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HYPOTHESIS

SUBJECT COLLECTION: AUTOPHAGY

A unifying model for the role of the ATG8 system in autophagy

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ABSTRACT

The formation of autophagosomes and their fusion with lysosomes are key events that underpin autophagic degradation of cargoes. The core ATG8 system, which consists of the ATG8 family of ubiquitin-like proteins and the machineries that conjugate them onto autophagosomal membranes, are among the most-studied autophagy components. Despite the research focus on the core ATG8 system, there are conflicting reports regarding its essential roles in autophagy. Here, we reconcile prior observations of the core ATG8 system into a unifying model of their function that aims to consider apparently conflicting discoveries. Bypass pathways of autophagy that function independently of the core ATG8 system are also discussed.

KEY WORDS: ATG8, Autophagosome, Autophagy, GABARAP, LC3

Introduction

Macroautophagy (hereafter autophagy) is a lysosomal degradative pathway that plays essential roles in cellular and organismal homeostasis. Autophagy typically targets cytoplasmic components, including excess or damaged organelles, protein aggregates and invading pathogens, for degradation and nutrient recycling (Gatica et al., 2018). Double-membrane vesicles, termed autophagosomes, are the central structures of autophagy. Autophagosomes function to encapsulate cytoplasmic cargoes before delivering them to lysosomes for degradation. These cargoes can be encapsulated by autophagosomes either in a non-selectively manner by bulk capture, or selectively via specific signals on the surface of the cargo (Dikic and Elazar, 2018; Melia et al., 2020). The ATG8 family of ubiquitin-like proteins, consisting of the LC3s (LC3A, LC3B and LC3C; also known as MAP1LC3A–MAP1LC3C) and GABARAPs (GABARAP, GABARAPL1 and GABARAPL2), together with their conjugation machineries (collectively referred to hereafter as the core ATG8 system), are widely recognized for their roles in both autophagosome formation and selective sequestration of certain cargoes (Gatica et al., 2018; Martens and Fracchiolla, 2020; Mizushima, 2020). However, there are inconsistencies in the literature regarding the role of the core ATG8 system, and this has raised new questions regarding its essentiality in autophagy. For example, some studies have reported that the core ATG8 system is indispensable for autophagosome formation (Komatsu et al., 2005; Sou et al., 2008), whereas other studies have been reported that, in contrast, it is not absolutely required for autophagosome formation (Collier et al., 2021; Nguyen et al., 2016; Tsuboyama et al., 2016). Although there are discrepancies with regard to its essentiality during autophagosome formation, it is generally accepted that the core ATG8 system is critical for the ultimate degradation of cargoes. However, the core ATG8 system is completely dispensable even for the autophagic degradation of some cargoes in certain contexts (Honda et al., 2014; Nishida et al., 2009; Ohnstad et al., 2020). In this Hypothesis article, we will take a closer look at the apparently conflicting studies and put forward a unifying model, which ties together previous observations and helps to more broadly define the role of the core ATG8 system in autophagy.

Before moving forward, we would like to acknowledge the recent description of ATG8 protein family attachment to membranes and proteins, a process termed atg8ylation (Agrotis et al., 2019; Carosi et al., 2021; Kumar et al., 2021; Nguyen et al., 2021). For simplicity, and to limit the number of new concepts in this article, we have kept the old terminology of lipidation. However, atg8ylation is a highly suitable term to describe events in which the ubiquitin-like family of ATG8s are attached to substrates, including in response to membrane stress and remodelling (Kumar et al., 2021).

Autophagosome formation and the core ATG8 system

To gain an understanding of the functional relevance of the ATG8 protein family and the conjugation machineries within the autophagy system, it is important to cover some of the core steps of autophagosome formation. Broadly speaking, autophagosome formation is a complex membrane expansion and remodelling process (Nakatogawa, 2020; Nishimura and Tooze, 2020). It incorporates lipid modification and transport, protein trafficking and many critical autophagy-related (ATG) factors that are largely conserved from yeast to mammals. Autophagosome biogenesis in mammals begins with the formation of a cup-shape precursor membrane called a phagophore, which expands and seals to form the completed autophagosome. There appear to be two main mechanisms of phagophore membrane expansion, one of which relies on the activity of the transmembrane protein ATG9A, whereas the other utilises the ATG8 system.

