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COMMENTARY

Membrane dynamics in autophagosome biogenesis

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ABSTRACT

Bilayered phospholipid membranes are vital to the organization of the living cell. Based on fundamental principles of polarity, membranes create borders allowing defined spaces to be encapsulated. This compartmentalization is a prerequisite for the complex functional design of the eukaryotic cell, yielding localities that can differ in composition and operation. During macroautophagy, cytoplasmic components become enclosed by a growing double bilayered membrane, which upon closure creates a separate compartment, the autophagosome. The autophagosome is then primed for fusion with endosomal and lysosomal compartments, leading to degradation of the captured material. A large number of proteins have been found to be essential for autophagy, but little is known about the specific lipids that constitute the autophagic membranes and the membrane modeling events that are responsible for regulation of autophagosome shape and size. In this Commentary, we review the recent progress in our understanding of the membrane shaping and remodeling events that are required at different steps of the autophagy pathway.

This article is part of a Focus on Autophagosome biogenesis. For further reading, please see related articles: 'ERES: sites for autophagosome biogenesis and maturation?' by Jana Sanchez-Wandelmer et al. (*J. Cell Sci.* **128**, 185-192) and 'WIPI proteins: essential PtdIns3*P* effectors at the nascent autophagosome' by Tassula Proikas-Cezanne et al. (*J. Cell Sci.* **128**, 207-217).

KEY WORDS: Atg, PtdIns3*P*, Autophagosome, Phagophore

Introduction

Macroautophagy, hereafter referred to as autophagy, is the best characterized form of autophagy and has been implicated in a diverse set of physiological and pathophysiological conditions. Autophagy was for a long time considered to be a non-selective cell survival process, involving random sequestration of cytoplasmic material into forming autophagosomes, but it is now evident that autophagy also plays an essential quality control function by selective removal of damaged or dysfunctional organelles, as well as protein aggregates and pathogens (Stolz et al., 2014). The pathway initiates with the nucleation of a double-membraned structure (the phagophore, also called the isolation membrane), which expands to engulf cytoplasm into a double-membraned vesicle (autophagosome). Fusion of the autophagosome with lysosomes leads to degradation of the sequestered material and the recycling of degradation products into new macromolecules (Klionsky and Codogno, 2013; Lamb et al., 2013) (Fig. 1).

A large number of autophagy-related (Atg) proteins have been identified and found to be essential for both the non-selective and selective types of autophagy (see Box 1) (Klionsky et al., 2011). Recent progress in the field has made it clear that Atg proteins act together with general membrane trafficking components, such as coat proteins, soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) proteins and RAB proteins. Membrane shaping and remodeling are required at several steps of the autophagy pathway (for general principles on the generation of membrane curvature, see Box 2) and these processes appear to be tightly regulated by lipid modifying enzymes, membrane sculpting and remodeling proteins, lipid-binding effector proteins and protein kinases and phosphatases. This Commentary will focus on recent progress in our understanding of the early membrane modeling events in phagophore biogenesis, such as those involved in the nucleation process and the role of phosphatidylinositol (PtdIns) 3-phosphate (PtdIns3*P*) and other lipids, as well as later steps of phagophore expansion and closure, including tethering and fusion reactions to obtain the membrane composition and curvature of the functional autophagosome. Finally, we briefly discuss the membrane dynamics and modeling events occurring in selective autophagy. For detailed reviews of the complex hierarchy of Atg proteins and their regulation upon induction of autophagy, see Lamb et al., 2013; Mizushima et al., 2011 and Box 1.

Membrane modeling events in the early autophagy response: formation of the phagophore

The phagophore membrane

The phagophore is a small cup-shaped membrane precursor formed upon induction of autophagy, and its origin and identity have been a matter of debate for decades (Tooze, 2013). Recent studies of Atg proteins in yeast and mammalian cells have contributed substantially to our understanding of the mechanisms involved in phagophore formation, but the constitutive lipid species and membrane modeling proteins involved in determining phagophore shape and size are still mostly unknown.

It is generally agreed that phagophores are formed *de novo* by nucleation on a preexisting membrane (for recent reviews, see Abada and Elazar, 2014; Hamasaki et al., 2013b; Lamb et al., 2013; Shibutani and Yoshimori, 2014). In yeast, the phagophore membrane has been found to originate from a single origin near the vacuole, called the pre-autophagosomal structure (PAS) (Suzuki and Ohsumi, 2010). In higher eukaryotes, phagophore nucleation might occur at different locations in the cell, but it is now largely accepted that, at least upon starvation, phagophores nucleate at an intricate membranous structure, termed the omegasome (due to its resemblance to the Greek letter omega in electron microscopy pictures). Omegasomes were originally identified as endoplasmic reticulum (ER)-associated spots labeled

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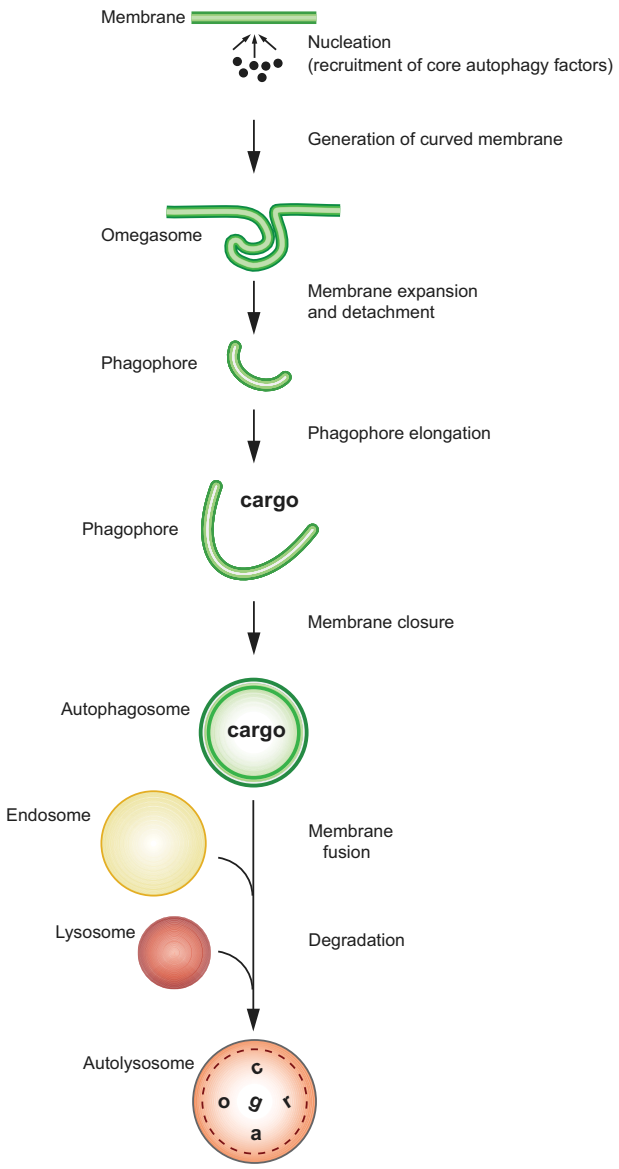

Fig. 1. Membrane structures and organelles in the autophagic pathway. Membrane sculpting and remodeling steps leading to the degradation of captured cargo in autolysosomes are indicated.

by the PtdIns3*P*-binding double-FYVE-containing protein 1 (DFCP1, also known as ZFYVE1) (Axe et al., 2008). The class III PtdIns3-kinase (PI3KC3, also known as PIK3C3; Vps34 in yeast) is required for omegasome formation (Axe et al., 2008) and forms an autophagy-specific complex with p150 (also known as PIK3R4; Vps15 in yeast), beclin 1 (Atg6 in yeast) and ATG14L (also known as BARKOR; Atg14 in yeast) (Itakura et al., 2008; Kihara et al., 2001; Sun et al., 2008) (see also Box 1).

