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Review

Current knowledge of the pre-autophagosomal structure (PAS)

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ABSTRACT

Autophagy is a system for degradation of bulk cellular components in lytic compartments, vacuoles, or lysosomes when eukaryotic cells face with nutrient starvation. In this review, we focus on the pre-autophagosomal structure (PAS), a functional entity responsible for autophagosome formation in *Saccharomyces cerevisiae*, and discuss its relevance to autophagy in mammalian cells.

1. Introduction

Autophagy is a system for degradation of bulk cellular components in lytic compartments, vacuoles, or lysosomes when eukaryotic cells face with nutrient starvation. Since the phenomenon of autophagy was discovered in mammals, autophagy has been studied mostly by electron microscopy. In the early 1990s, a method to monitor autophagy by light microscopy was established in the unicellular eukaryote, *Saccharomyces cerevisiae* [1]. Using *S. cerevisiae* as a model organism, the process of autophagy has subsequently been investigated in detail [2] (Fig. 1). Upon starvation, a double-membrane sac called an isolation membrane emerges in the cytoplasm. The isolation membrane expands and its leading edges fuse with each other to complete autophagosome formation. The outer membranes of the autophagosomes ultimately fuse with the vacuole and the cellular components enclosed by the inner membranes are released into the vacuolar lumen as autophagic bodies. Finally, the autophagic bodies are disintegrated and their contents are degraded by vacuolar hydrolases. Taking advantage of yeast genetics, genes involved in autophagy have been identified and analyzed by molecular biological and biochemical techniques. However, it has remained unclear how and where autophagosomes are generated. In this review, we focus on the pre-autophagosomal structure (PAS), a functional entity responsible for autophagosome formation in *S. cerevisiae*, and discuss its relevance to autophagy in mammalian cells.

2. Autophagy-related (*ATG*) genes

The most critical step in autophagy is the sequestration of contents to be degraded by an autophagosome. The autophagosome is a unique organelle bound by double lipid-bilayers. When eukaryotic cells are faced with nutrient starvation, autophagosomes are generated in the cytosol and enclose bulk cytoplasmic components for degradation. Using yeast genetics, a number of *apg* (*aut*ophagy) and *aut* (*aut*ophagocytosis) mutants defective in autophagy were identified [3,4]. In parallel, mutants defective in the transport of aminopeptidase I (Ape1) to the vacuole were isolated as *cvt* (*c*ytoplasm-to-vacuole *t*argeting) mutants [5]. Subsequent analysis revealed that the *APG*, *AUT*, and *CVT* genes largely overlap [6,7]. Recently, the *apg*, *aut*, *cvt*, and other genes essential for selective organelle degradation have been unified as *ATG* (*aut*ophagy-related) to avoid confusion [8].

In the Cvt pathway, Ape1 is selectively transported to the vacuole under nutrient-rich conditions; in contrast, bulk cytoplasmic materials are non-selectively transported to the vacuole by autophagy under starvation conditions. Electron microscopy provided clues to the genetic overlap of these pathways (Fig. 1). Ape1 assembles into a large complex, called the Cvt complex, in the cytoplasm; this complex is subsequently sequestered into a double-membrane-bound vesicle termed a Cvt vesicle [9]. The Cvt vesicle fuses with the vacuole and the inner membrane-bound Cvt body is released into the vacuolar lumen [10]. These processes are essentially analogous with processes in the autophagic pathway; the exception is that Cvt vesicles are about 150 nm in diameter whereas autophagosomes are 400–900 nm in diameter. In

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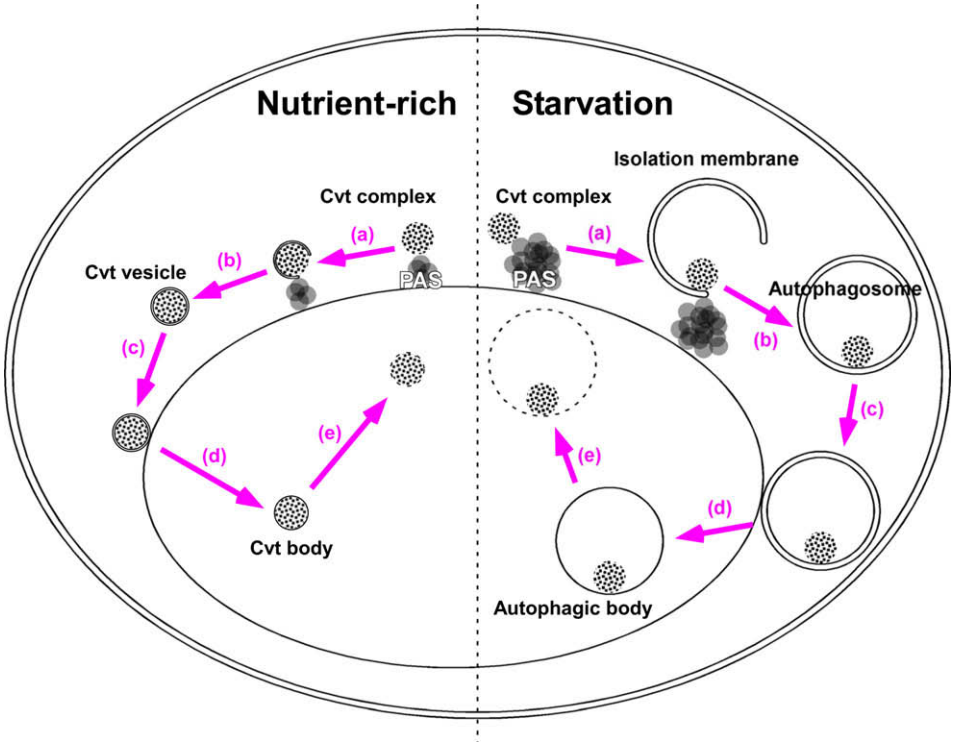

