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.

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doi: 10.1534/genetics.112.149013
Manuscript received December 21, 2012; accepted for publication March 7, 2013
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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

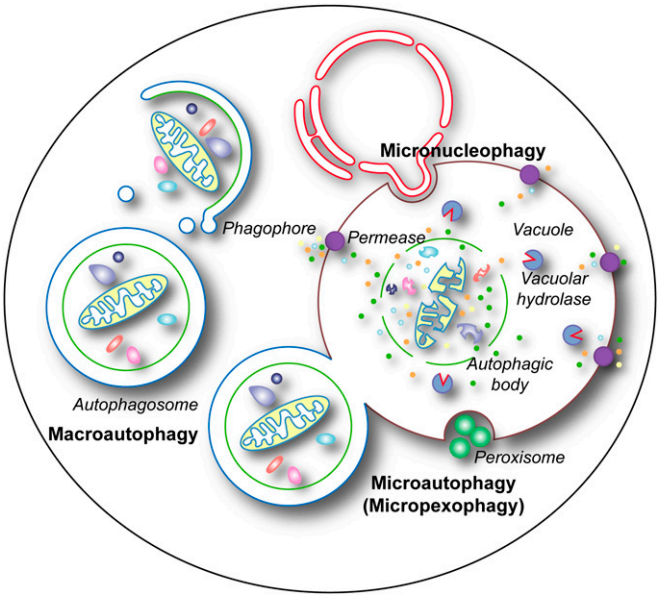

Figure 1 The principal types of autophagy in yeast. Macroautophagy entails the sequestration of bulk cytoplasm or specific structures into autophagomes. Autophagosomes are formed by expansion of a precursor compartment known as the phagophore, which initiates the sequestration of the cargo. Upon completion, 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

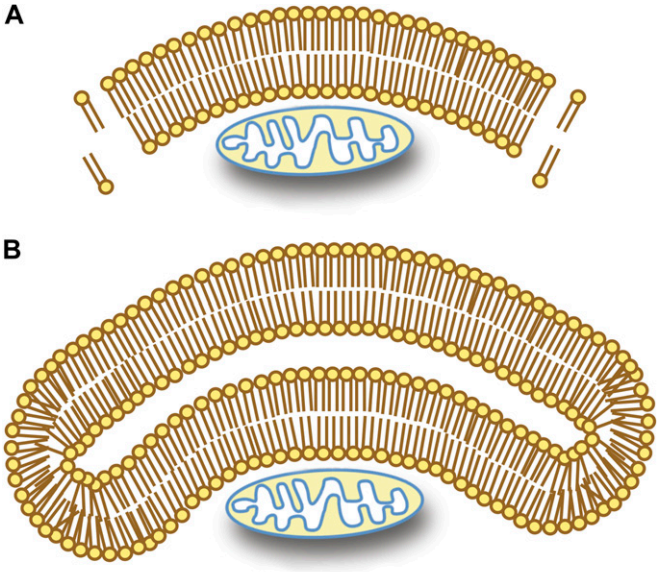

Figure 2 Sequestration of cytoplasmic cargo requires a double-membrane compartment. (A) Exposure of the hydrophobic core of a lipid bilayer to the aqueous cytosol would make it energetically unfavorable to use a single-bilayer membrane to sequester a cytoplasmic cargo. In this scenario, it would also 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.

