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Review

Advances in Autophagy Regulatory Mechanisms

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Abstract: Autophagy plays a critical role in cell metabolism by degrading and recycling internal components when challenged with limited nutrients. This fundamental and conserved mechanism is based on a membrane trafficking pathway in which nascent autophagosomes engulf cytoplasmic cargo to form vesicles that transport their content to the lysosome for degradation. Based on this simple scheme, autophagy modulates cellular metabolism and cytoplasmic quality control to influence an unexpectedly wide range of normal mammalian physiology and pathophysiology. In this review, we summarise recent advancements in three broad areas of autophagy regulation. We discuss current models on how autophagosomes are initiated from endogenous membranes. We detail how the uncoordinated 51-like kinase (ULK) complex becomes activated downstream of mechanistic target of rapamycin complex 1 (MTORC1). Finally, we summarise the upstream signalling mechanisms that can sense amino acid availability leading to activation of MTORC1.

Keywords: autophagy; amino acids; ULK; MTOR; autophagosome; isolation membrane; Beclin 1; VPS34; ATG14L

1. Introduction

All cells are challenged to adapt their metabolic pathways in response to nutritional levels and the resulting states of homeostasis go on to direct downstream decisions on growth, arrest or death. Macroautophagy, hereafter simply referred to as autophagy, is the intracellular recycling process that supports survival during times of energy stress and nutrient starvation. Under low nutrient conditions, cellular membranes elongate and sequester portions of the cytoplasm to capture a range of targets including proteins, organelles or foreign matter. This cargo capture event can be non-specific or directed by a family of adaptor proteins that recognise components labelled for degradation by ubiquitination. Engulfment of material then leads to formation of a nascent double-bilayer enclosed vesicle known as an autophagosome. Formation of the autophagosome represents early phases of autophagy in contrast to the late maturation stages that include the transport of autophagosomes and subsequent fusion with lysosomes or late endocytic compartments. Content mixing of autophagosomes and lysosomes marks transition to the degradative phase of autophagy in which metabolic building blocks are recycled back to the cytoplasm.

As a fundamental degradation and stress-response pathway, the function of autophagy is well conserved from plant and yeast, all the way through mammalian physiology. In humans, autophagy has become appreciated to play prominent roles in maintaining normal health at the cellular and organismal level from birth onwards, supporting essential homeostatic pathways that counteract slow deleterious events associated with aging [1,2]. The intracellular catabolism driven by autophagy influences an immense range of downstream functions in tissues, affecting outcomes in a number of medical fields including cancer, neurodegeneration and immunity, as reviewed elsewhere [3–7].

Research on autophagy can be historically traced back to the work of Christian de Duve during characterisation of the lysosome, leading to our current molecular era focusing on regulatory and

membrane trafficking mechanisms [8]. While our understanding has steadily progressed, important questions still remain on how rates of autophagy are regulated in response to different extracellular cues and stress [9]. In many respects, it is helpful to be reductionist and consider just the rapid autophagy response following acute nutrient starvation, as this canonical form has been the most widely observed and is controlled by the core pathways. As the main nutrient-sensitive routes, starvation stress generally leads to activation of AMPK and inactivation of MTOR complex 1 (MTORC1) pathways [10]. AMPK and MTORC1 signals converge by phosphorylating and regulating the kinase, ULK1. ULK1 (or its close family member ULK2) represent the central components of the ULK1/2 autophagy regulatory complex that includes additional factors ATG13, ATG101 and FIP200 (RB1CC1). Once activated, the ULK1/2 complex drives initiation of autophagosome formation, leading to increased levels of autophagy. As such, the AMPK–MTORC1–ULK1/2 mechanism represents a critical upstream control point of the autophagy cascade and a key target for developing strategies to manipulate autophagy in biomedical contexts.

Discoveries in autophagy mechanisms continue to be uncovered even as we are now two decades post isolation of the initial yeast ATG genes. Undoubtedly, progress in mammalian autophagy has been essentially founded on studies of the homologous pathways in yeast and other model systems. From these concerted experiments, the field has defined how core features of the regulatory network are conserved while additional layers of complexity have joined during the evolution to higher organisms. Indeed, function of ULK1 in driving early stages of autophagy is directly parallel to roles of the homologous yeast ATG1 complex. Here, we aim to summarise three broad areas in mammalian autophagy that have undergone dramatic levels of revision in recent years. We discuss models for formation and expansion of autophagosome membranes. We next discuss how the ULK1/2 complex is activated to signal downstream autophagsome formation. Finally, we shift towards signalling mechanisms further upstream and discuss the emerging network of sensor pathways that link amino acid availability to the activation of MTORC1.

2. Formation of Autophagy Isolation Membranes

Once starvation signals are transmitted, the cell responds within minutes to increase formation of pre-autophagosomes, also known as isolation membranes (IM). These changes demonstrate a substantial rate of organelle biogenesis that is driven by a combination of membrane transport and remodelling. Since early morphological studies, the mechanism for autophagosome formation has been a fundamental question that still remains elusive in key details [11]. In yeast, autophagosomes are generated from a single perivacuolar membrane compartment termed the pre-autophagosomal structure (PAS) ([12]). In mammalian cells, however, the analogous IM assembly incorporates more complexity, with typically tens of autophagsosome initiation sites per cell upon activation. The key issues are: where are the initiation sites formed and from which source is the IM derived? These inter-related questions continue to be controversial, although the consensus is that autophagosome initiation sites are most often associated with microdomains of the endoplasmic reticulum (ER). The mechanisms of autophagosome formation have been reviewed in detail elsewhere [13–15]. Below, we summarise key features of ER-centralised autophagosome initiation and its stimulation by coordination of the ULK1/2 and Beclin 1/VPS34 complexes (summarised in Figure 1).

Earlier morphological and biochemical data from starved hepatocytes first indicated that ER was predominantly associated with autophagosomes [11]. Since these observations, evidence with fluorescent-tagged autophagy molecular components further supports a close link between IM formation and the ER. One significant advance was the demonstration of autophagosome assembly on regions of membrane derived from the ER marked by the protein DFCP1 (double FYVE domain-containing protein 1) [16]. Via its phosphatidylinositol-3 phosphate (PI3P) binding FYVE domains, DFCP1 was observed to translocate onto distinct PI3P-enriched regions of the ER following amino acid starvation and autophagy induction. Because these PI3P-containing DFCP1-labelled structures had a cup shape (like the uppercase letter-Omega), they were named

omegasome membranes. While the functional role of DFCP1 for autophagosome formation remains enigmatic [16], DFCP1 remains a robust marker of IM initiation sites in a range of cell models.

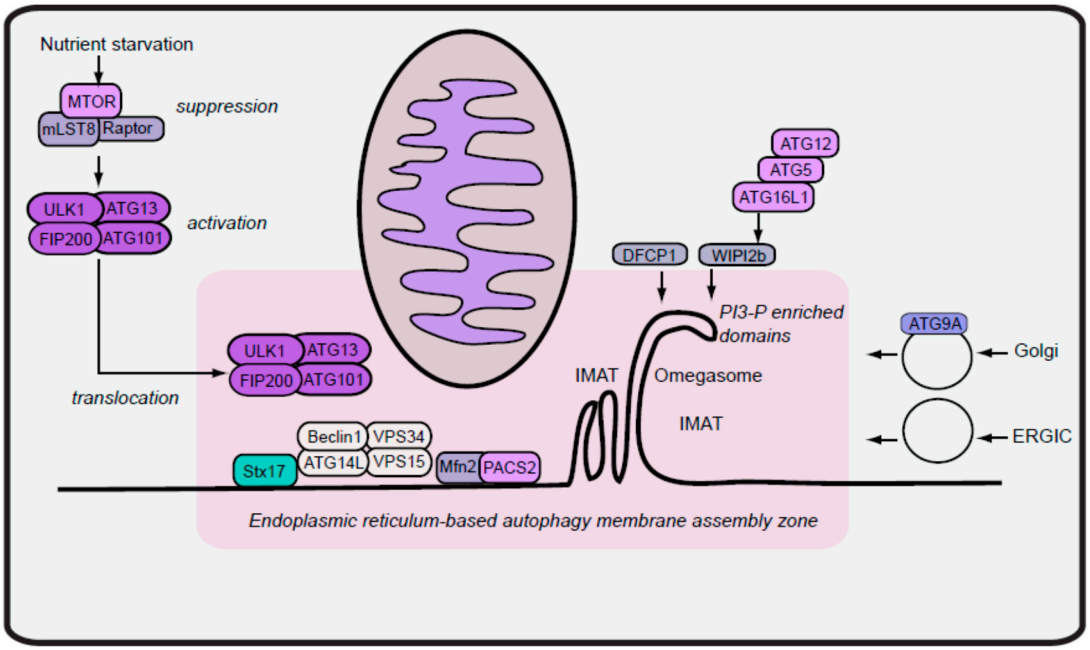

Figure 1. Assembly of autophagy isolation membranes. Starvation of nutrients leads to suppression of MTOR complex 1 resulting in downstream activation of the ULK1 autophagy initiation complex. The activated ULK1 complex translocates to an ER-based early autophagy membrane assembly zone associated with mitochondria contact sites, maintained in part via mitofusin 2 (Mfn2) and phosphofurin acidic cluster sorting protein-2 (PACS2). This assembly zone corresponds to early autophagy initiation puncta visualised by light microscopy. Activated ULK1 signals downstream by phosphorylating and activating the Beclin 1–VPS34–ATG14L–VPS15 complex, driving the generation of isolation membrane associated tubules (IMAT) and the omegasome. PI3P-enriched microdomains recruit markers like DFCP1 and machinery like WIPI2b along with further associated factors such as the ATG16L1–ATG5–ATG12 complex. After initial stages of membrane assembly, Golgi-derived vesicles containing ATG9A- and ERGIC-derived vesicles contribute further membranes and assembly machinery.

2.1. Early Isolation Membranes from ER-Mitochondria Contacts

Ultrastructural studies have helped characterise the intimate spatial relationship between IM initiation sites and the ER. Conventional transmission electron microscopy first suggested this close association [17,18], which has been resolved to finer detail using 3D electron tomography. For example, in amino acid-starved normal rat kidney (NRK) cells, multiple direct interconnections could be observed between the ER and forming autophagosome, in particular near the growing edge of the IM [19]. In addition, tomography has been able to reveal intimate structural features, such as the close association between the IM/ER interface and lipid droplets, consistent with proposed roles for lipolysis-derived neutral lipids to support autophagosome membrane expansion [20,21]. Tomography carried out using NIH3T3 cells revealed how cup-shaped bilayer regions of the ER can partially support both sides of the forming IM, leading to the conception of the ER cradle model for autophagosome formation [22]. Other experiments combining electron microscopy and tomography have characterised the presence of DFCP1-positive 30 nm tubular membrane structures associated with the ER that join to edges of the IM [23]. These IM-associated tubules (IMATs) were widely observed, for example, in HeLa, retinal epithelial, hepatoma and NRK cell lines. Moreover, IMAT could be detected in ATG3, ATG5, ATG7 or ATG16L1 −/− MEFs, which are deficient in different components of the autophagy

ATG8/LC3 conjugation pathway. In contrast, IMAT formation was blocked in MEFs lacking the FIP200 component of the ULK complex. These data support a model in which early IM formation, driven by tubulated ER projections, precedes involvement of membrane fusion events driven by activated ATG8 proteins. The common aspect was that membrane assembly was occurring on or in direct proximity to the cradle-shaped omegasome sites associated with DFCP1, and importantly, ULK/FIP200 function was essential for this step.

What are the upstream pathways signalling IM formation? Organisation of DFCP1-omegasomes are driven by increases in local concentrations of PI3P, formed by the VPS34 Class III PI3-kinase. VPS34 is part of the Beclin 1 autophagy signalling complex, which includes subunits ATG14L (also known as Barkor) and p150/VPS15 [24]. The multi-functionality of the Beclin 1 complex needs to be noted. While the core complex containing ATG14L drives autophagy membrane initiation, additional related Beclin 1 complexes are formed through modulatory subunits UVRAG, Rubicon and Ambra1 to regulate distinct trafficking stages of the formed autophagosome or endosome to the lysosome [25,26]. As expected, inhibition of ATG14L or its correct translocation to the ER upon starvation prevents formation of omegasomes [27]. Correct targeting of ATG14L is also key for anchoring Beclin 1/VPS34 complexes at IM assembly sites on ER microdomains. A conserved region within ATG14L, termed BATS (Barkor/ATG14L autophagosome targeting sequence), has been identified, that preferentially binds PI3-P and curved membranes [28]. As such, ATG14L may also have structural roles in shaping or stabilising the forming IM. Interestingly, other cell biology and biochemical approaches suggested that mitochondria were also critical in providing lipids for autophagosomes [29]. Further analysis of ATG14L during membrane assembly was able to provide some clarification in the model by highlighting the involvement of ER-mitochondria contact sites during autophagy initiation [30].

The ER is a vast membrane network, serving as the primary location for synthesis of proteins to be targeted towards the Golgi and further downstream secretion or lysosome maintenance. The ER shares connectivity with the nuclear envelope and most other major classes of cellular membranes, including mitochondria. ER-mitochondria contact sites have recently become better characterised as membrane microdomains with specific regulatory factors, enzymatic activities and functions for lipid and calcium homeostasis [31]. Components of ER-mitochondria contact sites are particularly enriched in mitochondrial-associated ER membranes (MAM), a biochemical fraction associated with markers such as the enzyme fatty acid CoA ligase 4. With this biochemical approach, it was first noted that ATG14L was not present in MAM isolated from cells maintained under full nutrients. Upon starvation, ATG14L, along with DFCP1 and other subunits of the Beclin 1/VPS34 complex, became enriched in the MAM, indicating a dramatic nutrient-dependent recruitment of autophagy factors. Live-cell imaging, indeed, could show ATG5, a marker for forming IM [32], associating with ER and mitochondrial markers. Quantification revealed that ATG5-labelled IM were almost entirely associated with ER markers, while association with the mitochondrial marker was dynamic and transient. These observations are consistent with a refined model in which the ER forms the predominant stable scaffold for forming IM, with more rapid membrane contributions from mitochondria contacting IM assembly sites. Knockdown of genes critical for the organisation of ER-mitochondria contact points (such as phosphofurin acidic cluster sorting protein-2 or mitofusin 2) blocked proper translocation of ATG14L and formation of functional autophagosomes. Interestingly, knockdown of the ER-associated SNARE protein, syntaxin 17, also disrupted proper localisation of ATG14L at ER–mitochondria contacts and an accumulation of arrested autophagy membranes. Thus, SNARE syntaxin 17 has important organisational roles for the ATG14L complex during the early stages, before acting at later during autophagosome maturation [33].

Compilation of all data leads to a consensus mechanism. Assembly of ATG14L-anchored Beclin 1/VPS34 complexes at the ER–mitochondria interface generates concentrated pools of PI3-P that then drive membrane tubulation to form and expand the IM. Lipid-dependent IM formation depends on PI3-P interacting effectors such as members of the beta-propellers that bind phosphoinositides (PROPPIN)/WIPI protein family (summarised elsewhere [34]). The WIPI2b member, specifically,

has been shown to have a downstream effector mechanism by binding ATG16L1, thus recruiting the ATG5–ATG12–ATG16L1 oligomeric complex to the initiation site [35]. The ATG5–ATG12 moiety of the complex in turn promotes lipidation of ATG8/LC3 by directly binding and stimulating activity of ATG3 [36,37]. As such, a step-wise mechanism is formed linking Beclin 1/VPS34 recruitment, PI3-P generation and ATG8 activation.

2.2. Maturation of Isolation Membranes via Vesicles from ER Exit Sites

Overall, the model with ATG14L–Beclin 1–VPS34 complexes on ER–mitochondria contact sites may reflect the central pathway for IM initiation. Multiple pathways likely feed into this basic scheme during the elongation phase forming the complete autophagosome. Much evidence supports the contribution of a wide range of cellular membranes (besides the ER and mitochondria) towards autophagosomes, including endosomes, the Golgi and the plasma membrane [38–41]. The long existing controversy on this topic seems to indicate that autophagosome formation involves the core pathway, with ER-based initiation, coupled with additional non-mutually exclusive membrane interactions that drive membrane growth to form the complete autophagosome. Other work has further suggested that ER exit sites (ERES) may play a critical role for autophagy, particularly at the elongation stage ([42], and reviewed in [43]). ERES are specialised platforms on the ER where proteins are sorted into COP II coatomer vesicles for downstream trafficking to the Golgi [44]. In yeast, mutation of SEC12 (GEF for the Sar1 GTPase) disrupts ERES and prevents proper localisation of ATG14 and downstream assembly steps [45].

Studies of mammalian autophagy have highlighted particularly important functions for the ER–Golgi intermediate compartment (ERGIC) [46], which is the membrane structure receiving vesicles from the ERES for further trafficking towards the *cis*-Golgi. Cell-free biochemistry approaches that reconstituted the ATG8/LC3 lipidation step indicated that membranes from the ERGIC fraction were enriched in LC3 lipidation activity, representing the presence of ATG5–ATG12–ATG16L and ATG3. In contrast, MAM fractions did not contain any detectable LC3 lipidation activity. These data suggest that IM may initially form from ER–mitochondria exit sites, while ATG8 lipidation activity joins later via transport vesicles from the ERGIC. Generation of COP II vesicles carrying ATG8/LC3 lipidation activity is dependent on PI3-P formation [47]. As such, the ATG14L–Beclin 1–VPS34 complex has been proposed to remain associated to IM initiation sites that then later mature into membranes associated with ERES and ERGIC. An alternative is that ATG14L–Beclin 1–VPS34 joins into COP II vesicles *en route* to the ERGIC, but current evidence cannot discriminate between these two possibilities.

The model above features IM from ER–mitochondria contact sites gaining further membranes and autophagy machinery via ERGIC-derived vesicles. While pared down schemes can be useful, it is widely acknowledged that multiple pathways are needed for a holistic view of autophagosome formation. Morphological analysis of membranes in starved NRK cells shed further light on the relative balance of pathways involved, even within the same cell type and stimulus [48]. Quantification of immuno-electron microscopy has aimed to measure sites of LC3-labelled newly formed autophagosomes. In agreement with the emerging model, early autophagosomes were observed most frequently with ER (59%). Autophagosomes were also observed in proximity with other membranes, but with lower frequency (close to: mitochondria (22%), recycling or late endosomes (16%) and Golgi (14%)). These data are consistent with live-cell imaging indicating that IM form with most stable support from the ER with additional transient contact of mitochondria [30]. The fewer instances of Golgi overlap may reflect lower relative amounts or shorter half-lives of these contacts, while the co-localisation with late endosomes may represent the maturation stage. Higher resolution tomography could reveal further clues, specifically on early IM formation. These data similarly supported the ER as the foundation membrane for autophagy initiation. In addition, tomography captured further evidence for IM closely tethered to a range of cellular membranes, in some cases with one IM simultaneously contacting multiple types of membranes. More strikingly, 100% of IM observed were in close proximity to the ER. IM were associated, but less frequently, with mitochondria (29%)

and endosomes/lysosomes (17%), while IM were rarely captured next to ERES (0.5%) or Golgi (0.5%), which could represent either fast membrane contacts or relatively lower contributions.

2.3. Outlook on Autophagy Membrane Initiation

To conclude, the data together support a model in which IM are formed from an initial ER-based membrane site (Figure 1). Following, multiple types of membranes dynamically interact with the ER to further donate membranes for IM initiation and subsequent elongation, although mitochondria and endosomes seem to be the major contributors. Vesicles from the ERGIC donate membranes and further factors such as ATG8 lipidation machinery to the forming autophagosome. Rapidly trafficking vesicles containing ATG9A (also known as mATG9) also contribute essential membranes to the IM [49,50]. The ATG9A pathway provides a mechanism to incorporate traffic from endosomes, Golgi and the plasma membrane biogenesis [40,51]. A recurring theme is that factors can play key roles at multiple steps. The ER SNARE syntaxin 17, appears to coordinate events at IM formation, autophagosome completion and degradative maturation. Vacuolar membrane protein 1 (VMP1) is another ER protein that may function at both early and later stages of autophagy [49,52]. ATG14L, which has been highlighted here to assemble Beclin 1/VPS34 complexes to initiate the IM, plays additional roles during maturation via syntaxin 17 [53]. ATG5 is a protein that assembles early at IM formation sites [32], yet appears to be essential for later elongation stages [49], possibly by promoting ATG8 lipidation activity via interactions with ATG3 [37]. The ATG3 pathway can further incorporate membrane-sensing mechanisms, directing ATG8-lipidation activity towards highly curved bilayers such as on the IM edges [54]. In this regard, a number of pathways are likely necessary to help properly shape the curved membranes encountered during autophagy. The BATS domain in ATG14L can provide structural support at highly curved bilayers or tubules [28]. Localised punctate assemblies of polymerised actin may also help provide scaffolding to shape IM initiating from the ER [55]. While IM formation is ER-based, other data highlight that autophagy can be turned around on itself during ER-phagy—to capture and degrade portions of the ER via receptor-mediated mechanisms [56,57]. Importantly, all models for autophagosome formation feature a central role for PI3-P. Reflecting this, there has been long-running focus on targeting VPS34 as a strategy to inhibit autophagy, even though this approach would be expected to affect a wide range of other vesicular pathways. Compounds such as wortmannin, LY294002 and 3-methyladenine have been generally effective in blocking autophagy, but there was a critical lack of specificity since all these agents can target class I PI3 kinases in addition to VPS34. On this front, recent work has led to the development of several specific and potent VPS34 inhibitors such as VPS34-IN1 (25nM IC_{50}), PIK-III (18 nM IC_{50}) and SAR405 (IC_{50} 1.2 nM) [58,59], which should be more precise tools to target VPS34-dependent autophagy (as reviewed further in [60]). The elucidation of key structural features within the VPS34 complex will further facilitate refinement of compounds and their mechanisms [61].

3. Regulation and Role of the ULK1 Complex during Autophagy Initiation

3.1. Phosphorylation of ULK1

In considering upstream pathways, several core mechanisms link ULK1 to the regulation of VSP34 during autophagy. Since activity of the ULK1/2 complex is coordinated by AMPK and MTORC1, signalling steps directly connect nutrient-dependent cues to autophagy initiation at the ER. The related family members, ULK1 and ULK2, show strong sequence similarity and appear to be functionally redundant *in vivo* [62,63], although regulatory events still remain better characterised for ULK1. In the current model, amino acid starvation leads to inactivation of MTORC1 and lower levels of phosphorylation on ULK1. MTORC1-mediated phosphorylation of ULK1 produces inhibition, in part by inducing conformational changes that prevent ULK1–AMPK interactions [64]. AMPK-dependent phosphorylation of ULK1 has the overall effect of promoting function in the ULK1 complex [64,65]. While this mechanism provides a framework for understanding ULK1 and its regulation by nutrients,

the model does not fully incorporate the full range of complexity that feeds into the pathway. In terms of post-translational modifications, over 70 phosphorylation sites are currently listed for mammalian ULK1 in databases (e.g., phosphosite.org). Phosphorylation events have been detected along all three major domains of the protein (N-terminal kinase, internal spacer region, and the C-terminal early autophagy targeting (EAT) domain), which represent the action of a number of kinases in addition to auto-phosphorylation. With this vast set of modifications, understanding of the functional roles of specific sites is still lacking and it is unclear how all the modifications are coordinated, as summarised elsewhere [66]. In addition, ULK1 can also receive ubiquitination and acetylation modifications during autophagy and these need to be involved in the overall model [67–71].

3.2. Regulation of ULK1 by MTORC1

At present, some clarity is gained by focusing just on the ULK1 sites regulated by MTORC1 and AMPK, which have been the best characterised. MTORC1 phosphorylates ULK1 on serine 757 (using mouse ULK1 amino acid annotation (PVVFTVG**S**PP)) and levels of this modification closely correlate with MTORC1 activation under amino acid-replete conditions [64,72]. ULK1-S637 (mouse annotation) is the other known MTORC1-regulated site [72,73]. Currently, modifications on ULK1-S757 have been the most widely reported, both in cell models and *in vivo*, with decreased P-S757 generally correlating with autophagy activation [74–80]. Conversely, ULK1-S757 is phosphorylated in response to type-I interferon signalling [81]. Functionally, mutation of S757 alters the kinetics of the autophagy response [72] and also decreases ULK1–AMPK binding [64], suggesting a model in which MTORC1 regulates the ability of AMPK to bind and activate ULK1.

An interesting development has been the identification of protein phosphatase 2A (PP2A) as a regulator for ULK1, specifically on the S637 site [73]. Wong *et al.* first noted that nutrient starvation triggered a more rapid dephosphorylation of the MTORC1 sites on ULK1 (and more rapid autophagy) as compared to MTORC1 inhibitory drugs. Rapamycin or Torin1 treatments, which are widely used tools, did indeed induce autophagy and changes on the ULK1 sites, but not as robustly as acute nutrient starvation. These observations thus suggested a starvation-induced phosphatase that dephosphorylated the MTORC1 sites to trigger autophagy, which was subsequently identified to be PP2A.

The inhibitory effects of okadaic acid on autophagy have indeed been observed earlier in studies of hepatocytes and neurons [82,83]. Other work from yeast, *Drosophila* and *C. elegans* systems lend further support for a conserved pathway linking PP2A to ATG1-dependent autophagy [84–87]. Wong *et al.* build upon these earlier studies to define the nutrient-dependent mechanism directing PP2A activity to ULK1 dephosphorylation. Since specificity of PP2A catalytic activity is controlled by its associated regulatory B-subunit, and at least 15 B isoforms are present in the human genome (see [88] for review), it was an important further advance that the authors were able to identify B55-alpha as the key regulatory subunit directing activity onto ULK1. The starvation-dependent mechanism for PP2A regulation involved a further factor. Activity and stability of PP2A are regulated in part via interaction with the alpha 4 protein (Tap42 in yeast) that keeps the catalytic subunit in an inactive state and prevents ubiquitination [89]. Nutrient starvation disrupted interactions between alpha 4 and PP2A catalytic subunits and this effect could not be mimicked by Torin1 treatment. Thus, only a starvation signal is able to promote PP2A activity, ULK1 dephosphorylation and the maximal, rapid autophagy response. Consistent with this model, mutation of Tap42 is able to induce ATG1-dependent autophagy in yeast [87]. The work on PP2A has also been valuable for shedding light on the relative roles of the ULK1-S637 and -S757 sites. Okadaic acid produced a clear effect, blocking the formation of autophagosomes. However, okadaic acid primarily inhibited dephosphorylation on the S637 site, while changes on S757 were not affected. These data suggest that dephosphorylation of S637 may be the key regulatory signal during formation of autophagosomes. Since the other MTORC1-sensitive site, S757, has potential to modulate interactions with AMPK [64] and autophagy [72], several non-mutually exclusive mechanisms may integrate multiple signalling pathways. Combined phospho-mimetic

or phosphorylation-incompetent mutations at these 2 MTORC1 sites might create constitutively inactive- or active-ULK1, respectively, although this needs to be shown experimentally and may be an oversimplification in light of the other modifications on ULK1.

3.3. Regulation of ULK1 by AMPK

Binding of AMPK to ULK1 has been widely observed, both from focused and larger proteomic approaches [64,72,90–93]. The AMPK–ULK1 interaction leads to a more complex set of modifications, with phosphorylation of at least seven serine/threonine residues in ULK1 [64,65,72]. It remains unclear how all AMPK signals integrate together to regulate ULK1, as all combinations from multi-site modification have not yet been explored. Simultaneous mutation of four AMPK sites within the ULK1 internal spacer region (mouse S467, S555, T574 and S637) impaired cellular responses including mitophagy and survival following prolonged nutrient stress [65]. Phosphorylation on S555 site controls direct binding to 14-3-3 proteins and ULK1 function, for example, during regulation of ATG9A trafficking [65,92,93]. In a separate study, dual mutation of sites ULK1-S317 and -S777 impaired the autophagy and cell survival response following prolonged glucose starvation [64]. Of these AMPK sites, modification of ULK1-S555 has so far been most widely observed, correlating with AMPK activation following glucose starvation, pharmacologic agents, or knockdown of the MAGE-A3/6-TIM28 ubiquitin ligases that down-regulate AMPK [65,76,92,94,95].

Consistent with earlier work on ULK1-S555, more recent results further show how this site is phosphorylated by activated AMPK following hypoxia stress to direct mitophagy [96]. Phosphorylation of S555 played a role in directing translocation of ULK1 onto mitochondria following hypoxia, and mutation of S555 blocked this regulation. Moreover, ectopic expression of an ULK1-S555D active variant could drive constitutive mitophagy, indicating a mode of directing ULK1 localisation by just a single AMPK site. Once on damaged mitochondria, the active ULK1 complex drives mitophagy by phosphorylating the mitochondrial resident protein FUNDC1 on serine 17 [97]. Phosphorylation of FUNDC1 promotes its interaction with LC3, providing one mechanism underlying increased ULK1-dependent mitophagy. Another mitophagy mechanism involves ULK1-mediated phosphorylation of ATG13 on serine 318 [98]. This phosphorylation signals for ATG13 to dissociate from the ULK1 complex and translocate onto damaged depolarised mitochondria to promote parkin-dependent mitophagy. In this context, ATG13 plays a more direct role during the mitophagy response, as compared to ULK1 which does not translocate to mitochondria following protonophore-mediated mitochondrial damage. Interestingly, ATG13-specific roles have also been suggested in TNF alpha-induced caspase-dependent cell death from studies of an ATG13 knockout model [99], although this could represent another pathway unrelated to mitochondria. Taken together, several distinct ULK1-mediated mitophagy mechanisms thus appear to control the response, depending on the severity of damage.

So far, the data on the S555 and S637 sites have illustrated the potential of phosphorylation to control sub-cellular localisation of ULK1. In this regard, regulation of ULK1 localisation has been a longstanding question in the autophagy field. Following acute nutrient starvation, the ULK1 complex rapidly translocates to punctate ER-associated autophagosome/IM assembly sites and this event is considered an early checkpoint during the initiation cascade [32,52]. Translocation has been widely observed for ULK1 and other components of the complex following amino acid starvation [52,100–106]. In mammalian cells, ULK1 is bound to its co-factors ATG13, FIP200 and ATG101, forming oligomers with a molecular weight near three megaDaltons [102]. Furthermore, this higher-order assembly was not altered following starvation. As such, the ULK1 complex was not remodelled or disassembled during autophagy and so post-translational signalling thus seemed critical.

How could modifications on ULK1 drive translocation? Phosphorylation of S555 and S757 can modulate protein interactions [64,65,92]. Analysis of kinase-inactive mutants also suggested that ULK1 is a protein that can adopt different conformations [103]. Speculatively, phosphorylation of ULK1 on S555 may cause a conformation that interacts with factors on damaged mitochondria.

Dephosphorylation of S637 might signal a distinct conformation that favours association with starvation-induced IM sites on the ER. It remains unclear what may constitute ULK1 interaction targets during the distinct mitophagy and autophagy initiation recruitment steps. For nutrient-dependent autophagy, one candidate is the protein VMP1, which is localised at punctate sites on the ER that precede recruitment of the ULK1 complex [32]. Interaction with protein factors may bring specificity that combines with the intrinsic membrane-binding activities of the ULK1 complex. The C-terminal EAT sub-domain of ULK1 is both necessary and sufficient to bind membranes and localise to autophagosomes [101,103]. Lipid-binding residues in mammalian ATG13 have been identified [105] and FIP200 can provide further recruitment activity by directly binding ATG16L [107,108]. Functional evidence supports all these mechanisms, but relative contributions of each are unclear and may be context-dependent. Interestingly, both ATG13 and FIP200 are phosphorylated via ULK1- and MTORC1-dependent mechanisms [98,100,102,109–111]. These signals might promote recruitment of ATG13 and FIP200, but the functions of specific phosphorylation sites in these proteins are still not defined.

3.4. Outlook on ULK1 Regulation

While translocation of the ULK1 complex to membrane sites has been widely observed, it remains unclear how catalytic function of the complex is activated. Activation of ATG1 kinase activity following nitrogen starvation of yeast could be clearly detected [109]. More recently, activation of ULK1 in cells following starvation could be detected, for example, by probing phosphorylation of the S318 site of ATG13 [41,111]. Specific modifications (e.g., phosphorylation) that are sufficient to drive ULK1 catalytic activity have not yet been identified. However, additional associated proteins have emerged with potential to regulate ULK1 kinase activity. In one model, the protein huntingtin has been shown to bind ULK1 and this interaction was mutually exclusive with MTORC1–ULK1 binding [112]. Thus, it was proposed that huntingtin would compete and prevent MTORC1-mediated inhibition of ULK1, thereby leading to ULK1 activation. An additional model has been proposed highlighting how ULK1 activity may require other regulatory interacting proteins, such as the GABARAP member of the mammalian ATG8 family [41]. The ULK1–GABARAP mechanism utilises a dynamic interplay of interactions that involves formation of an inactive complex containing GABARAP and GM130 localised on the Golgi. An additional protein, WAC, is then able to shift GABARAP to a distinct active pool that binds and activates ULK1 kinase function to promote autophagy. Several features of this model are unexpected, for example, that binding of ULK1 was specific for the non-lipidated form of GABARAP and did not involve other ATG8 family members. Also, ULK1–GABARAP binding was not starvation-dependent leading to a model in which translocation of the ULK1–GABARAP complex to the IM site is the critical event to drive autophagosome formation forward. Thus, all the evidence indicates that ULK1 function is controlled via the concerted action of post-translational modification, interacting proteins and sub-cellular translocation.

Future progress in ULK1 mechanisms may be further driven by structural elucidation of the components following advances for yeast ATG1. The regulatory mechanisms between yeast and mammalian ATG1/ULK1 are not identical but key features are conserved. Consistent with mammalian ULK1, the budding yeast *Kluyveromyces* EAT domain is able to bind and remodel liposomes in membrane tethering assays [113]. Also, the EAT domain of both yeast and mammalian ATG1/ULK1 binds its corresponding ATG13 co-factor [103,110,114]. The crystal structure of this interaction has been solved using *Kluyveromyces* ATG1 EAT in complex with an ATG13 sub-domain and these results highlight a six alpha-helix fold within the EAT, modelled to resemble tandem MIT (microtubule interacting and transport) domains [115]. A solution structure has been solved for the pentameric complex containing the ATG1 EAT and portions of ATG13, ATG17, ATG31 and ATG29 from budding yeast *Lachancea thermotolerans* [116]. All these data have helped form a model in which the pentameric complex oligomerises as a scaffold at the yeast PAS. Single-particle electron microscopy has been an independent approach to study purified pentameric complexes from budding yeast *S. cerevisiae* [117].

This complementary approach has been able to visualise the S-shaped ATG17–ATG31–ATG29 dimeric complex and further refine positioning of interactions with the ATG1–ATG13 subcomplex.

It remains unclear the extent that mammalian ULK1 structures might resemble the pentameric networks proposed for yeast ATG1. This issue is beginning to be resolved through studies of the mammalian ATG13–ATG101 HORMA (Hop1/Rev7/Mad2) domains in complex. Comparison of the human and fission yeast *S. pombe* crystal structures has revealed that overall architecture and interaction interfaces are conserved [118]. The *S. pombe* structure was used as a basis to successfully design mutants in human ATG101 that then disrupted ATG13-binding and autophagy function [106]. It has been speculated that the ATG13–ATG101 HORMA interaction found in *S. pombe* may represent the ancestral form of this functional module which has been conserved all the way to mammals [118]. The accumulating evidence suggests that, through evolution, budding yeast lost function of ATG101, while gaining novel regulatory factors such as ATG29 and ATG31, in addition to other mechanisms not found in mammals [106,119]. As such, mammalian ULK1 structure may be more similar to *S. pombe* ATG1. Mammalian ULK1 and ULK2 are between 1036–1051 residues in length, which has so far brought challenges in structural studies of full-length proteins. However, the crystal structure of the ULK1 kinase domain in complex with an ATP-site inhibitor has been solved [120]. In this context, the kinase structure was useful for rationale design of more potent inhibitory compounds and this approach should serve as a basis for future refinement of compound specificity.

3.5. Downstream Targets of ULK1

Once the ULK1 complex translocates to membrane-associated assembly sites, downstream substrates of ULK1 are phosphorylated to signal autophagy activation. Kinase-inactive ULK1 does not promote, but rather inhibits autophagy, indicating that downstream phosphorylation is critical [101,103]. Several ULK1 kinase inhibitors (for example MRT68921 and SBI-0206965) have recently been developed that block autophagy, bringing the field closer towards targeting this pathway as a therapeutic [74,111,120]. Another major question is: how does ULK1 signal autophagy downstream? The full range of ULK1-directed pathways will be driven by downstream effector molecules and interaction partners. Protein interaction databases [121] collating low- and high-throughput data currently list over 40 binding partners for ULK1 (summarised in Figure 2), which include GABARAP, p62/Sequestosome1 (SQSTM1, and the MTORC1 and AMPK complexes. Such dynamically updated databases will be useful to organise and visualise the networks of primary strong core interactions and more transient regulatory binding partners. A large number of substrates have already been reported for ULK1 (summarised in [66]). Overall, the large set of proposed ULK1 substrates overlaps substantially with the ULK1 interactome. These include factors from (or interacting with) autophagy-signalling networks such as p62, AMBRA1, AMPK and RAPTOR [122–125]. ULK1 has been reported to phosphorylate other autophagy regulatory proteins such as ATG9, ZIP kinase and folliculin [126–128]. Substrates for *C elegans* unc-51 (homologue of ATG1/ULK1) have been suggested, such as unc-14, unc-76 and VAB-8L, which function during endocytic trafficking [129,130]. ULK1 also phosphorylates STING (stimulator of interferon genes) to limit its activity as part of a negative feedback mechanism [94]. As mentioned above, ULK1 phosphorylates ATG13 and FUNDC1 during the mitophagy response [97,98].