Autophagosome formation in mammalian cells begins with the concurrent recruitment of the Unc-51 like autophagy activating kinase 1 and 2 (ULK1/2) complex and ATG9A, which is trafficked through Golgi-derived vesicles by factors, including TBC1D5, sorting nexin 18 (SNX18), arfaptin 2 (ARFIP2), lipopolysaccharide-responsive and beige-like anchor protein (LRBA) and ATG4s (ATG4A–ATG4D) (Judith et al., 2019; Nguyen et al., 2021; Popovic and Dikic, 2014; Soreng et al., 2018; Young et al., 2006). ATG9-containing vesicles supply factors required for autophagosome formation (Judith et al., 2019), and together with the lipid-transfer protein ATG2 (ATG2A and/or ATG2B), establish contact sites between phagophores and the endoplasmic reticulum (ER), which promotes lipid transfer and phagophore membrane expansion. (Gómez-Sánchez et al., 2018; Maeda et al., 2020; Matoba et al., 2020; Osawa et al., 2019; Sawa-Makarska et al., 2020; Valverde et al., 2019). The ULK1/2 kinase

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complex functions by recruiting and activating the class III phosphatidylinositol 3-kinase complex (Beclin-1–ATG14–VPS15–VPS34; VPS15 is also known as PIK3R4, and VPS34 as PIK3C3), which produces the lipid phosphatidylinositol-3-phosphate (PI3P) on phagophores (Russell et al., 2013). The PI3P-binding effector protein WIPI2b subsequently recruits the ATG8 lipid conjugation machinery, which includes an E3-like complex consisting of ATG5, ATG12 and ATG16L1 (Dooley et al., 2014). ATG7 and ATG3 serve as the E1- and E2-like enzymes, respectively (Mizushima et al., 1998a,b). Together, the conjugation machineries drive the attachment of ATG8 molecules onto the lipid phosphatidylethanolamine (PE) on autophagosomal membranes. Lipidation of ATG8 correlates with phagophore membrane expansion, which is followed by its closure, mediated by the ESCRT machinery (Takahashi et al., 2018; Zhou et al., 2019), completing autophagosome formation. Apart from autophagosome formation, the ATG8 family members have many reported functions, ranging from roles in selective recognition of certain cargoes to lysosome biogenesis (Gatica et al., 2018; Kumar et al., 2020; Martens and Fracchiolla, 2020; Mizushima, 2020; Nakamura et al., 2020). However, for the purpose of this Hypothesis, we will focus on their roles during autophagosome formation and the maturation steps that involve fusion with lysosomes.

Historical perspective on the function of ATG8 protein family members

Prior to the advent of modern gene-editing technologies, knockout of all six mammalian ATG8s was prohibitively difficult. However, yeast, which expresses a single Atg8 gene, were amenable to genetic modification, and therefore the role of mammalian ATG8s was initially extrapolated from studies carried out in yeast. Yeast Atg8 was discovered as an essential autophagy protein through genetic screens, which became the foundation of the autophagy field (Scott et al., 1996; Tsukada and Ohsumi, 1993). Analyses of yeast lacking Atg8 led to the conclusion that it is essential for autophagosome formation, although it is worthwhile noting that small autophagosome-like structures are occasionally observed in these cells (Kirisako et al., 1999; Xie et al., 2008). Nevertheless, in the absence of Atg8, there is a clear autophagosome formation defect, which includes failure to form autophagosome intermediates, indicating a role for Atg8 during the early initiation stages of autophagosome formation (Kirisako et al., 1999; Xie et al., 2008). Downstream of initiation, the levels of Atg8 protein were shown to be directly linked to autophagosome size (Abeliovich et al., 2000; Xie et al., 2008), and taken together with the observation that Atg8 is involved in membrane tethering and hemifusion (Nakatogawa et al., 2007), it was concluded that Atg8 must also play a role in autophagosome membrane expansion.