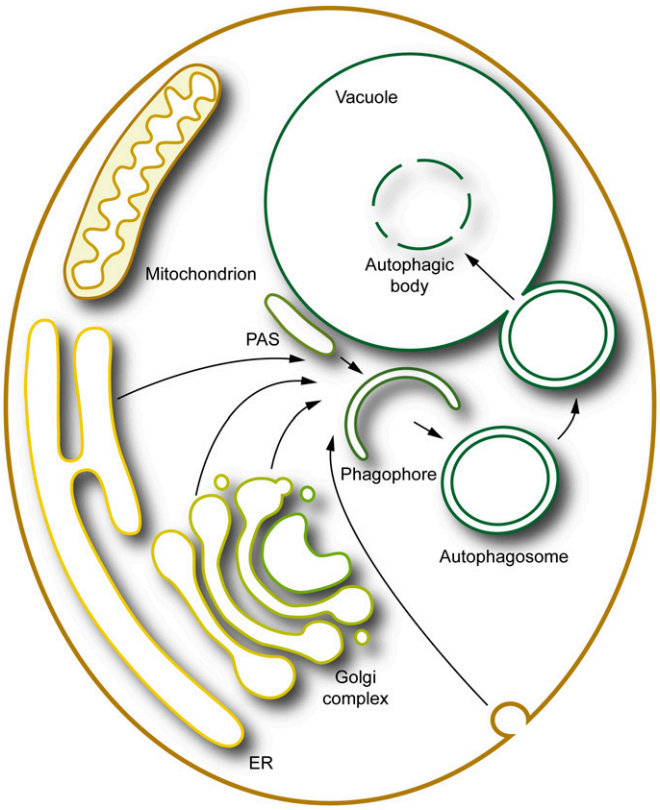

Figure 3 Multiple membrane sources may contribute to formation and expansion of the phagophore. Various compartments including the ER, the Golgi apparatus, and the plasma membrane may contribute to the nucleation and/or expansion of the phagophore. See the text for details.

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

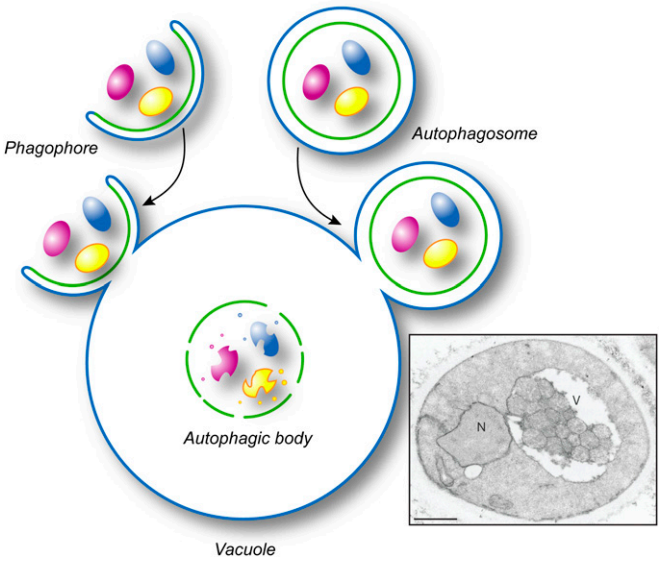

Figure 4 Topology of autophagosome fusion with the vacuole. Fusion of an expanding phagophore (*i.e.*, an incomplete autophagosome) with the vacuole (or lysosome in higher eukaryotes) does not allow delivery of the cytoplasmic content into the interior of the degradative organelle (left side of the drawing). 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

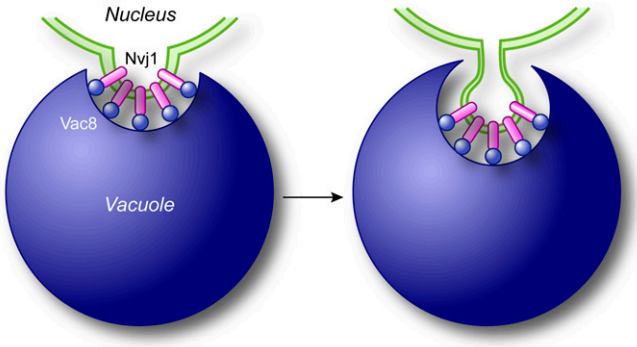

Figure 5 Mechanism of micronucleophagy. During micronucleophagy (also called piecemeal microautophagy of the nucleus), small portions of the nucleus, including the nuclear double membrane and part of the nucleoplasm, protrude into the vacuole lumen though a process that requires the association between 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

