental functions of eukaryotic cells, and well conserved from yeasts to humans. The most remarkable feature of autophagy is newly building up double membrane-bound compartments that sequester materials to be degraded in lytic compartments, which seems mechanistically distinct from conventional membrane traffic. The discovery of autophagy in yeast and the genetic tractability of this organism allowed us to identify genes responsible for this process, leading to the today's explosive growth of this research field. Analyses of those gene products, Atg proteins, have unveiled dynamic and diverse aspects of mechanisms underlying membrane formation during autophagy.

Introduction

Autophagy (or macroautophagy) was defined in mammalian cells more than 50 years ago as a delivery system of the cell's own cytoplasmic components and organelles into lysosomes for degradation[1-3]. The most critical event in autophagy is the sequestration of these materials by forming a new compartment. Induction of autophagy leads to *de novo* formation of cup-shaped membranes called isolation membranes in the cytoplasm, which expand while becoming spherical and eventually seal to become double membrane-bound structures called autophagosomes (Fig. 1). As a natural consequence of this process, a portion of the cytoplasm is confined within the autophagosome. The outer membrane of the autophagosome is subsequently fused with the lysosomal membrane to allow degradation of the contents together with the inner membrane. Therefore, in contrast to the ubiquitin-proteasome system, autophagy mediates primarily non-selective and bulk degradation of a large amount of intracellular proteins in one swoop.

Whereas autophagy is drastically induced in response to a shortage of nutrients, it is also be regulated by various physiological signals such as hormones, growth factors, and pathogen infection, and occurs even constitutively at a basal level[2, 3]. As aforementioned, cytoplasmic components are, in principle, non-specifically engulfed by autophagosomes under starvation conditions. By contrast, recent studies have revealed that autophagy can also be selective in other situations, where specific 'cargoes' including disease-related inclusions, superfluous or damaged organelles, and even invasive bacteria are enwrapped by autophagosome-like membranes. Autophagy is now used as a collective term for these related phenomena. Reflecting these diversities, in addition to its essential role for cell survival under nutrient-deprived conditions, autophagy is involved in a wide variety of physiological and pathological processes in eukaryotic organisms[3, 4]. However, molecular mechanisms of the formation of

autophagosomal membranes as well as of selective incorporation of cargoes into those membranes still remain largely unknown.

For a long time since its discovery, autophagy had been beyond the limits of molecular dissection, because electron microscopy was the only way to detect autophagy, and biochemical analysis of lysosomes was technically quite difficult. Under such situations, the budding yeast *Saccharomyces cerevisiae* proved to be an ideal organism to gain insights into genes that are essential for autophagy, and thereafter has played a role as a torch bearer for the recent vast of studies in this field. Yeast autophagy was discovered by observing vacuolar proteinase-deficient cells with a light microscope[5]. Upon shifting the cells to nutrient starvation media, within a few hours, vacuoles were filled with vesicles containing cytoplasmic components, which were termed autophagic bodies. It was then shown that the autophagic body was derived from the autophagosome by its fusion with the vacuolar membrane[6, 7]. The whole process to the vacuole proved to be essentially the same as macroautophagy, which had been described in mammalian and plant cells. Detailed analyses of autophagosomal membranes by electron microscopy showed that these membranes look thin compared with other organelle membranes[6]. Moreover, asymmetric compositions between the outer and inner membranes of the autophagosome were suggested by freeze-fracture electron microscopy: while the outer membrane contains a few particles perhaps including machinery for targeting to and fusion with the vacuole, almost no particle is observed in the inner membrane[7]. This suggested that the autophagosome is an organelle specialized for the sequestration of cytoplasmic components and their delivery into lytic compartments, and that its biogenesis involves an unconventional mechanism.

The discovery of yeast autophagy (starvation-induced autophagy) enabled a genetic screen of mutants deficient for this pathway. Autophagy-defective mutants were efficiently obtained by light microscopic selection of cells without autophagic body accumulation from a pool of mutants showing loss of viability phenotype under nitrogen starvation[8]. This screening provided us with 14 *APG* genes required for autophagy. At almost the same time, Daniel Klionsky's group started to work on the Cvt (cytoplasm-to-vacuole targeting) pathway, which mediates biosynthetic transport of a vacuolar protein, aminopeptidase I (Ape1), from the cytoplasm to the vacuole via a membrane dynamics that turned out to be quite similar to autophagy[9], and they isolated *cvt* mutants defective in this pathway[10]. Other genetic approaches also identified genes involved in autophagy as well as 'pexophagy', an autophagic degradation pathway for peroxisomes in yeasts[11-15].

These independent screens of mutants yielded different gene names (*AUT*, *GSA*, *PAG*, *PAZ*, and *PDD*), which have been unified to be *ATG* (autophagy-related gene) to avoid confusion[16]. Whereas total 31 *ATG* genes have been reported so far, 15 genes are commonly required for all of the above

pathways (starvation-induced autophagy, the Cvt pathway, and pexophagy), which are hereafter referred to as 'core *ATG*', that encode fundamental machinery for the biogenesis of autophagy-related membranes (Fig. 2). Characterization of these 15 gene products, Atg proteins, revealed that they consist of five subgroups — the Atg1 kinase and its regulators[17], the autophagy-specific phosphatidylinositol (PtdIns) 3-kinase complex[18], the Atg12 conjugation system[19], the Atg8 conjugation system[20], and yet functionally unknown proteins that interact with each other (Table 1)[21-24]. In addition to these core components, Atg proteins include those specifically required for each pathway (Fig. 2). In this review, we describe the present knowledge on the mechanisms of autophagy obtained from yeast studies, especially focusing on starvation-induced autophagy, which is the evolutionally-conserved and most fundamental mode of autophagy (see below). More specifically, we first describe the identification and basic characterization of the assembly of Atg proteins called the preautophagosomal structure (PAS), then how Atg subgroups work there to build the autophagosome, and finally dynamic aspects of the PAS revealed by recent studies.

Identification of the PAS

Immunoelectron microscopy of Atg8, one of the core Atg proteins, revealed that this protein localizes to both isolation membranes and autophagosomes; thus Atg8 now serves as a good marker for membrane dynamics during autophagy (also see below)[25]. Whereas, fluorescence microscopy showed that usually one dot per cell labeled with GFP–Atg8 is observed in close proximity to the vacuole (Fig. 3)[26]. This dot was observable even in several *atg* mutants, suggesting that it represents neither an isolation membrane nor an autophagosome. Time-lapse microscopy of GFP–Atg8 in the temperature-sensitive *atg1* strain suggested that autophagosomes are generated at or around this dot[26]. It was also found that most Atg proteins are at least partly co-localized at this peri-vacuolar dot[26]. From these results, it was likely that the dot represents the assembly of the Atg proteins responsible for autophagosome formation, and thus was termed the pre-autophagosomal structure (PAS).

