Membrane supply and remodeling during autophagosome biogenesis

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Membrane supply and remodeling during autophagosome biogenesis

Rubén Gómez-Sánchez[1], Sharon A. Tooze[2] and Fulvio Reggiori[1]

Abstract

The *de novo* generation of double-membrane autophagosomes is the hallmark of autophagy. The initial membranous precursor cisterna, the phagophore, is very likely generated by the fusion of vesicles and acts as a membrane seed for the subsequent expansion into an autophagosome. This latter step requires a massive convoy of lipids into the phagophore. In this review, we present recent advances in our understanding of the intracellular membrane sources and lipid delivery mechanisms, which principally rely on vesicular transport and membrane contact sites that contribute to autophagosome biogenesis. In this context, we discuss lipid biosynthesis and lipid remodeling events that play a crucial role in both phagophore nucleation and expansion.

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Autophagy, Phagophore, Isolation membrane, Lipids, Membrane origin, Lipid source, Membrane contact site, Vesicular transport, Omegasome, ATG proteins.

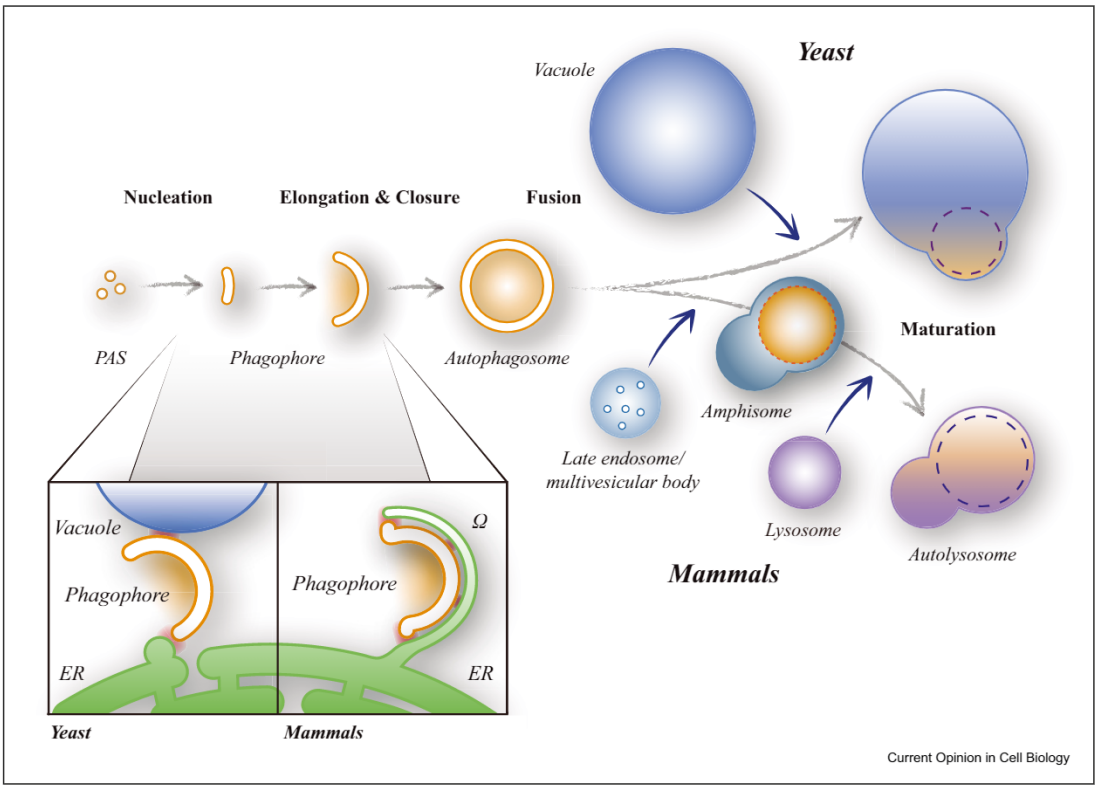
Introduction

Macroautophagy (hereafter autophagy) is an intracellular evolutionarily conserved lysosomal degradation process, which is essential to maintain cellular homeostasis by eliminating unwanted cytoplasmic structures and recycling their basic components. This intracellular catabolic pathway targets excess or dysfunctional proteins, protein complexes, and organelles but also invading pathogens by sequestering them into double-membrane vesicles called autophagosomes and delivering them to lysosomes/vacuoles for turnover [1]. The interest in autophagy has grown exponentially due to a series of discoveries revealing its involvement in numerous physiological processes and pathological conditions [2].

