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HYPOTHESIS

ERES: sites for autophagosome biogenesis and maturation?

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ABSTRACT

Autophagosomes are the hallmark of autophagy, but despite their central role in this degradative pathway that involves vesicle transport to lysosomes or vacuoles, the mechanism underlying their biogenesis still remains largely unknown. Our current concepts about autophagosome biogenesis are based on models suggesting that a small autonomous cisterna grows into an autophagosome through expansion at its extremities. Recent findings have revealed that endoplasmic reticulum (ER) exit sites (ERES), specialized ER regions where proteins are sorted into the secretory system, are key players in the formation of autophagosomes. Owing to the morphological connection of nascent autophagosomes with the ER, this has raised several questions that challenge our current perception of autophagosome biogenesis, such as are ERES the compartments where autophagosome formation takes place? What is the functional relevance of this connection? Are these compartments providing essential molecules for the generation of autophagosomes and/or are they structural platforms where these vesicles emerge? In this Hypothesis, we discuss recent data that have implicated the ERES in autophagosome biogenesis and we propose two models to describe the possible role of this compartment at different steps in the process of autophagosome biogenesis.

This article is part of a Focus on Autophagosome biogenesis. For further reading, please see related articles: 'Membrane dynamics in autophagosome biogenesis' by Sven R. Carlsson and Anne Simonsen (*J. Cell Sci.* **128**, 193-205) and 'WIPI proteins: essential PtdIns3*P* effectors at the nascent autophagosome' by Tassula Proikas-Cezanne et al. (*J. Cell Sci.* **128**, 207-217).

KEY WORDS: Phagophore assembly site, Pre-autophagosomal membrane, Phagophore, Isolation membrane, Autophagosome, Autophagy, Endoplasmic reticulum exit site

Introduction

Autophagy is a highly conserved catabolic process that is activated in eukaryotic cells mostly in response to life-threatening situations, including oxidative stress, nutrient scarcity, cellular damage and pathogen invasion. In multicellular eukaryotes, this pathway is also crucial for key physiological functions, such as development, cell differentiation and immunity (Mizushima, 2005). Autophagy is characterized by the selective or unselective sequestration of cytoplasmic components by autophagosomes (Fig. 1). These large vesicles subsequently fuse with lytic compartments and expose their content to resident hydrolases, which then degrade the cargoes into their basic constituents (Fig. 1). The resulting metabolites are transported back in the cytoplasm to be used as either building blocks for the synthesis of new macromolecules or as a source of energy.

Although several aspects of the molecular mechanism underlying the biogenesis of autophagosomes have been unveiled, the source(s) of the membrane of these carriers is still a topic of intense debate. Several genetic and microscopy studies mostly performed in yeast have identified the phagophore assembly site or pre-autophagosomal structure (PAS) – a locus where autophagy-related (ATG) proteins assemble (Suzuki et al., 2007) – as the earliest membranous precursor (Mari et al., 2010; Yamamoto et al., 2012). At the PAS, the coordinated action of ATG proteins mediates the formation and expansion of a cisterna termed the phagophore or isolation membrane, the extremities of which will eventually close, thereby leading to the generation of an autophagosome (Fig. 1). Autophagosomes are not able to fuse with lysosomes until the ATG machinery at their surface has disassembled (Cebollero et al., 2012) (Fig. 1).

The current model, at least for mammalian cells under conditions of nutrient deprivation, is that autophagosomes emerge at sites in close proximity to the ER where the ATG machinery associates. These sites have been named omegasomes (Axe et al., 2008) and could represent the PAS (Fig. 1). Several cellular compartments have been proposed to be the membrane donor for the initial membranes required for the formation of the PAS and/or the phagophore, including mitochondria, recycling endosomes and plasma membrane (Hailey et al., 2010; Hamasaki et al., 2013; Moreau et al., 2011; Puri et al., 2013; Ravikumar et al., 2010). Recent evidence supports the notion that the membranes and proteins (such as ATG16L1 and ATG9A) that are essential for the formation of autophagosomes are derived from the plasma membrane and that they coalesce in recycling endosomes where they acquire other ATG proteins including ULK1 (the mammalian homolog of yeast Atg1) (Moreau et al., 2011; Puri et al., 2013). Recycling endosomes (or subdomains of them) could thus represent an early PAS or phagophore intermediate that, through its positioning in the proximity of the ER, triggers the subsequent steps of autophagosome biogenesis (Karanasios et al., 2013; Knævelsrud et al., 2013). These steps probably include the establishment of a direct membranous connection between the PAS or phagophore and the ER (see below). As a result, the ER is probably the major donor of lipid bilayers for the forming autophagosome and the omegasome could represent the means to supply lipids from the ER to the growing phagophore. According to this model, mitochondria participate in autophagosome biogenesis primarily at the level of the omegasomes, by also providing newly synthesized lipids (Böckler and Westermann, 2014; Hailey et al., 2010; Hamasaki et al., 2013; Karanasios et al., 2013). The role of the ER in

