

The autophagosome: current understanding of formation and maturation

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Abstract: Autophagy is an important and highly conserved catabolic process with roles in development, homeostasis, and cellular stress responses. It describes various distinct pathways for the delivery of cytoplasmic materials (including misfolded protein aggregates and some organelles) to the lysosome for degradation and component recycling. The best understood form of autophagy (macroautophagy) describes the de novo assembly, maturation, and trafficking of a unique double membrane-bound organelle – the autophagosomes – that sequesters cytoplasmic materials and ultimately merges with the lysosomal compartment to form a degradative autolysosome. To rapidly assemble such a structure in response to stimuli, cells express a family of dedicated autophagy-related (ATG) gene products that act sequentially to control membrane events leading first to the nucleation of an isolation membrane or phagophore, followed by phagophore expansion, and sealing to form an autophagosome that traffics to – and ultimately fuses with – the lysosome. These molecules are activated in response to upstream signaling pathways (notably, the mechanistic target of rapamycin [mTOR] pathway), and comprise protein and lipid kinases, putative membrane coats, and unique ubiquitin-like conjugation systems. In concert, a barrage of accessory proteins involved in various membrane trafficking pathways focused on the endosomal compartment are co-opted at the assembly site to facilitate autophagosome biogenesis. Understanding the integrated pathways that coordinate autophagosome assembly at the molecular level will be crucial if we are to realize the potential for autophagy manipulation in future disease therapies.

Keywords: autophagy, ATG proteins, lysosome, phagophore, omegasome, autolysosome, membrane trafficking, ULK1, mTOR, PI(3) kinase, PI3P, LIR motif

Introduction: an overview of autophagy

The term autophagy was coined from ancient Greek, meaning to “self-eat”. It describes several distinct catabolic recycling pathways through which cytoplasmic material is degraded in the lysosome, with the resulting macromolecular building blocks liberated for re-use in the cytoplasm.¹ The most widely studied autophagy pathways involve the formation of double-membrane bound vesicles (autophagosomes) that encapsulate cytoplasm and organelles for trafficking to the lysosome (accurately described as “macroautophagy”), but there are other routes for delivery of cytoplasmic material into this degradative compartment; these are known as “microautophagy” and “chaperone-mediated autophagy” (CMA). In microautophagy, the lysosomal membrane invaginates to engulf small portions of cytoplasm containing proteins that are often specifically targeted by Hsc70, as well as a small part of the bulk cytoplasm.^{2,3} CMA describes a process by which the same chaperone, Hsc70, targets proteins directly (via a consensus

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KFERQ sequence) for degradation by directly importing them through the lysosomal Lamp2A receptor.⁴

This review focuses on mammalian macroautophagy, which we will hereafter refer to simply as “autophagy”. During this process the canonical double membrane-bound autophagosomes form at nucleation sites known as an isolation membranes or phagophores (Figure 1 for the basic terminology). Importantly, autophagy can be further classified into basal and inducible forms, with basal autophagy serving a housekeeping role, whereas inducible autophagy accounts for the engulfment of bulk cytoplasm in times of stress (eg, during nutrient deprivation). To add to the complexity, both basal and inducible forms can contribute to the sequestration and degradation of selective cellular components. Indeed, there is probably an element of selectivity in all types of autophagy.⁵ Thus, the terms “selective” and “selective and exclusive” autophagy have recently been introduced to more accurately classify the process.⁶

Autophagy in homeostasis and disease

Autophagy primarily functions as a stress response pathway that liberates amino acids and other cellular building blocks in times of paucity.⁷ It was first described in the 1960s,⁸ but has only been studied beyond a purely descriptive level much more recently,⁹ leading on from the extensive knowledge gained on the key molecular players through genetic screens in yeast.^{10,11} Autophagy was long regarded as an alternative cell death pathway (type II cell death), but more recently autophagy has come to be viewed as being much more of a cellular survival mechanism.¹² That said there are multiple molecular links between the pathways of apoptosis and autophagy (eg, Marino et al)¹³ meaning that in some

circumstances the regulation of these apparently opposing cellular responses cannot be regarded separately.

Most cells maintain a low level of basal autophagy whose role is probably to clear damaged and damaging proteins that would otherwise accumulate as a consequence of normal cell function.¹⁴ Such a role is highlighted by studies on calorie restriction, a trigger that has been shown to extend lifespan in a range of organisms,¹⁵ most likely through restricting the buildup of toxic cellular waste. In accordance with these important roles in maintaining normal cellular function, autophagy has been implicated in a wide range of diseases. These include infectious diseases, where autophagy is required to clear invading pathogens and contributes to the acquired immune response, as well as degenerative diseases (eg, neurodegenerative conditions; heart disease; diabetes), and cancer. Jiang and Mizushima¹⁶ have provided an excellent general overview of autophagy in disease, while a number of other recent reviews detail the roles of autophagy in immunity and specific types of diseases.^{16–18} Autophagy impairment affects neurons in particular – presumably because the extended lifespan and poor renewability of this highly specialized cell type makes them especially susceptible to accumulating toxicity, and the links between autophagy and neurodegenerative disease is expertly covered elsewhere.^{19,20} The role of autophagy in cancer is somewhat more complex.^{21,22} On the one hand, cells toward the center of an expanding solid tumor are likely to rely on autophagy to maintain a pool of cellular building blocks as nutrients and oxygen become restricted.²³ Here, a mechanistic block in autophagy might result in a tumor being unable to sustain itself, thereby making autophagy a potential therapeutic target. On the other hand, as autophagy is important for keeping damaging materials in the cell to a minimum, its downregulation might contribute to tumorigenesis through the buildup of genotoxic stress.²²

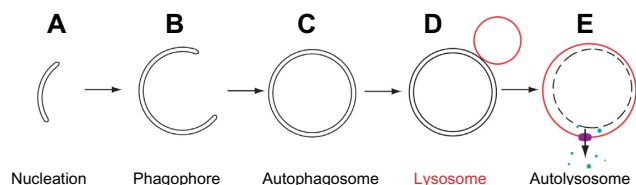


Figure 1 Terminology used to describe the stages of autophagosome assembly during (macro)autophagy.

Notes: (A) Nucleation: the first autophagy proteins begin to assemble at a membrane site that will subsequently mature to become and/or template a single autophagosome. (B) The isolation membrane or phagophore develops: this cytoplasmic sequestering structure is effectively an incomplete/unsealed autophagosome which grows, is shaped, and ultimately seals to form a 3D structure with double membrane (thereby engulfing parts of the cytoplasm and/or cargo) that is the completed autophagosome (C). The autophagosome is a motile transport vacuole that leaves the site of nucleation/assembly (which in mammalian cells is rapidly dismantled) and traffics to the lysosome (D), where it fuses to form an autolysosome (E). Here, macromolecules are degraded into their constitutive building blocks by acidic hydrolases that are released back into the cytoplasm via specific retrograde pumps (E).

Selective autophagy

There are several known types of selective autophagy that play roles in cellular homeostasis and responses to infectious challenge. Xenophagy describes the process through which invading microorganisms are specifically targeted for degradation via recognition and engulfment by the autophagy machinery.²⁴ This type of autophagy is important in the immune response to some pathogens.¹⁸ Aggrephagy is responsible for the sequestration and degradation of aggregated proteins too large to be accommodated in the proteasome.²⁵ Notably, a number of human diseases have been linked to the inefficient clearance of aggregate-prone proteins,

for which treatment could entail the appropriate stimulation of autophagy (eg, Sarkar and Rubinsztein).²⁶ Damaged, surplus, or redundant organelles are also specifically marked for autophagic degradation; an excellent example of this being the mitophagy pathway for mitochondrial degradation.²⁷ Mitophagy is a highly selective process by which only the damaged or redundant parts of the mitochondrial network are specifically exiled before being sequestered into autophagosomes by the actions of a variety of adapter molecules that recruit the autophagy machinery.²⁸ This process is seen during normal cellular homeostasis, and is also upregulated during erythroid terminal differentiation (eg, Betin et al).²⁹ The importance of regulated mitochondrial clearance by mitophagy is evident in Parkinson's disease, where early onset (hereditary) forms are often associated with loss-of-function mutations in mitophagy-linked proteins, including PINK1 (PTEN-induced putative kinase 1) and Parkin.²⁸ Cell-based *in vitro* studies have provided a molecular explanation for the cooperative actions of PINK1 and Parkin during mitophagy, in which PINK1 interrogates mitochondria for energetic functionality through a pathway of constitutive import and degradation. This pathway is abrogated in damaged mitochondria, leading to the targeted recruitment of Parkin and subsequent Parkin-mediated ubiquitylation of mitochondrial surface proteins.³⁰ Key to efficient mitophagy is the regulated exile of damaged organelles from the network coordinated by the mitochondrial membrane fission machinery, and in conditions where mitochondrial dynamics are suppressed mitophagy is strongly blocked.^{31–33} Other organelles can be targeted for autophagic degradation when damaged; for example, parts of the endoplasmic reticulum are sequestered into autophagosomes under endoplasmic reticulum stress conditions (reticulophagy),³⁴ while a pathway for the selective autophagic degradation of peroxisomes (known as pexophagy) also exists.³⁵ Further details on the molecular regulation of selective autophagy can be found in a number of excellent recent reviews (eg, Lamark and Johansen,²⁵ Svenning and Johansen³⁶).

