The Role of Atg Proteins in Autophagosome Formation

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Keywords

autophagy, autophagosome, Atg, preautophagosomal structure, isolation membrane

Abstract

Macroautophagy is mediated by a unique organelle, the autophagosome, which encloses a portion of cytoplasm for delivery to the lysosome. Autophagosome formation is dynamically regulated by starvation and other stresses and involves complicated membrane reorganization. Since the discovery of yeast Atg-related proteins, autophagosome formation has been dissected at the molecular level. In this review we describe the molecular mechanism of autophagosome formation with particular focus on the function of Atg proteins and the long-standing discussion regarding the origin of the autophagosome membrane.

Contents

INTRODUCTION

The lysosome is the key organelle for intracellular degradation (Kornfeld & Mellman 1985, Saftig & Klumperman 2009). Extracellular materials and plasma membrane proteins are delivered to the lysosome via the endocytic pathway. By contrast, cytoplasmic materials (e.g., proteins, lipids, glycogens, and organelles) are delivered to the lysosome through various types of autophagy (**Figure 1**). Of the various modes of autophagy, macroautophagy is believed to be the major one and is the most extensively analyzed. In canonical macroautophagy, a small part of the cytoplasm is sequestered by a membrane sac, the so-called isolation membrane (also termed the phagophore), which results in formation of a double-membrane structure, the autophagosome. The autophagosome matures as it fuses with endosomes and then finally fuses with lysosomes. Following fusion, the inner membrane and enclosed cytoplasmic materials are degraded by lysosomal enzymes. The resultant degradation products, such as amino acids, can be reused for many purposes. In addition to canonical macroautophagy, special types of macroautophagy have been discovered. These include autophagy directed to organelles, such as mitochondria (mitophagy) and peroxisomes (pexophagy), and to intracellular bacteria (xenophagy) (Klionsky et al. 2007; **Figure 1**). It is now also clear that autophagosomes can even recognize certain soluble proteins, such as p62 (Johansen & Lamark 2011, Kraft et al. 2010). Through these diverse modes, autophagy is involved in many physiological processes: generation of amino acids during starvation, quality control of intracellular proteins and organelles (related to suppression of cellular degeneration and tumorigenesis), regulation of expression levels of selective substrates, degradation of pathogens, and antigen presentation, all of which are described in recent comprehensive reviews (Cecconi & Levine 2008, Deretic & Levine 2009, Levine & Kroemer 2008, Menzies et al. 2011, Mizushima & Levine 2010, Mizushima et al. 2008, Virgin & Levine 2009, White et al. 2010, Wong & Cuervo 2010).

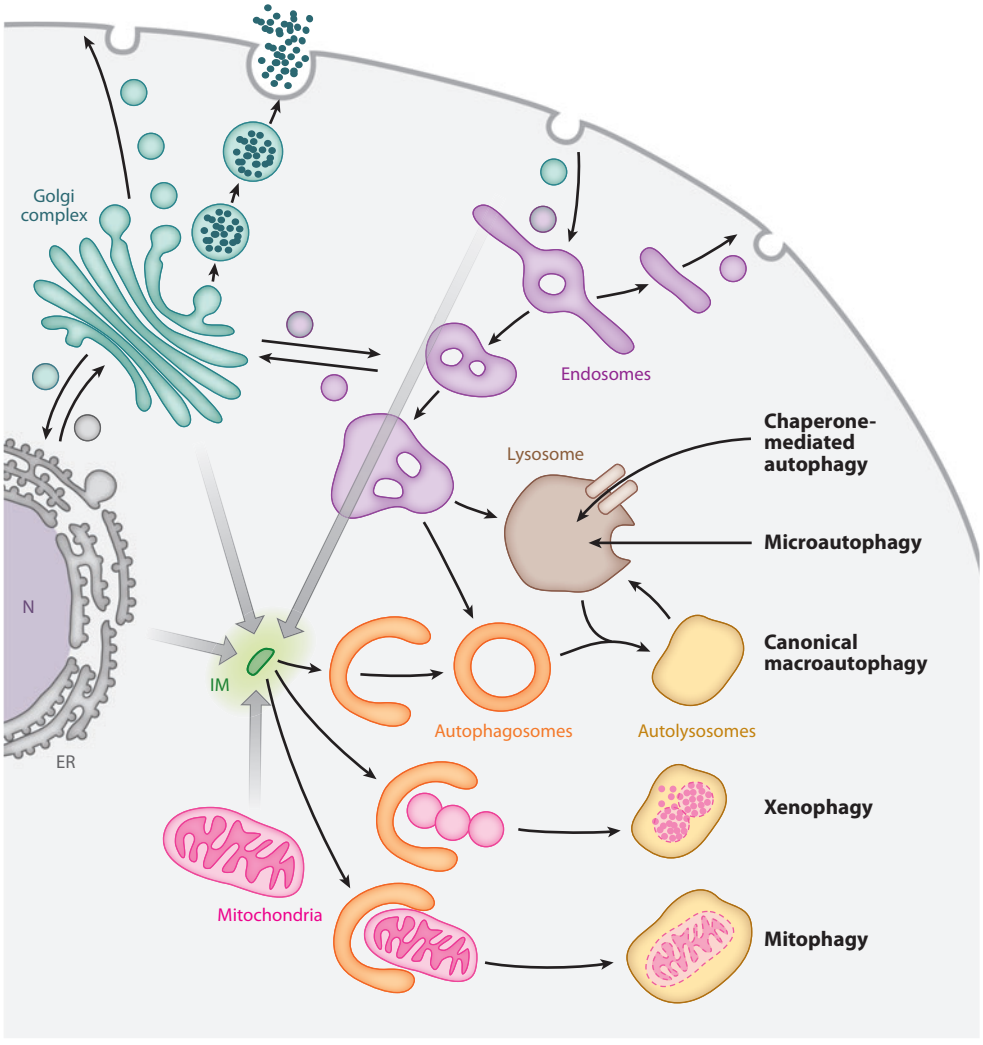


Figure 1

Membrane dynamics of autophagy in yeast and mammals. This figure shows various types of autophagy in both in yeast and mammals, including selective types of autophagy (e.g., against bacteria and mitochondria). Macroautophagy is mediated by a unique organelle called autophagosome, by which a portion of the cytoplasm is enclosed. The outer membrane of the autophagosome then fuses with the lysosome, which allows lysosomal enzymes to degrade the sequestered cytoplasmic materials in autolysosomes. Special types of macroautophagy include autophagy against organelles such as mitochondria (mitophagy) and against intracellular bacteria (xenophagy). In chaperone-mediated autophagy, substrate proteins are recognized specifically by cytosolic chaperones and directly transported across the lysosomal membrane to the lumen. Microautophagy occurs by invagination of the lysosomal membrane itself into the lumen. Abbreviations: ER, endoplasmic reticulum; IM, isolation membrane; N, nucleus.

The second type of autophagy is chaperone-mediated autophagy, which is not mediated by the autophagosome (Arias & Cuervo 2011; **Figure 1**). In this pathway, cytoplasmic substrates are recognized by the chaperone protein Hsc70 and directed toward translocation into the lysosomal lumen for degradation. The third type is microautophagy, in which the lysosomal membrane directly engulfs small parts of the cytoplasm. Unfortunately, the mechanism and role of microautophagy remain largely unknown, particularly in mammalian cells.

Autophagosome: a double-membrane structure enclosing cytoplasmic materials

ER: endoplasmic reticulum

***ATG*:** genes involved in various types of autophagy, originally identified in yeast, where *ATG* and *atg* represent dominant and recessive genes, respectively

Preautophagosomal structure (PAS): a yeast perivacuolar structure to which most Atg proteins gather to form the isolation membrane and autophagosome

Atg: autophagy-related protein

PI3K: phosphatidylinositol 3-kinase

TORC1: target of rapamycin (TOR) complex 1

In this review we focus on the mechanism of autophagosome formation, which is the key event in macroautophagy (referred to simply as autophagy hereafter). The autophagosome is a unique organelle both in its structure and in its dynamic regulation. The autophagosome is highly inducible; numbers can increase by more than tenfold during starvation. Autophagosomes are rapidly consumed by fusion with lysosomes, and their half-life can be 10–25 min in the liver (Hailey et al. 2010, Pfeifer 1978, Schworer et al. 1981). Elongation of the isolation membrane generates autophagosomes. However, the source of this membrane, how it elongates, and how it completes sealing remain unknown (Tooze & Yoshimori 2010). As the endoplasmic reticulum (ER) is frequently observed in close proximity to autophagosomes, the ER has been proposed as the source of the autophagosome or the platform for autophagosome formation (**Figure 1**). As we discuss in more detail below, recent ultrastructural and molecular studies further support this hypothesis. Nonetheless, autophagosome formation does not appear to be simple. A contribution from other organelles and membranes has also been proposed.