3.6. ULK1 Phosphorylation of the Beclin 1–ATG14L–VPS34 Complex

Which ULK1 substrate promotes IM formation? On this point, a major step was the finding that ULK1 phosphorylates and activates Beclin 1 during autophagy induction [131]. The modification was identified on serine 14 (S15 in humans) which was conserved in other Beclin 1 homologues, for example, from *Drosophila* and *C elegans*. Phosphorylation of S14 correlated with upstream signalling activity and was increased following starvation or MTORC1 inhibition. S14 phosphorylation was important to promote full activation of associated VPS34 to produce PI3P. Furthermore, mutation of S14 to alanine blocked Beclin 1 function during autophagy, while conversely, a phospho-mimetic mutant

at position 14 was sufficient to drive autophagy. ATG14L played a critical role in this mechanism by binding ULK1, promoting ULK1–Beclin 1 interaction and the subsequent phosphorylation event. Moreover, mutation of the BATS membrane-targeting domain blocked the ability of ATG14L to regulate the ULK1–Beclin 1 mechanism, which suggested that the ULK1 and ATG14L-Beclin 1 complexes needed to be interacting together at the autophagosome assembly site in order for the phosphorylation to occur. Overall, these findings defined a clear mechanism linking nutrient-dependent signalling to ULK1 and downstream activation of Beclin 1–ATG14L–VPS34.

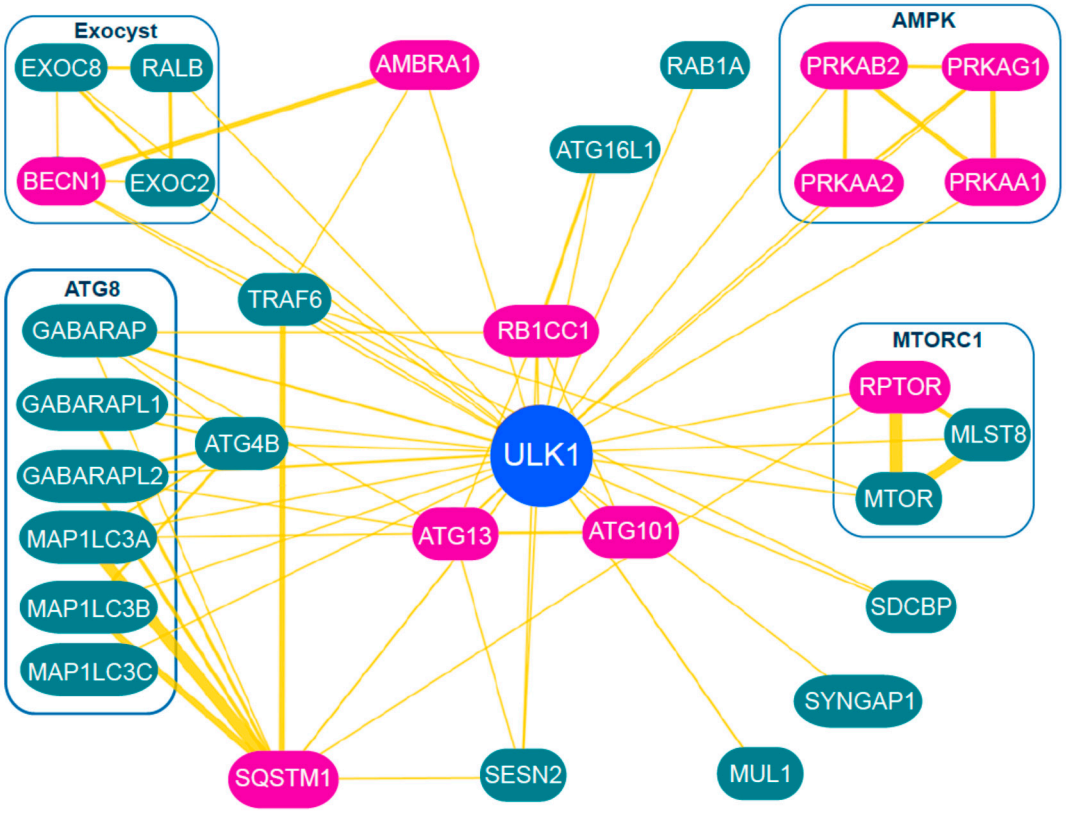

Figure 2. Summary of the ULK1 interactome. A subset of ULK1 protein interactions collated by the BioGRID server was selected for representation. Proteins characterised to also serve as ULK1 substrates are highlighted pink. Thickness of connectors corresponds to numbers of experimental entries supporting interaction. Note: this interaction database does not capture all known ULK1-binding partners including, for example, ATG9, ATG14L1 or FUNDC1.

A following study further highlighted additional mechanisms that regulate the ULK–VPS34 pathway. As the starting point, these experiments focused on ATG13 and its role in stabilising interactions between ULK1 and Beclin 1 complexes [132]. Mapping approaches indicated direct binding between the N-terminal HORMA domain of ATG13 and an internal region of ATG14L, consistent with the ATG13–ATG14 interaction detected in yeast [133]. The bridging function of ATG13 promoted ULK1-dependent phosphorylation on ATG14L, as seen by electrophoretic mobility shifting. A number of phosphorylated sites could be detected on ATG14L by mass spectrometry, but further work with mutagenesis and phospho-specific antibodies so far only focused on the serine 29 site, which was responsive to starvation or MTORC1 inhibition. Phosphorylation on ATG14L-S29 could be detected in mouse tissues, which was also responsive to dietary modulations, validating this pathway *in vivo*.

Functionally, phosphorylation on S29 was required for full starvation-dependent activation of VPS34 and autophagy. Consistent with a key role, ATG14L with an activating mutation at

position 29 was also able strongly stimulate activity of associated VPS34. Modification of S29 might possibly induce a conformational change in ATG14L that then activates VPS34. However, the ATG14L-S29D-activating mutation only partially promoted autophagy indicating that other cooperating mechanisms are required for a full response. Considering the findings so far, multiple pathways seem to integrate towards VPS34 regulation. Further work indicated that Beclin 1 phosphorylation functioned independently from that of ATG14L and so both these ULK1-driven phosphorylation events may synergise for combined spatial and catalytic regulation of VPS34. Roles for the other sites phosphorylated in ATG14L are still unclear. ATG14L is also phosphorylated by MTORC1, leading to suppression of associated VPS34 and autophagy [134].

Overall, details are still unclear on how the multiple signalling mechanisms coordinate in order to control autophagy. However, the predominant model shows MTORC1 and AMPK converging toward the regulation of ULK1 leading to subcellular translocation and downstream activation of the Beclin 1–ATG14L–VPS34 complex. Additionally, we need to keep in mind other key data that demonstrate distinct VPS34 functional complexes [135]. VPS34 complexes containing Beclin 1, along with either ATG14L or UVRAG, directed autophagy. On the other hand, VPS34 alone or in complex with just Beclin 1 regulate distinct pathways in vesicular trafficking or cellular stress responses. In this system, AMPK specifically regulates the non-autophagy pathway by phosphorylating VPS34 (on T163/S165). AMPK activated via glucose starvation also regulates the pro-autophagy complexes by phosphorylating Beclin 1 (on a distinct set of sites: S91 and S94). Beclin 1-S91 and S94 may also be sensitive to amino acid starvation pathways [136]. As such, key questions still remain as to how the multiple pathways feed into the Beclin 1–VPS34 complex together. In fact, the wider scope of evidence indicates that Beclin 1 forms a pleiotropic-signalling hub that integrates phosphorylation events from AMPK and ULK1, in addition to AKT, DAPK, ROCK1, MAPKAPK2/3 and the EGF receptor [137–141].

3.7. Outlook on Signalling Downstream of ULK1

From all available autophagy data, key features of the MTORC1, AMPK, ULK1 and VPS34 nutrient-dependent mechanism have now become better defined leading all the way to formation of the initiation membrane. What is now needed in this area? The field already has some pharmacological approaches for inhibiting ULK1/2 [74,111,120] and these tools could be further refined. To interpret effects of inhibitors *in vivo*, we need to understand the full range of pathways regulated by ULK1. Is ULK1 differentially regulated to control non-specific starvation-induced autophagy *vs.* specific pathways like mitophagy and xenophagy? How does ULK1 coordinate autophagy with roles in other pathways such as growth regulation and vesicular trafficking [130,142,143]? The range of ULK1 functions will be related to its interactome and set of substrates, but the full scope of these pathways is unclear. Along this line, recent efforts to identify ULK1 substrates in an unbiased manner have somewhat clarified the scope of ULK1 signalling and also opened new lines of study [74]. Egan *et al.* aimed to define the consensus phosphorylation motif that is recognized by ULK1 by screening a peptide substrate library *in vitro* with active purified ULK1 complex. This approach led to the formulation of an optimal ULKtide sequence (YANWLAA**S**IYLDGKKK) that features, for example, preferences for hydrophobic residues Met or Leu at the −3 position and aromatic residues like Tyr at the +2 position. Properties of the ULKtide bear some resemblance, for example, at the −3 and +2 positions, with the consensus motif described for yeast ATG1 [128]. Although the 10-residue-long ULKtide sequence still displays considerable variation, it provides a starting point to search genomic databases and also within given candidate proteins. Experimentally, robustness of the ULKtide consensus could be confirmed through cross-comparison with phospho-mass spectrometry data generated following co-expression of ULK1 with candidate substrates. In doing so, the authors were able to identify two new sites in ATG101 (along with multiple sites in ATG13 and FIP200) phosphorylated by ULK1. Similar approaches identified phosphorylation events on Beclin 1 (including S15, human), Ambra1 and VPS34. Generally these ULK1-directed phosphorylation events occurred on sites resembling the ULKtide, for example, with hydrophobic residues at −3. These results underscore how a network of

phosphorylation events emanate downstream of ULK1, even within the subunits of the ULK and Beclin complexes. While these phosphorylation events have been identified (and characterised to a certain degree by mutagenesis), functional roles for the majority remain unclear, highlighting the challenge in defining specific events when multiple signals likely cooperate together. Nonetheless, definition of phosphorylation recognition patterns and specific inhibitors, coupled with existing genetic approaches, will allow for more thorough investigations of the full range of ATG1/ULK1 function.

4. Amino Acid Signalling and MTORC1-Dependent Autophagy

To better understand the full context, it is necessary to consider how autophagy regulation is integrated into cellular energy homeostasis. Our discussion has highlighted the overall primacy of amino acid-dependent MTORC1 signalling for the negative regulation of autophagy. Amino acid availability activates MTORC1, which stimulates protein translation and cell growth, while suppressing at the same time autophagy (see [144] for overall review). The area of MTORC1 regulation, in particular, has witnessed a tremendous expansion in molecular detail and inter-connectivity fuelled in part by unbiased proteomic searches. Below, we discuss major advances in MTOR signalling, focusing on the positive and negative regulatory networks that sense amino acid availability (summarised in Figure 3).

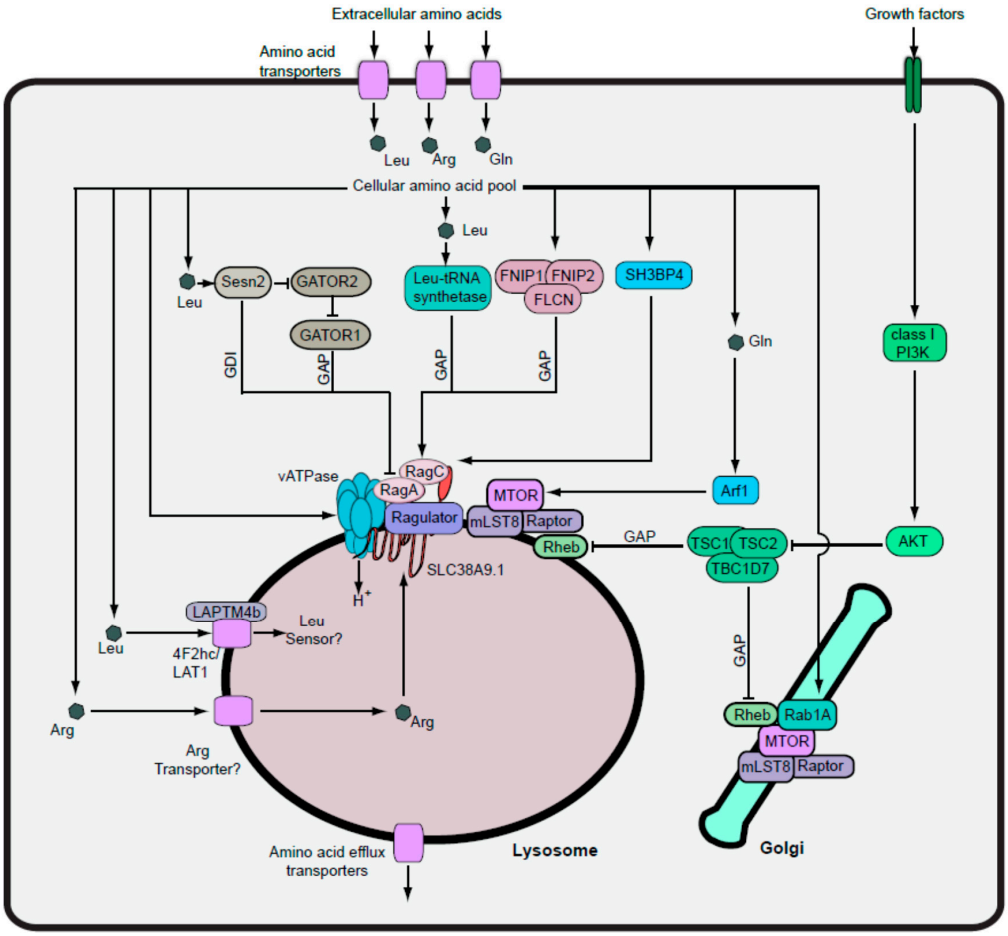

Figure 3. Circuitry of amino acid signalling to MTORC1. Extracellular amino acids are first transported into the cell. Regulatory amino acids such as Leu and Arg are further transported into the lysosome. Lysosomal Arg is sensed via SLC38A9.1, leading to activation of the vATPase-Ragulator complex and MTORC1. Leu is sensed via Sesn2 to regulate the GATOR1 pathway. Gln activates MTORC1 via an Arf1-mediated pathway. Amino acids also activate MTORC1 at the Golgi via Rab1A. Growth factor signalling leads to activation of PI3K and AKT, which controls the TSC1/2 complex and Rheb.

4.1. Uptake of Regulatory Amino Acids into Cells

In the basal state, resting mammalian cells encounter full, saturating levels of amino acids, glucose and growth factors (at least in the laboratory culture). Under these conditions, multiple pathways are engaged which converge, leading to MTORC1 activation. First, external amino acids enter the cell predominantly through the concerted function of members of the solute-linked carrier (SLC) family of transporters. Within the broad superfamily of membrane transporters, members have been historically categorised based on functional contexts, although HUGO more recently has provided the systematic SLC nomenclature. In the regulation of MTORC1 and autophagy, it had been recognised earlier that amino acids can greatly differ in their potency [101,145]. Recent work has provided mechanistic insight into the stimulatory effects of Leu, Gln and Arg, which are three of the most potent amino acids that activate MTORC1 via distinct sensing pathways.

The import of Leu into cells is primarily driven by the L-type transporter family (LAT1-4) [146]. LAT1 and LAT2 represent the SLC7 sub-family and function as a heterodimer with 4F2hc (4F2 antigen heavy chain). In contrast, LAT3 and LAT4 represent the SLC43 sub-family and function as low affinity transporters. Arg import is driven by cationic transporters, such as CAT-1, -2A, -2B and -3, which are all part of the SLC7 sub-family [147]. Also, Arg is transported via other routes such as the system y+L 4F2hc/y+LAT2 heterodimer [148]. On the other hand, Gln is transported through the concerted action of at least four different systems, some of which overlap with the transport of other amino acids. From these mechanisms, there is redundant, ubiquitous and thus robust maintenance of cytoplasmic Gln levels across wide cell contexts [149]. Transport of the regulatory amino acids can be inter-dependent. For example, sufficient cytoplasmic Gln levels are required by 4F2hc/LAT1 (SLC7A5) to drive uptake of Leu via bi-directional amino acid exchange [150]. As such, with Gln deprivation, cytoplasmic Leu subsequently becomes depleted leading to MTORC1 inactivation and autophagy, highlighting the complexity that needs to be recognised when attempting to pinpoint specific causal relationships within the metabolic network.

4.2. MTORC1 Activation via the Lysosomal Inside-Out Mechanism

Once cells achieve sufficient levels of cytoplasmic Leu, Gln and Arg, one of the major routes for activating MTORC1 occurs via an inside-out mechanism that involves coordinated input from multiple protein complexes in the lysosomal membrane [151]. MTORC1 itself contains a core module of MTOR, Raptor (regulator-associated protein of mTOR), and mLST8 (mammalian lethal with SEC13 protein 8). Understanding regarding the 3D organisation of this core complex has been particularly advanced via cryo-electron microscopy that illustrates how MTORC1 assembles with a symmetrical dimeric architecture and how specificity and access to the active site is controlled via interactions with Raptor [152,153]. The core MTORC1 is further regulated by additional non-core subunits, such as PRAS40 (proline-rich Akt substrate, 40 kDa). PRAS40 normally binds and inhibits MTORC1 but phosphorylation of PRAS40 by AKT outlines an additional mechanism linking growth factors to MTORC1 activation [154]. Tti1 and Tel2 are other accessory subunits that can regulate the protein stability of MTORC1 [155].

In the current consensus model, MTORC1 activation is associated with relocation of the complex onto the cytoplasmic surface of the lysosome through interaction with Rag family GTPases [156,157]. These Rag proteins function as heterodimeric complexes that in their active state, contain GTP-bound Rag A (or B) associated with GDP-bound Rag C (or D), which then binds the Raptor subunit of MTORC1. Once at the lysosome, MTORC1 receives further signals by interacting with another stimulatory GTPase, Rheb (Ras homolog enriched in brain). One critical aspect is that Rag heterodimers are normally kept localised on the lysosome by binding the lysosomal-resident Ragulator complex consisting of: p18 (LAMTOR1/C11orf59); p14 (LAMTOR2/ROBLD3); MP1 (LAMTOR3/MAPKSP1); LAMTOR4 (C7orf59); and LAMTOR5 (HBXIP) [158]. Together, the Rag and Ragulator complexes, along with vacuolar-ATPase, coordinate to form an amino acid-sensitive docking site responsible for anchoring MTORC1 onto the lysosome [151]. This involvement of v-ATPase in MTORC1 signalling

is intriguing in light of its more widely appreciated role as the proton pump maintaining lysosomal acidification. The precise mechanism remains uncertain but v-ATPase forms multiple interactions with Ragulator subunits. Under high amino acid availability, binding between the Ragulator and the v-ATPase V1 domain is disrupted, which speculatively might free the Ragulator to regulate Rags. Thus, v-ATPase provides one mechanism that is able to sense high amino acid levels leading to GTP-loading on RagA/B via GEF activity from the Ragulator [159]. Consistent with this model, the robust role of nucleotide binding on RagA/B has been demonstrated: knock-in mice that express constitutively GTP-bound RagA display nutrient-insensitive MTORC1 activation and are unable to activate a normal autophagy survival response following post-natal fasting [77].

The pathway involving Rags and Ragulator was termed inside-out since amino acid levels were being sensed within the lumen to activate MTORC1 on the cytoplasmic face of the lysosome [151]. In recent years, dissection of the components that sense intra-lysosomal amino acids have further uncovered a novel role for SLC38A9, a member of the amino acid transporter superfamily. Two independent studies were able to identify this connection using proteomic searches for factors co-precipitating with Ragulator components and Rag proteins [79,160]. A third group focused their attention on SLC38A9 based on its prior association to lysosomal fractions from proteomic studies [78]. Using SLC38A9 as bait, a proteomic search for interacting proteins thereby identified all five members of the Ragulator complex as well as the four Rag proteins. Critically, interaction with Rags and the Ragulator were specific to the SLC38A9.1 isoform, which contains a cytosolic-facing conserved N-terminal 110 amino acid region [79]. Other members, such as SLC38A9.2 and SLC38A9.4, which lack this N-terminal sequence, did not bind Ragulator subunits. In agreement, mass spectrometry analyses with other related transporter members such as SLC36A1, SLC38A1, SLC38A2 or SLC38A7 failed to pick up any Ragulator or Rag proteins [78,79]. Thus, interactions with the Ragulator pathway were specific for SLC38A9.1.

Functionally, multiple lines of evidence illustrate SLC38A9.1 to be a robust stimulator of Ragulator-dependent MTORC1 signalling. For example, knockdown of SLC38A9.1 suppressed MTORC1 activation by amino acids. On the other hand, forced expression of SLC38A9.1 sustained MTORC1 activity, even following amino acid starvation. Interestingly, the stimulatory effects from SLC38A9.1 overexpression were fully blocked by the dominan negative RagB/C heterodimer, indicating that SLC38A9.1 acted upstream of the Rag complex [79]. In contrast, inhibition of v-ATPase only partially blocked effects from SLC38A9.1 overexpression. Thus, SLC38A9.1 and v-ATPase might represent independent or parallel pathways that converge on the Ragulator.

For mechanistic details, SLC38A9.1 bound more strongly to mutant forms of RagB that were constitutively GDP-associated [79]. Also, amino acid stimulation tended to loosen interactions between SLC38A9.1 and the Ragulator complex. These data help construct a model in which high amino acid availability frees the Ragulator to act as a GEF for RagA/B. Intriguingly, further studies of SLC38A9.1 binding were able to delineate more precise roles for the regulatory amino acids Arg and Leu. Addition of either of these amino acids disrupted SLC38A9.1–Ragulator binding. However, CRISPR-mediated deletion of SLC38A9.1 only blocked MTORC1 activation in response to Arg stimulation. Surprisingly, cells lacking SLC38A9.1 were still responsive to Leu. Thus, evidence supports SLC38A9.1 to be preferentially an Arg sensor for MTORC1.

4.3. Transport of Amino Acids into the Lysosome

For the inside-out pathway, levels of regulatory amino acids were sensed from within the lysosomal lumen. As summarised above, basic amino acids such as Arg are imported into the cell via cationic family transporters. What are the mechanisms that further transport cytosolic amino acids into the lysosome and, secondly, do these play any regulatory roles? The SLC38A9.1 was shown *in vitro* using liposomes to indeed be capable of transporting Arg, Gln, (and also Asn) into the lumen, which suggests a potential role in equilibrating cytosolic and lysosomal amino acids [78,79]. However, rates and affinity of SLC38A9 were only moderate, suggesting that this pathway might not be able to

fully account for transport in cells. These data suggest that SLC38A9 may function primarily as the Arg sensor/receptor rather than a high efficiency transporter, and so the route of Arg entry into the lysosome remains unclear.

As one conundrum, interactions between SLC38A9.1 and the Ragulator were responsive to Leu [79]. However, since Leu still activated MTORC1 in cells lacking SLC38A9.1, other sensor pathways existed. Leu import into cells is driven by the LAT1 and LAT2 transporters, and other recent data have been able to outline further components of the Leu-sensing pathway. In this mechanism, transport of cytosolic Leu into the lysosome was mediated by 4F2hc/LAT1, which is recruited into the lysosomal membrane through the action of lysosome-associated transmembrane protein 4b (LAPTM4b) [161]. Knockdown of LAPTM4b decreased transport of Leu into lysosomes, which resulted in decreased activation of MTORC1. Thus, mechanisms that ensure import of regulatory amino acids, such as Leu, into the lysosomal lumen are required for MTORC1 activation. In agreement with this, overexpression of PAT1 (also known as lysosomal amino acid transporter (LYAAT-1) or SLC36A1) promoted efflux of amino acids out of the lysosome lumen and suppressed MTORC1 [151]. Overall, lysosomal amino acid transport has better characterised in term of export into the cytoplasm following proteolytic digestion, for example, in the context of lysosomal storage disorders [162,163]. The intra-lysosomal Leu sensor has yet to be defined. However, we now better appreciate that MTORC1 activation is dependent on the balance of export and import of regulatory amino acids such as Leu and Arg into the interior of the lysosome where they interact with their respective sensor proteins.

In the larger context, we need to integrate the other amino acid-sensing mechanisms to coordinate with the internal lysosomal pathways. For example, leucyl-tRNA synthetase has been shown to act as a Leu-specific sensor by acting as a GAP to generate the active GDP-bound form of RagD [164] (presumably on the cytosolic surface of the lysosome). High amino acid availability also recruits the folliculin (FLCN)-FNIP1/2 complex to the lysosome where it acts as a GAP for RagC/D [165]. The FLCN-FNIP complex may have additional roles for the regulation of MTORC1 localisation at the lysosome [166]. SH3-binding protein 4 (SH3BP4) can also bind and regulate the Rag complex in response to amino acid availability [167]. The GATOR complex discussed below contains another Leu sensor for the Rag pathway. Together with the inside-out mechanism, these findings illustrate how the Rag complex integrates a wide range of amino acid-dependent signals towards MTORC1.

4.4. Rag-Independent MTORC1 Pathways

While Rag complexes form a central route towards MTORC1, several further studies have outlined the wider range of amino acid-signalling pathways. One set of work from Jewell *et al.* investigated MEFs lacking both Rag A and Rag B. In this way, Rag A/B double knockout blocked the ability of Leu and Arg to activate MTORC1, but unexpectedly, Gln was still able to trigger signalling. CRISPR/Cas9-mediated double inactivation of Rag A and B in HEK293 cells also produced the same selective inhibition of just Leu and Arg signalling. Gln still induced lysosomal translocation of MTORC1 in Rag A/B KO MEFs, but this was blocked by Bafilomycin A or concanamycin A. Thus, Gln signalling to MTORC1 still required v-ATPase and the lysosomal docking aspects of the mechanism. Further investigation of this Gln-signalling pathway were based on earlier observations from the same research group implicating the Golgi-regulatory GTPase protein, Arf1, in MTORC1 signalling. Inhibition of Arf1 by knockdown or Brefeldin A treatment blocked the glutamine signalling in RagA/B-deficient cells. In contrast, other methods that disrupted general Golgi trafficking (such as Golgicide A) did not block amino acid signalling, which suggested that Arf1 had a Golgi-independent function to sense Gln and direct MTORC1 to the lysosome.

Complementary to the Arf1 mechanism, an independent report highlighted an alternative pathway involving the Rab1 GTPase during amino acid signalling [168]. Similarly, this work initiated from analyses of Rag-independent contexts, in this case, using yeast deficient in either of the Gtr1 or Gtr2 Rag homologues. Using this system, a screen based on sensitivity to rapamycin was able to identify Ypt1 (yeast homologue of Rab1A) in the TOR pathway. Interestingly, Ypt1 was required for

amino acid-dependent activation of TORC1. Furthermore, Ypt1 co-precipitated with yeast TOR1 in an amino acid-dependent manner and amino acid stimulation promoted GTP loading of Ypt1. Based on these data, knockdown approaches using HEK cells demonstrated that mammalian Rab1A was essential for MTORC1 activation by amino acids. A role for Rab1A transmitting amino acid signals is intriguing given its better known role in coordinating membrane traffic from the ER [169]. However, the amino acid–Rab1A mechanism seems highly conserved. Similar to yeast, amino acid stimulation of mammalian cells promoted GTP-loading of Rab1A– and Rab1A–MTORC1 interactions.

4.5. Coordination of MTORC1-Activation Pathways

How does the Rab1A mechanism fit in with the other established MTORC1 pathways? As one clue, Rab1A knockdown inhibited MTORC1 activation driven by overexpression of Rheb. Conversely, knockdown of Rheb blocked the ability of Rab1A to stimulate MTORC1, thus showing mutual co-dependencies. Mutant versions of Rab1A that were either locked in the GDP-bound inactive state or lacking membrane localisation signals failed to activate MTORC1. A proximity ligation assay further demonstrated that Rab1A–MTORC1 interaction preferentially occurred at Golgi membranes. This approach also highlighted Rheb and MTORC1 interactions at the Golgi. Lastly, Rab1A knockdown specifically disrupted the Rheb–MTORC1 interaction (but had no effect on MTORC1-binding RagC). All these data suggest another layer to the MTORC1 signalling model in which Rab1A drives recruitment of MTORC1 and Rheb to form an activated complex on the Golgi. From this, we can conclude that at least two highly conserved mechanisms can promote MTORC1 activation but at distinct membrane sites, namely the lysosome and Golgi.

The relative contributions of lysosomal *vs.* Golgi MTORC1 pathways are still unclear. However, both these mechanism include key roles for GTP-bound Rheb during MTORC1 activation. There is indeed evidence supporting Rheb at various membrane locations, including the Golgi and lysosomes, but this issue is also controversial [156,170,171]. The Arf1 mechanism is interesting as it seems to be preferentially linked to the lysosomal MTORC1 pathway. Further below, we discuss the TSC pathway which also focuses on Rheb and MTORC1 at the lysosome. In the wider context, both the Arf1 and Rab1A mechanisms were discovered through searches of Rag-deficient cellular systems, thus outlining three pathways controlled by small GTP-binding proteins that work independently. However, Rab1A knockdown also had inhibitory effects on RagB/C-driven MTORC1 signalling [168], indicating some crosstalk not yet fully understood. It also remains unclear how the Rab1A–MTORC1 pathway might sense different amino acids. Rab1A has been shown to bind ATG1/ULK1 in both yeast and mammalian systems and this may reflect another mechanism distinct from MTORC1 [172]. Lastly, Arf1 and Rab1A would be predicted to control autophagy initiation via MTORC1, but how this is coordinated with the trafficking roles of these two GTPases remains unclear.

4.6. MTORC1 Shutdown via the GATOR Complex

As nutrient availability decreases, MTORC1 signalling is suppressed, leading to autophagy. Part of this mechanism involves reduction of the positive regulatory signals from the Ragulator, Arf1 and Rab1A pathways. In addition, pathways that negatively regulate MTORC1 have been identified in recent years revealing the network of counterbalance systems that also can sense amino acid availability. For example, Rag proteins receive critical negative regulation from the GATOR (GTPase-activating protein activity towards Rags) super-complex, which is comprised of the GATOR1 and GATOR2 sub-complexes. GATOR1 (consisting of subunits DEPDC5, Nprl2 and Nprl3) functions as a GAP for Rags A/B to inhibit MTORC1 signalling [173]. The importance of DEPDC5 and Nprl2 were underpinned by cases of mutation in their respective genes from glioblastoma and ovarian cancers. Loss of heterozygosity observed with these further suggested that GATOR1 functioned as a tumour suppressor. Thus, in tumour cells without GATOR1 (in agreement with GATOR1 knockdown), RagA/B and MTORC1 signalling were hyperactive and resistant to shutdown, even in the absence of amino acids.

The GATOR2 complex (consisting of Mios, WDR24, WDR59, Seh1L and Sec13) brings an additional layer of regulation by binding GATOR1 [173]. Through this interaction, GATOR2 suppresses the GAP function of GATOR1 towards RagA/B, thereby positively regulating MTORC1. A series of further reports that combine biochemistry, cell biology and structural biology have outlined a mechanism in which GATOR2 controls RagA/B and MTORC1 activation in response to Leu levels sensed via Sestrin family members [174–178]. Sestrins (Sesn1–Sesn3) are a group of highly conserved proteins that are induced as part of the p53- and FoxO-dependent stress-response (see [179] for review). As one role, Sesn1/2 lower levels of ROS accumulation inside cells, although the mechanism for this remains controversial [177,180,181]. From earlier data, Sesn1/2 were also implicated in MTORC1 signalling by decreasing the GTP-loading ratio of its activator, Rheb [182]. In this pathway, Sesn1/2 promoted AMPK-mediated phosphorylation of the TSC2 (tuberous sclerosis 2) protein and activation of the TSC1–TSC2 GAP complex towards Rheb.

Later studies indicated further novel aspects to the mechanism as Sesn2 overexpression suppressed MTORC1 function even in AMPK-null cells. Searches for Sesn2-interacting proteins recovered GATOR2 components [174,176]. Conversely, an independent group searching for GATOR2-interacting protein identified Sesn2 (in addition to Sesn1 and Sesn3) [175]. Consistent with overexpression trends, when Sestrin expression was targeted, MTORC1 tended to show higher levels of activation, both under amino acid-depleted and replete conditions [174,175]. Thus, all evidence point to Sestrins as negative regulators or MTORC1 signalling. Fitting into the larger mechanism, Sesn2 overexpression required functional GATOR1 to inhibit MTORC1. In addition, Sesn2 overexpression was able to disrupt interactions between GATOR2 and GATOR1 complexes, and furthermore, promote formation of GDP-bound RagB [176]. These findings help define a model in which Sestrins bind GATOR2, thereby releasing GATOR1 and enabling GAP activity towards RagA/B (leading to shut-down of MTORC1).

How is this mechanism regulated by amino acids? As one indication, Sesn2–GATOR2 interaction was strengthened following starvation of amino acids [175]. Further work narrowed this down to show that add-back of Leu alone could robustly disrupt binding between GATOR2 and Sesn2 (or Sesn1) [178]. This effect could be demonstrated following addition of Leu either to cells or *in vitro* on purified Sens2–GATOR2 complexes. Other amino acids similar to Leu (such as methionine and isoleucine) could also disrupt Sesn2–GATOR2 binding but with much less potency. Importantly, biochemical approaches could show direct binding of Leu to purified Sesn2 and a crystal structure was solved for Sesn2 in complex with Leu [177], illustrating the network of interactions leading to specific recognition of Leu at a single binding pocket of Sesn2. Mutant versions of Sesn2 (with either L261A or E451A substitutions) were found to bind Leu poorly and, as predicted, these Sesn2 variants could no longer activate MTORC1 following stimulation with Leu. While Sesn2 may act as a Leu sensor upstream of GATOR2, additional pathways integrate into the larger scheme. A complementary mechanism has been suggested, as Sestrins also have the ability to directly bind Rag complexes and act as a guanine nucleotide dissociation inhibitor (GDI) for RagA/B to overall suppress MTORC1 activation [183]. For this function, a conserved motif could be identified in mammalian and *Drosophila* Sestrins that was similar to that of Rab GDI, and mutation of key charged residues within this motif abrogated Sestrin GDI activity and the ability to suppress the MTORC1 pathway. Altogether, a rapidly expanding body of evidence indicates that Sestrins may have separate AMPK-, GATOR- and GDI-dependent signalling mechanisms that integrate to suppress the MTORC1 pathway.

As Sestrins inactivate RagA/B and MTORC1 following amino acid starvation and other types of stress, they would be postulated to promote autophagy. Indeed, forced expression of Sesn2 stimulates autophagy in renal tubule cells [184]. Conversely, knockdown of Sesn2 in p53-containing cells such as HCT116 and U2OS suppressed starvation-induced autophagy [185]. Interestingly, Sesn2 knockdown also suppressed MTORC1-independent autophagy caused by lithium, which reinforces the multiple pathways linked to Sestrins. Another intriguing finding is that Sesn2 also binds the EAT region of ULK1 and this interaction can promote ULK1-directed phosphorylation of p62/SQSTM1 at a

critical serine 403 regulatory site [124]. Speculatively, the Sestrin–ULK1 interaction may be promoting p62/SQSTM1-mediated autophagy of Keap1 as part of an oxidative stress response [186]. ULK1 also can phosphorylate Sesn2 on multiple sites along the protein, although the function of these events remains unclear. These data suggest that Sestrins have the potential to modulate autophagy via MTORC1-dependent and -independent routes, which may be linked to the wide range of Sestrin function-sensing amino acids and other forms of cellular stress.

4.7. MTORC1 Shutdown via the TSC Complex

A central component in the model for MTORC1 activation features interaction with active GTP-bound Rheb. In agreement, pathways that lead to Rheb inactivation also serve as negative regulators of MTORC1 signalling. The intrinsic GTPase activity of Rheb is promoted by the TSC complex, made up of TSC1, TSC2 and TBC1D7 (tre2-bub2-cdc16-1 domain family member 7) [187]. TSC2 contains the GAP activity for Rheb while TSC1 serves as a scaffold for TSC2 and TBC1D7, stabilising the protein complex. This mechanism is widely recognised as the basis for growth factor-mediated regulation of MTORC1. For example, insulin stimulation activates the PI3K/AKT pathway leading to phosphorylation of TSC2 on at least five regulatory sites thereby inhibiting its function [188,189]. More recently, it was shown that these phosphorylation events cause dissociation of the TSC1/2 complex from the lysosome, thereby displacing its activity away from Rheb [171]. As such, growth factors in effect protect Rheb from inactivation, thereby promoting MTORC1. Conversely, growth factor (or serum) deprivation allows Rheb inactivation, MTORC1 shutdown and autophagy.