Fig. 1. A model for the function of the PAS in autophagy and the Cvt pathway. Under nutrient-rich conditions, the autophagosome-generating machinery known as the pre-autophagosomal structure (PAS) acts in the Cvt pathway (left), which selectively transports the Cvt complex mainly containing aminopeptidase I (Ape1) to the vacuole. On the other hand, when cells face nutrient-starvation, autophagy delivers cytoplasmic components to the vacuole to be degraded (right). These pathways proceed through mechanistically common steps: (a) generation of isolation membranes around a Cvt complex; (b) expansion of the isolation membrane and completion of Cvt vesicle/autophagosome formation; (c) fusion of the outer membrane of the Cvt vesicle/autophagosome with the vacuolar membrane; (d) subsequent release of Cvt/autophagic bodies, whose membranes are derived from the inner membranes of Cvt vesicles/autophagosomes; and (e) disintegration of the Cvt/autophagic body. Cvt vesicles (about 150 nm in diameter) and autophagosomes (400–900 nm in diameter) are distinguishable by their sizes and contents.

addition, Cvt complexes are frequently found in autophagosomes [9]; thus, Ape1 has been shown to be selectively enclosed in autophagosomes.

A subset of the Atg proteins is required for all types of autophagy. These Atg proteins are thought to be essential for the membrane biogenesis of Cvt vesicles and autophagosomes [6,7], and they are referred to as 'core' Atg proteins [11] (Fig. 2). 'Specific' Atg proteins modulate the function of the core Atg proteins and act in each type of autophagy. For example, Atg17, Atg29, and Atg31 are specific factors for starvation-induced autophagy [12–15], which we hereafter refer to simply as autophagy. Atg11 and Atg19 are dispensable for autophagy but are crucial for the Cvt pathway and play a role in targeting Ape1 to Cvt vesicles and autophagosomes [16–20]. Atg11 is also required for peroxisome degradation (called pexophagy) and mitochondrial degradation (called mitophagy) [16,21]. Recently, Atg32 and Atg33 were identified as mitophagy-specific Atg proteins [22–24]. In the following sections, we focus on the Atg proteins required for autophagy.

3. Atg proteins function during autophagosome formation

Atg proteins required for autophagosome formation are categorized into several subgroups (reviewed in [25]): the Atg1–Atg13 complex [26], the Atg17–Atg29–Atg31 complex [27], Atg9 [28], the phosphatidylinositol 3 kinase complex specifically required for autophagosome formation (PtdIns(3) kinase complex I) [29], the Atg2–Atg18 complex [30], the Atg12 system [31], and the Atg8 system [32,33].

In the yeast, *Sacchromyces cerevisiae*, two PtdIns(3) kinase complexes are known [29]. One is specifically required for autophagosome formation (complex I) and the other is specifically required for the vacuolar protein sorting (VPS) pathway (complex II).

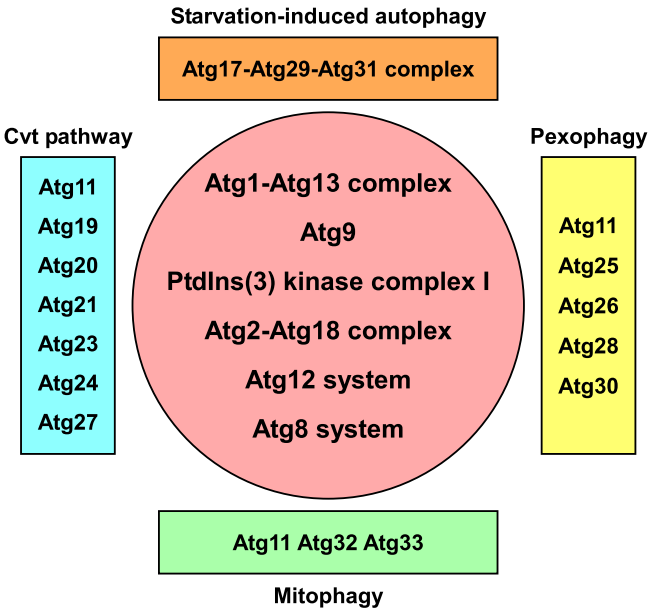

Fig. 2. Atg proteins required for various types of autophagy. 'Core' Atg proteins, which act in membrane biogenesis, are listed in the center circle. Several groups of Atg proteins are additionally required to specify the mode of autophagy; so-called autophagy, the Cvt pathway, pexophagy, and mitophagy. Atg11 is a common factor required for various types of selective autophagy.

PtdIns(3) kinase complex I includes Vps15, Vps30/Atg6, Vps34, and Atg14. Atg14 is thought to be a suitable marker protein for monitoring the localization of the PtdIns(3) kinase complex I [34]. In complex II, Vps38 is included instead of Atg14.

Atg8 and Atg12 are ubiquitin-like proteins. Atg12 is activated by Atg7 (an E1-like protein) [35] and conjugated to Atg5 by Atg10 (an E2-like protein) [36] to generate the Atg12–Atg5 conjugate. This conjugate associates with Atg16 to organize the Atg16–Atg5–Atg12 dimeric complex [37,38]. Atg8 is conjugated to the phospholipid phosphatidylethanolamine (Atg8–PE) by serial reactions of Atg4 (an Atg8 processing enzyme), Atg7 (an E1-like protein), Atg3 (an E2-like protein), and Atg12–Atg5 (an E3-like conjugate) [39]. The two products of the conjugation systems, the Atg16–Atg5–Atg12 complex and Atg8–PE, are necessary for autophagosome generation.