Further analyses, in which the localization of each Atg protein to the PAS under autophagy-inducing conditions was systematically examined in cells that lacked one of the other Atg proteins, demonstrated that the Atg proteins organize the PAS according to hierarchical relationships among the subgroups (Fig. 3)[27, 28]. If one *ATG* gene is deleted, the PAS localization of the hypostatic Atg proteins in the hierarchy is significantly impaired. By contrast, epistatic Atg proteins, in some cases, accumulate at the PAS in the absence of the hypostatic one (see below). Among Atg proteins that belong to the same subgroup, not only hierarchical relationships but also interdependent ones are also seen. These observations suggest that the Atg proteins coordinately act to generate the autophagosome at the

PAS while interacting with each other both within and among the subgroups. It seems that the hierarchy in the PAS localization of the Atg proteins substantially represents their order of action in autophagosome formation. In the following sections, we overview the characteristics and function of each Atg subgroup in this order.

Atg1 kinase and its regulators

Autophagy in yeast is mainly a response to nutrient starvation[5]. Target of rapamycin (Tor), a master regulator of nutrient signaling, is involved in induction of autophagy, because rapamycin, a Tor inhibitor, mimics starvation and induces autophagy even under nutrient-rich conditions[29]. Although the Tor protein forms two distinct complexes, Tor complex 1 (TORC1) and TORC2[30], only TORC1 function is sensitive to rapamycin, indicating that the TORC1 branch is responsible for controlling autophagy. The addition of cAMP suppresses induction of autophagy by nutrient starvation or rapamycin treatment, suggesting that the cAMP-dependent protein kinase (PKA) also has an inhibitory role in autophagy[29, 31, 32].

The Atg1 kinase and its regulators, Atg13, Atg17, Atg29, and Atg31, are collaboratively function in the initial step of autophagosome formation downstream of TORC1 (Table 1)(Fig. 4). These proteins consist of one of the Atg subfamilies, which is the most epistatic in the hierarchy of the PAS localization of the Atg proteins (Fig. 3). Atg1 is a Ser/Thr protein kinase, whose activity is essential for autophagy[33] and largely enhanced upon nutrient starvation or the addition of rapamycin[17]. This regulation involves Atg13[17]. While Atg13 is phosphorylated in a TORC1-dependent manner under nutrient-replete conditions, it is immediately dephosphorylated in response to starvation or rapamycin treatment (Fig. 4)[17, 34]. Dephosphorylated Atg13 associates with Atg1, somehow leading to upregulation of the kinase activity of Atg1. Since dephosphorylation of Atg13 normally occurs in any single mutant of non-essential phosphatases (Funakoshi and Ohsumi, unpublished results), multiple phosphatases may dephosphorylate Atg13. Phosphorylation of one or more factors by Atg1 is expected to trigger a downstream event in autophagosome formation. Although several Atg proteins are phosphorylated in an Atg1-dependent manner both *in vivo* and *in vitro*, the physiological significance of phosphorylation of these proteins and thus an authentic substrate(s) of Atg1 still remain elusive (Kamada and Ohsumi, unpublished results).

Whereas Atg1 and Atg13 are among the core components, Atg17, Atg29, and Atg31 are specifically required for starvation-induced autophagy (Fig. 2) and form a ternary complex (Fig. 4)[17, 35-38]. Although this ternary complex seems to be formed constitutively, it associates with the Atg1-Atg13 complex in response to nutrient starvation, which is important for the activation of Atg1[35, 39]. This association is also prerequisite for the recruitment of the other core Atg proteins to the PAS, suggesting

the function of this subgroup as a trigger of autophagosome formation (also see below)[35, 38-40]. The Atg1 kinase activity is dispensable for both the complex formation of these five Atg proteins and the recruitment of a number of Atg proteins to the PAS[38, 39]. However, some Atg proteins abnormally accumulate at the PAS, and others such as Atg2 become absent from the PAS in the kinase-dead allele of Atg1[38, 39, 41], suggesting that the Atg1 kinase activity is involved in the dynamics of the Atg proteins at the PAS probably through phosphorylation of one or more Atg proteins.

Although it was proposed that dephosphorylation of Atg13 is one of the initial events in autophagy, it has remained unanswered whether Atg13 is a direct target of TORC1, or whether dephosphorylation of Atg13 is sufficient for induction of autophagy[42]. These questions have recently been addressed in order to understand the mechanism by which TORC1 signaling regulates autophagy (Kamada and Ohsumi, unpublished results). It was shown that Atg13 is directly phosphorylated by TORC1 *in vitro* at multiple serine residues. Expression of an unphosphorylatable Atg13 mutant can at least partially induce autophagy in non-starved cells, suggesting that dephosphorylation of Atg13 is sufficient for autophagy induction. As mammalian homologues of the Atg1 complex have been reported, the TORC1-Atg1 signaling module is thought to be conserved across most eukaryotes to regulate autophagy[43-45].

The PtdIns 3-kinase complex

The Atg subgroups contain a complex of the PtdIns 3-kinase that phosphorylates the D-3 position of the inositol ring in PtdIns to produce PtdIns(3)-phosphate (PtdIns(3)*P*) (Table 1). In addition to this complex, we describe another subgroup that contains a possible effector for PtdIns(3)*P* in this section.

The activity of the PtdIns 3-kinase is essential for autophagy[18, 46, 47]. Vps34 is the sole PtdIns 3-kinase in *S. cerevisiae*[48] and forms two distinct complexes, I and II, which play essential roles in autophagy and the vacuolar protein sorting (Vps) pathway, respectively[18]. Complex I is composed of Vps34, Vps15, Vps30/Atg6 and Atg14, whereas complex II contains Vps38 instead of Atg14 (Fig. 5). These specific subunits, Atg14 and Vps38, direct the localization of complexes I and II to the PAS and the endosomal membrane, respectively, in addition to the vacuolar membrane (Fig. 5)[49]. PtdIns(3)*P* that is produced by complex I is suggested to recruit effector proteins required for autophagosome formation to the PAS.

Atg18 can bind to both PtdIns(3)*P* and PtdIns(3,5)P_2[50, 51], and is therefore a potent candidate for their effectors. A portion of Atg18 forms a complex with Atg2 and functions in autophagosome formation[27, 47], whereas this protein also regulates the size of the vacuole and PtdIns(3,5)P_2 homeostasis in complex with other proteins (Fig. 5)[50, 52, 53]. While the former function of Atg18 involves PtdIns(3)*P*,

the latter depends on PtdIns(3,5)P_2[47, 52, 53]. Atg14, but not Vps38, is required for the localization of the Atg2-Atg18 complex to the PAS, suggesting that the production of PtdIns(3)*P* at the PAS is important for the PAS localization of this complex and thus for its function in autophagosome formation (Fig. 3)[27, 47]. Although the precise location where complex I produces PtdIns(3)*P* is still unknown, recent studies showed that PtdIns(3)*P* is enriched on isolation membranes and autophagosomal membranes, which is finally transported into the vacuole[47].