DFCP1-positive omegasomes appear to concentrate at or near the connected mitochondria-associated ER membrane (Hamasaki et al., 2013a), in line with a study showing that the outer mitochondrial membrane provides lipids for the phagophore (Hailey et al., 2010). It is, however, clear that the phagophore also receives input from other membrane sources. The ER exit sites (ERES) (Graef et al., 2013; Zoppino et al., 2010), the ER–Golgi intermediate compartment (ERGIC) (Ge et al., 2013), the Golgi (Bodemann et al., 2011; Geng et al., 2010; van der Vaart et al., 2010), the plasma membrane (Ravikumar et al., 2010) and recycling endosomes (Knævelsrud et al., 2013; Longatti et al., 2012; Puri et al., 2013) have all been implicated as membrane sources for the phagophore (Fig. 2E). It has been difficult to differentiate between membranes contributing to phagophore nucleation and those contributing to phagophore expansion, but the recent elucidation of the time- and function-dependent hierarchy among the core autophagy proteins involved in phagophore nucleation has provided markers for the different steps of phagophore formation (Karanasios et al., 2013; Koyama-Honda et al., 2013), which might allow elucidation of the

Box 1. The core autophagosomal machinery

The Atg1 and ULK complexes

The yeast serine/threonine protein kinase Atg1 forms a complex with Atg13, Atg17, Atg29 and Atg31 upon induction of autophagy. This complex functions in the recruitment and release of other Atg proteins from the PAS. The ULK (Unc51-like kinase) kinases ULK1 and ULK2 are functional homologs of Atg1 in higher eukaryotes and form a complex with ATG13, FIP200 and ATG101. Although there are no human orthologs of yeast Atg29 and Atg31, nor a yeast ortholog of ATG101, FIP200 is thought to be the functional homolog of yeast Atg17, and the overall similarities of these complexes makes it likely that their function is conserved. The ULK kinases are activated upon induction of autophagy, and are tightly regulated by the mammalian target of rapamycin complex 1 (mTORC1) and the AMP-activated protein kinase (AMPK) (for recent reviews, see Mizushima, 2010; Wirth et al., 2013).

The PI3KC3 and Vps34 complexes

The catalytically active PI3KC3/Vps34 kinase and the associated p150/Vps15 and beclin 1/Atg6 proteins can engage in two distinct PI3KC3/Vps34 complexes in mammalian and yeast cells; complex I contains the autophagy-specific subunit ATG14L/Atg14 (Itakura et al., 2008; Kihara et al., 2001; Sun et al., 2008), and complex II contains UVRAG (Atg38 in yeast), which is involved in autophagosome maturation and endocytosis. The PI3KC3/Vps34 complex I produces PtdIns3*P* at the site of phagophore nucleation, which recruits downstream factors such as WIPIs/Atg18 and might also influence membrane structure through its bulky negatively charged head group (for recent reviews, see Dall'Armi et al., 2013; Simonsen and Tooze, 2009).

The ATG12–ATG5–ATG16L1 complex

ATG12/Atg12 is a ubiquitin-like protein that is conjugated to ATG5/Atg5 through the action of ATG7/Atg7 and ATG10/Atg10, which have E1 and E2-like activities, respectively. Atg16L1/Atg16 binds to the ATG12–ATG5/Atg12–Atg5 conjugate, and the complex can then facilitate the conjugation of LC3/Atg8 proteins to PtdEtn in the membrane (for recent reviews, see Hamasaki et al., 2013b; Mizushima et al., 2011).

Atg8 proteins and their conjugation system

Although yeast has only one Atg8 protein, seven Atg8 homologs have been identified in mammalian cells; they belong to the LC3 (LC3A, -B, -B2 and -C) or GABARAP [GABARAP, -L1, -L2 (also referred to as GATE-16)] subfamilies. In a well-nourished healthy cell, LC3/Atg8 mostly exists as a soluble protein (LC3-I). When autophagy is triggered, LC3/Atg8 becomes covalently modified with PtdEtn (the lipidated form is called LC3-II) through the action of ATG7/Atg7, ATG3/Atg3 and the ATG12–ATG5–ATG16L1/Atg12–Atg5–Atg16 complex, which have E1, E2 and E3-like activities, respectively (for recent reviews, see Hamasaki et al., 2013b; Mizushima et al., 2011).

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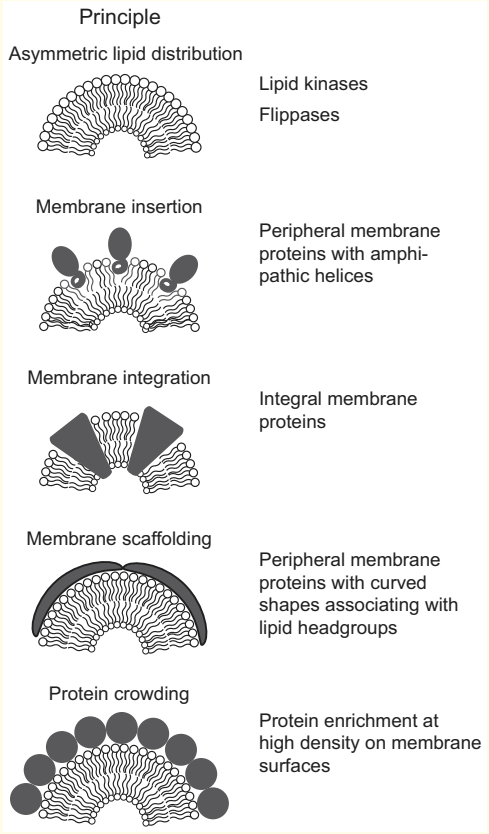
Box 2. Principles for the generation of membrane curvature by protein action

Generally, the modeling of cell membranes to yield curvature and to shape organelles, vesicles and tubules is thought to depend on several different principles (see box figure) (for recent reviews, see Derganc et al., 2013; Kirchhausen, 2012; Kozlov et al., 2014). An asymmetric distribution of membrane lipids causes membrane bending if the head groups on the two sides are of different sizes. An unequal distribution of lipids can be also caused by flippases that actively shuttle lipids across the membrane, and by the action of head-group-modifying enzymes such as PtdIns kinases. Partial insertion or integration of protein domains into the hydrophobic core of the membrane can create a wedging effect that forces the phospholipids to tilt. In addition, certain peripheral membrane proteins can, depending on their own shape or on the oligomeric complexes they create, stabilize the generated geometry by forming a membrane scaffold. Also, the enrichment of membrane-binding proteins at high density on one side of the membrane is thought to generate curvature by a crowding effect (Stachowiak et al., 2012). Although not yet elucidated in detail, several of the above mentioned principles are likely to be at work in the autophagy pathway. Table 1 lists autophagy-related proteins with proposed functions in membrane-binding and modeling events.

