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## Autophagy proteins in macroendocytic engulfment

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### Abstract

Eukaryotic cells must constantly degrade both intracellular and extracellular material in order to maintain cellular and organismal homeostasis. Two engulfment pathways, autophagy and phagocytosis, mediate the turnover of intracellular and extracellular substrates by delivering material to the lysosome. Historically these were thought to be separate pathways, but recent studies have revealed the direct participation of autophagy proteins in phagocytosis. Autophagy proteins lipidate LC3 onto phagosomes and other macroendocytic vacuole membranes, and are required for lysosomal degradation of engulfed cargo, demonstrating an autophagosome-independent role for autophagy proteins in mediating the turnover of extracellular substrates. This review discusses the biological systems where autophagy proteins have been found to regulate lysosome fusion to non-autophagic membranes.

### Keywords

autophagy; phagocytosis; entosis; engulfment; lysosome

### Pathways of lysosomal degradation

The capacity to degrade both intracellular and extracellular material is a critical function of eukaryotic cells. Lysosome-mediated turnover of damaged organelles and protein aggregates, as well as the clearance of diverse extracellular substrates including dying cells and pathogenic organisms, contributes to maintaining the fitness of individual cells and to the development and homeostasis of multicellular organisms. Through endocytic pathways, plasma membrane and extracellular material are internalized into vesicles that undergo maturation to target internalized cargo either to lysosomes for degradation, or alternatively back to the plasma membrane for recycling [1]. Macroscale endocytic (macroendocytic) pathways, such as phagocytosis and macropinocytosis, are defined by the relatively large sizes (>0.2 μm diameter) of the vesicles formed by mechanisms that generally require rearrangement of the actin cytoskeleton to promote engulfment [2]. Through autophagy pathways, intracellular material is degraded by lysosomes [3]. By macroautophagy (hereafter autophagy), intracellular substrates are engulfed into double-membrane vesicles called autophagosomes that deliver material to lysosomes for digestion, or alternatively to the plasma membrane for secretion. Autophagosomes can envelop cargo non-specifically, during bulk turnover of cytoplasm that enables the survival of nutrient-deprived cells, or specifically, to target damaged organelles, protein aggregates, or specific proteins for

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lysosomal degradation or secretion [4–6]. Recently evidence has emerged that proteins from the autophagy pathway control lysosome fusion to macroendocytic vacuoles, in an autophagy-independent manner, suggesting that the proteins that control this well-known pathway of intracellular substrate turnover more generally control the degradation of extracellular substrates as well. In this review we will discuss this emerging role for autophagy proteins in mediating lysosome fusion that is separable from the process of autophagy.

## Canonical autophagy machinery

A large body of work generated over the past decade in both yeast and mammalian systems has identified AuTophagy-related (*Atg*) genes and their interacting proteins, which comprise a complex hierarchy that controls each step of autophagosome formation, including initiation, elongation and closure (Figure 1A, the details of autophagosome formation have been reviewed elsewhere [4, 7]). Autophagy proteins generally function in four major groups: the Atg1 kinase complex, the Vps34 class III phosphatidylinositol 3-kinase complex, two ubiquitin-like conjugation systems involving Atg8 and Atg12, and a membrane-trafficking complex involving Atg9. In mammalian cells, the key upstream kinase that regulates induction of most forms of autophagy is the Atg1 homolog Ulk1, which forms a complex with Atg13, Fip200 and Atg101 [8–10]. Under nutrient-replete conditions, the activity of the Ulk1 complex is inhibited by the mammalian target of rapamycin (mTor), which phosphorylates Ulk1 and Atg13 to block autophagy induction [11]. Also essential for the induction of autophagy is the generation of phosphatidylinositol 3-phosphate (PI3P) at sites of autophagosome formation, through the action of Vps34 [12, 13]. Vps34 forms a complex with Beclin 1 and Vps15, and these core proteins associate with additional binding partners to form at least two mutually exclusive complexes: i) complex one includes Atg14L, which is implicated in autophagy; ii) complex two contains UVRAG (UV irradiation resistance-associated gene), which is functionally linked to autophagy and also endosomal trafficking [14]. Downstream of Ulk1 and Vps34, autophagosome formation is controlled by two ubiquitin-like conjugation systems that function in expansion of phagophore membranes, which are the precursors to autophagosomes [11]. In one conjugation system, Atg12 is conjugated to Atg5 in an Atg7- and Atg10-dependent manner [15]; the resulting conjugation binds to Atg16L, forming the Atg5-12:16L multimeric complex. In the other system, Atg3 and Atg7 conjugate LC3-I, which is formed by the cleavage of LC3 by Atg4, to phosphatidylethanolamine (PE) to form LC3-II. The site of LC3 lipidation is determined by the E3 enzyme-like activity of the Atg5-12:16L multimeric complex, directed by PI3P [16]. Lipidated LC3 and its other family members (e.g. GATE-16) stimulate elongation and finally closure of autophagosomes, after which Atg proteins are removed, and LC3-II is recycled from the outer autophagosomal membrane by Atg4 upon lysosome fusion [17–19]. The conversion of LC3-I to LC3-II, identified by localization to autophagosome-associated puncta, or by increased mobility via SDS-PAGE, is one of the main methods used to monitor autophagy in cells [20]. Recent demonstrations of LC3 lipidation to non-autophagic membranes however suggests that not all vesicles in cells associated with LC3 may be autophagosomes.

