# Chapter 2
# The Core Molecular Machinery of Autophagosome Formation

**Meiyan Jin and Daniel J. Klionsky**

**Abstract** Autophagy is a conserved cytoplasmic process from yeast to mammals, by which cells degrade and recycle their intracellular components. During macroautophagy, a unique compartment, named the autophagosome, is formed to engulf the cargos and send them to the vacuole or lysosome. Whether the cargos are nonspecifically sequestered, as occurs in most types of macroautophagy, or specifically selected, such as in the cytoplasm-to-vacuole targeting pathway or selective mitochondria degradation, a common set of molecular machinery is required for the formation of the autophagosome. In this chapter, we summarize our knowledge about the roles and regulation of these core machinery components in autophagosome formation, in both yeast and mammalian systems.

**Keywords** Autophagy • Lysosome • Phagophore • Protein degradation • Stress • Protein trafficking • Vacuole

## 1 Introduction

Macroautophagy, hereafter referred to as autophagy, is primarily a degradation pathway through which cells turn over and recycle intracellular materials through lysosomal/vacuolar hydrolysis. During autophagy, portions of the cytosol and even entire organelles, such as mitochondria, are sequestered by an expanding cup-shaped double-membrane structure, termed the phagophore (Xie and Klionsky 2007). After elongation and closure, the phagophore generates a double-membrane vesicle, called an autophagosome. Upon completion, the outer membrane of the

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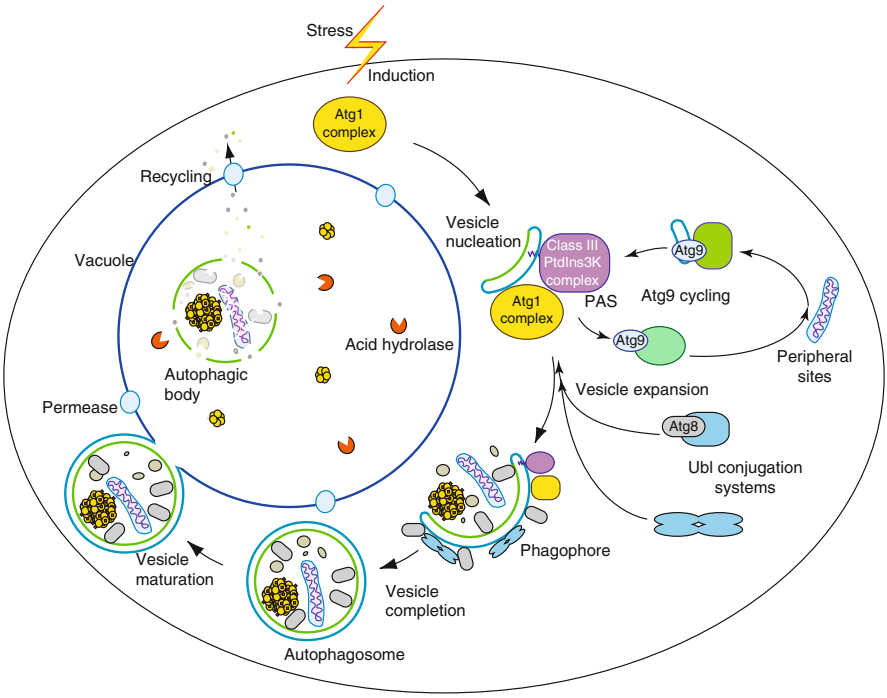


**Fig. 2.1** Overview of autophagy in yeast. The process of autophagy can be broken down into several steps, including induction, vesicle nucleation, Atg protein cycling, vesicle expansion and completion, vesicle maturation, vesicle breakdown, and recycling. The core machinery components can be grouped into several functional units, and they are responsible for the different steps of autophagy. The Atg1 complex is important for induction and also other downstream steps including Atg protein recruitment and cycling to the PAS, the PtdIns3K complex I has an essential role in vesicle nucleation, and vesicle expansion requires membranes transported to the PAS presumably through the Atg9 cycling system and regulation by two Ubl conjugation systems. The process is largely conserved in mammalian cells, and the slight differences in each complex are described in the text

autophagosome fuses with a lysosome, forming an autolysosome in mammalian cells, or releases the inner vesicle into the vacuole lumen, in yeast and plants. In either case, the autophagosome inner membrane, along with the enclosed cargos, is typically broken down by lysosomal/vacuolar hydrolases (Fig. 2.1). Thus, in mammalian cells, the morphology of autophagy is largely the same as that in yeast; however, in mammalian cells a specific kind of phagophore, termed an omegasome, was identified, which is a membrane structure that extends from the ER upon autophagy induction, and contains ZFYVE1/DFCP1 as one of its marker proteins (Axe et al. 2008).

Although autophagy was first described in mammalian cells more than 60 years ago (Stromhaug and Klionsky 2001), an understanding of the molecular mechanism began with the discovery of autophagy-related (*ATG*) genes by yeast genetic studies

**Table 2.1** The core molecular machinery of autophagosome formation

| | Yeast | Mammal | Characteristics |
|---|---|---|---|
| Atg1/ULK kinase complex | Atg1 | ULK1/2 | Serine/threonine protein kinase |
| | Atg13 | ATG13 | Regulator of the Atg1 complex through phosphorylation |
| | Atg17 | RB1CC1/FIP200 (functional homolog) | Component of the Atg1 complex |
| | Atg29 | | Component of the Atg1 complex |
| | Atg31 | | Component of the Atg1 complex |
| | | C12orf44/ATG101 | Component of the ULK complex in mammals |
| Atg9 and its cycling system | Atg2 | ATG2 | Peripheral membrane protein |
| | Atg9 | ATG9 | Transmembrane protein |
| | Atg18 | WIPI1/2 | Peripheral membrane protein |
| Class III PtdIns3K complex | Vps15 | PIK3R4/VPS15/p150 | Serine/threonine protein kinase |
| | Vps34 | PIK3C3/VPS34 | PtdIns3K |
| | Vps30/Atg6 | BECN1 | Component of PtdIns3K (complex I and II in yeast) |
| | Atg14 | ATG14 | Component of PtdIns3K (complex I in yeast) |
| Ubiquitin-like conjugation systems | Atg3 | ATG3 | E2-like enzyme for Atg8 conjugation |
| | Atg4 | ATG4A to ATG4D | Cysteine proteinase |
| | Atg5 | ATG5 | Conjugated with Atg12 |
| | Atg7 | ATG7 | E1-like enzyme |
| | Atg8 | LC3A,B,B2,C, GABARAP,L1,L2 | Ubl, conjugated to PE |
| | Atg10 | ATG10 | E2-like enzyme for Atg12 conjugation |
| | Atg12 | ATG12 | Ubl |
| | Atg16 | ATG16L1,L2 | Forms Atg12—Atg5–Atg16 complex |