4. Identification of the pre-autophagosomal structure (PAS)

We can easily imagine that indiscriminate autophagy would be catastrophic; therefore, autophagosome formation must be stringently and spatiotemporally regulated. How and where autophagosomes are generated in living cells has been a fundamental question for the field. In the face of nutrient starvation, the machinery for autophagosome formation must be assembled and/or activated in order to form autophagosomes. Electron and immunofluorescence microscopy using Atg8 as a marker showed that Atg8 is localized to isolation membranes, mature autophagosomes, and autophagic bodies [40]. GFP–Atg8 also labels these autophagic structures and further enables the monitoring of autophagy as GFP fluorescence in the vacuolar lumen in living cells [41]. Under nutrient-rich conditions, GFP–Atg8 is detected as a dot structure proximal to the vacuole, and the dot is still observable in the some *atg* disruptants [41], suggesting that this dot structure is autophagy-related but is not an autophagosome. We analyzed the colocalization of Atg8 and Atg1, Atg2, Atg5 and Atg16 by fluorescence microscopy; this analysis clarified that these Atg proteins are at least partially colocalized with Atg8 at the dot structure. Time-lapse microscopy demonstrated that the dot structure plays a central role in autophagosome formation; thus, it is named the pre-autophagosomal structure (PAS) [41]. During autophagosome formation, the fluorescence intensity of the PAS labeled by GFP–Atg8 oscillates with about 10-min duration [42,43], reflecting translocation of GFP–Atg8 from the PAS to autophagosomes.

5. Hierarchical organization model of the PAS

We have visualized almost all of the Atg proteins in *S. cerevisiae* under fluorescence microscopy [44]. Systematic disruption of each *ATG* gene and morphometric analysis clarified the interrelationships of Atg protein assembly at the PAS (Fig. 3). Under autophagy-inducing conditions, localization of Atg proteins at the PAS is severely impaired in the absence of Atg17, suggesting that Atg17 acts as a scaffold for downstream Atg protein assembly at the PAS. PAS localization of Atg1 depends on Atg13; this is consistent with a biochemical result that shows Atg13 is required for binding of Atg1–Atg17 [13]. Atg13 is also responsible for Atg14 recruitment to the PAS, suggesting that Atg13 physically interacts with the PtdIns(3) kinase complex I.

Atg9 is the sole integral membrane protein among the core Atg proteins [28]. Microscopic analyses have shown that Atg9–GFP is localized both to the PAS and to a few dozen dot structures moving around the cytoplasm [45]. The PAS localization of Atg9 is partially defective in *atg11Δ* and *atg17Δ* cells, and is abolished in the *atg11Δatg17Δ* double disruptant [44], suggesting that Atg9 associates with both Atg11 and Atg17. Indeed, Atg9 physically interacts with Atg11 and Atg17 via its N-terminal regions, and the interactions with Atg11 and Atg17 are required for the Cvt pathway and autophagy, respectively, by targeting Atg9 to the PAS [45–47]. Atg9 is proposed to cycle between the cytoplasmic pool and the PAS during Cvt vesicle/autophagosome formation [48], and it is an attractive hypothesis that Atg9 supplies lipids or membranes from the cytoplasmic pool to the PAS through its cycling. Electron microscopy shows that Atg9 is localized to membranous structures surrounding the Cvt complex in *atg1Δ* cells [49]. Analysis of the localization of Atg9 on the expanding isolation membrane will provide clues to the membrane sources of Cvt vesicles and autophagosomes.

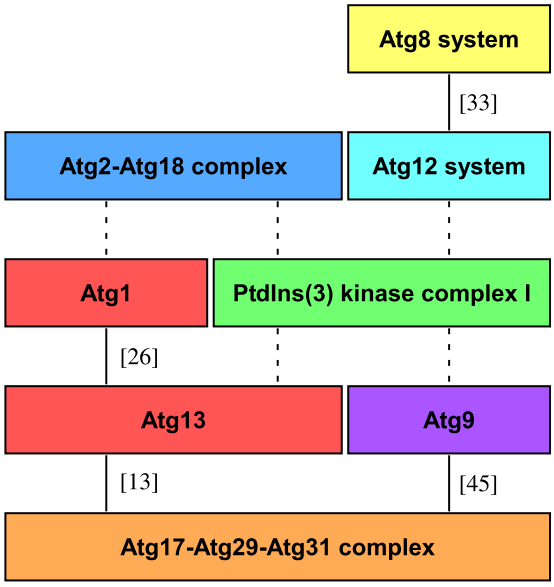

Fig. 3. Hierarchy diagram of Atg proteins. The Atg proteins/complexes are recruited to the PAS on the basis of this hierarchy. The Atg17–Atg29–Atg31 complex behaves as a scaffold for PAS organization. Solid lines are physical interactions described elsewhere. Dotted lines are genetically suggested interactions that have not yet been physically validated. The PtdIns(3) kinase complex includes Vps15, Vps30/Atg6, Vps34, and Atg14. The Atg12 system consists of Atg12, Atg7, Atg10, Atg5, and Atg16. The Atg8 system is composed of Atg8, Atg4, Atg7, and Atg3. Atg7 is involved in both systems.

PtdIns(3) kinase complex I is localized to the PAS, indicating that PtdIns(3)*P* required for autophagosome formation is produced at the PAS. PtdIns(3)*P* is localized to the inner surface of autophagosomes and autophagic bodies [50]. Atg18 was identified as a phosphoinositide binding protein [51,52]. An *atg18* mutant unable to bind to phosphoinositide ($Atg18^{FTTG}$) forms a complex with Atg2, as normal, but does not localize to the PAS, resulting in insufficient autophagic activity [30]. The Atg2–Atg18 complex is thus the most plausible functional unit acting as an effector of PtdIns(3)*P*. The Atg16–Atg5–Atg12 complex is another candidate effector of PtdIns(3)*P* in our hierarchy (Fig. 3). However, to date, there is no evidence for a direct interaction between PtdIns(3)*P* and the Atg16–Atg5–Atg12 complex.

The Atg12–Atg5 conjugate shows an E3-like activity in conjugation of Atg8 to PE in vitro, whereas no further acceleration is observed by the addition of Atg16 [39]. It is probable that Atg16 instead acts to target the Atg12–Atg5 conjugate to the PAS in vivo. In fact, PAS localization of the Atg12–Atg5 conjugate is abolished in the absence of Atg16 [41,44]. Atg8–PE is located most downstream in our hierarchy. The mechanisms of Atg8–PE retention to the PAS are largely unknown. One possible explanation is that Atg8 is produced at the PAS, as the Atg16–Atg5–Atg12 complex (an E3-like protein complex) is localized there. Analysis of the localization of

Atg3 will clarify the site of Atg8 lipidation. In the absence of upstream Atg proteins, Atg8–PE fails to localize to the PAS. Atg8–PE may interact with upstream Atg proteins and/or membrane structures produced by the upstream Atg proteins.