## Autophagy proteins promote lysosome fusion to single-membrane vacuoles

Macroendocytic vacuoles such as phagosomes, macropinosomes and entotic vacuoles can be targeted by autophagy proteins that lipidate LC3 to these single-membrane compartments, in an autophagosome-independent manner. Considering that autophagosomes are formed from pre-cursor single-membrane sources and expand by fusion of single-membrane vesicles [21], it may seem semantic to specify these LC3-associated compartments as single-

membrane structures, but the final structure of the LC3-associated membrane to which lysosomes fuse is a useful descriptor to differentiate these non-canonical roles of autophagy proteins from canonical autophagy. A non-autophagic role for autophagy proteins in facilitating lysosome fusion to single-membrane compartments has recently been described in a variety of cell systems.

### Phagocytosis of pathogenic organisms

Many pathogens are engulfed by phagocytosis and subsequently destroyed by lysosomal enzymes during phagosome maturation. Some pathogenic organisms can be targeted by the canonical autophagy pathway through a process termed xenophagy (see [22, 23] for review). Pathogens residing in phagosomes (e.g. *M. tuberculosis*) or those that escape into the cytosol (e.g. *L. monocytogenes*) can be wrapped by double-membrane autophagosomes and subsequently delivered to lysosomes for degradation. Consistent with a role for canonical autophagy in pathogen clearance, the induction of autophagy through starvation or inhibition of mTor can in some circumstances increase pathogen destruction [24, 25]. Alternatively, autophagy induction can favor the viability of some pathogenic organisms, such as *C. burnetii*, which resides in an acidified parasitophorous vacuole and derives nutrients through autophagosome fusion [26, 27].

But whereas some pathogens are targeted by canonical autophagy, there is now accumulating evidence that autophagy proteins can also directly modify single-membrane phagosomes in an autophagosome-independent manner (Figure 1B). The transient recruitment of autophagy proteins LC3 and Beclin 1 to phagosomes housing *E. coli*, yeast or LPS-coated latex beads was first demonstrated [28] in a process termed LC3-associated phagocytosis (LAP), which has since been reported by other groups [29, 30]. GFP-LC3 was shown by time-lapse microscopy to recruit to phagosomes after Beclin 1 translocation and PI3P formation, followed by acidification and lysosome fusion. Using macrophages from knockout mice, the autophagy proteins Atg5 and Atg7 were shown to be required for LAP, in an autophagy-independent manner. Double-membrane autophagosome structures were not detected at phagosome membranes by transmission electron microscopy (TEM), and stimulation of canonical autophagy by rapamycin treatment or starvation was insufficient to induce LC3 recruitment. LAP was required for the efficient acidification of phagosomes and also for the killing of phagocytosed yeast. Proteomic analysis of phagosomal membranes has also demonstrated an interaction with autophagy proteins, including endogenous lipidated LC3 [30]. However, these authors concluded that phagosomes acquired autophagy proteins through fusion with autophagosomes, a conclusion based on the use of the Vps34 inhibitor 3-methylalanine (3-MA), which is predicted to inhibit both canonical autophagy and LAP. Interestingly, this study also used uncoated latex beads as targets for the phagocytic engulfments that recruited LC3, whereas examination of similar phagosomes by time-lapse microscopy of GFP-LC3-expressing cells has failed to reveal such a recruitment, potentially owing to the different sensitivities of these approaches [28, 29, 31].

Interestingly, the roles of the canonical autophagy pathway and LAP in targeting engulfed pathogens may not always be mutually exclusive. *Salmonella* is engulfed by cells into single membrane vacuoles termed *Salmonella*-containing vacuoles, or SCVs, which have previously been reported to be targeted for destruction by autophagosomes [32]. Recent work has shown that the ATG5-12:16L complex is recruited directly to the SCV, where it promotes the lipidation of LC3 onto the forming autophagosome. However, in the absence of the Ulk complex and autophagosome formation, the ATG5-12:16L complex can lipidate LC3 directly onto the single-membrane SCV in a process resembling LAP [33]. Therefore, whereas in most systems the lipidation of LC3 onto single-membrane vacuoles occurs in the absence of evidence for double-membrane autophagosome structures, in some

circumstances these seemingly distinct mechanisms may collaborate, or potentially LAP may act as a compensatory mechanism in the absence of autophagy.

### Apoptotic cell phagocytosis

A role for autophagy in apoptotic cell engulfment was originally reported during embryoid body cavitation. Cells destined to die by apoptosis required canonical autophagy to generate ATP in order to express 'come find me' signals (lysophosphatidylcholine) and 'eat me' signals (phosphatidylserine) [34]. More recently a number of groups have reported a role for autophagy proteins within phagocytes in regulating the degradation of apoptotic cells. It was thought that LAP might be restricted to pathogen-containing phagosomes, yet LC3 was shown to recruit to phagosomes containing both apoptotic and necrotic corpses (Figure 1B) [31, 35]. Importantly, in these studies LAP and canonical autophagy could be distinguished genetically. While apoptotic cell LAP is dependent on the downstream lipidation factors Atg5, Atg7 and Beclin 1, it occurs independent of the upstream Ulk kinase complex that is required for autophagy. Correlative light electron microscopy (CLEM) was also used to demonstrate that the LC3-positive phagosome remained a single-membrane vacuole, adding further evidence that autophagosome structures are not involved in recruitment of LC3 [31]. Like pathogen LAP, autophagy proteins facilitated the maturation of apoptotic phagosomes, and inhibition of LC3 recruitment hindered the degradation of corpses. Inefficient corpse clearance in Atg7 knockout macrophages, as a consequence of LAP inhibition, promoted increased secretion of pro-inflammatory cytokines (IL-1b, IL-6), whereas wild-type cells generated anti-inflammatory cytokines (IL-10) in response to apoptotic corpse engulfment [35].