In addition to these morphological and biochemical approaches, the breakthrough in elucidation of the molecular machinery in autophagy came from yeast genetic studies, in which 35 autophagy-related (*ATG*) genes have been identified. Among them, *ATG1–10*, *12–14*, *16–18*, *29*, and *31* are essential for formation of canonical autophagosomes (**Table 1**; Nakatogawa et al. 2009).

Discovery of these molecules has enabled the analysis of autophagosome formation in detail, even in living cells. These approaches have identified a specific structure for autophagosome formation, the preautophagosomal structure (PAS), in yeast (Suzuki et al. 2001). The PAS is a single punctate structure close to the yeast vacuole membrane, where almost all Atg proteins are colocalized (**Figure 2**). An equivalent structure may be present on the ER in mammalian cells (Itakura 2010). Analyses of the function of these Atg and Atg-related proteins have provided fundamental information on the molecular mechanism of autophagosome formation.

ATG PROTEINS IN AUTOPHAGOSOME FORMATION

The Atg proteins involved in autophagosome formation consist of several functional units: Atg1 kinase and its regulators, the PI3K complex, Atg9, the Atg2-Atg18 complex, and two ubiquitin-like conjugation systems (**Table 1**). Mammals contain counterparts for most yeast Atg proteins as well as some additional factors that are specific to higher eukaryotes (**Table 1**). Although most Atg proteins seem to be involved mainly in autophagy, some of them have been shown to exhibit nonautophagic functions, which are discussed elsewhere (Kroemer & Levine 2008, Radoshevich et al. 2010).

Regulation by Target of Rapamycin Complex 1

Nutrient starvation can upregulate autophagy in all organisms and cultured cells. Of the various types of starvation, depletion of amino acids and/or growth factors (e.g., insulin) is most effective for inducing autophagy. These two signaling pathways converge (at least partially) at target of rapamycin (TOR), a central protein kinase of the nutrient-sensing pathway (Sengupta et al. 2010). TOR forms two distinct complexes that differ in their subunit composition, TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1 negatively regulates autophagy in yeast (Noda & Ohsumi 1998), mammals (Blommaart et al. 1995, Ravikumar et al. 2004), and other species (Diaz-Troya et al. 2008, Scott et al. 2004; **Figure 3**). Rapamycin and other mTOR kinase inhibitors have been widely used to induce autophagy even under nutrient-rich conditions.

The Atg1/Unc-51-Like Kinase Complex

Atg1/Unc-51-like kinase (ULK) is the only protein kinase among the Atg-related proteins.

The major subunits of the yeast Atg1 complex include Atg1, Atg11, Atg13, Atg17, Atg29, and Atg31. Although Atg11 is not essential for canonical autophagy, it is required for the cytoplasm-to-vacuole targeting (Cvt) pathway (an autophagy-related constitutive transport system in yeast that delivers some vacuolar enzymes) and forms a scaffold with Atg17

Table 1 Atg and Atg-related proteins involved in autophagosome formation

	Yeast	Mammals	Features	Function
Atg1/ULK complex	Atg1	ULK1/2	Ser/Thr kinase; phosphorylated by mTORC1	This complex is negatively regulated by (m)TORC1 in a nutrient-dependent manner. In yeast, Atg17 forms a complex with Atg29 and Atg31, and further interacts with Atg1 and Atg13 upon starvation to mediate PAS organization. By contrast, ULK1, Atg13, FIP200, and Atg101 form a stable complex irrespective of nutrient conditions and translocate to the autophagosome formation site upon autophagy induction in mammalian cells. Although FIP200 and Atg13 are phosphorylated by ULK1, physiologically relevant substrates of Atg1/ULK1 remain largely unknown. FIP200 and Atg101 may have functions similar to yeast Atg17, Atg29, and Atg31, although they show no sequence similarity with each other.
	Atg13	Atg13	Phosphorylated by (m)TORC1	
	Atg17	–	Ternary complex with Atg29 and Atg31	
	Atg11	–	Scaffold for the PAS organization in selective autophagy	
	Atg29	–	Ternary complex with Atg17 and Atg31	
	Atg31	–	Ternary complex with Atg17 and Atg29	
	–	FIP200	Scaffold for ULK1/2 and Atg13	
	–	Atg101	Interacts with Atg13	
Class III PI3K complex	Vps34	Vps34	PI3K	This complex produces PI3P in the PAS or on the ER. Vps34, Vps15 and Atg6/Beclin 1 are shared with the Vps38/UVRAG complexes, which function in the endocytic pathway. Rubicon negatively regulates autophagosome-lysosome fusion through interaction with the UVRAG complex. Bcl-2 inhibits autophagy by sequestering Beclin 1 from the PI3K complex.
	Vps15	Vps15	Ser/Thr kinase; myristoylated	
	Vps30/Atg6	Beclin 1	BH3-only protein; interacts with Bcl-2	
	Atg14	Atg14(L)/Barkor	Autophagy-specific subunit	
	–	AMBRA1	Interacts with Beclin 1	
Others	Atg2	Atg2A/B	Interacts with Atg18	DFCP1 localizes to the omegasome on the ER, where other Atg proteins are assembled. DFCP1 itself is not essential for autophagy. Atg9, WIPIs, and VMP1 are present on the autophagic membrane. Atg9 also exists in other compartments such as endosomes and the *trans*-Golgi network.
	Atg9	Atg9L1/2	Multispan transmembrane protein	
	Atg18	WIPI1/2/3/4	PI3P-binding proteins	
	–	DFCP1	PI3P-binding FYVE-containing protein	
	–	VMP1	Multispan transmembrane protein	
Atg12 conjugation system	Atg12	Atg12	Ubiquitin-like; conjugates to Atg5	The Atg12-Atg5-Atg16(L) dimer is important for Atg8/LC3-PE conjugation. This complex is present on the outer side of the isolation membrane and is essential for proper elongation of the isolation membrane. The function of Atg16L2 remains uncharacterized.
	Atg7	Atg7	E1-like enzyme	
	Atg10	Atg10	E2-like enzyme	
	Atg5	Atg5	Conjugated by Atg12	
	Atg16	Atg16L1/2	Homodimer; interacts with Atg5	

(*Continued*)

Table 1 *(Continued)*

	Yeast	Mammals	Features	Function
Atg8/LC3 conjugation system	Atg8	LC3A/B/C, GABARAP, GABARAPL1/2/3	Ubiquitin-like; conjugates to PE (GABARAPL2 = GATE16)	The formation of Atg8/LC3-PE conjugates and their deconjugation by Atg4 are important for isolation membrane elongation and/or complete closure. Atg8/LC3 is present on both inner and outer membranes of autophagosomes and also interacts with receptors/adaptors such as Atg19 and Atg32 in yeast, and p62, NBR1, and Nix in mammals, for selective cargo incorporation into the autophagosome.
	Atg4	Atg4A-D	LC3/Atg8 C-terminal hydrolase; deconjugating enzyme	
	Atg7	Atg7	E1-like enzyme (shared with Atg12)	
	Atg3	Atg3	E2-like enzyme	

Abbreviations: DFCP1, double FYVE-containing protein 1; ER, endoplasmic reticulum; FIP200, focal adhesion kinase family interacting protein of 200 kDa; LC3, microtubule-associated protein 1 light chain 3; mTORC, mammalian target of rapamycin complex; PAS, preautophagosomal structure; PE, phosphatidylethanolamine; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; ULK, Unc-51-like kinase; VMP1, vacuole membrane protein 1; WIPI, WD-repeat protein interacting with phosphoinositides.

(Cheong et al. 2008, Kawamata et al. 2008). Atg17, Atg29, and Atg31 form a stable protein complex (a 2:2:2 complex) irrespective of nutrient conditions, and Atg1 and Atg13 are incorporated into the Atg17-Atg29-Atg31 complex under autophagy-inducible conditions (Kabeya et al. 2005, 2009; Kamada et al. 2000). The association of Atg13 and Atg17 is important for Atg1 kinase activation during starvation. A systematic hierarchical analysis performed in yeast revealed that the Atg1 complex is the farthest upstream unit (Suzuki et al. 2007; **Figure 2**).