This table was modified from table 1 of Mizushima et al. (2011)

only within the last couple of decades (Harding et al. 1995; Klionsky et al. 2003; Thumm et al. 1994; Tsukada and Ohsumi 1993). To date, over 30 *ATG* genes have been identified in yeast, and many of them have orthologs in higher eukaryotes. Among these *ATG* genes one subset, including *ATG1* to *ATG10*, *ATG12* to *ATG14*, and *ATG16* to *ATG18*, is required for efficient autophagosome formation, and the corresponding gene products are referred to as the core machinery for autophagosome formation (Table 2.1) (Nakatogawa et al. 2009; Xie and Klionsky 2007). The core machinery can be grouped into several functional units: (1) the Atg1 kinase

complex (Atg1 and Atg13, which also interact with Atg17, Atg29, and Atg31); (2) Atg9 and its cycling system (Atg9, Atg2, and Atg18); (3) the phosphatidylinositol 3-OH kinase (PtdIns3K) complex, which includes Vps30/Atg6 and Atg14 together with two other vacuolar protein sorting (Vps) proteins, Vps34 and Vps15; and (4) two ubiquitin-like protein (Ubl) conjugation systems: the Atg12 conjugation system (Atg12, Atg5, Atg7, Atg10, Atg16) and the Atg8 conjugation system (Atg8, Atg3, Atg4, Atg7) (Mizushima et al. 2011; Xie and Klionsky 2007).

In yeast, a single peri-vacuolar punctate structure, to which almost all of the molecular core machinery and other Atg proteins are recruited, is termed the phagophore assembly site (PAS). One widely accepted model is that the PAS is the expansion and nucleating site for the phagophore, the proposed autophagosome precursor (Yang and Klionsky 2009). In the mammalian system, a single specialized site equivalent to a yeast PAS has not been defined. Instead, colocalization of ATG proteins has been observed in multiple sites throughout a mammalian cell, which may correspond to multiple PASs (Mizushima et al. 2001, 2003; Yamada et al. 2005; Young et al. 2006). Recent studies suggest that most of the core machinery proteins are recruited to the PAS, and this occurs in a hierarchical manner, with a similar order of assembly seen in mammalian cells (Itakura and Mizushima 2010; Suzuki et al. 2007). Briefly, the Atg1 kinase complex, which acts in part as the induction regulator for autophagosome formation, is recruited to the PAS as one of the initial Atg proteins (although it is worth noting that Atg11, a scaffold protein, may be instrumental in dictating the site of the PAS in vegetative conditions, even though it is not a core component of the autophagy machinery); assembly of the Atg1 complex at the PAS is required for Atg9, a putative membrane carrier, and the class III PtdIns3K complex, which is responsible for vesicle nucleation, to subsequently localize to the PAS. Proper localization of the two Ubl conjugation systems, which have roles in vesicle expansion, requires the activity of the PtdIns3K complex at the PAS (Suzuki et al. 2007).

## 2 The Yeast Atg1 Complex

### *2.1 Yeast Atg1 Kinase Complex*

The yeast Atg1 kinase complex contains the only kinase of the core machinery, Atg1, the regulatory subunit Atg13, and additional components including the Atg17–Atg31–Atg29 complex, which is required for nonspecific macroautophagy (Cheong et al. 2005; Kabeya et al. 2005, 2007; Kawamata et al. 2005). Atg1–Atg13 is also required for another subtype of autophagy, the cytoplasm-to-vacuole targeting (Cvt) pathway, when it associates with Atg11–Atg20–Atg24 instead of Atg17–Atg31–Atg29 (Fig. 2.2a) (Nice et al. 2002). The Atg1 complex is required for the most upstream induction of autophagosome formation, regulated by