While regulation of TSC1/2 by AKT and growth factors has been established, other findings indicate additional levels of inter-connectivity, with amino acid-dependent recruitment of the TSC complex to the lysosome. Proteomic approaches discovered that TSC2 precipitated with RagA + RagC heterodimers [190]. Interestingly, binding was strongest with inactive Rag conformations associated with amino acid starvation (*i.e.*, RagA-GDP and RagC-GTP). In agreement, amino acid starvation promoted translocation of the TSC1/2 complex to the lysosome, and functionally, this recruitment was critical to drive MTORC1 release. In TSC2 −/− MEFS, MTORC1 remained stuck on the lysosome and MTORC1 activation persisted even following amino acid starvation. How does TSC2 control MTORC1 release? Since TSC2 is a Rheb GAP, the authors tested this potential link and were able to demonstrate that the persistent MTORC1 localisation at the lysosome following TSC2 loss could be reversed by knockdown of Rheb. These results thus suggest a revised model in which both Rag and Rheb proteins contribute towards anchoring MTORC1 at the lysosome for activation. Critically, under starvation conditions, inactive Rag heterodimers, help recruit the TSC1/2 complex to then inactivate Rheb and allow MTORC1 dissociation. Further results in this study illustrate how the TSC complex is essential to mount a proper pro-survival autophagy response following extended periods of amino acid starvation. Requirements for the TSC1/2 complex to suppress MTORC1 and promote autophagy have been observed in a number of contexts [191,192]. With the more recent data, it is better understood how the TSC complex also senses amino acid signals via the Rag pathway to regulate the lysosomal Rheb-MTORC1 pathway upstream of autophagy.

5. Summary of MTORC1 Pathways and Autophagy

Recent years have witnessed substantial development in our understanding of the nutrient-dependent pathways that control MTORC1. This finer detailing of the cellular metabolic networks thus outlines the wide range of pathways that channel in signals to modulate the autophagy homeostatic mechanism. Major advancements have been made in the identification of specific proteins that sense levels of regulatory amino acids such as Leu and Arg. A prominent concept is the role of the lysosome as a focal point for MTORC1 activation with the refinement of an inside-out mechanism in which amino acids from the lysosome interior are detected. A key new player in this pathway is the SLC38A9 membrane transporter protein which senses Arg and then transmits signals via v-ATPase and the Ragulator complex to activate MTORC1 on the lysosome outer surface. Pivotal to the Ragulator

pathway are the Rag proteins localised on the lysosome, but other MTORC1 pathways can function independently, such as the Arf1-dependent mechanism, which preferentially senses Gln levels, while a Rab1A-dependent pathway preferentially activates Golgi-localised MTORC1. While mechanisms were identified that activate MTORC1, equally intricate counter pathways that suppress MTORC1 signalling were mapped out, including the GATOR1 GAP complex that inactivates RagA/B. Dissection of the GATOR1 function thus led to characterisation of the GATOR2 complex and its direct link to the Sestrin proteins which comprise another Leu-specific sensing pathway. The other key MTORC1 suppressor is the TSC1/2 complex which acts as a GAP for Rheb and the recent evidence indicates deeper levels of crosstalk, with inactive Rag proteins recruiting TSC1/2 to further inactivate Rheb.

Overall, these advances in nutrient-directed signalling pathways provide a more comprehensive list of molecular players that may serve as drug targets or genetic polymorphism candidates, for example, in the context of autophagy and cancer. Given the wide array of pathways that integrate amino acid and growth factor signals to MTORC1, it would be somewhat surprising if the link to autophagy initiation is confined to a small set of phosphorylation events on ULK1/2. It was outside the scope here, but several MTORC1-dependent mechanisms have been described for the up-regulation of lysosomal capacity, including the TFEB gene expression programme [193–195]. It is also intriguing that MTORC1 regulation is centred on the lysosome while autophagy membrane traffic ultimately funnels into the lysosome. Through prolonged autophagic flux and the re-generation of nutritional building blocks, the lysosomal network has also been characterised to tubulate, re-model and re-form via the autophagic lysosome reformation (ALR) programme driven by MTORC1 [196]. Thus, the lysosome is well-placed to provide a feedback mechanism that coordinates late stages of autophagy degradation with regulation during the early stages. Golgi-localised MTORC1 might have a different flavour of regulation, at least in terms of autophagy. Also, complementary to the amino acid-sensing pathways of MTORC1, decreased cellular energetics and AMPK are also accepted activators of autophagy [65,80,135], although this area is more controversial [197–199]. However, novel mechanisms have been proposed that link AMPK to the Ragulator [200], and as discussed earlier, AMPK signals are integrated with MTORC1 at the ULK1/2 complex and also at the Beclin 1-VPS34 complex. As such, AMPK is a critical regulator of autophagy but its role may be more context-specific.

We have structured our summary here into three broad areas, but nonetheless, all the mechanisms discussed work seamlessly in mammalian cells to activate and then terminate the autophagy response. If we zoomed out, we would see just the salient features: how starvation of key amino acids is sensed by a network of mechanisms to result in lower MTORC1 activity and how decreased MTORC1 activity leads to modified phosphorylation on ULK1 that triggers a translocation to autophagosome assembly sites associated with the endoplasmic reticulum. Activated ULK1 would then phosphorylate downstream substrates including members of the Beclin1 complex to drive further membrane trafficking and autophagosome assembly. From our discussion, we also appreciate that by zooming in, we uncover immense mechanistic diversity and complexity, reflecting the wide range of physiologic roles for autophagy under different contexts in mammalian cells. Teleological beauty continues to be discovered in the expanding world of autophagy biology. Pragmatically, our improved understanding of the regulatory circuitry also facilitates the development of pharmaceutical and genetic strategies for targeting autophagy, which will undoubtedly need fine-tuning across different biomedical contexts.

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Autophagic Processes in Yeast: Mechanism, Machinery and Regulation

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ABSTRACT Autophagy refers to a group of processes that involve degradation of cytoplasmic components including cytosol, macromolecular complexes, and organelles, within the vacuole or the lysosome of higher eukaryotes. The various types of autophagy have attracted increasing attention for at least two reasons. First, autophagy provides a compelling example of dynamic rearrangements of subcellular membranes involving issues of protein trafficking and organelle identity, and thus it is fascinating for researchers interested in questions pertinent to basic cell biology. Second, autophagy plays a central role in normal development and cell homeostasis, and, as a result, autophagic dysfunctions are associated with a range of illnesses including cancer, diabetes, myopathies, some types of neurodegeneration, and liver and heart diseases. That said, this review focuses on autophagy in yeast. Many aspects of autophagy are conserved from yeast to human; in particular, this applies to the gene products mediating these pathways as well as some of the signaling cascades regulating it, so that the information we relate is relevant to higher eukaryotes. Indeed, as with many cellular pathways, the initial molecular insights were made possible due to genetic studies in *Saccharomyces cerevisiae* and other fungi.

TABLE OF CONTENTS

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ALTHOUGH, historically, greater attention has focused on biosynthetic processes, it is clear that cellular homeostasis requires a balance between anabolism and catabolism. Thus, cells have an array of processes for breaking down proteins and other macromolecules, as well as organelles, and each of these distinct processes differ with regard to the machinery involved, the nature of the substrate, and the site of sequestration. The two primary mechanisms for subcellular degradation are the ubiquitin-proteasome system (UPS) and autophagy. A third, less-well-characterized, mechanism is the vacuole import and degradation (Vid) pathway (Hoffman and Chiang 1996), for which the most critical substrate is fructose-1,6-bisphosphatase (Fbp1), the key enzyme in gluconeogenesis, but other target proteins include Pck1, Mdh2, and Icl1 (Hung *et al.* 2004; Brown *et al.* 2010). Degradation of Fbp1 in the absence of glucose prevents futile cycling where the cell attempts to generate glucose under conditions where the carbon source is limiting. In the Vid pathway, which occurs under conditions of prolonged glucose starvation, Fbp1 is translocated into 30-nm cytosolic vesicles that subsequently fuse with the vacuole, releasing their contents into the lumen, where Fbp1 is degraded (Huang and Chiang 1997). The mechanism by which Fbp1 is translocated into the completed Vid vesicles remains unknown. The UPS can also target Fbp1 (Horak *et al.* 2002; Regelmann *et al.* 2003; Hung *et al.* 2004) and many other proteins, principally those with a short half-life (Ravid and Hochstrasser 2008). The targets are again individual proteins, but in this case they are tagged with ubiquitin chains and are not sequestered within a vesicle, but rather are recognized by, and degraded within, the proteasome, a multisubunit protein channel that includes deubiquitinating enzymes and proteases. In contrast with the Vid pathway and autophagy, UPS-mediated degradation occurs in the cytosol (or the nucleus), not in the vacuole.

Autophagy can be divided into two main types, microautophagy and macroautophagy (Figure 1), and both of these include nonselective and selective processes (Shintani and Klionsky 2004a). Nonselective microautophagy is not well defined with regard to the machinery involved or its physiological role. In this process the vacuole membrane invaginates and scissions to produce intravacuolar vesicles that are subsequently degraded (Kunz *et al.* 2004; Uttenweiler and Mayer 2008). This type of microautophagy does not rely directly on the cellular components involved in selective types of microautophagy or macroautophagy. In contrast, selective microautophagy has more in common with macroautophagy since both processes share most of the same machinery (Table 1). Selective microautophagy is used in the turnover of mitochondria (Deffieu *et al.* 2009), parts of the nucleus (Krick *et al.* 2008b), and peroxisomes (Dunn *et al.* 2005). In this case, the cargo is recruited and sequestered directly by the vacuole membrane, and the following invagination or protrusion/septation leads to its delivery into the vacuole lumen. Whereas selective microautophagy involves uptake of the cargo directly at the limiting membrane of the vacuole, the morphological hallmark of macroautophagy is the sequestration of the targeted cargo within cytosolic double-membrane vesicles that subsequently fuse with the vacuole, allowing (in most cases) breakdown of the

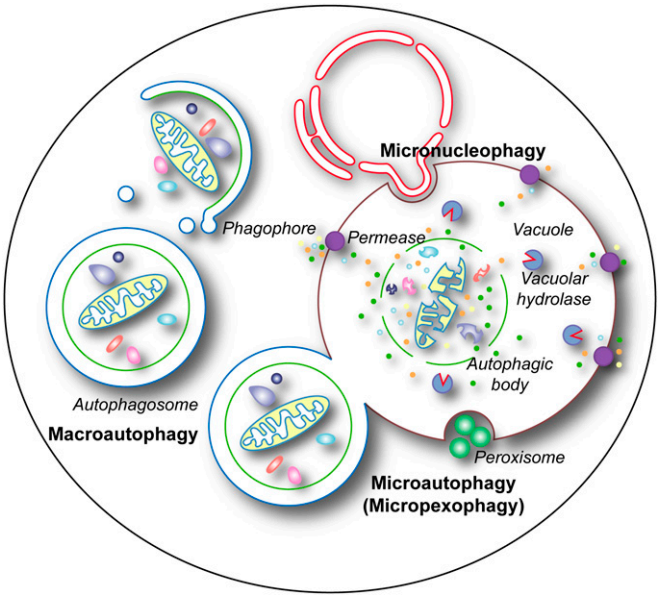

Figure 1 The principal types of autophagy in yeast. Macroautophagy entails the sequestration of bulk cytoplasm or specific structures into autophagomes. Autophagosomes are formed by expansion of a precursor compartment known as the phagophore, which initiates the sequestration of the cargo. Upon completion, the autophagosome fuses with the vacuole, releasing the inner autophagosome vesicle into the vacuole lumen, where it is now termed an autophagic body. During microautophagy (here micropexophagy is illustrated as an example), the structures targeted to degradation are recruited in proximity to the vacuole membrane. Protrusion/septation and/or invagination of this membrane, followed by scission, allows the cargo to be transported into the vacuolar lumen. Via a similar mechanism, micronucleophagy mediates the turnover of part of the nuclear envelope and content. In most cases, the components delivered by macroautophagy, microautophagy, and micronucleophagy into the interior of the vacuole are degraded by resident hydrolases. The resulting metabolites, *i.e.*, amino acids, sugars, and nucleotides, are subsequently transported into the cytoplasm by permeases (although these have been identified only for amino acids) and used either as a source of energy or as building blocks for the synthesis of new macromolecules.

cargo and recycling of the resulting macromolecules (Eskelinen *et al.* 2011). In this review, we focus on the mechanism and regulation of selective microautophagy and both selective and nonselective macroautophagy in yeast.

Physiological Roles of Autophagy

Autophagy is typically considered to be a degradative process that plays a role in the turnover of bulk cytoplasm (Mizushima and Klionsky 2007). While this pathway is primarily degradative, this view is not an adequate representation of the many functional roles of autophagy. Certainly autophagy is important as a response to starvation as cells are frequently confronted by these conditions in the wild. Thus, it is not surprising that a complex system such as autophagy is in place to allow the cell to maintain viability during nutrient depletion. Organelles can be eliminated by nonselective autophagy, but they can also be specifically targeted for degradation. This type of selective organellar autophagy may occur in response to organelle damage or dysfunction, but may also be the result of cellular adaptation to changing nutrient conditions. For example, when yeast cells are shifted from conditions under which they need peroxisomes, such as growth on methanol or oleic acid, to a preferred carbon source such as glucose, they rapidly turn over these organelles that are now in surplus (Tuttle *et al.* 1993; Titorenko *et al.* 1995). This type of degradation is beneficial to the cell because organelles are costly to maintain, and they can damage the cell when dysfunctional. Moreover, autophagy can even be involved in a biosynthetic process. The cytoplasm-to-vacuole targeting (Cvt) pathway is used for the delivery of several resident hydrolases to the vacuole, their ultimate site of function (Lynch-Day and Klionsky 2010). The machinery used for the Cvt pathway and the morphology of the process overlap extensively with that of selective macroautophagy (Harding *et al.* 1996; Scott *et al.* 1996; Baba *et al.* 1997). Finally, although more investigation is needed, initial observations also point to autophagosomes being able to deliver specific signaling molecules into the extracellular space through fusion with the plasma membrane.

Morphology

As discussed above, one of the ways to think about the different types of autophagy is in relation to the substrate and the mechanism through which the substrate is separated

Table 1 Types of autophagy in yeast

Name	Target	Characteristics/requirements
Microautophagy	Bulk cytosol, vacuole membrane	Uptake by direct invagination
Microautophagy, selective	Mitochondria	Uptake by direct invagination or protrusion/septation
	Peroxisomes	Uptake by direct invagination or protrusion/septation
	Nuclear membrane	Invagination requires Nvj1 and Vac8
Macroautophagy, nonselective	Bulk cytoplasm	Sequestration by autophagosomes
Macroautophagy, selective		Sequestration by autophagosomes. Uses a ligand on the cargo, and an autophagy receptor/adaptor system:
Cvt pathway	Resident hydrolases	Signal in the cargo. Atg19, and Atg34 are receptors, Atg11 is a scaffold
Mitophagy	Mitochondria	Atg32 is a receptor, Atg11 is a scaffold
Pexophagy	Peroxisomes	Atg30 and Atg36 are receptors, Atg11 and Atg17 are scaffolds
Ribophagy	Ribosomes	Ubp3–Bre5
Reticulophagy	Endoplasmic reticulum	Atg19
Vid pathway	Fbp1, Icl1, Mdh2, Pck1	Cargo uptake into 30-nm vesicles

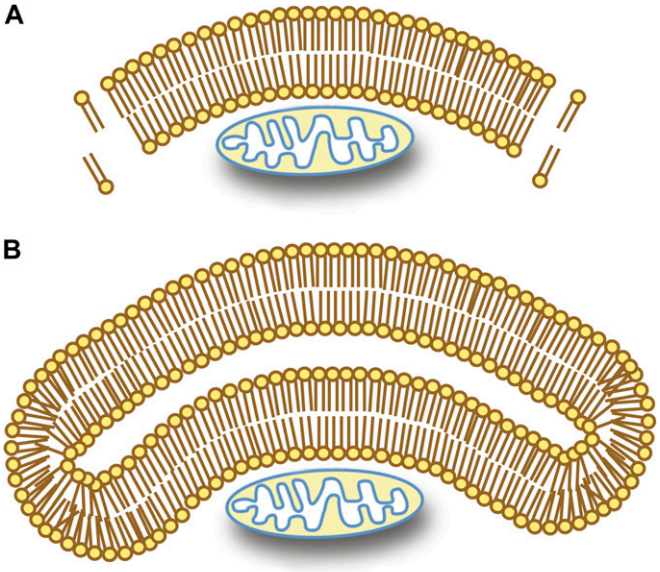

Figure 2 Sequestration of cytoplasmic cargo requires a double-membrane compartment. (A) Exposure of the hydrophobic core of a lipid bilayer to the aqueous cytosol would make it energetically unfavorable to use a single-bilayer membrane to sequester a cytoplasmic cargo. In this scenario, it would also be unclear how the phagophore membrane would expand by lipid addition. (B) The use of a double-lipid bilayer maintains thermodynamic energy requirements, while allowing the cargo to be sequestered by expansion of the double membrane. The expansion of the phagophore could occur by lateral movement or translocation of lipids from an attached organelle, or by vesicular fusion.

from the remainder of the cytoplasm and targeted for degradation. Both micro- and macroautophagy involve the movement of macromolecules and organelles from the cytosol into the vacuole lumen. Thus, these components must be translocated from the intracellular space (*i.e.*, the cytosol) to the topological equivalent of the extracellular space. An even simpler way to look at this problem is that during autophagy folded proteins, macromolecular complexes and intact organelles must be moved across a membrane, a process that represents a substantial thermodynamic barrier. Therefore, particularly with regard to an organelle, the question is, What process can the cell use to accomplish this requirement?

During transport throughout the secretory pathway, cargo transits within vesicles from one compartment to another. The critical issue then is getting the cargo within a vesicle. The cargo must first translocate across the ER membrane, a process that requires the protein to be unfolded and to move through a specialized channel inserted into the ER membrane. Although there are cytosolic chaperones that can unfold proteins, it is not feasible to do this on the scale needed for macroautophagy. In addition, such a mechanism cannot be used with organelles. For these reasons, during macroautophagy the vesicle must be formed around the existing cargo. A final point is that it is not possible to sequester a cytoplasmic cargo within a single-membrane vesicle; exposure of the hydrophobic core of the bilayer during the sequestration event would be thermodynamically unfavorable (Figure 2). As a consequence, this type of sequestration necessitates the use of a double membrane.

Nonselective macroautophagy

Formation of the autophagosome is described as *de novo* to distinguish it from what happens in the secretory pathway because these double-membrane vesicles do not form by budding from a preexisting organelle (Noda *et al.* 2002; Kovacs *et al.* 2007). The process of autophagosome biogenesis is perhaps the least understood part of macroautophagy, and many aspects remain to be fully elucidated. The first issue concerns the nature of the nucleation process. The initial sequestering compartment is termed the phagophore (Figure 1) (Klionsky *et al.* 2011). Accordingly, the phagophore assembly site (PAS) is the name given to the presumed nucleating site. The PAS is located next to the vacuole, although it is not known if there is any significance to this particular localization. The majority of the autophagy-related (Atg) proteins (Klionsky *et al.* 2003) that constitute the machinery of autophagy localize at least transiently to this site based on fluorescence microscopy of fluorophore-tagged chimeras (Suzuki *et al.* 2001, 2007; Kim *et al.* 2002). This observation has led to the circular definition of the PAS as the site of Atg protein localization, with the site where Atg proteins localize being defined as the PAS. At least part of the reason for this confusion is that the PAS is otherwise uncharacterized; it is not known whether it is a membrane structure, whether it is permanent, or whether it is literally converted into a phagophore as opposed to playing a role in the formation of a separate phagophore. Nonetheless, for the purposes of this review we consider the PAS as the dynamic precursor structure that nucleates into a phagophore. In a wild-type strain only ~30% of the cells have a detectable PAS (based on the localization of a fluorescent-tagged protein such as GFP–Atg8), whereas essentially the entire population displays a PAS when macroautophagy is blocked in an *atg* mutant (Shintani and Klionsky 2004b). Thus, either the PAS is a transient structure or localization of the Atg proteins to the PAS is dynamic, with proteins such as Atg8 cycling on and off.

A general model for the generation of an autophagosome involves the expansion of the phagophore by the addition of lipid bilayers from one or more donor sources. Presumably the membrane is delivered to the phagophore through vesicular trafficking events in yeast, but direct translocation from an adjacent organelle cannot be excluded. SNARE proteins, which play a role in membrane fusion, are implicated in macroautophagy (Nair *et al.* 2011), perhaps functioning at multiple steps of the pathway, including PAS/phagophore assembly and/or phagophore expansion, in addition to the fusion of the completed autophagosome with the vacuole. The origin of the membrane(s) that allow the phagophore to be formed and expand, that is, the membranes that ultimately form the autophagosome, is another highly controversial topic (Reggiori 2006). Molecular genetic studies have implicated protein components that normally function throughout the cell.

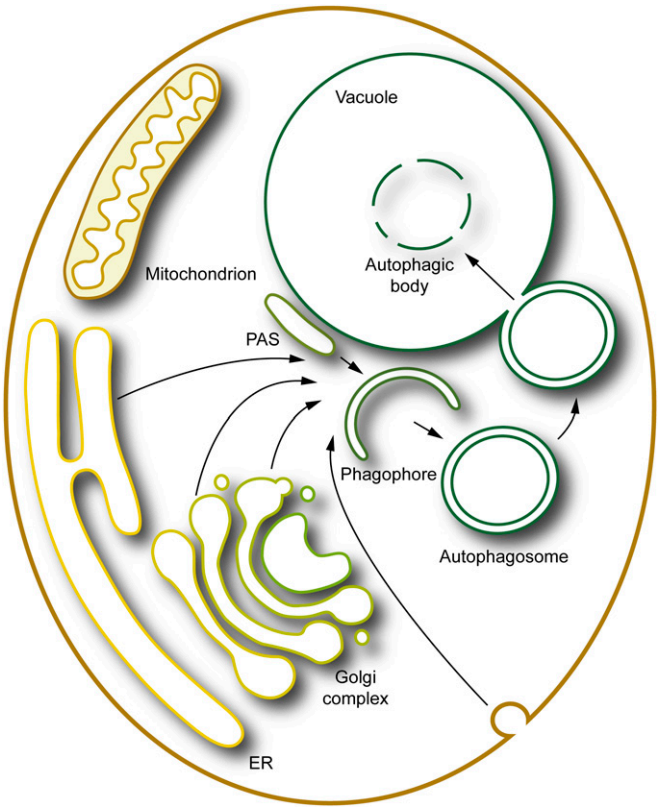

Figure 3 Multiple membrane sources may contribute to formation and expansion of the phagophore. Various compartments including the ER, the Golgi apparatus, and the plasma membrane may contribute to the nucleation and/or expansion of the phagophore. See the text for details.

For example, the involvement of Ypt1 and the autophagy-specific TRAPPIII complex (Lynch-Day *et al.* 2010) suggest that the membrane is delivered to the phagophore from the endoplasmic reticulum (ER). This would not be surprising considering the role of this organelle in synthesis of phospholipids, the major component of autophagosomal membranes. However, the requirement for the conserved oligomeric Golgi (COG) complex that acts as a tether, and several components that function in protein secretion from the *trans*-Golgi including Sec2, Sec4, Sec7, Arf1/2 and Pik1, suggests that the Golgi apparatus is also an important membrane donor (Geng *et al.* 2010; Mari *et al.* 2010; van der Vaart *et al.* 2010; Yen *et al.* 2010; Wang *et al.* 2012). It is also possible that other compartments, including the plasma membrane (Taylor *et al.* 2012), provide material for phagophore expansion. The cell may in fact mobilize membrane from multiple sources to meet the substantial demands of macroautophagy (Figure 3).

Another issue concerns the curvature of the sequestering membrane. In yeast, no proteins containing BAR domains have been clearly associated with autophagosome formation. Similarly, this process does not appear to involve the use of a canonical protein coat such as clathrin or COPII, which may not be surprising considering that the huge size of the autophagosome would require an unusually large amount of coat components. In the case of selective autophagy the cargo may determine the curvature. One observation in favor of this possibility is the interaction between the autophagy receptors (see below) and Atg8, which is also present on the phagophore membrane. This type of protein–protein interaction may allow the forming membrane to essentially wrap around the cargo. With nonselective macroautophagy, however, this mechanism cannot be invoked. Instead, biophysical parameters may be responsible for the curvature, including the potential unequal distribution of lipids, such as phosphatidylethanolamine (PE) or phosphatidylinositol-3-phosphate (PtdIns3P), or proteins—in particular on what will become the convex surface—which would cause the lipid bilayers to form a curved structure. Autophagosomes typically fall within a particular size range of ~400–900 nm and only the presence of an extremely large cargo could promote bending of the membrane.

The ultimate goal of phagophore expansion is the complete sequestration of the cargo, which requires the phagophore to seal, thus forming the autophagosome. The necessity of this step can be visualized by considering the outcome of fusion between a phagophore *vs.* an autophagosome and the vacuole (Figure 4). In the former event, the cargo is not delivered into the vacuole lumen, whereas in the latter the inner, now separate, vesicle of the autophagosome enters the vacuole lumen, where it is subsequently degraded. This inner vesicle, when present in the vacuole lumen, is termed an autophagic body. It is thus critical to prevent premature fusion between a phagophore and the vacuole. Although the mechanism involved is unknown, there are indications that release of the Atg machinery from the phagophore could be a critical regulatory step (see below). Similarly, it is not understood how the phagophore opening is closed, an event that presumably requires scission or fusion to separate the inner and outer membrane.

In most situations, sequestration of the cargo is not the end point of macroautophagy. A possible exception is seen with reticulophagy (Klionsky *et al.* 2007), the selective degradation of the ER that is induced by extreme stress in this organelle due to extensive protein misfolding (Yorimitsu *et al.* 2006). In this case, sequestration of a portion of the ER is sufficient to restore secretory capacity at a level that allows maintaining cell viability (Bernales *et al.* 2006), essentially providing additional time for the stress to dissipate and/or be handled by other systems. Under conditions where macroautophagy is induced by nutrient depletion, the final critical step of the process requires lysis of the autophagic body membrane, breakdown of the cargo, and efflux of the resulting metabolites for reuse in the cytosol. Although some vacuole membrane amino acid permeases have been identified or biochemically characterized (Klionsky *et al.* 1990), there is no information regarding the mechanism by which other types of macromolecules such as carbohydrates, nucleotides or lipids might be transported out of the vacuole.

Selective macroautophagy

The overall morphology of selective macroautophagy is largely the same as that of nonselective macroautophagy with one

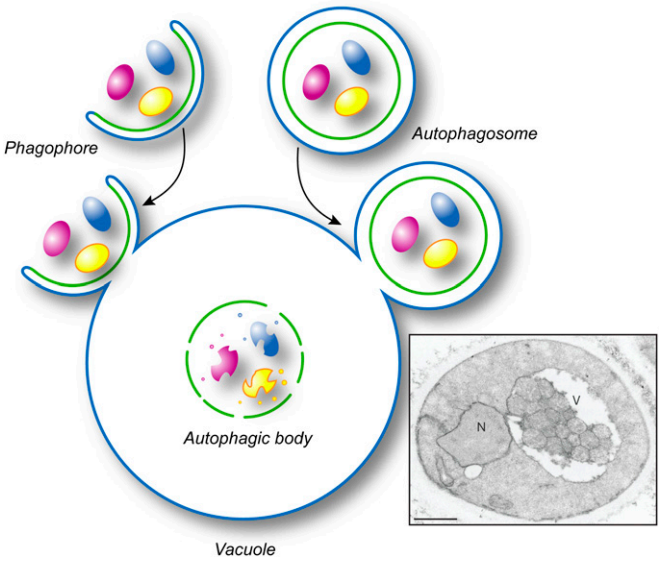

Figure 4 Topology of autophagosome fusion with the vacuole. Fusion of an expanding phagophore (*i.e.*, an incomplete autophagosome) with the vacuole (or lysosome in higher eukaryotes) does not allow delivery of the cytoplasmic content into the interior of the degradative organelle (left side of the drawing). In contrast, the fusion of a sealed autophagosome with the vacuole permits the delivery of its internal vesicle and cargo into the lumen making it accessible for subsequent degradation (right side of the drawing). The mechanism that prevents premature fusion of a phagophore with the vacuole is not known. The electron micrograph shows the presence of autophagic bodies in the vacuole. Scale bar, 1 μm. This image was modified from data previously published in Scott *et al.* (2000) and is reproduced by permission of the American Society for Biochemistry and Molecular Biology and Elsevier, copyright 2000.

primary distinction—in the former, the sequestering membrane is in close apposition to the cargo, excluding bulk cytoplasm. Partly for this reason, the completed sequestering double-membrane vesicles are given different names, whereas phagophore is a common term used in all cases. Thus, in the Cvt pathway the initial vesicle is termed a Cvt vesicle rather than an autophagosome (see below), and the resulting single-membrane vesicle in the vacuole lumen is a Cvt body (Baba *et al.* 1997). The terms mitophagosome and pexophagosome have similarly been used when referring to mitophagy and pexophagy, respectively (Ano *et al.* 2005b; Kim *et al.* 2007).

In nonselective macroautophagy the cargo is considered to be random cytoplasm. Thus, there is almost no effective size limit to the cargo, although the cytoskeleton is not sequestered within autophagosomes. In contrast, there appears to be a limit to the size of the sequestering vesicles formed during selective types of macroautophagy, which is dictated in part by the volume of the cargo. It is not clear why there is a size limit to the sequestering vesicle of selective autophagy if the membrane forms by wrapping around the cargo due to interactions between the autophagy receptor and Atg8, but the levels of this latter protein could be the limiting factor; higher amounts of Atg8 are required during nonselective macroautophagy to sustain the formation of large autophagosomes (see below).

Nonselective microautophagy

Nonselective microautophagy has been studied both *in vitro* and *in vivo* (Muller *et al.* 2000; Sattler and Mayer 2000). During this process a portion of the vacuole membrane invaginates to form a long, narrow tube-like structure. The sides and/or tip of the tube bud off to form an intravacuolar vesicle. The budding tip is devoid of membrane proteins and thus the resulting vesicle is similar in size to, and indistinguishable from, an autophagic body. In the last step of this process, as well as in selective microautophagy (see below), the intravacuolar vesicles must be degraded. In contrast to macroautophagy, however, the vesicle membrane is derived from the vacuole. It is not known how these membranes are now distinguished from the vacuole limiting membrane such that they can be degraded without disrupting the integrity of the vacuole.

Selective microautophagy

In microautophagy the sequestration of the cargo occurs directly at the vacuole-limiting membrane. The mechanism through which the vacuole membrane is induced to invaginate or protrude/septate to sequester the cargo is unknown. The closest parallel may be seen in the multivesicular body (MVB) pathway, in which the endosomal membrane invaginates to generate intralumenal vesicles. The MVB pathway requires the function of a series of large protein complexes, but the components of these complexes do not appear to play a role in yeast macroautophagy (Reggiori *et al.* 2004b), and hence presumably not in microautophagy either. In general, the protein machinery needed for macroautophagy is also needed for microautophagy. The simplest way to view this overlap is that one of the important roles for these proteins is the rearrangement of intracellular membrane to form a sequestering double-membrane structure, whether it involves the *de novo* generation of the phagophore or utilizes the sequestering arms of the vacuole.

The morphological details of selective microautophagy have been the most thoroughly explored in the case of micropexophagy in methylotrophic yeast such as *Pichia pastoris* and *Hansenula polymorpha*. A unique structure, the micropexophagic apparatus (MIPA) (Oku *et al.* 2003), which does not have an obvious functional equivalent in macropexophagy, characterizes this process. The MIPA is a membranous cistern that may operate as a scaffold for completion of the sequestering membrane and it is located at the open end of the vacuolar sequestering membranes (Oku *et al.* 2006). After the completion of sequestration, the peroxisomes are enclosed within a single-membrane intralumenal vesicle (Sakai *et al.* 1998), which is similar to the outcome of macropexophagy. One distinct difference between micro- and macropexophagy, however, is that during the former, multiple peroxisomes are sequestered, compared to a single peroxisome being the target during macropexophagy (Dunn *et al.* 2005). This difference may reflect the membrane source(s) used in these respective modes of sequestration. In particular, the vacuole is a relatively

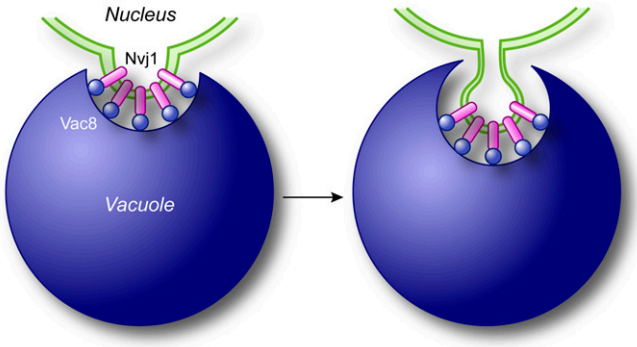

Figure 5 Mechanism of micronucleophagy. During micronucleophagy (also called piecemeal microautophagy of the nucleus), small portions of the nucleus, including the nuclear double membrane and part of the nucleoplasm, protrude into the vacuole lumen though a process that requires the association between Nvj1 in the nuclear membrane and Vac8 on the surface of the vacuole. Subsequently, a scission event mediated by Atg proteins leads to the generation of a subvacuolar vesicle that is degraded by resident hydrolases.

large organelle, and accordingly it may be possible to use a substantial amount of membrane during sequestration.

Another example of selective microautophagy is seen with micronucleophagy, also called piecemeal microautophagy of the nucleus (Roberts *et al.* 2003; Krick *et al.* 2008b). Small portions of the nucleus, including the nuclear double membrane and part of the nucleoplasm, protrude into the vacuole lumen (Figure 5). A scission event separates the membrane from the nucleus and the vacuole-limiting membrane, again generating a single-membrane intralumenal vesicle. There is no apparent specificity for which part of the nucleus is degraded, but this is still a selective process because it targets only the nucleus.

There is also evidence for micromitophagy, but this process has been less well characterized. Electron microscopy studies suggest that portions of the vacuole membrane may protrude to sequester mitochondria (Kissova and Camougrand 2009).

Noncanonical autophagy

Cytoplasm-to-vacuole targeting pathway: The morphology of the Cvt pathway is similar to that of other types of selective autophagy. The primary cargo protein of the Cvt pathway, precursor aminopeptidase I (prApe1), is synthesized in the cytosol and assembles into a dodecamer, which subsequently associate into an oligomer of a supra-order that is termed the Ape1 complex. This complex in combination with the receptor protein Atg19 and smaller oligomers formed by Ams1 (see below) is named the Cvt complex (Shintani *et al.* 2002). As mentioned above, the double-membrane sequestering vesicle is called a Cvt vesicle, and the intravacuolar single-membrane vesicle is called a Cvt body. The Cvt vesicle has a diameter of ~140–160 nm, which corresponds with the size of the Cvt complex that is generated under physiological conditions. In contrast, when prApe1 is overproduced, a larger cytosolic complex is formed (Baba *et al.* 1997). This complex can no longer be sequestered within a Cvt vesicle, and as a consequence the major part of the prApe1 complex accumulates in the cytosol. If nonselective macroautophagy is induced, however, even these large complexes can be sequestered within an autophagosome and efficiently delivered into the vacuole (Scott *et al.* 1996).

Compartment for unconventional protein secretion: Studies in *Saccharomyces cerevisiae* and *P. pastoris* have revealed that extracellular delivery of the cytosolic acyl coenzyme A-binding protein (Acb1), which occurs under starvation conditions, is not mediated by the secretory pathway (Duran *et al.* 2010; Manjithaya *et al.* 2010a). This transport route depends on Atg proteins, leading to the suggestion that autophagosomes could be the hallmark of this type of unconventional secretion (Duran *et al.* 2010; Manjithaya *et al.* 2010a). This conclusion, however, is not supported by ultrastructural observations, and therefore, the nature of the carriers transporting Acb1 remains to be deciphered. Nonetheless, electron microscopy work in *S. cerevisiae* has revealed that the initial precursor structure of this transport route is a cluster of membranes and vesicles, which morphologically resemble the precursor structures involved in autophagy (Mari *et al.* 2010), and they are positive for both Atg8 and Atg9 (Bruns *et al.* 2011).

Protein Machinery

Macroautophagy

Most of the machinery used in macroautophagy and selective microautophagy is conserved between these pathways (Kraft *et al.* 2009; Li *et al.* 2012). During the initial identification of the genes encoding the Atg proteins, several were classified as being specific for nonselective macroautophagy, the Cvt pathway and/or pexophagy. For example, Atg11 was originally characterized as being a Cvt pathway-specific protein (Harding *et al.* 1996; Oda *et al.* 1996). These denotations, however, reflected the different screens used to identify the corresponding genes and the limited analyses available at that time. We now know that Atg11 is involved in most or even all types of selective micro- and macroautophagy (Kim *et al.* 2001; Kanki and Klionsky 2008; Krick *et al.* 2008b). Furthermore, Atg11 even plays a role in the transition from the vegetative PAS to a starvation-specific PAS (Cheong *et al.* 2008). Here, we briefly review the current information on the functions of, and interactions among, the Atg proteins.