The molecular mechanisms of apoptotic corpse clearance have been extensively studied in *C. elegans* development. Genetic studies have identified key proteins involved in the engulfment and maturation processes [36–40]. Mutants of the Beclin 1 homolog *bec-1* were first shown to increase the appearance of apoptotic corpses in the *C. elegans* embryo [41]. This was attributed to loss of BEC-1 promoting increased apoptosis, although the authors acknowledged that other mechanisms, including reduced corpse degradation, could also play a role. Later, another group observed a similar increase in apoptotic corpses in the germ line of hermaphrodites mutant for *bec-1* or using *bec-1* RNAi [42]. Their data suggested that increased corpse number was not due to increased apoptosis but rather a defect in phagosome maturation and degradation of corpses, an effect attributed to either defective autophagy or potentially disrupted retrograde trafficking. These results were reproduced in a separate study showing that *bec-1* RNAi worms were not deficient for apoptotic corpse engulfment in the germ line, but instead phagosomes failed to acidify resulting in the prolonged appearance of apoptotic corpses. These authors attributed the effect of BEC-1 loss to its participation in LAP [35]. The recent demonstration that a GFP-tagged homologue of LC3 (GFP-LGG1) recruits to apoptotic cell phagosomes in *C. elegans* embryos has provided further evidence for LAP *in vivo*. Inhibition of *bec-1* by RNAi reduced LGG1 recruitment and inhibited corpse degradation while having no effect on engulfment [31]. Apoptotic cell phagosomes in the Q neuroblast lineage in *C. elegans* were also recently shown to recruit GFP-LGG-1 in a ring-like pattern at some phagosomes, which could be suggestive of LAP, although other phagosomes appeared to recruit GFP-LGG1 in the form of puncta, and still others appeared to recruit no GFP-LGG1 at all [43]. Curiously, neither ATG-1 nor ATG-7 were required for the degradation of Q neuroblast corpses. Altogether, these data point to a role for some autophagy proteins in controlling the degradation of apoptotic cell corpses in *C. elegans*, in a manner consistent with LAP in some phagocytes, but the finer details of the role of autophagy proteins in regulating phagocytosis in *C. elegans* will clearly require further studies.

## Entosis – cell-in-cell formation

Not all macroendocytic cell engulfments involve active phagocytosis [44]. One mechanism whereby live cells can become engulfed is called entosis. Observations of live cell engulfments like those resulting from entosis, which are often referred to as cell cannibalism or ‘cell-in-cell’ formation, have been documented in human tumors [45]. Unlike phagocytosis, the engulfment mechanism of entosis appears to be controlled by internalizing cells through an invasion-like mechanism involving adherens junctions and Rho-mediated contractile force. Once engulfed, internalized cells are housed within an entotic vacuole, that ultimately matures and becomes acidified like a phagosome, leading to the non-apoptotic death of internalized cells that are killed by their hosts in a manner resembling the killing of pathogenic organisms by macrophages [46]. The maturation of entotic vacuoles and hence the death of internalized cells was recently shown to be dependent on autophagy proteins in a process resembling LAP (Figure 1B) [31]. GFP-LC3 is recruited to entotic vacuoles after PI3P formation and prior to lysosome fusion, and, like phagosomes, the GFP-LC3-labeled entotic vacuole has a single-membrane structure when assessed by CLEM. Also, GFP-LC3 recruitment is dependent on downstream lipidation machinery including Atg5, Atg7 and Vps34, but is independent of the upstream Ulk complex, because depletion of Fip200, which blocked autophagy, had no effect on LC3 recruitment to entotic vacuoles. Moreover, the site of action of autophagy proteins was localized to the host cells; inhibition of Atg5 in internalized cells had no effect on LC3 recruitment, whereas Atg5 knockdown in host cells reduced the frequency of GFP-LC3 recruitment and entotic cell death. These experiments demonstrated a non-canonical role for autophagy proteins in a non-cell autonomous death mechanism of mammalian cells that resembles pathogen destruction. It is tempting to speculate that similar mechanisms of autophagy protein recruitment to vacuoles could also contribute to cell death in other contexts where viable cells are killed by neighboring engulfers, such as in *C. elegans*, where phagocytes can contribute to the death of cells harboring partial loss-of-function *ced-3* alleles [47, 48], or where overactive Rac can contribute to the death of cells rendered sick by sublethal cytotoxic treatments [49].