Membranes can undergo two fundamentally different types of transformations: (1) shape changes without disruption of the bilayer. This type of structural alteration is referred to in the literature as membrane bending, curvature generation or membrane sculpting; (2) transient distortions of the continuity of the bilayer, leading to the formation of new membrane surfaces, such as those occurring during membrane fusion and fission reactions. This type of process is referred to as membrane remodeling. The two types of shape transformations can collectively be termed membrane modeling (Campelo et al., 2010; Kozlov et al., 2014).



membrane source hierarchy. However, as most investigations have been performed in cells undergoing starvation-induced autophagy, it cannot be ruled out that, depending on the autophagy-inducing signal and the nature of the cargo to be sequestered, different membrane sources contribute to phagophore nucleation and/or expansion, as discussed below.

Phagophore nucleation

Several of the factors known to be crucial for phagophore nucleation have membrane-interacting domains that are thought to be important for their targeting to the appropriate membrane and/or their ability to mediate membrane shaping (see Table 1). Based on recent live-cell imaging and genetic and biochemical studies, we propose that the following events lead to phagophore nucleation upon induction of autophagy. Firstly, the mammalian ULK (UNC51-like) kinases and their associated complex partners [ATG13, FIP200 (also known as RB1CC1) and ATG101] are recruited to the membrane at the site of phagophore nucleation (for a recent review, see Wirth et al., 2013) (Fig. 2A). This might be mediated by the C-terminal domain of ULK (and its yeast ortholog Atg1), which is called the early autophagy targeting/tethering (EAT) domain, as it appears to be essential for recruitment to the site of phagophore nucleation (Chan et al., 2009; Ragusa et al., 2012). Moreover, a cluster of positive amino acids in the N-terminus of ATG13 has been found to interact with acidic phospholipids and to be important for the translocation of ATG13 to omegasomes (Karanasios et al., 2013). ULK1 has been found to be targeted to pre-existing structures containing the ER-resident multi-membrane spanning protein vacuole membrane protein 1 (VMP1) (Koyama-Honda et al., 2013). Secondly, PI3KC3 is recruited to phagophore nucleation sites to generate PtdIns3*P* (Fig. 2A). A recent study found that the N-terminal domain of yeast Atg13 forms a HORMA domain that is required for autophagy and for recruitment of the PI3KC3 subunit Atg14 to the PAS (Jao et al., 2013). ATG14L contains an ER-binding motif in its N-terminal domain, which appears to be essential for its function in autophagy and for the recruitment of the other PI3KC3 subunits (see Box 1) to the sites of phagophore formation (Matsunaga et al., 2010). An interaction between VMP1 and beclin 1 (Molejon et al., 2013) might further stabilize the association of the PI3KC3 complex with the ER membrane. Finally, the ATG12–ATG5–ATG16L1 complex (see Box 1) is recruited to the phagophore (Fig. 2A,B). ATG5 was found to be recruited to VMP1-positive structures concomitant with ULK1 (Koyama-Honda et al., 2013), which is rather surprising as the ATG12–ATG5–ATG16L1 complex has been placed downstream of the ULK1 and PI3KC3 complexes in the functional hierarchy of autophagosome formation. The synchronized recruitment of ATG5 with ULK1 might be mediated by a recently described interaction between the ULK complex subunit FIP200 and ATG16L1 (Gammoh et al., 2013; Nishimura et al., 2013). The VMP1–beclin 1 interaction was also found to facilitate the association of ATG16L1 and LC3 (also known as microtubule-associated protein 1 light chain 3) with the autophagosomal membranes (Molejon et al., 2013). As VMP1 expression is known to trigger autophagy (Ropolo et al., 2007) and because it seems to be the most ‘temporally’ upstream factor identified to date, it is tempting to hypothesize that VMP1 acts as a recruitment platform that determines the site of phagophore formation by recruitment of the early core ATG proteins. Further characterization of the VMP1-positive structures is required to understand why phagophores or omegasomes form from such spots, and why not all VMP1 spots appear to be involved in phagophore formation. The beclin-1-interacting transmembrane ER

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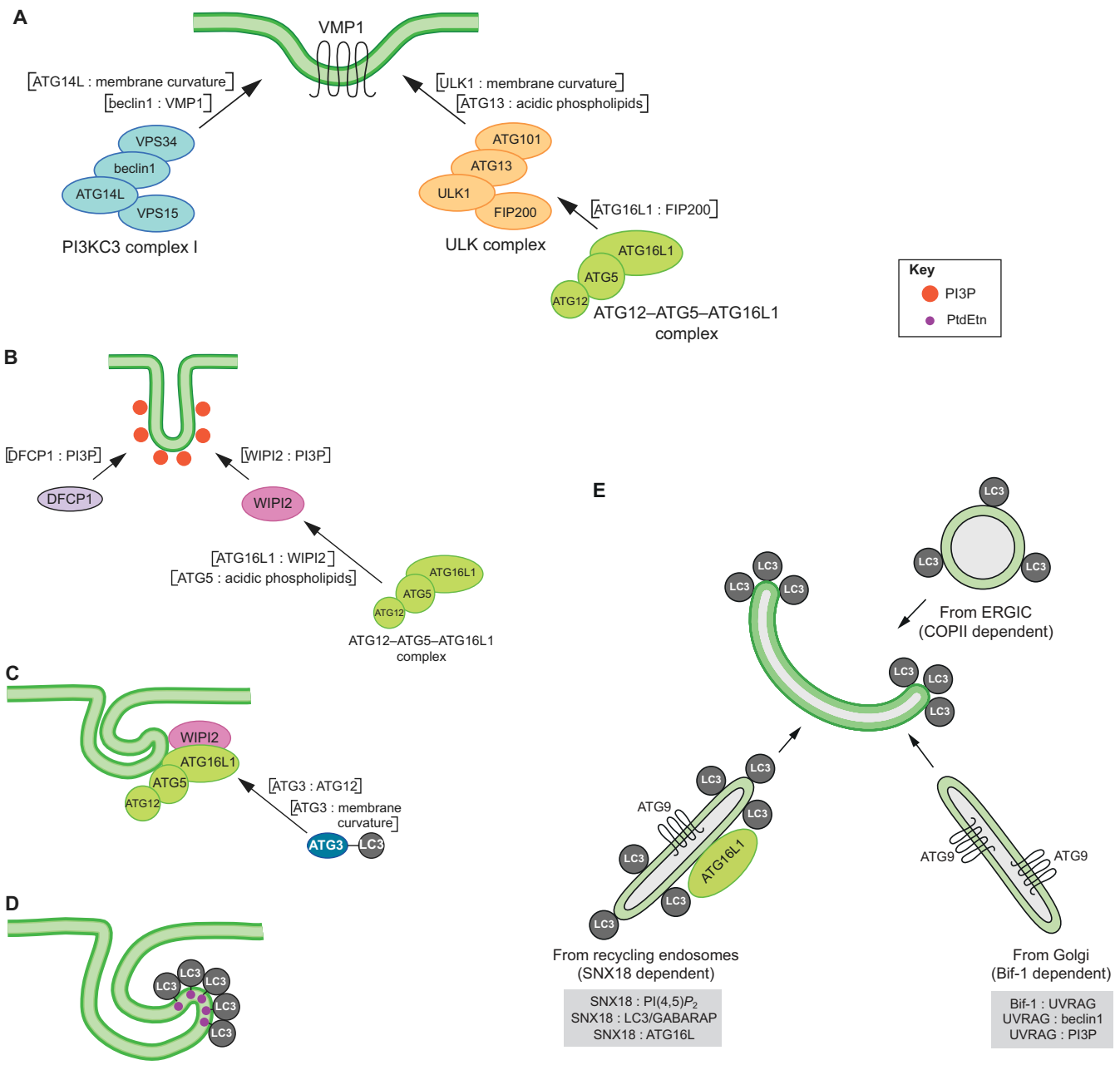