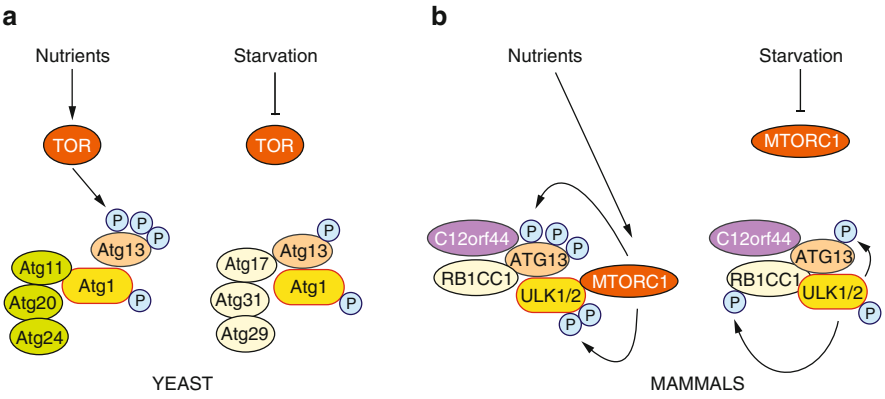


**Fig. 2.2** Regulation of the Atg1 complex in yeast and the ULK1/2 complex in mammals. (**a**) The Atg1 complex in yeast. In yeast, the Atg1 kinase may form a complex with different components depending on the nutrient status (the details of these complexes are still being determined). Under nutrient-rich conditions TOR (as part of TORC1) phosphorylates Atg13, and Atg1 forms a complex with Atg11–Atg20–Atg24 to function in the Cvt pathway. Upon starvation, TOR is inactivated, Atg13 is partially dephosphorylated, and Atg17–Atg31–Atg29 is substituted for Atg11–Atg20–Atg24 to induce macroautophagy. (**b**) The ULK1/2 complex in mammals. In mammalian cells ATG13 forms a complex with ULK1/2. Under nutrient-rich conditions, mechanistic TOR complex 1 (MTORC1) also binds with the ULK1/2 complex and phosphorylates ATG13 and ULK1/2. Upon starvation MTORC1 activity is inhibited and disassociated from the ULK1/2 complex. ULK1/2 is activated and phosphorylates ATG13 and RB1CC1, the mammalian functional homolog of yeast Atg17. The mammalian ULK1/2 complex contains a component, C12orf44, which does not have a known yeast homolog, and whose function remains unclear

several signaling inputs, such as the target of rapamycin (TOR), protein kinase A (PKA), and Sch9 pathways (Mizushima 2010; Yang and Klionsky 2009; Yorimitsu et al. 2007).

## 2.2 *Atg1*

Atg1 is a serine–threonine protein kinase required for both autophagy and the Cvt pathway (Matsuura et al. 1997; Straub et al. 1997). Autophosphorylation of the Atg1 Thr266 residue in the activation loop is required for Atg1 kinase activity, and other components of the complex, Atg13 and Atg17, are required for this autophosphorylation (Yeh et al. 2010). Kinase activity of Atg1 is upregulated upon autophagy induction, and this requires the interaction of Atg1 with Atg13 and Atg17 (Kabeya et al. 2005; Kamada et al. 2000). Both autophagy and the Cvt pathway require the kinase activity of Atg1, but Atg1 also has other non-kinase roles in autophagy. Atg1's role in protein recruitment to the PAS is independent of its kinase activity, indicating a presumable structural function, although its roles in membrane organization during autophagy are kinase dependent (Abeliovich et al. 2003; Cheong et al. 2008).

### 2.3 *Atg13*

Atg13 is a key regulatory subunit of the complex, whose phosphorylation is regulated by TOR complex 1 (TORC1) (Kamada et al. 2000). Atg13 serves as the linker between Atg1 and Atg17, binding both of these proteins; in *atg13*Δ yeast cells an Atg1–Atg17 interaction is not observed even when autophagy is induced by rapamycin (Kabeya et al. 2005). The binding affinity between Atg13 and Atg1 or Atg17 may be modulated by Atg13 phosphorylation. It has been proposed that in nutrient-rich conditions, Atg13 is highly phosphorylated, which prevents it from efficiently binding Atg1 and Atg17, whereas upon starvation- or rapamycin-induced autophagy, Atg13 is dephosphorylated, which restores its interaction with these proteins (Kabeya et al. 2005; Kamada et al. 2000).

### 2.4 *Atg17–Atg31–Atg29*

Atg17–Atg31–Atg29 forms a stable ternary complex, which is an autophagy-specific component of the larger Atg1 complex (meaning that these proteins are not strictly required for the Cvt pathway), whereas Atg11–Atg20–Atg24 is Cvt pathway specific (Kabeya et al. 2005, 2007; Kawamata et al. 2005; Nice et al. 2002). Smaller autophagosomes are observed in *atg17*Δ yeast cells, even though the Atg1–Atg13 interaction is maintained. The Atg17–Atg31–Atg29 complex interacts with Atg13, and this interaction is required for Atg1 kinase activity (Kabeya et al. 2005). Atg17 may function as a scaffolding protein (replacing Atg11 under autophagy-inducing conditions) to recruit other Atg proteins to the PAS (Suzuki et al. 2007). Atg29 is not required for the two Ubl conjugation systems or Atg1 kinase activity, but its interaction with Atg17 is essential for efficient autophagy (Kawamata et al. 2005, 2008). Atg31 also associates with Atg17, and its localization to the PAS depends on the latter (Kabeya et al. 2007). The functions of Atg29 and Atg31 are not known.

## 3 Mammalian ULK1/2 Complex

### 3.1 *The Mammalian Atg1 Homolog*

Among the mammalian Atg1-like proteins that have been identified, unc-51-like kinase 1 (ULK1) and ULK2 appear to be most similar to yeast Atg1, compared to the other related proteins ULK3, ULK4, and STK36 (Mizushima 2010). Since *ulk1*$^{-/-}$ mice do not display a significant autophagy defect, ULK2 might have a redundant function with ULK1 in autophagy regulation, although in some cases, siRNA knockdown of either *ULK1* or *ULK2* represses autophagy in cultured

mammalian cells (Chan et al. 2007; Jung et al. 2009; Kundu et al. 2008). The conserved C-terminal domain of ULK1, which contains a membrane-binding signal, has a dominant negative role in autophagy, and ATG protein recruitment to the phagophore is ULK1/ULK2 kinase activity dependent (Chan 2009; Hara et al. 2008).