Atg1 kinase complex: Atg1 is a serine/threonine protein kinase (Matsuura *et al.* 1997). It carries out autophosphorylation and presumably also phosphorylates other targets. The key substrate(s) of Atg1 with regard to autophagy, however, is unknown. Atg13 is required for optimal Atg1 kinase activity (Kamada *et al.* 2000), and Atg13 is hyperphosphorylated under nutrient-rich conditions, while being largely dephosphorylated under starvation conditions (Scott *et al.* 2000). Initial studies suggested that hyperphosphorylated Atg13 interacts with Atg1 with low affinity (Kamada *et al.* 2000), leading to

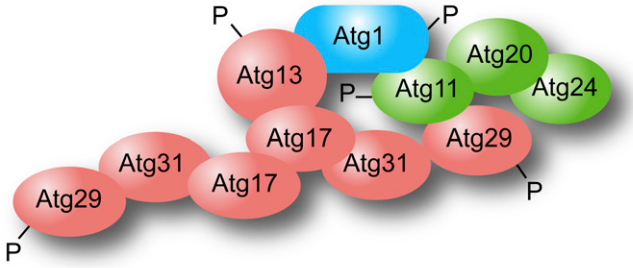

Figure 6 The interactome of the Atg1 complex. Note that there is no indication that all the depicted interactions occur simultaneously, and not all of the known interactions are shown; the Atg1 complex interactors could vary depending on both the step in the formation of the double-membrane vesicle and the type of autophagy.

a model whereby the Atg1 kinase complex functions in part as a switch between the constitutive Cvt pathway and nonselective autophagy. More recent data, however, suggest that Atg13 is always in a complex with Atg1 (Kraft *et al.* 2012), which would be in agreement with the interactions of the homologous proteins in higher eukaryotes. Atg17, Atg29, and Atg31 form a stable ternary complex, with Atg31 bridging the other two proteins (Kawamata *et al.* 2008; Cao *et al.* 2009; Kabeya *et al.* 2009). Atg17 most likely binds Atg13 directly, but in addition interacts with the complex via Atg29 and Atg11 (Yorimitsu and Klionsky 2005), a scaffold protein that also binds Atg1 (Figure 6). Atg17 (and hence, the Atg17–Atg31–Atg29 subcomplex) is also required for maximal Atg1 kinase activity (Kamada *et al.* 2000), although the mechanism through which Atg17 or Atg13 regulate Atg1 is not known. Atg17 may also play a role in organizing the recruitment of Atg proteins to the PAS, particularly under autophagy-inducing conditions (Cheong *et al.* 2008). This possibility is supported by the Atg17 crystal structure, which reveals that this protein assembles into a dimer with an extended coiled-coil domain that could regulate the intrinsic ability of Atg1 to tether membranes, but also acts as a scaffold (Ragusa *et al.* 2012). In the absence of Atg1 or Atg13 function Atg9–GFP localizes primarily at the PAS (Reggiori *et al.* 2004a), suggesting a role in regulating the movement of this protein, a step in autophagosome formation and/or completion. Atg13 (and also Atg1), however, are phosphorylated by the target of rapamycin (TOR) and/or protein kinase A (PKA) (Budovskaya *et al.* 2005; Kamada *et al.* 2010); post-translational modification by these upstream nutrient sensors suggests that these proteins act as a core regulator that functions at an early step in autophagy induction.

Atg20 and Snx4/Atg24, two sorting nexins, were identified on the basis of the Cvt-defective phenotype of the corresponding null strains (Nice *et al.* 2002). Atg20 interacts with Atg11, and both proteins bind Atg17. Atg20 and Snx4 also bind PtdIns3P via PX domains and localize to the PAS. The function of these proteins in the Cvt pathway, however, is not known.

PtdIns 3-kinase complex: In yeast there are at least two protein complexes that direct the synthesis of PtdIns3P (Kihara *et al.* 2001). Both complexes include Vps15 (a regulatory kinase), Vps34 (the PtdIns 3-kinase), and Vps30/Atg6 (Herman *et al.* 1991; Stack *et al.* 1993; Stack *et al.* 1995; Kametaka *et al.* 1998; Kihara *et al.* 2001). Complex I also includes Atg14 and functions in autophagy, whereas complex II contains Vps38 and is involved in endosomal trafficking, endocytosis, and the vacuolar protein sorting pathway (Kihara *et al.* 2001). The role of Vps30 is unknown, but it interacts directly with Atg14 (Kametaka *et al.* 1998). The latter plays a role in directing the localization of the complex to the PAS (Obara and Ohsumi 2011). A few of the Atg proteins bind PtdIns3P (Nice *et al.* 2002; Reggiori *et al.* 2004a; Krick *et al.* 2006; Nair *et al.* 2010), suggesting that one function of this phosphoinositide is the recruitment of proteins that function in phagophore and autophagosome formation. However, it cannot be excluded that PtdIns3P is able to regulate the activity of one or more Atg proteins.

Atg9 complex: Atg9 is the only transmembrane protein that is absolutely required for autophagosome formation (Noda *et al.* 2000). Atg9 transits through a portion of the secretory pathway and can be detected at the ER and Golgi apparatus as well as the PAS (Mari *et al.* 2010; Ohashi and Munro 2010; Yamamoto *et al.* 2012). In wild-type cells, Atg9–GFP is found in multiple puncta, one of these corresponding with the PAS, and others with peripheral sites (Noda *et al.* 2000; Reggiori *et al.* 2004a). As noted above, in an *atg1*Δ (or *atg13*Δ) strain Atg9–GFP is localized exclusively at the PAS. In an $atg1^{ts}$ mutant shifted from the nonpermissive to the permissive temperature, Atg9–GFP puncta are seen initially at the PAS and then also appear at the peripheral sites. These observations led to an initial model whereby Atg9 transits between these peripheral sites and the PAS, delivering membrane from donor sites to the expanding phagophore. Recent real-time imaging data, however, have indicated that Atg9 is not cycling through the PAS (Yamamoto *et al.* 2012). Rather, Atg9 appears to act as a regulator of autophagy initiation possibly by providing at least part of the initial membranes essential to recruit Atg proteins and organize the PAS (Mari *et al.* 2010; Yamamoto *et al.* 2012). This latter idea of Atg9 being a landmark scaffold is supported by the fact that this protein can self-interact (Reggiori *et al.* 2005b; He *et al.* 2008). The possible regulatory function is also underlined by the observation that Atg9, which principally sits on the external surface of the growing phagophore, is retrieved from the autophagosomal membrane just before or after fusion of autophagosomes with the vacuole (Yamamoto *et al.* 2012).

The current data indicate that shortly after synthesis, Atg9 is translocated into the ER, and from there it reaches the Golgi where it is very likely sorted into vesicles (Geng *et al.* 2010; Wang *et al.* 2012). This hypothesis is supported by the observation that in mutants defective in the function of SNARE proteins involved in protein secretion, in particular an *sso1*Δ $sso2^{ts}$ strain, Atg9 is detected in small vesicles (Nair *et al.* 2011). High-resolution microscopy has also confirmed

the presence of a vesicular pool of Atg9 (Yamamoto *et al.* 2012). The fate of these vesicles continues to be a controversial issue. One idea is that they remain independent vesicles and one (or a few) will move in close proximity of the vacuole to become the PAS upon autophagy induction (Yamamoto *et al.* 2012). Another possibility is that the Atg9-positive vesicles assemble in a SNARE-dependent process (Nair *et al.* 2011) to generate larger structures corresponding to the peripheral sites and at least one of these structures relocates near the vacuole surface to become the PAS (Mari *et al.* 2010). Nevertheless, Atg9-positive membranes are probably maturing into the PAS/phagophores possibly by fusing together or with other membranes, as suggested by the colocalization of Atg9 with Ypt1 and the TRAPPIII complex (Lynch-Day *et al.* 2010; Lipatova *et al.* 2012) and the recruitment of these latter factors onto autophagosomal membranes by Atg9 (Kakuta *et al.* 2012).

Both Atg18 and Atg2 are peripheral membrane proteins that interact with each other and associate with Atg9 at the PAS (Reggiori *et al.* 2004a; Obara *et al.* 2008). The absence of either protein results in a defect in Atg9 localization similar to that seen in the *atg1*Δ strain (Reggiori *et al.* 2004a). The precise role of Atg2 and Atg18 in autophagosome biogenesis is unknown, as is the mechanism through which they mediate Atg9 retrograde transport from autophagosomal membranes and/or the vacuole. The recruitment and localization of Atg18 and Atg2 to the PAS depends on each other, Atg9 and the Atg1–Atg13 kinase complex, and also on the presence of PtdIns3P generated by the PtdIns 3-kinase complex I (Shintani *et al.* 2001; Reggiori *et al.* 2004a; Suzuki *et al.* 2007; C.-W. Wang *et al.* 2001). The main structural feature of Atg18 is that its 7 WD40 repeats fold into a seven-bladed β-propeller (Barth *et al.* 2001; Dove *et al.* 2004). Its predicted structure is very similar to the recently published crystal structure of *Kluyveromyces lactis* Hsv2, a homolog of Atg18 (Baskaran *et al.* 2012; Krick *et al.* 2012; Watanabe *et al.* 2012). Atg18 is also able to bind both PtdIns3P and phosphatidylinositol-3,5-bisphosphate [PtdIns $(3,5)P_2$] through a conserved phenylalanine–arginine–arginine–glycine (FRRG) motif within its β-propeller (Nice *et al.* 2002; Dove *et al.* 2004; Krick *et al.* 2006; Nair *et al.* 2010). Atg18 binding to PtsInd3P is essential for its localization to the PAS (Krick *et al.* 2006, 2008a; Obara *et al.* 2008; Nair *et al.* 2010). The PAS localization of Atg18 also depends upon Atg2 and *vice versa* (Guan *et al.* 2001; Suzuki *et al.* 2007; Obara *et al.* 2008), and it has been proposed that these two proteins constitutively form a complex (Obara *et al.* 2008). The ability of Atg18 to interact with Atg2 does not depend on its PtdIns3P-binding capacity but rather on residues positioned on the opposite surface from the FRRG motif on the β-propeller (Watanabe *et al.* 2012; Rieter *et al.* 2013), whereas the binding of Atg18 to PtdIns3P seems necessary for the appropriate targeting of the Atg18–Atg2 complex to the PAS (Obara *et al.* 2008).

Atg23 and Atg27 are nonconserved peripheral and integral membrane proteins (Tucker *et al.* 2003; Yen *et al.* 2007), respectively, which bind Atg9 and are needed for the efficient movement of this protein to the PAS (Legakis *et al.* 2007). In particular, they appear to play a key role in this trafficking step by mediating Atg9 sorting from the Golgi (Yamamoto *et al.* 2012).

Ubiquitin-like protein conjugation complexes: There are two unique ubiquitin-like protein conjugation complexes that participate in autophagy, involving Atg8 and Atg12 (Geng and Klionsky 2008). Based on the crystal structure of the mammalian Atg8 homolog MAP1LC3 (LC3), Atg8 has structural similarities with ubiquitin (Sugawara *et al.* 2004). It is initially synthesized with a C-terminal arginine that is removed by the Atg4 cysteine protease (Kirisako *et al.* 1999; Huang *et al.* 2000) (Figure 7). The processed Atg8 is next activated in an ATP-dependent reaction by the ubiquitin-activating enzyme homolog Atg7 and then transferred to Atg3, a ubiquitin-conjugating enzyme analog. Atg3 forms a covalent bond between the now-exposed C-terminal glycine residue of Atg8 and PE (Ichimura *et al.* 2000). Atg8 is initially located on both sides of the phagophore. Atg4 can subsequently cleave the amide bond to PE in a deconjugation step, liberating Atg8, particularly the population of the protein that is on the external surface of the autophagosome, from the membrane, and allowing it to cycle through the conjugation process again. Analysis of human ATG4B alone and in combination with LC3 indicates that the protease undergoes a substantial conformational change, which may be critical in gaining access to the lipidated (and hence membrane bound) LC3-II (Atg8—PE) molecule (Sugawara *et al.* 2005; Kumanomidou *et al.* 2006; Satoo *et al.* 2009).

Comparison to the crystal structure of *Arabidopsis thaliana* ATG12b (Suzuki *et al.* 2005) suggests that the yeast Atg12 homolog also contains ubiquitin folds and participates in a parallel conjugation pathway. This protein is also activated by Atg7, and structural studies have provided insight into the mechanism by which Atg7 can act as a common E1 for two different conjugating enzymes (Hong *et al.* 2011; Noda *et al.* 2011; Taherbhoy *et al.* 2011; Yamaguchi *et al.* 2012a). The activated Atg12 is then transferred to the Atg10-conjugating enzyme (Shintani *et al.* 1999), which catalyzes the formation of a covalent bond between the C-terminal glycine of Atg12 and an internal lysine of Atg5 (Mizushima *et al.* 1998), a protein that also contains two ubiquitin-like structural domains (Matsushita *et al.* 2007). The conjugation of Atg12 to Atg5 occurs independently of an E3 ligase, and structural studies may provide information on this unique aspect of Atg10-conjugating activity (Yamaguchi *et al.* 2012b). Atg5, and preferentially the Atg12—Atg5 conjugate, noncovalently binds Atg16, promoting Atg16 self-interaction (Mizushima *et al.* 1999), generating a dimer of the Atg12—Atg5–Atg16 complex (Kuma *et al.* 2002; Fujioka *et al.* 2010). This complex is proposed to function as an E3-like enzyme for Atg8 conjugation (Hanada *et al.* 2007; Noda *et al.* 2013; Otomo *et al.* 2013), but Atg8—PE can be generated in the absence of these proteins (Cao *et al.* 2008).

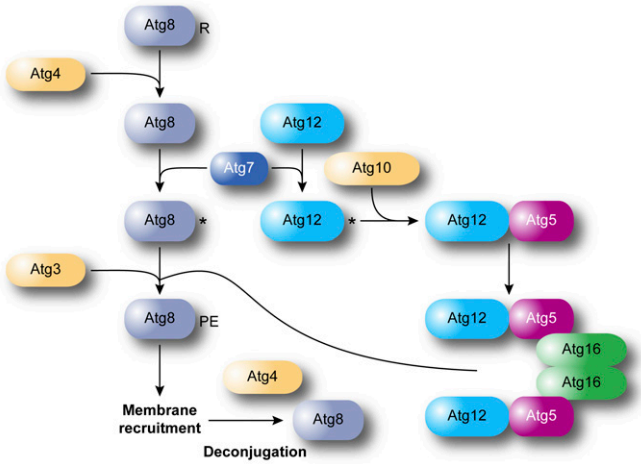

Figure 7 Schematic of the two ubiquitin-like conjugation systems involved in autophagy. Atg12, a ubiquitin-like molecule, is covalently conjugated to Atg5 through the activity of Atg7 and Atg10, an E1- and an E2-like enzyme, respectively. The Atg12—Atg5 complex subsequently associates with Atg16, and dimerization leads to the formation of a large complex. Atg8 is a second ubiquitin-like protein participating in autophagy. Atg8 is post-translationally processed by the specific cysteine protease Atg4, which removes the C-terminal amino acid (an arginine residue in yeast) exposing a glycine. Through another ubiquitination-like reaction mediated by Atg7 and the E2-like enzyme Atg3, Atg8 is covalently conjugated to PE. While it has been proposed that the Atg12—Atg5–Atg16 complex could be the E3 ligase catalyzing the formation of Atg8—PE, these proteins promote the linkage of Atg8 to PE, but they are not essential for it.

Thus, the function of the Atg12—Atg5–Atg16 complex remains unclear, but recent structural studies have revealed that it is probably acting as a platform to bring into close proximity the activated Atg8 in the Atg8–Atg3 conjugate to the acceptor PE (Kaiser *et al.* 2012; Noda *et al.* 2013; Otomo *et al.* 2013). In agreement with this model, recent data suggest that Atg5 contains a membrane-binding domain that is negatively regulated by Atg12, and mutations that interfere with Atg5 membrane binding inhibit macroautophagy (Romanov *et al.* 2012). Recruitment of the components of the Atg8 conjugation system, *i.e.*, Atg7 and Atg3, onto membranes depends on the Atg12—Atg5–Atg16 complex being able to associate with lipid bilayers (Romanov *et al.* 2012).

Atg8 shows the greatest change in synthesis of any of the Atg proteins upon autophagy induction in yeast (Kirisako *et al.* 1999; Huang *et al.* 2000). Experiments in which the amount of Atg8 is clamped at levels lower than that normally generated during autophagy induction indicate that the amount of this protein correlates with the size of the autophagosome (Xie *et al.* 2008). Atg8 that lines the concave side of the phagophore also plays a role in cargo recognition during selective types of autophagy by binding the receptors used in the Cvt pathway, pexophagy, and mitophagy (Shintani *et al.* 2002; Chang and Huang 2007; Mijaljica *et al.* 2012; Motley *et al.* 2012). X-ray crystallography, combined with NMR, has revealed that a hydrophobic pocket in Atg8 interacts with the Atg8-interacting motif (AIM, or, with regard to the mammalian homolog, an LC3-interacting region, LIR) in Atg19, providing insight into the mechanism of selective cargo recognition (Noda *et al.* 2008).

Cargo recognition during selective macroautophagy: The best-characterized process of selective macroautophagy is the Cvt pathway. Precursor Ape1 contains an N-terminal propeptide that may keep the enzyme inactive in the cytosol. In addition, this amino acid sequence binds a soluble receptor, Atg19 (Scott *et al.* 2001). The C terminus of Atg19 contains binding sites for Atg11 and Atg8 (Shintani *et al.* 2002). Localization and affinity isolation experiments suggest that Atg11 binds Atg19 after the latter interacts with the prApe1 propeptide. The extreme C terminus of Atg19 contains a WXXL motif (equivalent to the AIM) that allows binding to Atg8, thus linking the cargo complex with the phagophore (Figure 8) and its subsequent selective sequestration (Kraft *et al.* 2010). Atg19 also functions as a receptor for Ape4 (Yuga *et al.* 2011) and Ams1 (Hutchins and Klionsky 2001), two other resident vacuolar hydrolases that are part of the Cvt complex. In addition, Atg34 can substitute for Atg19 as an Ams1 receptor under starvation conditions (Watanabe *et al.* 2010).

Similar to Atg19, the mitochondria autophagy receptor Atg32 also interacts first with Atg11 (Kanki *et al.* 2009b; Okamoto *et al.* 2009) and then with Atg8 (Okamoto *et al.* 2009) via an AIM. Atg32 is a transmembrane protein residing in the outer mitochondrial membrane. Atg32 is phosphorylated (Aoki *et al.* 2011) and the interaction with Atg11 occurs only under conditions that induce mitophagy. Thus, an unknown alteration, perhaps in Atg32 conformation or the phosphorylation-dependent generation of a binding motif, presumably leads to its activation. Mitophagy induced at the post-log phase, but not that induced by starvation, also requires Atg33, a transmembrane protein residing in the outer membrane of the mitochondria (Kanki *et al.* 2009a). Similar to Atg32, *P. pastoris* Atg30 (PpAtg30) and *S. cerevisiae* Atg36 act as peroxisome autophagy receptors during pexophagy (Farre *et al.* 2008; Motley *et al.* 2012). Both proteins bind Atg11 and Atg8. Thus, Atg11 is a scaffold protein that is common to many types of selective autophagy. Atg11 interacts with receptors, the Atg1 kinase complex, and itself, thus playing a central role in organization of the Atg proteins at the PAS. In this regard, Atg11 interacts with Atg9 and mediates the relocation of part of the membranes positive for this protein from the peripheral pool to the perivacuolar site that will become the PAS (He *et al.* 2006; Mari *et al.* 2010). The movement of Atg9–Atg11 (and probably the cargo that must be sequestered into the nascent double membrane vesicles associated with them) is guided by actin filaments (Reggiori *et al.* 2005a) via a direct interaction between Atg11 and the Arp2/3 complex (Monastyrska *et al.* 2008). The cargo and ultimately Atg11, probably through its interaction with the Atg1 complex, could also dictate the kinetics

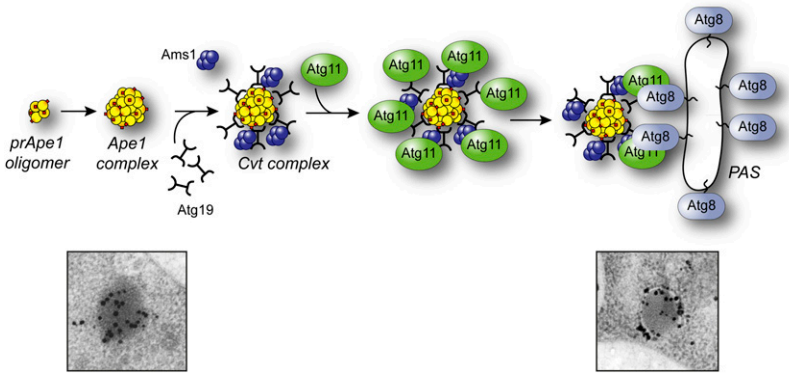

Figure 8 Mechanism of cargo recruitment during the Cvt pathway. Shortly after synthesis, prApe1 forms dodecamers that subsequently self-assemble in a larger oligomer that has been called the prApe1 complex. Association with the Atg19 autophagy receptor and oligomers of Ams1 (and additional cargo proteins) leads to the generation of the Cvt complex. The subsequent interaction between Atg19 and the autophagy adaptor Atg11 allows the movement of the Cvt complex within proximity of the vacuole through a mechanism that requires actin filaments and the Arp2/3 complex. This relocalization, which probably also coordinates the trafficking of Atg9-positive membranes, participates in the formation of the PAS. At this site, the interaction between Atg19 and Atg8 plays a key role in the sequestration of the Cvt complex into Cvt vesicles. One of the primary differences between selective and nonselective macroautophagy is that the sequestering vesicles of the former exclude bulk cytoplasm and contain primarily the targeted cargo. The electron micrographs depict the electron dense Cvt complex detected with antiserum to Ape1 (left) and a phagophore sequestering a Cvt complex marked with an antibody that detects GFP–Atg8 (right). The electron micrographs in this figure were modified from data previously published in Yen *et al.* (2010) and are reproduced by permission of the American Society for Cell Biology, copyright 2010.

of the autophagic process. The maturation time of precursor Ape1 is ~90 min, and this time presumably reflects the transport rate of the Cvt pathway (Klionsky *et al.* 1992). In contrast, monitoring GFP–Atg8 fluorescence at the PAS suggests that a cycle of autophagosome formation and fusion with the vacuole occurs in ~10 min (Xie *et al.* 2008), which is somewhat faster than the proposed 10 min half-life of mammalian autophagosomes (Mizushima *et al.* 2001).

Studies in methylotrophic yeast have identified additional *ATG* genes that are essential for micro- and/or macropexophagy in these organisms, *i.e.*, *ATG25*, *ATG26*, *ATG28*, and *ATG35*. Most of them do not have homologs in other yeast, and their precise molecular role has not yet been unveiled. Atg25 is a coiled-coil protein that localizes to the PAS (Monastyrska *et al.* 2005). This protein appears to be a regulator modulating the switch between selective types of pexophagy, because in its absence peroxisome turnover during glucose-induced macrophexophagy is abolished, whereas the cells constitutively degrade these organelles by micropexophagy. Atg28 and Atg35 form a complex that interacts with Atg17 and is essential for efficient MIPA formation during micropexophagy (Stasyk *et al.* 2006; Nazarko *et al.* 2011). As a result, these two proteins are required for this selective process, with Atg28 also being essential for macropexophagy, whereas Atg35 is not. The sterol glucosyltransferase Atg26 is also crucial for the generation of the MIPA (Oku *et al.* 2003). Upon micropexophagy induction, the synthesis of phosphatidylinositol-4-phosphate at the site where the MIPA will emerge leads to the recruitment of Atg26 through its GRAM domain, which specifically binds to this lipid, and permits the local production of ergosterol glucoside (Oku *et al.* 2003; Yamashita *et al.* 2006). The function of this molecule in micropexophagy, however, is unclear and it appears to be essential only for glucose-induced micropexophagy, but not when the same pathway is triggered by oleate or amine (Nazarko *et al.* 2007).

Late stages of macroautophagy: Completion of phagophore expansion and sealing of the autophagosome need to be regulated and also must provide some type of signal to allow subsequent fusion with the vacuole (Figure 4). One event that occurs at this time is the turnover of PtdIns3P, which is carried out mostly by the PtsIns3P-specific phosphatase Ymr1 (Cebollero *et al.* 2012b). The hydrolysis of this lipid is critical in releasing the Atg machinery from the completed autophagosome, and it appears to be requisite for the fusion of these vesicles with the vacuole (Cebollero *et al.* 2012b). Accordingly, autophagosomes with Atg8 on their surface fuse inefficiently with the vacuole (Nair *et al.* 2012; Nakatogawa *et al.* 2012). This fusion step involves components that are common to other transport processes that terminate at the vacuole. Thus, the Rab protein Ypt7, its GDP exchange factor complex Ccz1–Mon1, and SNARE proteins including Vam3, Vam7, Vti1, and Ykt6, along with the class C Vps/HOPS complex, are needed for tethering and fusion (Darsow *et al.* 1997; Rieder and Emr 1997; Ishihara *et al.* 2001; Meiling-Wesse *et al.* 2002; Wang *et al.* 2002; Nair *et al.* 2011; Polupanov *et al.* 2011). The fusion of the autophagosome outer membrane with the vacuole-limiting membrane releases the inner vesicle into the lumen (Figure 1). The membrane of the resulting autophagic bodies is lysed through the action of Atg15, a putative vacuolar lipase (Epple *et al.* 2001; Teter *et al.* 2001). While resident hydrolases such as prApe1 are activated in the vacuole lumen by removal of a propeptide, other cargoes are degraded through the action of the various hydrolases. The breakdown products are subsequently transported into the cytosol through vacuole membrane permeases including the amino acid transporter Atg22 (Yang *et al.* 2006).

Micronucleophagy

The key molecular machinery of micronucleophagy includes two proteins, Nvj1 and Vac8, which are localized in the nuclear and vacuolar membranes, respectively (Roberts *et al.* 2003). Sequestration occurs at the nucleus–vacuole junction (Figure 5) that is formed through the interaction

of these two proteins (Pan *et al.* 2000). The core Atg machinery is also needed for micronucleophagy; however, it is unknown what role they play in this process or how they become localized to the contact area between the nuclear and vacuolar membranes. In the absence of these core components, micronucleophagy is blocked at a very late step, after protrusion of a part of the nucleus into the vacuole, but prior to scission (Roberts *et al.* 2003; Krick *et al.* 2008b). Thus, micronucleophagy appears to differ from micropexophagy because protrusion of the vacuole membrane in the latter requires the Atg proteins. This lack of a requirement for the core machinery in the initial sequestration event actually suggests a fundamental difference between micronucleophagy and other types of autophagy. Micronucleophagy also requires the oxysterol-binding proteins (Kvam and Goldfarb 2004), as well as components involved in very-long-chain fatty-acid formation (Kvam *et al.* 2005; Dawaliby and Mayer 2010). Two separate stages of micronucleophagy are dependent on activity of the vacuolar ATPase and an electrochemical potential across the vacuole membrane: the initial invagination of the membrane at the nucleus–-vacuole junctions, and the late step of vesicle scission (Dawaliby and Mayer 2010).

Ribophagy

Because ribosomes are very abundant in the cytoplasm of a yeast cell and readily detectable in the interior of autophagosomes by electron microscopy during bulk macroautophagy (Takeshige *et al.* 1992; Baba *et al.* 1994), it has been assumed for a long time that they were randomly sequestered into autophagosomes. It has been revealed, however, that ribosomes are degraded through a selective type of macroautophagy, named ribophagy (Kraft *et al.* 2008), which could also be important for the disposal of defective or incorrectly assembled ribosomes (Cebollero *et al.* 2012a). Analysis of ribosome half-life under nutrient starvation conditions in *S. cerevisiae* has shown that these multiprotein complexes are more rapidly turned over compared to other cytoplasmic components, supporting the notion of a selective degradation process (Kraft *et al.* 2008). While it is clear that ribophagy depends on core components of the autophagy machinery such as Atg1 and Atg7, the molecular principles underlying the selectivity of this pathway remain to be elucidated. Several lines of evidence have indicated that ubiquitination/deubiquitination reactions are probably involved in determining the fate of ribosomes. In particular, the ubiquitin protease Ubp3 and its cofactor Bre5 are required for ribophagy, but not for bulk macroautophagy (Kraft *et al.* 2008). Interestingly, Ubp3 interacts with Atg19 and influences its ubiquitination status (Baxter *et al.* 2005), but it is still unclear whether Atg19 participates in ribophagy. Additional evidence for the possible involvement of ubiquitin modifications in ribophagy comes from the observation that a decrease of the cytoplasmic levels of the ubiquitin ligase Rsp5 together with the deletion of *UBP3* results in a defect in the turnover of ribosomes greater than that seen in *ubp3*Δ cells (Kraft and Peter 2008).

Reticulophagy

There are not many studies on reticulophagy. Furthermore, this process has been studied in various contexts. As a result, the data cannot be assembled into a single model because there may be significant differences in the process reflecting the way in which reticulophagy is stimulated. This selective type of macroautophagy has been investigated as a response to chemically induced ER stress, the accumulation of protein aggregates in the ER, starvation, and ER size recovery upon termination of an ER stress. How the ER is targeted for degradation and specifically sequestered into autophagosomes remains to be elucidated. In analogy with mitochondria and mitophagy, one possibility could be that fragments of the ER are fissioned off from the main ER body and are transported to the site where autophagosomes are generated. It is clear that the core Atg components are required for reticulophagy induced by both starvation and ER stress caused by treatment with dithiothreitol or tunicamycin (Hamasaki *et al.* 2005; Bernales *et al.* 2006; Yorimitsu *et al.* 2006), The fact that Atg19, Atg20, and the actin cytoskeleton are essential (Hamasaki *et al.* 2005; Bernales *et al.* 2006), however, supports the notion that reticulophagy is a selective type of macroautophagy, but also that the ER could be recruited to the site where it will then be incorporated into nascent autophagosomes. Interestingly, Atg proteins are necessary for cell survival, while vacuolar proteases are dispensable, under conditions of ER stress, indicating that the sequestration of the ER without degradation is sufficient to mitigate the effects of this type of stress (Bernales *et al.* 2006).

Vacuole import and degradation pathway

The Vid pathway involves the translocation of substrate proteins into 30-nm vesicles. The formation of these Vid vesicles is blocked in the absence of the ubiquitin-conjugating enzyme Ubc1 (Shieh *et al.* 2001), whereas import of Fbp1 into Vid vesicles requires the plasma membrane protein Vid22, the cyclophilin Cpr1, and the heat-shock protein Ssa2 (Brown *et al.* 2000, 2001, 2002). In contrast, the peripheral vesicle membrane protein Vid24 acts after the import step, because the *vid24-1* mutant accumulates Fbp1 within completed vesicles (Chiang and Chiang 1998). Association of Vid24 with these vesicles is dependent on the coatomer subunit Sec28 (Brown *et al.* 2008). Vid vesicles merge/cluster with endosomes at actin patches in a process requiring Vid30 (Alibhoy *et al.* 2012), and subsequent transport to and/or fusion with the vacuole is dependent on Vph1 (Liu *et al.* 2005), a subunit of the vacuolar H^+–ATPase, and both Rab and SNARE components that participate in most vacuolar fusion events including Ypt7, Ykt6, Vti1, and the class C Vps/HOPS complex (Brown *et al.* 2003).

Unconventional protein secretion

Genetic screens in *S. cerevisiae* and *P. pastoris* have revealed that genes involved in autophagy and endosomal trafficking, as well as the phospholipase D Spo14, are essential for the

unconventional secretion of Acb1 into the extracellular milieu (Duran *et al.* 2010; Manjithaya *et al.* 2010a). Another factor required for this process is Grh1, the yeast homolog of the mammalian Golgi reassembly and stacking protein (GRASP), which has been implicated in various types of unconventional secretion (Nickel and Rabouille 2009). As discussed above, while the nature of the carrier transporting Acb1 remains to be determined, a precursor structure named the compartment for unconventional protein secretion (CUPS) has been characterized (Bruns *et al.* 2011). This organelle, in close proximity to the ER exit sites and onto which Acb1 is recruited upon nitrogen starvation, is positive for Grh1, Atg8, Atg9, and Vps23, one of the components of the endosomal sorting complex required for transport (ESCRT), as well as PtdIns3P. The formation of the CUPS, however, does not depend on the Atg proteins or Vps23. Consequently it remains to be established how these structures are generated.

Regulation

One of the main differences between autophagy in yeast relative to other eukaryotes concerns the signals that induce the process beyond its basal level. In yeast, nutrient withdrawal is the primary stimulus that induces autophagy, whereas in mammals, numerous cues can regulate this pathway. One point to consider is that regulation is likely complex, in part because excessive—as well as insufficient—autophagy would be deleterious for the cell. In addition, multiple types of signals depending on the nature of the limiting nutrients need to be coordinated, suggesting an intricate network of interactions among the regulatory components.

Nitrogen-dependent regulation

The TOR kinase is considered to be the primary sensor of nitrogen (and amino acids), and the main negative regulator of macroautophagy (Noda and Ohsumi 1998; Cutler *et al.* 1999). TOR can directly regulate macroautophagy through the phosphorylation of Atg proteins including Atg13. However, TOR also acts through a signaling cascade. Tap42 is a TOR effector that is in a complex with the type 2A protein phosphatase Pph21/Pph22. Overexpression of either Pph21 or Pph22 inhibits macroautophagy, whereas inactivation of a temperature-sensitive *tap42* mutant or overexpression of the Tap42 interacting protein Tip41 results in macroautophagy induction under nutrient-rich conditions (Yorimitsu *et al.* 2009). The target(s) of Tap42–Pph21/Pph22 with regard to macroautophagy regulation is not known. One example of the complexity of the regulatory network is seen with the Ksp1 kinase. Ksp1 appears to positively regulate TOR (Umekawa and Klionsky 2012), but it is also a target of TOR phosphorylation (Huber *et al.* 2009), which suggests either a feedback or stimulatory feed-forward type of regulation. Furthermore, PKA, which is considered to be primarily a glucose sensor, could act upstream of TOR by regulating Ksp1 activity (Umekawa and Klionsky 2012).

Glucose depletion

Yeasts have a complex pathway for sensing and responding to glucose levels (Zaman *et al.* 2008). Here, we highlight the information known about the glucose response as it pertains to macroautophagy. High levels of glucose result in the production of cAMP, which binds to, and inactivates, Bcy1, the regulatory subunit of PKA. As a consequence, PKA is activated and inhibits macroautophagy (Budovskaya *et al.* 2004). PKA directly phosphorylates Atg1 and Atg13, at sites that are distinct from those targeted by TOR, and this post-translational modification regulates the association of these proteins with the PAS (Budovskaya *et al.* 2005; Stephan *et al.* 2009). Sch9 is a second glucose sensor that acts in parallel with PKA. Sch9 kinase activity is partly dependent on phosphorylation by TOR, but this is independent of the Sch9 phosphorylation that occurs in the presence of glucose. Similar to PKA, inactivation of Sch9 induces macroautophagy (Yorimitsu *et al.* 2007; Stephan *et al.* 2009). This regulation is mediated in part through the Rim15 kinase (a positive regulator of macroautophagy) and the Msn2/Msn4 transcription factors. As in the absence of nitrogen, the depletion of glucose serves as a positive signal for macroautophagy induction. In this case, the Snf1 kinase is involved in regulation (Z. Wang *et al.* 2001), although the details have not yet been elucidated.

Amino acid and phosphate starvation

Macroautophagy can be induced by nitrogen depletion, and one source of nitrogen is amino acids. Indeed, amino acid depletion is another stress that triggers macroautophagy. The general control of nutrient (GCN) pathway regulates amino acid biosynthesis and also modulates macroautophagy. The Gcn2 kinase is involved in sensing the level of intracellular amino acids and, when activated, initiates a cascade resulting in the activation of the Gcn4 transcription factor (although Gcn4 may also be able to sense amino acid levels through a Gcn2-independent mechanism). One outcome of this signal transduction is the activation of genes involved in amino acid synthesis, but there may also be an increase in the transcription of specific *ATG* genes (Natarajan *et al.* 2001). Thus, active Gcn2 stimulates macroautophagy, as does Gcn4. Negative regulation occurs through the degradation of Gcn4, which is mediated by Pho85-dependent phosphorylation when Pho85 is in a complex with the Pcl5 cyclin.

Pho85 is a cyclin-dependent kinase that has both inhibitory and stimulatory roles in macroautophagy regulation, depending on the particular cyclin to which it is bound (*e.g.*, Pho80 or Clg1). Under conditions of high phosphate, the Pho85–Pho80 complex inhibits the Pho4 transcription factor that is needed to induce genes involved in the generation, uptake, and storage of phosphate. Pho85–Pho80 also inhibits the Rim15 kinase (Yang *et al.* 2010). Conversely, the Pho85–Clg1 complex inhibits the cyclin-dependent kinase inhibitor Sic1, resulting in an activation of Rim15.

Mitophagy

Organelles that are involved in degradative processes such as peroxisomes, which carry out β-oxidation, or mitochondria, which utilize an electron transport chain, are prone to generating reactive oxygen species. Accordingly, these compartments need to be constantly repaired or degraded to prevent additional damage to the organelle or to the remainder of the cell. Maintaining organelle homeostasis is costly, and as a result, organelles that are superfluous, as well as those that are damaged, are subjected to selective degradation. When yeast grow on nonfermentable carbon sources such as glycerol or lactate, the mitochondria proliferate. A subsequent shift to glucose, particularly in medium lacking nitrogen, results in the selective degradation of a portion of the mitochondrial population through mitophagy. Growth of a yeast culture in a nonfermentable carbon source past the logarithmic phase can also induce mitophagy, through a mechanism that does not completely overlap with that stimulated by glucose in combination with nitrogen starvation. In contrast to the autophagic machinery where many components have been identified, however, relatively little is known about the proteins involved in regulating selective autophagy.