6. Two modes of the PAS under autophagy-inducing conditions

Under nutrient-rich conditions, Atg11 is proposed to constitutively act as a scaffold for PAS organization in the Cvt pathway [53], whereas Atg17 is dispensable [12,13]. Under starvation conditions, the activity of autophagy largely depends on Atg17 [12,13]. Even in the absence of Atg17, small and abnormal autophagosomes are generated [12,13], resulting in significant amounts of GFP–Atg8 localizing to the PAS and being transported to the vacuole during autophagy [44]. This remaining GFP–Atg8 transport depends on Atg11; double disruption of *ATG11* and *ATG17* leads to a severe synergistic defect in GFP–Atg8 transport to the vacuole [44]. We conclude that both Atg11 and Atg17 act as scaffolds for PAS organization under starvation conditions. By using *atg11Δ* cells to eliminate the Atg11-dependent PAS, formation of the Atg17-dependent PAS, termed the autophagy-specific PAS, can be monitored [54]. The Atg11-dependent PAS and the autophagy-specific PAS are genetically separable but exist as one functional entity in wild-type yeast cells. The Atg11-dependent PAS is organized mostly for selective autophagy, including Ape1 transport, whereas the autophagy-specific PAS acts in nonselective autophagy. Recent studies showed that the Atg1–Atg13 and Atg17–Atg29–Atg31 complexes are crucial for the organization of the autophagy-specific PAS [27,54].

In wild-type yeast cells, Ape1 has been used as a PAS marker for fluorescence microscopy studies. In principle, this is inappropriate, as Ape1 is just a cargo. In fact, careful observation of Ape1 and Atg14 shows that their localization is slightly different [34]. Under nutrient-rich conditions, Atg11 is a useful marker of the PAS [55]. As far as we know, the most suitable markers of the autophagy-specific PAS are components of the autophagy-specific PAS scaffold (Atg1, Atg13, Atg17, Atg29, and Atg31). Among these proteins, Atg1–GFP is less suitable, since it may localize to isolation membranes and autophagosomes in addition to the PAS [56].

7. Hierarchy of Atg proteins in mammalian cells

A majority of the core *ATG* genes are conserved in almost all eukaryotes. Conservation of the two ubiquitin-like conjugation systems was the first to be demonstrated in mammals [57–59]. ULK1/2 (mAtg1), mAtg13, FIP200, mAtg9, the class III PtdIns(3) kinase complex I (mVps15, mVps34, Beclin 1/mVps30/mAtg6, mAtg14), and WIPI-1 (mAtg18) have subsequently been reported; these proteins/complexes cover a majority of the functional units found in yeast. As far as we know, the hierarchy of mammalian Atg proteins is fundamentally consistent with that of yeast.

In mammalian cells, isolation membranes can be recognized by fluorescence microscopy using the mammalian Atg16–Atg5–Atg12 complex as a marker [58,59]. This complex dissociates from the isolation membrane immediately before or after autophagosome formation has been completed [58]. ULK1/2 [60], mAtg13 [61,62], FIP200 [60], and WIPI-1 [63] are exclusively localized to isolation membranes. LC3 is well characterized as a marker localized to autophagosomes as well as isolation membranes [57,58]. Similar to yeast, at least two types of class III PtdIns(3) kinase complexes containing Beclin 1/mAtg6 have been reported [64–66]. The class III PtdIns(3) kinase complex I, containing mAtg14, is localized to isolation membranes and the ER [65]. Under nutrient-rich conditions, mAtg9 is localized to the trans-Golgi network and peripheral endosomes [67]. Upon starvation, mAtg9 is partially colocalized with LC3 [63,68], but it is unclear whether mAtg9 is localized to autophagosomes.

FIP200 was reported as a functional counterpart of Atg17 [60]. In FIP200 knock-out cells, autophagy is severely blocked, probably because starvation-induced assembly of Atg proteins is deficient: ULK1/2, mAtg13, mAtg5, and LC3 are not recruited to isolation membranes [60,61]. These facts suggest that FIP200 acts as a scaffold for Atg protein organization in mammalian cells similar to yeast Atg17.

mAtg13 is required for autophagy in mammalian cells [61,62,69,70]. The characteristics of mAtg13 are similar to yeast Atg13; mAtg13 forms a complex with ULK and FIP200 [62], and mAtg13 mediates the interaction of ULK with FIP200 [61]. In mAtg13 knock-down cells, localization of ULK1 to isolation membranes is abolished [61]. Binding of mAtg13 and ULK1 is not affected by starvation or rapamycin treatment [62], whereas Atg1 associates with Atg13 in response to these treatments in *S. cerevisiae*. Behavior of the ULK1–mAtg13 complex is different from that of the Atg1–Atg13 complex in yeast in regard to the starvation/rapamycin response. Quite recently, Atg101 has been reported to interact with mAtg13 [71,72]. Atg101 is included in the ULK1–mAtg13–FIP200 complex and is localized to isolation membranes. Atg101 is thought to bind to free mAtg13 and protect it from proteasomal degradation [71]. In mAtg13-depleted cells, the ULK1–mAtg13–Atg101–FIP200 complex is disassembled and Atg101 is recovered as a monomer [72]. Atg101 is required for autophagy; further investigation will clarify its precise function.

It is well-known that the PtdIns(3) kinase inhibitors Wortmannin and 3-methyladenine inhibit autophagy in mammalian cells [73,74]. These treatments abolish the localization of mAtg5 to isolation membranes [58], suggesting that the activity of PtdIns(3)*P* is necessary for recruitment of the mammalian Atg16–Atg5–Atg12 complex to isolation membranes. Recently, a counterpart of Atg14 was identified as an essential protein for autophagy [64–66]. The lack of mAtg14 causes a defect in targeting the mammalian Atg16–Atg5–Atg12 complex to isolation membranes [65], possibly leading to a defect in LC3 localization to isolation membranes [65,66]. When PtdIns(3) kinase activity is inhibited by Wortmannin treatment, mAtg14 is still localized to autophagic membranes [66], suggesting that localization of the class III PtdIns(3) kinase complex I is independent of its kinase activity, whereas its activity is required for recruiting downstream Atg proteins, such as the mammalian Atg16–Atg5–Atg12 complex and LC3, to isolation membranes.