Fig. 2. A model for the events involved in autophagosome biogenesis in mammalian cells. The different steps at the autophagy initiation sites leading to the accumulation of membrane-bound LC3 and phagophore expansion are illustrated. Participating proteins, depicted as individual protein species or present in complexes, are shown. The protein–protein or protein–membrane interactions described here are indicated in square brackets, where ':' denotes a physical interaction occurring at or near the membrane. For protein domains involved in membrane interactions, see Table 1. (A) The ULK and PI3KC3 complexes are initially recruited to curved membrane sites containing the transmembrane protein VMP1, followed by recruitment of the ATG12–ATG5–ATG16L1 complex. (B) PtdIns3*P* (PI3P) produced by membrane-bound PI3KC3 further enhances membrane bending and forms a platform for the recruitment of WIPI2, which in turn recruits more ATG12–ATG5–ATG16L1. (C) Bound ATG12–ATG5–ATG16L1 recruits activated LC3 (ATG3–LC3), resulting in the lipidation of LC3 at the curved membrane forming the phagophore (D). (E) Vesicles and tubules originating from the ERGIC, Golgi and recycling endosomes, some of which presumably already contain lipidated LC3, are added to the phagophore to expand its size. It should be noted that the events depicted might not be strictly sequential, as recent studies indicate synergy between the reactions. The association and dissociation of proteins and protein complexes are known to be temporally regulated, but for clarity only membrane associations are shown. In E, protein–protein and protein–lipid interactions thought to be of relevance for the expansion of the phagophore are indicated in gray boxes. For further explanation, see text.

membrane protein complex subunit 6 (EMC6) might further contribute to the generation of the unique geometry of the phagophore, as it localizes to omegasomes and its depletion causes the accumulation of autophagosomal precursor structures and impaired autophagy (Li et al., 2013).

The role of PtdIns3*P* in phagophore formation

Although the initial recruitment of the early core autophagy components to sites of phagophore nucleation can act independently of PI3K activity (Itakura and Mizushima, 2010), PtdIns3*P* production appears to be important for their stable

Table 1. Autophagy proteins with membrane-modifying or membrane-modeling activity

Mammalian protein	Yeast ortholog	Protein function	Membrane-active domain	Proposed or putative membrane-active role	References
ULK1/2	**Atg1**	Ser/Thr kinase	C-terminal EAT domain	Membrane targeting; curvature sensing; vesicle tethering	Chan et al., 2009; Ragusa et al., 2012
FIP200[a]	**Atg17**	Scaffold for Atg1/ULK complexes	Elongated, crescent-shaped α-helical structure	Vesicle tethering	Ragusa et al., 2012
ATG13	Atg13	Scaffold for Atg1/ULK complexes	N-terminal cluster of positively charged amino acids	Membrane targeting	Karanasios et al., 2013
ATG14L	Atg14[b]	Autophagy-specific PI3KC3/Vps34 complex recruitment subunit	C-terminal amphipathic helix within a BATS domain	Curvature sensing and stabilization	Fan et al., 2011
PI3KC3	**Vps34**	Phospholipid kinase	Kinase domain	PtdIns phosphorylation (PtdIns3*P* generation)	Petiot, 2000; Kihara et al., 2001
WIPI1/2	**Atg18**	PtdIns3*P* effector	β-propeller structure	Membrane targeting and insertion	Krick et al., 2012; Baskaran et al., 2012; Watanabe et al., 2012
ATG3	Atg3	E2-like enzyme for Atg8/LC3–PtdEtn conjugation	N-terminal amphipathic helix	Curvature sensing	Nath et al., 2014
ATG5	**Atg5**	Part of an E3-like ligase for Atg8/LC3–PtdEtn conjugation	Cluster of positively charged amino acids	Membrane targeting	Romanov et al., 2012
Bif-1	–[c]	Membrane sculpting and protein scaffolding	N-BAR domain	Membrane curvature sculpting and stabilization	Takahashi et al., 2011
SNX18	–	Membrane sculpting and protein scaffolding	PX-BAR superdomain	Membrane curvature sculpting and stabilization	Knævelsrud et al., 2013
Class II PI3K	–	Phospholipid kinase	Kinase domain	PtdIns phosphorylation (PtdIns3*P* generation)	Devereaux et al., 2013
PI4K	**Pik1**	Phospholipid kinase	Kinase domain	PtdIns phosphorylation (PtdIns4*P* generation)	Wang et al., 2012
MTMR3 and MTMR14 (Jumpy)	Ymr1	Phospholipid phosphatases	Phosphatase domain	PtdIns3*P* dephosphorylation	Taguchi-Atarashi et al., 2010; Vergne et al., 2009
ATG9	Atg9	Integral membrane protein with unknown function	Transmembrane domain	Membrane wedging?	
VMP1	–	Integral membrane protein with unknown function	Transmembrane domain	Membrane wedging?	
LC3 family proteins	Atg8	PE-conjugated membrane protein	Phospholipid anchor	Membrane crowding?	

Orthologs for which a membrane-active function has been described or proposed are shown in bold. For some mammalian proteins no obvious yeast ortholog exists (–). [a]Mammalian FIP200 and Atg101 are viewed as functional counterparts to the yeast Atg17–Atg29–Atg31 complex without showing any sequence similarity (Mizushima et al., 2011). [b]Yeast Atg14 appears to lack a BATS domain. [c]A distantly related protein is present in *Schizosaccharomyces pombe* and some other fungi, but not in *Saccharomyces cerevisiae* (Takahashi, 2009).

