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REVIEW

Autophagosome biogenesis: From membrane growth to closure

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Autophagosome biogenesis involves de novo formation of a membrane that elongates to sequester cytoplasmic cargo and closes to form a double-membrane vesicle (an autophagosome). This process has remained enigmatic since its initial discovery >50 yr ago, but our understanding of the mechanisms involved in autophagosome biogenesis has increased substantially during the last 20 yr. Several key questions do remain open, however, including, What determines the site of autophagosome nucleation? What is the origin and lipid composition of the autophagosome membrane? How is cargo sequestration regulated under nonselective and selective types of autophagy? This review provides key insight into the core molecular mechanisms underlying autophagosome biogenesis, with a specific emphasis on membrane modeling events, and highlights recent conceptual advances in the field.

Introduction

Autophagosomes are double-membrane vesicles containing cytoplasmic components destined for lysosomal degradation in a process referred to as macroautophagy (hereafter autophagy). Autophagosome biogenesis involves nucleation, expansion, and closure of a cup-shaped membrane (called a phagophore or isolation membrane) to allow sequestration of cytoplasmic cargo, followed by their fusion with endolysosomal compartments to facilitate degradation of the sequestered material (Lamb et al., 2013). Autophagosomes form on demand, either to facilitate recycling of metabolic precursors to promote cell survival under conditions of cellular stress or to mediate clearance of damaged or surplus cellular components, thereby promoting cellular homeostasis (Dikic and Elazar, 2018). Knowledge about the molecular mechanisms involved in cargo sequestration and autophagosome biogenesis under various metabolic and pathological conditions is important to better understand the importance of this pathway in various pathophysiological conditions (Levine and Kroemer, 2019).

Autophagosomes are generally devoid of any transmembrane proteins (Baba et al., 1995; Fengsrud et al., 2000), which has made it difficult to trace the origin of the phagophore membrane and understand the dynamic events leading to phagophore elongation, bending, and closure. Indeed, the source of the autophagosomal membrane has been one of the major questions in the field for several decades and is still a topic of intense investigation. Our understanding of the mechanisms involved in autophagosome biogenesis have increased substantially over the last 20 yr, however. Pioneering screens in yeast identified several autophagy-related genes (*ATG*) as essential for autophagy (Harding et al., 1995; Thumm et al., 1994; Tsukada and Ohsumi, 1993), which later led to the characterization of mammalian *ATG* orthologues, commonly referred to as the core autophagy machinery (Inoue and Klionsky, 2010; Mizushima et al., 2011). Structural and functional decipherment of such ATG proteins has shown that they typically form multisubunit complexes that work together to coordinate the multiple membrane modeling events involved in autophagosome biogenesis (Dikic and Elazar, 2018; Hurley and Young, 2017; Mercer et al., 2018; Yin et al., 2016). In humans these include (a) the Unc51-like kinase (ULK) complex, (b) the autophagy-specific class III phosphatidylinositol 3-kinase complex I (PIK3C3-CI), (c) the transmembrane protein ATG9 and its cycling system, and (d) the ubiquitin-like proteins of the light chain 3 (LC3)/GABA type A receptor-associated protein (GABARAP) subfamilies (commonly referred to as ATG8) and ATG12, as well as their conjugation machineries (see Box 1 for details).

Sequestration of cargo for degradation by autophagy was long considered a random, nonselective process, but the identification of so-called autophagy receptors, which connect the cargo to be degraded to ATG8 proteins in the autophagic membrane, opened up the field of selective autophagy (Khaminets et al., 2016; Johansen and Lamark, 2020; Sánchez-Martín and Komatsu, 2020). As will be discussed, recent studies

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Box 1. An overview of the core autophagy complexes and their main functions in autophagosome biogenesis. See main text for key references.

Core Autophagy Complexes	Human components (Yeast)	Main functions
ULK complex	ULK1/ULK2, ATG13, FIP200, ATG101 (Atg1, Atg13, Atg17, Atg11, Atg29, Atg31)	Protein kinase ULK1 or ULK2 in complex with accessory proteins. Complex considered to be master regulator of autophagosome biogenesis.
PIK3C3-C1	VPS34, BECN1, p150, ATG14, NRFB2 (Vps34, Atg6, Vps15, Atg14)	Class III phosphatidylinositol 3-kinase complex I containing the lipid kinase VPS34 together with accessory proteins. The complex is responsible for PtdIns(3)P production during phagophore nucleation.
ATG12 conjugation machinery	ATG5, ATG7, ATG10, ATG12, (Atg5, Atg7, Atg10, Atg12)	ATG7 (E1-like) and ATG10 (E2-like) facilitate conjugation of ATG12 to ATG5
ATG8 conjugation machinery	ATG3, ATG4 A-D, ATG7, ATG12-ATG5, ATG16L1 (Atg3, Atg4, Atg7, Atg12-Atg5, Atg16) ATG8 proteins: LC3 subfamily: LC3A, LC3B, LC3C GABARAP subfamily: GABARAP, GABARAPL1, GABARAPL2 (Atg8)	ATG12-ATG5 binds ATG16L1 to form the ATG12-ATG5-ATG16L1 complex (E3-like) that together with ATG7 (E1-like) and ATG3 (E2-like) mediate covalent attachment of ATG8 family members of the LC3 and GABARAP sub-families to phosphatidylethanolamine (PE) in the autophagic membrane. Prior to their lipidation, newly synthesized pro-LC3 and pro-GABARAP proteins are cleaved by ATG4 proteases to expose a free C-terminal glycine residue that becomes covalently attached to PE. ATG4 proteases also cleave off LC3/GABARAP proteins conjugated to the outer autophagosome membrane prior to lysosomal fusion.
ATG2 complexes	ATG2, ATG9, WIPI4 (Atg2, Atg9, Atg18)	Lipid transfer protein ATG2 is in complex with the only transmembrane ATG protein, ATG9, or the WD repeat domain phosphoinositide-interacting protein 4 (WIPI4). In yeast the three proteins exist in the same complex.

indicate that autophagy receptors not only facilitate selective engulfment of cargo, but also recruit the core autophagy machinery to allow cargo-specific de novo autophagosome biogenesis. Our understanding of this process is still limited, however, and more investigation is needed.

This review focuses on the molecular mechanisms of the core autophagy components involved in autophagosome biogenesis during nonselective and selective types of autophagy, with a specific focus on membrane dynamics and the recent advances in the field. The intricate signaling pathways regulating autophagosome biogenesis and the mechanisms underlying fusion of autophagosomes with endolysosomal compartments have been extensively reviewed elsewhere (Walker and Ktistakis, 2019; Wesselborg and Stork, 2015; Zhao and Zhang, 2019) and are only briefly mentioned here. Finally, we point out some of the open questions that need further investigation.

Mechanisms of autophagosome formation

The origins of the membrane contributing to autophagosome formation and growth and the mechanisms that support membrane delivery have been contentious questions for >50 yr. The molecular mechanisms underlying autophagosome biogenesis have been elucidated mainly in cells subjected to nutrient starvation, when cells induce autophagy in a seemingly nonselective manner to provide breakdown products necessary to maintain cellular homeostasis. Less is known about regulation of the machinery and lipid mobilization involved in autophagosome formation during basal conditions, where autophagy plays an important role in cellular homeostasis by clearance of

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dysfunctional or surplus cellular components. In yeast subjected to nitrogen starvation, autophagosomes form from a single preautophagosomal structure located in close proximity to the vacuole (Ohsumi, 2014; Suzuki et al., 2001). In contrast, mammalian autophagosomes can originate concomitantly at several sites that are closely associated with specific phosphatidylinositol 3-phosphate (PtdIns(3)P)-enriched subdomains of the ER, referred to as omegasomes (Axe et al., 2008; Ylä-Anttila et al., 2009; Hamasaki et al., 2013). Further elongation of the phagophore membrane seems to involve several membrane sources. An individual autophagosome takes ∼10 min to form (Axe et al., 2008) and persists for ∼10–20 min after membrane conjugation of LC3 (Hailey et al., 2010; Kirisako et al., 1999; Xie et al., 2008). Autophagosomes can vary in size from a few hundred nanometers to more than a micrometer in diameter, depending on the size of the cargo being sequestered (Jin and Klionsky, 2014; Xie et al., 2008). Although the site of phagophore formation and the membrane sources involved may vary depending on the autophagy-inducing signal and the nature of the sequestered cargo, the core autophagy machinery required for autophagosome biogenesis is generally well conserved. The interconnections between these core ATG proteins are tightly regulated in space and time to allow phagophore nucleation (Fig. 1), elongation (Fig. 2), and closure (Fig. 3) to form an autophagosome.