Among the core Atg proteins, Atg9 is the sole integral membrane protein. Thus this protein has been extensively analyzed to obtain insights into a membrane source of the autophagosome[54-56]. In addition to its localization to the PAS[27], Atg9–GFP is also observed as a few dozen of small dots moving in the cytoplasm[54]. Disruption of actin filaments or microtubules does not disturb this movement; instead, energy depletion by the addition of sodium azide does inhibit the movement[41]. However, it is still unclear whether these cytosolic dots of Atg9 are relevant to autophagosome formation. Atg9 accumulates at the PAS at the non-permissive temperature in the *atg1*^{ts} strain[54]. It was therefore proposed that Atg9 shuttles between the PAS and the cytoplasmic pool during autophagosome formation. Similarly, deletion of *ATG2* and *ATG18* also causes the accumulation of Atg9 at the PAS[27]. The Atg2-Atg18 complex as well as the function of Atg1 may be involved in the dynamics of Atg9 at the PAS (also see below). In the hierarchical model of the PAS localization of the Atg proteins (Fig. 3), Atg9 is located just beneath the Atg1 subgroup. Consistent with this, it has recently been shown that Atg9 physically interacts with Atg17, a component of the subgroup[41].

Two ubiquitin-like conjugation systems

Among the Atg subgroups there are two protein conjugation systems, which are composed of two ubiquitin-like proteins, Atg12 and Atg8, and three enzymes, Atg7, Atg10, and Atg3, that are required for their conjugation reactions (Table 1) (Figs. 6a and 6b). Atg12 forms a conjugate with Atg5[19], whereas Atg8 is conjugated to phosphatidylethanolamine (PE)[20], a major component of various biological membranes. Both the conjugates localize to autophagy-related membranes (Fig. 6c)[57, 58], suggesting their direct involvement in the biogenesis of these membranes. Consistent with this notion, these conjugates are positioned hypostatically in the hierarchy of the PAS localization of the Atg proteins (Fig. 3). Recent *in vitro* studies have brought significant advances in our understanding of the functions of these ubiquitin-like protein conjugates.

Conjugation of Atg8. Atg8 is synthesized as a precursor with an additional sequence in its C terminus (a single Arg residue in *S. cerevisiae* Atg8), which is immediately clipped off by the cysteine protease Atg4 to expose the Gly residue that is essential for subsequent reactions (Fig. 6a)[58, 59].

Formation of the Atg8–PE conjugate is mediated by Atg7 and Atg3, which correspond to the E1 and E2 enzymes in the ubiquitination reaction, respectively[20]. The carboxyl group of the exposed Gly of Atg8 is activated by Atg7 with consumption of ATP to form a thioester intermediate with the active Cys residue of Atg7, then transferred to that of Atg3, and finally forms an amide bond with the amino group in PE (Fig. 6a). Atg8, probably in this lipidated form, localizes to the isolation membrane and the autophagosome[25].

Atg4 also acts as a deconjugation enzyme that cleaves Atg8–PE to liberate the protein from membranes (Fig. 6a)[58]. This reaction is thought to be important for recycling the Atg8 molecule that has fulfilled a role in membrane formation and/or for controlling the function of Atg8. A portion of Atg8 is left inside the autophagosome, delivered to and degraded in the vacuole (Fig. 6c)[25]. Both synthesis and lipidation of Atg8 are enhanced under autophagy-inducing conditions[58, 60]. These features of Atg8 allow us to use this protein and its homologs to trace progression of autophagy in various organisms[61-63].

The conjugation reaction of Atg8 is reconstituted *in vitro* with purified recombinant proteins (the Gly-exposed form of Atg8, Atg7 and Atg3), ATP and liposomes containing PE. In this mixture, Atg8 efficiently forms a conjugate with PE on the liposomes[64]. It was found that Atg8–PE forms an oligomer(s), and causes liposome clustering and hemifusion (Fig. 6d)[60]. Atg8 mutants deficient for clustering and hemifusion of liposomes exhibit significant defects in autophagosome formation, suggesting that these phenomena observed *in vitro* represent the authentic function of Atg8 *in vivo*. The size of the autophagosome decreases in cells that express Atg8 mutants with their functions partially impaired. Similar consequences are also observed when the expression level of Atg8 is genetically engineered to be decreased[65]. These results suggest that Atg8 is involved in the expansion of autophagosomal membranes. In addition, recent studies in mammals showed that lipidation of Atg8 homologs is essential for normal development of autophagosomal membranes; its abrogation causes accumulation of unclosed isolation membranes with anomalous morphology[66, 67].

On the basis of the *in vitro* observations, it can be assumed that Atg8–PE is involved in tethering and fusion of yet unidentified precursory structures of the autophagosome. Previous studies actually indicated the existence of such structures containing Atg8–PE. In immuno-electron microscopy, in addition to Atg8 signals on isolation membranes and autophagosomes, those enriched in electron less-dense regions, which appear to be abundant in lipids but free from evident membrane structures, are also observed[25]. Moreover, Atg8–PE increases under autophagy-inducing conditions even in cells deficient for autophagosome formation[27]; Atg8–PE may accumulate on precursors of autophagosomal membranes. Identification and characterization of these Atg8–PE-containing structures, including elucidation of their components, morphology, and formation process, will provide us with critical

information on molecular mechanisms of and a source of lipid supply for autophagosome formation.

Conjugation of Atg12. Similarly to the Atg8–PE system, the conjugation reaction of Atg12 is catalyzed by the common E1 enzyme Atg7 and the specific E2 enzyme Atg10. The C-terminal Gly residue of Atg12 forms the isopeptide bond with the specific Lys residue of Atg5 (Fig. 6b)[19]. It seems that neither a processing enzyme nor a deconjugation enzyme exists in the Atg12–Atg5 system, and that formation of this conjugate occurs constitutively. The Atg12–Atg5 conjugate further interacts with Atg16 and forms a complex of ~350 kDa (~800 kDa in mammals) by virtue of the oligomerization ability of Atg16 (Fig. 6b)[68-70]. Immuno-electron microscopic analyses of mammalian cells showed that while LC3 (a mammalian Atg8 homolog) is present on the both surfaces of the isolation membrane[61], the Atg12–Atg5-Atg16L (a mammalian Atg16 homolog) complex predominantly localizes on the outer surface of the membrane (Fig. 6c)[57, 70]. In addition, fluorescence microscopy showed that GFP-fused Atg5 dissociates from the membrane immediately before or after completion of the autophagosome[57]. Although these observations seem to imply that the Atg12–Atg5-Atg16 complex functions as a coat protein as observed in secretory vesicle formation, it has recently been estimated that the number of complex molecules participating in autophagosome formation is too low to assemble a coat surrounding the membrane[71].