Yeast Atg13 is hyperphosphorylated under growing conditions, and rapamycin treatment rapidly induces Atg13 dephosphorylation (Kamada et al. 2000). TORC1 directly phosphorylates Atg13 at multiple serine residues (Kamada et al. 2010). These phosphorylations are important for autophagy suppression; expression of an Atg13 mutated at these phosphorylation sites induces autophagy through Atg1 activation. Multiple phosphorylations also regulate Atg1 activity; phosphorylation of threonine 226 is particularly important for Atg1 kinase activity (Kijanska et al. 2010, Yeh et al. 2010).

Mammals possess two Atg1 homologs, ULK1 and ULK2, and one mAtg13 homolog (Ganley et al. 2009, Hosokawa et al. 2009a, Jung et al. 2009), but do not have homologs of Atg17, Atg29, and Atg31. Instead, focal adhesion kinase family interacting protein of 200 kDa (FIP200), also known as retinoblastoma 1-inducible coiled-coil 1 (RB1CC1) (Hara et al. 2008), and Atg101 (Hosokawa et al. 2009b, Mercer et al. 2009), which may be specific to higher eukaryotes, form a complex with ULK and Atg13 (Mizushima 2010). ULK1 could be the major Atg1 homolog (Chan et al. 2007, Young et al. 2006), as ULK2 seems to have a redundant function (Kundu et al. 2008). In contrast to the yeast Atg1 complex, nutrient-dependent complex disassembly is not observed in mammalian cells; the ULK1-mAtg13-FIP200-Atg101 complex is constitutively formed.

A hierarchical analysis using mammalian Atg proteins also suggested that the ULK complex functions at the most upstream step (Itakura 2010). The ULK1-mAtg13-FIP200-Atg101 complex is present mainly in the cytosol under nutrient-rich conditions and is inactivated by mammalian TORC1 (mTORC1) (Hosokawa et al. 2009a, Jung et al. 2009; **Figure 3**). In contrast to yeast, direct interaction between raptor (an mTORC1 subunit) and ULK1 can be detected under nutrient-rich conditions, and mTORC1 phosphorylates ULK1 and mAtg13

Cytoplasm-to-vacuole targeting (Cvt) pathway: a constitutive transport system in yeast that delivers some vacuolar enzymes by a macroautophagy-like process

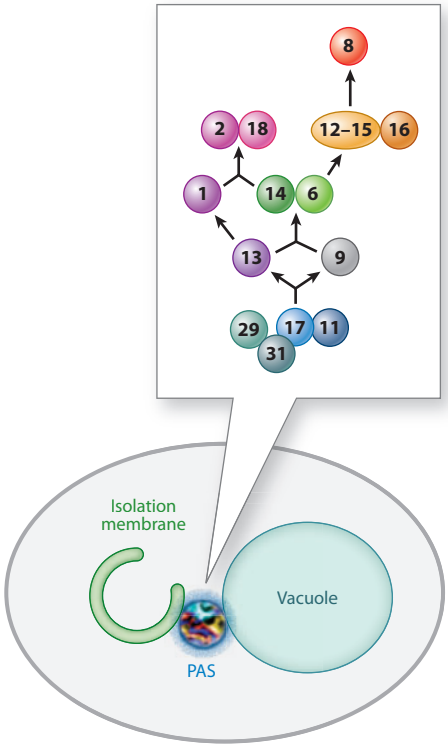


Figure 2

A model of the preautophagosomal structure (PAS) in yeast and the hierarchical relationship among yeast Atg proteins. The PAS is a dynamic assembly of Atg proteins at which the autophagosome is generated. Almost all Atg proteins assemble at the PAS in a well-organized manner (*inset*). The PAS is observed even under nutrient-rich conditions owing to the presence of the cytoplasm-to-vacuole targeting (Cvt) pathway, but it forms only under starvation conditions in Cvt-defective *atg11*Δ cells.

(Ganley et al. 2009, Hosokawa et al. 2009a, Jung et al. 2009). In *Drosophila*, dTor also phosphorylates dAtg1 and dAtg13 in a nutrient-dependent manner (Chang & Neufeld 2009). Under starvation conditions, mTORC1 no longer interacts with the ULK complex, which leads to dephosphorylation (activation) of ULK.

In addition to TORC1, the Atg1/ULK complex is regulated by multiple pathways including cAMP-dependent protein kinase A (Stephan et al. 2009) and AMP-activated protein kinase (Egan et al. 2011, Kim et al. 2011, Lee et al. 2010, Shang et al. 2011).

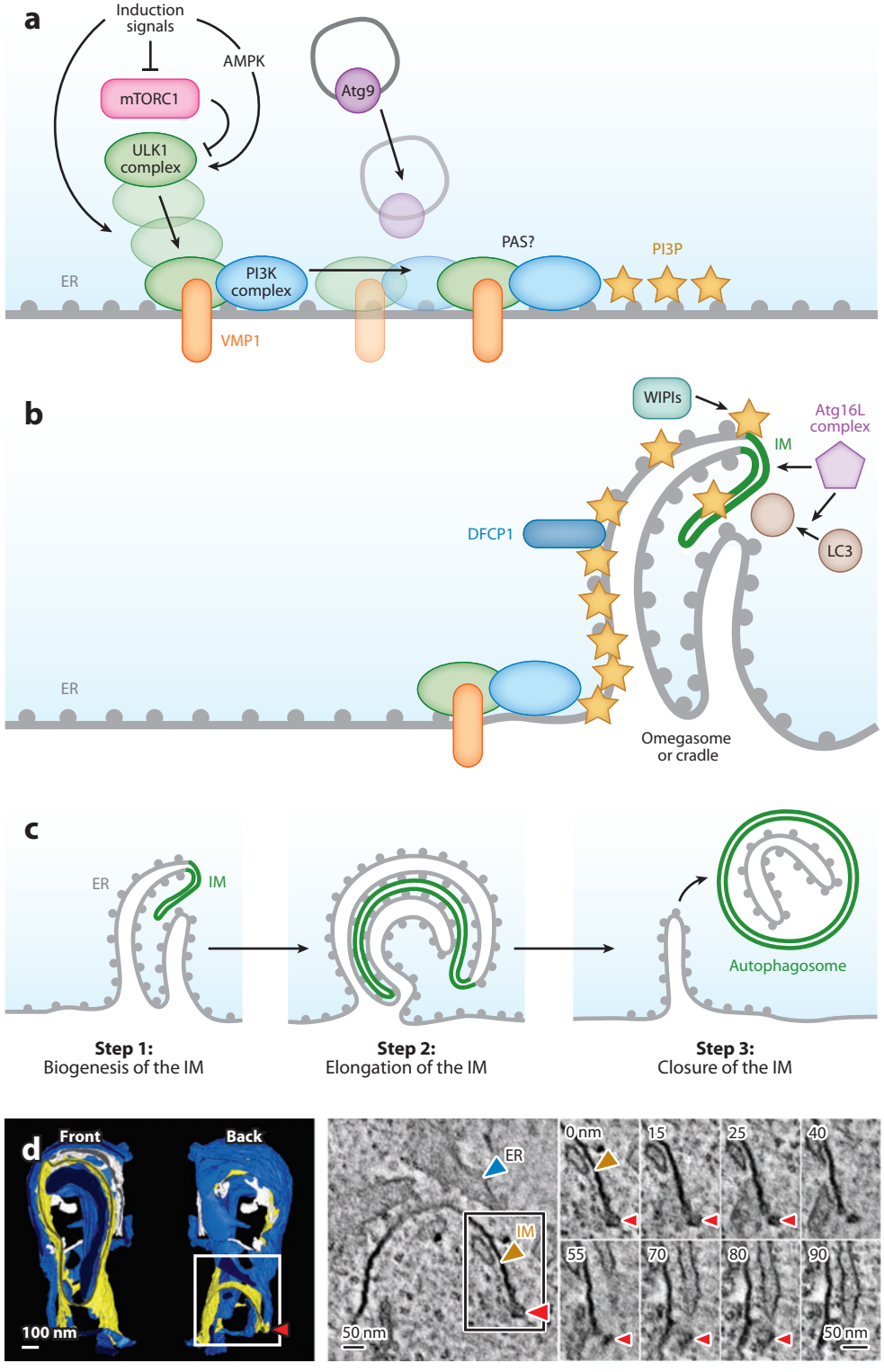
In yeast, the Atg1 complex is present at the PAS and has at least two distinct functions: (*a*) a kinase-independent recruitment of downstream Atg factors and (*b*) a kinase-dependent but unidentified role in autophagosome formation (Cheong et al. 2008, Kabeya et al. 2005, Kamada et al. 2000). In mammals, the ULK1 complex translocates from the cytosol to some domains of the ER, possibly forming PAS-like structures, upon autophagy induction (Itakura 2010; **Figure 3**). Whether ULK1 has kinase-independent roles is unknown. Identification of the physiologically relevant substrate(s) of Atg1/ULK will be essential to understand the precise role of this complex, but studies to identify such substrates are still ongoing. Mammalian Atg13 and FIP200 are phosphorylated by ULK1, but their significance remains unknown (Chan et al. 2009, Chang & Neufeld 2009, Hosokawa et al. 2009a, Jung et al. 2009). Recently, ULK1 was also shown to phosphorylate AMBRA1, a PI3K-interacting protein (see below) (Di Bartolomeo et al. 2010). Another recent study suggests that a myosin light chain kinase-like protein [Spaghetti-squash activator (Sqa) in *Drosophila*, or zipper-interacting protein kinase (ZIPK) in mammals] is a novel Atg1/ULK substrate and regulates Atg9-mediated autophagosome formation through myosin II activation (Tang et al. 2010). Therefore, Atg1/ULK may regulate several distinct pathways, which may or may not be related to its function in autophagy.