Phagophore nucleation

Induction of autophagosome biogenesis involves the coordinated activation and proper localization of the core autophagy machineries (Fig. 1 and Box 1). Their activity is tightly regulated by various posttranslational modifications, including phosphorylation and ubiquitination, which are discussed in more detail elsewhere (Mercer et al., 2018; Walker and Ktistakis, 2019). In general, signaling pathways that promote cell growth typically inhibit autophagosome formation, while those activated upon poor nutrient and energy status will stimulate autophagosome biogenesis. Prime examples include the mechanistic target of rapamycin (mTOR) kinase and AMP kinase (AMPK), which oppositely regulate autophagosome biogenesis by phosphorylation of key components of the core autophagy machinery, including subunits of the ULK and PIK3C3 complexes.

The ULK complex

\The multimeric ULK complex, comprising the kinase ULK1 (or ULK2), ATG13, ATG101, and FIP200 (FAK family kinase-interacting protein of 200 kD) is considered the master regulator of autophagosome biogenesis (Chan et al., 2007; Ganley et al., 2009; Hara et al., 2008; Hosokawa et al., 2009b; Jung et al., 2009; Mercer et al., 2009). The ULK complex is constitutively assembled and this is not regulated by nutrient conditions (Hosokawa et al., 2009a). It is not completely understood what causes translocation of the ULK complex to phagophore nucleation sites upon amino acid starvation, but it is independent of ULK1 kinase activity and does require the C-terminal EAT domain of ULK1 (Chan et al., 2009) and an interaction of the N-terminus of ATG13 with acidic phospholipids in the membrane, including PtdIns(3)P and PtdIns(4)P (Karanasios et al., 2013; Fig. 1 B). In line with this, ATG9-mediated delivery of the PI4-kinase PI4KIIIβ to phagophore nucleation sites and subsequent binding of ATG13 to PtdIns(4)P seems important for translocation of the ULK complex to such sites (Judith et al., 2019). Moreover, the ULK complex was found to localize to phosphatidylinositol synthase (PIS)-enriched ER subdomains (Nishimura et al., 2017). ULK complex activation and ER translocation is further promoted by binding to the ras-related protein 1 (RAB1) effector C9orf72 (Webster et al., 2016; Fig. 1 B).

The activated ULK1 complex phosphorylates several core autophagy components, various regulatory proteins, and itself, leading to recruitment of the PIK3C3-CI and PtdIns(3)P production, resulting in formation of PtdIns(3)P-positive omegasomes that function as platforms for phagophore elongation (Fig. 1 A). An overview of ULK1 substrates and their functions can be found in Mercer et al. (2018). Importantly, inactivation of mTOR in the absence of amino acids or growth factors is key to activation of ULK1. Active mTOR interacts directly with ULK1 via the mTOR complex 1 (mTORC1) subunit Raptor and inhibits autophagosome biogenesis via phosphorylation of ULK1 and ATG13 (Ganley et al., 2009; Hosokawa et al., 2009a; Jung et al., 2009; Kim et al., 2011; Puente et al., 2016; Shang et al., 2011; Fig. 1 A). In contrast, AMPK phosphorylates ULK1 and ATG13 to promote autophagosome biogenesis (Puente et al., 2016; Sanchez et al., 2012; Shang et al., 2011). Active AMPK and ULK1 both phosphorylate Raptor to inhibit mTORC1 activity (Gwinn et al., 2008; Kim et al., 2011), leading to further ULK1 stimulation (Fig. 1 A). In addition to this positive-feedback phosphoregulation of ULK1 activity, LYS-63–linked ubiquitination of ULK1 by the E3-ligase TRAF6 bound to AMBRA1 (autophagy and beclin 1 regulator 1, the activating molecule in beclin 1 [BECN1]-regulated autophagy) promotes ULK1 complex stabilization and function (Nazio et al., 2013). ULK1 ubiquitination by neural precursor cells expressing developmentally down-regulated protein 4–like (NEDD4) ligase (Lys-27, Lys-29 linked) or CULLIN3 ligase (Lys-48 linked) rather facilitate its degradation and down-regulation of the autophagic response (Liu et al., 2016; Nazio et al., 2016). ULK1 activation and ER targeting are also closely linked to recruitment and activation of the PIK3C3 complex I and trafficking of ATG9, the only transmembrane protein required for autophagosome biogenesis.

The PIK3C3-CI

The autophagy-specific PIK3C3 complex I (consisting of vacuolar protein sorting 34 [VPS34], p150, BECN1, ATG14, and nuclear receptor binding factor 2 [NRBF2]) is responsible for PtdIns(3)P production at omegasome structures (Axe et al., 2008). ULK1 enhances VPS34 activity by phosphorylation of several PIK3C3-CI subunits, including VPS34 itself, BECN1, and ATG14 (Mercer et al., 2018; Wold et al., 2016). Moreover, ULK1-mediated phosphorylation of the PIK3C3 regulator AMBRA1 causes release of BECN1-VPS34 from the cytoskeleton and their recruitment to the ER (Di Bartolomeo et al., 2010). Finally, a direct interaction between the ULK complex subunit ATG13 and ATG14 stabilizes membrane localization of the PIK3C3-CI (Park et al., 2016). Targeting of the PIK3C3-CI to the ER membrane is further facilitated by membrane-associated regions in PIK3C3-CI subunits, including an N-terminal

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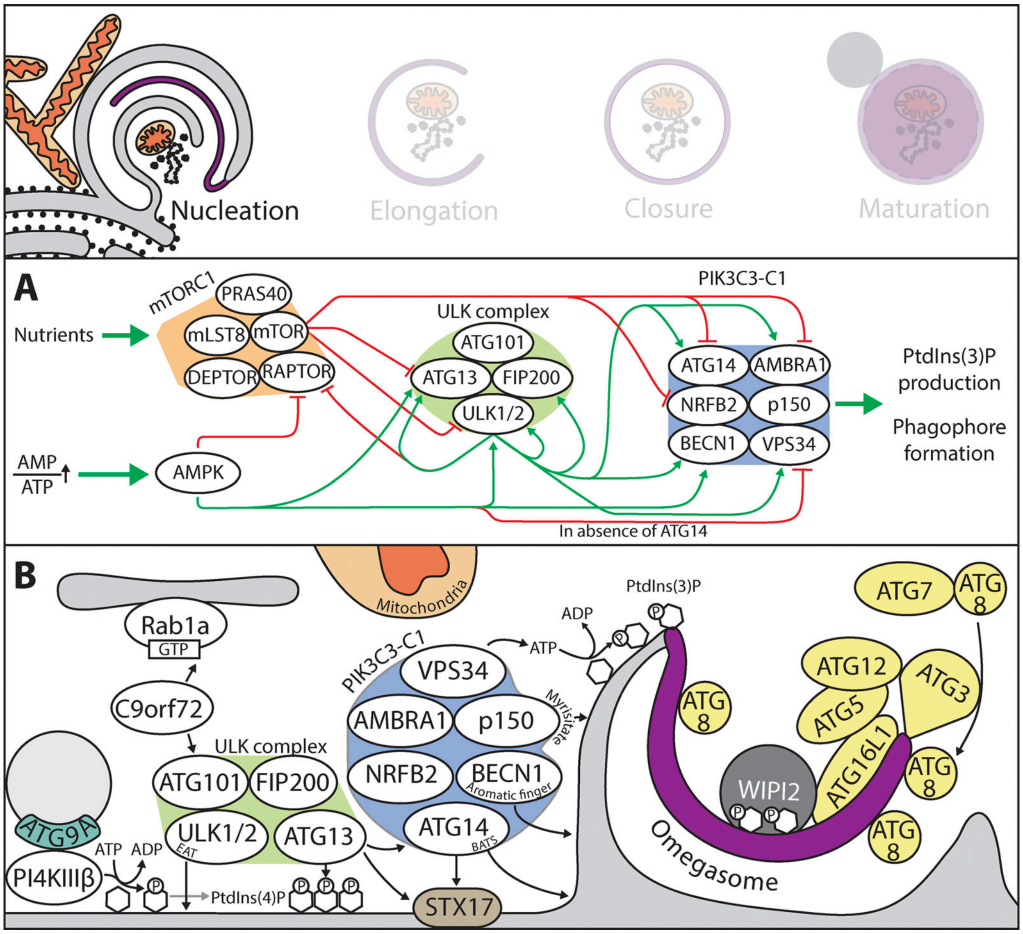