Crosstalk between the conjugation systems. The involvement of the Atg12–Atg5-Atg16 complex in the Atg8–PE system has been genetically suggested; mutations that abolish the complex significantly decrease Atg8–PE production[26, 72]. It has also been shown that purified Atg12–Atg5 conjugates drastically stimulate the formation of Atg8–PE *in vitro*[73, 74]. Atg12–Atg5 directly interacts with the E2 enzyme Atg3 and enhances its activity (Figs. 6a and 6b). Thus, Atg12–Atg5 exerts an E3-like function in the lipidation of Atg8. Atg8 can be conjugated to phosphatidylserine (PS) as efficiently as to PE in the *in vitro* reaction, although PE was identified as the sole target of Atg8 *in vivo*[64, 72, 75]. Unlike E3 enzymes in the ubiquitin system, Atg12–Atg5 is not involved in determining substrate specificity in lipidation of Atg8[73]. Instead, it has been proposed that Atg3 itself has an ability to discriminate PE from PS under physiological conditions[76].

Atg16 is dispensable for the E3-like function of Atg12–Atg5 *in vitro* in spite of its requirement for Atg8–PE formation *in vivo*[73]. This apparent contradiction could be explained by considering the spatial regulation of the Atg12–Atg5-Atg16 complex. In yeast, Atg16 is required for the localization of Atg12–Atg5 to the PAS[27]. In addition, forced localization of Atg16L to the plasma membrane in mammalian cells causes lipidation of LC3 on that membrane[77]. These results indicate that Atg16 is involved in specification of the site of Atg8 lipidation. The PAS localization of the Atg12–Atg5-Atg16 complex may imply that Atg8–PE is produced at the PAS. Lipidation of Atg8 on the isolation membrane

is also conceivable. As aforementioned, Atg8–PE, however, accumulates even in mutant cells where the isolation membrane is not formed. Therefore, it is possible that Atg8–PE is formed elsewhere, and then somehow transferred to the isolation membrane, or that lipidation of Atg8 occurs at both the sites. Thus, the answer to this long-standing question still awaits further investigation.

The role of Atg8 in selective autophagy. In addition to its function in autophagosomal membrane formation, Atg8 is involved in efficient incorporation of cargoes into autophagosomes in selective types of autophagy. Although the Cvt pathway exists only in yeast and serves as a biosynthetic pathway, its extensive studies have established a conceptual framework to understand the mechanism of selective autophagy (also see below). In this pathway, the cargo Ape1 self-assembles into an aggregate-like structure and interacts with the receptor protein Atg19[78, 79]. Atg19 also interacts with Atg8; this interaction is believed to link the cargo-receptor complex to the forming Cvt vesicle[80-82]. In mammalian cells, p62 is responsible for selective degradation of ubiquitin-positive, protein inclusions via autophagy and also binds to mammalian homologs of Atg8[83-86]. Therefore, although Atg19 and p62 are unrelated to each other in their entire sequences, these proteins are likely to function similarly in selective incorporation of the cargoes into vesicles. Interestingly, recent structural studies revealed a common interaction between these receptors and Atg8 homologs: the Trp-X-X-Leu motif in the receptors binds to the highly conserved, hydrophobic pocket in the Atg8 homologs in a quite similar manner[82, 87]. A similar interaction is possible to work broadly in recognition of various cargoes in selective autophagy.

Dynamic features of the PAS

In contrast to our earlier view that the PAS is a static and stoichiometric structure[26], recent studies have uncovered dynamic aspects of the PAS. The PAS can be versatile in its composition depending on the physiological situations. We here define the PAS as the dynamic assembly of the core Atg proteins that function as membrane-forming machinery and of ‘conductor’ proteins that spatiotemporally regulate the core proteins to determine the site for PAS organization and the mode of membrane formation. On the basis of this concept, we describe our present view on the PAS.

As aforementioned, yeast cells have the Cvt pathway, in which small autophagosome-like vesicles called Cvt vesicles are formed to deliver vacuolar enzymes, such as Ape1, to the vacuole. The assembly of the Atg proteins, which has also been called the PAS, is involved in the formation of the Cvt vesicle as well as that of the autophagosome[88]. In contrast to autophagy, the Cvt pathway is active under nutrient-rich conditions, and consistently the PAS that mediates Cvt vesicle formation is observed under those conditions, which had made it difficult to analyze Atg protein dynamics in response to induction of

autophagy. This problem has been circumvented by analyzing cells lacking Atg11[38, 39]; Atg11 is specifically required for the Cvt pathway, and is responsible for organization of the PAS under nutrient-rich conditions[89, 90]. In these cells, while the Atg proteins are totally dispersed in the cytoplasm under nutrient-rich conditions, they assemble into a perivacuolar dot in response to nutrient starvation[90], and the dot immediately disappears upon nutrient replenishment[38]. The lack of either Atg1, Atg13, Atg17, Atg29 or Atg31 completely abolishes the dot formation of all the other core Atg proteins as well as that of these five proteins[38, 39]. Therefore, the Atg17-Atg29-Atg31 complex is likely to act as a conductor together with the Atg1-Atg13 complex in organization of the PAS for autophagosome formation. Because in wild-type cells, the PAS already exists before autophagy induction, it may be more appropriate to describe the Atg17-Atg29-Atg31 complex as a 'reorganizer' of the PAS from the mode for the Cvt pathway to that for autophagy in response to nutrient starvation.

In addition to this dynamic PAS assembly during induction of autophagy, the following evidence suggests that Atg proteins are actively recruited to and dissociated from the PAS in a single round of autophagosome formation. The fluorescence intensity of GFP–Atg8 at the PAS periodically changes with about 10 minute intervals[39, 71], which corresponds to the time the single autophagosome is formed in mammalian cells[57]. By contrast, the intensity of Atg9–GFP is constant over a 30-minute time course[71]. However, as described above, Atg9–GFP markedly accumulates at the PAS in a number of *atg* mutant strains[27, 54], suggesting that the dynamics of Atg9 is in an equilibrium in wild-type cells. Similarly, comprehensive localization analysis revealed that the levels of most Atg proteins at the PAS are differently affected by disruption of other Atg proteins[27]. Therefore, it is assumed that the PAS is maintained by a dynamic equilibrium of intricate interactions among the Atg proteins.

In contrast to the PAS assembly in starvation-induced autophagy, it is remarkable that PAS assembly in the Cvt pathway depends on a large complex composed of the cargo Ape1 and the receptor Atg19[90]. It is thought that the cargo-receptor complex serves as a scaffold for the recruitment of Atg11[91]. Atg11 then recruits the core Atg proteins to allow the formation of the Cvt vesicle around the cargo-receptor complex. Thus, similarly to the Atg17-Atg29-Atg31 complex in starvation-induced autophagy, Atg11 behaves as a conductor together with the cargo-receptor complex in organization of the PAS in the Cvt pathway. In addition, it was reported that Atg11 is also involved in pexophagy and 'mitophagy', autophagic degradation of mitochondria, where Atg11 is suggested to act similarly but in cooperation with Atg30 and probably an unidentified factor, respectively, instead of Atg19[92, 93].