The Phosphatidylinositol 3-Kinase Complex and the Omegasome

Phosphatidylinositol 3-phosphate (PI3P) is essential for canonical autophagosome formation. Wortmannin and 3-methyladenine, which inhibit PI3K activity, are well-known autophagy inhibitors (Blommaart et al. 1997), and autophagosome membranes contain PI3P (Obara et al. 2008a). Vacuolar protein sorting protein 34 (Vps34) and class III PI3K (hVps34) produce PI3P for autophagy in yeast and

PI3P: phosphatidylinositol 3-phosphate

Vps: vacuolar protein sorting protein. *VPS*s are yeast genes essential for membrane traffic from the Golgi complex to the vacuole



mammals, respectively. In yeast, Vps34 forms two distinct protein complexes: Complex I consists of Atg14, Atg6/Vps30, Vps15, and Vps34, whereas complex II is composed of Vps38, Atg6/Vps30, Vps15, and Vps34 (Kihara et al. 2001). Only complex I functions in autophagy; complex II is involved in endosome-to-Golgi complex retrograde trafficking. The autophagy-specific factor, Atg14, is a determinant for recruitment of PI3K to the PAS and vacuolar membrane (Obara et al. 2006).

An autophagosome formation-specific PI3K complex has also been identified in mammals recently. It consists of Atg14L (also known as Atg14 and Barkor), Beclin 1 (Atg6 homolog), hVps15, and hVps34 (Itakura et al. 2008, Matsunaga et al. 2009, Sun et al. 2008, Zhong et al. 2009). Mammals have at least two other stable hVps34 complexes, which play roles in endosomal transport and autophagosome-lysosome fusion by including UVRAG (UV irradiation resistance-associated gene) and Rubicon (Run domain protein as Beclin 1 interacting and cysteine-rich containing) as subunits instead of Atg14L (one complex contains UVRAG alone, whereas the other contains both UVRAG and the negative regulator Rubicon) (Matsunaga et al. 2009, Zhong et al. 2009). Mammalian cells also possess other Beclin 1–binding proteins including Bcl-2, AMBRA1, Bif-1 (also known as Endophilin B1), and vacuole membrane protein 1 (VMP1; see below) (Funderburk et al. 2010); however, their binding seems to be transient. In addition to PI3K, phosphatidylinositol 3-phosphatases including myotubularin-related phosphatase 3 (MTMR3) and Jumpy (MTMR14) are implicated in autophagy (Taguchi-Atarashi et al. 2010, Vergne et al. 2009). They negatively regulate autophagosome formation and autophagosome size. Therefore, the balance between PI3K and phosphatidylinositol 3-phosphatase determines autophagy initiation through changing local PI3P levels.

Double-FYVE-containing protein 1 (DFCP1) has been identified as a downstream effector of PI3P (Axe et al. 2008). DFCP1 binds to PI3P via its FYVE domain. Although DFCP1 localizes to the ER membrane ubiquitously in a FYVE domain–independent manner and to the Golgi membrane in nutrient-rich conditions, upon starvation it concentrates in spots on the ER dependent on the FYVE domain, where it colocalizes partially with isolation membrane and autophagosome markers. Live imaging has revealed that these spots provide a platform for expansion of the isolation membrane and that the completed autophagosomes are separated from them. The term omegasome was coined for this PI3P-enriched ER subdomain with an Ω-like shape as the compartment specific to autophagosome biogenesis (the structure may be identical to the cradle, which has been identified by

Figure 3

Autophagosome formation. This figure illustrates a model of autophagosome formation on the endoplasmic reticulum (ER) and the localization of autophagy-related (Atg) proteins in mammalian cells. (*a*) Upon input of induction signals via mammalian target of rapamycin complex 1 (mTORC1) suppression, the Unc-51-like kinase 1 (ULK1) complex is activated and binds to the phosphatidylinositol 3-kinase (PI3K) complex. The ULK1 complex can also be positively regulated by AMP-activated protein kinase (AMPK). Then, these complexes translocate laterally to specific regions and form a putative mammalian preautophagosomal structure (PAS), possibly together with vacuole membrane protein 1 (VMP1) and Atg9, at which PI3K locally produces phosphatidylinositol 3-phosphate (PI3P). (*b*) In the next step, membrane deformation of the ER occurs, which leads to formation of the omegasome/cradle to create the isolation membrane (IM). At this stage, the PI3P effectors [double-FYVE-containing protein 1 (DFCP1) and WD-repeat protein interacting with phosphoinositides (WIPIs)] are recruited. Finally, the Atg16L complex induces microtubule-associated protein light chain 3 (LC3) lipidation, and lipidated LC3 (LC3–phosphatidylethanolamine) localizes to the isolation membrane. (*c*) The isolation membrane expands within the ER cisterna (omegasome/cradle) and is released from it by closure of the membrane. (*d*) Electron tomography of ER-IM complexes. (*Left*) A 3D model of an ER-IM complex. The ER, IM, and vesicular tubular cluster-like compartments are shown in blue, yellow, and white, respectively. (*Right*) Images of 1.5-nm tomographic slices of the boxed region in the left panel. The ER and IM are indicated by blue and gold arrowheads, respectively. Numbers indicate the depth of the tomographic slices. Red arrowheads indicate the continuity of the IM with the ER. Adapted from Hayashi-Nishino et al. (2009).

electron tomography; see below). Thus, DFCP1 is a marker for the omegasome, although its function remains unclear because silencing of DFCP1 does not inhibit autophagy (Axe et al. 2008). Because the ER usually does not contain PI3P, the study raised the enigma of how the omegasome acquires it. The answer was obtained by analysis of Atg14L, as described below.

Atg14L also localizes to the ER as well as to the isolation membrane and autophagosome (Matsunaga et al. 2009, 2010; **Figure 3**). Furthermore, it colocalizes well with DFCP1, and knockdown of Atg14L results in the disappearance of the omegasome, which suggests that it is upstream of omegasome formation (Itakura 2010, Matsunaga et al. 2010). As forced ectopic localization of Atg14L to the plasma membrane leads to translocation of Beclin 1 and hVps34 to the same membrane, Atg14L is likely a determinant of the membrane localization of the autophagy-specific PI3K complex (Matsunaga et al. 2010). The domain of Atg14L responsible for ER localization was determined to be the N-terminal region containing characteristic cysteine repeats completely conserved from yeast to mammals (Matsunaga et al. 2010). An Atg14L mutant in which these cysteines are converted to alanines lacks not only ER localization but also autophagosome formation ability. The addition of the ER-localizing motif of DFCP1 to this mutant fully complements these defects (Matsunaga et al. 2010). Thus, Atg14L recruits the autophagy-specific PI3K complex into the ER to form the omegasome (**Figure 3**). This finding explains how PI3P appears in the ER, which usually does not contain this lipid. ULK1 and its kinase activity are required for Atg14L localization to the omegasome (Itakura 2010, Matsunaga et al. 2010), which indicates that the mTORC1-ULK1 axis directs lateral translocation of the PI3K complex to the specialized part of the ER to produce PI3P. The ULK1-PI3K association can be promoted by an Exo84-containing exocyst complex, which is formed in a RalB-dependent manner (Bodemann et al. 2011). Nutrients also inactivate Beclin 1 through direct interaction with Bcl-2 (Wei et al. 2008). Presumably, an increase in the local PI3P content triggers omegasome formation and DFCP1 recruitment. The role of PI3P might be generation of the isolation membrane by changing the composition of the ER membrane (see below) and/or recruitment of the PI3P effectors required for isolation membrane formation.

