CELL BIOLOGY

LC3-associated endocytosis and the functions of Rubicon and ATG16L1

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LC3-associated endocytosis (LANDO) is a noncanonical function of the autophagy machinery, in which LC3 (microtubule-associated protein light chain) is conjugated to rab5-positive endosomes, using a portion of the canonical autophagy pathway. LANDO was initially discovered in a murine model of Alzheimer's disease as a critical regulator of amyloid-β receptor recycling in microglial cells, playing a protective role against neuronal loss and memory impairment. Recent evidence suggests an emerging role of LANDO in cytokine receptor signaling and innate immunity. Here, we discuss the regulation of two crucial effectors of LANDO, Rubicon and ATG16L1, and their impact on endocytosis, autophagy, and phagocytosis.

INTRODUCTION

Macroautophagy, henceforth referred to as autophagy, is a conserved process involving the nonspecific engulfment of cytoplasmic material or specific engulfment of intracellular misfolded proteins, aggregates, or damaged organelles in a double-membrane vesicle called the autophagosome. The autophagosome fuses with lysosomes for cargo degradation (*1*). A sequential activation cascade of autophagy-related (ATG) proteins allows the lipidation of microtubule-associated protein light chain 3 (LC3) family members and related molecules (herein, collectively called LC3) and their recruitment to a pre-autophagosomal structure known as the phagophore. The generation of gene-edited organisms revealed the role of ATG genes in stress responses, intracellular quality control, development, and cell differentiation, and studies have implicated autophagy in human diseases such as neurodegeneration, inflammatory disorders, and cancer (*1*).

Schematically, in canonical autophagy, a cascade of three sequentially engaged multiprotein complexes leads to autophagosome biogenesis: (i) a serine-threonine kinase ULK complex, (ii) a phosphoinositide 3-kinase (PI3K) complex containing VPS34, and (iii) ubiquitin conjugation–like systems for conjugation of ATG12 to ATG5 and conjugation of LC3 to phospholipids. In response to amino acid starvation, the activation of adenosine monophosphate–activated kinase (AMPK) and inactivation of mechanistic target of rapamycin complex 1 (mTORC1) result in the engagement of canonical autophagy by activation of a complex composed of the ULK1 or ULK2 serine-threonine kinase and the adaptor proteins ATG13, ATG101, and FIP200 (also called RB1CC1). ULK complex phosphorylation at the cytosolic face of the endoplasmic reticulum (ER) subsequently recruits the VPS34 complex (*2*, *3*). Phosphatidylinositol 3-phosphate (PI3P) is locally generated by the VPS34 complex consisting of a minimal tetrameric catalytic unit, the class III PI3K lipid kinase VPS34, Beclin 1, and VPS15. Two complexes known as complex I and complex II have been described wherein the minimal catalytic unit is either associated with the adaptor proteins ATG14 for complex I or UVRAG for complex II (*4*). PI3P-enriched membranes recruit, in turn, a complex of ATG12-ATG5 and ATG16L1, which acts as an E3 ligase to ligate LC3 to phosphoethanolamine. LC3 conjugation is crucial for cargo recognition, phagophore elongation, and its completion into a double-membrane vesicle organelle. The autophagosome ultimately fuses to lysosomes, in which the cargo is degraded by lysosomal hydrolases (Fig. 1).

Over the past 15 years, increasing evidence has supported roles of several autophagy-related proteins in nonautophagic processes such as phagocytosis, endocytosis, entosis, and micropinocytosis, in which LC3 is lipidated at single membranes (*5*). LC3-associated phagocytosis (LAP) was the first such nonautophagic pathway to be described (*6*). In murine macrophages, extracellular particles including killed yeast (zymosan), beads coated with lipopolysaccharide, PAM3csk4 or antibodies, *Escherichia coli*, or DNA–anti-DNA immune complexes that stimulate Fc receptors to mediate Toll-like receptor 9 (TLR9) and type I interferon signaling trigger LC3 recruitment on mature phagosomes engulfing those particles (*6*). A VPS34 complex containing UVRAG and Rubicon (RUN domain and cysteine-rich domain containing, Beclin 1 interacting protein) and the ATG12-ATG5-ATG16L1 conjugation system are necessary for LAP; however, all components of the ULK complex (including FIP200) and the VPS34 complex I adaptor protein ATG14 are dispensable (Fig. 1). Ablation of components of LAP in murine myeloid compartments demonstrated that LAP deficiency promotes an antitumor effect due to an improved T cell response to dying tumor cells (*7*). The role of LAP in efferocytosis and innate immunity has been reviewed elsewhere (*8*, *9*).