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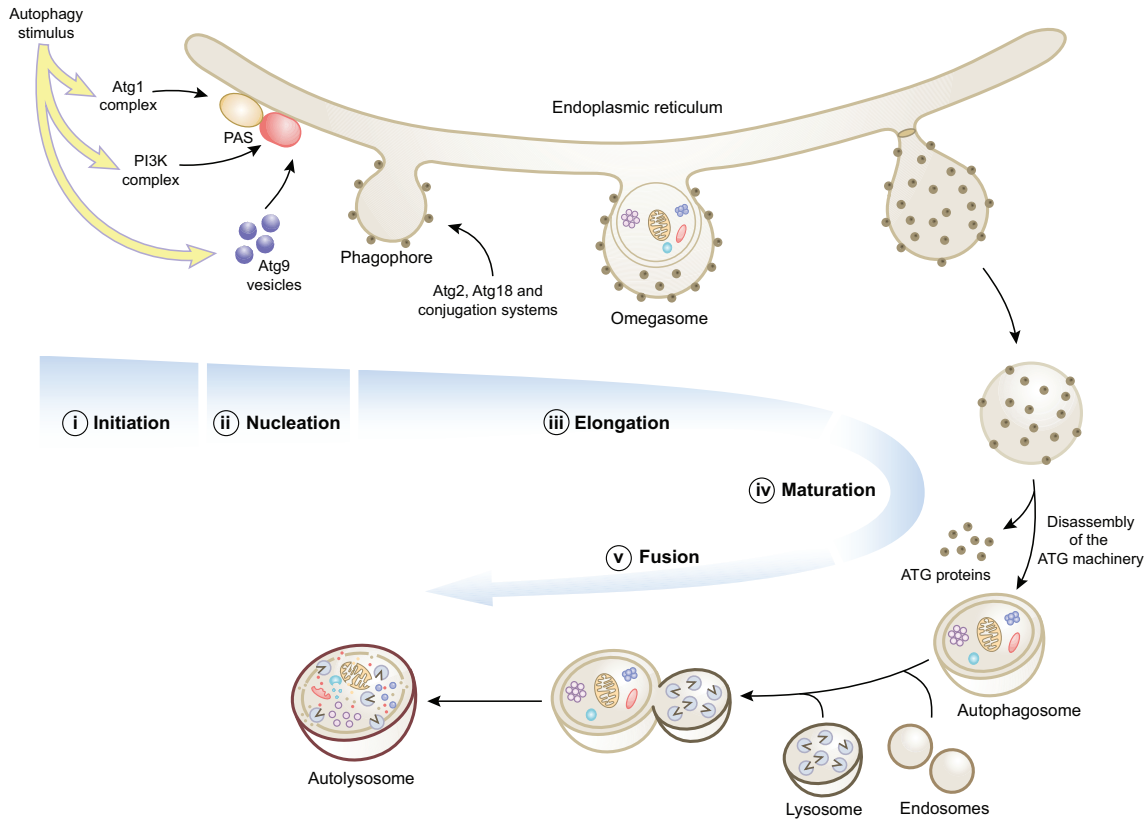

Fig. 1. Mechanistic model for the biogenesis of an autophagosome. When autophagy is induced, the Atg1/ULK1 complex is directed to the ER together with the PI3K complex and Atg9 vesicles during the initiation step (i). The assembly of the ATG machinery leads to the formation of the PAS, whereby the initial phagophore is likely formed through the nucleation (ii) of membranes from different origins. The subsequent recruitment of the ubiquitin-like conjugation systems and possibly the direct transfer of lipids from the ER mediate the elongation of the phagophore (iii) into an autophagosome on specialized ER subdomains that are called omegasomes. The material that is targeted for lysosomal degradation is sequestered during the autophagosome formation. Once complete, the autophagosome detaches from the ER and the autophagy machinery at its surface is released during a maturation step (iv), which involves PtdIns3*P* turnover, Atg4 delipidation activity and termination of Atg1/ULK1 activity. Mature autophagosomes then become autolysosomes through fusion first with endosomes and then with lysosomes (v). Finally, lysosomal hydrolases degrade the cargo material of the lysosomes into metabolites. These are then transported into the cytoplasm, where they are used either as a source of energy or as building blocks for the synthesis of new macromolecules.

autophagosome biogenesis, however, does not appear to be confined to establishing omegasomes, as an increasing number of recent publications have shown that another subdomain of this organelle, the ER exit site (ERES), plays a key role in autophagy.

In this Hypothesis, we will first discuss the most relevant findings supporting this notion and integrate the available data into two different models that illustrate how autophagosomes can be formed at the ERES. In the second part, we will propose the hypothesis that autophagosome maturation occurs at these ERES and explain potential mechanisms involved in governing these late steps of autophagosome formation.