How the ULK1 complex controls the PI3K complex remains to be elucidated. Analysis of the Beclin 1-binding protein AMBRA1 has suggested one possible mechanism. This protein anchors the PI3K complex to the microtubule via interaction with dynein light chains 1/2 under nutrient-rich conditions (Di Bartolomeo et al. 2010). In starvation-induced autophagy, ULK1 phosphorylates AMBRA1 to release the PI3K complex from dynein, which may enable its translocation into assembly sites for omegasome formation.

Atg18/Atg21/WD-Repeat Protein Interacting with Phosphoinositides and Atg2

The second category of PI3P effectors in the autophagy pathway is the Atg18 family proteins. Yeast Atg18/Svp1 and its related protein Atg21 are multi-WD-repeat-containing proteins. Neither protein has typical PI-binding motifs, but both have the Phe-Arg-Arg-Gly (FRRG) motif, through which they can bind PI3P and phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) (Dove et al. 2004, Stromhaug et al. 2004). Atg18 localizes to the PAS and endosomes and regulates the autophagy/Cvt pathway and vacuolar morphology through binding with PI3P and $PI(3,5)P_2$, respectively (Dove et al. 2004, Efe et al. 2007, Obara et al. 2008b). Atg2, an Atg18-interacting protein of ~200 kDa, is also important for the PAS localization of Atg18 (Obara et al. 2008b). Whereas Atg18 is essential for autophagy, the role of Atg21 is more important for the Cvt pathway (Barth et al. 2001, 2002). A third homolog, Ygr223c in yeast, may be important for micronucleophagy (Krick et al. 2008).

In the yeast hierarchical analysis (Suzuki et al. 2007), Atg2-Atg18 is placed downstream of the Atg1 and PI3K complexes, but in a branch separated from the Atg8 and Atg12 conjugation systems (see below). The results of a recent study suggest that the Atg2-Atg18 complex functions at a late step of autophagosome formation for efficient completion of sequestration, probably through facilitating recruitment of Atg8–phosphatidylethanolamine (PE) (– denotes covalent binding) to the PAS and/or its protection from deconjugation by Atg4 (Nair et al. 2010).

Mammalian cells have four Atg18 homologs, which are known as WD-repeat protein interacting with phosphoinositides (WIPI)1–4 (Jeffries et al. 2004, Polson et al. 2010, Proikas-Cezanne et al. 2004). A phylogenetic analysis revealed that WIPI1 and WIPI2 are similar to Atg18, and WIPI3 and WIPI4 are similar to Ygr223c rather than Atg21 (Polson et al. 2010). Whereas WIPI2 is expressed in many cell lines, WIPI1 expression is limited to a few (e.g., G361) (Polson et al. 2010).

During autophagosome formation, WIPI1 and WIPI2 almost completely colocalize with Atg14 and Atg16L1 (Itakura 2010, Polson et al. 2010). By contrast, DFCP1 often localizes adjacent to the WIPI1/2 structures (Itakura 2010, Polson et al. 2010). These observations suggest that although both DFCP1 and WIPIs are PI3P effectors, their precise localization is different; DFCP1 is on the omegasome, whereas WIPIs may be present on the isolation membrane developing from the omegasome (**Figure 3**). DFCP1-positive omegasomes accumulate in WIPI2-depleted cells, which suggests that WIPI2 plays a critical role in maturation of omegasomes into autophagosomes (Polson et al. 2010). Although formation of WIPI puncta depends on PI3K activity (Itakura 2010, Polson et al. 2010, Proikas-Cezanne et al. 2004), membrane association of WIPI2 does not require this activity (Polson et al. 2010), suggesting that WIPI2 on some membrane or on the ER translocates to autophagosome formation sites upon autophagy induction.

Two mammalian Atg2 proteins localize on autophagic membranes as well as lipid droplets (N. Ishihara, A. Kumar, E. Oita, T. Nishimura, & N. Mizushima, unpublished observation). Because it has been reported that autophagy is important for lipid metabolism, there may be an additional link at the molecular level.

PE: phosphatidylethanolamine

Atg9

Atg9 has attracted much attention because it is the only known transmembrane protein (spanning the membrane six times) among the core Atg proteins required for autophagosome formation (Noda et al. 2000). Although its function remains unknown, its dynamic behavior has been well characterized. In addition to the PAS in yeast, Atg9 localizes to cytoplasmic dot-like structures whose identity is as yet undetermined (Mari et al. 2010). The Atg9-containing structures are recruited to the PAS, and Atg9 finally localizes to the outer membrane of the autophagosome (H. Yamamoto & Y. Ohsumi, unpublished results). Atg9 localizes to the PAS in an Atg17-dependent manner upon starvation (Sekito et al. 2009). Physical interaction between Atg9 and Atg17 has also been shown. As mentioned above, Atg17 is a subunit of the Atg1 complex, and the hierarchical study indicated that Atg9 is just downstream of the Atg1 complex in PAS organization (**Figure 2**). Self-interaction of Atg9 is also essential for localization of Atg9 to the PAS (He et al. 2008). Atg9 translocates from the PAS to the cytoplasmic compartments by retrograde transport, which depends on the Atg1 complex, Atg2-Atg18 complex, and PI3K complex (Reggiori et al. 2004a).

In mammals, the Atg9 homolog Atg9L1 localizes to autophagy-related structures (or omegasomes) as well as to the *trans*-Golgi network and endosomes (Young et al. 2006). Atg9L1 seems to shuttle among these organelles, although its dependency on other Atg proteins has not been clarified fully. It has been suggested that a p38 mitogen-activated protein kinase (MAPK)-binding protein, p38IP, and a Beclin 1-binding protein, Bif-1, regulate

Atg9L1 trafficking (Takahashi et al. 2011, Webber & Tooze 2010).

On one hand, the anterograde transport of Atg9/Atg9L1 suggests a model in which Atg9/Atg9L1-containing vesicles are budded from the cytoplasmic membrane source and delivered to the PAS to supply some critical factors or lipid components of the isolation membrane. In this respect, the retrograde transport of Atg9 may mean its retrieval to the source membrane pool for reuse. On the other hand, Atg9 could also function by removing a component that is critical to autophagosome formation, e.g., a lipid or transmembrane protein, from the PAS or isolation membrane. Intriguingly, live cell imaging revealed that Atg9L1 appears on and disappears from the autophagic substrate prior to microtubule-associated protein 1 light chain 3 (LC3) appearance, which suggests that constant existence of Atg9 on the isolation membrane during autophagosome formation is unnecessary (Kageyama et al. 2011).

Vacuole Membrane Protein 1

VMP1 was originally identified as a protein highly upregulated in pancreatic exocrine cells during acute pancreatitis (Dusetti et al. 2002), but it was later shown to be required for autophagosome formation (Itakura 2010, Ropolo et al. 2007). *Dictyostelium discoideum* VMP1 (Calvo-Garrido et al. 2008) and *Caenorhabditis elegans* epg-3/VMP1 (Tian et al. 2010) are also essential for autophagy but not conserved in yeast. In addition to its involvement in general autophagy, VMP1 is required for selective degradation of pancreatic zymogen granules (termed zymophagy) (Grasso et al. 2011). VMP1 interacts with Beclin 1 (Ropolo et al. 2007) and tumor protein 53-induced nuclear protein 2 (TP53INP2) [also known as a product of diabetes- and obesity-regulated gene (*DOR*)] (Nowak et al. 2009), a nuclear factor that upon autophagy induction translocates to autophagic structures and interacts with LC3 and its homologs (Mauvezin et al. 2010, Nowak et al. 2009).

VMP1 is an ER- and Golgi complex–associated multispanning transmembrane protein (Dusetti et al. 2002, Itakura 2010). The precise role of VMP1 remains unknown. VMP1 partially colocalizes with autophagic structures during starvation (Calvo-Garrido et al. 2008, Itakura 2010). However, colocalization between VMP1 and early Atg proteins such as ULK1 is augmented in cells treated with wortmannin, a PI3K inhibitor (Itakura 2010). These observations suggest that VMP1 transiently localizes to early autophagic structures and dissociates from them in a PI3K-dependent manner.

However, this assumption does not easily explain the VMP1-deficient phenotype. VMP1 deficiency causes accumulation of abnormal autophagic structures containing ULK1, WIPI1, DFCP1, Atg16L1, and LC3 in mammalian cells (Itakura 2010, Tian et al. 2010), and LGG-1/Atg8, DFCP1, T12G3.1/p62, and SEPA-1 in *C. elegans* (Tian et al. 2010). *D. discoideum* VMP1 mutant cells also show accumulation of aggregates containing Atg8, ubiquitin, and p62 (Calvo-Garrido & Escalante 2010). Accumulation of these downstream Atg factors is not observed in wortmannin-treated cells, which suggests that VMP1 does not function primarily though PI3K regulation. Rather, these studies indicate that VMP1 may be an important factor at a downstream step in autophagosome formation.