Figure 1. **Overview of signaling events and protein–protein and protein–membrane interactions involved in phagophore nucleation. (A)** Nutrient-rich conditions promote the activity of mTORC1, which inhibits autophagy by mTOR-mediated phosphorylations of the ULK complex (ULK1/2 and ATG13) and PIK3C3-CI (NRFB2, ATG14, and AMBRA1). In contrast, low energy status (high AMP-to-ATP ratio) causes activation of AMPK, which positively regulates autophagy by phosphorylation of the mTORC1 complex (Raptor), the ULK complex (ULK1 and ATG13), and PIK3C3-CI (BECN1 in the presence of ATG14 and VPS34 in the absence of ATG14). Activation of the ULK complex facilitates autophagy by autophosphorylation (ULK1, FIP200, and ATG13), inhibitory phosphorylation on mTORC1 (Raptor), and activating phosphorylations of the PIK3C3-CI (BECN1, VPS34, ATG14, and AMBRA1). **(B)** The autophagy-inducing signaling events described in A lead to membrane recruitment of the ULK complex. This is promoted by its interaction with C9orf72 (Rab1 effector) and dependent on the EAT domain of ULK1 and the N-terminus of ATG13. The latter interacts with acidic phospholipids, including PtdIns(4)P, generated by the PI4KIIIβ, which interacts with ATG9, a transmembrane protein important for phagophore elongation (see Fig. 2). The ULK complex stabilizes the PIK3C3-CI through direct interactions between ATG14 and ATG13. ATG14 also interacts with the ER-resident protein STX17 at ER–mitochondria contact sites. PIK3C3-CI membrane binding is further mediated by ATG14 (N-terminal cysteine-rich domain and a PtdIns(3)P binding BATS domain), BECN1 (aromatic finger), and p150 (N-terminal myristate). Generation of PtdIns(3)P by the PIK3C3-CI facilitates recruitment of the PtdIns(3)P effector protein WIPI2 that promotes ATG8 conjugation to PE through recruitment of the ATG12-ATG5-ATG16L1 complex, an E3 of the ATG8 conjugation machinery. Lipidated ATG8 proteins can function as a scaffold for core autophagy machinery components and as membrane attachment sites for autophagic cargo receptors (see Fig. 4). The phagophore membrane is indicated in purple.

cysteine-rich domain and a PtdIns(3)P-binding BATS domain in ATG14 (Fan et al., 2011; Matsunaga et al., 2010; Tan et al., 2016), an aromatic finger in BECN1 (Huang et al., 2012), and an N-terminal myristate on p150 (Panaretou et al., 1997). Moreover, ATG14 interacts with the ER-resident SNARE protein Syntaxin-17 (STX17) at ER–mitochondria contact sites (Hamasaki et al., 2013) and NRBF2, which promotes VPS34 lipid kinase activity and complex assembly (Cao et al., 2014; Lu et al., 2014; Ohashi et al., 2016; Young et al., 2016; Fig. 1 B).

In addition to ULK1, several other kinases and scaffolding proteins have been found to regulate the activity of the PIK3C3-CI (Fig. 1 A). mTOR phosphorylates and inhibits ATG14 and NRBF2, as well as AMBRA1 (Egan et al., 2015; Ma et al., 2017). Interestingly, AMPK-mediated regulation of PIK3C3-CI activity is coordinated with the availability of ATG14, as AMPK inhibits VPS34 in the absence of ATG14, while its phosphorylation of BECN1 activates VPS34 activity in the presence of ATG14 (Kim et al., 2013; Zhang et al., 2016). Several PIK3C3-CI–interacting proteins are found to stabilize the complex or promote its activity, including dishevelled-interacting protein (Dapper1; Ma et al., 2014), progestin and adipoQ receptor family member 3 (PAQR3; Xu et al., 2016), and receptor for activated C kinase 1 (RACK1; Zhao et al., 2015). Moreover, as for ULK1, both the stability and activity of the PIK3C3 can be modulated by

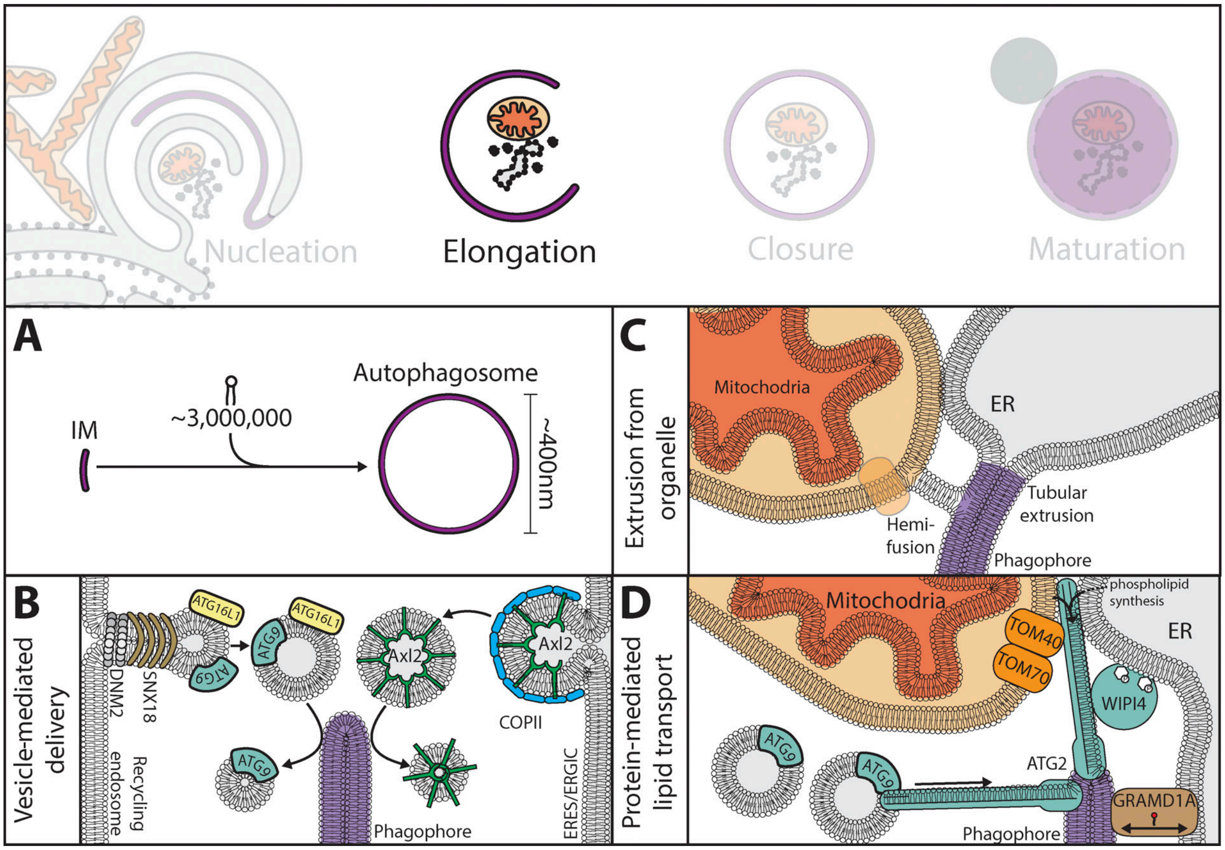


Figure 2. **Model of suggested mechanisms involved in phagophore elongation. (A)** A rough estimate shows that ∼3 million lipids could be required to produce an autophagosome of 400 nm (see Box 2). Three distinct mechanisms for delivery of lipids for phagophore elongation have been proposed: vesicle-mediated delivery, membrane extrusion from pre-existing organelles, and protein-mediated lipid transport. **(B)** For vesicle-mediated delivery, ATG9- and ATG16L1-positive vesicles formed from recycling endosomes (dependent on SNX18, DNM2, and adaptor proteins) and COPII vesicles from ER exit sites (ERES) and ERGIC have been implicated in phagophore elongation. **(C)** For membrane extrusion from preexisting organelles, tubular extrusions from the ER and mitochondria have been proposed to form the expanding phagophore. **(D)** For protein-mediated lipid transport, illustrated for ATG2A and GRAMD1A, ATG2A acts as a lipid tunnel with little or no lipid specificity, while GRAMD1A functions as a cholesterol transfer protein.

ubiquitination (Antonioli et al., 2014; Liu et al., 2016; Xia et al., 2013, 2014). Finally, PI3KC3-C1 stabilizes the ULK complex at the ER (Karanasios et al., 2013; Koyama-Honda et al., 2013), providing positive-feedback regulation of PtdIns(3)P production at phagophore nucleation sites.

The class II PI3-kinase α (PIK3C2A), also able to generate PtdIns(3)P, was recently implicated in autophagosome biogenesis following inhibition of mTOR with rapamycin (Merrill et al., 2017). PIK3C2A was found to interact with ATG9 and ATG14, and its depletion resulted in accumulation of RAB11- and transferrin-positive clathrin-coated vesicles. In line with a role for PIK3C2A-mediated PtdIns(3)P production at recycling endosomes, it was suggested that the PtdIns(3)P effector protein WD-repeat domain phosphoinositide interacting 2 (WIPI2), interacts with RAB11A and that autophagosomes can evolve from RAB11A-positive membranes (Puri et al., 2018).