Recently, a specialized PtdIns(3)*P*-rich ER region termed an omegasome has been identified using DFCP1 (*d*ouble *F*YVE domain-containing *p*rotein *1*) as a marker [75]. Fluorescence microscopy demonstrated that omegasomes are closely localized outside of isolation membranes. Further, the ultrastructure of omegasomes is analyzed by three dimensional electron tomography; this ER-isolation membrane complex is interconnected by a narrow membrane extension [76,77]. This fact suggests the involvement of the ER in autophagosome formation in mammalian cells. In yeast, the perinuclear ER is associated with the vacuole at the nuclear-vacuolar junction and the PAS is often localized there (our unpublished observation). It is possible that the PAS and the ER are associated. Although it is still unclear whether isolation membranes and the ER are physically connected, membrane trafficking between ER and Golgi is important for autophagosome formation [78].

As in yeast, mAtg12 is conjugated to mAtg5 and the conjugate subsequently associates with mAtg16 [59]. These three proteins are thought to be colocalized at isolation membranes [58,59]. In mammals, Wortmannin or 3-methyladenine treatment inhibits recruitment of the mammalian Atg16–Atg5–Atg12 complex to isolation membranes [59,66]. Moreover, knock-out of mAtg14 abro-

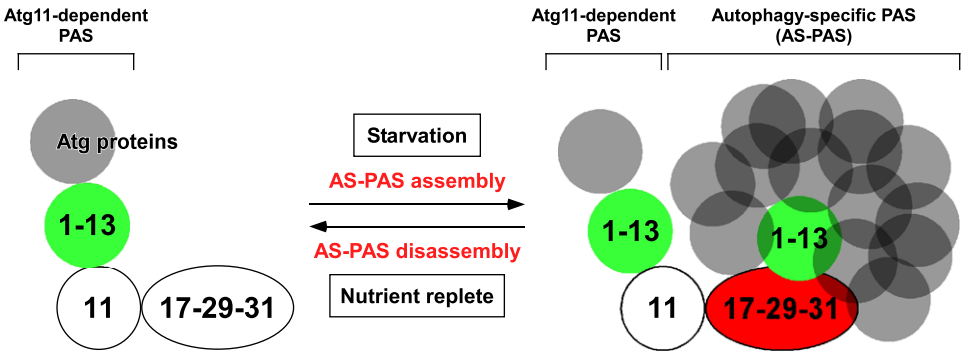

Fig. 4. Model for PAS organization in response to nutrient conditions. Atg proteins are assembled by the function of Atg11 to form the Atg11-dependent PAS irrespective of nutrient conditions. Upon starvation, the Atg17–Atg29–Atg31 complex is activated by binding to the Atg1–Atg13 complex, and organizes the autophagy-specific PAS. The autophagy-specific PAS is rapidly disassembled by addition of nutrients.

gates recruitment of the mAtg16 complex to isolation membranes [65]. Therefore, a common characteristic of both yeast and mammalian cells is that the class III PtdIns(3) kinase complex I is responsible for the localization of the Atg16–Atg5–Atg12 complex to autophagic membranes. In yeast, Atg16 and Atg5 are mutually required for targeting to the PAS [44]. This is also the case in mammalian cells [59,79].

Conjugation of LC3 with PE is necessary for its targeting to isolation membranes [58,80,81]. ULK1/2 [61,69], mAtg13 [61,62,69], FIP200 [60,61], the class III PtdIns(3) kinase complex I (mAtg14 [65,66]), the mammalian Atg16–Atg5–Atg12 complex [58,79], and mAtg9 [68] are required for LC3 localization. The requirement of the Atg2–Atg18 complex for localization of LC3 to isolation membranes has not yet been determined. *atg11Δ* yeast cells are available to monitor the autophagy-specific PAS; the autophagy-specific PAS, which is labeled with the Atg1–Atg13 and Atg17–Atg29–Atg31 complexes, emerges only upon starvation [54]. In the case of mammalian cells, isolation membranes, which are labeled with the ULK1–mAtg13–FIP200 complex, appear only during autophagy. This fact prompts us to hypothesize that isolation membranes in mammalian cells morphologically resem those in yeast but have a function that corresponds to the autophagy-specific PAS as well. [54]. If mammalian cells correspond to yeast *atg11Δ* cells, FIP200 would label autophagy-related structures/membranes corresponding to the PAS in cells defective in mAtg2, mAtg9, or mAtg14.

8. Induction of autophagy by organizing the PAS scaffold upon starvation

In yeast, TORC1 directly phosphorylates Atg13 to inhibit autophagy under nutrient-rich conditions [82]. Atg1 is a Ser/Thr protein kinase that phosphorylates itself [83]; the significance of autophosphorylation is still unclear. Upon starvation, Atg13 is dephosphorylated and binds to Atg1, forming the Atg1–Atg13 complex [26]. This interaction strongly enhances the kinase activity of Atg1, leading to autophagosome formation [26]. Initiation of autophagy is regulated in mammals in a similar way. TORC1 phosphorylates mAtg13 and ULK under nutrient-rich conditions and negatively regulates the kinase activity of ULK [61,62,69]. mAtg13 is partially dephosphorylated in response to starvation [62] and positively regulates ULK activity [69]. ULK1, mAtg13, and FIP200 form a protein complex of over 1 M Da [61,62]. TORC1 associates with the ULK1–mAtg13–FIP200 complex under nutrient-rich conditions and dissociates from the complex in response to starvation [62]; the association/dissociation of TORC1 with the ULK1–mAtg13–FIP200 complex presumably regulates ULK1 activity by modifying the phosphorylation status of mAtg13 [60].