The molecular mechanisms controlling starvation-induced autophagy

There are more than 30 proteins directly involved in autophagy, playing differing roles at the various stages of autophagosome assembly, maturation, and trafficking. Most of these key molecular regulators were first identified in yeast, and the proteins identified in the original screens^{10,11,37} are now collectively referred to as ATG (AuTophagy-related) genes.³⁸

For simplicity, the process of autophagosome biogenesis can be divided into a number of sub-stages: nucleation; elongation of the phagophore; closure of the phagophore to form an autophagosome; trafficking to the lysosome; and fusion of the outer autophagosomal membrane with the lysosome to deliver cargo³⁹ (Figure 1). All of these events involve complex, tightly localized and coordinated molecular signaling, recruitment, and trafficking steps, and the ATG proteins that drive these sequential processes are classed into functional groups corresponding to these substages of autophagy. Correspondingly, the sequential and largely hierarchical recruitment of these autophagy proteins to the site of autophagosome assembly has been well characterized in yeast (where a single assembly site, the pre-autophagosomal structure or "PAS" exists) and in mammals (where multiple assembly sites arise simultaneously and apparently stochastically throughout the cytoplasm). Several recent studies paint a more complex picture of the recruitment and actions of autophagy proteins at assembly sites, with the stability of "early" players positively reinforced by downstream factors whose recruitment critically depends on the former^{40–43} (Figure 2). The majority of autophagy proteins dissociate from the site of autophagosome assembly just prior to completion of the intact autophagosome (with [green fluorescent protein] GFP-labeled ATG5 structures remaining for no longer than 3–4 minutes during starvation-induced autophagy in human cells *in vitro*; our unpublished observations;^{40,42–44}), and it is likely that the coordinated removal of certain proteins and protein complexes is equally important for efficient, productive autophagosome assembly.

The nucleation stage: autophagy induction/nutrient sensing

Cellular responses to nutrient availability

In nutrient replete conditions, mTOR (mechanistic target of rapamycin) is held in a constitutively active state, and coordinates cell growth and proliferation, while inhibiting autophagy. Nutrient deprivation, particularly amino acid starvation, inactivates mTOR, which releases the brake on autophagy leading to rapid assembly of autophagosomes.⁴⁵ Information about nutrient levels in the cell is decoded by mTOR via the Rag (Ras-related small GTP-binding protein) GTPase family,⁴⁶ which in their GTP bound state (favored in the presence of amino acids) facilitate the translocation of mTOR to cellular locations enriched for the mTOR activator, Rheb (Ras homology enriched brain),⁴⁷ while simultaneously acting as part of a regulatory platform.⁴⁸ Energetic stress can be sensed by an increase in adenosine monophosphate (AMP)

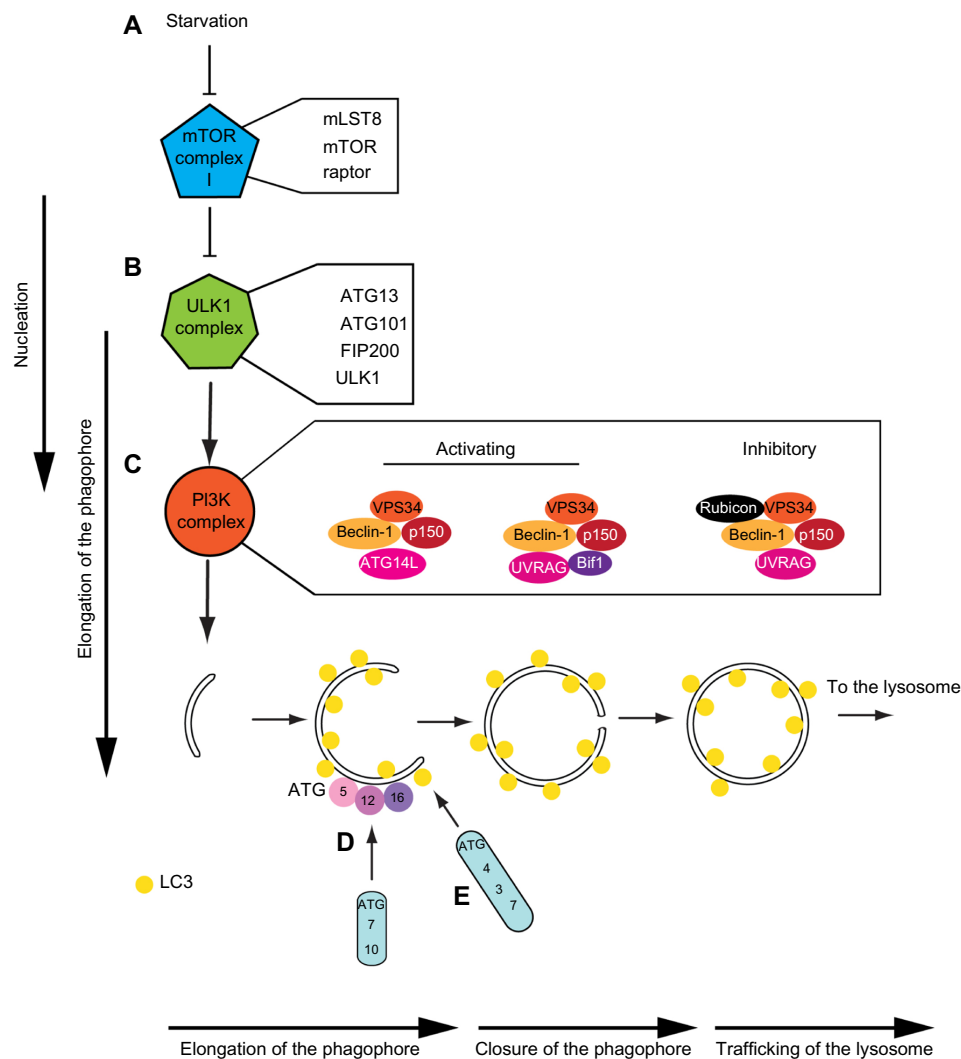


Figure 2 Protein complexes acting during starvation-induced autophagosome assembly.

Notes: (A) mTORC1 and its accessory partners. (B) The ULK1 complex and its constitutive components. (C) The autophagy PI(3) kinase complexes with autophagy activating and inhibiting actions. The core complex is shown in orange and red. (D) The conjugation apparatus that acts to form the ATG5-12 complex. (E) ATG proteins required for LC3 lipidation (a process that requires ATG5-12-16 targeting to the site of autophagosome nucleation).

through AMPK (AMP kinase). One of the consequences of nutrient limitation is a drop in cellular ATP levels (or more accurately, a change in the ATP:AMP ratio). This causes activation of AMPK, which inhibits mTor directly or indirectly by causing downstream inhibition of Rheb. AMPK can also directly activate ULK1 (uncoordinated 51-like kinase 1; the mammalian homologue of yeast ATG1 [see “ULK1 autophagy protein kinase complex” section]) in response to low ATP levels,^{49,50} further amplifying the autophagy response and providing an additional level of control.