Finally, studies in *D. discoideum vmp1* mutants revealed that the function of VMP1 is not limited to autophagy; VMP1 is required for integrity/biogenesis of secretory organelles such as the Golgi complex (Calvo-Garrido & Escalante 2010, Calvo-Garrido et al. 2008). Thus, VMP1 may regulate multiple pathways originating from the ER. One possibility is that VMP1 may provide a platform at the ER that promotes proper autophagosome formation; however, its precise role needs to be elucidated further.

The Atg8/LC3 Conjugation System

The hierarchical analysis among yeast Atg proteins suggested that two ubiquitin-like

conjugation systems function at a late step of autophagosome formation, i.e., expansion and closure of the membrane (Suzuki et al. 2007). The two conjugates, Atg12–Atg5 and Atg8–PE, serve as good markers for the detection of membrane structures during autophagy; the former localizes specifically to the isolation membrane, whereas the latter is useful in tracing the whole process of autophagy including the formation of the autophagosome and its fusion with lysosomes/vacuoles (Kabeya et al. 2000, Kirisako et al. 1999, Mizushima et al. 2001).

Atg8 and its mammalian homologs such as LC3, GABARAP, and GATE-16 (GABARAPL2) are ubiquitin-like proteins that are synthesized as precursors with additional sequences at their C termini, which are processed by the cysteine protease Atg4 (Kabeya et al. 2004, Kirisako et al. 2000). The resulting C-terminal glycine-exposed form of Atg8 is activated by Atg7 (E1 enzyme), transferred to Atg3 (E2 enzyme), and finally covalently linked to an amino group of PE, a major membrane phospholipid (Ichimura et al. 2000). Atg4 functions also as a deconjugating enzyme and may regulate the level of free Atg8 (Kirisako et al. 2000). Atg8–PE localizes on both the isolation membrane and the autophagosome (Kirisako et al. 1999). In several *atg* mutants defective in isolation membrane formation, Atg8–PE still accumulates, which suggests that lipidation of Atg8 occurs without isolation membrane formation (Suzuki et al. 2001). This is also the case for mammalian LC3 (Mizushima et al. 2010). Where Atg8 lipidation occurs and how Atg8–PE is recruited to the isolation membrane are still unknown. It will be critical to identify an Atg8–PE-containing membrane structure and characterize it biochemically.

In vitro studies have revealed that Atg8–PE has membrane tethering and hemifusion (fusion between outer leaflets of two opposing membranes while inner leaflets remain intact) activities (Nakatogawa et al. 2007). Additionally, Atg8 homologs are involved in other biological processes such as clustering of neurotransmitter receptors (Chen et al. 2000), intra-Golgi membrane trafficking (Legesse-Miller et al. 1998), and the regulation of vacuolar morphology in yeast (Tamura et al. 2010). Mutations at the residues required for these activities cause significant defects in autophagosome formation, indicating that these activities observed in vitro represent the actual function of Atg8 in the process in vivo. If these Atg8 activities are partially impaired, smaller autophagosomes are formed. Similarly, the size of autophagosomes becomes smaller as the Atg8 expression level decreases (Xie et al. 2008). These results suggest that Atg8 is involved in the expansion of the isolation membrane. In mammals, unclosed isolation membranes with abnormal morphology accumulate when knockout of *ATG3* or overexpression of an Atg4 dominant-negative mutant blocks lipidation of Atg8 homologs (Fujita et al. 2008a, Sou et al. 2008), which suggests that lipidation of Atg8 homologs is important for normal development of the isolation membrane, likely for its closing step. In this regard, the LC3 and GABARAP/GATE-16 subfamilies might play different roles in autophagosome formation with involvement in the expansion of the isolation membrane and at a later stage, respectively (Weidberg et al. 2010).

The Atg12 Conjugation System

Atg12 is synthesized as a C-terminal glycine-exposed form, is activated by Atg7 (E1 enzyme), is transferred to Atg10 (E2 enzyme), and finally forms a conjugate with the sole target protein Atg5 (Mizushima et al. 1998). The Atg12–Atg5 conjugate interacts with Atg16 (Atg16L in mammals) to form a complex with a 2:2:2 stoichiometry via homodimerization of Atg16/Atg16L1 (Fujioka et al. 2010, Fujita et al. 2008b). The Atg12 system has no deconjugating enzyme, and the Atg12–Atg5-Atg16 complex is formed constitutively irrespective of nutrient conditions. In yeast, Atg12–Atg5 conjugates reside on the PAS but not on the complete autophagosome (Suzuki et al. 2001). Likewise, in mammals, Atg12–Atg5-Atg16L1 predominantly localizes on the outer surface of

the isolation membrane and dissociates from the membrane immediately before or after the completion of autophagosome formation (Mizushima et al. 2001). Judging from the amounts of the complex, it is unlikely that the conjugates cover the membrane as a coat (Geng et al. 2008).

The two conjugation systems are closely related, as Atg12–Atg5-Atg16 is necessary for Atg8–PE formation in vivo. In vitro reconstitution experiments have revealed that Atg12–Atg5 interacts with Atg3 and intensively facilitates the transfer reaction of Atg8 from Atg3 to PE (Hanada et al. 2007; **Figure 3**). Although Atg16 does not affect the acceleration of Atg8–PE formation by Atg12–Atg5 in vitro, it is important for Atg8–PE formation in vivo as well as Atg12–Atg5 formation. In yeast, Atg16 is required for the PAS localization of Atg12–Atg5 (Suzuki et al. 2007). In mammalian cells, lipidation of LC3 occurs on the plasma membrane if Atg16L1 is artificially engineered to localize to this membrane (Fujita et al. 2008b). These findings suggest that the Atg12–Atg5-Atg16(L) complex is involved in determining the site of Atg8 lipidation. Further elucidation of the mechanism awaits identification of an interaction partner(s) of Atg16. The Golgi complex–resident small GTPase Rab33B was shown to interact with Atg16L; however, its role in autophagy is still unclear (Itoh et al. 2008).

When dissecting the mechanism of autophagosome formation, it is critical to understand the asymmetric features of the autophagosomal membrane. Atg12–Atg5-Atg16(L) localizes on the outer surface of the isolation membrane. Atg8–PE resides on both sides of autophagosomal membrane and is partly delivered to the lysosome/vacuole. Yeast Atg1 (but not mammalian ULK1) is transported to the vacuole via the autophagosome but its binding partner, Atg13, and Atg17, Atg29, and Atg31 are not. In vivo staining of PI3P revealed that it is concentrated in the inner surface of the autophagosome, but its predicted effector, the Atg18-Atg2 complex, is not delivered into the vacuole. The mechanism of this asymmetrical distribution is not known. How the class III PI3K complex regulates membrane association of Atg12–Atg5 and Atg8/LC3–PE also remains to be elucidated.

ORIGIN OF THE AUTOPHAGOSOME

The Preautophagosomal Structure

In yeast, autophagosomes are generated at or around the PAS. It is not known why and how the PAS is formed close to the vacuole. As the PAS is usually localized at the nuclear-vacuolar junction (K. Suzuki & Y. Ohsumi, unpublished observation), and ER to Golgi complex transport is necessary for autophagosome formation (Ishihara et al. 2001, Reggiori et al. 2004b), the PAS may be attached to the ER, which is the platform of autophagosome formation in mammals (see above).

The hierarchical relationship between functional units of Atg proteins in terms of PAS localization are shown in **Figure 2** (Suzuki et al. 2007, Suzuki & Ohsumi 2007). Atg17 and Atg11 are the farthest upstream factors for PAS organization. Atg11 is not essential for starvation-induced bulk autophagy but is required for selective modes of autophagy including the Cvt pathway, a biosynthetic pathway for some vacuolar enzymes. Therefore, the PAS is constitutively present even under nutrient-rich conditions. Dynamic features of the PAS for starvation-induced autophagy were elucidated using *atg11*Δ cells (Cheong et al. 2008, Kawamata et al. 2008). In *atg11*Δ cells, the upstream factors specifically required for starvation-induced autophagy, Atg17, Atg29, and Atg31, are dispersed in the cytoplasm under nutrient-rich conditions, but upon starvation these proteins immediately assemble into the PAS. Replenishment of nutrients rapidly disassembles the PAS. This indicates that the PAS is a dynamic assembly of Atg proteins sensitive to nutrient conditions. At present it is difficult to further dissect the isolation membrane from the PAS because of the limitations of the resolution of fluorescence microscopy and lack of

specific markers. It is critical to identify which Atg proteins localize at the PAS and which predominantly reside on the isolation membrane.