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membrane association and further phagophore growth, as almost all ULK1, ATG5 and ATG16L1 punctae disappeared upon inhibition of PI3K activity (Karanasios et al., 2013; Koyama-Honda et al., 2013). PtdIns3*P* is an inverted cone-shaped membrane lipid that, when clustered, can be seen to create a cytosol-facing bud in the membrane, which in turn can create a platform for the binding of membrane-active proteins. In line with this, ATG14L has been found to sense membrane curvature through an amphipathic α-helix located in its C-terminal BATS domain (Fan et al., 2011), which might facilitate its recruitment to already curved structures and/or be important for further stabilization of phagophore curvature. Interestingly, the C-terminal EAT domain of Atg1/ULK1 also appears to sense membrane curvature, as it binds to liposomes in a geometry-dependent manner (Ragusa et al., 2012). Recently, ULK1 was found to activate the PI3KC3 complex by phosphorylation of beclin 1 (Russell et al., 2013), and the ULK complex subunit ATG13 was found to bind to PtdIns3*P* (Karanasios et al., 2013), indicating that the ULK1 complex plays an important role in PtdIns3*P* production and stabilization at the phagophore. Furthermore, the crystal structure of yeast Atg17, which forms an Atg1-interacting complex with Atg29–Atg31, showed that it forms a crescent-shaped dimer reminiscent of curvature-sensing and inducing Bin-Amphiphysin-Rvs (BAR) domains, suggesting that Atg17 contributes to the stabilization of membrane curvature and vesicle tethering at the PAS (Ragusa et al., 2012). It is not known whether ULK proteins or mammalian ATG17 have similar functions, but it is interesting to note that the beclin-1-interacting protein AMBRA1 (activating molecule in beclin-1-related autophagy 1) mediates ULK1 dimerization (Nazio et al., 2013) and that ULK1-mediated phosphorylation of AMBRA1 is important for proper localization of the PI3KC3 complex to sites of phagophore nucleation (Di Bartolomeo et al., 2010). Taken together, these studies strongly support a model whereby the PI3KC3 and ULK complexes are recruited to specific membrane spots in the ER membrane where they act in a synergistic manner to stimulate PtdIns3*P* production and facilitate the elongation and initial membrane curvature of the phagophore membrane.

The LC3 lipidation reaction

In addition to a possible role for PtdIns3*P* as a cone-shaped lipid important for initial phagophore curvature, PtdIns3*P* facilitates the recruitment of autophagy proteins to the membrane (Fig. 2B). An important group of PtdIns3*P*-binding proteins are the PROPPINs (β-propellers that bind polyphosphoinositides), including the mammalian WD repeat domain phosphoinositide-interacting (WIPI) proteins and their yeast homolog Atg18 (Bakula et al., 2013), as well as yeast Atg21, which is involved in the cytoplasm-to-vacuole (Cvt) pathway (Krick et al., 2008) (for details on WIPI proteins, see Box 3).

A recent study shows that WIPI2 functions to recruit ATG16L1 to sites of phagophore formation (Dooley et al., 2014). A WIPI2-binding site in ATG16L1 that is different from the sites involved in binding to ATG5 and FIP200 has been identified. Interestingly, this WIPI2-binding site is absent from ATG16L2, an isoform of ATG16L1 that is not involved in autophagy, although it forms a complex with ATG5–ATG12 (Ishibashi et al., 2011). The ATG12–ATG5–ATG16L1 complex mediates the transfer of activated LC3 or GABARAP from the E2-like enzyme ATG3 to phosphatidylethanolamine (PtdEtn) and appears to determine the site of LC3 or GABARAP lipidation (Fujita et al., 2008b) (see Box 1; Fig. 2C). An amphipathic N-terminal helix in ATG3 was found to have curvature-sensing properties and to be necessary for its activity, suggesting that ATG3 might detect lipid-packing defects at the rim of the growing phagophore (Nath et al., 2014). This function would localize the lipidation reaction of LC3 or GABARAP to the highly curved surface at the edge of the growing phagophore where the addition of incoming vesicles is thought to occur (Fig. 2D). Therefore, the sequence of events leading to the production of lipidated LC3 or GABARAP at the phagophore could be explained by the following order of events: the ULK and PI3KC3 complexes are recruited to sites of phagophore nucleation, leading to PtdIns3*P* production and WIPI2 recruitment (as described above), followed by recruitment of the ATG12–ATG5–ATG16L1 complex, which again facilitates the transfer of LC3 or GABARAP from ATG3 to PtdEtn at the rim of the phagophore membrane. LC3/GABARAP-containing membrane might also be produced by ATG16L1 at other locations in the cell and transported in vesicular form to yield additional membrane for phagophore growth. One such source of membrane is recycling endosomes, which have been found to contain ATG16L1 and to contribute to the production of LC3/GABARAP-containing membrane by a process dependent on the PX-BAR protein sorting nexin 18 (SNX18) (Knævelsrud et al., 2013). In this context, ATG16L1 is likely to be recruited by other means than binding to WIPI2, as WIPI2 is not found on endosomes and the process was shown to be dependent on phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] rather than PtdIns3*P*.

PtdIns3*P* levels at the phagophore appear to be tightly regulated by phosphoinositide 3-phosphatases. Both MTMR14 (myotubularin-related protein 14) and MTMR3 were identified as negative regulators of autophagy that act by preventing recruitment of WIPI1 (WIPI2 not investigated) to phagophores (Taguchi-Atarashi et al., 2010; Vergne et al., 2009). Interestingly, smaller autophagosomes were seen in cells with overexpression of MTMR3 (Taguchi-Atarashi et al., 2010), indicating that PtdIns3*P* levels at the phagophore regulate autophagosome size. A likely explanation for this was provided by studies in yeast, showing that PtdIns3*P* hydrolysis (by the MTMR-related

Box 3. The WIPI proteins

WIPI proteins are recruited to the sites of autophagosome formation (Polson et al., 2010; Proikas-Cezanne et al., 2007), and recent structural analyses show that they have the ability to interact with two PtdIns3*P* molecules through a specific FRRG motif in their β-propeller structure (Baskaran et al., 2012; Krick et al., 2012; Watanabe et al., 2012). As PtdIns3*P* is found on many cellular membranes it was not known how these autophagy-specific PtdIns3*P*-binding proteins are specifically targeted to the phagophore. Recent elegant studies of Atg18 (the yeast ortholog of WIPI) show that binding to both Atg2 and PtdIns3*P* facilitates its association with the PAS and autophagy (Rieter et al., 2013). PtdIns3*P* and Atg18–Atg2 are also involved in cycling of the transmembrane protein Atg9 to the phagophore. Phosphorylation of Atg18 appears to determine its lipid-binding specificity, as increased binding to PtdIns(3,5)P_2 was detected upon Atg18 dephosphorylation (Tamura et al., 2013). Binding of Atg18 to PtdIns(3,5)P_2 is required for the localization of Atg18 to the vacuolar membrane where it regulates vacuolar morphology (Efe et al., 2007).

phosphatase Ymr1) is required for the dissociation of core Atg proteins before closure of the phagophore membrane to form an autophagosome (Cebollero et al., 2012).

Other lipids involved in phagophore formation

The roles of phospholipids and sphingolipids as signaling molecules for the regulation of autophagy are subjects of great current interest (for reviews, see Dall'Armi et al., 2013; Knævelsrud and Simonsen, 2012), but the influence of membrane lipid species on the structure and/or function of phagophores and autophagosomes has not been thoroughly studied. Owing to technical difficulties in the biochemical isolation of autophagic membranes in pure form, the exact lipid composition of these membranes is not known. As the interplay between constituent membrane lipids and proteins is vital for the behavior and biological function of a membrane (Sprong et al., 2001), future research into autophagy 'lipidomics' will likely provide novel insights into the processes of phagophore formation and growth. Knowledge about the membrane lipids that build up the autophagosomes is crucial for the interpretation of *in vitro* reconstitution experiments with purified proteins and liposomes that are increasingly being performed.