## Other models where autophagy proteins target single-membrane compartments

Non-canonical roles for autophagy proteins in lysosome fusion are not restricted to engulfment events targeting cells or pathogenic organisms. Macropinocytosis, or ‘cell drinking’, is an endocytic process whereby plasma membrane ruffles enclose portions of the extracellular milieu, internalizing them within vacuoles called macropinosomes [2, 50]. LC3 can be recruited to both constitutive and ligand-induced macropinosomes in multiple cell types, dependent on Atg5 but not Fip200 (Figure 1B) [31].

Interestingly, engulfment itself is also not a prerequisite for the non-canonical targeting of autophagy proteins to membrane compartments. LC3 recruitment to the ruffled border, a specialized region of the plasma membrane important for bone resorption, has now been reported in osteoclasts [51]. The ruffled border forms as an actin-rich sealing zone, effectively partitioning large portions of plasma membrane at the bone interface. Here, specialized lysosomes fuse and secrete hydrolytic enzymes that degrade the underlying bone. Debris formed during this process is then taken back into the cell. LC3 localizes to the ruffled border in an Atg5-dependent manner, and Atg5, Atg7 and Atg4 are required for lysosome secretion at the ruffled border and for optimal bone resorption [51].

## A potential role for LC3 in lysosome fusion?

Each of the reports discussed above shares the common theme that autophagy proteins regulate lysosome fusion to macroendocytic vacuoles or to specialized membrane compartments. The data point to a potential direct role for autophagy proteins, and in particular LC3, in promoting lysosome fusion. Any such role for LC3 in canonical

autophagy would be obscured by the fact that LC3 and its family members are required for autophagosome formation [16, 52]. LC3 proteins may simply promote membrane-membrane fusion directly, which is speculated to contribute to phagophore expansion during autophagy [53, 54]. LC3 and the related protein GATE-16 promote the tethering and fusion of liposomes *in vitro*, an effect attributed to several N-terminal amino acids in each protein that may directly interact with lipids [55]. Indeed, mutant LC3 or GATE-16 proteins harboring point mutations that disrupt liposome fusion fail to support autophagosome biogenesis in cells [55]. However, a recent report suggested that the sufficiency of LC3 in driving membrane fusion may be related to *in vitro* conditions such as non-physiological concentrations of PE [56]. Also, an *in vitro* assay of autophagosome-to-endosome fusion failed to reveal a requirement for LC3 [57]. Nevertheless, a role for LC3 in lysosome fusion to non-autophagic membranes in cells is speculated, based on the requirement of Atg5 and Atg7 proteins for GFP-LC3 recruitment to macroendocytic vacuoles and also for lysosome fusion. Such a role may be more broadly utilized in cells than currently appreciated given the variety of cellular contexts where autophagy proteins have been found to regulate lysosome fusion to single-membrane compartments.

## Mechanisms of targeting autophagy proteins to macroendocytic vacuoles

An important question raised by the recent studies identifying LC3 lipidation to non-autophagic membranes is whether the mechanisms that activate autophagy proteins in these contexts are shared or distinct, and how they may differ from mechanisms of induction of canonical autophagy. One key difference between the targeting of autophagy proteins to single-membrane compartments and canonical autophagy appears to be the independence of single-membrane targeting from upstream autophagy regulators such as mTor and the Ulk complex. LC3 recruitment to macroendocytic vacuoles occurs under nutrient-replete conditions, when mTorc1 is active and canonical autophagy is inhibited. Similarly, activation of canonical autophagy does not affect the ability of LC3 to recruit to phagosomes. What then are the mechanisms that control the activation of autophagy proteins to target LC3 lipidation to non-autophagic membranes?

## Signaling pathways involved in targeting autophagy proteins to pathogen phagosomes

Toll-like receptors (TLRs) are a family of pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) and activate the innate immune system. Ligation of TLRs transmits signals through adaptor proteins including MyD88, TRIF and TRAM, which activate transcription factors NF- $\kappa$ B and interferon regulatory factor (IRF)-responsive immune response genes, as well as mitogen-activated protein kinases p38 and JNK, which are essential for the inflammatory response [58]. TLR signaling facilitates the clearance of intracellular pathogens through two different mechanisms involving autophagy proteins. Pathogens that inhibit phagosome maturation in order to evade destruction (e.g. *M. tuberculosis*) can be enwrapped by autophagosomes that are formed following treatment with soluble TLR agonists [59, 60]. Autophagosome-lysosome fusion then facilitates pathogen destruction. The mechanisms that direct autophagosomes to these arrested phagosomes are unclear, but must be specific to pathogens, as neither starvation nor TLR-mediated autophagosome formation have any effect on the maturation of non-pathogen phagosomes [28, 59].

TLRs were also demonstrated to facilitate lysosome fusion to phagosomes containing pathogens that do not block maturation (e.g. *E. coli* and *S. aureus*) [61]. However, in these cases, the functional site of TLR signaling is restricted to the phagosome compartment itself. One mechanism that specifically directs phagosome maturation downstream of TLR signaling is the non-canonical autophagy protein process LAP, because *Tlr2*<sup>-/-</sup> macrophages are deficient for recruitment of LC3 to phagosomes housing zymosan, and

lysosome fusion is also delayed [28]. Thus, autophagy proteins can modulate pathogen phagosome maturation downstream of TLR signaling through either autophagy or LAP. It is tempting to speculate that in some circumstances LAP could be a default mechanism to target pathogens contained within phagosomes that might be inhibited in the presence of a phagosome maturation block, such as inhibition of PI3P formation as employed by *M. tuberculosis* [62–64]. Under these conditions, autophagosome formation could instead target phagosomes for destruction. It would be interesting to examine if overcoming the block in *M. tuberculosis* phagosome maturation could enable LAP as a mechanism of pathogen destruction.