Both starvation-dependent and post-logarithmic phase-induced mitophagy are controlled in part through two separate mitogen-activated protein kinase (MAPK) pathways. The *bck1*Δ mutant was identified in a screen for mitophagy-defective strains (Kanki *et al.* 2009a). Bck1 is a MAPK kinase kinase, and analysis of both upstream and downstream kinases demonstrated that Pkc1, Bck1, Mkk1/Mkk2, and Slt2 are all required for mitophagy, along with the cell surface sensor Wsc1 (Mao *et al.* 2011). Slt2 acts at an early stage of mitophagy induction, relative to the second MAPK, Hog1 (Mao *et al.* 2011). Mitophagy is also regulated by a cell surface sensor, Sln1, along with a two-component signal transducer that includes Ssk1 and the MAPK kinase Pbs2, both of which act upstream of Hog1 (Mao *et al.* 2011). Downstream targets for Slt2 and Hog1 that are involved in mitophagy have not been identified. Although these MAPKs target certain transcription factors, they may have other targets for controlling mitophagy, since both proteins appear to remain in the cytosol under mitophagy-inducing conditions (Mao *et al.* 2011). Finally, the regulation of mitophagy in yeasts is likely to be somewhat distinct from the mechanism (s) used in higher eukaryotes. For example, CCCP or poisons that interfere with the electron transport chain do not appear to be strong inducers of mitophagy in yeast, compared to mammalian cells. Such differences may reflect the fact that yeast have evolved to prefer fermentation to respiration, and unlike some mammalian cells, they can dilute out damaged or superfluous organelles by division.

Other factors also control selective mitochondria degradation. For example, starvation-dependent mitophagy is delayed in the absence of Uth1 (Kissova *et al.* 2004). In addition to Atg33, post-log-phase mitophagy is regulated by Aup1, a phosphatase that localizes to the mitochondrial intermembrane space (Tal *et al.* 2007). Aup1 function is mediated at least in part through its effect on the phosphorylation of Rtg3, a transcription factor that is a component of the retrograde signaling pathway, which is also required for post-log phase mitophagy (Journo *et al.* 2009).

Pexophagy

S. cerevisiae has evolved to grow on glucose as its preferred carbon source. Under standard laboratory conditions, peroxisomes are not abundant in this yeast. If forced to grow on oleic acid, however, peroxisomes proliferate because this is the only organelle in this organism that can carry out β--oxidation. A subsequent shift to glucose or to a medium lacking nitrogen results in the rapid and selective degradation of peroxisomes (Hutchins *et al.* 1999). Methylotrophic yeasts including *P. pastoris*, *H. polymorpha*, and *Yarrowia lipolytica* also synthesize a peroxisomal alcohol oxidase that is able to utilize methanol. When *P. pastoris* cells are shifted from methanol to glucose, micropexophagy is induced, whereas growth on ethanol results in elimination of the excess organelles through macropexophagy (Tuttle and Dunn 1995). This response, however, appears to directly correlate with ATP levels, rather than the actual carbon source, with higher ATP leading to micropexophagy (Ano *et al.* 2005a). The nutritional control of pexophagy is complex and varies depending on the specific organism and carbon source. For example, in contrast to *P. pastoris*, *H. polymorpha* induces macropexophagy when shifted from methanol to glucose (Till *et al.* 2012; van Zutphen *et al.* 2008).

Similar to mitophagy, pexophagy is regulated by the Slt2 pathway (Manjithaya *et al.* 2010b; Mao *et al.* 2011). In contrast, the Hog1 pathway does not appear to be involved in controlling this process (Mao *et al.* 2011).

Transcriptional control

Considering that the amount of Atg8 protein changes substantially upon autophagy induction, displaying as much as a 40-fold increase, and that *ATG8* mRNA shows a similar rapid upregulation within 30 min after shifting to starvation conditions (Kirisako *et al.* 1999), transcriptional control is an obvious component of autophagy regulation. The Ume6 transcription factor is part of a large complex that includes the Sin3 corepressor and the Rpd3 histone deacetylase. Deletion of any of the corresponding genes results in a dramatic increase in the amount of Atg8 prior to macroautophagy induction (Bartholomew *et al.* 2012). The *ATG8* promoter contains a consensus DNA binding sequence for Ume6. This protein is phosphorylated under nitrogen starvation conditions, and this modification is largely blocked in the absence of Rim15. As noted above, the Rim15 kinase appears to be regulated by several kinase sensors that act upstream primarily in inhibitory pathways. These findings support a model in which the nutrient-sensing kinases such as PKA inactivate Rim15 during nutrient-rich conditions, allowing active Ume6 to downregulate the synthesis of Atg8. The

lower level of this protein is sufficient to facilitate the formation of the smaller Cvt vesicles under growing conditions. When nutrients are depleted, the sensing kinases are no longer active, alleviating the suppression of Rim15 function, which in turn results in the phosphorylation and inhibition of Ume6. The subsequent increase in transcription of *ATG8* results in an increase in the Atg8 protein, allowing the formation of the larger autophagosome.

It is likely that transcriptional control is involved in the regulation of many additional *ATG* genes, but this has not yet been extensively explored. For example, *ATG14* transcription is regulated by the Gln3 transcription factor (Chan *et al.* 2001). Under conditions of nitrogen starvation, *ATG14* transcript levels increase more than 20-fold in a Gln3-dependent manner. Similarly, deletion of *URE2*, which encodes a negative regulator of Gln3, leads to constitutive expression of *ATG14* at a level similar to that seen with rapamycin treatment (Chan *et al.* 2001). Thus, the TOR pathway, which regulates the phosphorylation of Ure2 and the nuclear localization of Gln3, is also involved in regulation of macroautophagy via transcriptional control.

Inositols

Phosphoinositides, such as PtdIns3P, play a role in recruiting Atg proteins to the PAS and possibly modulating the activity of some of them, and thus might be considered to have a regulatory function in macroautophagy. Another type of inositol-containing macromolecule, the inositol polyphosphates, are also involved in controlling this process, although the mechanism remains to be elucidated. A screen of enzymes involved in inositol polyphosphate synthesis revealed a role for Ipk2 and Kcs1 in macroautophagy (Taylor *et al.* 2012). The phenotype of the *kcs1*Δ strain is consistent with a defect in autophagosome formation, which may reflect a failure to correctly localize PtdIns3P, and consequently Atg18, and/or generate PtdIns4P under macroautophagy-inducing conditions.

Conclusions

Autophagy, in all of its various modes, is a complex process devoted mostly to intracellular degradation. Considering that a characterization of the first *ATG* gene was published in 1997, our molecular understanding of autophagy has expanded tremendously in a relatively short period of time. Nonetheless, many fundamental questions remain to be answered. These include the identification of the membrane(s) used to generate the phagophore (along with a characterization of the machinery used to target the membrane into the macroautophagy pathway), the mechanism of phagophore formation and expansion (including the role of the PAS), the function of most of the Atg proteins, and the identification and characterization of additional regulatory elements that modulate the process, and the enzymes and permeases involved in the degradation and efflux of the vacuolar breakdown products. A more complete understanding of autophagy in yeast is likely to lead to additional breakthroughs in the analysis of this process in other organisms and holds the promise for advances that can lead to therapeutic uses for manipulating autophagy to treat disease.

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NOVEL SYSTEM FOR MONITORING AUTOPHAGY IN THE YEAST *Saccharomyces cerevisiae*

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The yeast *S. cerevisiae* imports cytosolic components into the vacuole non-selectively by autophagy and degrades them by vacuolar hydrolases under nutrient starvation conditions. We developed a novel system for monitoring autophagy by constructing cells in which modified vacuolar alkaline phosphatase is expressed as an inactive precursor form in the cytosol. Under starvation conditions, the processing of the precursor to the mature form and phosphatase activity appeared gradually, and the mature form was located in the vacuole. Disruption of *APG1*, an essential gene for autophagy, resulted in no processing or phosphatase activity. These results indicate that the precursor form in the cytosol is transferred to the vacuole by autophagy and converted to the active form by vacuolar proteinases. Thus, autophagy could be determined easily and accurately by measuring the phosphatase activity.

Under starvation conditions, cytosolic proteins are degraded mainly by autophagy in lysosomes of mammalian cells [2, 18]. Autophagy is associated with dynamic movement and interaction of intracellular membranes, indicating that there must be numbers of molecules involved in the process. However, the studies of autophagy in mammalian or plant cells remain mainly at morphological level. Further biochemical and genetic analyses will be necessary for understanding this complex and dynamic membrane interaction and underlying molecular mechanism involved in the autophagy.

In the yeast *S. cerevisiae*, the vacuole is equivalent to the lysosome in mammalian cell [11]. The vacuole is equipped with numbers of hydrolases and its lumen is acidic, so it functions as a lytic compartment. Previously we reported that autophagy in yeast involves dynamic changes of the membranes [1, 22]. Starvation induces formation of autophagosomes, which consist of a double membrane enclosing a portion of the cytosol non-selectively. The outer membrane of the autophagosome fuses with the vacuolar membrane and the resulting inner single membrane structure, the autophagic body, is delivered to the vacuole, where it is rapidly disintegrated depending on vacuolar proteinases, and then its cytosolic contents are degraded by vacuolar hydrolases.

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Abbreviations: ADH, alcoholdehydrogenase; ALP, alkaline phosphatase; CPY, carboxypeptidase Y; NPP, nitro-phenyl-phosphate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl.

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Since autophagic import of the cytosolic components into the vacuole is non-selective and no specific marker is available, monitoring of autophagy has been carried out by a combination of indirect ways such as measuring overall protein degradation, decrease of the long-lived cytosolic proteins, and detection of cytosolic proteins in the vacuolar fraction [3, 22, 25]. These methods, however, require a lot of process that prevent quantitative analysis on the autophagy, especially at the early stage of this process. We have developed a novel system for simple and sensitive monitoring of autophagy.

Materials and Methods

Strains, media for yeasts and bacteria . The yeast strains used were as follows: YW5-1B (*MAT***a** *leu2-3,112 trp1 ura3-52*); TN121 (*MAT***a** *leu2-3,112 trp1 ura3-52 pho8::pho8Δ60 pho13::URA3*); TN122 (*MAT***a** *leu2-3,112 trp1 ura3-52 pho8::pho8Δ60 pho13::URA3 prb1::TRP1*); TN123 (*MAT***a** *leu2-3,112 trp1 ura3-52 pho8::pho8Δ60 pho13::URA3 apg1::LEU2*), and TN124 (*MAT***a** *leu2-3,112 trp1 ura3-52 pho8::pho8Δ60 pho13::LEU2*). Bacterial strains were grown in standard media [17]. Yeast cells were grown in YPD, minimal essential medium SD, nitrogen starvation medium S(-N)D, carbon starvation medium S(-C), nitrogen and carbon starvation medium S(-N) or sulfate starvation medium S(-S)D as described previously [22].

Construction of plasmids and yeast strains. Manipulations of *E. coli* cells and DNAs were done essentially based on Sambrook et al. [17]. Plasmid pAL145 [7], which contains 4.4 kbp of *PHO8* region in the *Bam*HI site of pBR322 with *Bam*HI linker, was a gift from Dr. Kaneko of Osaka University. A *Kpn*I site was introduced at 181 bp downstream of the translational initiation codon ATG for *PHO8* by the PCR using a set of primers 5'-TCAGGTACCTCTGCATCACAC-AAGAAG-3', 5'-TTTGTCCTCATTGGAAGCT-T-3', and pAL145 as a template. The PCR product was subcloned into the *Kpn*I/*Hin*dIII sites of pBluescript II SK+ (Stratagene, CA) to generate pTN1. Plasmid pTN2, which contains the *pho8Δ60* lacking the region encoding the N-terminal and transmembrane segments of Pho8p, was constructed by subcloning of 0.8 kbp of *Bam*HI/*Hin*dIII fragment of pAL145 encoding the C-terminal and 3'-untranslated region of *PHO8* into the *Bam*HI/*Hin*dIII sites of pTN1. The *Kpn*I-*Kpn*I fragment (2.5 kbp) of pTN2 was inserted into the *Kpn*I site of pKT10 [21] to connect the *TDH3* promoter. The resulting plasmid, pTN3, was digested with *Bam*HI and *Sal*I, and 2.0 kbp fragment was subcloned into pRS306 [19] to obtain pTN7. Plasmid pTN9 was constructed by ligating the *Bam*HI-*Hin*dIII fill-in fragment (0.8 kbp) of 5' region of the *PHO8* gene in pAL145 into the *Bam*HI site in pTN7 with *Bam*HI linker. Plasmid pST100 contains disrupted *PRB1* gene with the *TRP1* gene [22]. Plasmid pYAP116 contains a disrupted *APG1* gene with the *LEU2* gene (Matsuura et al, manuscript in preparation). Plasmid pTN23, which contains *Eco*RI-*Hin*dIII (1.5 kbp) fragment of *PHO13* gene from pAL55 [9] in pUC119, was partially digested with *Pst*I, and 4.2 kbp of *Pst*I-*Pst*I fragment of the *LEU2* gene from YEp13 was inserted at the *PHO13* region to generate pTN24. Plasmids pAL55 and pPH13 were gifts from Dr. Harashima of Osaka University, and pKT10 was a gift from Dr. Toh-e of University of Tokyo. Yeast strain TN109 was generated by integrating pTN9 into the *PHO8* locus of wild-type strain YW5-1B, and the wild type *PHO8* gene was replaced with the *pho8Δ60* gene by counterselection of Ura$^-$ using 5-fluoro-orotic acid. TN121 and TN124 were generated by integrating pPH13 [9] and pTN24 into the *PHO13* locus of TN109 respectively. TN122 and TN123 were generated by integrating pST100 into the *PRB1* locus and pYAP116 into the *APG1* locus of TN121 respectively.

Preparation of antiserum of Pho8p. A *lacZ-PHO8* fusion gene was constructed by inserting a *Bgl*II-*Sal*I fragment of the *PHO8* gene, which does not contain C-terminal pro-region, into pUR278 [16], and was used to transform *E. coli* strain XL1-blue (Stratagene CA). The fusion protein produced was purified by SDS-PAGE and electro-elution. A rabbit was immunized with the purified fusion protein by Shibayagi (Gunma, Japan). The antiserum was purified on a cyanogen bromide activated Sepharose column coupled with the GST-Pho8p fusion protein. GST-Pho8p fusion protein was obtained by the similar procedure as described above, except for use of the *GST-PHO8* fusion gene in the pGEX-3X [5].

Subcellular fractionation, immunoblotting and alkaline phosphatase assay. Subcellular fractionation was carried out by the method of Herman and Emr [4]. Vacuoles were prepared as described before [22] except for incubation in S(-N) medium as starvation medium. SDS-PAGE was carried out by Laemmli's method [14] and the proteins separated were subjected to conventional immunoblotting analysis. Anti-Vma1p antibody was kindly provided by Dr. Anraku of University of Tokyo. Anti-ADH and anti-CPY sera were obtained as described elsewhere [1, 20] in collaboration with Dr. Nakano of University of Tokyo. Protein was determined with a BCA protein assay kit (Pierce, Ill). A yeast lysate was prepared by disrupting the cells with glass beads in ice-cold reaction buffer (50 mM Tris-HCl pH 9.0, 5 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml pepstatin A (Sigma, MI)) and removed the cell debris by centrifugation at 10,000 x g for 5 min. Alkaline phosphatase in the lysate was assayed with p-NPP (Wako, Japan) as a substrate by the method of Torriani [23] in the presence of 1 mM PMSF and 1 μg/ml pepstatin A. One micromole of p-nitro-phenol gives 11.7 units of ΔA_{420}.

Results and Discussion

The structure of Pho8p, vacuolar alkaline phosphatase, is as follows from the N-terminus: a cytosolic tail, a membrane spanning region, a catalytic region, and a pro-region at the C-terminus (Fig. 1) [10]. The analysis on various hybrid proteins of Pho8p and Suc2p showed that the membrane spanning region acts as an uncleavable signal sequence for translocation into the ER membrane and is necessary and sufficient for delivery of Pho8p to the vacuole [12]. Taking advantages of this structural and functional characteristics of Pho8p, for monitoring autophagy, we constructed a gene encoding a truncated form of Pho8p, Pho8Δ60p, which lacks 60 amino acid residues at the N-terminus including the membrane spanning region (Fig. 1). The modified version of Pho8p lacking the membrane spanning region should remain in the cytosol. The *PHO8* gene in wild-type cells (YW5-1B) was replaced with *pho8Δ60* connected under a strong promoter of *TDH3* gene, a gene for glyceraldehyde 3-phosphate dehydrogenase [15]. Disruption of the *PHO8* gene did not affect the accumulation and degradation of autophagic bodies or the growth phenotype (Shirahama and Ohsumi, unpublished observation). We examined the subcellular localization of

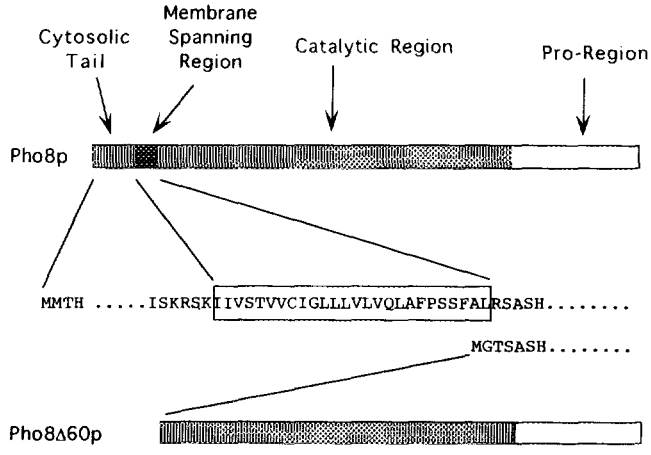


Fig. 1. Structure of Pho8Δ60p. Pho8Δ60p is truncated Pho8p deleted of 60 amino acid residues at the NH_2-terminus, including the membrane spanning region (box).

Pho8Δ60p. Affinity-purified antiserum against Pho8p recognized only Pho8p by immunoblotting (data not shown). A lysate of TN124 cells (*pho8::pho8Δ60*) grown in YPD, nutrient-rich medium, was subjected to centrifugation and proteins of the pellet and the supernatant were subjected to immunoblotting. An authentic Pho8p was localized within the membrane fraction (data not shown). While, Pho8Δ60p was detected in the supernatant fraction with the cytosolic enzyme alcoholdehydrogenase, but not in the pellet fraction with vacuolar H^+-ATPase (Fig. 2A). This result showed that Pho8Δ60p is expressed and remained at the cytosol.

On arrival at the vacuole via the secretory pathway, authentic Pho8p is converted to the mature form with a half time of about 6 min [10] as the result of cleavage of the pro-region at the C-terminus depending on the activity of proteinase A [6, 8, 10]. TN124 cells (*pho8Δ60*) grown in YPD were transferred to nitrogen starvation medium and the lysate was subjected to immunoblotting with anti-Pho8p serum. When grown in YPD, the Pho8Δ60p was detected at approximately 64 kDa, which corresponds to the size deduced from the total length of its open reading frame (Fig. 2B lane 1). When the cells were subjected to nitrogen starvation, a novel band appeared at approximately 59 kDa, and gradually increased in amount during prolonged incubation (Fig. 2B lanes 2 to 5). As the size of the pro-region of Pho8p is about 4 kDa, this processing of Pho8Δ60p coincided with cleavage of the C-terminal pro-region.

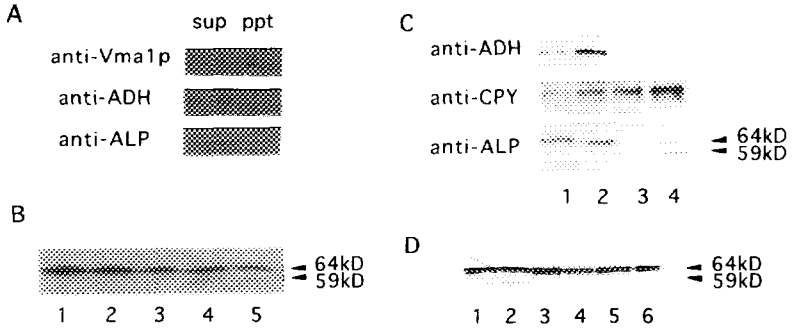
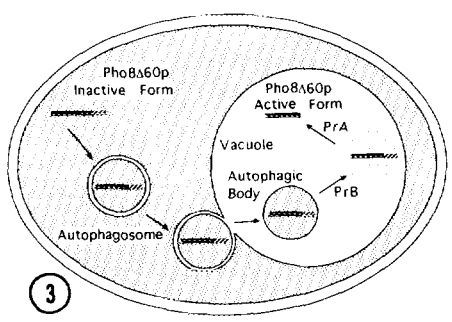


Fig. 2. A. Localization of Pho8Δ60p. TN124 (*pho8Δ60*) cells were grown in YPD medium and disrupted for subcellular fractionation. Sup and ppt indicate the supernatant and pellet fraction, respectively, obtained by centrifugation at 100,000 x g for 30 minutes. Fractions equivalent to the same numbers of the cells were loaded on 10% SDS-PAGE gel and immunoblotted with each antiserum. B. Processing of Pho8Δ60p under starvation conditions. TN124 (*pho8Δ60*) cells were grown in YPD and transferred to S(-N)D. At intervals during incubation, the cells were collected, disrupted and subjected to immunoblotting with anti-Pho8p serum. Samples of 20 μg of protein were loaded in each lane. Lanes 1, 2, 3, 4 and 5 are samples incubated in S(-N)D for 0, 1, 3, 5 and 7 hours, respectively. C. Localization of processed Pho8Δ60p. TN124 (*pho8Δ60*) cells were grown in YPD to a density of 2 x 10^7 cells/ml and transferred to S(-N). Vacuoles were prepared as described in Materials and methods. Samples of 10 μg of protein of each fraction were subjected to immunoblotting with anti-CPY or anti-ALP serum and samples of 0.5 μg of protein with anti-ADH serum. Lanes 1 and 2, spheroplast lysates; lanes 3 and 4, vacuolar fraction; lanes 1 and 3, starvation for 0 hour; lanes 2 and 4, starvation for 4.5 hours. D. TN124 (*pho8Δ60*) , TN122 (*pho8Δ60 Δprb1*) and TN123 (*pho8Δ60 Δapg1*) cells were grown in YPD, transferred to S(-N)D and subjected to immunoblotting with anti-Pho8p serum. Samples of 20 μg of protein were loaded in each lane. Lanes 1 and 2, TN124 (*pho8Δ60*) ; lanes 3 and 4, TN122 (*pho8Δ60 Δprb1*); lanes 5 and 6, TN123 (*pho8Δ60 Δapg1*). Lanes 1, 3 and 5, 0 hour; lanes 2, 4 and 6, 7 hours in S(-N)D.

The intracellular localization of the processed Pho8Δ60p was examined. Pho8Δ60p was not detected in the vacuolar fraction of cells grown in YPD (Fig. 2C). After incubation in starvation medium for 4.5 hours, processed Pho8Δ60p was concentrated in the vacuolar fraction as well as carboxypeptidase Y (Fig. 2C), and most of it was resistant to trypsin treatment (data not shown), indicating that it was located within the vacuole. Proteinase B, encoded by the *PRB1* gene, is localized in the vacuole and necessary for disintegration of the autophagic body [22]. Even after incubation of disruptant of *PRB1* for 7 hours in the nitrogen starvation medium, no processing of Pho8Δ60p occurred (Fig. 2D lane 4). These results indicate that the processing of Pho8Δ60p in starvation conditions is resulted from its delivery to the vacuole, and did not occur outside the vacuole. The *APG1* gene is shown to be essential for autophagy and a disruptant of the *APG1* gene does not accumulate autophagic bodies in the vacuole (24, Matsuura et al, manuscript in preparation). After nitrogen starvation for 7 hours, no processed Pho8Δ60p appeared in *Δapg1* cells (Fig. 2D). This finding indicates that delivery of Pho8Δ60p from the cytosol to the vacuole depends on autophagy (Fig. 3).

Pho8p acquires phosphatase activity when its pro-region is cleaved off in the vacuole. Besides Pho8p, wild-type cells possess Pho13p, a cytosolic alkaline phosphatase that is not essential for vegetative growth [9]. To measure the phosphatase activity derived only from Pho8Δ60p, we disrupted the *PHO13* gene. As shown in Fig. 4A, TN121 cells (*pho8::pho8Δ60 pho13::URA3*) grown in YPD showed negligible phosphatase activity, indicating that non-processed Pho8Δ60p, like authentic Pho8p, does not have phosphatase activity. While, the activity appeared after a lag period of 1 hour and increased during incubation in nitrogen-starvation medium for 7 hours. Furthermore, this induction of phosphatase activity was not seen in *Δapg1* or



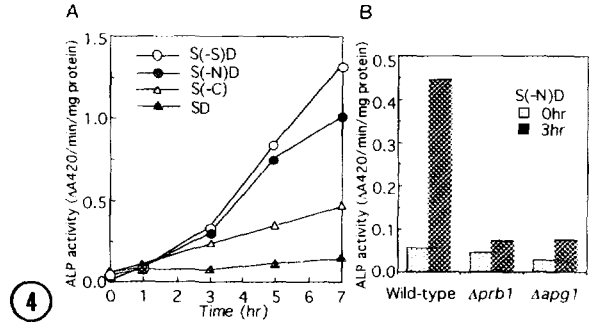


Fig. 3. Schematic drawing of import of Pho8Δ60p into the vacuole by the autophagic pathway. Under nutrient starvation conditions, an autophagosome enclosing Pho8Δ60p in the cytosol fuses with the vacuole. The resulting single membrane structure of the autophagic body is disintegrated depending on vacuolar proteinases. Pho8Δ60p is processed by vacuolar proteinases to acquire phosphatase activity. PrA, proteinase A; PrB, proteinase B.

Fig. 4. Induction of phosphatase activity of Pho8Δ60p under starvation conditions. A. TN121 (*pho8Δ60*) cells were grown in YPD medium and transferred to each medium at 0 hour. After indicated times of incubation, cells were lysed and pNPPase activity was measured as described in Materials and methods. B. Cells of TN121 (*pho8Δ60*), 122 (*pho8Δ60 Δprb1*) and 123 (*pho8Δ60 Δapg1*) were grown in YPD and transferred to S(-N)D. After incubation for 0 or 3 hours, cells were lysed and alkaline phosphatase activity was measured as described in Materials and methods.

Δprb1 cells (Fig. 4B), consistent with the finding that processing of Pho8Δ60p did not occur in these cells. Phosphatase activity emerged also under carbon starvation and sulfate starvation (Fig. 4A). We also observed accumulation of autophagic bodies in the vacuole under these starvation conditions [22]. From these results, we concluded that this phosphatase activity reflects autophagy-dependent processing of Pho8Δ60p.

During nitrogen starvation for up to 7 hours, the amount of the precursor form of Pho8Δ60p did not change significantly in *Δapg1* cells, as determined by immunoblotting (Fig. 2D). In these cells, Pho8Δ60p was not imported into the vacuole, and so its level in the cytosol remains constant during nitrogen starvation. Furthermore, processed Pho8Δ60p was stable in the vacuole, because the phosphatase activity induced under nitrogen starvation remained constant after further incubation in a medium supplemented with ammonium sulfate to stop the autophagy (data not shown). From these results, we concluded that the phosphatase activity shows the extent of autophagy under nitrogen starvation.

There have been several attempts to monitor autophagy so far. One method used was measurement of the radioactivity of amino acids released on degradation of pre-labeled protein [25]. However, by this method, it is hard to distinguish protein degradation by autophagy from the degradation by the other systems. Moreover, released amino acids may be reutilized for synthesis of proteins, which results in under-estimation of the degradation. Another method used was measurement of the decrease of long-lived proteins in the cytosol by immunoblotting [3]. However, this method is not sensitive enough to detect decrease by a few per cent in the total amount of protein in the initial phase of autophagy. There are also reports of quantitation of autophagy in mammalian or yeast cells by subcellular fractionation of autophagolysosomes and measurement of the cytosolic marker [3, 13]. However, this method of analysis involves quite many steps. In contrast, the system presented here allows not only sensitive but simple analysis of autophagy even in the early phase of autophagy.

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A protein conjugation system essential for autophagy

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Autophagy is a process for the bulk degradation of proteins, in which cytoplasmic components of the cell are enclosed by double-membrane structures known as autophagosomes for delivery to lysosomes or vacuoles for degradation[1–4]. This process is crucial for survival during starvation and cell differentiation. No molecules have been identified that are involved in autophagy in higher eukaryotes. We have isolated 14 autophagy-defective (*apg*) mutants of the yeast *Saccharomyces cerevisiae*[5] and examined the autophagic process at the molecular level[6–9]. We show here that a unique covalent-modification system is essential for autophagy to occur. The carboxy-terminal glycine residue of Apg12, a 186-amino-acid protein, is conjugated to a lysine at residue 149 of Apg5, a 294-amino-acid protein. Of the *apg* mutants, we found that *apg7* and *apg10* were unable to form an Apg5/Apg12 conjugate. By cloning *APG7*, we discovered that Apg7 is a ubiquitin-E1-like enzyme. This conjugation can be reconstituted *in vitro* and depends on ATP. To our knowledge, this is the first report of a protein unrelated to ubiquitin that uses a ubiquitination-like conjugation system. Furthermore, Apg5 and Apg12 have mammalian homologues, suggesting that this new modification system is conserved from yeast to mammalian cells.

In yeast, autophagy is induced by various starvation conditions, and its progression is easily monitored under a light microscope[1]: when wild-type cells were cultured under nitrogen-starvation conditions in the presence of phenylmethylsulphonyl fluoride (PMSF), autophagic bodies accumulated in the vacuoles (arrows in Fig. 1a). The *apg12-1* mutant did not accumulate autophagic bodies during starvation. We cloned the *APG12* gene by the method described previously[7,8]. *APG12* encodes a hydrophilic protein of 186 amino acids with a predicted relative molecular mass (M_r) of 21K (Fig. 1b).

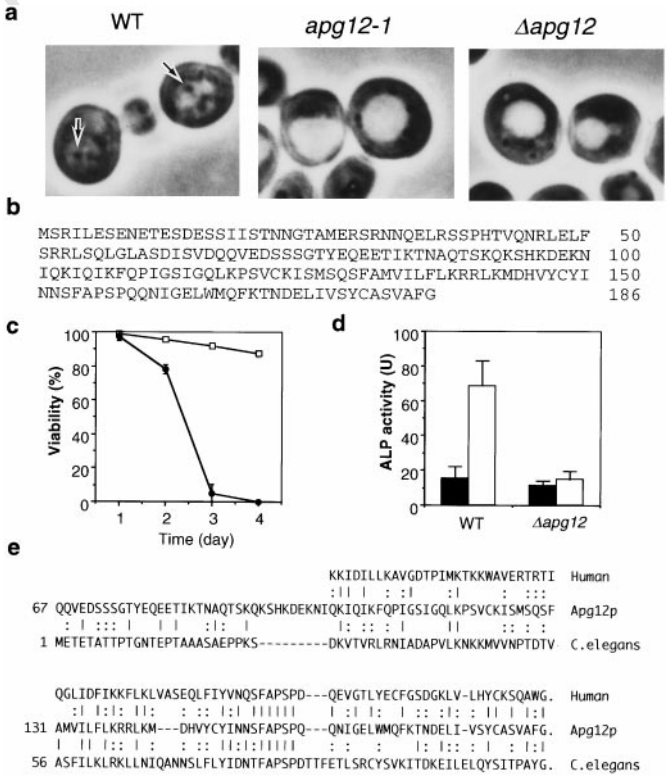

Figure 1 Cloning of *APG12* and phenotype of *apg12* disruptant. **a**, Wild-type, *apg12-1* mutant and Δ*apg12* cells were cultured in nitrogen-starvation medium containing 1 mM PMSF. After incubation for 6 h, cells were observed under a phase-contrast microscope. Arrows indicate autophagic bodies. **b**, Amino-acid sequence of Apg12. **c**, Wild-type (squares) and Δ*apg12* (circles) were cultured in nitrogen-starvation medium and their viability was determined by phloxine B staining[5]. **d**, Quantification of autophagic activity of wild-type and Δ*apg12* cells by alkaline phosphatase (ALP) assay before (black bars) and after (white bars) nitrogen starvation for 4 h. Error bars indicate s.d. of three independent experiments. **e**, Homology between Apg12 and potential human and *C. elegans* counterparts. *C. elegans* U32305 is 46% similar and 22% identical to amino acids 67–186 of yeast Apg12. A human cDNA (THC173313) encodes a protein that is 59% similar and 32% identical to amino acids 102–186 of Apg12.

Gene disruption experiments revealed that *APG12* is not essential for growth (data not shown) but is essential for autophagy (Fig. 1a) and for maintaining viability during starvation (Fig. 1c). We confirmed this in an assay system for measuring autophagic activity (Fig. 1d), in which a truncated form of pro-alkaline phosphatase expressed in the cytoplasm was delivered to vacuoles in an autophagy-dependent manner and processed to the active enzyme[10]. A vacuolar enzyme, aminopeptidase I, is delivered from the cytoplasm to vacuoles constitutively to yield the mature, active enzyme[11]. This 'Cvt pathway' is closely linked to the autophagic process[12], and all *apg* mutants[13], including Δ*apg12* cells, show defects in this pathway (see Fig. 3d). The amino-acid sequence of Apg12 did not provide any insight into its function, but a BLAST search identified a potential *Caenorhabditis elegans* homologue whose function is unknown (Fig. 1e). In addition, a search of the EST (expressed-sequence tag) database identified several cDNA fragments encoding parts of a potential human homologue (Fig. 1e).

To detect Apg12, we constructed a 3 × haemagglutinin(HA)-tagged APG12. On immunoblotting, Apg12 presented as a ladder of bands between 31K–32.5K (Fig. 2a). As phosphatase treatment of the lysate yielded a single band at 31K representing tagged Apg12 (data not shown), we concluded that Apg12 is phosphorylated *in vivo*. Furthermore, we found that about half of the Apg12 was present as a much larger band of ~70K (asterisked in Fig. 2a, b). Although the 31K Apg12 was detected in all *apg* mutant strains, the Δ*apg5*, *apg7-1* and *apg10-1* strains did not show the 70K band (Fig. 2b; Δ*apg1* is representative of the other mutants). These results indicate that these three *APG* products are essential for the generation of the 70K band.

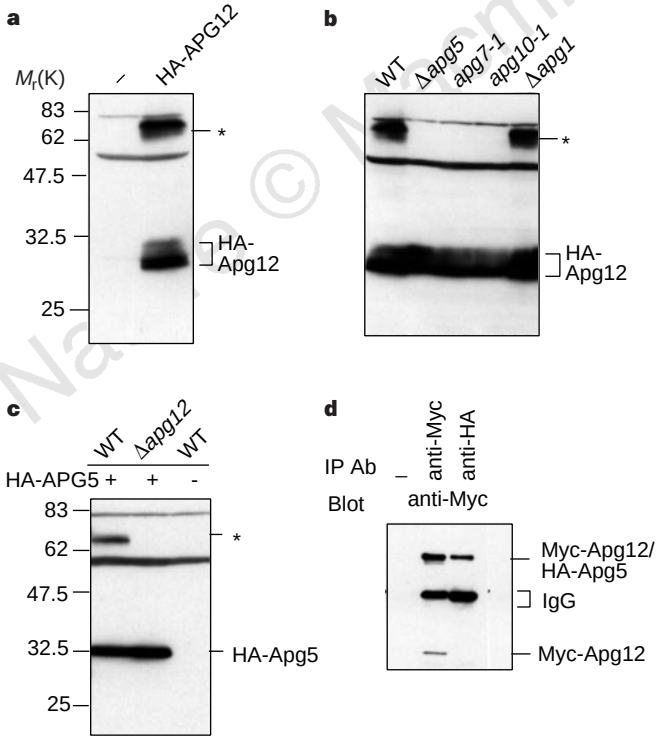

Figure 2 Apg12 is conjugated to Apg5. **a**, **b**, Lysates from Δ*apg12* cells carrying only vector or 3 × HA-APG12 (**a**), and Δ*apg5*, *apg7-1*, *apg10-1* and Δ*apg1* cells carrying 3 × HA-Apg12 plasmid (**b**) were immunoblotted using anti-HA antibody. The positions of 3 × HA-Apg12 and the larger product (asterisks) are indicated. **c**, Immunoblot analysis of wild-type and Δ*apg12* cells harbouring HA-APG5 plasmid. **d**, Δ*apg5* Δ*apg12* cells were co-transformed with Myc-APG12 and HA-APG5. Their lysates were immunoprecipitated with anti-Myc or anti-HA antibodies and detected by immunoblotting using anti-Myc antibody. The position of the crossreacting IgG heavy chain is indicated.

We have previously shown that the *APG5* gene encodes a 294-amino-acid protein[6]. Immunoblot analysis of 1 × HA-tagged Apg5 indicated that it also generated two bands in nearly equal amounts, one of the size of tagged Apg5 (32.5K) and the other at about 70K (Fig. 2c). In the Δ*apg12* strain, the higher band was not seen, whereas the 32.5K band of Apg5 was slightly increased (Fig. 2c). Immunoprecipitation analysis revealed that the 70K band included both Apg5 and Apg12 (Fig. 2d). We concluded that it was a one-to-one conjugate of Apg5 and Apg12.

To characterize the 70K band further, we did mutagenic analysis of Apg12 (Fig. 3a). We found that the carboxy-terminal portion of Apg12 was important for the conjugation (Fig. 3b: Δ57 and Δ121).