Determination of the site of PAS organization is the most fundamental question remaining to be solved. In the case of yeast, the mechanisms depend on the mode of autophagy. Under nutrient-rich conditions, the cargo-receptor complex plays a major role in Atg11-dependent PAS formation. Upon starvation, the Atg1–Atg13 complex associates with the Atg17–Atg29–Atg31 complex to trigger autophagy-specific PAS organization (Fig. 4), which does not require Atg11 [54]. By interacting with the Atg1–Atg13 complex, the Atg17–Atg29–Atg31 complex organizes a scaffold for the autophagy-specific PAS [54]. However, it remains to be elucidated how the scaffold complex determines the location at which it assembles.

9. Future directions

Since we identified the PAS, fluorescence microscopy has been the only way to analyze the structure. At present, we cannot distinguish the PAS and isolation membranes as both structures are detected just as dots under a fluorescence microscope. To understand the mechanisms of autophagosome formation, the next step we must undertake is to discriminate the isolation membrane from the PAS. Improvement of spatiotemporal resolution of fluorescence microscopy is a possible way to overcome this limit. The morphological and biochemical analysis of isolation membranes will enable us to analyze various aspects of the isolation membrane dynamics: how it expands, how its outer and inner membranes differentiate, and how its curvature and diameter are determined.

References

[1] Takeshige, K., Baba, M., Tsuboi, S., Noda, T. and Ohsumi, Y. (1992) Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. J. Cell Biol. 119, 301–311.
[2] Baba, M., Takeshige, K., Baba, N. and Ohsumi, Y. (1994) Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterization. J. Cell Biol. 124, 903–913.
[3] Tsukada, M. and Ohsumi, Y. (1993) Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. FEBS Lett. 333, 169–174.
[4] Thumm, M., Egner, R., Koch, B., Schlumpberger, M., Straub, M., Veenhuis, M. and Wolf, D.H. (1994) Isolation of autophagocytosis mutants of *Saccharomyces cerevisiae*. FEBS Lett. 349, 275–280.
[5] Harding, T.M., Morano, K.A., Scott, S.V. and Klionsky, D.J. (1995) Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway. J. Cell Biol. 131, 591–602.
[6] Harding, T.M., Hefner-Gravink, A., Thumm, M. and Klionsky, D.J. (1996) Genetic and phenotypic overlap between autophagy and the cytoplasm to vacuole protein targeting pathway. J. Biol. Chem. 271, 17621–17624.
[7] Scott, S.V., Hefner-Gravink, A., Morano, K.A., Noda, T., Ohsumi, Y. and Klionsky, D.J. (1996) Cytoplasm-to-vacuole targeting and autophagy employ the same machinery to deliver proteins to the yeast vacuole. Proc. Natl. Acad. Sci. USA 93, 12304–12308.
[8] Klionsky, D.J. et al. (2003) A unified nomenclature for yeast autophagy-related genes. Dev. Cell 5, 539–545.