Role of PtdIns(3)P

So what is the purpose of PtdIns(3)P production at phagophore nucleation structures? The ER contains very little PtdIns(3)P under basal conditions (Gillooly et al., 2000), so localized enhanced PtdIns(3)P levels would function to recruit specific PtdIns(3)P effector proteins. The PtdIns(3)P binding protein DFCP1 (double FYVE-containing protein 1, also known as ZFYVE1) localizes to omegasomes, but is itself dispensable for autophagosome biogenesis flux (Axe et al., 2008). More importantly, the presence of PtdIns(3)P at the omegasome allows recruitment of members of the WIPI1–4 protein family (Proikas-Cezanne et al., 2015), which are functionally related to yeast Atg18 (Barth and Thumm, 2001; Guan et al., 2001; Dove et al., 2004), containing a seven-bladed β-propeller structure that preferably binds two molecules of PtdIns(3)P, but also can bind PtdIns(5)P or PtdIns(3,5)P_2 (Jeffries et al., 2004). While all WIPI proteins localize to nascent autophagosome membranes (Bakula et al., 2017; Polson et al., 2010; Proikas-Cezanne et al., 2004), they seem to function at different stages of autophagosome formation. WIPI1 and WIPI2 localize to omegasomes, where WIPI2 promotes PE conjugation of ATG8 proteins by recruitment of the ATG12-ATG5-ATG16L1 E3-like complex through a direct interaction with ATG16L1 (Dooley et al., 2014; Fig. 1 B). WIPI3 and WIPI4 seem to link upstream regulatory pathways to PtdIns(3)P production and autophagosome biogenesis by interacting with the AMPK-activated TSC complex and the AMPK/

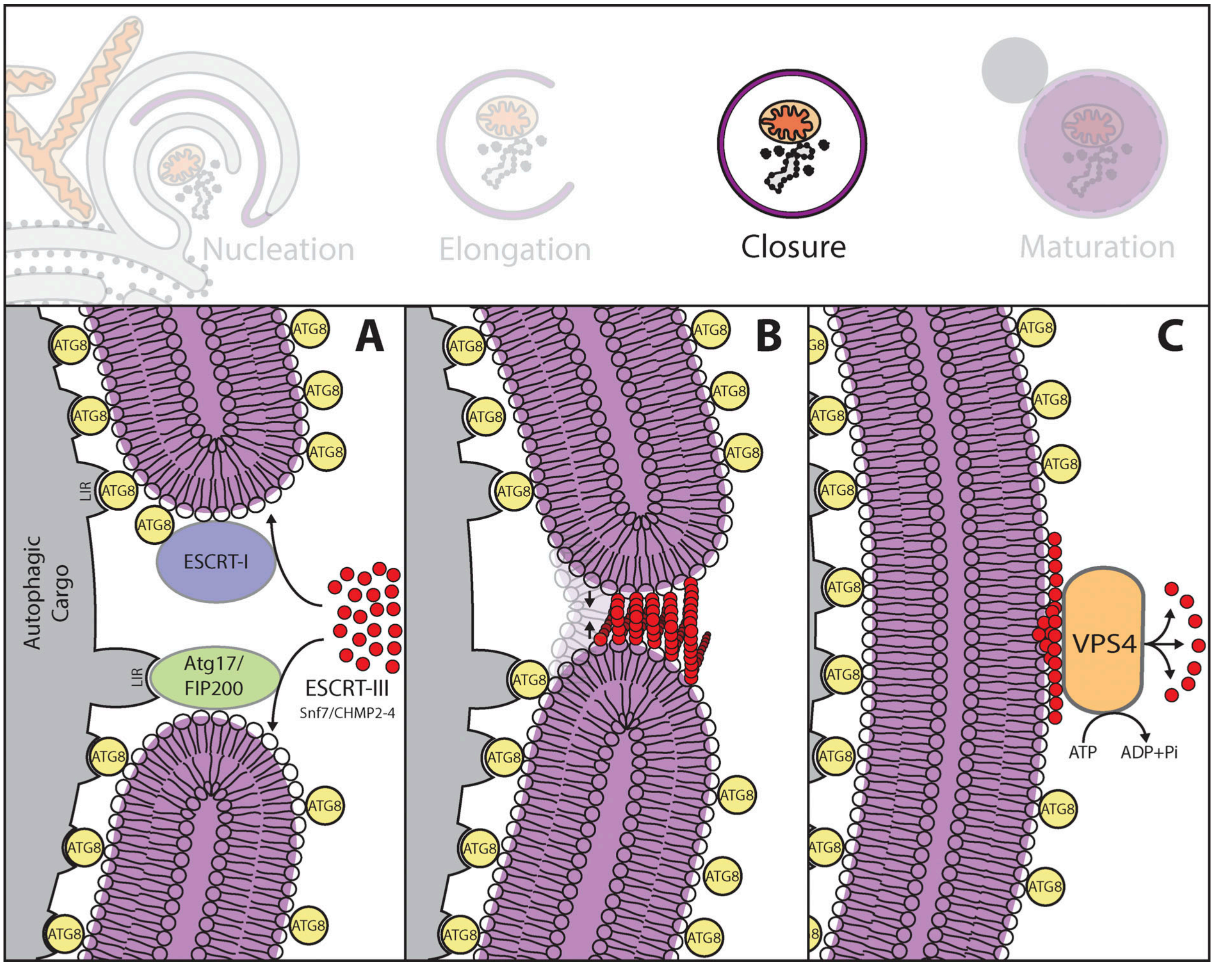


Figure 3. **Autophagosome closure is facilitated by the ESCRT machinery. (A)** ESCRT-I components are recruited to the phagophore by an unknown mechanism, followed by recruitment of the filament-forming ESCRT-III components CHMP2A and CHMP4B. In yeast, Atg17 (FIP200) interacts with the ESCRT-III subunit Snf7 (CHMP4), indicating a role for the ULK complex in recruitment of ESCRT-III for phagophore closure. **(B)** ESCRT-III polymerization leads to filament formation, bringing the leading edge of the phagophore into close apposition to allow membrane fission. **(C)** Recruitment of the AAA-ATPase VPS4 resolves the fission process and facilitates depolymerization of the ESCRT-III filament structure. ATG8 proteins are also implicated in phagophore elongation and closure, but the mechanisms involved are not clear.

ULK1 complex at lysosomes, respectively, followed by their translocation to nascent autophagosomes in response to glucose starvation (Bakula et al., 2017). WIPI4 also interacts with the lipid transfer protein ATG2 to promote phagophore elongation, as is discussed below.

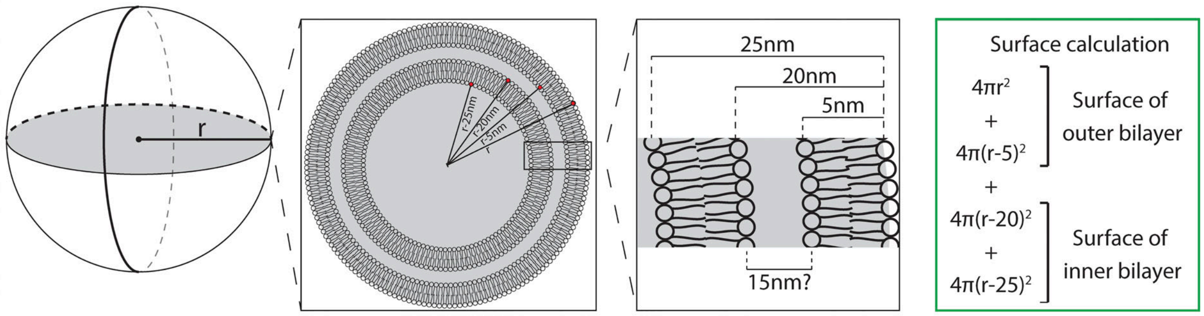
PtdIns(3)P turnover seems necessary for dissociation of the ATG machinery from the surface of autophagosomes before their fusion with the lysosome/vacuole (Cebollero et al., 2012). PtdIns(3)P levels at the growing phagophore are closely regulated by PtdIns(3)P phosphatases, which negatively regulate autophagy by dephosphorylation of PtdIns(3)P (Cebollero et al., 2012; Taguchi-Atarashi et al., 2010; Vergne et al., 2009). Moreover, additional phosphoinositide kinases facilitate phosphorylation of PtdIns(3)P, including the PtdIns 5-kinase FYVE-type zinc finger containing (PIKFYVE) that converts PtdIns(3)P into PtdIns(3,5)P_2, being involved in autophagosome maturation (Rusten et al., 2007). Interestingly, in addition to its negative regulation of autophagy by converting PtdIns(3)P to PtdIns(3,5) P2, PtdIns(5)P production by PIKFYVE was found to rescue WIPI2b recruitment to omegasomes in PIK3C3-deficient cells, particularly in glucose starvation conditions (Vicinanza et al., 2015).