Another receptor family important for the recognition and phagocytosis of opsonized pathogens are Fc $\gamma$  receptors. Ligation of Fc $\gamma$  receptors by phagocytosis of IgG-coated beads was sufficient to trigger LC3 recruitment in a similar manner to TLRs [29], demonstrating that there are multiple receptors that can activate LC3 recruitment through shared or distinct pathways. In addition to LC3, Atg12 localizes to IgG bead-containing phagosomes [29], suggesting that the Atg5-12:16L conjugation complex directs lipidation of LC3 at phagosome membranes during LAP.

Although TLRs and Fc $\gamma$  receptors are important for pathogen LAP, the downstream events that link these receptors to the recruitment of autophagy proteins are unclear. Reactive oxygen species (ROS) are required for recruitment of LC3 in some contexts [29]. Both TLRs and Fc $\gamma$  receptor activate Nox2 NADPH oxidase, generating phagosome-localized ROS [65, 66]. LC3 recruitment to LPS or IgG-coated bead-containing phagosomes was reduced by pharmacological inhibition of NADPH oxidase, or in *Nox2*<sup>-/-</sup> macrophages. Yet how ROS might influence the autophagy pathway in this process remains unclear. ROS can induce the upregulation of key autophagy genes [67], but this is unlikely to play a role due to the rapid kinetics of LC3 recruitment. Atg4 can also be inhibited by ROS, possibly through oxidation of a critical cysteine proximal to its catalytic site [68]. Interestingly, a recent report demonstrated the requirement of Atg4 for de-lipidation of Atg8 (LC3) from various organelle membranes, including ER and endosomes, in yeast under basal conditions. It is conceivable that localized inhibition of Atg4 could allow LC3-II accumulation through blockage of constitutive de-lipidation [69].

Another potential candidate in the induction of ROS and recruitment of autophagy proteins is SLAM, a member of the signaling lymphocyte-activation molecule family. SLAM binds to gram-negative bacteria and is present on phagosome membranes, where it interacts with the Vps34:Beclin 1 complex to promote PI3P formation, which is a prerequisite for the recruitment of p40phox and generation of ROS [70].

### **Signaling pathways involved in targeting autophagy proteins to non-pathogen macroendocytic vacuoles**

There are numerous surface receptors involved in the recognition and phagocytosis of apoptotic cells, including scavenger receptors, phosphatidylserine (PtdSer) receptors, integrins and complement receptors [71]. They transmit signals through shared and distinct pathways to regulate engulfment and phagosome maturation. Tim4, a recently identified receptor for PtdSer and mediator of apoptotic cell engulfment [72], promotes LC3 recruitment to apoptotic phagosomes [35]. Engulfment of PtdSer-containing liposomes also promoted LC3 recruitment showing sufficiency for this interaction. However, the exact role of Tim4 in LC3 recruitment is difficult to discern owing to the requirement of Tim4 for the initial engulfment that precedes recruitment of LC3.

Both pathogen and apoptotic cell phagocytosis are receptor-ligand mediated processes, which offers potential direct mechanisms for signaling to activate autophagy proteins. It is

unclear how these mechanisms may relate to other macroendocytic engulfments that also recruit LC3, such as macropinocytosis and entosis, because these are not thought to be ligand-receptor driven processes [44]. For entosis, it is possible that the accumulation of unknown secreted factors, including ROS, inside of entotic vacuoles initiates the recruitment of autophagy proteins after reaching a certain threshold. Alternatively, localized modifications to vacuole membrane proteins, such as ubiquitylation, could conceivably recruit ubiquitin-binding adaptor proteins (e.g. p62) that in turn bind to LC3, mimicking the xenophagy process that targets autophagosomes to pathogens that escape from phagosomes [73]. Indeed, ubiquitylation of IgG-coated latex bead phagosome membrane proteins has been previously reported [74]. The potential signaling mechanisms that might control LC3 recruitment to entotic vacuoles may be shared with phagocytic processes, but these await identification.

## Concluding remarks

From the recent work reviewed here, there is clearly accumulating evidence for autophagy-independent roles of core autophagy proteins in controlling lysosome fusion to macroendocytic vacuoles. Although once thought to be restricted to pathogen-containing phagosomes, it is becoming clear that this function of autophagy proteins is a more general mechanism that can control maturation events associated with lysosome fusion in a variety of contexts. The recruitment of GFP-LC3 even to macropinosome membranes does suggest that GFP-LC3-associated vesicles in cells may not always represent autophagosomes. Likewise LC3-II detected by Western blotting may not exclusively reflect autophagosome-derived pools of lipidated LC3, particularly under nutrient- and growth factor-replete conditions where autophagy may be generally suppressed and other pathways like macropinocytosis may be induced. In addition to macroendocytic engulfment, there are also other recently identified roles of core autophagy proteins in apoptotic cell death that are distinguishable from autophagy [75–77]. Further investigation into the molecular mechanisms underlying these autophagy-independent roles of autophagy proteins will increase our understanding of endocytosis, cell death and autophagy, and how these processes may be coordinately or antagonistically regulated.