Another way in which mTor can be inactivated in response to environmental change is through the inhibition of upstream regulatory inputs. For example, insulin or growth factor stimulation results in the activation of the PKB (protein kinase B)/Akt pathway, one downstream consequence of

which is Rheb activation.⁵¹ This means that in the absence of growth factors and/or insulin, a further obstruction to autophagy activation is removed. Furthermore, in glucose rich conditions, the cAMP (cyclic AMP)-dependent protein kinase A (PKA) signaling pathway suppresses autophagy. When glucose levels are limiting, elevated cAMP levels lead to inactivation of the Ras-PKA pathway, which is predicted to trigger ATG1 phosphorylation/activation (at least in yeast), meaning that the Ras-PKA pathway acts in concert with mTor to block autophagy under nutrient rich conditions.^{51,52} Additionally, LC3 (microtubule-associated protein 1 Light Chain 3), which is a relatively “late” autophagy protein (Figure 2 and following sections), has been shown to be a direct target of PKA in mammalian cells.⁵³ This indicates that the PKA pathway has the potential to regulate autophagy at

multiple levels, although it was noted that none of the other mammalian ATG8-related proteins (nor, indeed, yeast ATG8) possesses this PKA consensus sequence.⁵³

The mTor complex I (mTorC1)

mTor forms two distinct complexes in mammalian cells – mTorC1 and mTorC2 – with only mTorC1 having nutrient sensing capabilities.⁵⁴ The mTorC1 complex consists of mTor, mLST8 (mammalian lethal with SEC13 protein 8; also referred to as G protein beta subunit-like [GβL]), and Raptor (regulatory associated protein of mTOR)⁵⁴ (Figure 2A). Raptor is believed to be the subunit that controls the targeted localization of mTorC1 to signaling hubs, but the function of mLST8 is not known.⁴⁷ Under basal conditions, mTorC1 is associated with, and suppresses ULK1 and ATG13 activity by phosphorylation.^{55–57} This means that amino acid restriction results in the activation of ULK1 and ATG13 – two key players acting during early autophagy initiation that will be described in greater detail below.

The ULK1 autophagy protein kinase complex

In their activated states – and in concert with FIP200 – ATG101, the autophagy kinase ULK1 and its partner ATG13 form the ULK1-complex^{55–58} (Figure 2B). The ULK1 complex is the molecular machinery that defines the nucleation stage of autophagic induction (Figure 2B). Its assembly results in the recruitment of the class III phosphoinositol-3 kinase (PI(3)K) complex (see “The autophagy PI(3)K/VPS34 complex” section) – also known as the vacuolar protein sorting 34 (VPS34) complex – and this is required for downstream events in the autophagosome assembly pathway^{59,60} (Figure 2B). ULK1 complex activity is mechanistically linked to the recruitment and activation of the autophagic VPS34 complex, because Beclin1 (one of its core components [see “The autophagy PI(3)K/VPS34 complex” section]) is a substrate for ULK1 kinase-mediated phosphorylation, and this regulatory switch initiates the assembly of the VPS34 complex.⁶¹ Intriguingly, ULK1 is also capable of inactivating mTor, exemplifying a positive feedback loop in the autophagy pathway⁶² (Figure 3). ATG13 is thought to be required to support the interaction between ULK1 and FIP200 in mammalian cells,⁵⁷ as well as the association between ATG101 and ULK1,⁵⁸ and it may also be directly phosphorylated by mTor. FIP200 is believed to be the mammalian counterpart of yeast ATG17,⁶³ a protein considered to act as a scaffolding factor for the ULK1 complex, as well as potentially inducing local membrane curvature.⁶⁴ In addition to ULK1 activating downstream autophagy

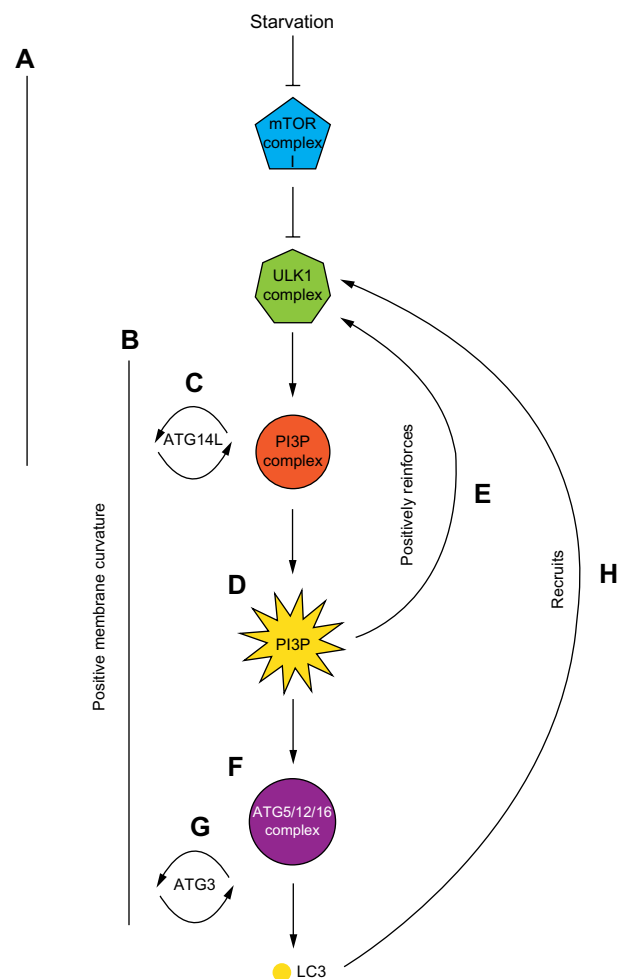


Figure 3 Positive reinforcement of the hierarchical recruitment of autophagy proteins during autophagosome assembly.

Notes: (A) Early stages of autophagosome assembly. (B) Positive membrane curvature at the autophagosome assembly site. (C) ATG14L has affinity for curved membranes. (D) The VPS34 PI(3)K complex triggers localized PI3P formation. (E) This platform of PI3P stabilizes the ULK1 complex at the phagophore through a PI3-binding region in ULK1. (F) The ATG5/12/16 complex is recruited to PI3P positive phagophores and WIPI2b. (G) ATG3 binds to and lipidates LC3 at curved membranes. (H) LC3 stabilizes/recruits the ULK1 complex due to a LIR motif in ULK1.

effector proteins, the ULK1 complex serves as a scaffold for ATG9 vesicles,⁶⁵ indicating that its function may be more important later relative to its time of assembly. Indeed, the yeast ATG1 complex is thought to sense membrane curvature, and thus its ATG17-dependent localization to the phagophore assembly site would be further stabilized by membrane reshaping.⁶⁴ Interestingly, there is evidence that ATG1 binds directly to ATG8 (LC3), and this has been shown to be required for complete activation of the ATG1 kinase complex⁶⁶ (Figure 3). Additionally, the ULK1 complex is stabilized by the presence of the phosphoinositide lipid PI3P (see “PI3P recruits effector proteins: WIPI2, ATGS5/12/16” section), which indicates that positive reinforcement between autophagy proteins is critical for optimal autophagy; a lack

of PI3P results in a reduction of ULK1 lifespan on the autophagosome initiation site⁴³ (Figure 3).

The ULK1 complex is also regulated by Rab proteins.⁶⁷ Rabs are small Ras-like GTPases that define membrane compartment identity to aid the specificity of membrane trafficking, and potentially facilitate vesicle docking.⁶⁸ Rab proteins bind GTP, and usually it is only the GTP bound form that is involved in vesicle/target recognition. Rabs can be activated through specific GEFs (guanine nucleotide exchange factors) and inactivated by GAPs (GTPase activating proteins). Every Rab GAP that has been identified thus far contains a TBC (Tre2/Bub2/Cdc16) domain, which dictates the Rab GAP nomenclature. In a screen for TBC domain containing proteins, Longatti and Tooze⁶⁷ identified the RabGAP TBC1D14 as a negative regulator of autophagosome formation. The authors went on to validate Rab11 – the Rab regulated by TBC1D14 – as an important mediator of early autophagy events. Rabs have also been found to play distinct roles during later stages of autophagosome biogenesis and maturation as will be detailed later.