Phagophore elongation

As the expanding phagophore is largely devoid of transmembrane proteins (Baba et al., 1995; Fengsrud et al., 2000), membrane expansion is primarily through the delivery of lipids. To estimate the magnitude of lipid demand needed to form a single autophagosome, we consider the number of lipids required to build a model autophagosome-like structure (Box 2). Using established physical dimensions for phosphatidylcholine to approximate a single lipid (i.e., a headgroup area of 65 Å^2 and a length of 20 Å; Kucerka et al., 2005) and an estimate of the luminal space between bilayers in an autophagosome (which various studies suggest is between 10 and 30 nm as described in Nguyen et al. [2017]), we calculate that for a ~400-nm-diameter autophagosome, expansion could require the delivery of as many as 3,000,000 lipids (Box 2). Furthermore, in mammals,

Box 2. Calculation of the approximate amount of lipids going into a model autophagosome of various sizes.

1. Calculating the membrane surface area of a model autophagosome

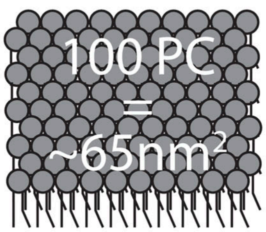


Surface area of a sphere = $4\pi r^2$

Autophagosomes have two bilayers, thus four lipid-headgroup surfaces

Estimated thickness of- and distance between- bilayers

2. Calculating the approximate number of lipids of model autophagosomes of different sizes



100 phosphatidylcholine lipids covers approximately $65nm^2$

Total number of lipids in a model autophagosome:

$$\frac{\text{Surface area of model autophagosome}}{\text{Surface area of one PC}}$$

Autophagosome Diameter (nm)	Total Surface Area (nm2)	Total Lipids
200	390,186	600,286
400	1,772,487	2,726,902
600	4,160,097	6,400,149
800	7,553,017	11,620,026
1,000	11,951,247	18,386,533

autophagosomes form continuously throughout the cytoplasm and, in periods of stress, can number close to 100 per cell, depending on cell type (Fass et al., 2006; Guo et al., 2012; Hailey et al., 2010). Thus, the macroautophagy stress response is fundamentally an organelle biogenesis event that, when stimulated, could require the mobilization of 100,000,000-plus lipids per cell. How lipid is harvested to support autophagosome growth and how it is delivered to the many forming structures remains unclear, but recent advances in cell biology, imaging, and protein biochemistry have highlighted three mechanisms by which lipids appear to reach the expanding phagophore: vesicle-mediated delivery (Fig. 2 B), direct extrusion from a preexisting organelle (Fig. 2 C), and direct protein-mediated transport of lipids (Fig. 2 D).

Vesicle-mediated delivery

The multispanning transmembrane protein Atg9 (yeast) or ATG9 (mammals) resides in vesicles that traffic to and from the developing autophagosome (Kakuta et al., 2012; Mari et al., 2010; Orsi et al., 2012; Reggiori et al., 2005; Takahashi et al., 2011; Yamamoto et al., 2012; Fig. 2). In yeast, these vesicles may cluster together via Atg1 complexes that drive initiation of autophagosome biogenesis (Matscheko et al., 2019; Rao et al., 2016), into a phase-separated compartment (Fujioka et al., 2020). Then, homotypic fusion of these vesicles (Yamamoto et al., 2012) gives rise to the phagophore. In mammals, the phagophore is surrounded by tubules and vesicles that are ATG9-positive, but the evidence for fusion into the phagophore is less clear (Orsi et al., 2012), and the precise role for ATG9-associated membranes is still under investigation. Thus, ATG9 vesicle flux to and from the phagophore during membrane expansion may serve additional purposes, such as scaffolding of protein complexes functioning in initiation of autophagosome biogenesis and organization of the machinery needed during expansion such as ATG2. ATG9 localizes to the Golgi complex under normal conditions but translocates to recycling endosomes and small vesicles referred to as the ATG9 compartment upon induction of autophagy in an ULK1-dependent manner (Mari et al., 2010; Orsi et al., 2012; Young et al., 2006; Zhou et al., 2017). Exactly where and when the ULK1–ATG9 interaction takes place is not clear, but it was found that small ATG9 vesicles colocalize with the ULK1 complex in regions that overlap with the ER-Golgi intermediate compartment (ERGIC; Karanasios et al., 2016). At the recycling endosome, ATG9A localizes to ATG16L1 and RAB11-positive recycling endosomes, which may either transform into a nascent phagophore or provide membrane to the growing phagophore by vesicle-mediated delivery (Knævelsrud et al., 2013; Longatti et al., 2012; Puri et al., 2013; Puri et al., 2018; Ravikumar et al., 2010; Søreng et al., 2018). Indeed the importance of continued ATG9 trafficking to and from the endosome is supported by studies demonstrating a key role in autophagy for adaptor proteins (Imai et al., 2016; Mattera et al., 2017; Popovic and Dikic, 2014; Zhou et al., 2017), Dynamin 2 (DNM2), and the DNM2 interacting protein SNX18 (Søreng et al., 2018; Fig. 2 B). Although Atg9/ATG9 vesicles are essential to the expansion phase, it is likely that lipid is also harvested from other sources.

A role for coat protein complex II (COPII) vesicles was first suggested by the discovery that the autophagy-specific trafficking protein particle (TRAPP)-III tethering complex engages the COPII machinery (Tan et al., 2013) and that yeast autophagosome biogenesis occurs precisely at ER exit sites normally dedicated to COPII vesicle production (Graef et al., 2013). In vitro experiments separating cellular membranes then demonstrated that COPII-related vesicles derived from the ERGIC can support LC3-II formation in vitro (Ge et al., 2014), indicating that the lipid composition and structure of these membranes is consistent with autophagosome-directed biochemistry. Most recently, the transmembrane COPII cargo protein Axl2 was found to colocalize with growing phagophores, supporting a direct role for these vesicles in phagophore expansion (Shima et al., 2019); however, the machinery that supports fusion of these vesicles into the phagophore has not yet been identified (Fig. 2 B). Consistent with this idea, a variety of studies have suggested that unique ER exit sites are engineered to produce vesicles dedicated to eventual consumption by autophagosomes or autophagy-related processes (Crawford et al., 2019; Ge et al., 2017). By controlling the formation of the vesicles destined for autophagosome expansion, these engineered ER sites might also regulate the accessibility of transmembrane proteins, providing a potential explanation for the relative dearth of these molecules on the mature organelle.

Direct extrusion from a preexisting organelle

In mammals, both immunobiochemistry (Dunn, 1990) and cryo-electron tomography (Hayashi-Nishino et al., 2009; Ylä-Anttila et al., 2009) suggest that phagophores form at ER subdomains termed omegasomes (Axe et al., 2008). At the omegasome, two groups have postulated that the phagophore might directly extrude from the ER itself (Hayashi-Nishino et al., 2009; Ylä-Anttila et al., 2009). Cryo-electron tomography and immunogold electron microscopy suggest possible continuity between the cup-shaped phagophore and the surrounding ER (Fig. 2 C). In such an instance, growth could be by continued extrusion and rely on the almost limitless supply of lipids in the ER. Moreover, the early core autophagy machinery components translocate to omegasomes, and several ER-localized proteins, including vacuole membrane protein 1, vesicle-associated membrane protein-associated proteins A and B (VAPA and VAPB), extended synaptotagmin, PIS, and transmembrane protein 41B (TMEM41B), have been implicated in autophagosome biogenesis (Ktistakis, 2020). Conversely, not all studies have been able to observe direct connections between isolation membranes and the ER (e.g., Kishi-Itakura et al., 2014), and thus the generality of extrusion from the ER remains uncertain.

Autophagosome biogenesis has been documented at sites other than the ER, and in cases of both the mitochondria (Hailey et al., 2010) and early recycling endosome (Puri et al., 2018), these results can also be interpreted as growth by extrusion. By combining superresolution fluorescence microscopy with engineered markers of the mitochondria, LC3-positive structures forming directly on extruded regions of the mitochondria outer membrane were detected (Hailey et al., 2010). These regions specifically excluded transmembrane proteins of the mitochondria, but could recruit proteins embedded in only a single cytoplasm-facing leaflet of the outer mitochondrial membrane, suggesting that, as for the ER exit sites, a selection process against transmembrane proteins may occur at the moment of membrane utilization (Fig. 2 C). Interestingly, omegasomes overlap with ER–mitochondria contact sites (Hamasaki et al., 2013; Karanasios et al., 2013) and specific ER exit sites, where also ATG9 vesicles seem to be recruited (Karanasios et al., 2016), suggesting a close interconnection between several membrane sources of autophagosome biogenesis.

Direct protein-mediated transport of lipids

At contact sites throughout the cell, lipid transport proteins function to move lipids from one organelle to another. In most cases, these proteins can bind one or two lipids; flux across the contact site is then regulated by either specific exchange for lipids on the acceptor membrane or by maintaining a gradient through the consumption of the transported lipid (Wong et al., 2019). Membrane expansion by such a mechanism has not yet been demonstrated and would likely require that lipids are transferred en masse in one direction.