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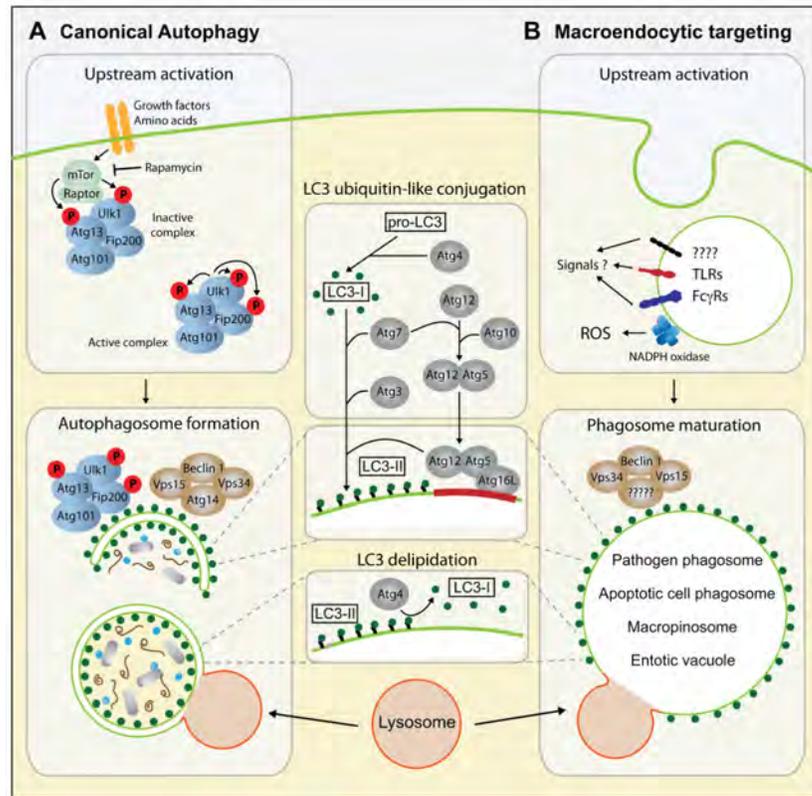
## References

1. Huotari J, Helenius A. Endosome maturation. *EMBO J.* 2011; 30:3481–3500. [PubMed: 21878991]
2. Kumari S, et al. Endocytosis unplugged: multiple ways to enter the cell. *Cell Res.* 2010; 20:256–275. [PubMed: 20125123]
3. Wong E, Cuervo AM. Integration of clearance mechanisms: the proteasome and autophagy. *Cold Spring Harb Perspect Biol.* 2010; 2:a006734. [PubMed: 21068151]
4. Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol.* 2010; 22:124–131. [PubMed: 20034776]
5. Burman C, Ktistakis NT. Autophagosome formation in mammalian cells. *Semin Immunopathol.* 2010; 32:397–413. [PubMed: 20740284]
6. Manjithaya R, Subramani S. Role of autophagy in unconventional protein secretion. *Autophagy.* 2010;6.
7. Rubinsztein DC, et al. Mechanisms of autophagosome biogenesis. *Curr Biol.* 2012; 22:R29–34. [PubMed: 22240478]

8. Hara T, et al. FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J Cell Biol.* 2008; 181:497–510. [PubMed: 18443221]
9. Ganley IG, et al. ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. *J Biol Chem.* 2009; 284:12297–12305. [PubMed: 19258318]
10. Jung CH, et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell.* 2009; 20:1992–2003. [PubMed: 19225151]
11. Chan EY. mTORC1 phosphorylates the ULK1-mAtg13-FIP200 autophagy regulatory complex. *Sci Signal.* 2009; 2:pe51. [PubMed: 19690328]
12. Kihara A, et al. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. *J Cell Biol.* 2001; 152:519–530. [PubMed: 11157979]
13. Suzuki K, et al. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J.* 2001; 20:5971–5981. [PubMed: 11689437]
14. Itakura E, et al. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Mol Biol Cell.* 2008; 19:5360–5372. [PubMed: 18843052]
15. Mizushima N, et al. Autophagosome formation in mammalian cells. *Cell Struct Funct.* 2002; 27:421–429. [PubMed: 12576635]
16. Fujita N, et al. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol Biol Cell.* 2008; 19:2092–2100. [PubMed: 18321988]
17. Kim J, et al. Membrane recruitment of Aut7p in the autophagy and cytoplasm to vacuole targeting pathways requires Aut1p, Aut2p, and the autophagy conjugation complex. *J Cell Biol.* 2001; 152:51–64. [PubMed: 11149920]
18. Kirisako T, et al. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J Cell Biol.* 2000; 151:263–276. [PubMed: 11038174]
19. Marino G, et al. Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. *J Biol Chem.* 2007; 282:18573–18583. [PubMed: 17442669]
20. Klionsky DJ, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy.* 2008; 4:151–175. [PubMed: 18188003]
21. Moreau K, et al. Autophagosome precursor maturation requires homotypic fusion. *Cell.* 2011; 146:303–317. [PubMed: 21784250]
22. Levine B, et al. Autophagy in immunity and inflammation. *Nature.* 2011; 469:323–335. [PubMed: 21248839]
23. Noda T, Yoshimori T. Molecular basis of canonical and bactericidal autophagy. *Int Immunol.* 2009; 21:1199–1204. [PubMed: 19737785]
24. Gutierrez MG, et al. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell.* 2004; 119:753–766. [PubMed: 15607973]
25. Nakagawa I, et al. Autophagy defends cells against invading group A *Streptococcus*. *Science.* 2004; 306:1037–1040. [PubMed: 15528445]
26. Gutierrez MG, et al. Autophagy induction favours the generation and maturation of the *Coxiella*-replicative vacuoles. *Cell Microbiol.* 2005; 7:981–993. [PubMed: 15953030]
27. Knodler LA, Celli J. Eating the strangers within: host control of intracellular bacteria via xenophagy. *Cell Microbiol.* 2011; 13:1319–1327. [PubMed: 21740500]
28. Sanjuan MA, et al. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature.* 2007; 450:1253–1257. [PubMed: 18097414]
29. Huang J, et al. Activation of antibacterial autophagy by NADPH oxidases. *Proc Natl Acad Sci U S A.* 2009; 106:6226–6231. [PubMed: 19339495]
30. Shui W, et al. Membrane proteomics of phagosomes suggests a connection to autophagy. *Proc Natl Acad Sci U S A.* 2008; 105:16952–16957. [PubMed: 18971338]
31. Florey O, et al. Autophagy machinery mediates macroendocytic processing and entotic cell death by targeting single membranes. *Nat Cell Biol.* 2011; 13:1335–1343. [PubMed: 22002674]
32. Birmingham CL, et al. Autophagy controls *Salmonella* infection in response to damage to the *Salmonella*-containing vacuole. *J Biol Chem.* 2006; 281:11374–11383. [PubMed: 16495224]