[9] Baba, M., Osumi, M., Scott, S.V., Klionsky, D.J. and Ohsumi, Y. (1997) Two distinct pathways for targeting proteins from the cytoplasm to the vacuole/lysosome. J. Cell Biol. 139, 1687–1695.
[10] Kim, J., Huang, W.P. and Klionsky, D.J. (2001) Membrane recruitment of Aut7p in the autophagy and cytoplasm to vacuole targeting pathways requires Aut1p, Aut2p, and the autophagy conjugation complex. J. Cell Biol. 152, 51–64.
[11] Xie, Z. and Klionsky, D.J. (2007) Autophagosome formation: core machinery and adaptations. Nat. Cell Biol. 9, 1102–1109.
[12] Cheong, H., Yorimitsu, T., Reggiori, F., Legakis, J.E., Wang, C.W. and Klionsky, D.J. (2005) Atg17 regulates the magnitude of the autophagic response. Mol. Biol. Cell 16, 3438–3453.
[13] Kabeya, Y., Kamada, Y., Baba, M., Takikawa, H., Sasaki, M. and Ohsumi, Y. (2005) Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. Mol. Biol. Cell 16, 2544–2553.
[14] Kabeya, Y., Kawamata, T., Suzuki, K. and Ohsumi, Y. (2007) Cis1/Atg31 is required for autophagosome formation in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. 356, 405–410.
[15] Kawamata, T., Kamada, Y., Suzuki, K., Kuboshima, N., Akimatsu, H., Ota, S., Ohsumi, M. and Ohsumi, Y. (2005) Characterization of a novel autophagy-specific gene, *ATG29*. Biochem. Biophys. Res. Commun. 338, 1884–1889.
[16] Kim, J. et al. (2001) Cvt9/Gsa9 functions in sequestering selective cytosolic cargo destined for the vacuole. J. Cell Biol. 153, 381–396.
[17] Scott, S.V., Guan, J., Hutchins, M.U., Kim, J. and Klionsky, D.J. (2001) Cvt19 is a receptor for the cytoplasm-to-vacuole targeting pathway. Mol. Cell 7, 1131–1141.
[18] Leber, R., Silles, E., Sandoval, I.V. and Mazon, M.J. (2001) Yol082p, a novel CVT protein involved in the selective targeting of aminopeptidase I to the yeast vacuole. J. Biol. Chem. 276, 29210–29217.
[19] Suzuki, K., Kamada, Y. and Ohsumi, Y. (2002) Studies of cargo delivery to the vacuole mediated by autophagosomes in *Saccharomyces cerevisiae*. Dev. Cell 3, 815–824.
[20] Shintani, T., Huang, W.P., Stromhaug, P.E. and Klionsky, D.J. (2002) Mechanism of cargo selection in the cytoplasm to vacuole targeting pathway. Dev. Cell 3, 825–837.
[21] Kanki, T. and Klionsky, D.J. (2008) Mitophagy in yeast occurs through a selective mechanism. J. Biol. Chem. 283, 32386–32393.
[22] Kanki, T., Wang, K., Cao, Y., Baba, M. and Klionsky, D.J. (2009) Atg32 is a mitochondrial protein that confers selectivity during mitophagy. Dev. Cell 17, 98–109.
[23] Okamoto, K., Kondo-Okamoto, N. and Ohsumi, Y. (2009) Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. Dev. Cell 17, 87–97.
[24] Kanki, T. et al. (2009) A genomic screen for yeast mutants defective in selective mitochondria autophagy. Mol. Biol. Cell 285, 1508–1515.
[25] Nakatogawa, H., Suzuki, K., Kamada, Y. and Ohsumi, Y. (2009) Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat. Rev. Mol. Cell Biol. 10, 458–467.
[26] Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M. and Ohsumi, Y. (2000) Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J. Cell Biol. 150, 1507–1513.
[27] Kabeya, Y., Noda, N.N., Fujioka, Y., Suzuki, K., Inagaki, F. and Ohsumi, Y. (2009) Characterization of the Atg17–Atg29–Atg31 complex specifically required for starvation-induced autophagy in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. 389, 612–615.
[28] Noda, T., Kim, J., Huang, W.P., Baba, M., Tokunaga, C., Ohsumi, Y. and Klionsky, D.J. (2000) Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. J. Cell Biol. 148, 465–480.
[29] Kihara, A., Noda, T., Ishihara, N. and Ohsumi, Y. (2001) Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. J. Cell Biol. 152, 519–530.
[30] Obara, K., Sekito, T., Niimi, K. and Ohsumi, Y. (2008) The Atg18–Atg2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function. J. Biol. Chem. 283, 23972–23980.
[31] Mizushima, N. et al. (1998) A protein conjugation system essential for autophagy. Nature 395, 395–398.
[32] Kirisako, T. et al. (2000) The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J. Cell Biol. 151, 263–276.
[33] Ichimura, Y. et al. (2000) A ubiquitin-like system mediates protein lipidation. Nature 408, 488–492.
[34] Obara, K., Sekito, T. and Ohsumi, Y. (2006) Assortment of phosphatidylinositol 3-kinase complexes–Atg14p directs association of complex I to the pre-autophagosomal structure in *Saccharomyces cerevisiae*. Mol. Biol. Cell 17, 1527–1539.
[35] Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y. and Kominami, E. (1999) Apg7p/Cvt2p: a novel protein-activating enzyme essential for autophagy. Mol. Biol. Cell 10, 1367–1379.
[36] Shintani, T., Mizushima, N., Ogawa, Y., Matsuura, A., Noda, T. and Ohsumi, Y. (1999) Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. EMBO J. 18, 5234–5241.
[37] Kuma, A., Mizushima, N., Ishihara, N. and Ohsumi, Y. (2002) Formation of the approximately 350-kDa Apg12–Apg5–Apg16 multimeric complex, mediated by Apg16 oligomerization, is essential for autophagy in yeast. J. Biol. Chem. 277, 18619–18625.
[38] Fujioka, Y., Noda, N.N., Nakatogawa, H., Ohsumi, Y. and Inagaki, F. (2009) The dimeric coiled-coil structure of *Saccharomyces cerevisiae* ATG16 and its functional significance in autophagy. J. Biol. Chem. 20, 4730–4738.
[39] Hanada, T., Noda, N.N., Satomi, Y., Ichimura, Y., Fujioka, Y., Takao, T., Inagaki, F. and Ohsumi, Y. (2007) The Atg12–Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. J. Biol. Chem. 282, 37298–37302.
[40] Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T. and Ohsumi, Y. (1999) Formation process of autophagosome is traced with Apg8/Aut7p in yeast. J. Cell Biol. 147, 435–446.
[41] Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T. and Ohsumi, Y. (2001) The pre-autophagosomal structure organized by concerted functions of *APG* genes is essential for autophagosome formation. EMBO J. 20, 5971–5981.
[42] Cheong, H., Nair, U., Geng, J. and Klionsky, D.J. (2008) The Atg1 kinase complex is involved in the regulation of protein recruitment to initiate sequestering vesicle formation for nonspecific autophagy in *Saccharomyces cerevisiae*. Mol. Biol. Cell 19, 668–681.
[43] Xie, Z., Nair, U. and Klionsky, D.J. (2008) Atg8 controls phagophore expansion during autophagosome formation. Mol. Biol. Cell 19, 3290–3298.
[44] Suzuki, K., Kubota, Y., Sekito, T. and Ohsumi, Y. (2007) Hierarchy of Atg proteins in pre-autophagosomal structure organization. Genes Cells 12, 209–218.
[45] Sekito, T., Kawamata, T., Ichikawa, R., Suzuki, K. and Ohsumi, Y. (2009) Atg17 recruits Atg9 to organize the pre-autophagosomal structure. Genes Cells 14, 525–538.
[46] He, C., Song, H., Yorimitsu, T., Monastyrska, I., Yen, W.L., Legakis, J.E. and Klionsky, D.J. (2006) Recruitment of Atg9 to the preautophagosomal structure by Atg11 is essential for selective autophagy in budding yeast. J. Cell Biol. 175, 925–935.
[47] Chang, C.Y. and Huang, W.P. (2007) Atg19 mediates a dual interaction cargo sorting mechanism in selective autophagy. Mol. Biol. Cell 18, 919–929.
[48] Reggiori, F., Tucker, K.A., Stromhaug, P.E. and Klionsky, D.J. (2004) The Atg1–Atg13 complex regulates Atg9 and Atg23 retrieval transport from the pre-autophagosomal structure. Dev. Cell 6, 79–90.
[49] He, C., Baba, M., Cao, Y. and Klionsky, D.J. (2008) Self-interaction is critical for Atg9 transport and function at the phagophore assembly site during autophagy. Mol. Biol. Cell 19, 5506–5516.
[50] Obara, K., Noda, T., Niimi, K. and Ohsumi, Y. (2008) Transport of phosphatidylinositol 3-phosphate into the vacuole via autophagic membranes in *Saccharomyces cerevisiae*. Genes Cells 13, 537–547.
[51] Dove, S.K. et al. (2004) Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors. EMBO J. 23, 1922–1933.
[52] Stromhaug, P.E., Reggiori, F., Guan, J., Wang, C.W. and Klionsky, D.J. (2004) Atg21 is a phosphoinositide binding protein required for efficient lipidation and localization of Atg8 during uptake of aminopeptidase I by selective autophagy. Mol. Biol. Cell 15, 3553–3566.
[53] Shintani, T. and Klionsky, D.J. (2004) Cargo proteins facilitate the formation of transport vesicles in the cytoplasm to vacuole targeting pathway. J. Biol. Chem. 279, 29889–29894.
[54] Kawamata, T., Kamada, Y., Kabeya, Y., Sekito, T. and Ohsumi, Y. (2008) Organization of the pre-autophagosomal structure responsible for autophagosome formation. Mol. Biol. Cell 19, 2039–2050.
[55] Kim, J., Huang, W.P., Stromhaug, P.E. and Klionsky, D.J. (2002) Convergence of multiple autophagy and cytoplasm to vacuole targeting components to a perivacuolar membrane compartment prior to de novo vesicle formation. J. Biol. Chem. 277, 763–773.
[56] Abeliovich, H., Zhang, C., Dunn Jr., W.A., Shokat, K.M. and Klionsky, D.J. (2003) Chemical genetic analysis of Apg1 reveals a non-kinase role in the induction of autophagy. Mol. Biol. Cell 14, 477–490.
[57] Kabeya, Y. et al. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 19, 5720–5728.
[58] Mizushima, N. et al. (2001) Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J. Cell Biol. 152, 657–668.
[59] Mizushima, N. et al. (2003) Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12–Apg5 conjugate. J. Cell Sci. 116, 1679–1688.
[60] Hara, T., Takamura, A., Kishi, C., Iemura, S., Natsume, T., Guan, J.L. and Mizushima, N. (2008) FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. J. Cell Biol. 181, 497–510.
[61] Ganley, I.G., Lam du, H., Wang, J., Ding, X., Chen, S. and Jiang, X. (2009) ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. J. Biol. Chem. 284, 12297–12305.
[62] Hosokawa, N. et al. (2009) Nutrient-dependent mTORC1 association with the ULK1–Atg13–FIP200 complex required for autophagy. Mol. Biol. Cell 20, 1981–1991.
[63] Vergne, I., Roberts, E., Elmaoued, R.A., Tosch, V., Delgado, M.A., Proikas-Cezanne, T., Laporte, J. and Deretic, V. (2009) Control of autophagy initiation by phosphoinositide 3-phosphatase jumpy. EMBO J. 28, 2244–2258.
[64] Zhong, Y., Wang, Q.J., Li, X., Yan, Y., Backer, J.M., Chait, B.T., Heintz, N. and Yue, Z. (2009) Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. Nat. Cell Biol. 11, 468–476.
[65] Matsunaga, K. et al. (2009) Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. Nat. Cell Biol. 11, 385–396.

