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

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. turberculosis*) or those that escape into the cytosol (e.g. *L. monocytogenes)* can be enwrapped 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

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,

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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

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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- 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. turberculosis*) 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−/−* 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. turberculosis*

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
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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 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.