ERES contribution to autophagy

ERES are specialized ER sites at which COPII-coated vesicles (Box 1) emerge to transport a large majority of newly synthesized ER proteins to the Golgi, from where they are distributed to various subcellular compartments. Although ERES have been considered to be exclusively involved in the secretory pathway, recent evidence highlights a functional crosstalk between these ER sites and autophagy. The first indications that the COPII machinery could be connected to the biogenesis of autophagosomes were already published more than a decade ago. Yeast *Saccharomyces cerevisiae* mutant strains with a defect in the early secretory pathway, including defective COPII vesicle formation, showed an impairment in autophagy under conditions of nitrogen starvation because they were unable to generate autophagosomes (Ishihara et al., 2001; Reggiori et al., 2004). These experiments, however, did not demonstrate a direct functional connection between COPII vesicles or the ERES and autophagy, for example, through the colocalization of marker proteins of both compartments; thus, at the time, an indirect involvement could not be excluded (Reggiori et al., 2004).

More recently, it has been shown that subcellular membranes that are enriched in components of the ER–Golgi intermediate compartment are necessary and sufficient to catalyze the *in vitro* lipidation of LC3, which is essential for autophagosome formation (Shpilka et al., 2011), and that these membranes lose this capacity when ER-to-Golgi transport inhibitors are added to the *in vitro* LC3 lipidation reaction (Ge et al., 2013). The use of the same inhibitors, as well as the use of a dominant-negative mutant of Sar1, a GTPase that is essential for COPII vesicle formation (Box 1), confirmed that the ERES also have a crucial role in autophagosome biogenesis *in vivo* (Ge et al., 2013; Zoppino et al., 2010). In particular, a decrease in the number of ATG14 and ATG16L1 puncta, which represent early autophagosomal precursors (Itakura and Mizushima, 2010), indicates that ERES are important during

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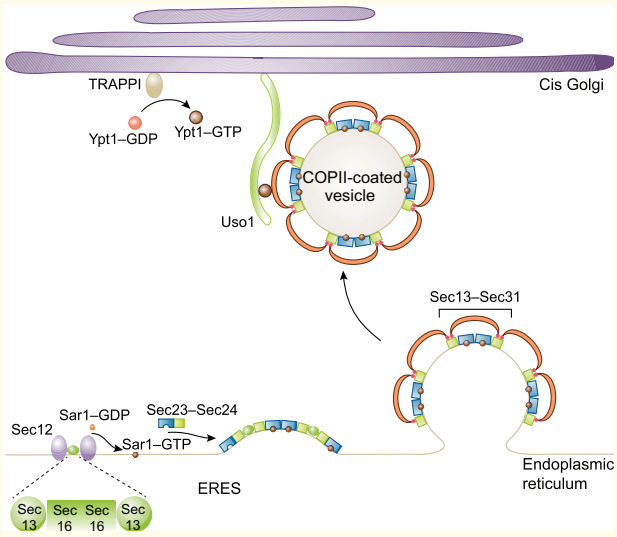
Box 1. COPII-coated vesicle structure and formation

The COPII coat consists of two proteinaceous layers that are formed by the coordinated action of a number of proteins (Jensen and Schekman, 2011). The Sec13–Sec16 tetramer is stably associated with the ER membrane, where it binds to the integral membrane protein Sec12 (Whittle and Schwartz, 2010) (see figure). The budding of a COPII vesicle begins with a switch of the small GTPase Sar1 from a GDP to a GTP-bound state that is mediated by its GEF Sec12 (Weissman et al., 2001) (see figure). When activated, Sar1 induces the generation of membrane curvature as well as the recruitment of Sec23 and Sec24, the components of the inner COPII coat (Bi et al., 2002). Together, the Sec13–Sec16 tetramer and the Sec23–Sec24–Sar1 complex trigger the association of the outer COPII coat components Sec13 and Sec31 (Whittle and Schwartz, 2010). The forming coat is progressively enriched with these two proteins and depleted of Sec13 and Sec16, thereby gaining membrane curvature and structural rigidity (Whittle and Schwartz, 2010). Finally, fission of the vesicle is driven by Sar1, and the newly formed COPII vesicle is delivered to the ERGIC (see figure). The uncoating of the COPII vesicles is thought to take place after they tether to the target compartment, i.e. the cis-Golgi (Sato and Nakano, 2005), an event that proceeds the fusion step and that is mediated by the Uso1 tether, the TRAPPI complex, the GTPase Ypt1 and SNARE proteins (Cao and Barlowe, 2000; Sacher et al., 2001) (see figure).



the early stages of autophagy, possibly during the generation of the PAS and/or phagophore (Ge et al., 2013). Interestingly, the number of omegasomes that can be detected using the specific marker protein DFCP1 (also known as ZFYVE1) (Axe et al., 2008) is also strongly diminished when COPII vesicle formation is inhibited (Ge et al., 2013). This functional connection and their association with the ER raises the possibility that omegasomes and ERES cooperate in autophagosome biogenesis, even at the structural level.