Autophagosomes are formed by nucleation, expansion, and sealing of a small cisterna termed the phagophore, which is generated at specific locations known as the phagophore assembly sites (PAS) in yeast, or omegasomes in mammalian cells (Figure 1). Autophagosome formation is mediated by autophagy-related (ATG) proteins, a subset of which is highly conserved from yeast to higher eukaryotes. This core machinery consists of six functional clusters: the Atg1/ULK kinase complex, Atg9/ATG9A-positive vesicles, the phosphatidylinositol-3-phosphate kinase (PtdIns3K) complex, the Atg2-Atg18/ATG2-WIPI4 complex, and the Atg12/ATG12 and Atg8/LC3 conjugation systems [1]. Autophagy can be both a non-selective and a selective process [3].

Autophagosomes are almost devoid of transmembrane proteins [4,5] but rich in lipids, in particular phospholipids such as phosphatidylcholine, phosphatidylethanolamine (PtdEtn), and phosphatidylinositol [6]. Protein and lipid composition changes over the course of autophagosome biogenesis regulate the different steps of this complex process. For instance, phosphatidylinositol-3-phosphate (PtdIns3P) is synthesized on the phagophore membranes by the PtdIns3K complex, and it is essential for the recruitment of PtdIns3P-binding Atg proteins [1]. Upon closure of an autophagosome, PtdIns3P dephosphorylation allows the Atg machinery to dissociate and in yeast is a prerequisite for the subsequent fusion with vacuole [7]. Autophagosomes can have a diameter of 300–900 nm in yeast and 500–1500 nm in mammalian cells, with their size being determined by the volume of the sequestered cargo [8]. In comparison to classical single membrane transport vesicles (30–80 nm), autophagosomes are huge, and therefore, their biosynthesis requires a massive lipid supply, which involves a major dynamic redirection of

Figure 1



Schematic overview of the process of autophagy. Autophagosome formation begins with the *de novo* generation of a cisterna, termed phagophore, through the fusion of vesicles. In yeast, this event takes place in specific cellular regions often called the PAS, and the phagophore is localized in between the ER and the vacuole (left inset). In mammalian cells, phagophores are formed within omegasomes (Ω), which are PtdIns3P-enriched subdomains of the ER-positive for DFCP1 (right inset). Phagophore elongation (see Figures 2 and 3) leads ultimately to its closure through a membrane fission event and the formation of an autophagosome. In yeast, complete autophagosomes directly fuse with vacuoles, delivering their internal vesicle and cargo into the lumen of this organelle, in which they are degraded by resident hydrolases. In mammalian cells, autophagosomes undergo a process of maturation. In particular, upon release of the ATG machinery (an event that also occurs in yeast), they fuse with late endosomes/multivesicular bodies forming the so-called amphisomes, and lysosomes through the same mechanism. This series of fusion events leads to both a lowering of the pH (also through the transfer of the vacuolar proton pump ATPase) and the delivery of hydrolases, which initiate the degradation of the autophagosomal internal membrane and cargo.

lipid fluxes within a cell coupled to specific lipid biosynthetic pathways.

We discuss here recent conceptual advances on membrane sources and lipid delivery mechanisms that play a major role during the formation of autophagosomes, focusing on yeast *Saccharomyces cerevisiae* and mammalian cells.