To date, whether the PAS also exists in mammals is unclear. However, as the functional units and their hierarchy are well conserved from yeast to mammals (Itakura 2010), the PAS also might be conserved despite some differences in details. It is speculated that the mammalian PAS forms on or close to the ER (see below).

Role of the Endoplasmic Reticulum in Autophagosome Formation

The PAS is believed to generate the isolation membrane, but the PAS does not directly change into the isolation membrane. Over the past half-century, the membrane source of the autophagosome has been the topic of much debate. Many suggestions have been proposed, including de novo synthesis or supply from preexisting organelles such as the ER, Golgi complex, or endosomes. Although we do not yet have the final answer, recent studies have provided new insight into this important issue.

As mentioned above, the molecules in the cascade from ULK1 to PI3P effectors are essential for autophagosome nucleation to localize to the ER, which suggests that this organelle is the best candidate for the membrane source and/or the scaffold for autophagosome formation. Additionally, the ER has often been observed in close vicinity of the isolation membrane. Two recent independent electron tomography studies delineated the 3D architecture of the convoluted relationship between the ER and the isolation membrane (Hayashi-Nishino et al. 2009, Yla-Anttila et al. 2009). The reconstituted 3D image showed that a portion of the ER forms a cradle-like curvature encircling the isolation membrane (**Figure 3*d***). The isolation membrane is sandwiched between two ER cisternae. The associated ER differs from the normal ER in its spherical structure but continues to the regular ER network, so it can be regarded as a specialized subdomain of the ER. Furthermore, a narrow membrane extension from the isolation membrane connects it to the associated ER. In immunoelectron micrographs, DFCP1 was localized to this structure, which suggests strongly that the cradle is identical to the omegasome (Hayashi-Nishino et al. 2009). Given these observations, it is possible that the isolation membrane elongates inside the cradle formed by the two ER cisternae, which act as a template for the spherical shape of the autophagosome and also provide lipids to the isolation membrane through the membrane extension, which acts like an umbilical cord. Although this is simply a hypothesis based not on dynamic but on static electron microscopy (EM) images, the idea requires much further examination.

In yeast, membrane trafficking from the ER seems to be involved in autophagy. Autophagosome formation requires Sec12, Sec16, Sec23, Sec24, and Sar1, all of which have functions in the formation of COPII-coated vesicles delivering cargo from the ER to the Golgi complex (Ishihara et al. 2001, Reggiori et al. 2004b). As overexpression of Sfb2, the Sec24 homolog, in *sec24*Δ cells suppresses defects in both ER-Golgi complex transport and autophagy, membrane flow rather than specific COPII vesicle components may play a role in autophagy (Hamasaki et al. 2003). By contrast, Huang et al. (2011) suggest that general ER-to-Golgi complex trafficking is not important for autophagy of *Salmonella typhimurium* in mammalian cells.

CONTRIBUTION OF OTHER ORGANELLES TO AUTOPHAGOSOME FORMATION

The Golgi Complex and the Endosome

In mammals, Rab33B, which interacts with Atg16L, and Atg9L1 localize to the Golgi complex in a steady state, and both appear in the autophagic membrane upon autophagy induction (Itoh et al. 2008, Young et al. 2006). These findings suggest that the Golgi complex is a potential membrane source for autophagy because Rab proteins are well-known membrane traffic regulators and Atg9 is

a transmembrane protein. Consistent with this, the rim of the isolation membrane is labeled with wheat germ agglutinin, a lectin that recognizes complex oligosaccharide chains, which are added to proteins in a secretory pathway at the Golgi complex; thus, wheat germ agglutinin is a marker for post-Golgi membrane compartments (Yamamoto et al. 1990).

A large body of evidence from yeast genetics strongly supports this view. Autophagosome formation involves components of the machinery for vesicle exit from the Golgi complex including COG subunits and the Golgi complex–resident ADP ribosylation factors Sec2, Sec4, Sec7, Gea1, and Gea2, perhaps through controlling Atg9-vesicle trafficking (Geng et al. 2010, van der Vaart et al. 2010, Yen et al. 2010). The observation that mammalian Bif1 (see above) is required for both Atg9 trafficking from the Golgi complex to the peripheral vesicles and Golgi membrane fission is also consistent with the idea that autophagy uses the Golgi membrane (Takahashi et al. 2011). The yeast Rab GTPase Ypt1 regulates ER–Golgi complex and intra-Golgi complex traffic. Lynch-Day et al. (2010) demonstrated the involvement of Ypt1 and a third complex of transport protein particle (TRAPP), the guanine nucleotide exchange factor for Ypt1, in autophagosome formation, which also suggests that membrane trafficking delivers the ER or Golgi membrane to the PAS. Consistently, Rab1, a mammalian Ypt1 homolog, is present on autophagic membranes and required for autophagosome formation (Huang et al. 2011).

Mammalian Atg9 localizes to endosomes as well as to the Golgi complex. Thus, endosomes could also be the membrane source. Early morphologic studies showed the close relationship between the endocytic pathway and autophagy (Liou et al. 1997, Tooze et al. 1990). Fusion between endosomes and autophagosomes has been observed under EM, and Gordon & Seglen (1988) suggested that the resultant compartment (termed the amphisome) is able to fuse with lysosomes. If the isolation membrane is also able to fuse with endosomes, lipids from endosomes may contribute to its elongation.

Collectively, we now have many pieces of evidence with which we can discuss the long-standing important question: what is the autophagosomal source membrane? We speculate that the Golgi complex–endosome system supplies some lipids and/or proteins to elongate the isolation membrane progenitor generated within the cradle in the ER. However, if recruitment of the core Atg machinery is the most important event, an alternative membrane could be utilized. To resolve this important question, in vitro reconstitution of the cradle and isolation membrane formation would be useful.

Mitochondria

Mitochondria are unlikely candidates for the membrane source in autophagy because of their independence from the endocytic and secretory pathways. However, in an interesting model, Hailey et al. (2010) recently proposed that the autophagosome forms directly from mitochondrial membranes under starvation conditions. Colocalization of the mitochondrial membrane markers with Atg5 and LC3 was shown as well as transfer of the fluorescent lipid NBD-PS [*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-YL)-phosphatidylserine] from mitochondria to autophagosomes. Furthermore, photobleaching demonstrated transient sharing of membranes between mitochondria and autophagosomes. The proteins anchored in the outer leaflet of the mitochondrial outer membrane, but not the outer or inner transmembrane proteins or the matrix proteins, colocalized with the autophagosome. From these observations, the authors speculated that sharp membrane curvature at an autophagosome budding site hinders mitochondrial transmembrane proteins from entering autophagosomes.

Furthermore, Hailey et al. (2010) showed that autophagosome formation is reduced in mitofusin 2 (Mfn2)-knockout cells). PE, which is a component of autophagosomes, is produced in mitochondria from PS, and the ER contains an abundance of PS. Thus, Hailey et al. suggested that an Mfn2-dependent connection between mitochondria and the ER is

indispensable for translocation of PS from the ER to mitochondria to convert it to PE for autophagosome formation. There is, however, an alternative possibility that PE produced in the mitochondria moves to the ER for autophagosome formation via a mitochondria-ER connection. The proteins anchored in the mitochondrial outer leaflet might be able to bypass the connection and enter the autophagosome. Although the mitochondrial origin hypothesis is not mutually exclusive to other models, it would be worth testing the ideas that the mitochondria-ER connection is important for autophagosome formation in the ER and that the autophagosome forms in or near the connection.

The Plasma and Nuclear Membranes

As the nuclear membrane is continuous with the ER membrane, the nuclear envelope could participate in autophagosome formation. Indeed, at a late phase of herpes simplex virus type 1 infection, LC3-positive autophagosome-like structures emerge from the nuclear envelope of macrophages (English et al. 2009). In addition, in yeast the PAS is often observed close to the nuclear-vacuolar junction, which suggests a possible contribution of the ER/nuclear membrane to PAS organization (Suzuki & Ohsumi 2010).

Finally, the results of a recent study suggest that even the plasma membrane contributes to formation of autophagosomal precursors. Ravikumar et al. (2010) found that Atg16L1 interacts with the clathrin heavy chain, and Atg16L1-positive, LC3-negative vesicles are generated at or close to the plasma membrane. Depletion of early endocytic factors such as the clathrin heavy chain, epsin 1, and AP2 partially suppresses autophagosome formation. It remains unknown whether these plasma membrane–derived vesicles can mature into autophagosomes or fuse with ER-derived membranes (or omegasomes).