Two recent mostly microscopy-based studies in *S. cerevisiae* have revealed that the PAS and phagophores localize in close proximity to both the ER and the vacuole (Graef et al., 2013; Suzuki et al., 2013). It is worth noting that the growing phagophores are associated with the ER in a precise orientation, i.e. the opened extremities face the proximal ER region (Graef et al., 2013; Suzuki et al., 2013). In addition, several COPII marker proteins such as Sec23 localize frequently to these potential sites of autophagosome–ER contact or proximity and also strongly colocalize with Atg8-positive autophagosomal membranes (Graef et al., 2013). Large-scale purification of ATG factors and their interacting proteins after autophagy induction further supports the intimate connection between autophagy and vesicular trafficking (Graef et al., 2013). Particularly significant is the density of interactions found between the ATG machinery and the COPII vesicle transport components. For example, Sec23 appears to interact with ATG proteins from each of the different functional clusters that orchestrate autophagosome formation (Graef et al., 2013). Taken together, these findings strongly support the notion of molecular links between ERES and autophagosome biogenesis.

How could ERES contribute to autophagosome formation?

Model I – membrane delivery by vesicular traffic

One obvious possibility is that ERES provide membranes and eventually other essential components for the formation of autophagosomes through COPII vesicles. In addition to having a role in the tethering of COPII vesicles to the Golgi and in intra-Golgi trafficking, the RAB GTPase Ypt1, the yeast counterpart of mammalian RAB1A, is also involved in autophagy (Huang et al., 2011; Lipatova et al., 2012; Lynch-Day et al., 2010; Wang et al., 2013). The role of Ypt1 in different pathways is finely regulated by three different guanine nucleotide exchange factors (GEFs) that form multifactorial complexes. These complexes are known as the transport protein particle I (TRAPPI), TRAPPII and TRAPPIII, and they act to dictate the site of action of Ypt1. TRAPPI and TRAPPII participate in ER-to-Golgi and intra-Golgi trafficking, respectively (Cai et al., 2007; Cai et al., 2005; Yamasaki et al., 2009), whereas TRAPPIII is the autophagy-specific GEF for Ypt1. During autophagy, TRAPPIII localizes to the PAS in yeast, to which it appears to be recruited by Atg17 (Lynch-Day et al., 2010; Tan et al., 2013; Wang et al., 2013). Once at this site, TRAPPIII mediates the recruitment of YPT1 (Kakuta et al., 2012; Lynch-Day et al., 2010; Wang et al., 2013), which, in its activated GTP-bound form, is thought to recruit Atg1 (Wang et al., 2013). Of further significance, the TRAPPIII–Ypt1 module has also been detected in immunopurified Atg9-containing vesicles in yeast (Kakuta et al., 2012), which have been shown to contribute to the generation of the PAS in this organism (Mari et al., 2010; Yamamoto et al., 2012) and could be part of the early PAS or phagophore intermediate observed at the recycling endosomes in mammalian cells (Nair et al., 2011). Atg9 was also found to interact with the COPII machinery (Graef et al., 2013) and to colocalize with Ypt1 during autophagy (Lynch-Day et al., 2010). Although this could reflect a requirement for the sorting of Atg9 to the Golgi before its delivery to its final compartments (Mari et al., 2010), it is also possible that vesicular transport of this protein out of the ERES to the PAS is a crucial early event in autophagy.

Interestingly, recombinant TRAPPIII retains the capacity to interact with Sec23 through its Trs33 subunit, implying that it might bind to COPII vesicles (Tan et al., 2013). Consistent with this, TRAPPC8, the mammalian counterpart of Trs85, a TRAPPIII-specific subunit, also interacts with the COPII coat component SEC31A (Bassik et al., 2013). However, more experimental evidence is needed to indisputably support the notion that the COPII complex has a direct role in providing