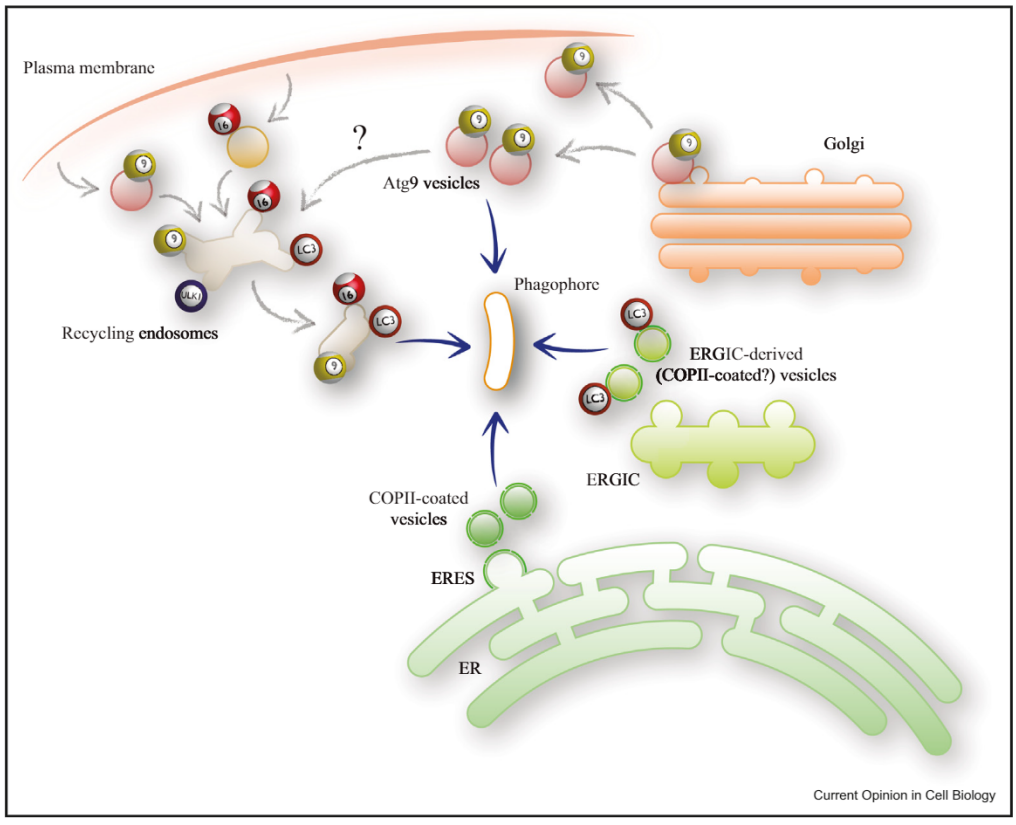
Phagophore nucleation

The phagophore is generated *de novo* as a small and flattened cisterna, very likely by the homotypic and heterotypic fusion of precursor vesicles of different origins (Figure 2). In yeast, this event occurs adjacent to the endoplasmic reticulum (ER), and in mammalian cells, it occurs on ER domains [9–11]. Recent studies suggest that the phagophore formation is promoted by the presence of a scaffold structure, which could be a liquid-like condensate and/or the cargo destined to degradation during selective types of autophagy (reviewed in Ref. [12]). In this section, we will describe the different membranes involved and their potential roles in phagophore nucleation.

Atg9/ATG9A vesicles

Atg9/ATG9A, the only transmembrane protein in the core ATG machinery, is present in post-Golgi clusters of vesicles and tubules [13,14]. Small vesicles and possibly tubules carrying Atg9/ATG9A are the key contributors to the phagophore [11,13,15]. While a few Atg9 vesicles initially become a part of the phagophore membrane in yeast [15], ATG9A-positive vesicles dynamically associate with the mammalian phagophore throughout the entire process of autophagosome formation [14]. Atg9/