These observations suggest that multiple membrane sources participate in autophagosome formation. An important question is whether autophagosome formation always requires all these membrane sources or utilizes only some of them, depending on conditions (basal or induced), sites and target substrates. The requirement for two membrane proteins, VMP1 and Atg9, suggests that at least the ER and an Atg9-containing membrane (Golgi complex or endosome) are essential for autophagosome formation. Studies on the diversity of the autophagosome will provide more information to answer this question.

ATG PROTEINS IN SELECTIVE AUTOPHAGY

Recruitment and Degradation of p62

An important function of the Atg8 family proteins is to serve as receptors for selective autophagy (Noda et al. 2010). They recognize the WXXL-like sequence in substrate proteins or adaptors such as Atg19 (an adaptor for the Cvt pathway) and Atg32 (mitochondrial cargo receptor) in yeast, and p62/SQSTM1 (sequestosome 1), the neighbor of *BRCA1* gene 1 (NBR1), and Nix (also called Bnip3L) in mammals, and thereby selectively incorporate these substrates into autophagosomes (Johansen & Lamark 2011).

Although this could be the primary mechanism of the selectivity, the Cvt cargoes, p62, and NBR1 could target the PAS or autophagosome formation site independently of Atg8/LC3 (Cao et al. 2008, Itakura & Mizushima 2011). Thus, the interaction between Atg8/LC3 and selective substrates may not be the first recognition event, and another unknown mechanism(s) for substrate recruitment may exist. In the Cvt pathway, the Cvt cargo prApe1 (precursor of aminopeptidase 1) can be delivered to the PAS by Atg19 (a cargo receptor) and Atg11 (Cao et al. 2008).

Mitophagy

Mitochondria are often found in autophagosomes. Some of them, particularly under starvation conditions, may be enclosed incidentally. However, growing evidence has shown that

autophagosomes can selectively recognize mitochondria. Yeast genetic analysis showed that, in addition to the core Atg proteins required for macroautophagy, mitophagy also requires Atg11, Atg20, and Atg24 (Kanki & Klionsky 2008). This pattern is similar to that observed for pexophagy, which suggests the presence of a mechanism for selective cargo recognition. Kanki et al. (2009a) and Okamoto et al. (2009) identified as the cargo receptor a mitochondrial protein, which was named Atg32. Another factor, Atg33, is also involved in yeast mitophagy (Kanki et al. 2009b).

Although Atg32 is not conserved in higher eukaryotes, selective elimination of damaged or obsolete mitochondria by autophagy also has been observed in mammals (Kim et al. 2007, Mortensen et al. 2010, Youle & Narendra 2011). Recently, Youle & Narendra (2011) highlighted a surprising link between mitophagy and the pathogenesis of Parkinson's disease. Mutations in *PARKIN/PARK2* and *PINK1/PARK6*, which encode an E3 ubiquitin ligase and a mitochondrial kinase, respectively, cause autosomal recessive juvenile Parkinson's disease. Cytosolic Parkin translocates to depolarized mitochondria in a Pink1 kinase activity–dependent manner and promotes mitophagy through ubiquitination of mitochondrial proteins (Narendra et al. 2008, Youle & Narendra 2011). Although several Parkin substrates such as VDAC1 (voltage-dependent anion channel 1) (Geisler et al. 2010) and mitofusin (Gegg et al. 2010, Poole et al. 2010, Tanaka et al. 2010, Ziviani et al. 2010) have been identified, the substrate required for mitophagy remains to be elucidated (Youle & Narendra 2011). The protein p62 is recruited to ubiquitinated mitochondria, but the requirement of p62 as a mitophagy adaptor is under debate (Youle & Narendra 2011). During erythrocyte development, Nix could serve as an adaptor between mitochondria and LC3 on the autophagosomal membrane (Novak et al. 2010).

Although conventional Atg proteins are required for mitophagy, whether additional factors are also needed remains to be determined. Isolation membranes enclosing mitochondria often associate with rough ER (C. Kishi & N. Mizushima, unpublished data), which suggests that mitophagy and canonical autophagy share fundamental autophagic processes. However, in mitophagy, particularly that mediated by Parkin, large autophagosomes (mitophagosomes) are generated that sometimes enclose more than ten mitochondria and are similar to those seen in xenophagy (C. Kishi & N. Mizushima, unpublished data). Further studies will be required for a better understanding of the mechanism of mitochondrial recognition and mitophagosome formation.

Xenophagy

Beyond its original meaning of self-eating, autophagy is able to target a variety of invading pathogens, especially bacteria; this type of autophagy has been termed xenophagy (Levine et al. 2011, Randow 2011, Shahnazari & Brumell 2011; **Figure 1**). Similar to mitophagy, xenophagy seems to be a selective process, and again ubiquitin has been suggested as a tag for targeting. However, the underlying molecular mechanisms are largely unknown.

Bacteria usually invade cells via the endocytic pathway, and many of them sabotage or escape from endosomes/phagosomes to avoid degradation in lysosomes. Several types of bacteria such as *Listeria monocytogenes* escape into the cytoplasm, where they colocalize with polyubiquitinated proteins. Consequently, one can speculate by analogy to mitophagy that surface proteins of these bacteria are ubiquitinated and recognized by autophagy. Neither the substrates nor the E3 enzymes, however, have been indentified to date. There is also an alternative possibility that endosomes containing bacteria are ubiquitinated and targeted by autophagy. For example, autophagosomes sequester ubiquitin-positive and the late endosome marker LAMP1-positive *S. typhimurium* (S. Kageyama & T. Yoshimori, unpublished data). Presumably damage to the membrane by the phagocytosed bacteria triggers ubiquitination; indeed the endosomal membrane remnants from which *Shigella flexneri* have escaped are

reportedly colocalized with polyubiquitinated proteins and engulfed by autophagosomes, which suggests that the autophagosome is able to surround the damaged endosome together with the phagocytosed bacteria (Dupont et al. 2009). An advantage of this strategy is that the common E3 ligase(s) is available irrespective of the diversity of the invading bacteria, which have very different surface components. It would be interesting to determine whether a common mechanism exists for screening damaged organelles for elimination by autophagy.

The adapter-LC3 hypothesis has also been considered for xenophagy. In addition to p62, NDP52, a newly discovered adapter linking polyubiquitinated protein and LC3, plays a pivotal role in autophagy against *S. typhimurium* (Thurston et al. 2009). Although the significance of the adapters is undoubted, the hypothesis does not completely explain the process. Even in xenophagy, LC3 is not the first Atg protein recruited to the target bacteria (see above for the hierarchy of Atg proteins). The upstream Atg proteins can be recruited to *S. typhimurium* prior to the emergence of or in the absence of lipidated LC3 (N. Fujita & T. Yoshimori, unpublished observation), which predicts the existence of an unknown recognition mechanism beside the adapter-LC3 interaction.

In addition to the core Atg machinery, formation of xenophagosomes against Group A streptococcus deploys an additional molecule, Rab7, that is not used in canonical autophagosome formation (Yamaguchi et al. 2009). Thus, xenophagy is regarded as an evolutionary variation of the prototype autophagy that is to eliminate invaders efficiently.

CONCLUDING REMARKS AND FUTURE ISSUES

During the 18 years since *ATG* genes were first identified in yeast, the molecular details of autophagosome formation mediated by core Atg proteins have been unveiled. However, many fundamental questions remain. Knowledge about the biochemical features of intermediates and autophagosomes is still lacking. The protein and lipid compositions of these membranes need to be determined to fully understand the origin and mechanism of autophagosome formation. We need to know how the membrane curvature of the isolation membrane and the size of the autophagosome are determined as well as how the inner and outer membranes are differentiated, which may provide an understanding of how the isolation membrane is formed and why the inner membrane is so susceptible to lysosomal/vacuolar enzymes.

Recently, the crystal structures of many Atg proteins and their complexes have been determined, and novel motifs and domains in Atg proteins have been elucidated (Noda et al. 2009). Further structural analyses will give us an important base for understanding the molecular mechanism of Atg protein functions.

As autophagy plays important roles in so many biological processes, diverse mechanisms likely have been developed for autophagosome formation in different cell types and organs and under different physiological conditions. Further molecular analyses of the function and dynamics of Atg proteins will unveil the unique mechanism of membrane dynamics during autophagy.

DISCLOSURE STATEMENT

T.Y. is a consultant for Kao Co. and for Merck Millipore.

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