In this way, conductor Atg proteins seem to regulate the core Atg proteins spatiotemporally and determine the mode of the PAS. The results obtained in studies on starvation-induced autophagy and the Cvt pathway clearly show that two factors, nutrient conditions and the existence of the cargo, can

regulate the mode of the PAS and thus the site of vesicle formation and the size of the vesicle. These factors could independently or cooperatively affect the mode of the PAS. In addition, there may exist yet unidentified conductors that respond to different environmental signals or recognize specific cargo complexes, which create the functional diversity of autophagy as exemplified by its versatile roles rapidly emerging in higher eukaryotes. Although the PAS has not yet been described in mammalian cells, foci that might correspond to the PAS have recently been reported in slime molds and higher plants[94, 95].

The PAS is currently observed as just a dot under a fluorescence microscope. We defined the PAS is an assembly of the Atg proteins, which can exist without the isolation membrane[96]. However, certain Atg proteins such as Atg8 and the Atg12–Atg5-16 complex localize to both the PAS and the isolation membrane. In addition, intriguingly, Atg1 but neither Atg13 nor the Atg17-Atg29-Atg31 complex is transported into the vacuole as well as Atg8 under starvation conditions, even though these five Atg proteins are interdependently assembled to the PAS[26]. Therefore, it is possible that a number of Atg proteins transit from the PAS to the isolation membrane as the membrane grows. Further analyses including detailed immunoelectron microscopy and fluorescence imaging for protein interactions in living cells will allow us to more definitely discuss the PAS as the spatial configuration of the Atg proteins to the isolation membrane and the cargo complex.

Concluding remarks

For unicellular organisms such as yeast, depletion of nutrients must be the most frequent and crucial stress in nature. Therefore, a starvation-induced mode of autophagy, which is essential for the maintenance of metabolite pool such as amino acids, would have been established first and conserved during evolution. In fact, all the core *ATG* genes are conserved in mammals and plants. Once the basic system for membrane formation had been established, additional molecules that endow cargo selectivity then may have been acquired, which have developed various functions of autophagy.

The machinery for starvation induced-autophagy is likely to be able to use special factors for selective autophagy in some cases. Certain cargoes for selective autophagy are more efficiently degraded by autophagy under starvation conditions in a manner dependent on such factors, where the cargoes should be incorporated into autophagosomes together with other cytoplasmic components[9, 97]. In addition, Ald6 (cytoplasmic acetaldehyde dehydrogenase) and ribosomes are known to be preferentially degraded via autophagy under nitrogen starvation conditions, even though they seem to be completely dispersed in the cytoplasm, suggesting a novel mechanism for these cases[98, 99]. Thus, the way in which materials that are to be degraded in autophagy are sequestered also varies. Finally, it should also be noted that Lap3 (leucine aminopeptidase III) has recently proved to be selectively transported to the

vacuole for degradation in glycerol-grown (non-starved) yeast cells[100](Kageyama and Ohsumi unpublished results). This process depends on Atg11 and Atg19, suggesting that the Cvt pathway also functions as a selective degradation system in yeast.

Recent studies especially in higher eukaryotes such as mammals and plants have rapidly unveiled the diversity and complexity of autophagy. Our paying attention to the diverse modes of autophagy as reviewed here is quite important for accurate understanding of not only the mechanism but also the significance of autophagy in each physiological or pathological situation. Studies on the mechanisms of autophagy have just begun and thus those at a molecular level will be increasingly critical.

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Figure legends

Figure 1 Schematic diagram of yeast autophagy. In starvation-induced autophagy, the isolation membrane principally non-selectively engulfs cytosolic constituents and organelles to form the autophagosome. The inner membrane-bound structure of the autophagosome (autophagic body) is released into the vacuolar lumen upon fusion of the outer membrane with the vacuolar membrane, and disintegrated to allow degradation of the contents by resident hydrolyases. In selective autophagy, specific cargoes (protein complexes or organelles) are enwrapped by membrane vesicles similar to autophagosomes, and delivered to the vacuole for degradation. Although the Cvt pathway mediates biosynthetic transport of vacuolar enzymes, its membrane dynamics and mechanism are almost the same as those of selective autophagy (see text).

Figure 2 Classification of Atg proteins. Atg proteins commonly required for three autophagy-related pathways, starvation-induced autophagy, the Cvt pathway, and pexophagy, are classified as the core machinery for membrane formation. Proteins specific for each pathway, which include conductor proteins (see text), are also shown. It should be noted that Atg11 is involved in both the Cvt pathway and pexophagy.

Figure 3 The PAS as the assembly of Atg proteins. (**a**) The fluorescence microscopic images of cells expressing both CFP-tagged Atg8 and YFP-tagged Atg5. The merged image is also presented (modified from ref. 31). (**b**) The hierarchical model of the PAS localization of Atg proteins in starvation-induced autophagy. If an Atg protein is genetically removed, the PAS localization of proteins placed in the upper position is significantly lost (see text for details); proteins positioned inferiorly in this scheme are epistatic in the PAS localization of Atg proteins.

Figure 4 The Atg1 subfamily in the initial step of autophagosome formation. When TORC1 is inactivated upon nutrient depletion or rapamycin treatment, Atg13 is dephosphorylated. This allows association of Atg1 subfamily proteins, followed by upregulation of the Atg1 kinase activity and recruitment of other core Atg proteins to the PAS to initiate autophagosome formation. These events are immediately reversed upon the addition of nutrients.

Figure 5 Two PtdInd 3-kinase complexes. Complex I and II are composed of common subunits, the PtdInd 3-kinase Vps34, Vps30/Atg6, and Vps15, and specific subunits, Atg14 and Vps38, respectively. The localization, downstream factors, and functions of these complexes are summarized.

Figure 6 Two ubiquitin-like conjugation systems. (**a**) The conjugation system of Atg8. First, Atg4 cleaves the C-terminal Arg of Atg8 to expose Gly at the new C terminus. Atg8 is then activated by Atg7 (E1 enzyme), transferred to Atg3 (E2 enzyme), and finally conjugated to PE. The transfer reaction of Atg8 from Atg3 to PE is stimulated by the other ubiquitin-like protein complex Atg12–Atg5-Atg16, which is thus suggested to determine the site of the production of Atg8–PE. Atg4 also cleaves the amide bond between Atg8 and PE to release the protein from membranes. (**b**) The conjugation system of Atg12. Atg12 is conjugated to the specific Lys residue of Atg5 similarly to the conjugation reaction of Atg8 except that Atg10 functions as the E2 enzyme in this system instead of Atg3. The Atg12–Atg5 conjugate interacts with Atg16 and forms an oligomer. (**c**) Localization of ubiquitin-like protein conjugates on autophagy-related membranes. While Atg8–PE is present on the both surfaces of the isolation membrane, and a part of the conjugate is left inside the autophagosome, delivered to the vacuole, and degraded, Atg12–Atg5-Atg16 preferentially localizes on the outer surface of the membrane and dissociates from the membrane upon completion of the autophagosome. (**d**) Membrane tethering and hemifusion functions of Atg8. Upon conjugated to PE on liposomes, Atg8 oligomerizes and tethers together the liposomes, leading to hemifusion of the membranes. Only lipids in outer leaflets interdiffuse in hemifused membranes.