It is worth noting that at least five potential mammalian ATG1 homologs have been identified thus far, some of which have been implicated in autophagy under specific settings. ULK1 was the first to be shown to play a role in autophagy, and it remains the most widely studied.^{69,70} In addition to its regulation by phosphorylation ULK1, may also be post-translationally regulated by ubiquitination and acetylation,⁷⁰ adding further complexity to autophagosome assembly control pathways.

Elongation, shaping, and sealing the phagophore

The autophagy PI(3)K/VPS34 complex

Inositol lipids and their phosphorylated derivatives, including PI3P, are important mediators of intracellular signaling, with different phosphorylated PIs marking varied membranous compartments; examples include PI(4)P and PI(4,5)P₂ that decorate the Golgi apparatus,⁷¹ and PI3P which be found on early endosomes and the nascent autophagosome. The term “omegasome” has been coined to describe the PI3P membrane patch that is generated on or close to the ER (see “PI3P recruits effector proteins: WIPI2, ATGS5/12/16” section) during mammalian autophagy initiation.⁷² The marking of membranous compartments by differing PIs to some extent defines the compartment, because the presence of a specific PI – in concert with the actions of additional targeting/stabilization factors – dictates the proteins that are recruited, and hence the associated biological activity that

is focused on that cytosol-facing patch of membrane.⁷³ As will become clear in the following sections, this scenario certainly holds true for the autophagy system.

In higher eukaryotes, three VPS34 complexes are present: I, II, and III. Complexes I and III regulate autophagy in a negative and positive fashion, respectively, while the class II complex functions in the endosomal network with no direct involvement in autophagy. The class I VPS34 complex is a plasma membrane-associated component of the insulin-signaling pathway that controls mTor activity, and thus indirectly and negatively regulating autophagy.^{54,74} The core of the autophagic class III VPS34 complex is composed of the catalytic VPS34 subunit, with p150 (VPS15 in yeast), and Beclin1 (VPS30/ATG6 in yeast).^{75,76} VPS15 is found on all VPS34 complexes in yeast, and is thought to anchor the VPS34 complex to the membrane through N-terminal myristoylation, and positively modulate the activity of VPS34 through an unknown mechanism.^{70,77} Beclin1 is considered to be the primary regulatory subunit of the class III VPS34 complex, with which both positive and negative modulators bind to determine its context-specific roles during autophagy (Figure 2C). The best-characterized VPS34 class III complex contains ATG14L/Barkor, a peripheral ER protein that concentrates into puncta at early autophagosome initiation sites in response to stimuli.⁷⁶ It is believed to have the capability to sense and maintain the membrane curvature, and preferentially binds membranes enriched for PI3P.⁷⁶ However, and as would be predicted from its involvement with the VPS34 complex, ATG14L concentrates at the nascent autophagosome before PI3P is formed,⁷⁵ implying that, like with ULK1, ATG14L may be stabilized at the nascent autophagosome by the generation of PI3P (Figure 3).

A second VPS34 class III complex that positively regulates autophagy contains UVRAG (UV-irradiation-resistance-associated gene) in the place of ATG14L.⁷⁸ Endophilin B1 (also known as Bif1 [Bax-interacting factor 1]) associates indirectly with Beclin1 via UVRAG, and in common with ATG14L, Endophilin B1 is thought to sense and induce membrane curvature.⁷⁹ Unlike the ATG14L-containing complex, the UVRAG class III VPS34 complex also contributes to autophagosome maturation, although this idea remains controversial.⁷⁰ Furthermore, an inhibitory class III VPS34 complex can assemble from the core elements VPS34, p150, and Beclin1, with UVRAG and “Rubicon” (RUN domain protein as Beclin1 interacting and cysteine-rich containing).⁸⁰ This complex is thought to prevent both autophagy induction and maturation, and probably plays a role in the endosomal compartment.⁸¹

An additional protein that is necessary for the assembly/translocation of the VPS34 complex III is Ambra1 (Activating Molecule in Beclin1-regulated autophagy).⁸² Under normal conditions, Ambra1 sequesters the VPS34 complex to microtubules via binding to cytoplasmic dynein, but this interaction is disrupted upon starvation allowing Beclin1 to translocate to the ER.⁶⁰ This putative regulatory system for AMBRA1-mediated autophagy control has been thrown into doubt following the recent demonstration that Ambra1 facilitates the association between ULK1 and TRAF6 (tumor necrosis factor receptor associated factor 6), an E3 ligase, with the ensuing ubiquitination activating ULK1.⁸³ The exact regulation of Ambra1 remains controversial, with different studies also reporting conflicting data with regard to the phosphorylation status of Ambra1.^{60,83}

PI3P generation is vital for autophagy, but in mammalian cells MTMR3 (myotubularin related protein 3; hereafter referred to as Jumpy) – a member of the myotubularin lipid phosphatase family – has been shown to be needed for productive autophagosome assembly by acting as an important limiter of localized PI3P production.⁸⁴ In support of this theory, yeast Ymr1 (myotubularin-related 1) – the only yeast myotubularin ortholog – has been shown to be needed for the completion of the nascent autophagosome.⁸⁵ It is possible that PI3P needs to be asymmetrically distributed at the isolation membrane for the purposes of autophagosome assembly. In yeast the inside membrane of the autophagosome is enriched for PI3P,⁸⁶ which could be achieved via targeted myotubularin-mediated dephosphorylation. Curiously, though, this asymmetric distribution pattern is reversed in mammals.⁸⁶

PI3P recruits effector proteins:WIPI2,ATG5/12/16

The localized formation of PI3P is an essential step for autophagosome assembly, because when this step is inhibited by drugs such as wortmannin, autophagy is blocked.^{87,88} One purpose of the generation of PI3P by the VPS34 class III complex is to recruit a number of effector proteins that translate the local PI3P signal into downstream membrane remodeling and autophagosome formation steps; however, until recently, a molecular outline of this process was missing. An essential PI3P effector protein family for autophagy is the WIPI (WD-repeat protein interacting with phosphoinositides) family, which in mammals comprises WIPI1–4 – the mammalian functional homologs of yeast ATG18.^{89–91} Importantly, in mammalian cells, WIPI2b has recently been shown to recruit ATG16L1,⁹² which in turn recruits the ATG5-12 conjugate to form the ATG5/12/16L1 complex.⁹³

The ATG5/12/16 complex is needed for ATG8 lipidation (see “Roles and regulation of ATG8 family members in autophagy” section), and is thought to act as a scaffold for the expanding autophagosome, which, in conjunction with ATG8, probably facilitates its growth.^{94,95} ATG5 is required for targeting ATG5-12 to the autophagosome formation site, and ATG5 can indeed arrive independently of ATG12; however, the presence of ATG12 is required for subsequent steps.⁹⁶ In mammalian cells, the ATG5/12/16L1 complex localizes to the isolation membrane via ATG16L1. Indeed, if ATG16L1 is mislocalized to the plasma membrane for example, LC3 is lipidated at and incorporates into this compartment.⁹³ ATG16L1 binds directly to FIP200 and this interaction has been shown to be required for ATG16L1 localization to the phagophore.⁹⁷ Additionally, ATG16 shows a preference for PI3P containing liposomes *in vitro*.⁹⁵ In combination with the study of Dooley et al,⁹² this may indicate that there are several parallel processes by which ATG16L1 localizes to the nascent autophagosome.