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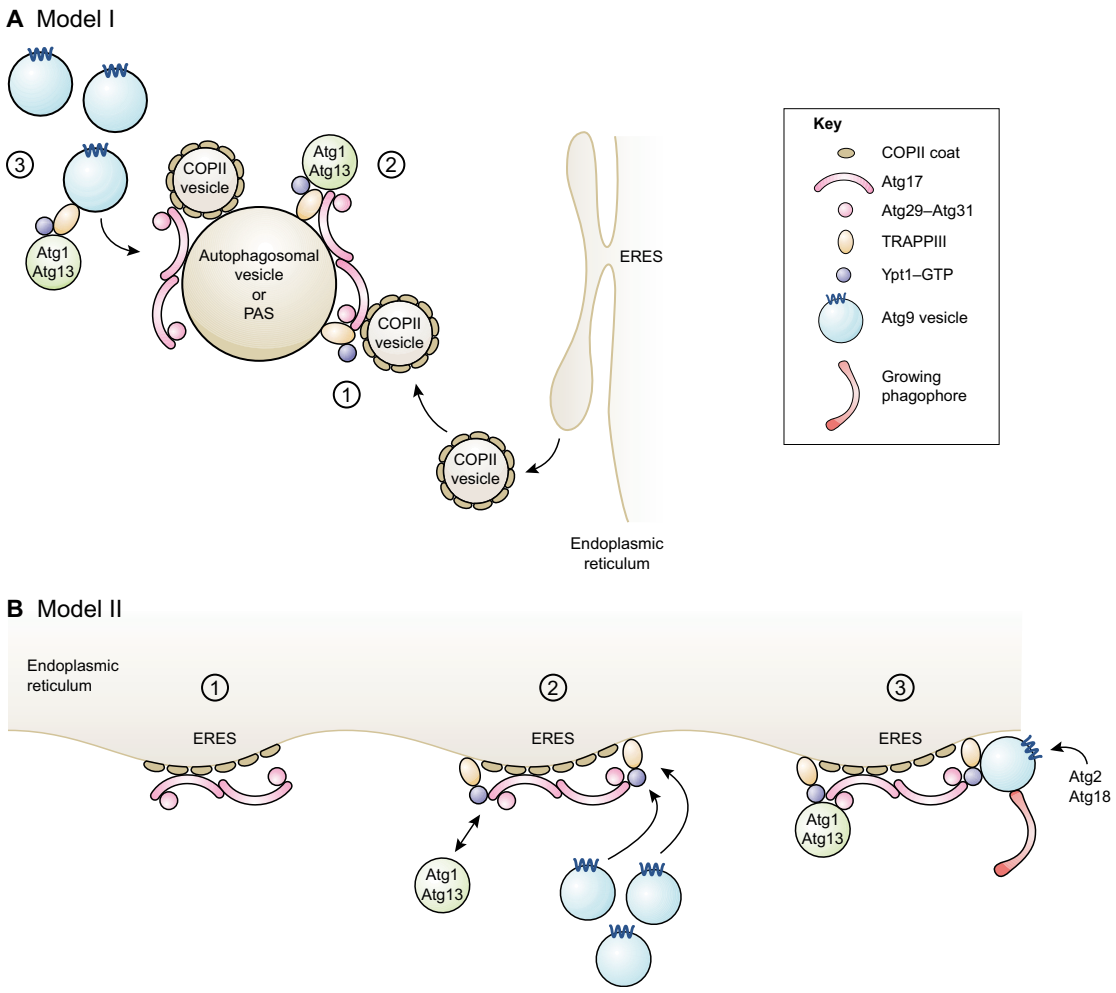

Fig. 2. Models for the involvement of ERES in autophagosome biogenesis. (A) Model I: Membrane delivery by vesicular traffic. Upon the induction of autophagy, COPII vesicles emerging from the ERES are recruited to the forming PAS and contribute to the generation of this structure. Here, targeting of COPII vesicles is mediated by the TRAPPIII–Ypt1 module, which is recruited to the PAS through its interaction with the Atg17–Atg29–Atg31 complex (1). Ypt1 and Atg17–Atg29–Atg31 are also likely to be involved in recruiting the Atg1–Atg13 complex to the PAS (2). In this model, Atg1 and Atg13, as well as the COPII machinery are both crucial for the recruitment of Atg9-positive vesicles to the PAS (3). (B) Model II: ERES as a scaffold for autophagosome formation. In the first step of autophagosome biogenesis, Atg17 is recruited to ERES through interactions with subunits of the COPII machinery (1). Subsequently, the TRAPPIII–Ypt1 module and the Atg1–Atg13 complex will associate at these sites and contribute to the establishment of the initial scaffold that is required for the assembly of a membranous PAS (2). Atg9-positive vesicles will then be directed to the nascent PAS by Ypt1 and Atg17 and contribute to the formation of an initial phagophore (2). Finally, the recruitment of Atg2 and Atg18 to the PAS by Atg9 establishes the connections between the phagophore and the ER for the transfer of lipids that are necessary for its expansion to an autophagosome.

membrane for autophagosome biogenesis through vesicular traffic.