Table 1. Subgroups of Atg proteins required for starvation-induced autophagy

subgroup and component	**known or putative function**
Atg1 kinase and its regulators	**triggering and regulating PAS assembly of other proteins**
Atg1	Ser/Thr kinase
Atg13	TORC1 substrate involved in regulation of Atg1 activity
Atg17	forming a starvation-induced autophagy-specific complex with Atg29 and Atg31, which further associates with Atg1 and Atg13
Atg29	
Atg31	
PtdIns 3-kinase complex	**producing PtdIns(3)*P* at the PAS**
Vps34	PtdIns 3-kinase
Vps15	Ser/Thr kinase required for Vps34 activity
Vps30/Atg6	component of unknown function
Atg14	recruiting this complex to the PAS
Atg12 conjugation system	**stimulation and site determination of Atg8 lipidation**
Atg12	ubiquitin-like protein conjugated to Atg5
Atg5	target of Atg12 and interacts with Atg16
Atg7	common E1 enzyme for Atg12–Atg5 and Atg8–PE formation
Atg10	specific E2 enzyme for Atg12–Atg5 formation
Atg16	required for the PAS localization of Atg12–Atg5
Atg8 conjugation system	**membrane tethering and hemifusion**
Atg8	ubiquitin-like protein conjugated to PE
Atg4	removing the C-terminal Arg and conjugated PE from Atg8
Atg7	common E1 enzyme for Atg12–Atg5 and Atg8–PE formation
Atg3	specific E2 enzyme for Atg8–PE formation
Atg2-Atg18 complex and Atg9	**unknown**
Atg18	binding to PtdIns(3)*P*
Atg2	forming a complex with Atg18, which is involved in Atg9 dynamics at the PAS
Atg9	membrane integral protein of unknown function

Glossary

Macroautophagy: In addition to classification as described in the text, autophagy is also classified into three types according to their difference in membrane dynamics: (i) macroautophagy as focused in this review, (ii) microautophagy where lysosomal or vacuolar membranes by themselves invaginate and pinch off the invaginated part to incorporate a portion of the cytoplasm into their lumen, and (iii) chaperon-mediated autophagy where specific proteins are translocated directly across the lysosomal membrane.

Autophagosome: A double membrane-bound vesicle newly formed during autophagy to sequester materials be degraded and deliver them to the lysosome in mammals or the vacuole in yeasts and plants.

Ubiquitin-proteasome system: The system that degrades selected proteins one by one, which are first marked with ubiquitin chains and then degraded by the multi-catalytic proteinase complex proteasome.

Autophagic body: The inner membrane-bound structure of the autophagosome released into the vacuolar lumen by fusion of the autophagosomal outer membrane with the vacuolar membrane.

Vps pathway: The pathway that mediates selective transport of a subset of proteins from the late Golgi compartment to the vacuole via the endosome.

E1 enzyme: Enzymes that activate ubiquitin and ubiquitin-like proteins using ATP and transfer them to E2 enzymes.

E2 enzyme: Enzymes that receive ubiquitin and ubiquitin-like proteins from E1 enzymes and conjugate them to target molecules.

Deconjugation enzyme: Enzymes that cleaves the isopeptide bond (the amide bond in Atg8–PE) formed in ubiquitin and ubiquitin-like protein conjugates.

Hemifusion: Fusion between outer leaflets of membranes while inner leaflets remained intact, which is regarded as a common intermediate state in biological membrane fusion events.

E3 enzyme: Enzymes that stimulate the conjugation reaction by E2 enzymes and are also involved in selection of target molecules.

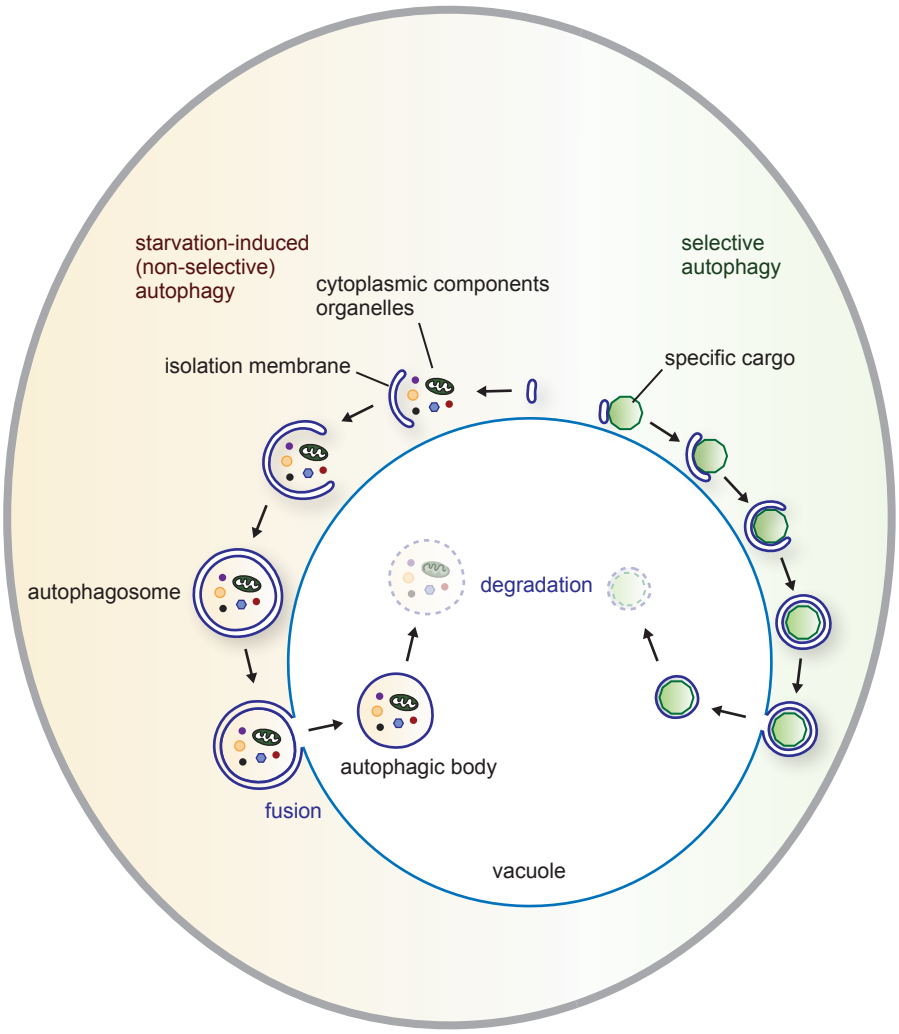

starvation-induced
(non-selective)
autophagy
cytoplasmic components
organelles
isolation membrane
selective
autophagy
specific cargo
autophagosome
degradation
autophagic body
fusion
vacuole