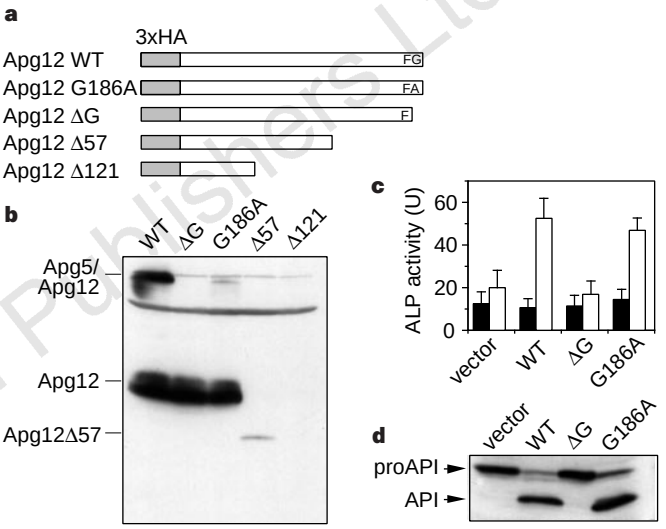

Figure 3 The C-terminal Gly residue of Apg12 is essential for interaction with Apg5 and for autophagy. **a**, Diagram of Apg12 C-terminal mutants. **b**, Δ*apg12* cells were transformed with the mutant plasmids and their lysates were immunoblotted with anti-HA antibody. **c**, Autophagic activity was measured as described for Fig. 1d. **d**, Transport of pro-API to the vacuole was examined by immunoblotting with anti-API antiserum. The positions of pro-API and mature API are indicated.

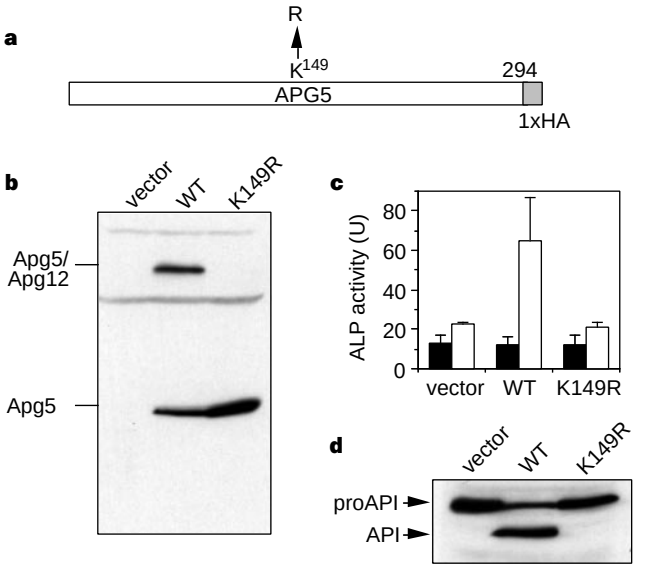

Figure 4 Apg5^{K149R} is unable to generate Apg5/Apg12 conjugate and is defective in autophagy. **a**, Position of the putative Apg12-interacting Lys residue. Δ*apg5* cells were transformed with vector alone, wild-type APG5 or APG5^{K149R}, and then immunoblotted with anti-HA (**b**) and anti-API (**d**). Autophagic activity was determined by alkaline phosphatase assay (**c**).

Even a single Gly 186 deletion at the C terminus (Apg12ΔG) caused complete loss of the Apg12/Apg5 conjugate, although free Apg12ΔG was detected in an amount comparable to that in the wild type (Fig. 3b: ΔG). $Apg12^{G186A}$, in which the Gly 186 is replaced by alanine, was incorporated into the higher band inefficiently, but still significantly (Fig. 3b: G186A). This indicates that Gly 186 is important for Apg5/Apg12 conjugation. We next assessed the functional activities of these mutants. Apg12ΔG showed an Apg-negative phenotype (Fig. 3c), and was also unable to produce mature aminopeptidase I (Fig. 3d), indicating that the Apg5/Apg12 conjugate is required for both autophagy and cytosol-to-vacuole targeting of this enzyme. The $Apg12^{G186A}$ mutant showed an almost normal phenotype for autophagy and for maturation of aminopeptidase I (Fig. 3c, d), suggesting that a small amount of Agp5/Apg12 conjugate is enough for it to function normally.

By analogy with ubiquitin[14–16], conjugation of Apg5 and Apg12 probably occurs through formation of an isopeptide bond between the C-terminal Gly 186 of Apg12 and an ϵ-amino group of one of the 19 lysine residues in Apg5. To test this, we systematically replaced each lysine residue of Apg5 with arginine. Both free Apg5 and the Apg5/Apg12 conjugate were detected in 18 mutants (data not shown). The $Apg5^{K149R}$ variant had no conjugate at all, but a higher amount of free $Apg5^{K149R}$ (Fig. 4a, b), indicating that the Lys 149 residue of Apg5 is the acceptor site for Apg12 conjugation. As expected, $Apg5^{K149R}$ was defective in both autophagy and in generating mature aminopeptidase I (Fig. 4c, d), whereas the other 18 mutants were normal (data not shown). Starvation did not alter the relative amounts of free Apg5, free Apg12 or of the Apg5/Apg12 conjugate. We conclude that the conjugate functions as a common machinery in both pathways: for the autophagic pathway during starvation and for the Cvt pathway in the growing phase.

As shown in Fig. 2, the *apg7* and *apg10* mutants failed to conjugate Apg5 and Apg12, suggesting that these two *APG* products may function as an enzyme system for conjugation. Cloning of the *APG7* gene revealed that it encodes a 630-amino-acid protein with predicted M_r of 71.4K (Fig. 5a). The region containing amino acids 322–392 of Apg7 shows significant homology with the corresponding region in E1, the ubiquitin-activating enzyme in *S. cerevisiae* (Fig. 5b) and in other species (data not shown). This region encompasses a putative ATP-binding site (GxGxxG)[17], suggesting that Apg7 may be an Apg12-activating enzyme. Although the sequence around the active-site cysteine is less conserved, alignments between Apg7 and other E1-like enzymes indicate that Cys 507 is a putative active-site cysteine (Fig. 5b). Apg10 might be an E2 ubiquitin-conjugating enzyme type of protein because its size is similar to various E2 enzymes and one of its cysteine residues is essential for its function (T. Shintani *et al.*, unpublished results). We reconstituted the conjugation reaction *in vitro*. Lysates of Δ*apg5* cells and Δ*apg12* cells were mixed *in vitro* and incubated with or without ATP. Figure 5c shows that the 70K band appeared in a time-dependent and ATP-dependent manner. The conjugation was sensitive to 1 mM *N*-ethylmaleimide (data not shown). These results show that the Apg12 conjugation pathway contains an ATP-dependent step, which is probably the activation of Apg12 by Apg7.

Autophagy involves a dynamic membrane rearrangement[2–4]. Morphological studies have indicated that all *APG* products function at or before the autophagosome formation step (M. Baba and Y.O., unpublished results). Some Apg proteins are present on membrane structures[9]. Most of the Apg5 and Apg5/Apg12 conjugate, and more than half of the free Apg12, were present in 100,000*g* pellet fractions (data not shown), suggesting that they associate with some membrane compartments. We therefore examined their intracellular localization by sucrose density-gradient centrifugation analysis and found that free Apg5 and the Apg5/Apg12 conjugate co-fractionated (Fig. 6); in contrast, most of the

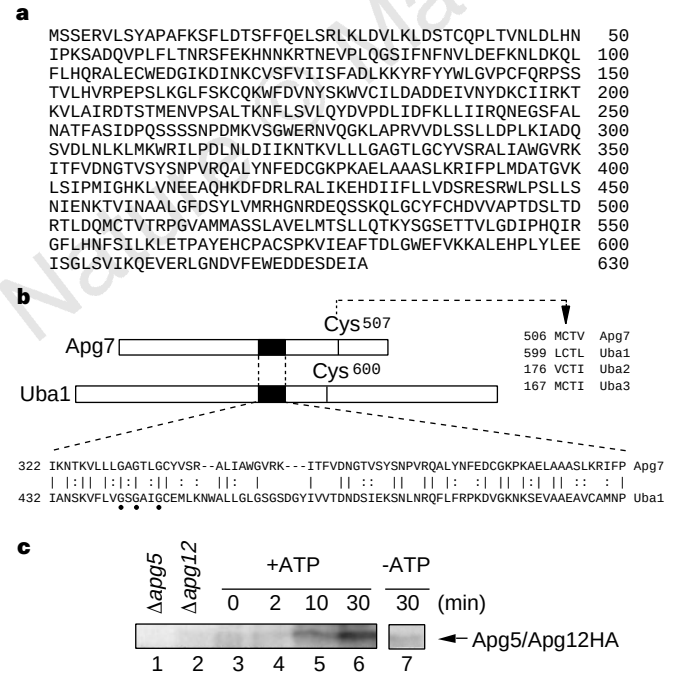

Figure 5 Apg7 is an E1-like protein and Apg12 is conjugated to Apg5 in an ATP-dependent manner. **a**, Amino-acid sequence of Apg7. **b**, Homology between Apg7 and Uba1, *S. cerevisiae* E1 enzyme. Black circles indicate a putative ATP-binding site (GxGxxG). The putative active-site Cys residue of Apg7 is indicated. **c**, *In vitro* conjugation of Apg5 and Apg12. A cell lysate of Δ*apg12* carrying HA-APG5 (lane 1) was incubated with an equal amount of lysate from Δ*apg5* carrying HA-APG12 (2 μ plasmid) (lane 2) at 30 °C with (lane 4–6) or without (lane 7) 5 mM ATP. Samples were mixed with SDS-sample buffer at the times indicated.

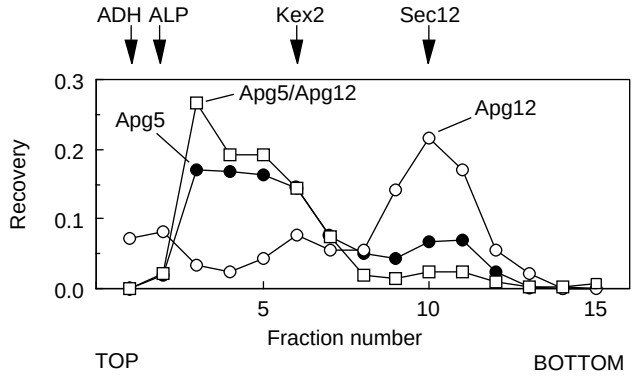

Figure 6 Apg5/Apg12 conjugate co-fractionates with free Apg5 but not free Apg12. Spheroplasts were generated from cells expressing either HA-Apg12 or HA-Apg5. Their lysates were mixed and layered on top of a 10-step (18–54 % w/w) sucrose gradient, and centrifuged at 174,000*g* for 2.5 h (ref. 29). Fifteen fractions were collected and the positions of free Apg5, free Apg12 and Apg5/Apg12 conjugate were examined by western blotting. The peak fractions of alcohol dehydrogenase (ADH)(cytosol), ALP (vacuole), Kex2 (Golgi) and Sec12 (endoplasmic reticulum) are indicated by arrows.

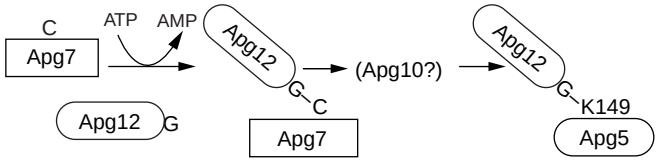

Figure 7 Model of the Apg12-conjugation system.

Apg12 was in the denser fractions. These results indicate that the conjugation of Apg5 and Apg12 is associated with a change in the subcellular localization of Apg12.

We have described a new covalent modification system that is required for autophagy in yeast. Four of 14 *APG* products function in this pathway. Our model is shown in Fig. 7: Apg12 is activated by binding to Apg7 via a high-energy thioester bond; through transfer to an E2-like molecule (possibly Apg10), Apg12 is finally conjugated to Lys 149 of Apg5 via an isopeptide bond. Although the steps in this conjugation pathway are similar to those that occur in ubiquitination[14–16] and in the modification by other ubiquitin-like proteins such as SUMO-1 (refs 18–21), Smt3 (ref. 22), Rub1 (refs 23, 24) and Nedd8 (ref. 25), Apg12 has several unique features. It has no significant homology to ubiquitin and is much larger than ubiquitin and ubiquitin-related modifiers[18–25]. Only a single specific substrate, Apg5, has been found. Apg12 homologues in human and *C. elegans* have a glycine residue at the C terminus (Fig. 1c). We have cloned human Apg12 and found that it is conjugated to human Apg5 (N.M., H. Sugita, T.Y. and Y.O., manuscript in preparation). Human Apg5 was recently cloned as 'apoptosis specific protein' by another group[26], although its physiological significance is not clear yet. This conjugation system is conserved from yeast to mammalian cells, and may be critical for autophagy in every eukaryote. □

Methods

Yeast strains. The *Saccharomyces cerevisiae* strains used for cloning and immunochemical analysis were MT3-4-4(*MAT*a *apg12-1 ura3*), MT87-4-5(*MAT*a *apg7-1 ura3*), MT91-4-2(*MAT*a *apg10-1 ura3*), SKD5-1D(*MAT*a *ura3 leu2 trp1* Δ*apg5::LEU2*) and YYK36(*MAT*a *ura3 leu2 trp1 his3* Δ*apg1::LEU2*). Gene disruptions of *APG5* and *APG12* were performed with YW5-1B(*MAT*a *ura3 leu2 trp1*) or KA31(*MAT*α *ura3 leu2 trp1 his3*).

Alkaline phosphatase assay. The *APG12* or *APG5* gene was disrupted in TN125(*MAT*a *ura3 leu2 trp1 his3 ade2 lys2 PHO8::pho8*Δ*60*), and the assay was done as described[27].

Immunochemical procedures. Whole-cell extracts were prepared by suspending cells in 0.2 M NaOH, 0.5% β-mercaptoethanol, and precipitated with acetone. Extracts were separated by SDS–PAGE, followed by immunoblotting using anti-HA antibody (16B12, BAbCO) or anti-API (aminopeptidase I) polyclonal antibody. Immunoprecipitation was done as described[28] using 16B12 or anti-Myc antibody (9E10).

Site-directed mutagenesis. Mutation and deletion constructs were generated by PCR-based site-directed mutagenesis and confirmed by automated DNA sequencing.

***In vitro* Apg12 conjugation assay.** Total cell lysates were prepared from Δ*apg12* strain expressing HA-Apg5 and Δ*apg5* strain expressing HA-Apg12 after spheroplasting. Both lysates (30 mg ml^{-1}) were mixed in 50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.3 mM PMSF and 2 μg ml^{-1} pepstatin, and incubated at 30 °C for the indicated times with or without 5 mM ATP. The reaction was stopped by mixing with SDS–PAGE buffer and boiling.

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Retinoid-X receptor signalling in the developing spinal cord

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Retinoids regulate gene expression through the action of retinoic acid receptors (RARs) and retinoid-X receptors (RXRs), which both belong to the family of nuclear hormone receptors[1,2]. Retinoids are of fundamental importance during development[2], but it has been difficult to assess the distribution of ligand-activated receptors *in vivo*. This is particularly the case for RXR, which is a critical unliganded auxiliary protein for several nuclear receptors, including RAR[1], but its ligand-activated role *in vivo* remains uncertain. Here we describe an assay in transgenic mice, based on the expression of an effector fusion protein linking the ligand-binding domain of either RXR or RAR to the yeast Gal4 DNA-binding domain, and the *in situ* detection of ligand-activated effector proteins by using an inducible transgenic *lacZ* reporter gene. We detect receptor activation in the spinal cord in a pattern that indicates that the receptor functions in the maturation of limb-innervating motor neurons. Our results reveal a specific activation pattern of Gal4–RXR which indicates that RXR is a critical *bona fide* receptor in the developing spinal cord.

Ligands for retinoid receptors are all-*trans* retinoic acid (RA), which binds to RAR, and 9-*cis* RA, which binds both RAR and

Dynamics and diversity in autophagy mechanisms: lessons from yeast

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Abstract | Autophagy is a fundamental function of eukaryotic cells and is well conserved from yeast to humans. The most remarkable feature of autophagy is the synthesis of double membrane-bound compartments that sequester materials to be degraded in lytic compartments, a process that seems to be mechanistically distinct from conventional membrane traffic. The discovery of autophagy in yeast and the genetic tractability of this organism have allowed us to identify genes that are responsible for this process, which has led to the explosive growth of this research field seen today. Analyses of autophagy-related (Atg) proteins have unveiled dynamic and diverse aspects of mechanisms that underlie membrane formation during autophagy.

Macroautophagy
The sequestration of cytosolic components in autophagosomes and their subsequent degradation when autophagosomes fuse with lysosomes.

Autophagosome
A double membrane-bound vesicle that is formed during autophagy. The vesicle sequesters materials to be degraded and delivers them to the lysosome in mammals or the vacuole in yeasts and plants.

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Autophagy (or macroautophagy) was defined in mammalian cells more than 50 years ago as a system that delivers the cytoplasmic components and organelles of a cell to lysosomes for degradation[1–3]. The most crucial 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 in the cytoplasm called isolation membranes (or phagophores), 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 in the autophagosome. The outer membrane of the autophagosome is subsequently fused with the lysosomal membrane to allow the degradation of the contents together with the inner membrane. Therefore, in contrast to the ubiquitin–26S proteasome system, autophagy mediates primarily non-selective and bulk degradation of many intracellular proteins in one swoop.

Whereas autophagy is drastically induced in response to a shortage of nutrients, it is also regulated by various physiological signals, such as hormones, growth factors and pathogen infection, and occurs constitutively at a basal level[2,3]. Cytoplasmic components are, in principle, nonspecifically engulfed by autophagosomes under starvation conditions. By contrast, recent studies have revealed that autophagy can also be selective in other situations, in which 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 range of physiological and pathological processes in eukaryotic organisms[3,4]. However, the molecular mechanisms that underlie autophagosomal membrane formation and the selective incorporation of cargoes into these membranes remain largely unknown.

Autophagy was beyond the limits of molecular dissection for a long time because it could only be detected by electron microscopy and biochemical analysis of lysosomes was technically difficult. The budding yeast *Saccharomyces cerevisiae* therefore proved to be an ideal organism to use to gain insights into genes that are essential for autophagy. Yeast autophagy was discovered by observing vacuolar proteinase-deficient cells with a light microscope[5]. Within a few hours of shifting the cells to nutrient starvation media, vacuoles were filled with vesicles containing cytoplasmic components, and these vesicles were termed autophagic bodies. It was subsequently shown that the autophagic body is derived from the autophagosome by its fusion with the vacuolar membrane[6,7]. This process 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 seem thin compared with other organelle membranes[6]. Moreover, freeze-fracture electron microscopy indicated that there are asymmetric compositions between the outer and inner membranes of the autophagosome: whereas

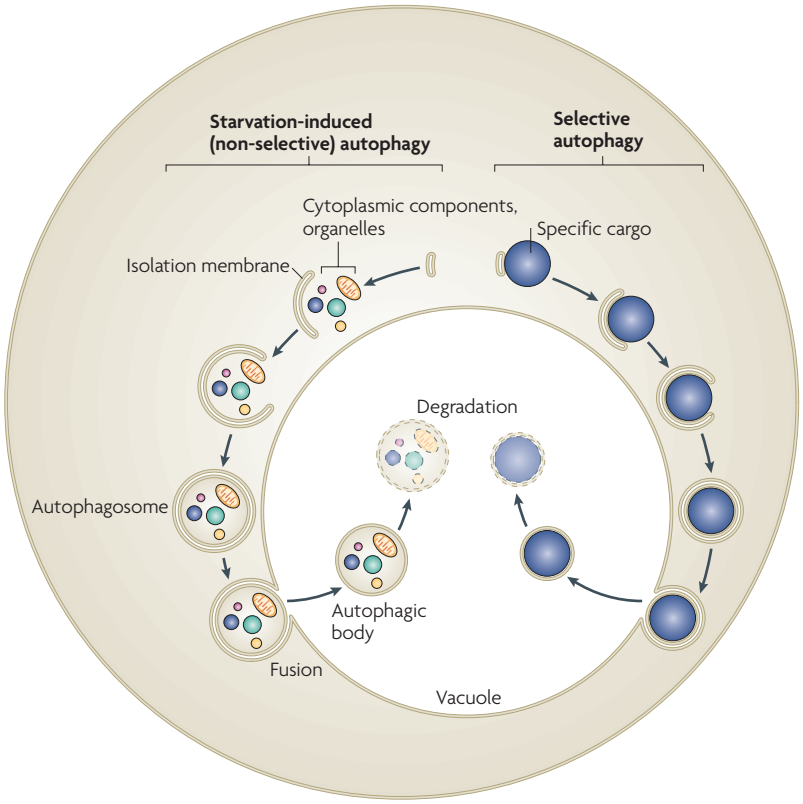

Figure 1 | **Autophagy in yeast.** In starvation-induced (non-selective) autophagy, the isolation membrane mainly non-selectively engulfs cytosolic constituents and organelles to form the autophagosome. The inner membrane-bound structure of the autophagosome (the autophagic body) is released into the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

the outer membrane contains a few particles, perhaps including the machinery for targeting to and fusion with the vacuole, almost no particles are observed in the inner membrane[7]. This suggests that the autophagosome is a specialized organelle for the sequestration of cytoplasmic components and their delivery into lytic compartments, and that its biogenesis involves an unconventional mechanism.

The discovery of autophagy in yeast enabled the genetic screening of mutants that are deficient for the starvation-induced autophagy pathway. From a pool of mutants showing a loss-of-viability phenotype under nitrogen starvation, autophagy-defective mutants were efficiently obtained by light microscopy-based selection of cells that lacked autophagic body accumulation[8]. This screening provided us with 14 *APG* genes that are required for autophagy. Around the same time, Daniel Klionsky's group started to work on the Cvt (cytoplasm-to-vacuole targeting) pathway, which mediates the biosynthetic transport of the vacuolar protein aminopeptidase 1 (Ape1) from the cytoplasm to the vacuole. This occurs through membrane dynamics that turned out to be similar to that of autophagy[9]. The group also isolated mutants of Cvt genes that are defective in this pathway[10]. Other genetic approaches identified genes involved in autophagy as well as pexophagy, an autophagic degradation pathway for peroxisomes in yeast[11–15].

These independent screens of mutants produced different gene names (*APG*, *AUT*, *CVT*, *GSA*, *PAG*, *PAZ* and *PDD*) that have since been unified to *ATG* (autophagy-related) to avoid confusion[16]. Although 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). These genes, hereafter referred to as 'core' *ATG* genes, encode the fundamental machinery for the biogenesis of autophagy-related membranes (FIG. 2). Characterization of these 15 *ATG* gene products 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 a subgroup of functionally unknown proteins that interact with each other[21–24] (TABLE 1). In addition to these core components, some Atg proteins are specifically required for each pathway (FIG. 2). In this Review, we describe the present knowledge on the mechanisms of autophagy that are obtained from yeast studies, especially focusing on starvation-induced autophagy, which is the most fundamental and evolutionally conserved mode of autophagy (see below). More specifically, we first describe the identification of the pre-autophagosomal structure (PAS), an assembly of Atg proteins, then describe how Atg subgroups function 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 both to isolation membranes and to autophagosomes; thus, Atg8 serves as a marker for membrane dynamics during autophagy (see below)[25]. However, fluorescence microscopy showed that one dot of fluorescently tagged Atg8 is usually observed in close proximity to the vacuole in each cell[26] (FIG. 3a). This dot was seen even in several mutants of *ATG* genes, suggesting that it represents neither an isolation membrane nor an autophagosome. Time-lapse microscopy of Atg8 fused with green fluorescent protein (GFP) in a temperature-sensitive *ATG1* strain suggested that autophagosomes are generated at or around this dot[26], and, indeed, most Atg proteins are at least partly colocalized here[26]. From these results, it is likely that the dot represents the assembly of the Atg proteins responsible for autophagosome formation, and thus the assembly was termed the 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, showed that the Atg proteins organize the PAS according to hierarchical relationships among the subgroups[27,28] (FIG. 3b). If one

Ubiquitin–26S proteasome system
The system that degrades selected proteins that are first marked with ubiquitin chains and then degraded by the multi-catalytic proteinase complex, the proteasome.

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

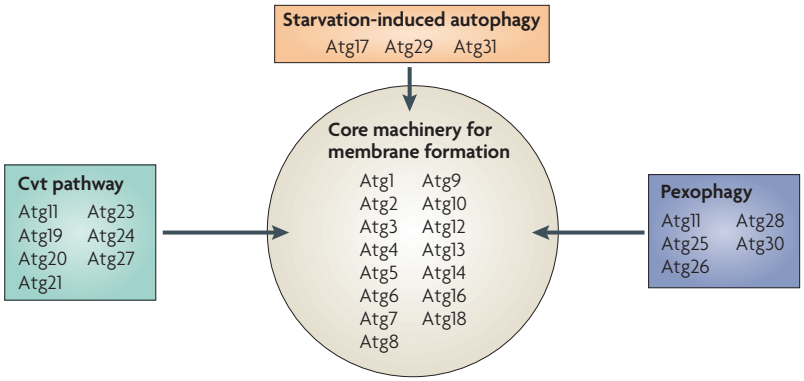

Figure 2 | **Classification of Atg proteins.** Autophagy-related (Atg) proteins that are commonly required for three autophagy-related pathways — starvation-induced autophagy, the Cvt (cytoplasm-to-vacuole targeting) pathway and pexophagy (an autophagic degradation pathway for peroxisomes in yeast) — are classified as the core machinery for membrane formation. Proteins that are specific for each pathway, which include conductor proteins (see the main text), are also shown. It should be noted that Atg11 is involved in both the Cvt pathway and pexophagy.

Atg protein placed upstream in the hierarchical model is genetically deleted, the PAS localization of the downstream Atg proteins is significantly impaired. By contrast, in some cases, upstream Atg proteins accumulate at the PAS in the absence of the downstream protein (see below). Among Atg proteins that belong to the same subgroup, not only hierarchical relationships but also interdependent relationships are also seen. These observations suggest that the Atg proteins function in a coordinate manner to generate the autophagosome at the PAS while interacting with each other both in and among the subgroups. It seems that the hierarchy in the localization of Atg proteins to the PAS represents their order of action in autophagosome formation. Below, we overview the characteristics and functions of each Atg subgroup.

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 signalling, is involved in the induction of autophagy because the Tor inhibitor rapamycin 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 (REF. 30), only TORC1 function is sensitive to rapamycin, indicating that TORC1 is responsible for controlling autophagy. The addition of cyclic AMP suppresses induction of autophagy by nutrient starvation or rapamycin treatment, suggesting that 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 — collaboratively function in the initial step of autophagosome formation, downstream of TORC1 (FIG. 4; TABLE 1). These proteins comprise the most upstream Atg subfamily in the hierarchy of the localization of Atg proteins to the PAS (FIG. 3). Atg1 is a Ser/Thr protein kinase, the activity of which is essential for autophagy[33] and is largely enhanced following nutrient starvation or the addition of rapamycin[17]. This regulation involves Atg13 (REF. 17). Although Atg13 is phosphorylated in a TORC1-dependent manner under nutrient-replete conditions, it is immediately dephosphorylated in response to starvation or rapamycin treatment[17,34] (FIG. 4). Dephosphorylated Atg13 associates with Atg1 and somehow leads to upregulation of the kinase activity of Atg1. As dephosphorylation of Atg13 occurs normally in the presence of any non-essential phosphatase mutant (T. Funakoshi and Y.O., unpublished observations), multiple phosphatases might 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 the phosphorylation of these proteins — and thus an authentic substrate or substrates of Atg1 — still remain elusive (Y.K. and Y.O., unpublished observations).

Whereas Atg1 and Atg13 are among the core components of autophagosome formation, Atg17, Atg29 and Atg31 are specifically required for starvation-induced autophagy (FIG. 2) and form a ternary complex[17,35–38] (FIG. 4) (Y. Kabeya and Y.O., unpublished observations). 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 (REFS 35,39). This association is also a prerequisite for the recruitment of other core Atg proteins to the PAS in the absence of Atg11, suggesting that this subgroup functions as a trigger for autophagosome formation (see below)[35,38–40]. The kinase activity of Atg1 is dispensable for both the formation of a complex of these five Atg proteins and the recruitment of a number of Atg proteins to the PAS[38,39]. In yeast that have the kinase-dead allele of *ATG1*, however, some Atg proteins abnormally accumulate at the PAS, and others, such as Atg2, become absent from the PAS (REFS 38,39,41). This suggests that the kinase activity of Atg1 is involved in the dynamics of the Atg proteins at the PAS, probably through the phosphorylation of one or more Atg proteins.

Although it was proposed that dephosphorylation of Atg13 is one of the initial events in autophagy, whether Atg13 is a direct target of TORC1 or whether dephosphorylation of Atg13 is sufficient for the induction of autophagy has remained unanswered[42]. These questions have recently been addressed to understand the mechanism by which TORC1 signalling regulates autophagy (Y.K. and Y.O., unpublished observations). It was shown that Atg13 is directly phosphorylated by TORC1 *in vitro* at multiple Ser 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 signalling module is thought to be conserved across most eukaryotes to regulate autophagy[43–48].

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

Subgroup components	Known or putative function	References
Atg1 kinase and its regulators*		
Atg1	Ser/Thr kinase	17,33,38,39,57
Atg13	TORC1 substrate involved in the regulation of Atg1 activity	17,34,38,39,57
Atg17	Forms a starvation-induced autophagy-specific complex with Atg29 and Atg31, which further associates with Atg1 and Atg13	17,35,38–41
Atg29	Component of the Atg17–Atg29–Atg31 complex	36,38
Atg31	Component of the Atg17–Atg29–Atg31 complex	37,38
***PtdIns 3-kinase complex*‡**		
Vps34	PtdIns 3-kinase	18,52
Vps15	Ser/Thr kinase required for Vps34 activity	18,52
Vps30 (also known as Atg6)	Component of unknown function	18,52
Atg14	Recruits the PtdIns 3-kinase complex to the PAS	18,52
***Atg12 conjugation system*§**		
Atg12	Ubiquitin-like protein that is conjugated to Atg5	19,72,73,76,77
Atg5	A target of Atg12 and interacts with Atg16	19,72,73,76,77
Atg7	Common E1 enzyme for Atg12–Atg5 and Atg8–PE formation	19
Atg10	Specific E2 enzyme for Atg12–Atg5 formation	19
Atg16	Required for the PAS localization of Atg12–Atg5	72,73
***Atg8 conjugation system*‖**		
Atg8	Ubiquitin-like protein conjugated to PE	20,25,62–64,68,69
Atg3	Specific E2 enzyme for Atg8–PE formation	20,63,68
Atg4	Removes the carboxy-terminal Arg and conjugated PE from Atg8	62–64
Atg7	Common E1 enzyme for Atg12–Atg5 and Atg8–PE formation	20,68
Atg2–Atg18 complex*¶ *and Atg9		
Atg2	Forms a complex with Atg18, which is involved in Atg9 dynamics at the PAS	21,22,50
Atg18	Binds to PtdIns3P	50,54,55
Atg9	Integral membrane protein of unknown function	23,41,57,60

*Trigger and regulate PAS assembly of other proteins. ‡Produces PtdIns3P at the PAS. §Stimulates and determines the location of Atg8 lipidation. ‖Controls membrane tethering and hemifusion. ¶The function of this complex is unknown. PAS, pre-autophagosomal structure; PE, phosphatidylethanolamine; PtdIns, phosphatidylinositol; TORC1, target of rapamycin complex 1; Vps, vacuolar protein sorting.

The PtdIns 3-kinase complex

One Atg subgroup forms a complex with the PtdIns 3-kinase that phosphorylates the D3 position of the inositol ring in PtdIns to produce PtdIns3P (TABLE 1). In addition to this complex, we describe another subgroup that comprises Atg18, a possible effector for PtdIns3P, Atg2 and Atg9.

The activity of PtdIns 3-kinase is essential for autophagy[18,49,50]. Vps34 is the sole PtdIns 3-kinase in *S. cerevisiae*[51] and forms two distinct complexes, I and II, that have essential roles in autophagy and the vacuolar protein sorting (Vps) pathway, respectively[18]. Complex I is composed of Vps34, Vps15, Vps30 (also known as Atg6) and Atg14, whereas complex II contains Vps38 instead of Atg14 (FIG. 5). The specific presence of Atg14 or Vps38 directs the localization of complexes I and II to the PAS and the endosomal membrane, respectively, in addition to the vacuolar membrane[52] (FIG. 5). PtdIns3P that is produced by complex I is thought to recruit effector proteins required for autophagosome formation to the PAS.

Atg18 can bind to both PtdIns3P and PtdIns(3,5)P_2 (REFS 53,54), and is therefore a potent candidate for the effector of these molecules. A portion of Atg18 forms a complex with Atg2 and functions in autophagosome formation[27,50], whereas this protein also regulates the size of the vacuole and PtdIns(3,5)P_2 homeostasis in complex with other proteins[53,55] (FIG. 5). Whereas the autophagosome formation function of Atg18 involves PtdIns3P, the regulatory function in vacuole morphology depends on PtdIns(3,5)P_2 (REFS 50,55). Atg14, but not Vps38, is required for the localization of the Atg2–Atg18 complex to the PAS, suggesting that the production of PtdIns3P by PtdIns 3-kinase complex I at the PAS is important for the localization of this complex to the PAS and thus for its function in autophagosome formation[27,50] (FIG. 3).

Vacuolar protein sorting
A pathway that mediates the selective transport of a subset of proteins from the late Golgi compartment to the vacuole through the endosome.

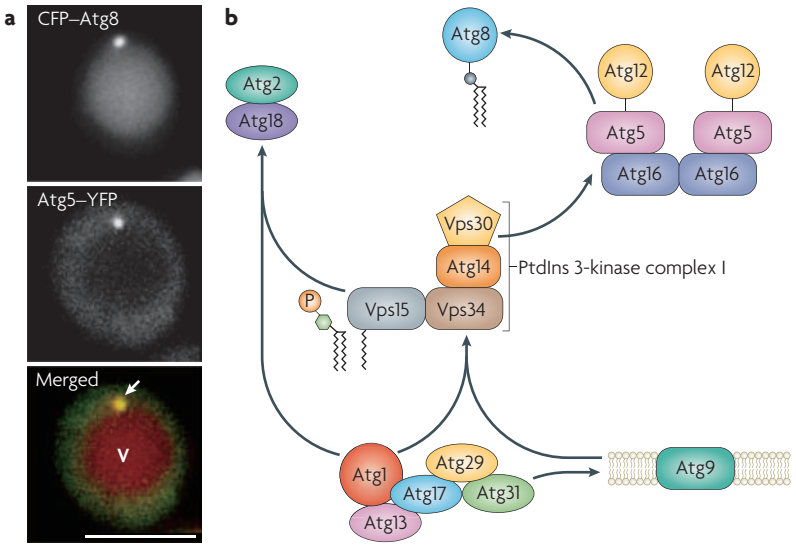

Figure 3 | **The PAS as the assembly of Atg proteins. a** | Fluorescence microscopy images of cells that express both cyan fluorescent protein (CFP)-tagged autophagy-related 8 (Atg8) and yellow fluorescent protein (YFP)-tagged Atg5, as well as the merged image. The arrow indicates the pre-autophagosomal structure (PAS). The vacuole is indicated by the letter V. Scale bar, 5 μm. **b** | A hierarchical model of the localization of Atg proteins to the PAS in starvation-induced autophagy. If an upstream Atg protein is genetically removed, the localization of downstream Atg proteins is lost. The Atg1–Atg13–Atg17–Atg29–Atg31 complex, which is formed in response to nutrient starvation, has an essential role in PAS organization in starvation-induced autophagy. This complex is involved not only in the recruitment of other Atg proteins to the PAS but also in their dissociation from the PAS. Mechanisms by which upstream proteins recruit downstream proteins are still largely unknown. It is suggested that phosphatidylinositol-3-phosphate (PtdIns3P) produced by PtdIns 3-kinase complex I at the PAS recruits the PtdIns3P-binding protein Atg18 and its associated protein Atg2. Vps, vacuolar protein sorting. Figure part **a** is modified, with permission, from *EMBO J.* REF. 26 © (2001) Macmillan Publishers Ltd. All rights reserved.

Although the precise location in which complex I produces PtdIns3P is still unknown, recent studies show that PtdIns3P is enriched on isolation membranes and autophagosomal membranes and is eventually transported to the vacuole[56].

Of the core Atg proteins, Atg9 is the sole integral membrane protein. Thus, this protein has been extensively analysed to obtain insights into a membrane source of the autophagosome[57–59]. In addition to its localization to the PAS[27], Atg9–GFP is also observed as small dots that move in the cytoplasm[57]. Whereas disruption of actin filaments or microtubules does not disturb this movement, energy depletion by the addition of sodium azide does[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 an *ATG1* temperative-sensitive strain[57]. 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 and the function of Atg1 might be involved in the dynamics of Atg9 at the PAS (see below). In the hierarchical model of the localization of Atg proteins to the PAS (FIG. 3), Atg9 is located just after the Atg1 subgroup. Consistent with this, it has recently been shown that Atg9 physically interacts with Atg17, a component of the Atg1 subgroup[41]. It has also been shown that Atg9 self-associates, which is important for its localization to the PAS[60].

E1
An enzyme that activates ubiquitin and ubiquitin-like proteins using ATP and transfers them to E2 enzymes.