### *3.2 ULK1/ULK2 Complex*

There are three main components in the ULK1/2 complex: ULK1/2, ATG13, and RB1CC1/FIP200. ATG13 is the conserved Atg13 homolog in mammals, and RB1CC1 is a putative functional homolog of Atg17 whose interaction with ULK1/2 is mediated by ATG13 (Hosokawa et al. 2009a; Jung et al. 2009). Unlike the yeast Atg1–Atg13–Atg17 interaction that is proposed to be nutrient dependent, in mammalian cells ULK1/2 forms a complex with ATG13 and RB1CC1 even in nutrient-rich conditions (Hara et al. 2008). The key upstream negative regulator of autophagy, MTORC1, forms a complex directly with ULK1/2–ATG13–RB1CC1 in nutrient-rich conditions, and phosphorylates ULK1/2 and ATG13. Upon starvation, MTORC1 is released from the complex, leading to partial dephosphorylation of its ATG substrates (Fig. 2.2b). ULK1/2 is subsequently activated and phosphorylates ATG13 and RB1CC1 (Ganley et al. 2009; Hosokawa et al. 2009a; Jung et al. 2009). In addition to phosphorylation, acetylation is another type of post-translational modification that regulates autophagy through ULK1 (Lin et al. 2012). There is also a mammalian protein, C12orf44/ATG101, that forms a complex with ULK1 and ATG13 and is essential for autophagy, although it does not have an identified yeast homolog, and the role of the ULK1–ATG13–C12orf44 complex in autophagosome formation is not known (Hosokawa et al. 2009b; Mercer et al. 2009).

## 4 Atg9 and Its Cycling System

### *4.1 Yeast Atg9 and Its Cycling Regulation*

Atg9 is the first, and so far the only, characterized transmembrane protein in the core machinery that is absolutely required for both autophagy and the Cvt pathway (Noda et al. 2000). In yeast cells, Atg9 localizes to multiple punctate structures, one of which is the PAS, and other peripheral sites [Atg9 reservoirs, or tubulovesicular clusters (TVCs)] that are in proximity to the mitochondria (Noda et al. 2000; Reggiori et al. 2004). Atg9 can associate with membranes, and the proposed dynamic movement of Atg9 between the PAS and the TVCs supports a model wherein Atg9 functions as a carrier that transports membrane to the

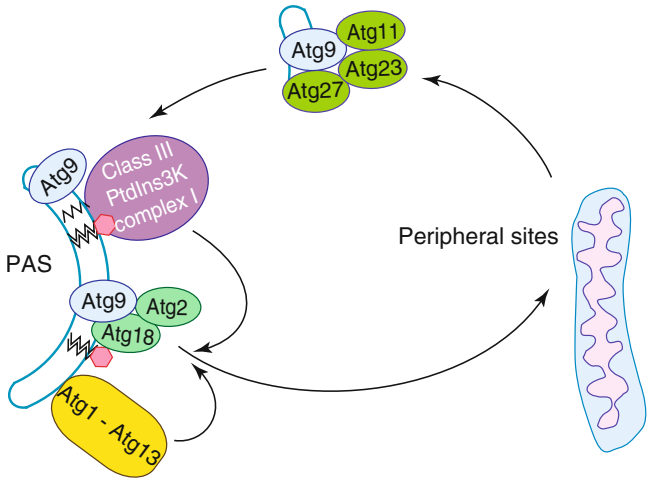


**Fig. 2.3** Atg9 cycling. The efficient anterograde transport of Atg9 from peripheral sites to the PAS requires Atg11, Atg23, and Atg27. Peripheral membrane proteins Atg2 and Atg18 form a complex with Atg9 and they are required for Atg9 retrieval from the PAS back to the peripheral sites. Atg1–Atg13 and class III PtdIns3K complex I are required for the localization of Atg2 and Atg18 to the PAS and the retrieval (retrograde transport) of Atg9

phagophore from other organelles (Reggiori et al. 2004, 2005). Several studies have shown that the cycling of Atg9 is required for autophagosome formation (Reggiori et al. 2004, 2005; Yen et al. 2007). Various proteins and complexes, which are involved in general intracellular trafficking, have essential roles in autophagy via the regulation of Atg9 cycling, such as Sec12, VFT, the COG Golgi tethering complex, Sec7, Sec2–Sec4, Arf1–Arf2, and Ypt1 (Weidberg et al. 2011). In addition, several Atg proteins have important roles in Atg9 cycling regulation (Fig. 2.3).

## *4.2 Atg9 Localization to the PAS*

Atg9 PAS localization upon autophagy induction is dependent on Atg17, and Atg9 transits to the PAS in a complex with Atg23 and Atg27 (Reggiori et al. 2004; Sekito et al. 2009; Yen et al. 2007). Movement to the PAS in nutrient-rich conditions, for Cvt vesicle formation, does not require Atg17, Atg13, or Atg1 kinase activity, but does depend on Atg11, Atg23, and Atg27 as well as actin (Chang and Huang 2007; He et al. 2006; Legakis et al. 2007; Monastyrska et al. 2008). Atg23 is required for the Cvt pathway, and efficient autophagy but not for the selective degradation of peroxisomes by autophagy (pexophagy). This component is a peripheral membrane protein that localizes on multiple punctate structures, one of which is the PAS, and its membrane association requires Atg9; Atg23 is diffuse in the cytosol without Atg9 (Tucker et al. 2003). Atg27 is a type I transmembrane protein containing an

N-terminal signal sequence. This protein is required for the Cvt pathway, pexophagy, and efficient autophagy. In addition to the PAS, Atg27 localizes to mitochondria and the Golgi complex, presumably transiting to the latter through the endoplasmic reticulum (Yen et al. 2007).

### *4.3 Atg9 Retrieval from the PAS*

Atg9 forms a complex with the peripheral membrane proteins Atg2 and Atg18, which are required for its retrograde trafficking from the PAS to the TVCs, and these proteins—and the presumed return movement of Atg9—are required for autophagy, the Cvt pathway, and pexophagy (Guan et al. 2001; Reggiori et al. 2004; Wang et al. 2001). Atg18 binds to PtdIns3P via two sites that are composed of parts of blade 5 and blade 6 of the WD-40 β-propeller domain, and a hydrophobic loop that inserts into the membrane (Baskaran et al. 2012; Krick et al. 2012). Atg2 and Atg18 PAS localization are dependent on each other, Atg1, Atg13, Atg9 and the PtdIns3K complex I (see below) (Guan et al. 2001; Shintani et al. 2001; Suzuki et al. 2007; Wang et al. 2001); the PtdIns3K and Atg1–Atg13 complexes are also essential for Atg9 retrieval (retrograde trafficking) from the PAS (Reggiori et al. 2005). The role of Atg18 in targeting Atg2 to the PAS may be important for its autophagosome formation function, as an engineered PAS-targeting Atg2 can restore autophagosome formation in yeast cells lacking Atg18 (Kobayashi et al. 2012).