Figure 2



Membrane contributions during phagophore formation. Atg9/ATG9A-containing vesicles are an important contributor of membranes and possibly key factors in generating the autophagosome precursors. Formation of Atg9/ATG9A-positive vesicles occurs at the Golgi, but they can also be generated from the PM through clathrin-mediated endocytosis and coalesce at the RE with ATG16L1-positive vesicles (originating, as well at the PM by clathrin-mediated endocytosis). At the RE, where ULK1 is also present, ATG16L1 recruits LC3 and promotes its conjugation to PtdEtn. Through an SNX18- and DNM2-dependent mechanism, ATG9A-, ATG16L1- and LC3-positive membranes tubulate from RE and relocalize to the omegasomes (not shown), contributing to the phagophore nucleation. The ER, in particular the ERES, also plays a critical role during this initial step of the autophagosome formation. COPII-coated vesicles generated at the ERES also provide membranes necessary for the phagophore nucleation. Another organelle that appears to be central in the formation of the phagophore is the ERGIC, from where vesicles, probably COPII-coated, able to acquire lipidated LC3 originate.

ATG9A vesicles also transport other factors for autophagosome formation, including the transport protein particle complex III (TRAPPIII) and the PtdIns 4-kinase PI4KIIIβ [16,17], which are important for the phagophore nucleation (see below), the assembly and/or regulation of the ATG machinery [11,18], respectively. A plausible mechanism underlying phagophore nucleation at the yeast PAS is through the homotypic fusion of Atg9 vesicles by the action of both the Atg1 complex, which is able to recruit and tether Atg9 vesicles *in vitro* [19,20], and/or SNARE proteins [21].

Atg9/ATG9A trafficking through different compartments, including Golgi, endosomes, and plasma membrane, requires multiple membrane transport components. In mammals, this includes the adapter protein complexes AP2 and AP4, various sorting nexins (SNX4, SNX7 and SNX18), the TBC1 domain family members TBC1D5 and TBC1D14, retromer and TRAPPIII complexes (reviewed in Ref. [22]). Thus, the relevance of organelles such as Golgi in the phagophore nucleation appears to be indirect and mostly linked to Atg9/ATG9A trafficking.

Recycling endosomes

RAB11-positive recycling endosomes (RE) are key in sorting membranes necessary for the phagophore nucleation and/or expansion [23–25]. ATG9A-positive membranes are released from RE during autophagy through a mechanism that requires SNX18 and BAR domain-containing BIF1, in concert with DNM2 [26,27]. Although transport from Golgi and post-Golgi compartments cannot be excluded, ATG9A appears to reach RE from the plasma membrane by endocytosis, where it

coalesces with ATG16L1-positive vesicles [28—30]. ATG16L1 can be recruited to RE by interacting with WIPI2, which associates through coincidence detection of RAB11 and PtdIns3P [25], or in a SNX18-dependent manner [27]. ATG16L1 on the RE drives conjugation of LC3 to PtdEtn. ATG16L1- and LC3-positive membranes are released from RE through a SNX18- and DNM2-dependent mechanism [23,27,31]. These membranes likely also transport ATG9A out of RE and contribute to the phagophore nucleation at the omegasomes [27]. The centrality of RE in the phagophore biogenesis is also underlined by the fact that ULK1 also localizes to this compartment [24].

COPII-coated vesicles and the ER-Golgi intermediate compartment (ERGIC)

COPII-coated vesicles are principally involved in membrane traffic from the ER to the Golgi and form at specialized subdomains of the ER, known as ER exit sites (ERES). Functional ERES and COPII-coated vesicles appear to be required for phagophore nucleation in yeast and mammals, since inhibition of COPII-coated vesicle formation blocks the assembly of the ATG machinery at the PAS [10,11,32,33]. Further, evidence in yeast shows that COPII-coated vesicles deliver membranes to the PAS and/or phagophore [34]. One possible scenario is that COPII-coated vesicles contribute membranes to the PAS/phagophore by direct fusion or via Atg9/ATG9A-positive vesicles.