E2
An enzyme that receives ubiquitin and ubiquitin-like proteins from E1 enzymes and conjugates them to target molecules.

Two ubiquitin-like conjugation systems

There are two protein conjugation systems among the Atg subgroups, each composed of two ubiquitin-like proteins (Atg8 and Atg12) and three enzymes (Atg3, Atg7 and Atg10) that are required for their conjugation reactions (FIG. 6a,b; TABLE 1). Atg12 forms a conjugate with Atg5 (REF. 19), whereas Atg8 is conjugated to phosphatidylethanolamine (PE)[20], a major component of various biological membranes. Both conjugates localize to autophagy-related membranes[25,61] (FIG. 6c), suggesting their direct involvement in the biogenesis of these membranes. Consistent with this notion, these conjugates are positioned downstream in the hierarchy of the localization of Atg proteins to the PAS (FIG. 3). Recent *in vitro* studies have substantially advanced 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 carboxyl terminus (a single Arg residue in *S. cerevisiae* Atg8). This is immediately cleaved by the Cys protease Atg4 to expose the Gly residue that is essential for subsequent reactions[62,63] (FIG. 6a). Formation of the Atg8–PE conjugate is mediated by Atg7 and Atg3, which are the E1 and E2 enzymes in the ubiquitylation reaction, respectively[20]. The carboxyl group of the exposed Gly of Atg8 is activated by Atg7, in a reaction that requires ATP, to form a thioester intermediate with the active Cys residue of Atg7. The Gly residue of Atg8 is then transferred to the active Cys residue of Atg3 and eventually 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].

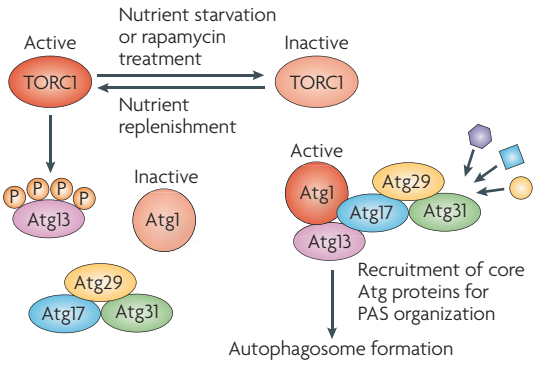

Figure 4 | **The Atg1 subfamily.** When target of rapamycin complex 1 (TORC1) is inactivated following nutrient depletion or rapamycin treatment, autophagy-related 13 (Atg13) is dephosphorylated. This allows the association of Atg1 subfamily proteins with Atg13, followed by the upregulation of the Atg1 kinase activity and recruitment of other core Atg proteins to the pre-autophagosomal structure (PAS) to initiate autophagosome formation. These events are immediately reversed on the addition of nutrients.

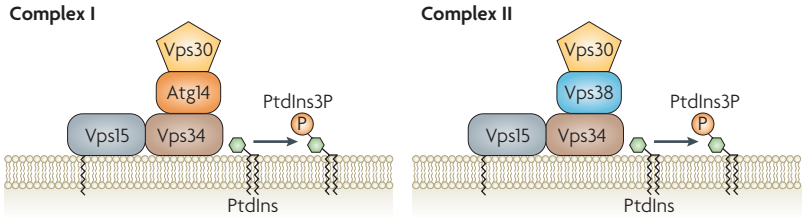

Figure 5 | **Two PI3K complexes.** Phosphatidylinositol (PtdIns) 3-kinase complexes I and II are composed of common subunits — the PtdIns 3-kinase vacuolar protein sorting 34 (Vps34), Vps30 (also known as autophagy-related 6 (Atg6)) and Vps15 — and specialized subunits, Atg14 and Vps38, respectively. Complex I localizes to the pre-autophagosomal structure (PAS) and the vacuole, its downstream factors include Atg2–Atg18 and it functions in autophagosome formation. By contrast, complex II resides in endosomes and the vacuole, its downstream factors include the Fab1–Fig4–Vac14 complex, Vac7 and Atg18, and it has a role in the regulation of vacuole morphology.

Atg4 is also a deconjugation enzyme that cleaves Atg8–PE to liberate the protein from membranes[62] (FIG. 6a). 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, and delivered to and degraded in the vacuole[25] (FIG. 6c). Both synthesis and lipidation of Atg8 are enhanced under autophagy-inducing conditions[62,64]. These features of Atg8 allow us to use this protein and its homologues to trace progression of autophagy in various organisms[65–67].

The conjugation reaction of Atg8 can be reconstituted *in vitro* with purified recombinant proteins (the Gly-exposed form of Atg8, Atg7 and Atg3), ATP and liposomes that contain PE. In this mixture, Atg8 efficiently forms a conjugate with PE on the liposomes[68]. It was found that Atg8–PE forms an oligomer and causes liposome clustering and hemifusion[64] (FIG. 6d). Atg8 mutants that are deficient for clustering and hemifusion of liposomes exhibit significant defects in autophagosome formation, suggesting that these phenomena observed *in vitro* represent the physiological functions of Atg8 *in vivo*. The size of the autophagosome decreases in cells that express mutant forms of Atg8, in which their functions are partially impaired. Similar consequences are also observed when the expression level of Atg8 is genetically engineered to be decreased[69]. These results suggest that Atg8 is involved in the expansion of autophagosomal membranes. In addition, recent studies in mammals showed that lipidation of Atg8 homologues is essential for normal development of autophagosomal membranes; its abrogation causes accumulation of unclosed isolation membranes with anomalous morphology[70,71].

On the basis of the *in vitro* observations, it can be assumed that Atg8–PE is involved in tethering and fusion of unidentified precursory structures of autophagosomes. In fact, previous studies indicated the existence of such structures that contain Atg8–PE. Using immunoelectron microscopy, Atg8 signals are not only observed on isolation membranes and autophagosomes, but also in less electron-dense regions, which seem to be abundant in lipids but free from evident membrane structures[25]. Moreover, the level of Atg8–PE increases under autophagy-inducing conditions, even in cells that are deficient for autophagosome formation[27]. Atg8–PE might accumulate on precursors of autophagosomal membranes in these cells. Identification and characterization of these structures containing Atg8–PE, including the elucidation of their components, morphology and formation process, will provide us with crucial information on molecular mechanisms of, and a source of lipid supply for, autophagosome formation.

Conjugation of Atg12. Similar to the Atg8–PE system, the conjugation reaction of Atg12 is catalysed by the common E1 enzyme Atg7 and the specific E2 enzyme Atg10. The C-terminal Gly residue of Atg12 forms an isopeptide bond with the specific Lys residue of Atg5 (REF. 19) (FIG. 6b). 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 (REFS 72–74) (FIG. 6b). Immunoelectron microscopic analyses of mammalian cells showed that while LC3 (a mammalian Atg8 homologue) is present on both surfaces of the isolation membrane[65], the ATG12–ATG5–ATG16L (a mammalian Atg16 homologue) complex predominantly localizes on the outer surface of the membrane[61,74] (FIG. 6c). In addition, fluorescence microscopy showed that GFP-labelled ATG5 dissociates from the membrane immediately before or after completion of the autophagosome[61]. 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 that participate in autophagosome formation is too low to assemble a coat that surrounds the membrane[75].

Crosstalk between the conjugation systems. The involvement of the Atg12–Atg5–Atg16 complex in the Atg8–PE system has been implied genetically, as mutations that abolish the complex significantly decrease Atg8–PE production[26,76]. It has also been shown that purified Atg12–Atg5 conjugates drastically stimulate the formation of Atg8–PE *in vitro*[77,78]. Atg12–Atg5 directly interacts with the E2 enzyme Atg3 and enhances its activity (FIG. 6a,b). 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*[68,77,79]. Unlike E3 enzymes in the ubiquitin system, Atg12–Atg5 is not involved in determining substrate specificity in lipidation of Atg8 (REF. 77). Instead, it has been proposed that Atg3 itself has an ability to discriminate PE from PS under physiological conditions[80].

Atg16 is dispensable for the E3-like function of Atg12–Atg5 *in vitro*, in spite of its requirement for Atg8–PE formation *in vivo*[26]. 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].

Deconjugation enzyme
An enzyme that cleaves the isopeptide bond (the amide bond between autophagy-related protein 8 (Atg8) and phosphatidylethanolamine) formed in ubiquitin and ubiquitin-like protein conjugates.

Hemifusion
Fusion between outer leaflets of membranes while inner leaflets remain intact. This is regarded as a common intermediate state in biological membrane fusion events.

E3
An enzyme that stimulates the conjugation reaction by E2 enzymes and is also involved in the selection of target molecules.

In addition, forced localization of ATG16L to the plasma membrane in mammalian cells causes lipidation of LC3 on that membrane[81]. These results indicate that ATG16 is involved in specification of the site of ATG8 lipidation. The localization of the Atg12–Atg5–Atg16 complex to the PAS might imply that Atg8–PE is produced at the PAS. Lipidation of Atg8 on the isolation membrane is also conceivable. As mentioned above, Atg8–PE, however, accumulates even in mutant cells in which 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 sites. Thus, this long-standing question requires further investigation.

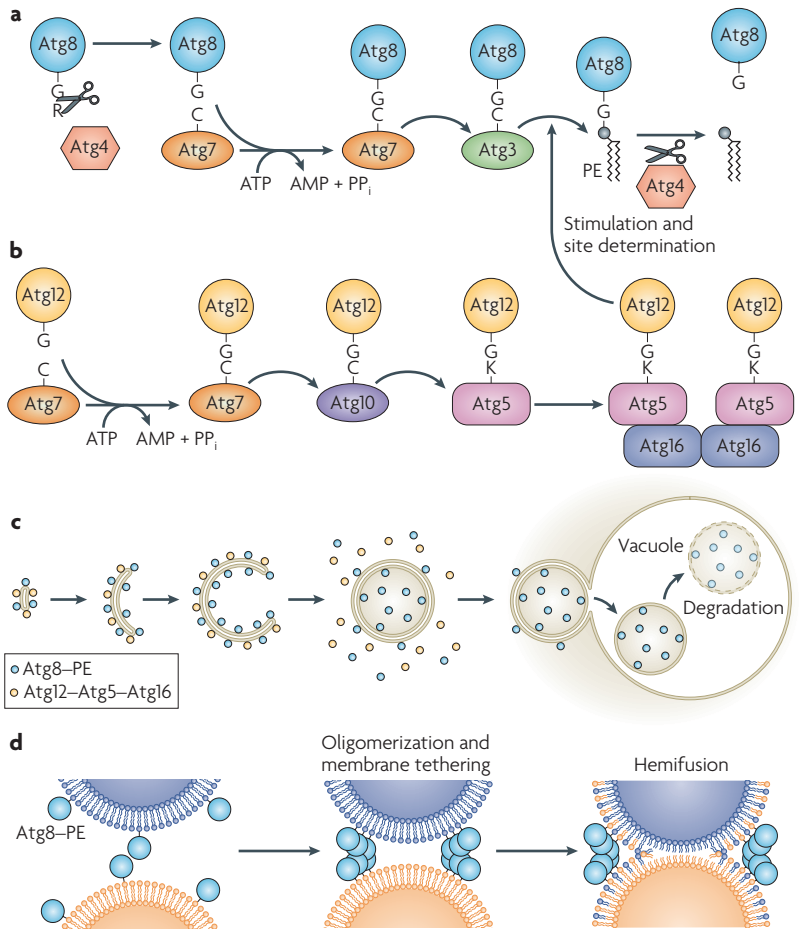

Figure 6 | **Two ubiquitin-like conjugation systems. a** | The conjugation system of autophagy-related 8 (Atg8). First, Atg4 cleaves the carboxy-terminal Arg (R) residue of Atg8 to expose Gly (G) at the new C terminus. Atg8 is then activated by Atg7 (an E1 enzyme), transferred to Atg3 (an E2 enzyme) and eventually conjugated to phosphatidylethanolamine (PE). The active site Cys (C) residues of Atg7 and Atg3 are indicated. 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 (K) residue of Atg5 in a similar manner to the conjugation reaction of Atg8, except that Atg10 functions as the E2 enzyme in this system instead of Atg3. An E3 enzyme for the Atg12 conjugation reaction has not been reported. The Atg12–Atg5 conjugate interacts with Atg16 and forms an oligomer. The Atg12–Atg5–Atg16 complex then exerts an E3 enzyme-like function on the Atg8 conjugation reaction; the transfer reaction of Atg8 from Atg3 to PE is stimulated by this complex. Atg12–Atg5–Atg16 is also suggested to determine the site of the production of Atg8–PE (see the main text for details). **c** | Localization of ubiquitin-like protein conjugates on autophagy-related membranes. Atg8–PE is present on both surfaces of the isolation membrane, and 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. On conjugation to PE on liposomes, Atg8 oligomerizes and tethers the liposomes, leading to hemifusion of the membranes. Only lipids in outer leaflets interdiffuse in hemifused membranes.

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, extensive studies on this pathway have established a conceptual framework to understand the mechanism of selective autophagy (see below). In this pathway, the cargo Ape1 self-assembles into an aggregate-like structure and interacts with the receptor protein Atg19 (REFS 82,83). Atg19 also interacts with Atg8, and this interaction is thought to link the cargo–receptor complex to the forming Cvt vesicle[84–86]. In mammalian cells, p62 is responsible for selective degradation of ubiquitin-positive protein inclusions through autophagy and also binds to mammalian homologues of Atg8 (REFS 87–90). Therefore, although Atg19 and p62 are unrelated to each other in their entire sequences, these proteins probably function in similar ways in selective incorporation of the cargoes into vesicles. Interestingly, recent structural studies revealed a common interaction between these receptors and Atg8 homologues: the Trp-X-X-Leu motif in the receptors binds to the highly conserved, hydrophobic pocket in the Atg8 homologues in a similar manner[86,91]. A similar interaction might work broadly in the 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, which can be versatile in its composition depending on the physiological situations. Here, we define the PAS as a dynamic assembly of the core Atg proteins, which function as membrane-forming machinery, and of 'conductor' proteins that spatio-temporally regulate the core proteins to determine the site for PAS organization and the mode of membrane formation. On this basis, we describe our present view on the PAS.

As mentioned above, 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[92]. 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 such conditions — this made it difficult to analyse Atg protein dynamics in response to induction of autophagy. The problem has been circumvented by analysing cells that lack Atg11 (REFS 38,39); Atg11 is specifically required for the Cvt pathway, and is responsible for organization

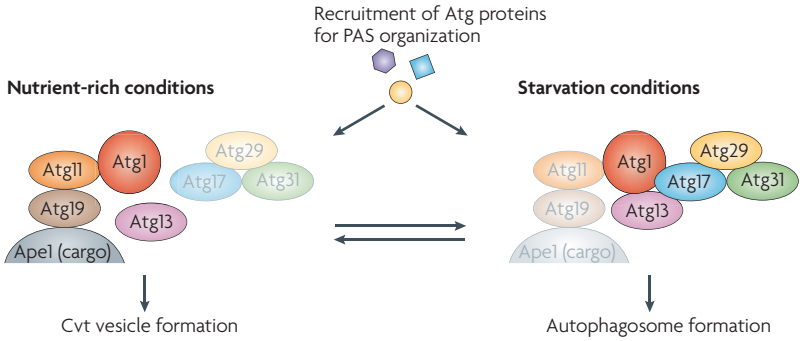

Figure 7 | **Model for the role of conductor proteins in PAS organization.** Under nutrient-rich conditions, autophagy-related 11 (Atg11) serves as a conductor, together with a large assembly of aminopeptidase 1 (Ape1; a cargo protein) and Atg19 (a receptor), to recruit other Atg proteins, including core machinery components and Cvt (cytoplasm-to-vacuole targeting)-specific proteins. This recruitment organizes the pre-autophagosomal structure (PAS) to mediate Cvt vesicle formation around the cargo (left panel). Although the Atg17–Atg29–Atg31 complex, which is specifically required for starvation-induced autophagy, also localizes to the PAS under these conditions, this complex is dispensable for the Cvt pathway. By contrast, under nutrient-deprived conditions, Atg17–Atg29–Atg31, together with Atg1 and Atg13, acts as a conductor in a manner that is independent of Atg11. By these means the PAS is reorganized to a mode for autophagosome formation (right panel). The precise interactions among these proteins under each condition are elusive.

of the PAS under nutrient-rich conditions[93,94]. Whereas the Atg proteins in these cells are totally dispersed in the cytoplasm under nutrient-rich conditions, they assemble into a perivacuolar dot in response to nutrient starvation[94]. The dot immediately disappears upon nutrient replenishment[38]. The lack of either Atg1, Atg13, Atg17, Atg29 or Atg31 completely abolishes dot formation of all other core Atg proteins, as well as these five proteins[38,39]. Therefore, the Atg17–Atg29–Atg31 complex is likely to function as a conductor together with the Atg1–Atg13 complex in organizing the PAS for autophagosome formation (FIG. 7). Because the PAS already exists before autophagy induction in wild-type cells, it might be more appropriate to describe the Atg17–Atg29–Atg31 complex as a 'reorganizer' of the PAS from its mode for the Cvt pathway to that for autophagy in response to nutrient starvation (FIG. 7).

In addition to 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 ~10 min intervals[39,75], which corresponds to the time it takes for a single autophagosome to form in mammalian cells[61]. By contrast, the intensity of Atg9–GFP is constant over a 30 min time course[75]. However, as described above, Atg9–GFP markedly accumulates at the PAS in a number of *ATG* mutant strains[27,57], suggesting that the dynamics of Atg9 is in 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 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 (REF. 94) (FIG. 7). It is thought that the cargo–receptor complex serves as a scaffold for the recruitment of Atg11 (REF. 95). Atg11 then recruits the core Atg proteins to allow the formation of the Cvt vesicle around the cargo–receptor complex. Thus, similar to the Atg17–Atg29–Atg31 complex in starvation-induced autophagy, Atg11 behaves as a conductor together with the cargo–receptor complex in the organization of the PAS in the Cvt pathway (FIG. 7). In addition, it was reported that Atg11 is also involved in pexophagy and 'mitophagy', the autophagic degradation of mitochondria, in which Atg11 is suggested to function in a similar manner but in cooperation with Atg30 and probably an unidentified factor, respectively, instead of Atg19 (REFS 96,97).

In this way, conductor Atg proteins seem to regulate the core Atg proteins spatio-temporally 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, unidentified conductors might exist that respond to different environmental signals or recognize specific cargo complexes. This creates the functional diversity of the roles of autophagy that is rapidly emerging in higher eukaryotes. Although the PAS has not been described in mammalian cells, foci that might correspond to the PAS have recently been reported in slime moulds and higher plants[98,99].

The PAS has only been observed as a dot under a fluorescence microscope. We defined the PAS as an assembly of the Atg proteins that can exist without the isolation membrane[26]. However, certain Atg proteins, such as Atg8 and the Atg12–Atg5–Atg16 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 to the vacuole, even though these five Atg proteins are interdependently assembled onto 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 discuss the PAS more definitively 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 a pool of metabolites such as amino acids, would have been established first and has been conserved during

evolution. In fact, all of 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 might have been acquired, which have developed various functions of autophagy.

The machinery for starvation-induced autophagy is likely to utilize specialized factors for selective autophagy in some cases. Certain cargoes for selective autophagy are more efficiently transported to lytic compartments by autophagy under starvation conditions in a manner that is dependent on such factors, in which the cargoes should be incorporated into autophagosomes together with other cytoplasmic components[9,100,101]. In addition, cytoplasmic acetaldehyde dehydrogenase 6 (Ald6) and ribosomes are known to be preferentially degraded through autophagy under nitrogen starvation conditions, even though they seem to be completely dispersed in the cytoplasm, suggesting a novel mechanism for these cases[102,103]. Thus, the way in which materials that are to be degraded in autophagy are sequestered also varies. Finally, it should also be noted that Leu aminopeptidase 3 (Lap3) has recently proved to be selectively transported to the vacuole for degradation in glycerol-grown (non-starved) yeast cells[101] (T. Kageyama and Y.O., unpublished observations). This process depends on Atg11 and Atg19, which are specifically required for the Cvt pathway, indicating that the Cvt pathway also functions as a selective degradation system.

Recent studies, especially in higher eukaryotes such as mammals and plants, have rapidly unveiled the diversity and complexity of autophagy. An in-depth knowledge of the diverse modes of autophagy is quite important for accurate understanding of not only the mechanism but also the significance of autophagy in each physiological or pathological situation.

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DATABASES

UniProtKB: http://www.uniprot.org

Ape1 | Atg1 | Atg2 | Atg3 | Atg5 | Atg7 | Atg8 | Atg9 | Atg10 | Atg11 | Atg12 | Atg13 | Atg14 | Atg16 | Atg17 | Atg18 | Atg29 | Atg31 | Tor | Vps15 | Vps30 | Vps34 | Vps38

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Review

Autophagy in health and disease: A comprehensive review

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ABSTRACT

Autophagy, a conserved catabolic process, plays an immensely significant role in a variety of diseases. However, whether it imparts a protective function in diseases remains debatable. During aging, autophagy gradually subsides, manifested by the reduced formation of autophagic vacuoles and improper fusion of these vacuoles with the lysosomes. Similarly, in neurodegenerative disorders, accumulation of tau and synuclein proteins has been attributed to the decline in the autophagic removal of proteins. Equivalently, lysosomal disorders show an impairment of the autophagic process leading to the accumulation of lipid molecules within lysosomes. On the other hand, activation of the autophagic pathway has also proved beneficial in evading various foreign pathogens, thereby contributing to the innate immunity. In the context of cancer, autophagy has shown to play a puzzling role where it serves as a tumor suppressor during initial stages but later protects the tumor cells from the immune system defense mechanisms. Similarly, muscular and heart disorders have been shown to be positively and negatively regulated by autophagy, respectively. In the present review, we, therefore, present a comprehensive review on the role of autophagy in various diseases and their corresponding outcomes.

1. Introduction

Autophagy is defined as a catabolic process that is conserved among all eukaryotic organisms. The study of autophagy has gained immense importance in the past decade by defining the basic functioning of cellular and organismal metabolism. From regulating the fundamental metabolic functions inside the cells to varied diseases, namely, aging, cancer, neurodegenerative disorders, and lysosomal disorders, autophagy has become the central regulating point in controlling the homeostasis of the human body. As an evolutionarily conserved process, autophagy, through the breakdown of proteins and peptides, has assisted the cells to adapt to myriad stress conditions by providing a pool of amino acids. Hence, autophagy maintains the cellular homeostasis, thereby enabling the cells to stride past the crisis situations. Autophagy, at the basal level, regulates the intracellular conditions through cytoplasmic turnover of proteins and organelles. In some cases, accumulation of ubiquitinated proteins has been reported inside the cells upon deletion of certain autophagic proteins, such as Atg5 and Atg7, suggesting the indispensable role that autophagy plays in regulating the protein turnover of the cells [1]. Nonetheless, autophagy is important in maintaining cell growth and development. For instance, some mutant yeasts defective in autophagy displayed impaired spore formation [2]. Moreover, recent investigations have articulated the concept of selective autophagy. Autophagy has also been revealed to recognize specific substrates, mitochondria, lipid droplets, and peroxisomes resulting in their overall turnover. Despite its role in controlling the protein turnover of the cell, autophagy has also been investigated to play a crucial role in innate immunity. Autophagy facilitates the binding of endogenous antigens with major histocompatability complex-II (MHC-II) molecules that are recognized by CD4 + T cells. For example, in an experiment where influenza antigen was fused to LC3, it was efficiently incorporated into the autophagolysosomes and presented along with MHC-II class [3].

Our current knowledge on autophagy broadly differentiates it into three types: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy mainly involves the sequestration of cytoplasmic contents in a double-walled membrane followed by the fusion with the lysosomes. The lysosomal enzymes facilitate the degradation of the sequestered products. Microautophagy is categorized by the direct engulfment of cytoplasmic cargo by the lysosomes. The last type is the only one where proteins are specifically targeted to lysosomes via signal peptides and coordinated by chaperones located on both sides of the targeted membrane. Selective autophagy including mitophagy, ERphagy, lipophagy, xenophagy to clear mitochondria, endoplasmic reticulum, lipid droplets and invading pathogens respectively are degraded by for maintaining cellular homeostasis [4,5]. In the present review, we focus on the implications of different types of autophagy in the maintenance of health and progression of diseases,

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thereby deciphering the relevance of autophagy in the human system.

2. Autophagy: self-eating mechanism

Autophagy as a cytoprotective mechanism is triggered by different stimuli including nutrient deprivation, oxidative stress, hypoxia, protein aggregates and toxic molecules to mitigate stress. The process of autophagy initiates with the formation of the isolating membrane and phagophore. During stress conditions, the mammalian target of rapamycin (mTOR) is inactivated, which consequently activates Atg1 (Ulk1 and Ulk2 mammalian homolog) kinase activity. The activation of Atg1/ULK1-2, in turn, causes phosphorylation of Atg13 and FIP200 and autophosphorylation of ULK proteins. The phosphorylation and the formation of Atg13-ULK-FIP200 complex recruit other Atg proteins, thereby resulting in the initiation of autophagosome formation [4–7]. The formation of the phagophore membrane is regulated by the class-III phosphatidylinositol 3-kinase (PtdIns3K) complex. This complex comprises PtdIns3KVps34, Vps15/p150, Atg14/mAtg14, and Vps30/Beclin1 [4]. The PtdIns3K complex forms phosphatidylinositol-3-phosphate from phosphatidylinositol, and this complex targets the formation of pre-autophagosomal structure (PAS) through binding of several yeast autophagic proteins, such as Atg18, Atg20, Atg21, and Atg24, to phosphatidylinositol-3-phosphate. The PtdIns3K complex along with the Atg proteins then recruits two ubiquitin-like conjugation systems, namely Atg12-Atg5-Atg16 and Atg8-phosphatidylethanolamine (Atg8-PE), to PAS. These are, in turn, involved in the phagophore membrane elongation and expansion. In the first system, Atg7 activates Atg12, which is then transferred to Atg10 and linked to Atg5. The Atg12-Atg5 complex then binds to the Atg 16, followed by the recruitment of this complex to the phagophore membrane [4]. The Atg5-Atg12 conjugation system is not linked to the activation of autophagy; therefore, as the autophagosome is formed, the Atg5-Atg12-Atg16 complex gets dislodged from the membrane making it a poorer marker for autophagy [8]. Another ubiquitin pathway involves the lipidation of LC3 (Atg8). LC3 is synthesized as pro-LC3, which is cleaved by Atg4 at the C-terminus to form LC3-I. The LC3-I moiety is conjugated to PE with the help of Atg7 and Atg3 to form LC3-II. LC3-II is then recruited on both the membranes of autophagosomes and enables the fusion with the lysosomes [9]. The connection of autophagosomes to the microtubule proteins facilitates their transport to the lysosomes. Moreover, Rab GTPase, involved in membrane trafficking are localized to the late endosomes and lysosomes for their motility and fusion [10]. After the fusion of autophagosome with the vacuole, the cytosolic cargo is broken down by the acidic lysosomal hydrolases [6] (Fig. 1).

3. Aging: an unhealthy autophagic recipe

Aging in all multicellular organisms is characterized by the decreased ability to combat the environmental stresses that lead to the loss of cellular homeostasis and make the organism prone to many old-age associated diseases. It has been observed that global proteolysis decreases with age. Most of the proteins whose degradation is hindered during aging have been found to be the substrates of lysosomal degradation. This observation connects autophagy to the process of aging. With the onset of aging, macroautophagy gradually subsides, consequently leading to the reduced formation of autophagic vacuoles and improper fusion of the vacuoles with the lysosomes. This finally causes a significant impairment in the protein flux with an accumulation of autophagic vacuoles in old tissues [11,12]. Additionally, the diminished fusion of vacuoles with lysosomes is also attributed to the oxidation of lipids and proteins in the membrane of the lysosomes that make them fragile and unable to fuse with the vacuoles. Intriguingly, the inactivation of many autophagic proteins, such as VPS30/ATG6/beclin1, has been shown to diminish the lifespan extension in *C. elegans* suggesting that autophagy is essential for increasing the lifespan [11].

Many reports have well documented that signaling pathways play a pivotal role in controlling longevity. The most studied of these pathways is the IGF-1 pathway [11,12]. A disruption of this pathway has been found to extend longevity in different groups of species starting from *Caenorhabditis elegans (C. elegans)* to humans via the mitigation of stress responses [11]. The IGF-1 pathway includes PtdIns 3-kinase, tyrosine kinase receptor, and Akt/PKB. Interestingly, Akt/PKB has been discovered to be a potent positive regulator of mTOR, which is an autophagic inhibitor. Therefore, the deregulation of Akt/PKB pathway may induce autophagy confirming the linking of the IGF-1 pathway to autophagy, which is extensively connected with the aging process. To further support the role of autophagy in aging, a report demonstrated that the lifespan could be extended when TOR kinase/let-363 is depleted [13]. In the context of the role of insulin in aging, it has been found that high levels of insulin in the plasma have a profound effect on aging. In an experiment, the administration of an anti-lipolytic drug, ACIPIMOX, which is known to decrease the insulin level and plasma-free fatty acids, was shown to decrease aging by enhancement of autophagy [14]. Recent reports illustrate the role of sirtuins in autophagy during aging. Sirtuins belong to the family of NAD-dependent deacetylases that are associated with an increase in longevity. The decline in the role of sirtuin1 (Sirt1) has been related to the decline in turnover of autophagic activity. Interestingly, increase in the expression of Sirt1 has been shown to accelerate autophagy as depicted through the accumulation of LC3 II and the formation of GFP-LC3 puncta to regulate aging, indicating a sirtuin-mediated strong relation between autophagy and aging. Moreover, experimental evidence shows that Sirt1-/- mouse has increased p62 levels, which is a marker for impaired autophagy [15].

Consistent with the above findings, skeletal muscles of aged mice displayed decreased autophagic activity as reflected by the reduction in the conjugation of LC3B-I with PE upon decreased expression of Atg12-Atg5 and Atg3 protein levels. Apart from the alteration in conventional autophagy, selective autophagy gets affected during aging. For example, the expression of LAMP2A, Hsc70, and Drp1, PINK1 and PGC1α that regulate mitochondrial autophagy has been reported to decline with the progress of aging [16]. Similarly, recent experiments have demonstrated a decline in the mitochondrial biogenesis and autophagy in *triceps brachii* muscle of aged horses. The autophagy marker proteins, Atg5 and LC3-II, were found to decline in aged muscles than in young ones along with a decline in LAMP2, suggesting an impairment in autophagosome-lysosome fusion [17]. Further, the detrimental effect of aging on autophagy was experimented to be exaggerated by the overexpression of mitochondrial aldehyde dehydrogenase (ALDH2), an enzyme with a single-point mutation in humans. The overexpression of ALDH2 decreased longevity and compromised autophagy that included a decline in the phosphorylation of IKKβ, AMPK, and TSC2, and an increase in the mTOR phosphorylation [18]. The impairment in autophagy is also observed in old stromal vascular fractions (SVFs) that are related to the release of pro-inflammatory cytokines (MCP-1 and IL-6) [19]. Moreover, an experiment on mice has demonstrated that glycogen synthase kinase-3β (GSK-3β) has a role to play in aging and found to be inter-related with autophagy. GSK-3β has been found to diminish the cardiac aging via Ulk1 [20].

4. Cancer: the autophagic dilemma

Autophagy has been found to play an interesting role in cancer biology. It is considered to be a tumor-suppressive mechanism during tumor initiation and malignant transformation. It exerts its inhibitory effect on cancer by removing damaged cells and organelles, thereby limiting cell proliferation and genomic instability. An effective link between autophagy and tumorigenesis is accomplished by tumor suppressor p53 gene, a mutation in which significantly facilitates the progression of cancer. Earlier reports revealed that the gain of function of mutant p53 counteracts the autophagic process through Akt/mTOR pathways. Therefore, p53 shows an inverse relationship with autophagy, thereby regulating cancer progression [21]. Among the

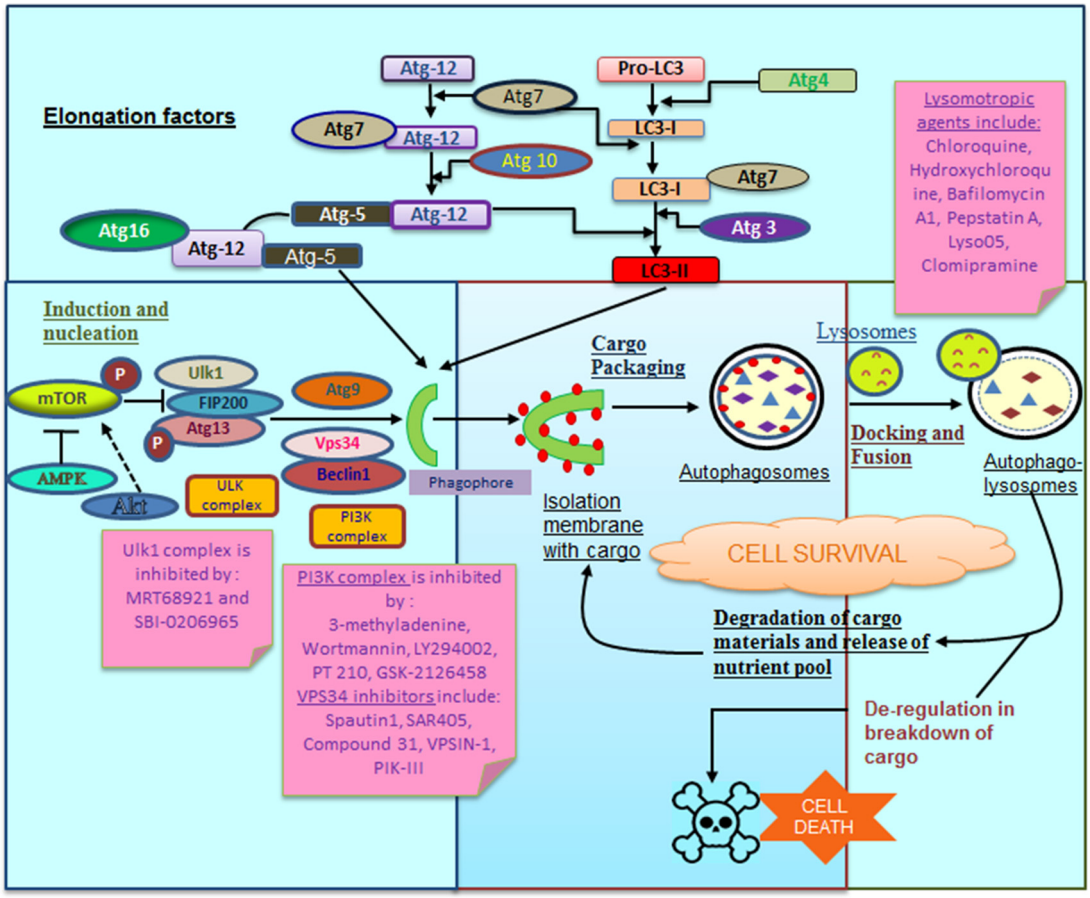

Fig. 1. The general process of macroautophagy.
The initiation of autophagy is regulated by Ulk1-FIP200-ATg13 complex which is further assisted by Vps34, Beclin-1 and Atg9 leading to the formation of a phagophore. The elongation of the phagophore is facilitated by two ubiquitin-conjugation systems, Atg12-Atg5-Atg16 complex and PE conjugated-LC3II (Atg8) system transforming into autophagosome with the accumulation of cytosolic contents. The autophagosomes then fuses with the lysosomes, which thereby degrades the autophagosomal contents. Deregulation in the breakdown of autophgosomal contents may sometimes lead to cell death.

autophagy genes, Beclin 1 is reported to be a tumor-suppressor gene. It is observed that Beclin1 locus is deleted up to 75% in ovarian cancers and up to 50–70% in breast cancers [22]. It has also been reported that epidermal growth factor receptor (EGFR) inhibits autophagy by binding to Beclin1, which is involved in autophagic induction, thus allowing the cancer cells to survive against stress conditions. Additionally, it has been witnessed that administration of cetuximab drug inhibits EGFR through suppression of miR-216b that is involved in the inhibition of translation of Beclin1. Therefore, deactivating EGFR favors the up-regulation of Beclin1, thereby favoring autophagy in cancer cells [23]. A knockdown of Atg4 has also been shown to increase the susceptibility to fibrosarcoma in response to carcinogens [24].

On the contrary, evidence also suggests that autophagy plays a striking protective role in cancer cells. Highly burgeoning cancer cells require cellular building blocks for their metabolism and energy production. At this stage of cancer development, autophagy acts as a friend providing all the essential cellular intermediates to satisfy the metabolic demands of proliferating tumorigenic cells. To validate the protective role of autophagy in cancer cells, it has been demonstrated that tongue squamous cell carcinoma (TSCC) shows cisplatin resistance via autophagy activation. The treatment of TSCC with chloroquine (CQ) and Beclin1 siRNA increased cisplatin sensitivity, strengthening the fact that autophagy inhibition can be a potential target for treating TSCC [25]. Nonetheless, the resistance property of the oral squamous cell carcinoma (OSCC) against cisplatin has also been shown to be regulated by increased autophagic flux. The Fadu-CDDP-R (Fadu cisplatin resistant) cells showed increased autophagic markers, such as Beclin1, Ulk1, Atg5, Atg7, and Atg14. In the same context, the surface resistant marker, CD44 was found to be decreased in Atg14-deficient Fadu cells [26]. Other reports show that the over-expression of phospholipase C (PLC) reduced autophagy in response to anti-cancer drug oxaliplatin and induced cell death. These observations emphasize the negative effect of PLC on autophagy and attest that autophagy promotes tumorigenesis. Additionally, miRNA has been found to induce autophagy in colorectal cancer, thereby enhancing cancer progression as well as chemotherapeutic resistance. miR-18a and miR-124 induces autophagy, promoting cancer progression whereas miR-210 provides chemoresistance by inducing autophagy [27]. As another example, in prostate cancer (PC3) cell lines, celecoxib has been found to induce apoptosis. Additionally, it exerts its protective role against prostate cancer cells by stimulating JNK-mediated autophagy. Therefore, the obstruction of the JNK-mediated autophagy can be a strategy to inhibit prostate cancer [28].