### *4.4 Mammalian ATG9*

In mammalian cells, the yeast Atg9 homolog, ATG9A, localizes to the *trans*-Golgi network and endosomes in nutrient-rich conditions, whereas in yeast this protein localizes to the PAS and TVCs as noted above (Noda et al. 2000; Reggiori et al. 2005; Young et al. 2006). Another Atg9 ortholog, ATG9B, has a similar subcellular localization as ATG9A, and is functionally redundant with the latter, but has a different tissue expression pattern. In adult human tissue, ATG9A is ubiquitously expressed, whereas ATG9B displays significant expression only in the placenta and pituitary gland (Yamada et al. 2005). ATG9 may interact with the phagophore membrane in a very dynamic and transient manner, and may not actually be a component of the forming autophagosome, suggesting a conserved role as a membrane carrier that transports lipid from donor sources to the phagophore (Orsi et al. 2012). In mammalian cells, the cycling of ATG9 between the phagophore and non-phagophore sites has not been as well studied as in yeast. Upon starvation, a population of ATG9 translocates to LC3-positive autophagosomes, in a ULK1- and PIK3C3/VPS34 kinase activity-dependent manner (Young et al. 2006). Retrieval of ATG9 from the phagophore is dependent on WIPI2, a mammalian Atg18 homolog, but is ULK1 independent (Orsi et al. 2012). In WIPI2 knockdown cells, ZFYVE1-positive, but LC3-negative, omegasomes accumulate in both fed and starved conditions, and ATG9 localizes at these sites (Orsi et al. 2012; Polson et al. 2010).

# 5 The PtdIns3K Complexes

## 5.1 *Yeast PtdIns3K Complex I*

Different types of phosphoinositide phosphates (PIPs) localize at specific membrane compartments, and have important roles in the recruitment of molecular machinery and in signal transduction (Skwarek and Boulianne 2009). On the phagophore membrane, there is one type of PIP, PtdIns3P, which is essential for autophagy, possibly through the recruitment of PIP-binding proteins, such as Atg18, to the PAS (Juhasz et al. 2008; Stromhaug et al. 2004). Vps34 is the only PtdIns3K in yeast, and there are two distinct Vps34 complexes in this organism (Fig. 2.4):

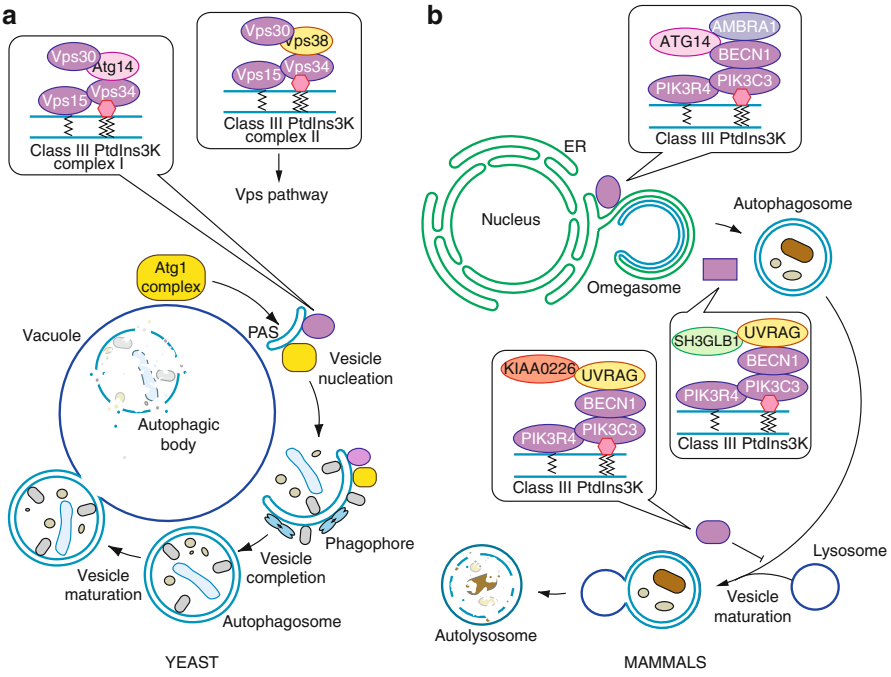


**Fig. 2.4** PtdIns3K complexes in yeast and mammals. (**a**) Yeast PtdIns3K complexes. In yeast, there are two separate class III PtdIns3K complexes that play roles in autophagy and the Vps pathway. Both complexes contain Vps15, Vps30, and Vps34. Complex I, which has important roles in multiple steps of autophagy, including vesicle nucleation and Atg protein cycling, contains Atg14 as its autophagy-specific component, while complex II, which functions in the Vps pathway, contains Vps38. (**b**) Mammalian PtdIns3K complexes. There are at least three distinct class III PtdIns3K complexes that regulate autophagy in the mammalian system, and PIK3C3/VPS34, BECN1, and PIK3R4 are the core components in all of them. One complex containing ATG14 and an additional modulating protein, AMBRA1, has roles in phagophore formation, while a second complex with UVRAG and SH3GLB1 has roles in autophagosome formation and probably maturation. The third complex, which contains UVRAG and KIAA0226, negatively regulates autophagosome maturation

complex I, which is specific for autophagy, composed of Vps34, Vps15, Vps30/Atg6, and Atg14, and complex II, for the endosomal/Vps pathway, which contains Vps34, Vps15, Vps30, and Vps38 (Kihara et al. 2001).