ATG16L1 is found in pre-autophagosomal vesicles that derive from the plasma membrane, traffic through the endocytic compartment, and undergo homotypic fusion prior to their integration into the expanding phagophore.^{98–100} Furthermore, ATG16L1 is believed to be a Rab effector for Rab33A/B, possibly explaining how the autophagy proteins are initiated to arrive at the nascent autophagosome.¹⁰¹ Interestingly, the Rab33 GAP, RUTBC1 (RUN And TBC1 Domain Containing 1), has also been identified as an autophagy modulator.¹⁰²

Ubiquitin-like conjugation systems in autophagy: ATG5-12 and LC3-PE

In addition to the aforementioned complexes that regulate distinct, temporal membrane-associated events during early autophagosome assembly, there are two ubiquitin-like conjugation systems that are essential for autophagy (Figure 2D and E). The first conjugation reaction involves the covalent attachment of ATG5 to ATG12, and the second reaction consists of ATG8 family members being conjugated to PE (phosphatidylethanolamine)^{103–105} – a reversible membrane anchor that is required for function of ATG8 in autophagy.^{104,106–109} Neither ATG8 nor ATG12 display sequence homology with ubiquitin, yet each of their crystal structures reveals obvious ubiquitin-like folding.¹¹⁰ The respective conjugation reactions employ separate E2-like enzymes – ATG10 for ATG5 conjugation to ATG12, ATG3 for the lipidation of ATG8 – but ATG12 and ATG8 share ATG7 as their common E1-like enzyme. In fact, ATG7 binds to either ATG8 or

ATG12 in a mutually exclusive manner.¹¹¹ One interesting difference between these two conjugation systems is that ATG5-12 appears to be constitutively conjugated, whereas the covalent attachment of ATG8 to PE is upregulated following autophagy stimulation.^{105,112} Importantly, the product of the “upstream” conjugation reaction – the ATG5-12 complex – acts as an E3-like ligase (in association with ATG16) to aid the covalent attachment of ATG8 to PE, which means that the ATG5/12/16 complex acts to speed up the transfer of ATG8 from ATG3 to PE without conveying any additional specificity.¹¹³

Membrane vesicle trafficking: ATG9 and ATG16

ATG9 acts immediately downstream of FIP200 in the autophagosome assembly pathway. It localizes to poorly characterized membrane reserves near the trans-Golgi network (TGN) or the mitochondria in nonstarvation conditions, upon which it translocates to the autophagosome formation site during starvation.^{114–116} This transport step requires actin in yeast,¹¹⁷ and is dependent on myosin II in *Drosophila*,^{118,119} however it is unclear how transport of ATG9 is regulated in mammalian cells. At the yeast phagophore assembly site, an estimated number of three roughly 30 nm ATG9-containing vesicles fuse to form the phagophore,¹¹⁴ in a process that is dependent on ATG1 and ATG13 for retrieval of ATG9 from the phagophore assembly site.¹²⁰ ATG9 is not found on the autophagosome itself – most likely ATG9 interacts with the phagophore only briefly in a “kiss and run” fusion event, perhaps to donate membrane.¹²¹ Thus ATG9 cycling forms a separate membrane trafficking event – both the anterograde and retrograde movement of ATG9 are required for efficient autophagy.¹²⁰ Intriguingly, ATG9 has also been found to be a direct target for ATG1 in yeast, with ATG9 phosphorylation critical for the recruitment of ATG2 and ATG18 (the yeast equivalents of mammalian WIPI proteins) to the assembling autophagosome.¹²² This again shows that there is positive reinforcement within the recruitment hierarchy of autophagy proteins. Interestingly, ATG9 feeds into the MAPK pathway via p38 interacting protein (p38IP), which is regulated by p38 α MAPK: p38IP binds to ATG9 and is required for starvation-induced ATG9 trafficking.¹²³

Autophagosome biogenesis requires the actions of SNARE proteins (soluble *N*-ethylmaleimide-sensitive fusion protein attachment receptors). SNAREs function to aid the docking and ultimately the fusion of transport vesicles with target organelles. In order to do this, SNAREs assemble specific binding pairs – one on each of the compartments

that are to fuse – which means that only vesicles with the correct cognate SNARE will dock with the desired target compartment. One of the SNARE-requiring fusion steps is thought to be the homotypic fusion of ATG9 vesicles to create a tubule that turns into the phagophore, another being during ATG9 cycling.^{99,124} In addition to ATG9 trafficking to the forming autophagosome in membranous vesicles, ATG16L1 has more recently been identified to arrive at the forming autophagosome from the plasma membrane on vesicular carriers.¹²⁵ In fact, even though ATG16L1 and ATG9 display distinct trafficking pathways, they coincide in the recycling endosome from where they are proposed to traffic together to autophagosomes.¹²⁵ Interestingly, autophagosome precursor maturation has also been shown to require ATG16L1 vesicle fusion, which is also demonstrably dependent on SNAREs.⁹⁹

Roles and regulation of ATG8 family members in autophagy

Yeast have a single ATG8 gene, whereas the situation in mammalian cells is more complicated, with human cells expressing at least eight ATG8 paralogs separated into LC3 and GABARAP (gamma aminobutyric acid receptor associated protein) subfamilies. In the LC3 family there are LC3A, LC3B, LC3B2, and LC3C, while the GABARAP family comprises GABARAP, GABARAP-L1/GEC1/ATG8L, GABARAP-L2/GATE-16, and GABARA-L3. All ATG8 family proteins share structural similarities with ubiquitin,¹²⁶ and all are cleaved shortly after synthesis to reveal a conserved C-terminal glycine (G120 for LC3B and G116 for all the GABARAP family members).^{104,106,127,128} This exposed glycine is later covalently linked to PE (see “Ubiquitin-like conjugate systems in autophagy: ATG5-12 and LC3-PE” section) as an essential step in autophagosome assembly. The ATG8 family – particularly LC3B in mammals – is widely used as a marker for the forming and completed autophagosome.¹²⁹ It is the only autophagy protein that remains attached to the autophagosome following its completion, and has multiple proposed roles during autophagosome formation and maturation. LC3 was initially identified as a microtubule binding protein, and LC3B and GABARAPs display microtubule-binding capabilities at their N-termini.^{130–133} Despite this, no direct microtubule binding functions have yet been found to mediate autophagy.

ATG8 has membrane tethering and hemifusion properties *in vitro*,¹³⁴ suggesting that it, and possibly also some members of the expanded family of mammalian ATG8s, may directly facilitate the fusion of the phagophore to form the

completed autophagosome; however, it has been noted that ATG8-mediated hemifusion does not occur in liposomes containing physiological concentrations of PE.¹²⁴ The findings of Nakatogawa et al¹³⁴ have been confirmed to some extent in experiments using certain mammalian ATG8 family members, both in a cell free system and in cells, where the deletion of the N-termini of GATE-16 and LC3 – responsible for the membrane fusion capabilities of the ATG8 family – resulted in the accumulation of immature phagophores and/or a failure in sealing liposomes.¹³⁵ Interestingly, the amino acids that mediate membrane fusion are not conserved between LC3 and GATE-16, with positive amino acids responsible for fusion in LC3 and neutral amino acids in GATE-16.¹³⁵ Furthermore, in mammalian cells the fusion activity of LC3 is dispensable for fusion of autophagic vacuoles with early or late endosomes,¹³⁶ meaning that it is unlikely that this capability of LC3 is required for autophagosome maturation.

In yeast, ATG8 protein concentration is one factor that has been found to influence autophagosome size, but this has no apparent effect on steady state autophagosome numbers or their rate of assembly.¹³⁷ The density of ATG8 on the convex surface of the yeast phagophore corresponds with the density expected for a coat protein leading to the proposal that ATG8 acts as a coat adaptor in concert with the ATG12-ATG5 complex.¹³⁸ This finding has been supported by an *in vitro* study by Kaufmann et al⁹⁴ suggesting that ATG8 is part of a coat complex during autophagosome formation, which is subsequently disassembled by ATG4 deconjugase activity. ATG8 also serves a further scaffolding role of recruiting the ATG1 (ULK1) complex to the forming autophagosome,¹³⁹ this both positively reinforces autophagosome formation (Figure 3) and may support ULK1 targeting of proteins at precise locations on the forming autophagosome. LC3 has been shown to be required for autophagosome expansion, whereas GATE16/GABARAP is involved in autophagosome sealing.¹⁴⁰ This important study highlights how regulated and sequential employment of different ATG8 family members in mammalian cells has the potential to fine-tune the autophagosome assembly pathway to meet diverse cellular needs.

LC3 binds readily to the cargo adaptor p62 (sequestosome1 or SQSTM1).⁵ This means that as mentioned above “nonselective” forms of autophagy have an element of specificity, since p62 binds tightly to ubiquitinated protein targets. In mammalian cells, the various ATG8 family members show differing affinities for p62; LC3 efficiently incorporates p62 into autophagosomes, whereas GABARAP lacks this capability.¹⁴¹ Importantly, p62 is not the only autophagy-associated cargo adaptor; neighbor of BRCA1 gene 1 (NBR1), which acts as

a cargo receptor during selective autophagy,¹⁴² also interacts directly with the main members of the ATG8 family.¹⁴³ Conversely, Nix – a mitophagy cargo adapter protein – binds preferentially to GABARAP family members.¹⁴⁴ Interestingly, an extensive autophagy network analysis identified 67 ATG8 family interactors, and 31 of these were found to be specific for a single subtype of the ATG8 family.¹⁴³ Once again, this shows how selective utilization of different classes of mammalian ATG8 proteins can have a profound effect on autophagosome cargo, providing further functional flexibility in the autophagic response.