A role for the PI3KC3 complex in the initial phagophore formation is very convincing, but the class II PI3K (PI3KC2, also known as PIK3C2) was also found to regulate autophagosome biogenesis by contributing to PtdIns3*P* generation at autophagosome nucleation sites (Devereaux et al., 2013). Apart from PtdIns3*P*, no specific lipid has been identified that marks the phagophore nucleation site or autophagic membranes in general. However, other phosphoinositides might come into play during phagophore elongation, as shown for phosphatidylinositol 4-phosphate (PtdIns4*P*) (Wang et al., 2012), PtdIns(4,5)P_2 (Knævelsrud et al., 2013) and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] (Ho et al., 2012). Although these lipid species are mainly viewed as recruitment platforms for autophagy-related proteins in this context, they might, in addition, influence the structure and geometry of the membrane when clustered, owing to their inverted cone shape.

Cone-shaped membrane lipids have a propensity to form negative membrane curvature, such as that required for the sharp negative curvature of the lumenal membrane leaflet at the edges of the growing phagophore. One such lipid, phosphatidic acid, might have a role in autophagy, as the phosphatidic-acid-producing enzyme phospholipase D1 was found to localize, in a PtdIns3*P*-dependent manner, to autophagosome-related structures upon starvation (Dall'Armi et al., 2010). Other specific lipids, such as sphingosine-1-phosphate and ceramide, are also suggested to have roles in autophagy (Dall'Armi et al., 2013; Knævelsrud and Simonsen, 2012), but it is not yet known whether these lipids are utilized for the recruitment of other factors or whether they have a role in the generation of membrane structure.

PtdEtn is the co-substrate for the lipidation reaction of LC3 or GABARAP (or the yeast homolog Atg8) and is required in the membrane at the nucleation site and elsewhere (Mizushima et al., 2011). PtdEtn is produced from phosphatidylserine (PtdSer) in mitochondria, a fact that has been used as the basis of the argument that mitochondria might supply membrane for autophagosome biogenesis (Hailey et al., 2010). However, PtdEtn is also shuttled from mitochondria to ER, through mitochondria–ER contact sites, and can be delivered efficiently from there to other organelles (Rowland and Voeltz, 2012). The plasma membrane and recycling endosomes also contain PtdEtn, and the latter organelle is, in addition, rich in PtdSer (Gagescu et al., 2000; Taguchi, 2013). PtdSer is highly exposed on the cytosolic side of the recycling endosome membrane, possibly by the action of a flippase (Uchida et al., 2011), and has been described as being important for endosomal trafficking in yeast and higher eukaryotes (Uchida et al., 2011; Xu et al., 2013). It is not yet known whether PtdSer is involved in the biogenesis of autophagosomes.

Autophagosomal membranes appear to be rich in unsaturated lipids (Reunanen et al., 1985), and a recent study indicated that the nature of the hydrocarbon chains of membrane lipids influences autophagy (Ogasawara et al., 2014). Inhibition of a stearoyl-CoA desaturase resulted in the suppression of autophagy at an early stage, indicating that unsaturated fatty acids are required for autophagosome formation. Genetic depletion of the corresponding enzyme in *Drosophila* also affects autophagy (Köhler et al., 2009). These findings point to the possibility that membrane fluidity is an important factor in the intricate dynamics and curvature of the autophagic membrane.

Later steps in autophagosome biogenesis: generation of new membrane

Phagophore elongation

The size of autophagic cargoes varies from small precursor aminopeptidase I (prApe1) particles of the Cvt pathway in yeast (~150 nm) (Jin and Klionsky, 2014) to large bacteria and mitochondria (up to several micrometers) in selective autophagy (Stolz et al., 2014). The process of phagophore elongation and closure must therefore be flexible and regulated in a cargo-specific manner.

Elucidating the localization and trafficking of ATG9, the only transmembrane protein that is recruited to the omegasome and that is required for autophagy (and that of yeast Atg9 to the PAS), might provide insight into the process of phagophore elongation (Mari and Reggiori, 2007; Webber and Tooze, 2010; Zavodszky et al., 2013). The majority of yeast Atg9 exists on highly dynamic Golgi-derived vesicles with a diameter of 30–60 nm that appear to be generated upon induction of autophagy (Mari et al., 2010; Yamamoto et al., 2012). These vesicles then accumulate at the PAS in an Atg1-dependent manner, where Atg1-mediated phosphorylation of Atg9 further facilitates the recruitment of Atg8 and Atg18 and subsequent phagophore expansion (Papinski et al., 2014). Induction of autophagy also leads to the dispersal of mammalian ATG9 from the Golgi into tubo-vesicular compartments, including recycling endosomes, in an ULK1-dependent manner (Orsi et al., 2012; Young et al., 2006). Moreover, the N-BAR-containing protein Bif-1 (Bax-interacting factor 1, also known as endophilin B1) (Takahashi et al., 2011) and binding of its interaction partner UVRAG (ultraviolet irradiation resistance-associated gene) (Takahashi et al., 2007) to PtdIns3*P* appear to regulate the transport of ATG9 from the Golgi to the phagophore (He et al., 2013). ATG9 was recently found to traffic through the plasma membrane into recycling endosomes, where it colocalizes with ATG16L1 (Puri et al., 2013). Vesicular or tubular trafficking from recycling endosomes might feed ATG9- and ATG16L1-positive membrane onto the growing phagophore in a process that appears to be regulated by the PX-BAR protein SNX18 and by the RAB11 effector protein TBC1D14 in an opposite manner (Knævelsrud et al., 2013; Longatti et al., 2012). Interestingly, yeast Atg9 was found to be embedded in the outer membrane of the autophagosome, and, after fusion with the vacuole, new Atg9-positive vesicles bud off from the vacuolar

membrane (Yamamoto et al., 2012). This is in contrast to mammalian ATG9, which associates only transiently with the forming phagophore (Orsi et al., 2012), but these differences could also be explained by methodological and/or model system differences. The exact function of ATG9 is not known, but being a multi-spanning integral membrane protein, it is possible that it directly or indirectly participates in the formation of phagophore curvature, for example, by wedging of the membrane. It is also possible that ATG9 could function as a lipid transfer protein.

In addition to ATG9 and ATG16L1, recycling-endosome-derived membrane might contain membrane-anchored LC3 or GABARAP proteins (Knævelsrud et al., 2013) As several transport pathways converge at recycling endosomes, such trafficking would efficiently supply phagophores with membrane of the correct composition. LC3-containing membranes formed at other cellular locations could also be sources for phagophore elongation, and, in a recent study, the ERGIC membrane was found to stimulate LC3 lipidation both *in vitro* and *in vivo* (Ge et al., 2013). It is tempting to speculate that phagophore-bound proteins that have a LC3-interacting region (LIR) could mediate the docking of such incoming LC3-containing membranes. The ULK1 complex subunits ULK1, ATG13 and FIP200 all have an LIR motif that preferentially binds to GABARAP family proteins *in vitro* (Alemu et al., 2012). Although the ULK1 complex functions in early phagophore nucleation, recent studies have found that the LIR motifs of ULK1 and Atg1 are not required for this process but rather for their recruitment into autophagosomes and their colocalization with WIPI2- and LC3B-positive structures (Alemu et al., 2012; Kraft et al., 2012; Nakatogawa et al., 2012). Thus, the interaction of Atg1/ULK1 with Atg8 homolog proteins might facilitate the recruitment of membrane to the forming phagophore.