33. Kageyama S, et al. The LC3 recruitment mechanism is separate from Atg9L1-dependent membrane formation in the autophagic response against *Salmonella*. *Mol Biol Cell*. 2011; 22:2290–2300. [PubMed: 21525242]
34. Ravichandran KS. Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. *Immunity*. 2011; 35:445–455. [PubMed: 22035837]
35. Martinez J, et al. Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proc Natl Acad Sci U S A*. 2011; 108:17396–17401. [PubMed: 21969579]
36. Ellis RE, et al. Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. *Genetics*. 1991; 129:79–94. [PubMed: 1936965]
37. Gumienny TL, et al. CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. *Cell*. 2001; 107:27–41. [PubMed: 11595183]
38. Hedgecock EM, et al. Mutations affecting programmed cell deaths in the nematode *Caenorhabditis elegans*. *Science*. 1983; 220:1277–1279. [PubMed: 6857247]
39. Wu YC, et al. *C. elegans* CED-12 acts in the conserved crkII/DOCK180/Rac pathway to control cell migration and cell corpse engulfment. *Dev Cell*. 2001; 1:491–502. [PubMed: 11703940]
40. Zhou Z, et al. The *C. elegans* PH domain protein CED-12 regulates cytoskeletal reorganization via a Rho/Rac GTPase signaling pathway. *Dev Cell*. 2001; 1:477–489. [PubMed: 11703939]
41. Takacs-Vellai K, et al. Inactivation of the autophagy gene *bec-1* triggers apoptotic cell death in *C. elegans*. *Curr Biol*. 2005; 15:1513–1517. [PubMed: 16111945]
42. Ruck A, et al. The Atg6/Vps30/Bec1 ortholog BEC-1 mediates endocytic retrograde transport in addition to autophagy in *C. elegans*. *Autophagy*. 2011; 7:386–400. [PubMed: 21183797]
43. Li W, et al. Autophagy genes function sequentially to promote apoptotic cell corpse degradation in the engulfing cell. *J Cell Biol*. 2012; 197:27–35. [PubMed: 22451698]
44. Doherty GJ, McMahon HT. Mechanisms of endocytosis. *Annu Rev Biochem*. 2009; 78:857–902. [PubMed: 19317650]
45. Overholtzer M, Brugge JS. The cell biology of cell-in-cell structures. *Nat Rev Mol Cell Biol*. 2008; 9:796–809. [PubMed: 18784728]
46. Overholtzer M, et al. A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion. *Cell*. 2007; 131:966–979. [PubMed: 18045538]
47. Hoepfner DJ, et al. Engulfment genes cooperate with *ced-3* to promote cell death in *Caenorhabditis elegans*. *Nature*. 2001; 412:202–206. [PubMed: 11449279]
48. Reddien PW, et al. Phagocytosis promotes programmed cell death in *C. elegans*. *Nature*. 2001; 412:198–202. [PubMed: 11449278]
49. Neukomm LJ, et al. Loss of the RhoGAP SRGP-1 promotes the clearance of dead and injured cells in *Caenorhabditis elegans*. *Nat Cell Biol*. 2011; 13:79–86. [PubMed: 21170032]
50. Kerr MC, Teasdale RD. Defining macropinocytosis. *Traffic*. 2009; 10:364–371. [PubMed: 19192253]
51. DeSelm CJ, et al. Autophagy proteins regulate the secretory component of osteoclastic bone resorption. *Dev Cell*. 2011; 21:966–974. [PubMed: 22055344]
52. Weidberg H, et al. LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. *EMBO J*. 2010; 29:1792–1802. [PubMed: 20418806]
53. Xie Z, et al. Atg8 controls phagophore expansion during autophagosome formation. *Mol Biol Cell*. 2008; 19:3290–3298. [PubMed: 18508918]
54. Nakatogawa H, et al. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell*. 2007; 130:165–178. [PubMed: 17632063]
55. Weidberg H, et al. LC3 and GATE-16 N termini mediate membrane fusion processes required for autophagosome biogenesis. *Dev Cell*. 2011; 20:444–454. [PubMed: 21497758]
56. Nair U, et al. SNARE proteins are required for macroautophagy. *Cell*. 2011; 146:290–302. [PubMed: 21784249]
57. Morvan J, et al. In vitro reconstitution of fusion between immature autophagosomes and endosomes. *Autophagy*. 2009; 5:676–689. [PubMed: 19337031]