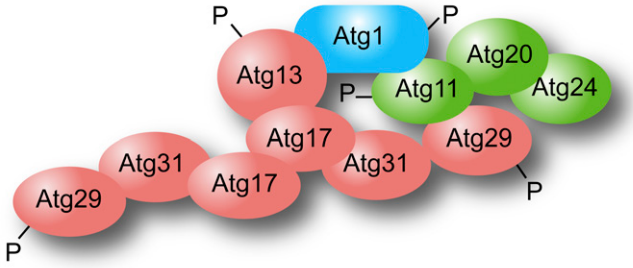

Figure 6 The interactome of the Atg1 complex. Note that there is no indication that all the depicted interactions occur simultaneously, and not all of the known interactions are shown; the Atg1 complex interactors could vary depending on both the step in the formation of the double-membrane vesicle and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

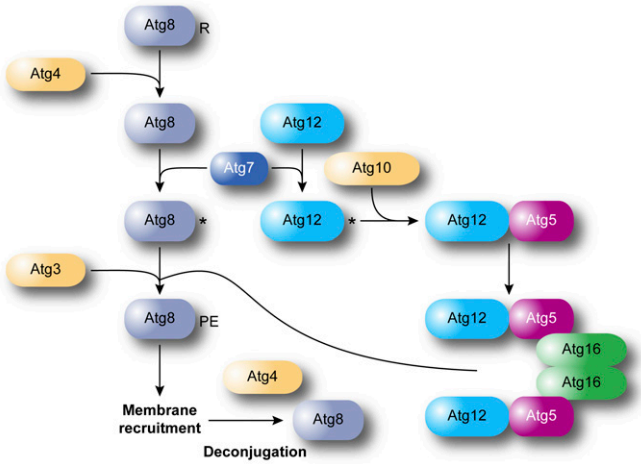

Figure 7 Schematic of the two ubiquitin-like conjugation systems involved in autophagy. Atg12, a ubiquitin-like molecule, is covalently conjugated to Atg5 through the activity of Atg7 and Atg10, an E1- and an E2-like enzyme, respectively. The Atg12—Atg5 complex subsequently associates with Atg16, and 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

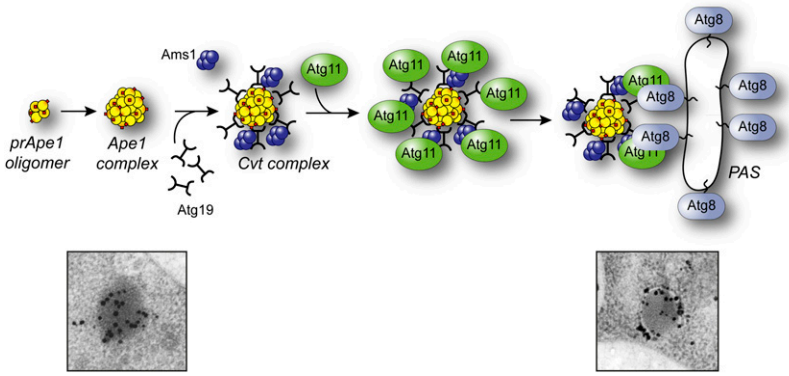

Figure 8 Mechanism of cargo recruitment during the Cvt pathway. Shortly after synthesis, prApe1 forms dodecamers that subsequently self-assemble in a larger oligomer that has been called the prApe1 complex. Association with the Atg19 autophagy receptor and oligomers of Ams1 (and additional cargo proteins) 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.

Acknowledgments

F.R. is supported by Chemical Science (CW) ECHO (700.59.003), Earth and Life Sciences (ALW) Open Program (821.02.017), Deutsche Forschungsgemeinschaft–Netherlands Organization for Scientific Research (NWO) cooperation (DN 82-303), and Netherlands Organization for Health Research and Development (ZonMW) VICI (016.130.606) grants. D.J.K. is supported by National Institutes of Health grant GM053396.

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Communicating editor: T. N. Davis