All of these observations can be integrated into a working model (Fig. 2A) for some of the earliest events during PAS or phagophore formation. This model is inspired by the structural characterization of the Atg17–Atg31–Atg29 complex, which has revealed that this complex forms a dimer that assumes a crescent-shape structure; this has led to the proposition that it could tether vesicles and/or membranes during the initial nucleation of the phagophore (Ragusa et al., 2012). This model also takes into account the large-scale analysis of ATG interactors, which suggests that the Atg17–Atg31–Atg29 complex could interact with the COPII machinery during autophagy (Graef et al., 2013), although no functional connection has been confirmed so far. Nonetheless, one possible scenario is that the Atg17–Atg31–Atg29 complex would target COPII-coated vesicles to tethered autophagosomal vesicles making those contributing to the formation of an early intermediate of autophagosome biogenesis. Mechanistically, Atg17 would recruit the TRAPPIII complex through its interaction with Trs85 (Wang et al., 2013), which, in turn, would lead to the tethering of COPII-coated vesicles through both Ypt1 activation and direct binding between TRAPPIII and COPII subunits (Bassik et al., 2013; Tan et al., 2013; Wang et al., 2013) (Fig. 2). Ypt1 is also important in recruiting Atg1 (Wang et al., 2013) and its interacting partner Atg13. Together, these interactions could mediate the assembly of the entire Atg1 complex (which, in yeast, is composed of Atg1, Atg13, Atg17, Atg29 and Atg31; Cheong et al., 2008; Kawamata et al., 2008), which is necessary to initiate the nucleation of the phagophore, possibly through the fusion of vesicles of different origins.

Atg17 also interacts with the transmembrane protein Atg9, and this factor could thus mediate the recruitment of Atg17 and TRAPPIII to early autophagic membranes (Sekito et al., 2009).

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However, such a scenario is probably too simple because Atg9-positive membranes that have not yet reached the PAS appear to be negative for Atg17, and it is known that – as is the case for Atg1 and TRAPPIII – Atg9 localization to the PAS depends on Atg17 (Sekito et al., 2009; Suzuki et al., 2007; Yamamoto et al., 2012). Nonetheless, Atg9-positive vesicles are able to recruit TRAPPIII, even in the absence of Atg17 (Kakuta et al., 2012). So potentially, Atg1 could be recruited to these Atg9-positive membranes through the TRAPPIII–Ypt1 module. This is consistent with the observation that ATG9A and ULK1, the mammalian homolog of Atg1, colocalize on autophagosomal precursor membranes in mammals (Puri et al., 2013). Subsequently, membranes containing Atg9, Atg1, TRAPPIII and Ypt1 could be recruited to the PAS (Sekito et al., 2009; Suzuki et al., 2007; Yamamoto et al., 2012) by the Atg17–Atg29–Atg31 complex through one or more of the interactions described above. Finally, the resulting membranous platform could serve as a docking point for COPII vesicles, which would provide additional membranes to the forming phagophore. However, these are currently only speculative ideas that need to be further tested. Although they are mostly based on documented interactions, their spatiotemporal regulation is still unknown. For example, it cannot be discarded *a priori* that Atg9-containing vesicles, the Atg17–Atg29–Atg31 complex and the COPII vesicles assemble simultaneously rather than in a successive manner. Moreover, whether or not these interactions are sufficient to provide the minimum molecular environment to support phagophore formation also requires further study. Finally, it is worth noting that Atg29 and Atg31 have no homologs in mammals, and Atg17 appears to only have a functional counterpart, FIP200 (also known as RB1CC1), but not an equivalent protein. Therefore, it remains to be determined how this proposed model could be adapted to higher eukaryotes.

Model II – ERES as a scaffold for autophagosome biogenesis

An alternative scenario that cannot be excluded based on available data is that the ERES and/or COPII vesicles could provide a scaffold structure that is required for autophagosome formation, without being directly involved in membrane transport (Fig. 2B). In this context, it is noteworthy that one subunit of the COPII coat, Sec13, is also part of the nuclear pore, indicating that some COPII proteins could have a scaffolding role that is unrelated to their function in transport (Siniossoglou et al., 1996). The recruitment of the molecular machinery that organizes the assembly and expansion of the phagophore needs to be precisely regulated, and the proteins involved in the process have to be targeted to their specific site of function. ERES, probably through COPII components, could be part of one of these anchoring points, which could hypothetically also mediate the relocalization of the early PAS or phagophore intermediate that is formed at the recycling endosomes in mammals. The Atg17–Atg31–Atg29 complex lacks liposome-binding properties *in vitro* and, consequently, it is likely to need at least one binding partner at the membranes to which is recruited (Ragusa et al., 2012). Because Atg17 is known to interact with COPII proteins (Graef et al., 2013), an alternative model is that, when autophagy is induced, the Atg17–Atg31–Atg29 complex associates with ERES through its interaction with the COPII machinery. The subsequent recruitment of the TRAPPIII complex to these sites through the association between Atg17 and Trs85, and the TRAPPIII interaction with Sec23 and Ypt1 (Tan et al., 2013), would strengthen this scaffold and produce the anchoring point that supports some of the initial steps of PAS formation.