To clarify how the ATG8 protein family functions in mammalian cells, researchers focused on cell and murine models lacking components of the conjugation machinery, including ATG5, ATG3 and ATG7 (Kishi-Itakura et al., 2014; Komatsu et al., 2005; Mizushima et al., 2001; Sou et al., 2008; Uemura et al., 2014), or cells expressing a dominant-negative mutant of the cysteine protease ATG4B, which inhibits lipidation of ATG8 family proteins (Fujita et al., 2008). Collectively, these studies led to the overall conclusion that the core ATG8 system is necessary for autophagosome formation, and, therefore, the function of ATG8s was assigned to this role. However, unlike the initiation defect observed in yeast lacking Atg8, phagophore formation and expansion could be observed in the absence of the core ATG8 system. Notably, smaller autophagosome-like structures were also observed in such cells (Kishi-Itakura et al., 2014; Komatsu et al., 2005; Sou et al., 2008), which raised the possibility that the key defect of lack of the core ATG8 system in mammalian cells could be a decreased autophagosome formation efficiency as opposed to an essential requirement leading to a defect in autophagosome formation. The downstream effect of inefficient autophagosome formation would be a defect in autophagic turnover of substrates.

Analysis of the conjugation machineries, while was highly informative, was nevertheless only an indirect approach for assessing the function of ATG8s. However, with the recent development of gene-editing technologies, such as CRISPR/Cas9, that can be used in human cells (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013), it became possible to knockout the entire ATG8 protein family and so directly study its function. Genetic knockout of all six ATG8 family members in human cells to generate a hexa-KO cell line revealed that ATG8s are important for efficient autophagosome formation, and like yeast Atg8, important for autophagosomal membrane expansion (Nguyen et al., 2016). Precisely how ATG8s promote autophagosome growth remains unclear. It is possible that ATG8s are delivered to the phagophore via vesicles derived from the ER-Golgi intermediate compartment (ERGIC), where lipidation is proposed to occur (Ge et al., 2013, 2014), and they facilitate the fusion of these vesicles with the phagophore. Alternatively, the recently reported interaction between ATG8s with ATG2 (Bozic et al., 2020) might contribute to autophagosome growth by providing a mechanism of lipid transfer from ATG8-containing vesicles to the phagophore. However, ATG8s are not absolutely required for autophagosome formation. Instead, the essential role of ATG8s is to drive the fusion of autophagosomes to lysosomes, and here, the GABARAPs have a prominent role (Nguyen et al., 2016; Vaites et al., 2018). Autophagosome–lysosome fusion is governed by several factors, including SNAREs (Itakura et al., 2012; Matsui et al., 2018) and the HOPS complex (Jiang et al., 2014). GABARAPs preferentially interact with the adaptor protein PLEKHM1, recruiting it to autophagosomal structures, where it functions to promote assembly of HOPS and the SNARE membrane fusion machineries (McEwan et al., 2015; Nguyen et al., 2016; Rogov et al., 2017).

The discovery of the SNARE protein syntaxin 17 as an autophagosomal marker (Itakura et al., 2012) created an opportunity to revisit autophagosome formation in lipidation-defective lines, given that previous work was hindered by the lack of an autophagosome marker in the absence of ATG8 family lipidation. Analysis of syntaxin 17-labelled autophagosomes led to the discovery that autophagosomal structures can indeed form in the absence of ATG8 lipidation, and that these structures can go on to fuse with lysosomes, although this stage appeared to be slowed (Tsuboyama et al., 2016). The primary defect observed was during maturation where the degradation of the inner autophagosomal membrane was delayed (Tsuboyama et al., 2016). The defect in inner membrane degradation is downstream of fusion with lysosomes and therefore appears to be inconsistent with the hypothesis that ATG8s are essential for autophagosome–lysosome fusion. In fact, there are several apparent conflicting results between studies of lipidation-defective cells and those in which the ATG8 family has been depleted. However, we believe that the unifying model of the core ATG8 system during autophagy presented below can help to reconcile these apparently conflicting observations.

Reconciling apparently conflicting observations into a unifying model for the core ATG8 system

If cells lacking all ATG8 family members can form autophagosomes, albeit smaller and with less efficiency (Nguyen