The LIR motif as an important interacting module in autophagy that confers selectivity to the autophagy response

Many cargo adaptor and other proteins that bind to the ATG8 family contain the consensus sequence WXXI/L, which is known as the LIR (LC3-interacting region) motif.^{36,145} In most cases, LIR motifs facilitate cargo receptor binding to ATG8 family members, and are therefore important for cargo incorporation into the nascent autophagosome – examples include p62 and NBR1; however, LIR motifs have been identified in ULK1 and ATG12, and these facilitate targeting and/or stabilization of the autophagy machinery at the autophagosome assembly site.^{66,94} Competition for LIR binding between cargo and the autophagosome assembly machinery has been demonstrated *in vitro*, revealing a plausible molecular mechanism for the different functions of ATG8 on the inside and outside of the autophagosome (in cargo recognition and as a putative coat, respectively).⁹⁴

Within the known LIR motifs, there are different classes with differential binding affinities for ATG8 family members. In the case of the LIR motif in NBR1, the binding sequence is YXXI, and exchanging the tyrosine at P1 to tryptophan increases the LC3 binding affinity of this motif, implying that additional factors might facilitate/strengthen this particular interaction.¹⁴⁶ The atypical LIR motif identified in ATG12 includes residues Phe185 and Ile111 which are well separated in the primary sequence.⁹⁴ Consequently, this LIR motif is tertiary structure-dependent; that is to say, it is formed in 3D space by precise folding of the polypeptide chain.⁹⁴ Importantly, the LIR motif described for the cargo adaptor NDP52 is also atypical, in that it does not conform to the WXXI/L consensus, but nevertheless it binds readily to mammalian LC3C due to compensatory alterations in the aromatic pocket of its cognate ATG8 family member.¹⁴⁷ Perhaps most significantly, switching the native isoleucine at position 133 in the NDP52 LIR for tryptophan dramatically

increased its affinity for LC3C (into the nanomolar range), but simultaneously destroyed its specificity for LC3C.¹⁴⁷

Based on these findings, it can be assumed that other noncanonical LIR domain-containing proteins exist in diverse cargo proteins, meaning that sequence analysis approaches that rely on classical properties in the primary structure alone (eg, iLIR; <http://repeat.biol.ucy.ac.cy/iLIR/>) will fail to identify these. To add to this complexity, LC3 binding to LIRs may be occluded and/or stimulated by posttranslational modifications; for example, the LIR motif of the mitophagy adaptor, FUNDC1 (FUN14 Domain Containing 1), contains a phosphorylation-competent tyrosine, and dephosphorylation at this site during hypoxia is required to activate LC3 binding.¹⁴⁸ Interestingly, FUNDC1 mitophagy activity is also regulated by phosphorylation/dephosphorylation at a two further sites, S13 and S17, by CK2/PGAM5 and ULK1 respectively,^{149,150} meaning that there are likely to be multiple regulatory influences on LIR motif activity.

Further roles for ATG8 family members during autophagosome maturation

LC3 plays key roles on the outside as well as the inside of the autophagosome. By acting as a Rab GAP adaptor, LC3 integrates autophagosomes into the global membrane trafficking network, enabling them to be sorted to and fuse with the correct endomembrane components.¹⁵¹ Here, the Rab GAP TBC1D5 was found to bind competitively to retromer components on the recycling endosome and to LC3. GABARAP is believed to act as a scaffold to mediate signal transduction through the RabGAP, TBC1D25 (also known as ornithine aminotransferase-like 1 or Oat1),¹⁵² and TBC1D25 is required for fusion of the autophagosome with the lysosome.¹⁵² It has GAP activity toward Rab33, and active Rab33 is thought to recruit the ATG5/12/16 complex to the phagophore. This could mean that Rab33 is inactivated as soon as GABARAP is recruited to the phagophore. As the ATG8 family members have been shown to play different roles in autophagosome biogenesis, and TBC1D25 preferentially binds GABARAP,¹⁵² it is possible that the regulated removal of other ATG8 family members (eg, LC3B) from the outside of the autophagosome is required for optimal TBC1D25 binding to the mature autophagosome. It may also be that Rab33 needs to be deactivated by GATE16 to complete a phagophore, as GATE16 is required for autophagosomal sealing; thus Rab33 inactivation could be a prelude to autophagosome completion. Rab7 has been shown to be required in conjunction with LC3 and FYCO1 (FYVE and coiled-coil domain containing 1) to form an adaptor protein

complex, essential for plus end directed, kinesin-mediated microtubule transport.¹⁵³ Subsequently Rab7 is required for autophagosome-endosome/MVB (multivesicular body) fusion and a depletion of Rab7 leads to an accumulation of autophagosomes.¹⁵⁴ This also means that at least some ATG8 is required on the outside of the autophagosome to mediate autophagosome trafficking.

Roles and regulation of the ATG4 endopeptidase family

ATG4 is a cysteine endopeptidase that is essential for controlling ATG8 processing during autophagy. It serves two distinct roles, priming and delipidation.¹²⁸ Initially, ATG4 cleaves newly synthesized ATG8 – which is produced in an immature pro-form – to reveal a C-terminal glycine (the priming step). The exposed glycine is subsequently conjugated to PE at the expanding isolation membrane, meaning that the priming step is indispensable for autophagy.^{128,155} The second role of ATG4 is to deconjugate membrane-bound ATG8 in order for ATG8 to disassociate from the membrane (the delipidation step).^{107,128} ATG8 delipidation has been shown to be required for the completion of autophagosome assembly in yeast,^{137,156,157} although it is not yet known whether this holds true in mammalian cells.

Akin to the situation regarding mammalian ATG8 divergence, ATG4 is represented by four paralogs in mammalian cells; namely ATG4A, ATG4B, ATG4C, and ATG4D. These are collectively known as the ATG4 cysteine endopeptidase family, or the autophagins.¹⁵⁸ The structure of ATG4 resembles that of the papain family of proteases, despite low sequence homology.^{159,160} Each of these ATG4 family members displays subtly differing substrate affinities for the various mammalian ATG8 family members, thus potentially allow fine-tuning of the autophagy response with respect to autophagosome form and content in mammalian cells. ATG4B displays the broadest substrate specificity,^{161–164} while ATG4A is selective for GATE16,¹⁶⁵ and ATG4D shows activity exclusively against GABARAP-L1 *in vitro*.^{161,163}

To add an additional level of regulatory complexity, ATG4B (but not other ATG4 family members) is down-regulated by RNF5 (Ring Finger Protein 5), an E3 ubiquitin protein ligase on membranous compartments.¹⁶⁶ Reduced RNF5 levels leads to an increase in ATG4B, and subsequently lipidated LC3-II levels, but does not result in a change in the levels of the GABARAPs. Another example of ATG4 regulation was demonstrated by Scherz-Shouval et al¹⁶⁷ who showed that reactive oxygen species inhibit ATG4A and ATG4B, by modifying an essential cysteine located close to the catalytic

site triad of these family members. The authors hypothesized that ATG4s are active in a reducing environment; autophagy induction releases a burst of the oxidizing agent hydrogen peroxide, which inhibits ATG4A/B locally until the newly formed autophagosome leaves the oxidizing environment. At this point, ATG4 becomes active again and delipidates the appropriate ATG8 family member before the autophagosome fuses with the lysosome.¹⁶⁷ Atg4C and Atg4D both have a caspase cleavage site, and immediately downstream, a mitochondrial-targeting sequence, suggesting that apoptotic cleavage triggers mitochondrial import.¹⁶⁸