At least three lipid-transport proteins are now known to support autophagosome biogenesis. GRAM domain–containing 1A (GRAMD1A) is a cholesterol transfer protein in the StART domain family, and recent work developing small-molecule inhibitors of its cholesterol-binding activity unexpectedly revealed a role in autophagy (Laraia et al., 2019). These inhibitors specifically delayed the recruitment of ATG5 to WIPI2-positive puncta, suggesting a role for GRAMD1A-mediated cholesterol transport in the membrane expansion step (Fig. 2 D). TipC (Dictyostelium) and its human homologue VPS13A were previously each shown to support efficient autophagosome production in model systems (Muñoz-Braceras et al., 2015) through an unknown mechanism. In 2018, VPS13A was established as the first in a new class of lipid-transport proteins (Kumar et al., 2018), which have the notable distinctions of binding large numbers of lipids at once and appearing to exhibit little to no lipid specificity during in vitro lipid transport. Thus, this class of proteins could be ideally suited for bulk delivery of lipid in support of dramatic membrane expansion. Intriguingly, VPS13A shares two short stretches of homology with ATG2, called chorein domains. In a series of papers last year, it was demonstrated that Atg2 (yeast) and ATG2A (humans) harbor the same high lipid-binding capacity and in vitro lipid transport activity as VPS13A (Maeda et al., 2019; Osawa et al., 2019; Valverde et al., 2019). The crystal structures of the N-terminal chorein domains of VPS13A and Atg2 reveal a similar shovel-like fold in which the "scoop" region of the shovel is covered entirely in hydrophobic amino acids, likely comprising the lipid-binding surface (Kumar et al., 2018; Osawa et al., 2019). In VPS13A, this scoop region extends down the length of the structure as a series of β-sheets (Li et al., 2020). Likewise, cryo-electron reconstruction of full-length

ATG2A revealed a continuous cavity down the length of the 1,900-aa protein of the same width as this scoop (Valverde et al., 2019), suggesting that in both ATG2A and VPS13A, a lipid-binding surface is extended along the entire length and essentially forms a lipid tunnel (Fig. 2 D).

ATG2 lipid transfer activity during phagophore expansion

Overexpression of GFP-ATG2A in non-starved cells dramatically labels lipid droplets (Pfisterer et al., 2014; Velikkakath et al., 2012), and knockout of ATG2A has been implicated in dysfunctional lipid droplet homeostasis (Velikkakath et al., 2012), suggesting that this protein family might have a role outside of autophagy at these organelles. Direct immunostaining of endogenous ATG2A, however, shows very little (Velikkakath et al., 2012) or no (Valverde et al., 2019) staining of lipid droplets. Instead, all of the easily observable puncta colocalize with early markers of the phagophore and both the numbers and colocalization increase following starvation, consistent with its role in membrane expansion. Knockout of ATG2A blocks autophagy flux and inhibits membrane expansion (Kishi-Itakura et al., 2014; Tamura et al., 2017; Valverde et al., 2019), although a few small and apparently closed autophagosomes have been reported (Tang et al., 2019). Thus, ATG2A is an essential component of the phagophore membrane expansion apparatus.

Precise determination of which organelles and which contact sites are associated with the lipid transport activity of ATG2 has been complicated. ATG2A appears to reside predominantly at organelle–organelle contact sites, as the phagophore-associated protein in mammals lies perfectly along the ER (Valverde et al., 2019). In yeast, Atg2 is needed to form the phagophore–ER contact site (Kotani et al., 2018), and this activity is coordinated with Atg9 (Gómez-Sánchez et al., 2018). Studies on elongated phagophores in yeast further reveal that Atg2 is restricted to the highly curved rim of the phagophore (Suzuki et al., 2013). Thus, fluorescence imaging of ATG2A and Atg2 suggests a simple lipid transport model in which the proteins move lipids from the ER to the expanding phagophore. However, ATG2 association with the phagophore is coincident with ATG9 vesicle recruitment (Papinski et al., 2014), and at least in yeast, both proteins are part of the larger complex defining the ER–phagophore contact site (Gómez-Sánchez et al., 2018). Thus, it is possible that this contact site actually involves at least three distinct membranes (ER, isolation membrane, and ATG9 vesicle; Fig. 2 D). Furthermore, autophagosome biogenesis is tightly associated with ER–mitochondria contact sites (Hamasaki et al., 2013), suggesting another level of potential complexity.

In fact, ATG2A could potentially engage each of these membranes. Specific recruitment of Atg2/ATG2B and its binding partner Atg18/WIPI4 to the phagophore requires Atg9 (Gómez-Sánchez et al., 2018) and TRAPP-II (Stanga et al., 2019). In addition, ATG2A has been shown to interact with the translocase of outer mitochondrial membrane 40 (TOM40)/TOM70 complex on mitochondria (Tang et al., 2019), WIPI4 at the omegasome (Zheng et al., 2017), and GABARAP likely decorating the phagophore (Bozic et al., 2020). The very large size of ATG2A suggests it could engage many of these contacts simultaneously (Fig. 2 D); alternatively, these different contacts might allow relocalization of ATG2 in a stress-dependent manner, similar to the way in which yeast VPS13 moves to different contact sites depending on the local needs for lipid mobilization (Bean et al., 2018).

In vitro, ATG2A is sufficient to tether separate liposomal membranes, provided they exhibit a very strong curvature (Chowdhury et al., 2018), and in yeast, Atg2 is essential to the tethering of phagophore membranes and the neighboring ER (Gómez-Sánchez et al., 2018; Kotani et al., 2018). Thus, ATG2A itself could be the key component of the contact site. However, in ATG2A/B double knockout cells, autophagy can be rescued by strong overexpression of relatively short N-terminal fragments of ATG2A harboring the chorein domain (Valverde et al., 2019). This suggests two other key elements of ATG2A function: (a) As in other sites of lipid transport, there is already a contact site machinery that maintains close organelle apposition independent of ATG2A. Moving forward, it will be essential to establish with which other contact-site protein complexes ATG2A associates, including, for example, proteins already thought to play a role in maintaining ER–phagophore interfaces in mammals (i.e., VMP1 or VAPA/B proteins; (Zhao et al., 2017, 2018). (b) The tunnel architecture is not itself essential (as the short N-terminal fragment probably cannot span between two membranes), but rather the tunnel dramatically increases the efficiency of lipid transport to allow endogenous levels of ATG2A to meet the demands of cell biology.

High lipid mobilization is likely coupled to increased local lipid synthesis

The ER is the site of most lipid synthesis in the cell, and several studies have suggested that autophagosome biogenesis may be localized to regions on the ER where lipid synthesis enzymes are concentrated. Mizushima and colleagues first described how PIS1 colocalizes with key early autophagy markers including ULK1 and FIP200 (Nishimura et al., 2017). PIS1 was later shown to more broadly colocalize with the ER contact site protein VMP1, found at interfaces with both autophagosomes and endosomes, and also colocalized with the choline/ethanolamine phosphotransferase enzyme (Tábara et al., 2018). VMP1 also interacts with the ER-localized transmembrane protein TMEM41B, which plays a role in lipid mobilization or homeostasis. Knockout of TMEM41B phenocopies VMP1 and ATG2 knockouts, blocking autophagosome biogenesis at an early stage consistent with a failure in expansion (Moretti et al., 2018; Morita et al., 2018; Shoemaker et al., 2019). Thus, the production and mobilization of bulk lipids is physically coupled to sites where autophagosomes and the ER make contact. Sustained phospholipid synthesis requires available pools of fatty acids, and intriguingly, recent work in yeast found the acyl-CoA synthetase Faa1 accumulating directly on growing phagophores (Schutter et al., 2020). Critically, the efficiency of autophagosome growth depended not only on the presence of Faa1 in the cell, but also on its precise localization to autophagosome biogenesis sites. Active phospholipid synthesis has also been observed as essential in mammalian autophagy, with at least some newly synthesized lipids becoming directly integrated into the growing phagophore (Andrejeva et al., 2019). How these locally produced lipids are specifically used in autophagosome membrane expansion is not yet known, but could involve direct coupling to the transfer machinery (like ATG2) or reflect a uniquely available lipid pool that is stably associated with contact sites (King et al., 2020). Likewise, how these lipids might be moved in one direction across ATG2 (to support expansion) is not yet understood. Finally, if ER-derived lipids are moved via lipid transport proteins or hemifusion-like extrusion structures, they would be expected to populate only the outer leaflet of the growing phagophore. How these lipids might reach the inner leaflet for the purpose of membrane expansion remains to be determined. Alternatively, one or both of these lipid-exchange mechanisms could be used to control lipid homeostasis, perhaps allowing for the rapid redistribution of a key lipid on the cytosol-facing leaflet.