The ERGIC is a network of tubulovesicular membrane clusters involved in bidirectional trafficking between ER and Golgi. Upon autophagy induction by nutrient starvation, a fraction of the ERES is remodeled and associates with the ERGIC [35], suggesting a functional reorganization of these compartments to sustain autophagosome formation. Hence, studies in mammalian cells have revealed that ERGIC acts as a membrane source for the autophagosomes, possibly supplying some of the membrane composing the phagophore [32]. In particular, the COPII-coat is recruited to the ERGIC in a PtdIns3P-dependent manner leading to the budding of LC3 lipidation-competent vesicles [36]. The exact contribution of ERGICs to the phagophore nucleation remains to be understood, as early autophagic markers, including ATG16L1, are associated with scattered ERGIC-derived vesicles rather than the ERGIC compartments [11]. These ERGIC-derived vesicles may well be the LC3 vesicles derived from the COPII-coated vesicles exiting from the ERGIC [35].

Phagophore elongation

The phagophore is a membrane platform with a dual function. First, it is an acceptor compartment for lipids required for expansion into an autophagosome. Indeed, it has recently been shown *in vitro* that Atg9-positive membranes can act as a platform for lipid influx [37]. Secondly, by recruiting tethering factors, it mediates the establishment of the lines of lipid supply from other organelles through both vesicular traffic and membrane contact sites (MCSs). A 3D EM study has revealed that the phagophore physically interacts with several subcellular compartments, including ER, mitochondria, Golgi, late endosomes, and lysosomes [38], supporting this notion (Figure 3).