In vivo studies of ATG4 deletion in mouse

The apparent specificity of ATG4 endopeptidases for ATG8 family members reported in vitro is not supported by genetic deletion studies in mouse, which generally reveal a milder effect on autophagy than might be expected.^{169–171} This is perhaps due to adaptive redundancy in the developing mouse, nevertheless the ATG4B and ATG4C knockout models have provided clues as to the individual roles of these family members in vivo. The ATG4C knockout mouse is viable and fertile, showing only an increased susceptibility to fibrosarcoma, decreased locomotor activity after prolonged starvation, and a mild decrease in autophagic activity measured by LC3B lipidation in the cells of the diaphragm.¹⁷⁰ Thus, ATG4C is dispensable under normal conditions, but under stress conditions such as prolonged starvation or in the presence of carcinogens, a protective role is revealed. The ATG4B knockout mouse, despite showing a pronounced defect in ATG8 family member lipidation throughout its tissues, also has a mild phenotype with a loss of equilibrium due to the abnormal formation of otoconia (organic calcium carbonate crystals) in the inner ear.¹⁶⁹ It is noteworthy that some tissues showed partial lipidation of LC3B, meaning that another factor may functionally compensate for ATG4B under certain conditions.¹⁶⁹ When the ATG4B knockout mouse was further studied, abnormal spheroid-like bodies were found in the neuropil of the deep cerebellar nuclei.¹⁷¹ These abnormal structures were interpreted as lesions, and this translated into slightly poorer performance on a rotarod, giving a second physiological explanation to the impaired locomotion of the mutant mice.¹⁷¹ Additionally, homozygous ATG4B^{-/-} mice were born at slightly lower incidence than Mendelian genetics would predict, possibly indicating an issue in fetal development upon ATG4B deletion.¹⁷¹

Many questions remain about the control of autophagosome assembly and function by ATG4 family members. There is clear evidence that ATG8 priming and delipidation are

needed for efficient autophagosome formation, maturation, and vacuolar delivery in yeast,^{10,107,128,156,172,173} but the picture is much less clear in mammalian cells. It has been shown that overexpression of wild-type and dominant-negative mutant (C74A) ATG4B arrests autophagosome formation at the ATG8 lipidation step,¹⁷⁴ and apparently at the lysosomal fusion stage in human erythroid cells,²⁹ but more sophisticated tools will be needed to decipher contribution of the delipidation of ATG8 family members during phagophore closure, autophagosome maturation and trafficking, and lysosomal fusion.

Other factors implicated in the control of autophagosome assembly

VMP1 (Vacuole Membrane Protein 1) is a transmembrane, Beclin1-binding protein that has been shown to be required for autophagy.¹⁷⁵ VMP1 localizes to the ER and generates punctate structures upstream of the VPS34 complex upon autophagy stimulation,¹⁷⁵ although other work has described constitutive ER-associated VMP1 puncta that do not colocalize with early autophagy markers (ULK1).¹⁷⁵ VMP1 competes with BCL-2 for binding to Beclin1, suggesting that it may release Beclin1 at local ER-associated sites.¹⁷⁵ VMP1 silencing blocks autophagic flux, and causes an accumulation of abnormally large early autophagy structures (positive for ULK1, WIPI1, DFCP1), suggesting that it might act downstream of the assembly site initiation stage.¹⁷⁵ In this respect, it is noteworthy that VMP1 silencing did not increase steady state ATG16L1 puncta numbers,¹⁷⁵ arguing that its action is restricted to the PI3P generation step, perhaps to coordinate the turnover of PI3P.

As mentioned above, Rab33B/A is required to localize ATG16 to the phagophore,¹⁰¹ but there are additional Rabs that are believed to function in autophagy, at least in yeast. Ypt1 (yeast protein two 1), and its GEF, TRAPP III (transport protein particle III) – also a multi-protein tethering complex – are needed for autophagy, although with the exact stage and mechanisms of action unknown.^{176,177} Notably, studies of selective autophagy have implied that Ypt1 recruitment occurs downstream of ATG9.¹⁷⁸

Autophagosome maturation

Fully formed autophagosomes travel along the microtubule network toward the lysosome, fusing en route with early endosomes and late endosomes/MVBs, to form a hybrid structure termed an amphisome.^{179–181} The autophagosome/amphisome acidifies as it matures, either due to the pH of the structures it fuses with and/or through the actions of proton pumps. Indeed, the autophagosome is known

to attain a proton pump on its outer membrane, as well as lysosomal LAMP2 (lysosomal associated membrane protein 2), well before the formation of a bona fide autolysosome.^{179,182,183} Despite autophagosomes having been shown to fuse to a similar extent with early and late endosomes *in vitro*,¹³⁶ these different steps appear to use distinct fusion machineries.

Autophagosomes fuse with endosomes and/or MVBs to form amphisomes

Rab11 has been shown to be required for the fusion of autophagosomes to MVBs in K562 cells.¹⁸⁴ Rab11 has however also been shown to be required for autophagosome formation,¹⁰² meaning that Rab11 may serve two separate roles during assembly and maturation. The Rab GAP TBC1D5 has also been potentially implicated during autophagosome maturation. It binds both LC3 and the endosome competitively, and has two distinct LIR motifs.¹⁵¹ This means that TBC1D5 may form an important bridge between the endosome and the autophagosome, potentially aiding the tethering of these two compartments.

A second function for UVRAG – this time independent of Beclin1, and thus the VPS34 complex in general – is in binding to the endosomal tether C-VPS, to mediate fusion of autophagosomes with endosomes and/or lysosomes.¹⁸⁵ Interestingly, it is an entirely different subdomain on UVRAG that binds Beclin1 when in conjunction with the VPS34 complex, than when binding C-VPS; thus Beclin1 and C-VPS do not compete for access to UVRAG.¹⁸⁵ UVRAG is probably involved in regulating/mediating fusion within the endosomal network, independently of autophagy.¹⁸⁵ The C-VPS complex is the core of either the HOPS (homotypic fusion and vacuole protein sorting) or the CORVET (class C core vacuole/endosome tethering) complex involved in endosome-endosome or endosome-lysosome fusion.¹⁸⁶ A SNARE implicated in the autophagosome-endosome fusion stage is VAMP3 (vesicle associated membrane protein 3)/cellubrevin. VAMP3 has been shown to be required for the fusion of autophagosomes with MVBs in erythroid maturation.¹⁸⁷ Here, it is worth noting that VAMP3 has also been shown to be required for homotypic fusion of ATG9 and ATG16L1 containing pre-autophagic membrane structures during autophagosome initiation.⁹⁹

Autophagosome fusion with the lysosome

Fusion of autophagosomal structures with the lysosomal compartment depends upon defined membrane identity, as dictated by the phosphoinositide PI(3,5)P₂. This phosphoinositide

is required for endomembrane acidification, and for autophagosome maturation in mammalian cells,¹⁸⁸ but whether it plays a direct role in autophagy has yet to be confirmed.⁷⁴ Other phosphoinositides and lipids have been identified as having autophagy roles in a variety of studies, and these are summarized well elsewhere.⁷⁴ Rab24 has been implicated in autophagy because its position changes drastically upon starvation, to colocalize with autophagic vacuoles.¹⁸⁹ Rab24 is thought to influence autophagosome-lysosome fusion with the aid of the *dr*s tumor suppressor.¹⁹⁰ Rab7 is implicated in autophagosome/lysosome fusion,¹⁵⁴ possibly in conjunction with HOPS complex GEF activity.^{191–193}

The ESCRT (endosomal sorting complex required for transport) machinery has also been implicated during late stages of autophagosomal maturation. ESCRT is required for autophagosome-lysosome fusion in human cells and in *Drosophila*.^{194–196} The ESCRT complex was originally identified through its involvement in MVB biogenesis and the sorting of ubiquitinated proteins to MVBs and lysosomes.¹⁹⁷ It is possible that the ESCRT machinery is directly involved in autophagosome maturation, but the data also support a situation where autophagy requires functional MVBs.