58. Akira S, et al. Pathogen recognition and innate immunity. *Cell*. 2006; 124:783–801. [PubMed: 16497588]
59. Delgado MA, et al. Toll-like receptors control autophagy. *EMBO J*. 2008; 27:1110–1121. [PubMed: 18337753]
60. Xu Y, et al. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity*. 2007; 27:135–144. [PubMed: 17658277]
61. Blander JM, Medzhitov R. Regulation of phagosome maturation by signals from toll-like receptors. *Science*. 2004; 304:1014–1018. [PubMed: 15143282]
62. Fratti RA, et al. Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *J Cell Biol*. 2001; 154:631–644. [PubMed: 11489920]
63. Vergne I, et al. Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca<sup>2+</sup>/calmodulin-PI3K hVPS34 cascade. *J Exp Med*. 2003; 198:653–659. [PubMed: 12925680]
64. Vergne I, et al. Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A*. 2005; 102:4033–4038. [PubMed: 15753315]
65. Nauseef WM. Biological roles for the NOX family NADPH oxidases. *J Biol Chem*. 2008; 283:16961–16965. [PubMed: 18420576]
66. Rada B, et al. Role of Nox2 in elimination of microorganisms. *Semin Immunopathol*. 2008; 30:237–253. [PubMed: 18574584]
67. Mitroulis I, et al. Regulation of the autophagic machinery in human neutrophils. *Eur J Immunol*. 2010; 40:1461–1472. [PubMed: 20162553]
68. Scherz-Shouval R, et al. Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J*. 2007; 26:1749–1760. [PubMed: 17347651]
69. Nakatogawa H, et al. Atg4 recycles inappropriately lipidated Atg8 to promote autophagosome biogenesis. *Autophagy*. 2012:8.
70. Berger SB, et al. SLAM is a microbial sensor that regulates bacterial phagosome functions in macrophages. *Nat Immunol*. 2010; 11:920–927. [PubMed: 20818396]
71. Lauber K, et al. Clearance of apoptotic cells: getting rid of the corpses. *Mol Cell*. 2004; 14:277–287. [PubMed: 15125832]
72. Miyanishi M, et al. Identification of Tim4 as a phosphatidylserine receptor. *Nature*. 2007; 450:435–439. [PubMed: 17960135]
73. Deretic V. Autophagy in immunity and cell-autonomous defense against intracellular microbes. *Immunol Rev*. 2011; 240:92–104. [PubMed: 21349088]
74. Lee WL, et al. Role of ubiquitin and proteasomes in phagosome maturation. *Mol Biol Cell*. 2005; 16:2077–2090. [PubMed: 15703218]
75. Radoshevich L, et al. ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. *Cell*. 2010; 142:590–600. [PubMed: 20723759]
76. Rubinstein AD, et al. The autophagy protein Atg12 associates with antiapoptotic Bcl-2 family members to promote mitochondrial apoptosis. *Mol Cell*. 2011; 44:698–709. [PubMed: 22152474]
77. Yousefi S, et al. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat Cell Biol*. 2006; 8:1124–1132. [PubMed: 16998475]



**Figure 1.** Autophagy proteins involved in autophagy and macroendocytic degradation pathways. **(A)** Autophagy pathway. In the presence of growth factors and amino acids, mTor associates with and inactivates the Ulk complex by phosphorylating Atg13 and Ulk1. Upon starvation and release of mTor inhibition, the active complex localizes to a membrane source and acts in concert with the Vps34-ATG14L complex to recruit and activate components of the LC3 and Atg12 ubiquitin-like conjugation systems. LC3 is lipidated onto forming double-membrane autophagosomes. After lysosome fusion, LC3 is de-lipidated and recycled by Atg4. **(B)** Macroendocytic engulfment. Following phagocytosis or related macroendocytic engulfment mechanisms, signals dependent on activation of TLRs, Fc $\gamma$ R, or other uncharacterized receptors, which are not fully understood but include ROS, are transmitted across the vacuole to recruit and activate a Vps34 complex and the LC3 and Atg12 conjugation systems. LC3 is lipidated directly to the single-membrane vacuole, followed by lysosome fusion. Both degradation pathways in (A) and (B) utilize common Vps34 and LC3 and Atg12 conjugation machinery, but differ in upstream activation mechanisms.