Phagophore membrane curvature

Theoretical modeling of autophagosome biogenesis suggests that the phagophore forms de novo and grows as a flattened double-membrane sheet that, upon relaxation of membrane curvature energy, bends into a spherical autophagosome (Agudo-Canalejo and Knorr, 2019). According to such studies, mechanisms must exist to prevent premature bending of the phagophore rather than a machinery to drive their bending. Several factors can affect membrane curvature, including the lipid composition of the two leaflets of the bilayer membrane or the binding of proteins to lipids in one bilayer. Conical lipids such as PE and phosphatidic acid induce membrane curvature, while other lipids, such as PI, phosphatidylcholine, and phosphatidylserine, promote bilayer formation (Carlsson and Simonsen, 2015). PtdIns(3)P is an example of a cone-shaped lipid that, when clustered, can create a cytosol-facing bud in the membrane that serves as a platform for recruitment of the autophagic machinery via WIPI2 (Dooley et al., 2014). The membrane curvature of autophagic membranes can also be affected by binding of various proteins, including proteins containing specific lipid-binding domains; a membrane inserted helix; or being covalently conjugated to a lipid. There are several examples of lipid binding and curvature sensing proteins involved in autophagy. Prime examples include the BAR domain–containing proteins SNX18 and BIF-1/Endophilin-1, as well as the fission yeast proteins Atg20 and Atg24 (Knævelsrud et al., 2013; Takahashi et al., 2011; Zhao et al., 2016); the ATG8 conjugation machinery proteins ATG3 and ATG16L1, containing membrane inserted helixes essential for ATG8 lipidation (Lystad et al., 2019; Nath et al., 2014); and LC3 itself, being covalently conjugated to PE (Knorr et al., 2014). Moreover, several components of the ULK and PIK3C3 complexes contain specific regions that likely facilitate membrane recruitment in a geometry-dependent manner, including an EAT domain in Atg1/ULK1 (Chan et al., 2009) and a BATS domain in ATG14 (Fan et al., 2011). ATG12-ATG5-ATG16L1–mediated conjugation of LC3 or GABARAP to PE is also highly curvature sensitive, being more efficient on liposomes with high curvature (25–65 nm) than those with relatively low curvature (∼400 nm; Lystad et al., 2019). This is likely due to an amphiphatic helix in the N-terminus of the E2-enzyme ATG3 that facilitates lipidation preferentially on membranes with local lipid packing defects (Nath et al., 2014), but an amphiphatic helix in ATG16L1 is also required for its membrane binding and function in ATG8 protein lipidation (Lystad et al., 2019). Thus, lipidation of ATG8 family proteins likely occurs at the highly curved ends of the phagophore, which may explain their function in membrane elongation, but may also facilitate their interaction with cargo-bound receptors upon de novo autophagosome biogenesis during selective autophagy, as is discussed below. It is possible that the highly bent rim of the phagophore functions as a diffusion barrier for lipids and conjugated ATG8 proteins, thereby facilitating asymmetric lipid compositions in both membranes, but this concept is yet to be tested.

The various roles of ATG8 family proteins

Recent studies have found that while autophagosomes can form in cells depleted of components of the ATG conjugation machinery (Engedal and Seglen, 2016; Nguyen et al., 2016; Tsuboyama et al., 2016), they are formed at a reduced rate, are unable to fuse properly with lysosomes, and, critical to expansion, they are smaller. In yeast expressing little or no Atg8 (Abeliovich et al., 2000; Kirisako et al., 2000; Xie et al., 2008), in mammals depleted of a single LC3/GABARAP subfamily (Weidberg et al., 2010), or in a complete knockout of both mammalian LC3 and GABARAP families (Nguyen et al., 2016), autophagosome size is reduced. Because Atg8/ATG8 proteins can tether or fuse small liposomes in vitro (Nair et al., 2011; Nakatogawa et al., 2007; Weidberg et al., 2011), one possible model is that ATG8 proteins are part of the machinery needed to fuse vesicles to drive membrane expansion. Notably, in vitro, these proteins tether membranes only in a topologically restricted trans conformation (Motta et al., 2018), and they drive lipid-mixing only if the membranes exhibit strongly destabilized lipid packing (Nair et al., 2011). Thus if they drive fusion in vivo, lipidated Atg8 would need to be present on both the incoming vesicle and expanding phagophore at sites where one or both of these membranes were "prone" to fuse, perhaps because of high local curvature or high surface densities of fusogenic lipids.

Interestingly, in mammalian cells depleted for ATG8 proteins, apparently "open" autophagosomes accumulate (Fujita et al., 2008; Weidberg et al., 2010), and depletion of the conjugation machinery leads to a significant delay in degradation of the inner autophagosomal membrane and cargo (Tsuboyama et al., 2016), likely because of incomplete closure of the autophagosomal edge. Thus, these proteins may also be needed for efficient closure. How they contribute to closure is uncertain, but it is tempting to speculate that ATG8 proteins could be involved in the recruitment of LC3-interacting region (LIR)-containing proteins required for phagophore closure (see below and Fig. 3 A).

While ATG8 proteins are needed throughout autophagosome growth, they must be recycled by the ATG4 family of proteases to support efficient fusion into the lysosome (Sánchez-Wandelmer and Reggiori, 2017). For some ATG8 homologues, ATG4 proteolysis proceeds much more slowly on membranes than in solution (Hill et al., 2019; Kauffman et al., 2018) and could function as a kind of timer-based mechanism to limit rapid turnover of lipid-attached proteins. In addition, both the ATG4 protease and the ATG8 substrate are subjected to posttranslational modifications that allow strict temporal control over ATG8-family protein removal. In yeast and mammals, the Atg1/ULK1 kinase can inhibit total Atg4/ATG4B activity (both priming and delipidation; Pengo et al., 2017; Sánchez-Wandelmer et al., 2017), by phosphorylating serines within the catalytic site. Thus, the local accumulation of Atg1/ULK1 at expanding phagophores will naturally suppress active ATG4 proteins. In mammals, recycling of ATG8 proteins depends on their interaction with a C-terminal LIR on ATG4B (Kauffman et al., 2018; Skytte Rasmussen et al., 2017), and phosphorylation of serine residues near this motif has been implicated in slowing protein removal and promoting autophagic flux (Huang et al., 2017). In addition to phosphorylation, these proteases are controlled by oxidation (Pérez-Pérez et al., 2014; Scherz-Shouval et al., 2007), ubiquitination (Kuang et al., 2012), *O*-GlcNacylation (Jo et al., 2016), and *S*-nitrosylation (Kuk et al., 2009; Li et al., 2017), potentially allowing for varying controls depending on the cellular stress condition. Direct modification of the ATG8 proteins is also possible: it was recently discovered that TBK1-mediated phosphorylation of LC3C and GABARAPL2 regulates their delipidation by specifically disrupting the ability of ATG4 proteins to recognize these substrates (Herhaus et al., 2020). Finally, both Atg1 and ULK1 can bind ATG8 family proteins (Alemu et al., 2012; Grunwald et al., 2020; Kraft et al., 2012), implying a continuing role for these kinases on maturing autophagosomes.

Autophagosome closure

Recent technical advances and the identification of STX17 as a marker for closed autophagosomes (Itakura et al., 2012) have made it possible to study the mechanisms involved in phagophore closure, a process involving fission of the inner and outer membrane of the phagophore edge (Knorr et al., 2015; Fig. 3). Several recent studies have implied a role for the endosomal sorting complexes required for transport (ESCRT) machinery in closure of the phagophore to form an autophagosome. This process shares topology with canonical ESCRT-dependent processes, including multivesicular body formation, virus budding from the plasma membrane, and cytokinesis (Vietri et al., 2020). A possible role for the ESCRTs in phagophore closure was previously suggested, as autophagosomes were found to accumulate in ESCRT-depleted cells (Filimonenko et al., 2007; Lee et al., 2007; Rusten et al., 2007), but owing to technical limitations, it has been difficult to distinguish fully closed autophagosomes from those containing small holes in ESCRT-depleted cells. Recently, several elegant imaging studies using advanced fluorescent probes, such as HaloTag-LC3 in combination with membrane-impermeable and -permeable HaloTag ligands (Takahashi et al., 2018) or LC3 tagged with a pH-sensitive red-fluorescent protein (pHuji; Zhen et al., 2020), have established a direct role for the ESCRT machinery in autophagosome closure, during both starvation-induced autophagy and mitophagy (Takahashi et al., 2018, 2019; Zhen et al., 2020; Zhou et al., 2019). Targeting of ESCRT-I components (VPS37A and VPS28) to the phagophore seems to facilitate transient recruitment of ESCRT-III components, including chromatin-modifying protein/charged multivesicular body protein 2A (CHMP2A) and the filament-forming subunit CHMP4B, bringing the two membranes of the phagophore leading edge in close proximity to allow membrane abscission, followed by VPS4 (an AAA-ATPase)-mediated depolymerization of ESCRT-III (Fig. 3). It is not completely understood how the ESCRT machinery is targeted to the unsealed phagophore, but in budding yeast, it was found that the ESCRT-III subunit Snf7 (CHMP4) interacts with Atg17 (FIP200) in a Vps21 (RAB5)-dependent manner (Zhou et al., 2019), suggesting that the ULK complex may regulate ESCRT recruitment and phagophore closure (Fig. 3 A). Interestingly, an N-terminal putative ubiquitin E2 variant domain in VPS37A seems required for autophagosome closur, but is dispensable for multivesicular body formation (Takahashi et al., 2019). As the ESCRT-I subunit tumor susceptibility gene 101 (TSG101) also contains a ubiquitin E2 variant domain and is implicated in autophagy (Filimonenko et al., 2007), it is tempting to speculate that ESCRT-I might be recruited to sites of autophagosome closure through binding to ubiquitinated cargo proteins. It is important to point out that ESCRTs can promote only the very last scission step of a highly constricted membrane neck (or opening), implying that other yet unknown mechanisms must exist to constrict the phagophore rim to this stage.