Proteins acting at the lysosomal membrane have also been implicated in autophagosome maturation; LAMP1 and LAMP2 deficient mouse embryonic fibroblasts (MEFs) have been shown to accumulate immature and mature autophagosomes.^{198,199} Surprisingly, lysosomal protease activity in these cells appeared to be normal, but the lysosomes showed some structural abnormalities.^{198,199} This led to the hypothesis that LAMPs may be involved in autophagosome-lysosome fusion, although it is very difficult to tell whether this is a specific effect of inhibition of fusion, or merely due to attenuated lysosomal function.^{198,199} Impaired phagosome fusion with lysosomes in LAMP1 and LAMP2 double knockout MEFs has been shown to be due to an inability of phagosomes to move toward the lysosome.²⁰⁰ This means that the same could hold true for autophagosome-lysosome fusion; an absence of LAMP1/2 would mean a defect in trafficking, and thus a coincidental fusion defect rather than a mechanistic one.

The autophagosome-lysosome fusion event itself is likely to be governed by SNARE proteins. In yeast there are reports of a number of SNAREs being implicated,²⁰¹ for example syntaxin 17.²⁰² Upon starvation, syntaxin 17 translocates to fully formed autophagosomes, where it mediates fusion of the autophagosome with the lysosome through its partner SNAREs, SNAP-29 (soluble *N*-ethylmaleimide-sensitive factor attachment protein 29) and VAMP8.²⁰² Indeed, both Vti1b (vesicle transport through interaction with the t-SNAREs

homologue 1B) and VAMP8 have been shown independently to be required for autophagosome-lysosome fusion,^{203,204} and TI-VAMP/VAMP7 has been implicated in amphisome-lysosome fusion.¹⁸⁷ Mechanistically, syntaxin 17 has been shown to directly interact with the HOPS complex, providing HOPS with a second potential role in autophagosome maturation.²⁰⁵ That differing SNAREs are required for different steps in the autophagosome maturation process indicates a high level of regulation. It is worth noting that these same SNAREs have been identified as a requirement for the maturation of the autophagosome precursors.⁹⁹ Lastly, and perhaps surprisingly, the ATG5-12 conjugate has also been implicated in autophagosome lysosome fusion; TECPR1 (Tectonin Beta-Propeller Repeat Containing 1), which localizes to autolysosomes, is suggested to bind to PI3P and to ATG5-12 in a manner that excludes ATG16, to mediate tethering and fusion.²⁰⁶

Membrane sources for the autophagosome

There are two models for the source of the membrane in the early autophagosome: one is the maturation model and the other is the assembly model. The maturation model proposes that the membranes forming the early autophagosome are derived from the ER.²⁰⁷ The assembly model states that the membranes needed for autophagosome assembly either stem from a separate, dedicated pool, or are synthesized *de novo* at the assembly site.²⁰⁸ These two models are not mutually exclusive, and most probably autophagy utilizes both. In addition to the two hypotheses regarding membrane origins, there are two theories on the involvement of the ER in autophagosome biogenesis. One of them states that the ER is merely a scaffold on which the autophagosome forms, whereas the other claims that the autophagosome is contiguous with, and therefore an extension of the ER.

The ER as a cradle for autophagosome biogenesis

Using a reporter protein that binds selectively to PI3P (double FYVE-containing protein 1; DFCP1) Axe et al⁷² demonstrated that PI3P defines a subdomain of the ER that is formed upon autophagy induction. They showed that this domain forms a cup-like structure, the center of which progressively recruits LC3, implying that the autophagosome forms in a cradle of ER membrane.⁷² The early stages of autophagosome formation have also been described by electron microscopy tomography. Independent studies found that autophagosomes are synthesized upon the ER, and provided evidence that the nascent autophagosome is cradled

between two ER cisternae.^{209,210} These high-resolution studies suggested that there might be a physical connection between the autophagic isolation membrane and the ER. This would imply that the ER is both a scaffold for autophagosome formation, and a possible donor of membranes. Whilst compelling, it must be pointed out that these observations were based on EM tomography reconstructions, which are necessarily subjective. Interestingly, 70% of autophagosomes have been shown to contain a portion of ER membrane, implying that during the formation process the inner ER scaffold might become enclosed by the nascent autophagosome.²⁰⁹ It is also interesting to note that the isolation membrane often appears unusually electron dense when visualized by electron microscopy following aldehyde fixation, implying that the double membranes might be in very close proximity (at least in fixed samples), which hints at a mechanism for luminal and membrane protein exclusion from the expanding isolation membrane. At present very little is known about how the ER is reorganized to accommodate the nascent phagophore, and a molecular explanation is needed.

Membranes of diverse organelles contribute to the forming autophagosome

Aside from the ER, many other organelles have been implicated in membrane delivery to the expanding autophagosome. Determining the origin of the autophagosomal membrane is problematic, though, because the autophagosomal membrane appears to be devoid of ancillary markers.²¹¹ Early autophagy markers (ATG2, ATG18, and ATG9) have often been observed in close proximity to ER exit sites (ERES) in yeast.²¹² The involvement of ERES in autophagosome formation has been confirmed by a proteomics study, which showed that phagophores and ERES were physically and functionally linked.²¹³ The authors speculated that in yeast, autophagosome biogenesis is linked to the generation of a COPII compartment that is similar to the ERGIC (ER-Golgi intermediate compartment) in mammals, which agrees with a separate study showing involvement of the ERGIC in autophagosome assembly.^{213,214} Another finding that backs up the requirement for the ERGIC in mammals or the ERES in yeast is that specific COPII components are required for autophagy.²¹⁵ The aforementioned study showed that the ERGIC compartment is needed for LC3 lipidation.²¹⁴ Pharmacological disruption of the ERGIC prevented efficient generation of ATG14 puncta,²¹⁴ suggesting that a functional ERGIC may be required for very early initiation stages of autophagosome biogenesis (specification

of the assembly site itself). If membrane contributions to the phagophore from the ERGIC were a limiting factor, then one might have expected to record an accumulation and not a depletion of ATG14 puncta. Finally, ATG9 has been proposed to traffic from, or at least through the Golgi and the ERGIC, leading to the hypothesis that Golgi membranes contribute to the expanding autophagosome.^{103,216} Golgi-derived membranes have further been proposed to contribute to the phagophore, as Golgi derived lectins localize to the expanding rims of the phagophore.²¹¹

Mitochondria have been suggested to contribute lipids directly to the forming autophagosome.²¹⁷ This could be either through direct lipid exchange or by a more complex autophagy-specific remodeling event. More recently, the MAM (mitochondrial-associated membrane at the ER) has been identified as a possible site of the autophagosome initiation.²¹⁸ Here, important autophagosome assembly molecules including Beclin1 and ATG14L have been shown to concentrate,²¹⁸ and the implications for this with respect to autophagy and ER-to-mitochondrial calcium homeostasis are covered elsewhere.²¹⁹

The endosomal system is increasingly being implicated as a major contributor to the process of autophagosome assembly. A need for a functional early endosomal system for autophagosome progression has been widely shown (eg, (184)). The recycling endosome may contribute membrane to the autophagosome, as ULK1 has been shown to initially relocate to the recycling endosome upon starvation.^{67,102} This study is supported in part by evidence that endosomal sorting nexin 18 is required for autophagy through its role in tubulating the endosome as a prelude to membrane delivery to the autophagosome.²²⁰ Additionally, both ATG9 and ATG16L1 have been shown to cycle through the recycling endosome, potentially acquiring membrane en route to contribute to the autophagosome expansion.^{100,125} In a similar fashion, the plasma membrane has been suggested to donate membrane to the autophagosome via ATG16L1 vesicles.^{98,221} Furthermore, ATG9 has more recently been shown to also localize to the plasma membrane prior to moving to the recycling endosome, which means that ATG9 could also bring part of the plasma membrane with it. Although internalized separately, ATG9 and ATG16L1 coalesce within the same endocytic subcompartment upon starvation.¹²⁵ Lipid droplets have been shown to donate lipid to the nascent autophagosome by proximity.²²² Further to this, the mobilization of lipids with the aid of a lipase was shown to be required for autophagy. Dupont et al²²² identified PNPLA5 (patatin-like phospholipase domain containing 5) as such a lipase. Finally, the

plasma membrane has been proposed to act as a reservoir for autophagy proteins that become anchored to connexin 43.²²³ Upon starvation, connexin 43 is degraded releasing ATG16L1 to amplify the autophagy response.²²³ As autophagy is such a crucial facet of cellular stress response pathways, it is likely that additional regulatory pathways that converge with autophagy will be revealed in the coming years.

Disclosure

The authors report no conflicts of interest in this work.

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