Autophagy has been shown to play a prominent role in cancer progression by favoring cellular metabolites and redox homeostasis. During hypoxic conditions, cancer cells consume glucose through anaerobic glycolysis, known as Warburg effect to provide unlimited pool of glycolytic intermediates. For example, glycolytic enzyme pyruvate kinase M1 (PKM1)-activated autophagy contributes to tumor malignancy in $Kras^{G12D}$ mouse model. Moreover, PKM1-Atg7 knockout mice showed decreased tumor growth as compared to wild type ATG7 cells. [29]. In addition, cancer cells exhibit higher glutamine utilization leading to autophagy induction through mTOR inactivation for survival of the cancer cells in harsh microenvironmental conditions. [30].

Autophagy in endothelial, stromal and immune cells present in tumor microenvironment has been documented to play prominent role in modulation of cancer growth and progression. The features of the tumor microenvironment including deprived nutrient condition, limited energy, hypoxia are responsible for inducing autophagy through different pathways leading tumor development to metastasis. It showed that autophagy in innate immune cell including NK cells, macrophages and neutrophil play a dual role in regulating tumor growth and progression in context dependent manner [31]. Moreover, autophagy in dendritic cells (DCs) facilitates the efficient processing and presentation of the intracellular antigens on MHC class I or II [32]. In addition, autophagy promotes the liberation of ATP and damage-associated molecular patterns (DAMPs) from dying tumor cells and therefore presenting it to the $CD8^+$ cytotoxic lymphocytes that results in the clearance of the tumor cells [33]. Cancer associated adipocytes (CAAs) and cancer associated fibroblasts (CAFs) has found to exhibit increase in autophagic flux in response to hypoxia and ROS production by malignant cells as compared to normal counterparts [32]. On the other hand, it was demonstrated that lncRNA CPS-IT suppressed metastasis and epithelial-mesenchymal transition by inhibiting hypoxia-induced autophagy in colorectal carcinoma suggesting the fact that inhibition of autophagy inhibits metastasis [34].

5. Diabetes and obesity: fit or fat; sugar or not: the autophagic verdict

Type 2 diabetes and obesity are among the most emerging health problems worldwide. The factors that are responsible for the establishment of diabetes include decreased insulin production, rise in adiposity, and an increase in insulin resistance in skeletal muscles. The increase in adiposity and insulin resistance are primarily attributed to defective mitochondria characterized by impaired beta-oxidation, accumulation of lipids, oxidative stress, and hence mitochondrial damage. Autophagy, specifically mitophagy, at this stage eliminates oxidative stress and damaged mitochondria, thus playing a protective role against the development of insulin resistance and increase in adiposity [35,36].

Endoplasmic reticulum (ER) stress resulting from accumulation of misfolded proteins in ER lumen stimulates a chain of adaptive responses termed as the unfolded protein response (UPR). Glucose related protein (GRP78/BiP), key ER chaperone essential for the activation of the ER-transmembrane signaling molecules. The three major transducers of ER stress-PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) facilitate for sensing the presence of unfolded proteins and signal transduction to the cytosol or nucleus [37]. The UPR of pancreatic beta cells is regulated by autophagy. It has been observed that the autophagy-deficient pancreatic cells are susceptible to ER stress that is involved in the progression of diabetes. The UPR machinery is compromised in autophagy-deficient beta cells making them prone to ER stress in vitro. The autophagy-deficient mice, when bred with obese mice to induce ER stress, were found to develop severe diabetes with the reduction in beta cell survival and accumulation of ROS as shown by nitrotyrosine staining [38]. For example, an increase in the ER stress and a defect in insulin signaling pathway have been found both in vivo and in vitro during the repression of Atg7 expression. While restoring the Atg7 expression, the ER stress was limited with the enhancement in the effect of insulin in obese mice [35]. Thus, autophagy is essential for UPR machinery, and autophagy-deficient cells can result in the development of diabetes from obesity. The expression of UPR genes *Eif2α, Chop, Ero1α, Bip, Grp94, Erp72, Ubc7, Hrd1, Edem, Erdj4* was found to be significantly downregulated in autophagy deficient pancreatic beta cells compared to rat insulin promoter autophagy deficient mice, thereby suggesting that basal UPR is downregulated in autophagy deficient mice [38]. It was experimentally found that mice with only obesity or autophagy-deficient beta cells developed hyperglycemia but not diabetes. On the contrary, mice with both obesity and defective autophagy in pancreatic beta cells developed severe diabetes [38]. Recent investigations have found the role of autophagy in controlling insulin signaling and lipid metabolism. Improper processing of the lipid affects the functioning of the liver and may reduce the effect of insulin [39]. Whereas constitutive adipogenesis has been studied to be regulated by autophagy, suppressing the autophagic activity has an anti-obesity effect and is sensitive to insulin. Atg7 depletion in the adipose tissue of high-fat diet resulted in sensitivity toward insulin with the resistance to obesity [40]. Nevertheless, an increase in the accumulation of ubiquitinated protein aggregates was observed in INS-1 cells (a mouse insulinoma cell line) when they were treated with high glucose. Upon the inhibition of proteasome machinery, there was no alteration in the ubiquitinated proteins in INS-1 cells. When the cells were treated with an autophagic inhibitor 3-methyladenine (3-MA), the level of aggregated proteins increased in the presence of high glucose [36]. These observations, therefore, suggest that autophagy is the sole process for the regulation and degradation of ubiquitinated proteins in INS-1 cells. Similarly, the depletion of Atg7 causes accumulation of protein aggregates [41]. In an experiment, a type 2 diabetes drug, metformin, was shown to decrease the formation of autophagic vacuoles in type 2 diabetes as well as NEFA-treated beta cells. Metformin enhances AMP kinase activity that is known to inhibit mTOR pathway. Inhibition of mTOR pathway, in turn, leads to the removal of autophagic vacuoles. Therefore, metformin helps in the removal of autophagic vacuoles by inhibiting the action of mTOR on autophagy [42]. Similarly, exendin-4 that controls the level of glucose has also been found to regulate the autophagic markers, such as mTOR, LC3-II, LAMP1, parkin, Atg7, and p62, further validating the fact that autophagy plays a crucial role in regulating diabetes [43]. Another study put stress on the contribution of autophagy on diabetes where the lack of autophagy in skeletal muscles activated Atg4 that helped in the induction of Fgf21 expression. The activation of Fgf2 enhanced oxidation of fatty acids and therefore energy expenditure along with the white adipose tissue (WAT) browning. This resulted in resistance to high fat diet induced (HFD-induced) obesity. Therefore, the Atg7 mutant HFD-mice showed a decline in insulin concentration and better glucose homeostasis [44]. Interestingly, it has been investigated that exercise increases the autophagic turnover and mitochondrial fission in type 2 diabetes with an increase in the levels of Atg7 and p62/SQSTM1 and decrease in LC3-II protein [45].

6. Microbial pathogenesis, inflammation, and immunity: fight or flight: the autophagic response

Autophagy controls the intracellular pathogens in response to different types of infections. In many cases, it has been noticed that eukaryotic pathogens use their autophagic machinery for successful pathogenesis. On the other hand, intracellular pathogens can be sequestered in autophagosome for their degradation by lysosomes. This selective elimination of invading pathogens through autophagy is termed as xenophagy. The induction of xenophagy results with protein ubiquitination present on the pathogen-associated phagosomes. The adapter proteins including NDP52, optineurin and p62 binds to the ubiquitinated protein which further escorts the autophagic proteins required for the autophagosome formation. NDP52 also cooperates with a β-galactose-binding lectin, galectin-8. As a result of rupture of phagosomal membrane by pathogens, NDP52 and galectin-8 access the β-galactose chain of phagosomes resulting in the induction of xenophagy [46,47]. This specific pathway can be linked to various aspects of adaptive and innate immunity, including antigen presentation, cytokine and interferon production, and lymphocyte development. The relationship between classical autophagic response and microbial infection to adapt inside the host system rules the consequence of host-microbe interaction for disease growth and progression [46]. Autophagy is activated in most bacterial infections. During *Listeria monocytogenes* and *Staphylococcus aureus* infections, it has been reported that TLR2

stimulation induces autophagy [47]. TLR9 recognizes the CpG motifs of bacteria, which activate AMPK signaling pathway thus stimulating autophagy [48]. A very recent experiment validated the anti-mycobacterial effect of calcimycin against *Mycobacterium bovis* BCG as a result of autophagic up-regulation through an increase in Atg1, Atg7, Atg3, and LC3-II conversion. The effect of calcimycin was observed to decrease in the presence of an autophagic inhibitor 3MA [49]. In contrast, there are certain microorganisms, such as *Brucella abortus* and *Porphyromonas gingivalis*, where autophagy plays a protective role. Here, autophagosomes are prevented from the fusion with the lysosomes so that the bacterial content is protected against the lysosomal hydrolases and the bacteria use their hydrolases to process the autophagosome as components for energy. The action of *Legionella pneumophila* is even more critical. The bacteria reach the autophagolysosomes that already contain hydrolytic enzymes such that the lytic enzymes process the host-sequestered products that are used by the bacteria for to live [50]. It has been inspected that the infection of Herpes simplex virus stimulates autophagy to remove the viral particles [51]. An experiment confirmed the aforesaid statement where deletion in the dendritic cell Atg5 of mice showed impaired CD4 + T cell priming of the viral antigen after Herpes infection resulting in rapid disease condition [52].

Microbial infection is accompanied by an activation of the immune system of the host system where autophagy serves as a part of innate immunity, thereby eliminating the foreign pathogens [53]. Inflammasomes are cytosolic proteins that are produced in response to the invading pathogens. After activation, it proteolytically cleaves the pro-inflammatory cytokines IL1B. The canonical inflammasome comprises of PYCARD/ASC adaptor, pro-CASP1 (caspase 1) and some proteins for sensing microbial products including NLRC4, NLRP1 and NLRP3 along with endogenous agonists especially mitochondrial ROS and lysosomal damage. After the assembly of inflammasome components, CASP1 cleaves pro-IL1B to make ready to be secreted from the cells. It has shown that autophagy inhibits inflammasome activation through clearance of the damaged mitochondria and other organelles thereby ROS generation cannot activate the inflammasomes [54]. The anti-microbial defensive role of autophagy is found to be controlled by Th1/Th2 polarization. The Th1 cytokines induce autophagy, whereas Th2 cytokines inhibit it [53]. The pathogen-associated molecular patterns (PAMPS), which are present on the surfaces of microbial pathogens, have been found to activate the autophagy through regulation of Toll-like receptors (TLRs), present on the surfaces of macrophages and dendritic cells, cellular sensors for PAMPS. Thus, the invading pathogens are removed by autophagy response [55]. Autophagy is also induced by the bacterial LPS and TLR4 ligands, which belong to the diverse group of PAMPs, in macrophages [56]. Contrary, LPS has been shown to induce the secretion of IL1-b and IL-18 during deficiency of Atg161L, an essential component for autophagosome formation [57]. Similarly, autophagy inhibitors have been found to increase the activation of LPS-induced inflammasomes [58]. Further evidence proves the fact that Atg5 deletion causes the enhancement of both interferon-alpha and interferon 1 in response to single-stranded RNA viruses [59]. Atg16l1 and Atg7 deletions, with a deficiency in Beclin1 and LC3B in monocytes and macrophages of the mouse, cause the enhanced secretion of IL-1beta and IL-18, the pro-inflammatory cytokines in response to LPS. However, deletions of Atg16l and Atg7 had no effect on the production of TNF and IFN-beta [60]. The Epstein-Barr virus infection demonstrated reduced MHC-II antigen presentation when Atg12 was genetically altered [61].

Crohn's disease, an inflammatory bowel disease that affects the gastrointestinal (GI) tract, is characterized by a polymorphism in ULK1 autophagy gene due to the presence of single nucleotide polymorphisms (SNPs). Thus, the autophagic process is impaired during the disease. The mutations in the leucine-rich domain of nucleotide oligomerizing domain-containing protein2 (NOD2) contain three mutations that are also associated with Crohn's disease. NOD2 plays an effective role in the recruitment of ATG16L in the plasma membrane during bacterial invasion. Therefore, a mutation in NOD2 causes impairment in autophagy induction, antigen presentation, and bacterial trafficking. These defects could, therefore, cause persistence of bacterial inflammation [62]. Accumulating evidence has thus suggested autophagy to play a major role in Crohn's disease. Genome studies have revealed the presence of SNPs in Atg genes, e.g., Atg161L, which affects the autophagic process in the disease. Atg161L plays a major role in the formation of autophagosome. Therefore, deletion of Atg161L leads to impaired autophagosome formation; moreover, in macrophages, it enhances the production of IL-1beta in response to LPS [63,64]. MAP1S has been recently identified as an autophagy-related protein that interacts with LC3 and undergoes biogenesis of autophagosomes. It has also recently been found to be significantly upregulated with an increase in autophagic flux and the activation of Wnt/beta-catenin signaling [65].

7. Lysosomal storage disorders: the autophagic management issues

Lysosomes are intracellular organelles with an acidic pH and play an integral role in the clearance of cellular materials and numerous other cellular processes, such as those involved in maintaining cholesterol homeostasis, degradation of cellular constituents, fighting against invading microorganisms, and plasma membrane repair. A defect in their functioning may result in lysosomal storage disorders (LSDs) manifested by the inability of the lysosomes to remove undigested molecules. Therefore, a major restraint in the cellular functions is observed in LSDs. Lysosomes have also been found to play a prominent role in an autophagic process characterized by their fusion with autophagosome followed by the digestion of its contents. Considering the profound effect that lysosomes have on autophagy, LSDs are expected to have a major impact on autophagy and vice-versa [66,67].

Various LSD models have revealed the association of defective lysosomal functioning to impaired autophagy, which includes an increase in the number of autophagosomes, reduced organelle turnover, and defective clearance of autophagic substrates. The impairment of autophagosome-lysosome fusion in LSD is attributed to the defect in the vesicular trafficking. Moreover, it is observed that the lipid composition changes during LSDs. For instance, cholesterol and sphingolipids primarily accumulate in LSDs, resulting in the formation of lipid rafts, which, in turn, affect the dynamics of the lysosomal membranes leading to the impaired fusion with the autophagosomes [66]. It has been investigated that autophagy block leads to an accumulation of toxic proteins and dysfunctional mitochondria, the consequent result of which is apoptosis. Moreover, the cells manifesting defective autophagy are prone to mitochondria-mediated apoptosis. This subject has grasped significant attention such that administration of bafilomycin A1, an attenuator of lysosomal acidification, causes blockage of autophagosome-lysosome fusion, for example, in LSDs, deciphering the role of lysosomal dysfunction as a feedback system to inhibit the fusion between autophagosome and lysosome [68].

Danon disease occurs due to the deficiency of lysosomal-associated membrane protein 2 (LAMP2). LAMP 2 has three isoforms, namely LAMP2a, LAMP2b, and LAMP2c, among which LAMP2a serves the dominating role of a receptor in chaperone-mediated autophagy. LAMP, in general, has been found to be involved in the formation of mature vacuoles from early autophagic vacuoles, suggesting the involvement of LAMP in the fusion of autophagic vacuoles with endosomes and lysosomes. Therefore, deficiency of LAMP2a disturbs the autophagic process [67]. It has been experimentally proved that LAMP1 and LAMP2 double-deficient cells delay the recruitment of RAB7, a regulator of endo-lysosomal trafficking, to the late endocytes, thereby disrupting the autophagic process [69]. Another interesting experiment demonstrated the impact of Danon disease on selective autophagy where Danon human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) demonstrated an accumulation of defective mitochondria,

impaired mitochondrial flux, and an impaired mitochondrial respiratory capacity. Restoring the LAMP2a recovered all the defective mitochondrial outcomes strongly ascertaining about the association of Danon disease with impaired mitophagy [70].

Pompe disease is accompanied by the deficiency of the lysosomal α-1,4-glucosidase that makes the cells unable to hydrolyze glycogen to glucose, thereby resulting in the accumulation of glycogen in lysosomes, especially in skeletal muscles and cardiac tissues. This accumulation results in the structural disorganization of the cells, cellular dysfunction, and an impaired autophagy. The impairment of autophagy is a consequence of enlarged glycogen-filled lysosomes and their inability to fuse with the autophagosome, thereby causing an accumulation of autophagic debris. This becomes one of the major hindrances in the enzyme replacement therapy (ERT) [71]. A recent experiment on α-glucosidase knockout (GAA KO) mice model with Atg7 deficient established successful ERT to clear the lysosomal glycogen in expanding muscle fibers [72]. Yet in another interesting experiment where autophagic debris was avoided through the transfection of shRNA-TSC2 in GAA KO mice, the mTOR activity was activated, thereby providing a successful outcome for ERT [73].

Gaucher disease is caused due to a mutation in GBA1 gene and is associated with defective glucocerebrosidase, an enzyme involved in the degradation of glucosylceramide and glucosylsphingosine [74]. Therefore, it results in the toxic accumulation of glycolipids in the neuronal cells. The autophagy-lysosome pathway, responsible for the clearance of aggregated materials, is defective in GBA1-mutant cells. In an experiment, GBA1-mutant neuronal stem cells showed a decrease in transcription factor EB (TFEB), leading to a decrease in the number of lysosomes and promoting autophagic block. Additionally, TFEB is regulated by mTORC1 at the surface of lysosomes. It has been witnessed that the activity of mTORC1 is elevated in GBA1 mutants, thus regulating TFEB and hence deteriorating the autophagic flux [75].

8. Muscular disorders and heart disease: fatigue: the autophagic hesitation

The role of autophagy in maintaining muscle homeostasis has recently been studied. Autophagy, with its vast array of functions, plays an interesting and dual role in protecting and damaging the myofibrils. Similar to neuronal cells, muscle cells are also non-proliferative that causes excess accumulation of damaged materials within them. Therefore, an increase in the levels of autophagic vesicles serves as the diagnostic marker in any muscular atrophy. Autophagy-related genes that are up-regulated/down-regulated in different muscular disorders belong to the class of “atrogenes” or atrophy-related genes [76].

The protective role of autophagy has been validated in many publications. The muscle protein, myotubularin, regulates the concentration of phosphatidylinositol that is required for vesicular trafficking during autophagy. Myopathy is manifested by a defect in the muscle protein demonstrating the dysregulation of autophagy [77]. In an experiment where lysosomal membrane protein (LAMP2) was knocked out during myopathy showed an impairment in autophagy. Despite the accumulation of autophagic vacuoles in liver and heart cells, the protein degradation was not achieved owing to the reduced fusion of autophagosomes with lysosomes [78]. The administration of autophagic drugs further validates the role of autophagy in muscular disorders. For instance, chloroquine, a lysosomotropic agent, resulted in the induction of myopathy in cultured cells. Chloroquine is responsible for the elevation of lysosomal pH that disrupts the fusion of autophagosome with the lysosome and consequently reduced protein degradation [79]. Conversely, it has been demonstrated that chloroquine treatment results in the increase in expression of Atg5-Atg12 and Beclin1 protein of rat pulmonary artery smooth muscle cells [80]. Hence, the accumulation of autophagic vacuoles in myopathy may be due to reduced autophagy. In accordance with the context, a decline in ULK1 in skeletal muscles during fasting has been shown to accumulate LC3-I causing muscle atrophy, suggesting impairment in the conversion of LC3-I–LC3-II, which is indispensable for the autophagic process. Whereas knockdown of Ulk-2 did not cause any change in LC3, it resulted in the accumulation of p62 protein [81].

Various cardiovascular diseases show a high occurrence of autophagosomes. Whereas basal autophagy is required for the proper functioning of cardiomyocytes [82], the deletion of Atg5 gene in myocardium shows a high risk of cardiomyopathy [83]. Hamacher et al. observed that an upregulation of Bnip3, a mitochondrial protein, stimulates apoptotic cell death in cardiomyocytes. Its activation elevates autophagy that counteracts the apoptotic cell death by diminishing it [84]. Additionally, Munteanu et al. have recently identified VMA21 as a chaperone of V-ATPase, which facilitates proton pump and acidifies the organelles. Mutation in VMA21 increases the lysosomal pH, and therefore affects the final autophagic degradation and accumulation of autophagic debris. This forms the basis of many cardiac diseases, and hence loss of function of VMA21 gene has been characterized as one of the reasons for a cardiac disease linked through disrupted autophagy [85]. Recently, the role of small RNAs in cardiovascular diseases has also emerged to be promising. For instance, overexpression of miR-199a evoked cardiac myopathy by suppressing the autophagy through mTOR activation. This finding was further validated by the accumulation of p62 and decrease in lipidation of LC3-II with miR-199a overexpression [86].

The damaging role of autophagy is also very fascinating. The ischemic heart disease results in elevated levels of calcium in the cells, a potent activator of autophagy. The calcium mobilizing factors, such as ATP, ionomycin, and thapsigargin, inhibit mTOR, resulting in the accumulation of autophagosomes in a Beclin-1- and Atg7-mediated manner [87]. ROS is also produced during ischemic heart disease, which is a potent activator of autophagy. Moreover, LPS treatment in cardiomyocytes resulted in an increase in both autophagy and ROS levels in newborns [88]. Some studies have also pointed that down-regulation of Beclin1 and 3MA treatment caused reduced cell death in I/R cardiomyocytes [89].

9. Neurodegenerative disorders: the autophagic wiring

As neurons are post-mitotic cells and are unable to undergo cytokinesis, aggregated proteins cannot be diluted by cell division. Hence, the common basis of neurodegenerative disease that portrays the accumulation of abnormal protein aggregates inside the neuronal cells can be avoided through autophagic degradation. Thus, activation of autophagy plays a critical role in neurodegenerative therapeutics providing an enthralling platform in the field of medical science [90].

9.1. Parkinson’s disease

The clearance of misfolded proteins in the healthy cells is mediated mainly through the ubiquitin-mediated or autophagic pathway. In Parkinson’s disease (PD), both these pathways are disturbed leading to the accumulation of misfolded proteins [90]. PD has been examined to contain mutations in six genes, namely, SNCA, glucocerebroside (GBA), Parkin (PARK2), PTEN-induced putative kinase 1 (PINK1), leucine-rich repeat kinase 2 (LRRK2), and DJ1, which are responsible for the early occurrence of the disease [91]. One of the important distinguishing features of the disease is the presence of round, intracytoplasmic bodies called Lewy bodies that are present in the nucleus of the neurons. These bodies consist of an insoluble aggregated protein called α-synuclein, which is susceptible to degradation through chaperone-mediated autophagy (CMA) [92,93]. Familial PD is characterized by the inability of the lysosomes to take up mutant α-synuclein due to the very high affinity of the LAMP-2A, a lysosomal receptor. The high-binding affinity does not allow the substrate to be translocated properly and blocks the uptake of substrates for CMA, thus preventing degradation [94]. SNCA is also observed to be degraded by the CMA pathway through the

recognition by LAMP2A receptor and HSC70 chaperone, as SNCA contains a KFERQ motif. The mutant forms of SNCA can bind to LAMP2A receptor but fail to localize to the lysosomes [95]. Overexpression of a key component of autophagosome assembly, RAB1A, has shown to improve the shortcomings of the SNCA-expressing neurons [96]. Experimental evidence in *Drosophila* shows that SNCA-induced neuronal effect is reduced by one of the components of autophagic machinery, such as histone deacetylase 6 (HDAC6), involved in the autophagosome maturation [97]. A decline in the levels of HSC70 chaperone and LAMP2A receptor has been observed in the brain of patients with PD [98]. Moreover, SNCA proteins contain two missense mutations at A53T and A30P positions, respectively, that block the uptake by lysosomes thus inhibiting autophagy [99]. It has been investigated that increase in the level of intracellular α-synuclein impairs the autophagic process by inhibiting Rab-1A, a small GTPase and an autophagosome assembly protein [100]. Down-regulation of CMA proteins by micro-RNA increased the level of SNCA, further validating the fact that degradation of SNCA proteins can be regulated by autophagy [101]. Interestingly, it has been detected that with increasing age, neurons become more susceptible to accumulation of SNCA owing to disability in the removal of aggregated proteins by autophagy [90].

PD is also characterized by a mutation in another gene, known as the Parkin gene, which is an E3 ligase that targets proteins for degradation. PINK1, along with Parkin, is required for targeting of the damaged mitochondria [102]. Moreover, PINK1 gene, a regulator of autophagy, acts by interacting with Parkin and controls the mitochondrial balance [103]. Adding to the observation, mutations in the Parkin gene alone is also responsible for the establishment of PD; the mutant Parkin does not allow it to ubiquitinate voltage-dependent anion channel 1 (VDAC1), thereby non-clearance of damaged mitochondria [102].

Intriguingly, the expression of LC3-II in PD has been found to be induced by thioredoxin-interacting protein (TXNIP). Whereas it could not degrade p62, a substrate of autophagy, it repressed the expression of a lysosomal membrane protein, ATP13A2. The accumulation of α-synuclein was found to be induced by TXNIP that was attenuated by ATP13A2 overexpression. These suggest that TXNIP promotes the accumulation of α-synuclein by inhibition of ATP13A2 activity proving TXNIP to be one of the causes for PD [104]. PD is considered to be a disease of debate where a specific cause has not yet been deciphered. It was examined, in yet another experiment, that an alteration of NAD + metabolism may exist in PD promoting the activation of sirtuin-2 with a decline in the levels of acetylated-α tubulin. The increase in acetylated α-tubulin protein facilitated the clearance of misfolded proteins, caused by the inhibition of sirtuin-2 deacetylase expression. These suggest that sirtuin-2 regulates the autophagic process through acetylation [105].

9.2. Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia that is characterized by the accumulation of two proteins, namely Tau and amyloid β-peptide. It is well-established that deficiency in autophagic pathway forms the hallmark of AD. The Tau protein plays a role in microtubule stabilization and its hyperphosphorylation results in the loss of affinity toward microtubules, thereby tangling it. The removal of these proteins by autophagy is then required to suppress neurodegeneration [106,93]. Moreover, treatment of cells with 3-methyladenine, an autophagic inhibitor, results in more accumulation of tau protein, suggesting that autophagic system plays an evident role in the removal of aggregated tau protein [107].

The other protein amyloid β-peptide results from the degradation of the amyloid precursor protein (APP). The accumulation of amyloid β-peptide results in an impaired fusion of autophagosomes with the lysosomes, which, in turn, results in the accumulation of the protein. Additionally, a mutation in the gene that encodes presenilin 1 (PSEN1) forms one of the major characteristics of Alzheimer's disease. The mutation results in the impaired lysosome function and accumulation of amyloid β-peptide [93]. The accumulation of amyloid β-peptide and PSEN1 mutations are protected by UPR and autophagy in the AD. Only intracellular amyloid β-peptide and PSEN1 mutations trigger the ER stress by releasing calcium ions from ER via RYR and ITPR. Interestingly, PSEN1 mutations block the ER stress sensors, thus making the neuronal cells more susceptible to ER stress. Therefore, UPR plays a protective role in the AD though it can be disrupted either by PSEN1 mutations or accumulation of β-amylopeptide [108]. In addition to the ER stress, change in lysosomal pH also has an effective role in the AD. V0-ATPase is essential for the lowering of pH in the autolysosomes. It has been found that PSEN1 mutations cause a defect in the targeting of a1 subunit of Vo-ATPase from the ER to the lysosomes. Therefore, PSEN1 mutations lead to reduced proteolysis of substrates through autophagy in patients with AD, as the pH is not properly maintained in patients with PSEN1 mutation [109]. Phagocytosis of β-amyloid peptide causes disruption of lysosomes, leading to the release of cathepsin B (CTSB), a lysosomal proteolytic enzyme, which, in turn, activates NLR family, pyrin domain containing 3 (NLRP3) inflammasomes. Therefore, it leads to the release of neurotoxic factors through the activation of interleukin 1b (IL1B) pathway. Consequently, induction of the autophagic pathway through deletion of cystatin B (CSTB) reduces the deposition of β-amyloid peptide in AD mouse models [92]. Administration of different drugs that affect the distinct phases of the autophagic pathway has shown to be effective against AD. Rapamycin, an inhibitor of TORC1, enhances the clearance of amyloid β-peptide and tau protein in AD mouse models, validating the importance of autophagy in the removal of aggregated proteins [110]. Another experiment showed that use of the drug latrepirdine (commonly known as dimebon), which induces Atg5-dependent autophagy in the mouse brain cells, reduced β-amyloid pathology [95].

9.3. Huntington's disease

Huntington's disease is characterized by the CAG trinucleotide repeat that is expanded in the gene encoding for the huntingtin (HTT) protein. The HTT protein is involved in the microtubule function and vesicle formation. Mutation in the gene results in the aggregation of the protein. Other manifestations of this disease include impaired activity in the degradation of the autophagosome, accumulation of ubiquitinated protein aggregates, and therefore reduced levels of autophagy [111,112]. Dynein motor machinery is affected by certain mutations that damage the fusion of autophagosome-lysosome. This results in the non-clearance of abnormally aggregated proteins and an increment in the toxicity of mutant HTT protein in different disease models and human beings [113]. Moreover, in the autophagic process, ATG14 is phosphorylated at serine 29 by Ulk1 in an mTOR-dependent pathway. The phosphorylation of ATG14 regulates autophagic flux through the ATG14-Vps34 complex that mediates the clearance of polyQ residues removing HTT protein. This phosphorylation is compromised in mouse HD models strengthening the fact that autophagy is necessary in HD [114].

ER stress causes the release of HTT protein from the membrane and translocation into the nucleus. During the absence of ER stress, HTT protein is released from the nucleus and is reassociated back with the ER. The release of HTT protein is inhibited in HD when the protein consists of a polyglutamine expansion. Therefore, cells with mutant HTT protein show an increase in autophagic vacuoles along with deformed ER [115]. Treatment with trehalose reduces the pathologic effect of HD by binding to the polyglutamine expansion, thereby balancing the mutant HTT protein [116]. Moreover, mTOR-dependent autophagy is induced through the sequestration of mTOR into the HTT protein that inhibits the kinase activity, thus promoting the degradation of HTT and its clearance [117]. Insulin signaling pathway involving beclin1 and Vps34 also plays a role in the clearance of mutant HTT protein. Even during the activation of mTOR and Akt, the activation of

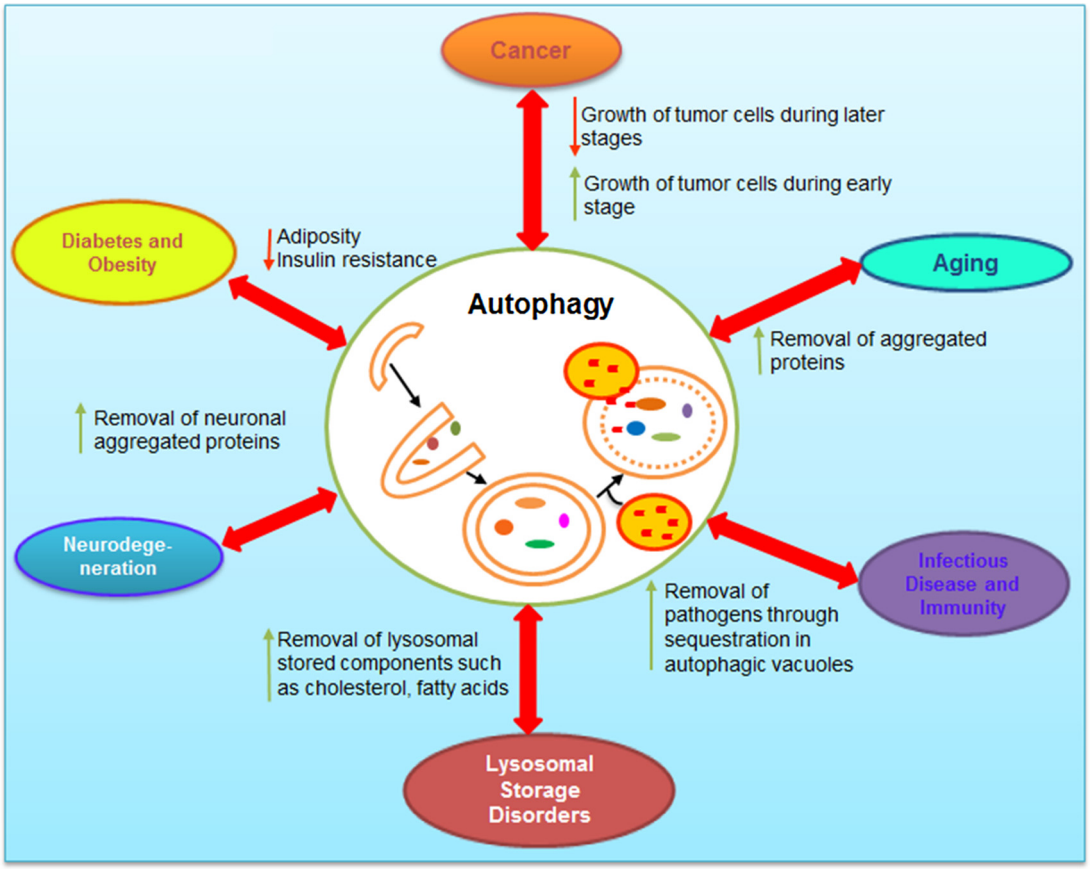

Fig. 2. Role of autophagy in various diseases.
The role of autophagy in aging is manisfested by the removal of aggregated proteins. Similarly, in neurodegenerative disorders, accumulation of neuronal proteins has been attributed to be removed by autophagy. Equivalently, autophagy show a protective role in the removal of lysosomal stored components thereby demeliorating lysosomal storage disorders. On the other hand, autophagy has also proved beneficial in evading various foreign pathogens, thereby contributing to the innate immunity. The role of autophagy in diabetes and obesity has been shown to decrease adiposity and insulin resistance. In the context of cancer, autophagy has shown to play a dual role where it serves as a tumor suppressor during initial stages but protects the tumor cells during the later stages.

Table 1
Role of autophagy in different diseases.

Diseases	Functional autophagy	Defective autophagy
Aging	The removal of aggregated proteins prolongs lifespan [11]	The reduced formation of autophagic vacuoles and the improper fusion of the vacuoles with the lysosomes lead to significantly impaired protein flux [11,17]
Cancer	Restricts the growth of the tumor [21].	Tumor growth is enhanced making the cells unable to undergo autophagic cell death [22].
• Early Stage • Later Stage	Favors survival of low-vascularized tumor cells [25].	Blocks the growth of the low-vascularized tumor cells [24].
Infectious Disease and Immunity	The removal of bacterial and viral pathogens through sequestration in autophagic vacuoles and degradation by lysosomal hydrolases, thereby conferring immunity against pathogens [49,51].	Provides a protective environment for pathogens due to the impairment of phagosomes with lysosomes [50].
Lysosomal Storage Disorders	The removal of lysosomal stored materials such as fatty acids, cholesterol, invading pathogens [66,67].	Increase in the number of autophagosomes, reduced organelle turnover and defective clearance of autophagic substrates [66,68].
Neurodegeneration	The removal of neuronal protein aggregates [91,92,95,109].	Accumulation of protein aggregates in neurons [92,112]
Diabetes and Obesity	Protection against the development of insulin resistance and increase in adiposity [35,36,40].	–

insulin receptor substrate 2 (IRS2) that mediates the insulin and IGF1 signaling pathway results in the clearance of the aggregated mutant proteins through autophagy [118].

10. Conclusion

The discovery of autophagy has emerged as a major breakthrough in both physiological and pathological conditions. Studies in various models and genetic approach have clearly pointed out the implications of lysosomes in diverse diseases, including neurodegenerative diseases, cancer, aging, heart diseases, lysosomal disorders, diabetes, and obesity. We aimed to provide a better understanding of how autophagy may contribute a new perspective to human diseases (Fig. 2) (Table 1). The impact of autophagic molecular mechanisms on diseases provided a surprising, sometimes contradictory, view of the autophagic effect. In a recent experiment it showed that, genetic manipulation of the Atg5 gene with lox*P* sequences before exon 3 in mice showed worsening of the clinical manifestations associated with Pompe disease [119]. Thus,

the activators and inhibitors of autophagy have become an exciting area for the drug development with the hope that modulating autophagic pathway could be a useful approach for the treatment of diseases. Certainly, the impact of autophagy on various diseases has shown promising results and become the new arena of research. Despite the role of autophagy in diseases, there are still some areas that need to be resolved. Moreover, the precise role of autophagy has still not been explored. Many experiments have shown that autophagy is either diminished or activated during a disease. But what is the exact process of autophagy by which this organelle regulates the progression of the disease? What is the basis of such differences in response and what extra studies should be undertaken to nullify these differences? Based on what we have reviewed, we hope that we have been able to provide a comprehensive view to reveal the role of autophagic modulators in diseases, apart from the apoptotic drugs, thus opening a new horizon in the treatment of lysosomal diseases.

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