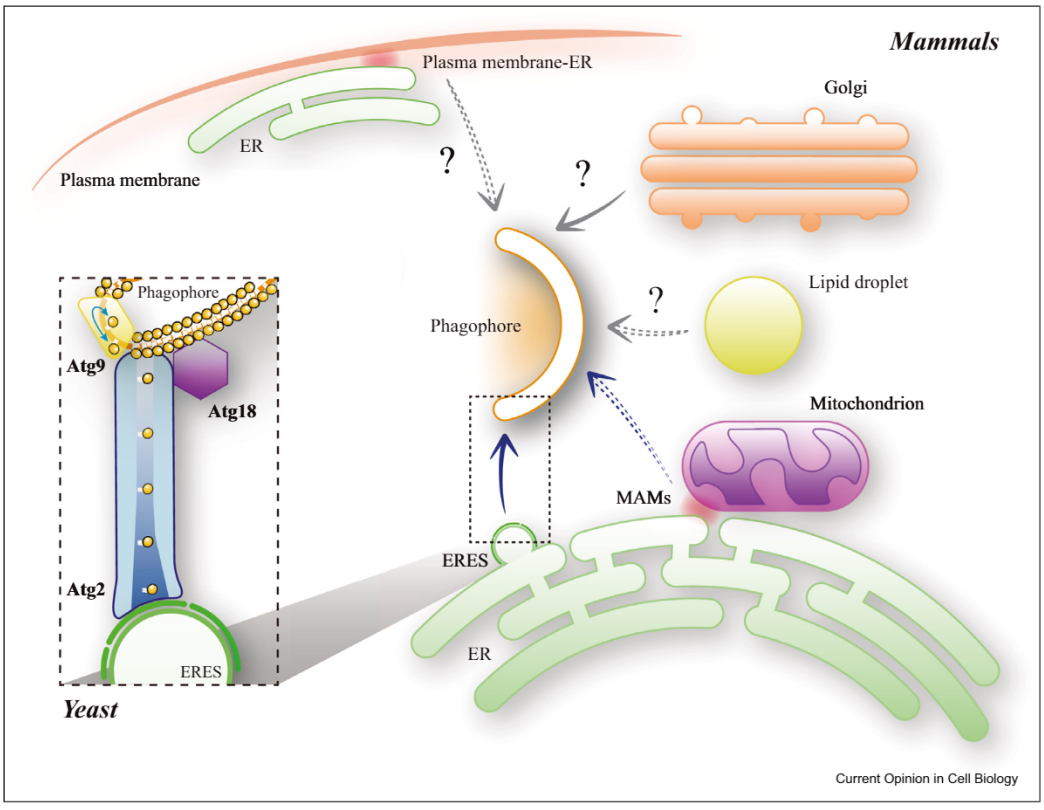
ER and ERES

Phospholipids are principally synthesized in the ER, which also acts as a platform for *de novo* biogenesis of organelles, such as peroxisomes and lipid droplets [39]. Over the years, it has become evident that the ATG machinery assembly and autophagosome formation take place adjacent to the ER. In mammals, the phagophore elongation often occurs within omegasomes, which are PtdIns3P-enriched subdomains of the ER characterized by both their Ω shape and the concentration of ER resident and PtdIns3P-binding protein DFCP1 [40—42] (Figure 1). In yeast, although nascent autophagosomes are in close contact with the ER network, they are not enveloped in an ER subdomain [9,10]. Lipophilic fluorescent dyes have shown that the ER supplies lipids for the phagophore elongation [43—45].

A connection between the ER and phagophore, which involves contact between the high curvature extremities of the phagophore and the ER [9,10,40,41,46], appears to be central for the lipid supply from the ER to the nascent autophagosome. In yeast, at least one of the phagophore edges is tethered to the ERES [9,10]. Thus, the ERES may be a central organizational hub of autophagosome formation by coordinating both the nucleation and expansion of phagophores. At the molecular level, Atg9 localizes to the tips of phagophores [9,10]. Atg2 and Atg18 are sequentially recruited at these sites through direct and indirect binding to Atg9, respectively, and PtdIns3P [47]. This series of events leads to the establishment of MCSs between the phagophore extremities and the ER [47,48] (Figure 3). The formation of MCSs by Atg2-Atg18 is also supported by the finding that this complex and ATG2A-WIPI4 tether liposomes *in vitro* [48,49]. One of the recognized functions of MCSs is to transfer lipids between organelles, and indeed, Atg2 proteins can transfer lipids between liposomes *in vitro* [50—52], supporting the notion that Atg2 conveys lipids from the ER to the phagophore to promote its expansion into an autophagosome. Atg2-mediated lipid transfer is probably promoted by the Atg9/ATG9A phospholipid scramblase activity [53,54]. It remains to be determined whether ATG2 proteins, their binding partners, and ERES also establish a MCS between the phagophore and ER in mammalian cells.

Ultrastructural investigations have revealed that the mammalian phagophores may have additional MCSs

Figure 3



Membrane contribution during phagophore elongation. ER is very likely the major source of lipids during the phagophore expansion. The ER is connected with the phagophore through MCSs, allowing direct lipid transfer (*blue arrow*). One of these MCSs has been characterized in detail in yeast (inset). Atg2 appears to be central in establishing ER-phagophore MCSs by interacting with the ERES/ER, Atg9 and PtdIns3P pools on phagophores. Atg9-Atg2-Atg18 complexes mediate the flow of lipids from the ERES/ER to the phagophore. Specifically, Atg2 is a lipid transfer protein whose activity is promoted by Atg18, and Atg9 acts as a phospholipid scramblase on the phagophore membrane. The mammalian counterparts of these proteins probably have a similar function. During starvation, mammalian STX17 interacts with ATG14, facilitating the positioning of MAMs adjacently to nascent phagophores. The proximity of MAMs and the mitochondria indicates that they may indirectly contribute, i.e. via the ER, specific lipid species that are necessary for the phagophore elongation (*dashed blue arrow*). Other organelles, including lipid droplets and Golgi, but also ER-plasma membrane MCSs, have been postulated as putative direct (*grey arrows*) and indirect (*dashed grey arrows*) membrane sources for the phagophore expansion.

with ER [40,41] (Figure 1). ER integral membrane proteins (such as VMP1, its interactor TMEM41B and VAP proteins) modulate ER-phagophore MCSs. However, it remains to be determined whether they regulate the ATG machinery and/or the lipid supply at the extremities of the expanding phagophore [55–57].