## 5.2 *Vps34–Vps15–Vps30*

Vps34 and Vps15, its presumed regulatory protein, are essential for autophagy (Kihara et al. 2001). Vps34 presumably functions downstream of TOR signaling, and its PtdIns3K activity is required for autophagy (Kihara et al. 2001; Obara et al. 2008). The kinase activity of Vps34 requires its C-terminal helix, which controls cycling between membrane and cytosolic pools (Weidberg et al. 2011). Membrane association and the lipid phosphorylation activity of Vps34 require the kinase activity of Vps15 (Stack et al. 1993, 1995), but Vps15 does not directly phosphorylate Vps34 (Stack and Emr 1994). Vps30 is not essential for Vps34 activity and its role in the PtdIns3K complex is not well understood (Kihara et al. 2001).

## 5.3 *Atg14*

Atg14 is a PtdIns3K complex I-specific component, which directs the complex to the PAS and is required for the PtdIns3K complex to function in autophagy and the Cvt pathway (Kametaka et al. 1998; Obara et al. 2006). Atg14 is thought to be a connector between Vps30 and Vps34–Vps15 via the N-terminal half of the protein, which contains coiled-coil domains (Yang and Klionsky 2009).

## 5.4 *Mammalian Class III PtdIns3K*

In mammalian cells, there are two types of PtdIns3K, class I and class III; the class III enzymes are the orthologs of yeast Vps34 (Fig. 2.4). The components of class III PtdIns3K complexes are conserved between yeast and mammal. PIK3C3/VPS34, BECN1 (the mammalian homolog of yeast Vps30), and PIK3R4/p150 (the homolog of Vps15) are the core components in two different complexes. One complex additionally contains the homolog of Atg14, ATG14/ATG14L/Barkor, and is required specifically for autophagy, whereas the other complex includes the homolog of Vps38, ultraviolet irradiation resistance-associated gene (UVRAG), and mediates endocytosis but also regulates autophagy in several ways (Itakura et al. 2008). In contrast to yeast, in mammals there is a third class III PtdIns3K complex that contains the protein KIAA0226/Rubicon (see below).

### 5.5 *PIK3C3 and PIK3R4*

Mammalian PIK3C3 can interact with either ATG14 or UVRAG through the same C2 domain. In nutrient-rich conditions PIK3C3 forms puncta that colocalize with UVRAG almost completely, but upon starvation, a portion of the PIK3C3 puncta colocalize with ATG14 (Itakura et al. 2008). PIK3C3 is required for autophagy, and its kinase activity is essential both in the fly and mammalian systems (Axe et al. 2008; Juhasz et al. 2008; Petiot et al. 2000). In mammals, membrane targeting and optimal activity of PIK3C3 require PIK3R4, which is needed for the activation of PIK3C3 by BECN1 and UVRAG (Yan et al. 2009).

### 5.6 *BECN1*

BECN1 was first identified as a BCL2-interacting protein, and later shown to directly bind with PIK3C3 (Furuya et al. 2005). The interaction between BECN1 and PIK3C3 is regulated by phosphorylation of PIK3C3 by CDK1 during mitosis (Furuya et al. 2010). Unlike yeast Vps30, which is essential for Vps34's role in both autophagy and endocytic trafficking, BECN1 is only required for autophagy but not other PtdIns3K-dependent trafficking, suggesting a role for BECN1 in engaging PIK3C3 in autophagy (Furuya et al. 2005; Zeng et al. 2006). Binding with BCL2 impairs BECN1's binding with PIK3C3, thus inhibiting autophagy, which has important implications with regard to the role of BECN1 in preventing tumor formation; BECN1 is proposed to function as a tumor suppressor by promoting autophagy activity (Liang et al. 1999). A study of the crystal structure of BECN1 reveals that it binds to phospholipids through an aromatic finger (Huang et al. 2012); this is interesting considering that BECN1 is part of a complex that generates PtdIns3P, suggesting that it may be recruited to membranes containing this phospholipid, and then subsequently participate in amplifying the PtdIns3P level. There are three BECN1 complexes in mammals: ATG14–BECN1–PIK3C3–PIK3R4, UVRAG–BECN1–PIK3C3–PIK3R4, and KIAA0226–UVRAG–BECN1–PIK3C3–PIK3R4 (the latter being a negative regulator, see below) control autophagy at different steps of the process through differential regulation by ATG14, UVRAG, and KIAA0226 (Matsunaga et al. 2009; Zhong et al. 2009). BECN1-mediated autophagy is also positively regulated by activating molecule in BECN1-regulated autophagy (AMBRA1), which has essential roles in embryonic neural development in mammals (Fimia et al. 2007).

### 5.7 *ATG14*

ATG14 has an important role in mammalian autophagosome formation. The coiled-coil region of ATG14 is required for its binding with PIK3C3 and BECN1 (Itakura et al. 2008). Under nutrient-rich conditions, most ATG14 is dispersed in the cytosol,

whereas starvation induces ATG14 puncta formation at the phagophore, and some of the ATG14 puncta are colocalized with ER markers (Matsunaga et al. 2009). Depletion of ATG14 suppresses ATG16L1 and LC3 puncta formation, which are markers of phagophores, and both phagophores and autophagosomes, respectively (Matsunaga et al. 2009). Overexpression of ATG14 increases the kinase activity of PIK3C3 and induces autophagy, whereas knockdown of ATG14 impairs PIK3C3 activity and suppresses autophagy (Sun et al. 2008; Zhong et al. 2009).

### *5.8 UVRAG, SH3GLB1, and KIAA0226*

UVRAG is a mammalian homolog of Vps38, and it regulates autophagy in several ways (Liang et al. 2006). First, UVRAG competes with ATG14 to bind with PIK3C3, forming a UVRAG–BECN1–PIK3C3–PIK3R4 complex, directing the PtdIns3K to function in autophagosome maturation, whereas the ATG14–BECN1–PIK3C3–PIK3R4 complex has a function in early phagophore formation. Second, UVRAG interacts with SH3GLB1/Bif-1, which is required for autophagy, and the interaction of SH3GLB1 with BECN1 through UVRAG activates the class III PtdIns3K complex to stimulate autophagy (Takahashi et al. 2007). Third, UVRAG is part of a KIAA0226–UVRAG–BECN1–PIK3C3–PIK3R4 complex, which localizes to the late endosome and negatively regulates autophagosome maturation (Matsunaga et al. 2009; Zhong et al. 2009).