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et al., 2016), this raises the question of why early studies of the mammalian conjugation machinery and cells expressing an ATG4B-dominant negative mutant show expanded but not fully formed autophagosomes (Fujita et al., 2008; Sou et al., 2008)? In addition, why did Tsuboyma et al. observe that autophagosomes fuse with lysosomes in the absence of ATG8 lipidation (Tsuboyama et al., 2016), whereas others concluded that ATG8s are essential for this same process (Nguyen et al., 2016), and why did RNAi knockdown studies of LC3 and GABARAP subfamilies (Weidberg et al., 2010) lead to the conclusion that ATG8s are essential for autophagosome formation? We would like to propose a unifying model that accommodates and reconciles these apparently conflicting observations. This model proposes that the core ATG8 system (incorporating ATG8s and their lipidation machineries) functions during two key phases of autophagy – (1) during autophagosome formation and (2) during autophagosome–lysosome fusion. During the first phase, the ATG8 system has an important, but non-essential, role to drive the efficiency of autophagosome formation (Fig. 1A). The reason why the ATG8 system is not essential is that membrane expansion can also occur via the ATG9A-ATG2 axis, although relying solely on this pathway results in a reduced efficiency of autophagosome formation and reduced autophagosome size (Nguyen et al., 2016). In the second phase, ATG8s themselves play an essential role in autophagosome–lysosome fusion, whereas their lipidation has an important but not-essential role in driving fusion efficiency (Fig. 1B). Our reasoning is that lipidation increases the local concentration of ATG8 molecules at sites of autophagosome formation and autophagosome–lysosome fusion, which allows for a maximum efficiency of their function; this also implies that ATG8s might still have an activity in the absence of their lipidation. This hypothesis can be tested by comparing the mitophagy rate of hexa-KO cells expressing a conjugation-defective GABARAP mutant (lacking the C-terminal glycine) with those expressing native GABARAP. Nevertheless, it