Fig. 1

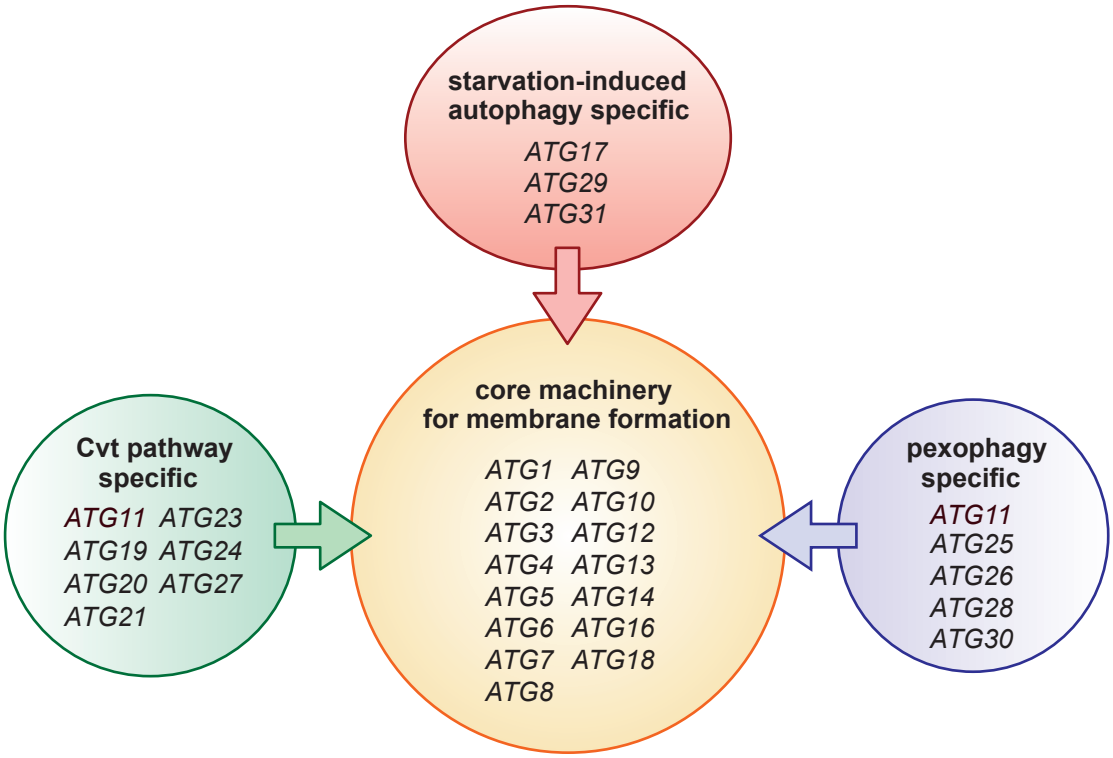

starvation-induced autophagy specific
ATG17
ATG29
ATG31
core machinery for membrane formation
ATG1 ATG9
ATG2 ATG10
ATG3 ATG12
ATG4 ATG13
ATG5 ATG14
ATG6 ATG16
ATG7 ATG18
ATG8
Cvt pathway specific
ATG11 ATG23
ATG19 ATG24
ATG20 ATG27
ATG21
pexophagy specific
ATG11
ATG25
ATG26
ATG28
ATG30

Fig. 2

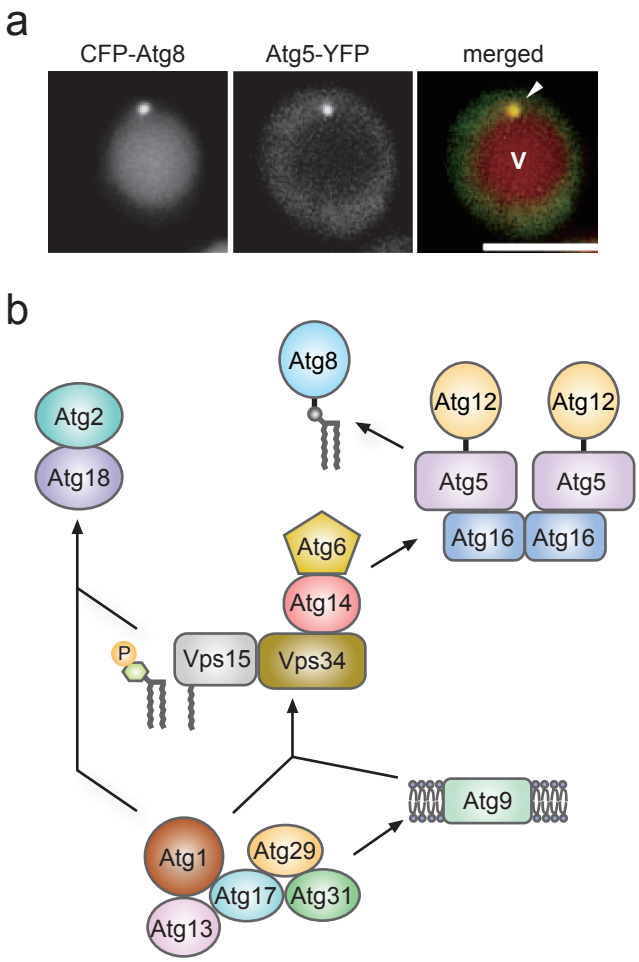

a
CFP-Atg8
Atg5-YFP
merged
V
b
Atg8
Atg12
Atg12
Atg2
Atg18
Atg5
Atg5
Atg16
Atg16
Atg6
Atg14
P
Vps15
Vps34
Atg9
Atg1
Atg29
Atg17
Atg31
Atg13