As mentioned above, the expanding phagophores face the ERES in yeast (Graef et al., 2013; Suzuki et al., 2013), and, interestingly, the extremities of these cisternae are enriched in Atg9, Atg2 and Atg18, but not Atg17 (Graef et al., 2013; Suzuki et al., 2013). Thus, one hypothesis is that these factors replace the Atg17–Atg31–Atg29 complex at the extremities and, together with ERES and/or COPII components, anchor the phagophore to the ER. During this factor exchange, Atg9-containing vesicles would first dock to the initial scaffold at ERES by interacting with Atg17, and possibly through the TRAPPIII–Ypt1 module on their surface. Thus, although Atg9 is confined to the phagophore–ERES contact sites in this model, Atg17 (and the Atg1 complex) would relocate from their initial location and become more homogenously distributed on this cisterna or concentrated in a different area, such as the phagophore–vacuole contact site (Graef et al., 2013; Suzuki et al., 2013). This relocation, however, remains to be demonstrated. Electron tomography examinations of the omegasome ultrastructure have revealed that the extremities of the expanding phagophore are indeed physically connected with the ER, supporting this idea (Hayashi-Nishino et al., 2009; Ylä-Anttila et al., 2009). In addition, two more recent studies that combined correlative light and electron microscopy have shown that omegasomes arise from hotspots in the ER and connect to it through thin tubular membranes (Duke et al., 2014; Uemura et al., 2014). The association between these two organelles could be essential for the efficient supply of the enormous quantities of lipids that are required to form autophagosomes. But this then raises the question of why ERES and autophagy machinery components are kept in these specific contact regions. Autophagosomes are almost exclusively composed of lipids (Baba et al., 1995; Fengsrud et al., 2000), and therefore the presence of a proteinaceous constriction, in part formed by ERES components, could ensure the sole passage of lipids (and not proteins). It is worth noting that similar scaffold functions have been proposed for the COPII coat during the initial formation of procollagen-VII-containing megacarriers at the ER (Nogueira et al., 2014) and also for the COPI coat in connecting the ER with lipid droplets (Wilfling et al., 2014).

Although COPII vesicles could also contribute to phagophore expansion by vesicular traffic (as outlined above), one important consideration is that these vesicles typically transport proteins and lipids – with cargo proteins being important regulators of the nucleation of the vesicles on ER membranes. Thus, even if it cannot be excluded, it is difficult to imagine COPII vesicles that are devoted to the exclusive delivery of lipids in an autophagy-specific role, unless retrograde transport at the autophagosomal membrane facilitates retrieval of COPII-vesicle cargo proteins. However, the putative vesicular and scaffold roles of COPII proposed here are not mutually exclusive and could contribute to different steps of autophagosome biogenesis.

Disassembly of the ATG machinery

Autophagosome maturation, i.e. the closure and subsequent disassembly of the ATG machinery from the forming autophagosomes, has to be precisely regulated, and these maturation steps are essential for the fusion of autophagosomes with lysosomes or vacuoles (Cebollero et al., 2012) (Fig. 1iv, Maturation). Therefore, keeping part of the ATG machinery confined to specific locations such as the phagophore–ERES/ER contact sites might facilitate the accurate coordination of these

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events. Below, we will discuss recent work examining these maturation steps and how the new insights could be integrated into the models we have proposed above.

The Atg1/ULK1 complex as regulator of the late steps of autophagosome biogenesis

ULK1 and ATG13 are among the first proteins to leave the membranes of the completing autophagosome (Karanasios et al., 2013), an event that appears to be preceded only by the release of Atg5 (Graef et al., 2013). In particular, ATG13 dissociation occurs before the detachment of autophagosomes from the ER and their subsequent fusion with lysosomes (Karanasios et al., 2013). In yeast, a kinase-dead mutant form of Atg1 causes accumulation of Atg8 on autophagosomal membranes (Cheong et al., 2008), suggesting that a phosphorylation event by Atg1 could be one of the key steps that regulates the disassembly of the ATG machinery. In this putative context, four possible scenarios can be envisioned for how the Atg1/ULK1 complex could mediate the release of the ATG proteins from complete autophagosomes. One possibility is that Atg1/ULK1 phosphorylates and activates the factor(s) that mediate the release of ATG proteins from complete autophagosomes. Alternatively, it could phosphorylate and inactivate an inhibitor of ATG machinery disassembly. Similarly but in an opposite way, it cannot be excluded that Atg1/ULK1 inactivation is the signal that leads to the release of ATG proteins from the complete autophagosome. In this case, dephosphorylation of an activator or an inhibitor would activate or repress them, respectively. Whatever the situation might be, another intriguing question concerns the identity of the signal that regulates Atg1/ULK1 activity prior to the autophagosome closure, especially because Atg1/ULK1 activity is also required for the initiation of autophagosome biogenesis.