## 6 Ubiquitin-Like Conjugation Systems

### *6.1 Two Ubl Conjugation Systems in Yeast*

Like other kinds of posttranslational modification, ubiquitination is a well-studied process, which is important for protein function and stability. During ubiquitination, a series of enzymes conjugate the substrate proteins with ubiquitin. In a similar way, some enzyme cascades catalyze the attachment of proteins that share identity with ubiquitin to other proteins, and those proteins that are similar to ubiquitin are called Ubls (Hochstrasser 2009). There are two Ubls among the Atg proteins, Atg8 and Atg12, which have no clear sequence homology with ubiquitin, but both contain a ubiquitin fold at their C terminus (Sugawara et al. 2004; Suzuki et al. 2005). These proteins are part of two distinct Ubl conjugation systems, and function to form Atg8—phosphatidylethanolamine (Atg8—PE) and Atg12—Atg5, respectively, both of which are essential for autophagy (Fig. 2.5) (Ichimura et al. 2000; Mizushima et al. 1998). The conjugation systems participate in phagophore expansion, and Atg8 can regulate the size of autophagosomes, as smaller autophagosomes are observed in yeast cells expressing Atg8 at levels lower than wild type (Mizushima et al. 2001; Xie et al. 2008).

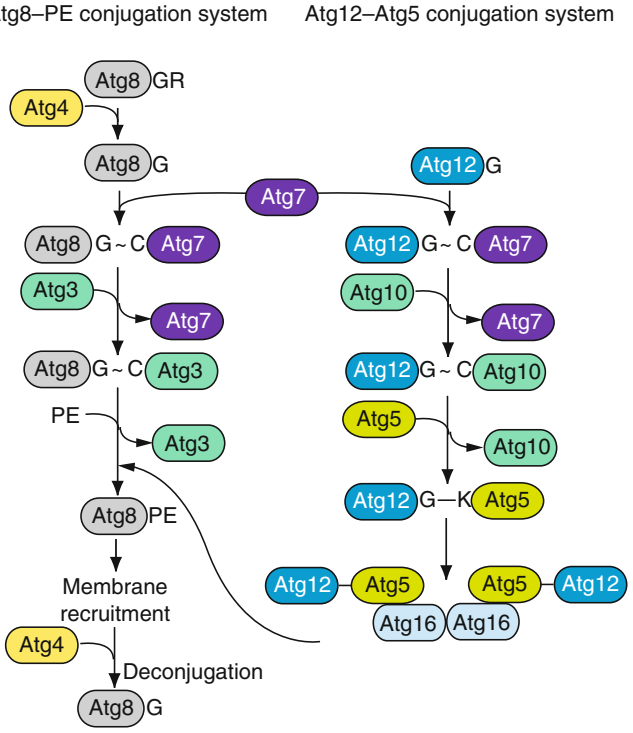


**Fig. 2.5** Two Ubl conjugation systems in yeast. The ultimate C-terminal amino acid of Atg8 is removed by Atg4, and the resulting truncated Atg8 protein, with an exposed C-terminal glycine residue, is conjugated to PE in a cascade mediated by Atg7 (E1-like enzyme), and Atg3 (E2-like enzyme), and facilitated by Atg12—Atg5–Atg16 (E3-like enzyme). This conjugate can subsequently be cleaved by Atg4. The Atg12—Atg5 conjugate is also formed via Atg7 (E1-like enzyme), but utilizes a separate conjugating enzyme, Atg10 (E2-like enzyme). Atg12—Atg5 further forms a complex with Atg16, which dimerizes

## *6.2 Atg12—Atg5 Conjugation System*

Atg12 is stoichiometrically conjugated with Atg5, in a process that is essential for both autophagy and the Cvt pathway (Mizushima et al. 1998). This conjugation event occurs in a manner that is similar to canonical ubiquitination. First, the E1-like enzyme of this conjugation system, Atg7, activates Atg12 by forming a thioester bond between Atg7 Cys507 and Atg12 Gly186 (Tanida et al. 1999). Second, Atg12 is transferred to the Cys133 residue of the E2-like enzyme Atg10 to form an Atg12—Atg10 thioester, and finally conjugated with its target protein Atg5 at Lys149 (Mizushima et al. 1998; Shintani et al. 1999). There is no E3-like enzyme for Atg12—Atg5 conjugation so far identified. Atg12-conjugated Atg5 further forms a noncovalent complex with Atg16, a small coiled-coil protein, which is also essential for autophagy and the Cvt pathway (Mizushima et al. 1999). In yeast, an

Atg12—Atg5–Atg16 complex, which may be a dimer, is formed via Atg16 homo-oligomerization (Fujioka et al. 2010; Kuma et al. 2002). Unlike canonical ubiquitination, which is reversible, Atg12—Atg5 conjugation appears to be irreversible, and no enzyme that hydrolyzes the Atg12—Atg5 conjugate has been identified (Kirisako et al. 2000). In yeast, the Atg12—Atg5–Atg16 complex localizes on the outer membrane of the phagophore and is disassociated from the phagophore near the time of autophagosome completion (Mizushima et al. 2001, 2003).