Fig. 3

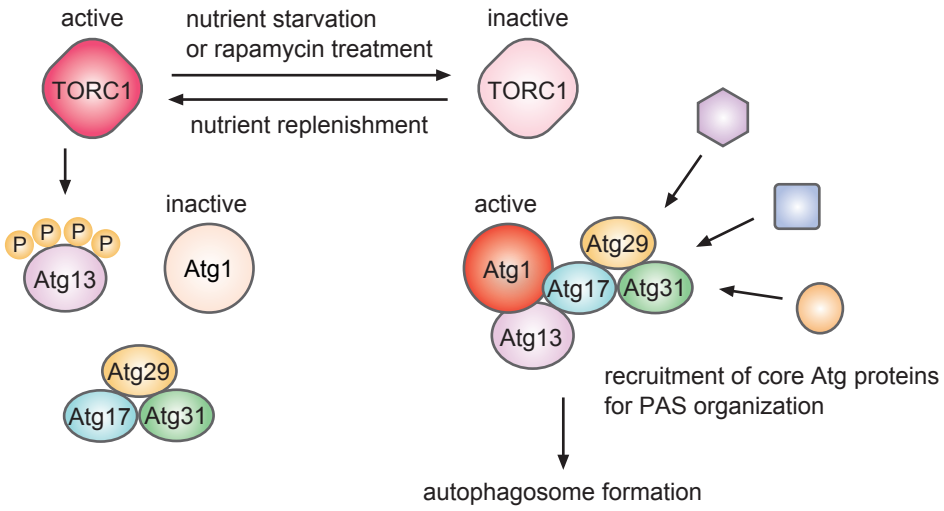
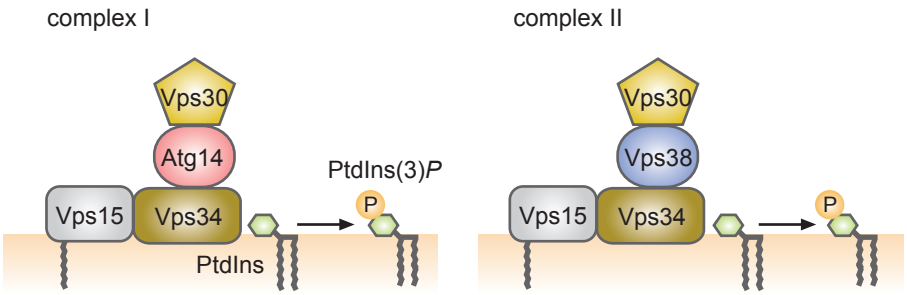

active
TORC1
nutrient starvation
or rapamycin treatment
nutrient replenishment
inactive
TORC1
P
P
P
Atg13
inactive
Atg1
Atg29
Atg17
Atg31
active
Atg1
Atg29
Atg17
Atg31
Atg13
recruitment of core Atg proteins
for PAS organization
autophagosome formation

Fig. 4



	complex I	complex II
localization:	PAS and vacuole	endosome and vacuole
downstream factors:	Atg2-Atg18	Fab1-Fig4-Vac14, Vac7, Atg18
function:	autophagosome formation	vacuole morphology

Fig. 5

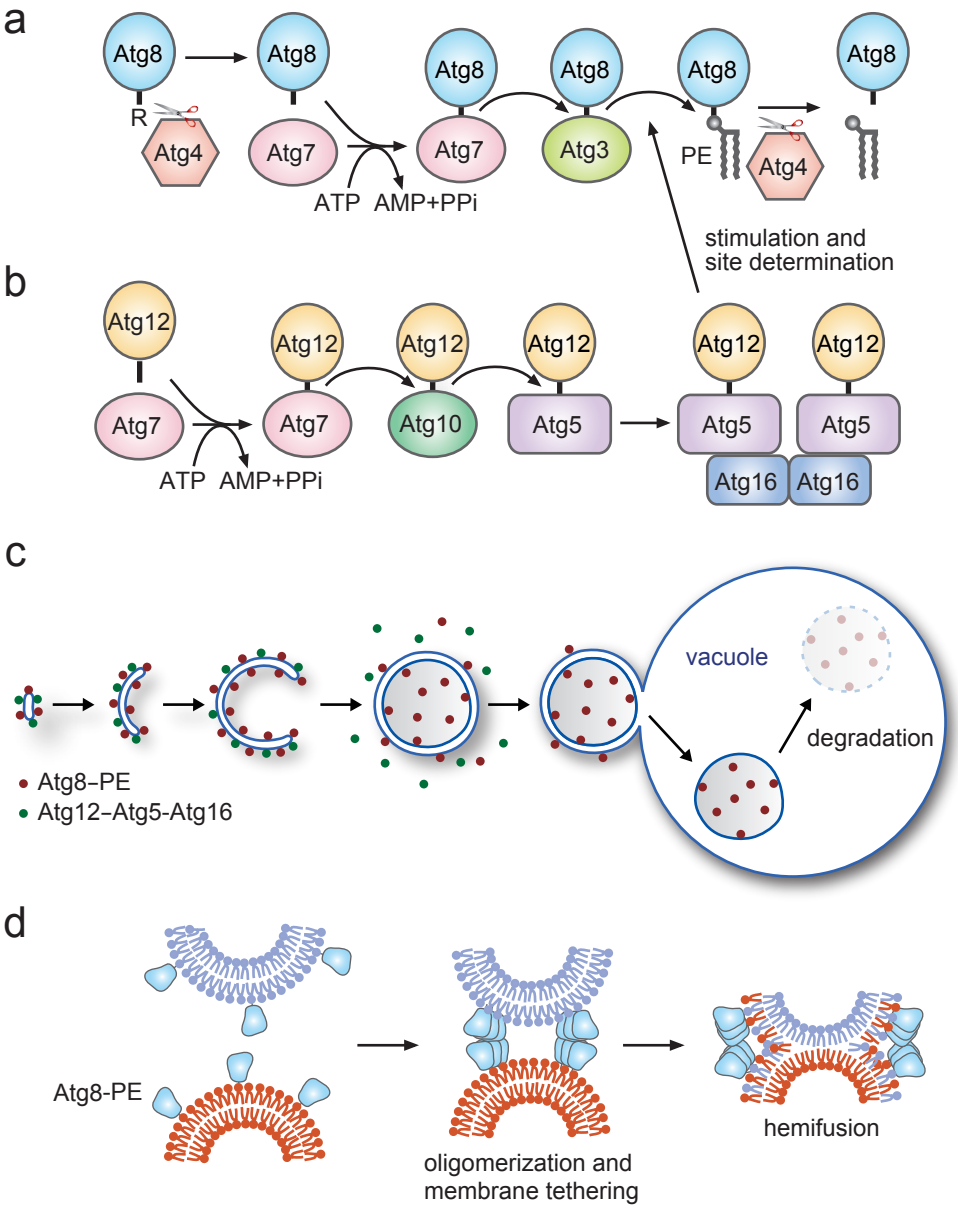

a
Atg8
R
Atg4
Atg8
Atg7
ATP
AMP+PPi
Atg8
Atg7
Atg8
Atg3
Atg8
PE
Atg4
Atg8
stimulation and
site determination
b
Atg12
Atg7
ATP
AMP+PPi
Atg12
Atg7
Atg12
Atg10
Atg12
Atg5
Atg12
Atg5
Atg12
Atg5
Atg16
Atg16
c
vacuole
degradation
Atg8–PE
Atg12–Atg5-Atg16
d
Atg8-PE
oligomerization and
membrane tethering
hemifusion

Fig. 6