Phosphatidylinositol 3-phosphate turnover: clearing the ATG machinery from closed autophagosomes

A key lipid for the biogenesis of autophagosomes is phosphatidylinositol 3-phosphate (PtdIns3*P*), which is generated on autophagosomal membranes by an autophagy-specific phosphatidylinositide 3-kinase (PI3K) complex (Lamb et al., 2013). Interestingly, the clearance of this phosphoinositide is also crucial for the release of the ATG machinery from complete autophagosomes. In particular, yeast strains lacking the PtdIns3*P* phosphatase Ymr1 show an accumulation of autophagosomes that are still associated with ATG proteins in the cytoplasm (Cebollero et al., 2012). Although PtdIns3*P* is generated to an equal extent in both membrane leaflets of the phagophore, it is absent from the surface of autophagosomes that form in wild-type yeast cells, but not from those that accumulate in the *ymr1* mutant (Cheng et al., 2014). One possible mechanism for how Ymr1 could regulate the disassembly of the ATG machinery is that hydrolysis of PtdIns3*P* into phosphatidylinositol leads to the dissociation of those ATG proteins that are able to bind to PtdIns3*P*, such as yeast Atg18 or its mammalian homologs, the members of the WIPI protein family (Strømhaug et al., 2004) and ATG13 (Karanasios et al., 2013). Interestingly, in yeast, Atg18 has been exclusively detected at the edges of the growing phagophore, adjacent to the ERES (Suzuki et al., 2013). This particular localization is likely to be mediated by the binding of both PtdIns3*P* and Atg2 (Rieter et al., 2013; Watanabe et al., 2012). As a result, hydrolysis of PtdIns3*P* could be one possible way to disconnect autophagosomes from the ER. Another not mutually exclusive scenario is that high local concentrations of PtdIns3*P* might directly or indirectly inhibit one or more regulators of ATG machinery disassembly.

The ATG4 paradox

Yeast Atg4 and the mammalian homologs ATG4A–ATG4D are cysteine proteases that are essential for the conjugation of a member of the Atg8/LC3 protein family to phosphatidylethanolamine by cleaving its terminal amino acids at the C-terminus to expose a glycine residue (Kirisako et al., 2000; Shpilka et al., 2011). The proteolytic activity of this protein is also crucial during the final steps of autophagosome formation when Atg4 cleaves Atg8 again to release it from its lipid anchor (Kirisako et al., 2000). Although it has been proposed that Atg4 processes lipidated Atg8 on all membranes except autophagosomal ones (Nakatogawa et al., 2012), recent *in vitro* data have shown that Atg4 activity is crucial for the disassembly of an eventual autophagosome protein coat formed by Atg8 and the Atg12–Atg5–Atg16 complex (Kaufmann et al., 2014). This scenario requires that Atg4 activity is tightly regulated to ensure that vesicle uncoating occurs precisely upon autophagosome completion. However, whether Atg4 is recruited to autophagosomal membranes only when a second Atg8 cleavage is required or whether it is on autophagosomal membranes but in an inactivated form remains to be understood. The structure of human ATG4B has been solved, and it revealed that an inhibitory loop blocks the catalytic site, thereby preventing the processing of LC3 (Sugawara et al., 2005). Therefore, interactions with other proteins or its post-translational modification are potential mechanisms that provide the switch for Atg4 to assume an active conformation. In this regard, reactive oxygen species (ROS) have been shown to specifically and negatively regulate yeast Atg4 and human ATG4B by targeting cysteine residues (Pérez-Pérez et al., 2014; Scherz-Shouval et al., 2007). Although ROS have been shown to be required for autophagy induction, it has been proposed that decreased ROS levels might also be necessary to allow the activation of Atg4 and ATG4B in order to release members of the Atg8 protein family from autophagosomal membranes (Pérez-Pérez et al., 2014; Scherz-Shouval et al., 2007). How the local levels of ROS at the site of autophagosome biogenesis are regulated needs to be investigated.

Conclusions

During the past ten years, the advances made in the field of autophagy have been remarkable. Many molecular aspects of the process, however, are not yet well understood. In particular, how autophagosomes are generated has been a topic of debate and intense investigation since the discovery of autophagy. Emerging evidence from independent laboratories has revealed that the ERES are involved in autophagosome biogenesis, and, here, we have discussed possible mechanistic scenarios. Despite the fact that we do not yet understand their exact contribution to autophagy, a role in the process is a newly described function of these specialized ER domains, which were previously thought to participate exclusively in the secretory pathway. ERES are thus a new element to be integrated into our descriptions of autophagosome biogenesis and, as eluded to here, their role might go beyond acting as a site of autophagosome formation. The ERES could also represent a hub to reorganize the secretory pathway in response to different nutrient conditions. We anticipate that further work on this aspect of ERES function will provide new and exciting insights into the regulation of eukaryotic cell physiology.

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Competing interests

The authors declare no competing interests.

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