Autophagosome biogenesis during selective autophagy

The term selective autophagy refers to turnover of specific cargo, including surplus or dysfunctional organelles and cellular proteins or invading pathogens (Levine and Kroemer, 2019). Cargo degradation by selective autophagy relies on autophagy receptors, which are LIR-containing proteins that facilitate interaction between a cargo (often ubiquitinated) and LC3/GABARAP in the autophagosomal membrane and themselves become degraded together with the cargo (Galluzzi et al., 2017). Autophagy receptors can be cytosolic proteins (such as p62, NBR1, or NDP52) or membrane-bound cargo-specific proteins (such as the mitochondria proteins BNIP3 and BNIP3L [BCL-interacting protein 3 and its ligand] and FUN14 domain-containing 1; Montava-Garriga and Ganley, 2020). The identification of cargo receptors initially offered a simple linear model of selective autophagy, in which cargo is recognized by specific autophagy receptors that further recruit LC3-containing phagophores to facilitate cargo sequestration. This model has been challenged, however, by recent studies showing that autophagy receptors (p62 and NDP52) interact with the ULK complex subunit FIP200 to initiate de novo autophagosome formation around the cargo to be degraded, including protein aggregates (p62), mitochondria, and bacteria (NDP52; Ravenhill et al., 2019; Turco et al., 2019; Vargas et al., 2019; Fig. 4 A). The interaction of NDP52 with FIP200 appears to be regulated by TBK1-mediated phosphorylation of NDP52 (Ravenhill et al., 2019; Vargas et al., 2019). In line with this model, a study using the lactone ivermectin to induce mitophagy found that ubiquitination of mitochondria was followed by activation of TBK1, leading to recruitment of FIP200 and the autophagy receptor optineurin, and later ATG13 and the other core autophagy components, including VPS34 and WIPI2, resulting in ATG8 lipidation (Zachari et al., 2019; Fig. 4 B). TBK1-mediated phosphorylation of STX17 was demonstrated to induce its translocation from the Golgi to phagophore nucleation sites

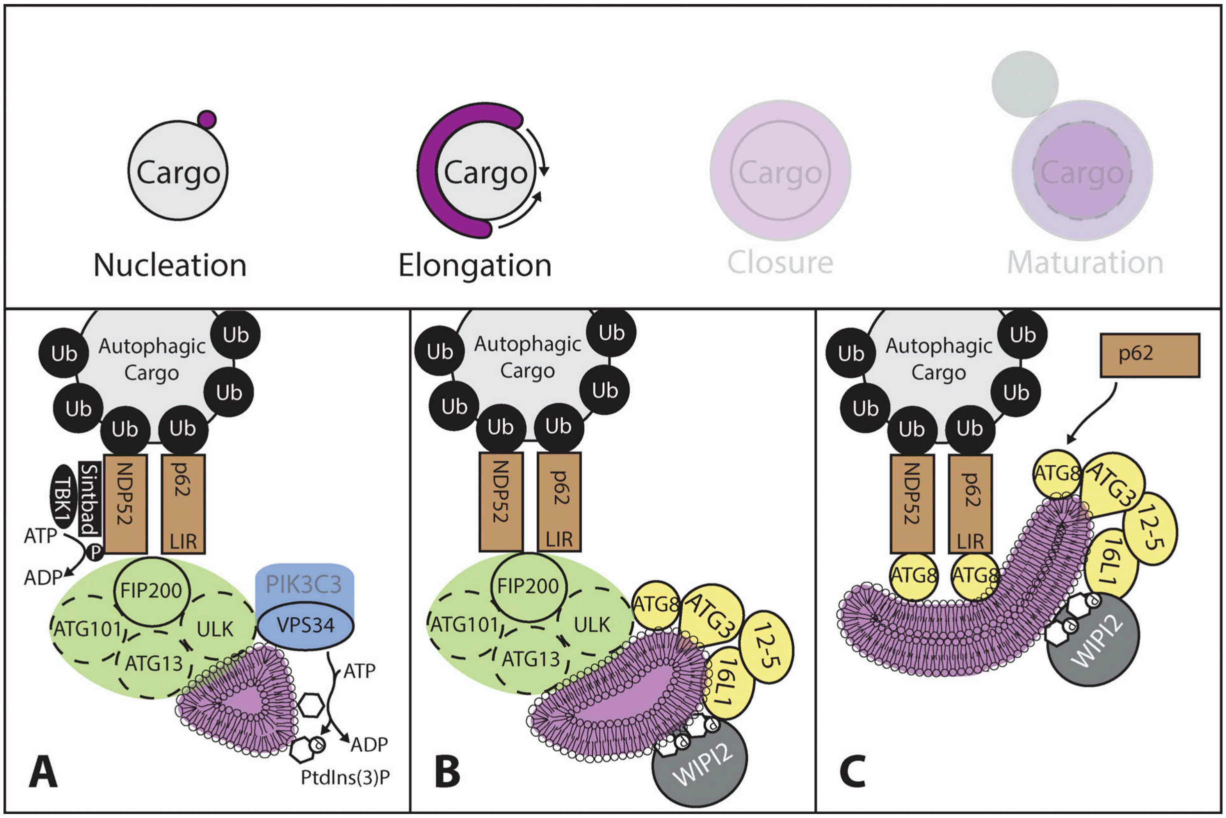


Figure 4. **Hypothetical model for de novo autophagosome formation during selective autophagy. (A)** Autophagy receptors (p62 and NDP52) bound to selective cargo interact directly with FIP200, leading to recruitment of the ULK complex as well as VPS34. TBK1-mediated phosphorylation of NDP52 stimulates the NDP52–FIP200 interaction. The p62–FIP200 interaction requires the LIR domain in p62. **(B)** PtdIns(3)P production by VPS34 causes recruitment of WIPI2 and the ATG8 conjugation machinery, leading to ATG8 lipidation. **(C)** The p62-FIP200 binding can be outcompeted by binding of p62 to ATG8, which facilitates further recruitment of autophagy receptors and expansion of the autophagic membrane tightly around the selective substrate.

and interaction with ATG13-FIP200 upon starvation-induced autophagy (Kumar et al., 2019). Whether STX17 has a similar role in de novo autophagosome formation during selective autophagy is not known, but it is clear that TBK1 is a central regulator of selective autophagy.

It is interesting to note that the p62–FIP200 interaction is outcompeted by LC3B (Turco et al., 2019), indicating a sequential order of p62 binding partners (Fig. 4 C). In line with this, LC3/GABARAP proteins can be recruited to damaged mitochondria independent of their binding to autophagy receptors, where they stimulate further ubiquitin-independent, LIR-dependent recruitment of autophagy receptors (OPTN and NDP52), suggesting that a LC3/GABARAP-dependent positive-feedforward loop enables phagophore expansion and mitophagy (Padman et al., 2019). Elegant live-imaging microscopy has demonstrated that phagophore initiation seems to occur at multiple mitochondria sites closely connected to the ER (Zachari et al., 2019). Thus, it is tempting to propose a zippering model for autophagosome biogenesis during selective autophagy, in which ubiquitination of cargo-specific proteins acts as an eat-me signal to initiate TBK1 activation and binding of autophagy receptors, leading to further recruitment of FIP200 and core autophagy components to facilitate lipidation of ATG8 proteins, which again can recruit more autophagy receptors. The high avidity of cargo receptors to membrane-localized ATG8 family proteins facilitates zippering of the cargo in a manner that likely excludes any non-targeted material from sequestration within autophagosomes.

Future perspectives

In this review, we discuss the various models that currently exist to explain autophagosome biogenesis during nonselective and selective types of autophagy. Whether different models for membrane delivery exist depending on the cargo sequestered or the autophagy-inducing signal still remains unknown. Future studies will be needed to address this. Likewise, to understand the molecular mechanisms of autophagy it is also essential to better describe the specific lipids that constitute the phagophore and autophagosome membranes, as these molecules will no doubt contribute to the multiple membrane modeling events involved in autophagosome biogenesis. Finally, although we here focused on the role of membranes in determining the local biochemistry driving autophagosome biogenesis, it is increasingly obvious that liquid condensates also play a significant role as an organizing principle during the early events of autophagy (Sun et al., 2020; Wang and Zhang, 2019). How membranes physically engage and assemble around these structures will be a key area moving forward.

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