[66] Itakura, E., Kishi, C., Inoue, K. and Mizushima, N. (2008) Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol. Biol. Cell 19, 5360–5372.
[67] Young, A.R. et al. (2006) Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. J. Cell Sci. 119, 3888–3900.
[68] Yamada, T., Carson, A.R., Caniggia, I., Umebayashi, K., Yoshimori, T., Nakabayashi, K. and Scherer, S.W. (2005) Endothelial nitric-oxide synthase antisense (NOS3AS) gene encodes an autophagy-related protein (APG9-like2) highly expressed in trophoblast. J. Biol. Chem. 280, 18283–18290.
[69] Jung, C.H., Jun, C.B., Ro, S.H., Kim, Y.M., Otto, N.M., Cao, J., Kundu, M. and Kim, D.H. (2009) ULK–Atg13–FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol. Biol. Cell 20, 1992–2003.
[70] Chan, E.Y., Longatti, A., McKnight, N.C. and Tooze, S.A. (2009) Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. Mol. Cell. Biol. 29, 157–171.
[71] Mercer, C.A., Kaliappan, A. and Dennis, P.B. (2009) A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. Autophagy 5, 649–662.
[72] Hosokawa, N., Sasaki, T., Iemura, S., Natsume, T., Hara, T. and Mizushima, N. (2009) Atg101, a novel mammalian autophagy protein interacting with Atg13. Autophagy 5, 973–979.
[73] Blommaart, E.F., Krause, U., Schellens, J.P., Vreeling-Sindelarova, H. and Meijer, A.J. (1997) The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur. J. Biochem. 243, 240–246.
[74] Seglen, P.O. and Gordon, P.B. (1982) 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc. Natl. Acad. Sci. USA 79, 1889–1892.
[75] Axe, E.L., Walker, S.A., Manifava, M., Chandra, P., Roderick, H.L., Habermann, A., Griffiths, G. and Ktistakis, N.T. (2008) Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J. Cell Biol. 182, 685–701.
[76] Hayashi-Nishino, M., Fujita, N., Noda, T., Yamaguchi, A., Yoshimori, T. and Yamamoto, A. (2009) A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat. Cell Biol. 11, 1433–1437.
[77] Yla-Anttila, P., Vihinen, H., Jokitalo, E. and Eskelinen, E.L. (2009) 3D tomography reveals connections between the phagophore and endoplasmic reticulum. Autophagy 5, 2683–2686.
[78] Ishihara, N. et al. (2001) Autophagosome requires specific early Sec proteins for its formation and NSF/SNARE for vacuolar fusion. Mol. Biol. Cell 12, 3690–3702.
[79] Saitoh, T. et al. (2008) Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 456, 264–268.
[80] Komatsu, M. et al. (2005) Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J. Cell Biol. 169, 425–434.
[81] Sou, Y.S. et al. (2008) The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. Mol. Biol. Cell 19, 4762–4775.
[82] Kamada, Y., Yoshino, K., Kondo, C., Kawamata, T., Oshiro, N., Yonezawa, K. and Ohsumi, Y. (2010) Tor directly controls the Atg1 kinase complex to regulate autophagy. Mol. Cell. Biol. 30, 1049–1058.
[83] Matsuura, A., Tsukada, M., Wada, Y. and Ohsumi, Y. (1997) Apg1p, a novel protein kinase required for the autophagic process in *Saccharomyces cerevisiae*. Gene 192, 245–250.