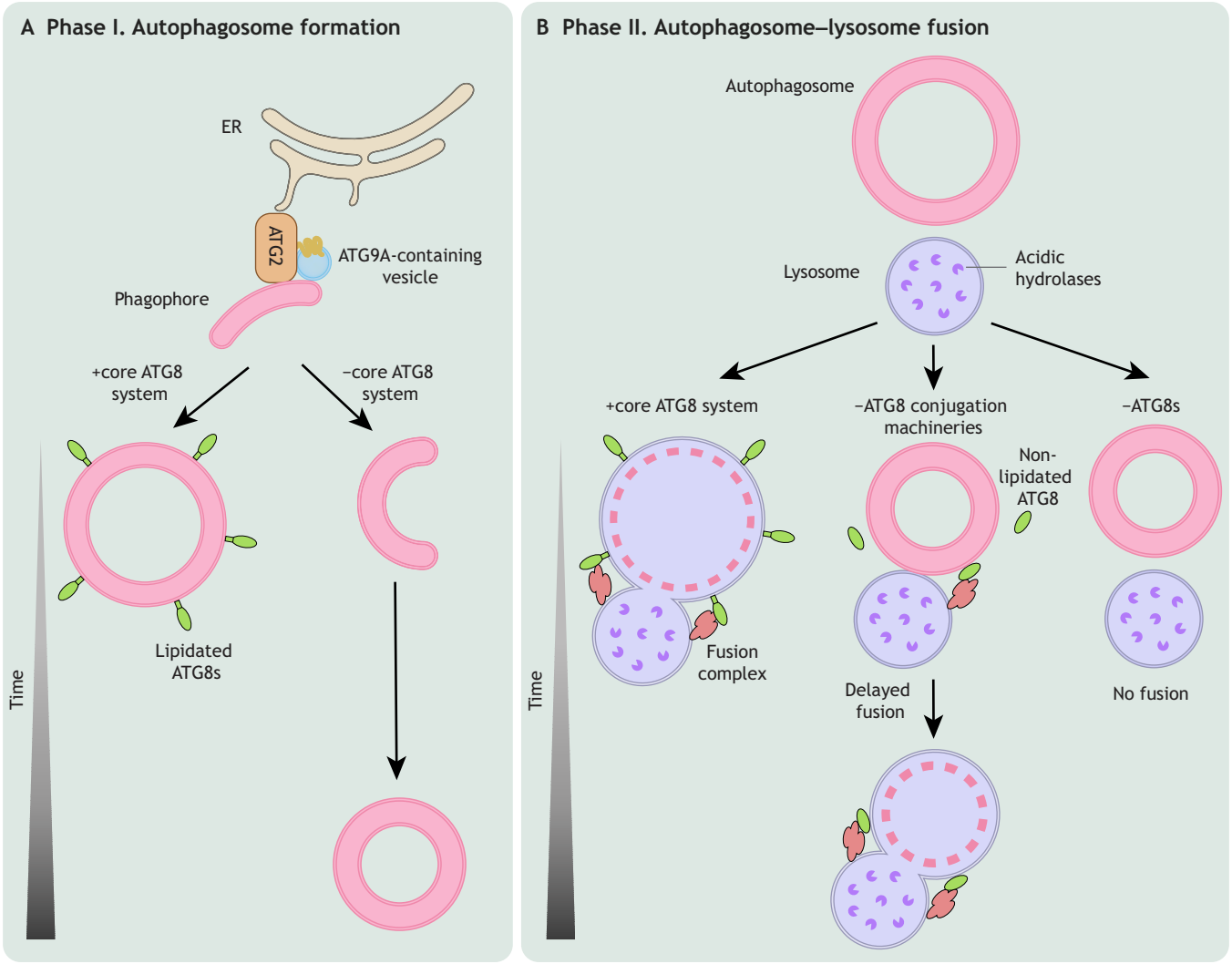

Fig. 1. A unified model for the role of the core ATG8 system in autophagy. The core ATG8 system (consisting of the ATG8 family proteins and their conjugation machineries) functions during two important stages of autophagy. (A) During autophagosome formation (phase I), the core ATG8 system promotes the efficiency of autophagosomal membrane expansion and autophagosome formation together with the ATG9A–ATG2 membrane expansion axis. (B) During autophagosome–lysosome fusion (phase II), ATG8s have an essential role during autophagosome–lysosome fusion, with GABARAPs playing a prominent role by recruiting PLEKHM1, which promotes the assembly of the fusion complex consisting of PLEKHM1, HOPS and SNARE complexes. Lipidation by ATG8 conjugation machineries ensures ATG8 molecules are concentrated at sites of autophagy where they function. In the absence of lipidation, autophagosome–lysosome fusion is less efficient.

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is possible that the delayed inner-membrane degradation observed in lipidation-deficient cells (Tsuboyama et al., 2016), is in part a result of the reduced efficiency of non-lipidated ATG8 in mediating autophagosome–lysosome fusion. The idea that lipidation governs the efficiency of ATG8 family function helps to explain why cells lacking ATG8s do not phenocopy cells that lack the conjugation machineries.

This unifying model also explains why incomplete autophagosomes are observed in studies that used conjugation-deficient cells, RNAi of ATG8 subfamilies or the ATG4B mutant (Fujita et al., 2008; Sou et al., 2008; Weidberg et al., 2010). These studies analysed early autophagy time points (after 1 to 2 h of starvation), compared to the 8 h starvation time point that was used to analyse ATG8-null cells (Nguyen et al., 2016). Given that autophagosomes form less efficiently in the absence of the core ATG8 system, analysis at early time points is likely to yield incomplete autophagosomal structures, which take longer to become fully formed autophagosomes (Fig. 1A). Consistent with this idea, lipidation-defective and ATG4B mutant cells were not completely devoid of what appeared to be fully formed autophagosome-like structures, but those were greatly reduced in number relative to controls (Fujita et al., 2008). The unifying model posited here therefore brings together previous observations and helps to clarify the essential and non-essential roles of the core ATG8 system in autophagy.