Mitochondria and mitochondria-associated membranes (MAMs)

Mammalian phagophore elongation also occurs in the vicinity of MAMs, which appears to be functionally connected to the ATG machinery via the association of STX17 with ATG14 [58]. Upon autophagy induction, MAMs, like the ERES, may create a specialized lipid raft platform for autophagosome biogenesis [59]. Alternatively, the positioning of mitochondria and MAMs in near proximity of the expanding phagophore could be important to supply phospholipids like PtdEtn, which is mostly produced in the mitochondria and is essential for the Atg8/LC3 conjugation system [1]. Mitochondria and MAMs likely play a part in lipid distribution rather than organizing the Atg machinery. In addition, the observation that ATG protein association with mitochondria is dynamic [18,58] also suggests these organelles may not represent a static platform. Finally, the presence of the lipid transfer protein ATG2A at the MAMs also corroborates this model [60].

Other organelles

Other organelles have been linked to autophagosome biogenesis, but their precise role remains to be fully understood (Figure 3). ER-plasma membrane MCSs positively regulate autophagosome generation by stabilizing the PtdIns3K complex at the ER, but whether the

plasma membrane provides lipids to the phagophore is not known [61]. Phagophore MCSs with Golgi complex have also been reported, but their relevance in autophagy is still enigmatic [38]. Lipid droplets, in contrast, are essential to generate autophagosomes, but it is unclear whether they represent a source for autophagosomal lipids and/or fulfill critical functions for ER homeostasis linked to autophagy regulation [62–64].

In yeast, the PAS and the phagophore make an MCS with the vacuole (Figure 1), and vacuolar tethering of the phagophores is regulated by the interaction between vacuolar Vac8 and Atg13 [65]. Since smaller autophagosomes are generated in the absence of *VAC8*, one possibility could be that the vacuole also provides lipids to the expanding phagophore, but it cannot be excluded that this phenotype is due to a mislocalization of the Atg machinery. Mammalian phagophores also have MCSs with late endosomes and lysosomes [38], indicating that an interaction with these degradative compartments and autophagosomal membrane may occur before autophagosome completion. The significance of this intriguing observation remains to be uncovered.

Lipid biosynthesis and concluding remarks

One of the major roles of the cellular endomembrane system during autophagy is to provide the lipids, in particular phospholipids, necessary to generate autophagosomes *de novo*. This requires the establishment of a lipid flux from membrane donor compartments to the phagophore, which appears to rely on vesicular trafficking during the phagophore nucleation and on MCSs during phagophore expansion. The latter requires directionality, but how this is established is unknown, especially considering that energy is scarce in most of the conditions that induce autophagy. Interestingly, the acyl-CoA synthetase Faa1 is recruited onto phagophores and produces the acyl-CoA required for the synthesis of phosphatidic acid from lysophosphatidic acid at the ER membrane, which is essential for the phagophore elongation [6]. Additionally, numerous phospholipid biosynthetic enzymes concentrate at the ER subdomains where the phagophore nucleates [66]. Local biosynthesis at specific MCSs in close proximity to the Atg2 transfer proteins may be important to guarantee the unidirectional transport of lipids from the ER membrane into the growing phagophore. However, additional mechanisms may be involved in promoting this lipid flux, including phospholipid modifications like PtdEtn conjugation to Atg8/LC3 proteins and/or molecular machines such as AAA+ ATPases that transform ATP hydrolysis into mechanical power.

Recent advances have provided insights into the compartments and the proteins regulating the convoy of lipids into nascent autophagosomes. Membrane remodeling, as well as dedicated MCSs mobilization, are aspects that are still poorly understood. Thus, the study of the lipid dynamics during autophagy will not only uncover the mechanism of autophagosome formation but could also provide the paradigm to understand multiple other cellular processes in molecular detail.

Credit author statement

Rubén Gómez-Sánchez, Sharon A. Tooze, Fulvio Reggiori: Conceptualization; Writing - Original draft; Writing - Reviewing and Editing.

Conflicts of interest

Nothing declared.

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