Tethering and fusion of incoming phagophore membranes

What are the mechanisms involved in the tethering and fusion of incoming membrane to the phagophore, and how is the curvature of the phagophore obtained? As discussed above, the class III PI3K and ULK complexes might contribute to the formation and stabilization of phagophore curvature, through production of PtdIns3*P*, but also as membrane-binding proteins with curvature-generating properties. Recent structural and functional analysis of the yeast Atg1 complex (also containing Atg17, Atg29 and Atg31) indicates that it forms a crescent-shaped dimer, where the Atg1 EAT domain is able to bind to and tether highly curved 20–30 nm vesicles, corresponding to the size of Atg9-positive vesicles (Ragusa et al., 2012). This, together with the findings that an interaction between Atg17 and Atg9 is required for Atg9 localization to the PAS (Sekito et al., 2009), that Atg17–At29–Atg31 can regulate the activity of the Atg1 EAT domain (Ragusa et al., 2012) and that Atg1-mediated phosphorylation of Atg9 is required for phagophore expansion (Papinski et al., 2014), indicate that the Atg1 complex is involved in the recruitment and tethering of Atg9 vesicles to the phagophore. Moreover, Atg11, a scaffold protein required for the recruitment of Atg9 to the PAS, was found to tether Atg9 vesicles (Lipatova et al., 2012), and Atg9 itself might facilitate transport of the TRAPP (transport protein particle) vesicle tethering complex to the PAS (Kakuta et al., 2012). The yeast SNARE proteins Sso1, Sso2, Sec9 and Tlg2 have all been implicated in the early steps of autophagy (Nair et al., 2011) and might mediate the fusion of these tethered Atg9 vesicles. In mammalian cells, small ATG16L1 vesicles accumulate upon depletion of the endosomal SNARE molecules VAMP7, syntaxin 7, syntaxin 8 and VT1B (also known as VTI1B or vesicle transport through interaction with t-SNAREs homolog 1B) (Moreau et al., 2011). The SNARE protein syntaxin 17 (STX17) appears to play a dual role in autophagy, being an upstream effector for ATG14L that is required for phagophore formation at the ER–mitochondria contact sites (Hamasaki et al., 2013a), but that is also important for the fusion of autophagosomes with lysosomes through the association of a C-terminal hairpin structure of two tandem transmembrane domains in STX17 with the outer membrane of completed autophagosomes (Itakura et al., 2012; Jiang et al., 2014). The latter is supported by the interaction of STX17 with SNAP-29 and the endosomal/lysosomal SNARE VAMP8 (Itakura et al., 2012), as well as the homotypic fusion and vacuole protein sorting (HOPS) tethering complex (Jiang et al., 2014).

Several members of the RAB (Ras-related protein) family of small GTPases have been implicated in autophagy. RAB proteins cycle between an active GTP-bound state and an inactive GDP-bound state and act by recruiting effector proteins to mediate trafficking between different compartments (Stenmark, 2009). RAB1 regulates vesicular transport between the ER and Golgi, and was recently found to be important for omegasome formation (Mochizuki et al., 2013). The yeast TRAPPIII complex acts as an autophagy-specific guanine nucleotide exchange factor (GEF) for Ypt1 (the yeast homolog of RAB1) (Lynch-Day et al., 2010). TRAPPIII is recruited to the PAS by Atg17, and the activated Ypt1 stimulates the recruitment of Atg1 to the PAS (Wang et al., 2013), suggesting that TRAPPIII and Ypt1/RAB1 might facilitate the tethering of Atg9 vesicles. Moreover, the conserved oligomeric Golgi (COG) complex, another Ypt1 effector, localizes to the PAS and is required for proper localization of Atg8 and Atg9 (Yen et al., 2010). Interestingly, the TRAPPIII complex interacts with a subunit of the coat protein complex (COP)II, suggesting that COPII vesicles might provide membrane for phagophore formation and/or elongation (Tan et al., 2013). It is not known whether TRAPP complexes are involved in autophagy in mammalian cells, but as RAB1 and functional ER exit sites (ERES) have been implicated in autophagosome biogenesis (Graef et al., 2013; Zoppino et al., 2010) it is likely that ERES-derived COPII-coated vesicles also provide membrane to the growing phagophore in a TRAPP-dependent manner. The Golgi-resident protein RAB33B interacts with ATG16L1 and modulates LC3 lipidation (Itoh et al., 2008), but has also been implicated in the fusion of autophagosomes with lysosomes (Itoh et al., 2011). Thus, further studies are needed to fully understand RAB33B-mediated membrane fusion events in autophagy. The endosomal RAB proteins RAB4 (Talaber et al., 2014), RAB5 (Ravikumar et al., 2008), RAB7 (Gutierrez et al., 2004; Jäger et al., 2004; Pankiv et al., 2010; Tabata et al., 2010) and RAB11 (Fader et al., 2008; Knævelsrud et al., 2013; Longatti et al., 2012; Puri et al., 2013) have all been shown to play a role in autophagy. However, as endosomes have a dual role in autophagy, because they fuse with autophagosomes after their closure and, at the same time, provide membrane input to the forming phagophore, it is difficult to interpret the function of the different endosomal RAB proteins in autophagy (for recent reviews, see Szatmári and Sass, 2014; Tooze et al., 2014).

Membrane curvature of autophagosomes

No typical ‘coat’ structure is visible in electron microscopy images, and it is possible that hitherto unknown curvature-generating proteins act to remodel and stabilize the autophagic membrane into the correct dimension. It is conceivable that Atg8

homolog proteins themselves participate in forming the geometry of the autophagosome. LC3 lipidation and accumulation at the membrane surface might create a crowding effect that leads to membrane bending (LC3-II can indeed be envisioned as a membrane lipid with a very large head group). In such a scenario, curvature might be the result of membrane tension caused by the unbalanced distribution of LC3-II between the concave and the convex side of the double bilayer. Moreover, as PtdEtn, the target of LC3 lipidation, is a cone-shaped lipid, it might further contribute to the membrane curvature and possibly to the regulation of autophagosome size. Indeed, in experiments where the level of Atg8 was manipulated, the autophagosome size was found to be affected (Xie et al., 2008). LC3-mediated recruitment of cargo-bound autophagy receptors to the concave surface of the phagophores might also influence the curvature of the expanding phagophore. Several such cargo-specific receptors have been identified that all possess LIR sequences that bind to LC3 and GABARAP proteins (Rogov et al., 2014). LC3-II molecules that are trapped inside the autophagosome together with receptors and cargo are eventually degraded, whereas LC3-II on the outer convex membrane is recycled to the cytosol by the action of the cysteine protease ATG4 (Kabeya et al., 2004). Little is known about the specific roles of the individual Atg8 homolog proteins in autophagy, but one study found that LC3 is important for phagophore elongation, whereas GABARAPL2 functions at a later stage of the process (Weidberg et al., 2010). LC3 family proteins have also been implicated in the fusion reaction that is required to seal the membrane (Fujita et al., 2008a; Weidberg et al., 2010).