Autophagy pathways independent of ATG8 family protein lipidation

In cell biology, there are almost always exceptions to the rule. Indeed, there are certain biological contexts in which lipidation of ATG8s is completely dispensable for autophagic activity. Mitophagy in mice lacking ATG5 proceeds normally in embryonic reticulocytes (Honda et al., 2014), through an alternative form of macroautophagy that can also be induced by DNA damage (Nishida et al., 2009). Autophagosome formation through such an alternative autophagy pathway is dependent on some of the canonical autophagy factors, including ULK1 and phosphoinositide 3-kinase (PI3K), but not on ATG9A nor the conjugation machineries (Nishida et al., 2009). Instead, autophagosomes are formed from trans-Golgi-derived membranes, with this process called Golgi-membrane-associated degradation pathway (GOMED) (Yamaguchi et al., 2016). More recently, whole-genome CRISPR/Cas9 screens using autophagy reporters led to the discovery of another pathway that bypasses ATG8 lipidation in which autophagosomes still form and autophagic degradation of certain cargo proceeds unperturbed (Ohnstad et al., 2020; Shoemaker et al., 2019). This pathway involves turnover of the autophagy adaptor NBR1, and unlike GOMED, it relies on all canonical autophagy factors including ATG9A, with the exception of the conjugation machineries (Ohnstad et al., 2020). This NBR1 turnover pathway is therefore distinct from GOMED, which functions independently of ATG9A. We hypothesise that the ATG9A–ATG2 axis becomes more active to drive efficient membrane expansion for lipidation-independent NBR1 turnover. It is unlikely that an alternative mechanism for membrane expansion exists, because apart from TAX1BP1 and TBK1, the genetic screening data did not identify many factors that had not already been associated with canonical autophagy (Ohnstad et al., 2020). The identity of the switch that increases the activity of the ATG9A–ATG2-mediated membrane expansion axis remains an interesting question to explore in the future. It also remains to be determined whether the ATG8s themselves play a role in NBR1 turnover, despite this process not absolutely requiring their lipidation.

Overall, the existence of these additional autophagy pathways demonstrates that there are multiple roads toward making autophagosomes. This can provide robustness to the autophagy system, while also providing routes for the degradation of specific cargoes or during certain biological contexts. To date, cargoes have been identified for GOMED, while cargoes associated with the NBR1-related process await further identification. Mammalian cells might have evolved different strategies to generate autophagosomes in order to overcome pathogens that target autophagy factors or to support other cellular processes during stress. For example, bypass autophagy pathways would be able to take place during infection with *Legionella pneumophila*, a pathogen that produces the protein RavZ, which irreversibly deconjugates ATG8, to counteract its own degradation by autophagy (Choy et al., 2012). Indeed, NBR1 turnover can proceed in cells expressing RavZ (Ohnstad et al., 2020). Lipidation of ATG8s, including onto single-membrane vesicles, can occur during activation of the pro-inflammatory cGAS-STING pathway (Fischer et al., 2020; Gui et al., 2019) and during LC3-associated phagocytosis (LAP) (reviewed by Heckmann and Green, 2019). Prolonged or chronic activation of cGAS-STING and/or high levels of phagocytosis might lead to a shortage of ATG8s, and thus a reduction in canonical autophagy pathways, and phagocytic cells, such as macrophages, might be more sensitive to such a scenario. By having autophagy pathways that can function independently of the core ATG8 system, especially during times of stress, cells can thus ensure that certain crucial cargoes continue to be degraded.

Conclusions and perspectives

By considering apparently conflicting reports, we have reconciled observations of the core ATG8 system into a unifying model of their function, with the affect on efficiency of autophagosome formation being the central pillar of the model (Fig. 1). The model defines the role of the core ATG8 system as an important (but non-essential) driver of autophagosome formation efficiency. During the latter stages of autophagy involving autophagosome–lysosome fusion, ATG8s play an essential role, and their lipidation affects the efficiency of fusion. The effect of the ATG8 system on the efficiency of the process is clearly biologically important since deletion of the conjugation machineries is neonatally lethal in mice, owing primarily to neuronal dysfunction (Yoshii et al., 2016), whereas humans with ATG7 mutations survive but with neurodevelopmental disorders (Collier et al., 2021).

Autophagosome formation is an intricate process that can take multiple paths to expand phagophore membranes, which can either utilise the core ATG8 system or bypass it. However, the requirement for ULK1-mediated initiation is conserved between canonical autophagy, GOMED and other pathways of autophagy that bypass ATG8 lipidation. It therefore appears that initiation and phagophore formation mechanisms are shared, whereas membrane expansion processes diverge. Although, as noted above, there are always exceptions to the rule, with ammonia- and hypoxia-induced autophagy identified as being independent of ULK1 (Cheong et al., 2011; Feng et al., 2019). Nevertheless, the mechanisms of phagophore formation appear to be largely conserved across multiple forms of autophagy. Much remains to be discovered regarding the steps underlying the expansion of the phagophore membrane around cargoes. Specifically, questions remain with regard to what these various mechanisms of membrane expansion are and how it is dictated which one(s) are utilised. In addition, further exploration into exactly how membrane expansion occurs via the ATG8 system is warranted, and whether its membrane

expansion activity differs between selective versus non-selective autophagy pathways.

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Competing interests
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