## *6.3 Atg8—PE Conjugation System*

Atg8, the second Ubl among the core machinery, is conjugated to a lipid molecule, PE, upon autophagy induction (Huang et al. 2000). In the initial step, the peptide bond of the C-terminal Arg117 of Atg8 needs to be cleaved by a cysteine protease, Atg4, to expose Gly116, the residue that will interact with the E1-like enzyme (Kirisako et al. 2000). The Atg8—PE conjugation system shares the same E1-like enzyme, Atg7, with the Atg12—Atg5 conjugation system. Atg8 is activated by Atg7, its exposed Gly116 forming a thioester bond with Atg7 Cys507 (Ichimura et al. 2000). Then, Atg8 is transferred to an E2-like enzyme, Atg3, and forms an Atg8—Atg3 intermediate also through a thioester bond, between Atg8 Gly116 and Atg3 Cys234 (Ichimura et al. 2000). Finally, Gly116 of Atg8 is conjugated to its target PE, which may involve the Atg12—Atg5–Atg16 complex, acting as an E3 ligase (Hanada et al. 2007; Ichimura et al. 2000). Although lacking the conserved E3 ligase domain, Atg12—Atg5–Atg16 displays some E3-like features; for example, it interacts with both the substrate, Atg8, and the E2-like enzyme, Atg3 (Fujita et al. 2008; Hanada et al. 2007). Besides its putative E3-like activity, Atg12—Atg5–Atg16 may also be required for PAS localization of Atg8 (Suzuki et al. 2007). In contrast to Atg12—Atg5 conjugation, Atg8—PE conjugation is reversible via a second Atg4-dependent cleavage (referred to as deconjugation), and the release of Atg8 from Atg8—PE by Atg4 is also essential for efficient autophagy, possibly through disassembly of Atg proteins from completed autophagosomes (Kirisako et al. 2000; Nair et al. 2012; Yu et al. 2012). Unlike Atg12—Atg5–Atg16, Atg8—PE localizes on both the outer and inner membrane of the phagophore, and some Atg8 on the inner surface remains inside the completed autophagosome and is further digested by the vacuole as part of the autophagic body (Huang et al. 2000; Kirisako et al. 2000). This characteristic makes Atg8, and in particular its mammalian homologs, a critical marker for following the autophagosome.

## *6.4 Mammalian Ubl Conjugation Systems*

Ubl conjugation systems are highly conserved between yeast and mammals, and the human and mice homologs of components of the conjugation systems have been characterized.

## 6.5 Mammalian Atg12 Conjugation System

Human Atg12 and Atg5 homologs also form an ATG12—ATG5 conjugate through the generation of an isopeptide bond between the ATG12 C-terminal glycine and the ATG5 Lys130 residue (Mizushima et al. 1998). Cys572 of ATG7 is the active-site cysteine residue that is essential for its interaction with ATG12 and the mammalian Atg8 homologs. Human ATG7 forms a homodimer, similar to yeast Atg7 (Tanida et al. 2001). A mammalian Atg10 homolog is identified in mice, and it interacts with ATG12 through Cys165, an interaction that requires activation of ATG12 by ATG7. Interestingly, mammalian ATG12 and ATG5 and yeast Atg7 and Atg10 cannot function heterologously to generate an ATG12—ATG5 conjugate, whereas reconstitution of mouse ATG12 conjugation in yeast can be achieved with ATG5, ATG7, ATG10, and ATG12, supporting the concept that an E3-like enzyme is not required for this event (Mizushima et al. 2002). A functional homolog of Atg16, autophagy-related 16-like 1 (ATG16L1) was identified in mice, and it forms a complex with ATG12 and ATG5. ATG5, but not ATG12, is required for membrane targeting of ATG16L1 (Mizushima et al. 2003).

## 6.6 Mammalian Atg8 Conjugation System

As with the yeast Atg8 conjugation system, mammalian ATG7 also acts as the E1-like enzyme, and site-directed mutagenesis shows that Cys264 of ATG3, the conserved E2-like enzyme, is essential for the formation of an intermediate conjugate between the Atg8 homolog and this enzyme. Overexpression of ATG3 facilitates ATG12—ATG5 conjugation, indicating a possible cross talk between the two conjugation systems (Tanida et al. 2002). Atg8 and Atg4 have multiple homologs in mammals. For Atg8, homologs including several isoforms of LC3 and GABARAP have been identified, and all of these undergo a conjugation process similar to that in yeast (Kabeya et al. 2004; Tanida et al. 2001, 2002, 2003, 2006). Among them, LC3 is the best-characterized autophagosome marker in mammalian cells. ATG4B removes the amino acids located C-terminally from the last glycine residue of the newly synthesized LC3, proLC3, to form cytosolic LC3-I; after activation of LC3-I by ATG7, it is conjugated with PE to form membrane-associated LC3-II, which can ultimately be cleaved by ATG4B in a deconjugation step (Kabeya et al. 2004; Tanida et al. 2006). Different Atg8 homologs have distinct, but essential, roles in autophagosome formation; LC3 functions at the stage of phagophore elongation, whereas the GABARAP subfamily has roles in later steps of autophagosome maturation (Weidberg et al. 2010). In the mammalian system, there are four ATG4 isoforms, and among them ATG4B is the one most involved in autophagy. A kinetic analysis of the Atg4 and Atg8 homologs shows that ATG4B has the broadest spectrum against different Atg8 homologs, followed by ATG4A, whereas ATG4C and ATG4D have minimal proteinase activity against these targets. Among the Atg8 homologs, GABARAPL2/GATE-16 is the best substrate, while LC3 is the weakest substrate of ATG4 although there are only minor differences (Li et al. 2011).

## 7 Conclusion

As a highly conserved process between yeast and human, autophagy plays a role in various human diseases, including certain types of neurodegeneration, metabolic disorders, liver and heart disease, and cancer. Thus, an understanding of the core molecular machinery, especially as this pertains to the most complicated step of autophagy, autophagosome formation, has a crucial physiological and therapeutic significance. Although major breakthroughs have been achieved in our understanding of autophagosome formation based on studies in yeast and more recently in higher eukaryotes, our knowledge of the organization and regulation of the core machinery, especially in the mammalian system where redundant homologs of the autophagy-related proteins exist, is still limited. Furthermore, the roles of noncore-machinery Atg proteins, which direct the core machinery to function in special types of autophagy, such as the Cvt pathway, pexophagy, and selective mitochondria degradation by autophagy (mitophagy), are also important and await a more detailed functional analysis.

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