In vitro studies with liposomes and purified autophagy proteins have suggested additional functions of the essential ATG12–ATG5–ATG16L1 complex. Yeast Atg5 was shown to have affinity for negatively charged lipids, and to mediate the tethering of membrane vesicles independently of Atg8 (Romanov et al., 2012). Such a mechanism might be at work in the early fusion of incoming vesicles, as suggested for Atg9-containing vesicles (Mari et al., 2010; Yamamoto et al., 2012). Another study, also using yeast proteins, showed that once lipidated Atg8 is formed, it can anchor the Atg12–Atg5–Atg16 complex to the membrane through a specific Atg8–Atg12 interaction, leading to the assembly of a 'coat-like' scaffold at the convex side of the phagophore (Kaufmann et al., 2014). At the concave side, binding of cargo or cargo adaptors through Atg8–LIR-sequence interactions would outcompete the binding of Atg8 to Atg12, providing an explanation as to why Atg16–Atg12–Atg5 is exclusively found on the outer phagophore membrane (Mizushima et al., 2011). It remains to be determined whether these interesting observations with purified proteins indeed reflect mechanisms that operate in the cell.

Membrane modeling in selective autophagy

When it comes to selective autophagy, it is likely that the cargo and its bound autophagy receptors contribute to phagophore expansion and curvature. A recent study found that the Cvt vesicle cargo prApe1 facilitates the interaction of the cargo receptor Atg19 with Atg8 in the membrane (Sawa-Makarska et al., 2014). Atg19 contains multiple Atg8-binding motifs in its C-terminus, whereas its paralog Atg34 contains only one Atg8-binding motif. Using an *in vitro* reconstituted system with giant unilamellar vesicles (GUVs) and prApe1 pro-peptide beads, it was found that the Atg8-positive GUV membrane closely wrapped around the beads in the presence of Atg19 and that this correlated with the density of Atg8 and the Atg8-binding activity of Atg19. A chimeric Atg19–Atg34 protein with one Atg8-binding site was unable to support prApe1 processing through the Cvt pathway, although binding to the prApe1 cargo was intact. In contrast, prApe1 processing was nearly normal upon rapamycin-induced bulk autophagy, leading the authors to suggest a model in which close membrane–cargo apposition (termed 'exclusive autophagy') requires the binding of Atg19 to several Atg8 molecules, whereas one Atg8-binding site in Atg34 (or the Atg19–Atg34 chimera) is sufficient for selective but not exclusive sequestration of prApe1.

A similar principle is likely to apply to other forms of selective autophagy. It is interesting to note that although the binding affinity between the LIR domain of the ubiquitin-binding autophagy receptor p62 (also known as SQSTM1) and LC3 or GABARAP is rather low (Maruyama et al., 2014), the avidity of the interaction is high, owing to PB1-domain-mediated self-polymerization of p62 (Bjørkøy et al., 2005). This likely contributes to the exclusive p62-mediated sequestration of cargo (e.g. protein aggregates). The p62-interacting adaptor protein ALFY (also known as WDFY3) has an LIR motif with a greater than tenfold higher affinity for GABARAP proteins than that of the p62 LIR (Lystad et al., 2014). Binding of ALFY to GABARAP facilitates the recruitment of LC3B to ALFY and p62-positive structures, further indicating that a high-affinity LIR–Atg8 protein interaction is required for exclusive autophagy. In line with this, interaction of the ubiquitin-binding autophagy receptor NDP52 (also known as CALCOCO2) with LC3C was found to be required for the recruitment of other Atg8 family members to cytosolic *Salmonella* Typhimurium (von Muhlinen et al., 2012). Taken together, these studies suggest that an initial interaction between an Atg8 protein and a LIR-containing receptor forms a platform for further phagophore expansion and cargo sequestration. Several key questions remain to be addressed, including whether the phagophore membrane of phagophores involved in selective autophagy also nucleates from ER-associated omegasomes.

Membrane dynamics during phagophore closure

Little, if anything, is known about the mechanisms involved in the closure of the phagophore membrane. The phagophore is a double-membraned structure, which infers that its closure involves the fusion of a narrow opening, different from most other membrane fusion events. The topology of the phagophore is similar to that of multivesicular bodies (MVB) formed upon invagination of the early endosome membrane, viral budding and cytokinesis, which all rely on the endosomal sorting complex required for transport (ESCRT) (Rusten et al., 2012). The ESCRTs and their associated proteins facilitate membrane budding away from the cytosol and subsequent cleavage of the bud neck (Hurley and Hanson, 2010). Several studies have shown that depletion of ESCRT subunits, as well as of their regulatory ATPase Vps4, causes an accumulation of autophagosomes (Filimonenko et al., 2007; Lee et al., 2007; Rusten et al., 2007), but it is not clear whether this is due to ESCRTs being required for autophagosome closure or autophagosome–endosome fusion. It is interesting to note that several ESCRT subunits are PtdIns3*P*-binding proteins. PtdIns3*P* turnover by the PtdIns3*P* phosphatases (MTMRs, Ymr1) was found to mediate the dissociation of the early core ATGs from the phagophore membrane and to be required for the final closure of the phagophore to form an autophagosome. A tight regulation of

PtdIns3*P* levels at the phagophore also appears to regulate autophagosome size (Taguchi-Atarashi et al., 2010). Thus, one can speculate that ESCRTs are recruited to closing phagophores through interactions with PtdIns3*P*, and that a tight regulation of PtdIns3*P* levels in the highly curved membrane at the remaining opening controls the final phagophore closure. In line with such a model, loss of Atg2, which forms a complex with the PtdIns3*P*-binding protein Atg18 was found to inhibit autophagosome closure (Velikkakath et al., 2012). Several ESCRT subunits interact with ubiquitin (Shields and Piper, 2011), and a recent study found that interaction of the ESCRT-III subunit vacuolar protein sorting 2.1 (VPS2.1) with the deubiquitylating enzyme AMSH1 is important for autophagy in *Arabidopsis*. Both AMSH1 and VPS2.1 mutants accumulate autophagosome markers and have fewer autophagic bodies in the vacuole (Katsiarimpa et al., 2013). It is not known whether ubiquitin is recycled before autophagosome closure, as is the case in the endocytic pathway, and future studies are needed to address this. Consistent with such a model, the ubiquitin-like proteins LC3 and Atg8, being conjugated to both sides of the phagophore membrane, are cleaved off the outer membrane by the activity of the Atg4 protease (Kabeya et al., 2000).

Concluding remarks

Our understanding of the membrane modeling events involved in autophagosome biogenesis has increased immensely during the last few years. However, the constitutive lipid species and membrane modeling proteins that are involved in determining phagophore curvature and autophagosome size are still mostly unknown and several fundamental questions remain to be answered. Where is the membrane coming from? What are the mechanisms involved in the regulation and execution of membrane tethering and fusion? How is the final closure mediated and regulated? Investigations of phagophore membrane properties and biogenesis in the near future are likely to reveal novel principles in membrane biology.

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