Endocytic pathways allow cells to sense their environment and regulate their signal transmission by internalizing various extracellular or surface resident molecules, which are either degraded, recycled back to the cell surface, or targeted to the trans-Golgi network (*10*). Endocytic transport consists of several sets of vesicles including early endosomes, recycling endosomes, late endosomes, and lysosomes, which dynamically interact with each other by fusion and fission events. To exercise this complex trafficking of molecules, each organelle is marked by specific Rab–guanosine triphosphatases (GTPases) and tether proteins. Beyond its role in autophagy, VPS34 lipid kinase activity is essential for endocytic vesicle trafficking (*11*–*14*). VPS34 complex II is recruited to Rab5-positive early endosomes and is involved in the maturation to Rab7-positive late endosomes and endolysosomal fusion (*15*). While multiple lines of evidence suggest that several proteins involved in the endocytic pathway are essential for efficient autophagy at different stages of the degradative process (*16*, *17*), here, we review emerging functions of a recent

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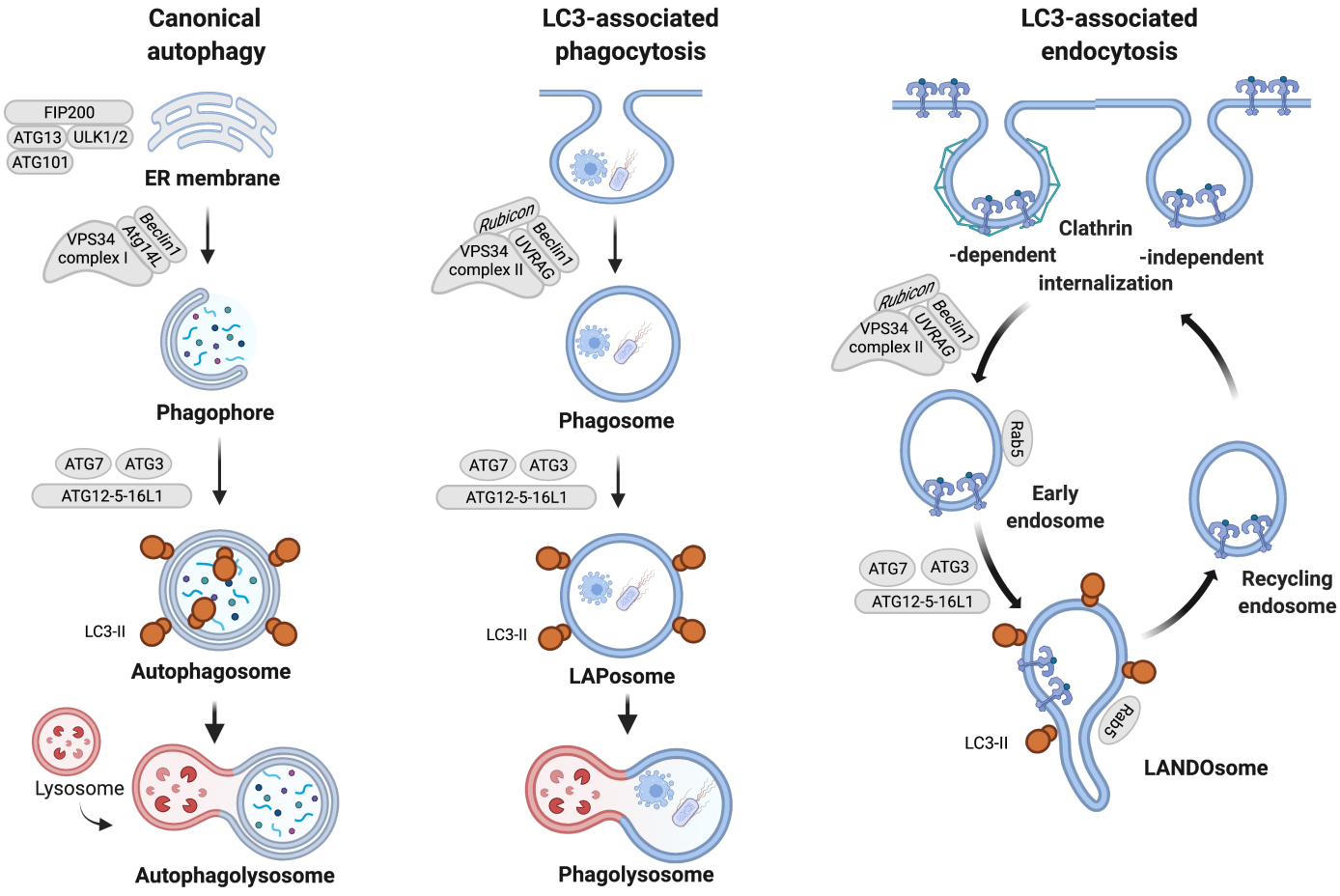

Fig. 1. Molecular machinery of canonical autophagy and noncanonical autophagy processes LC3-associated phagocytosis (LAP) and LC3-associated endocytosis (LANDO). Schematic illustration of the differences in membrane trafficking and cascades of autophagy-related (ATG) proteins. LC3-II (orange circle) represents the lipidated form of LC3 (including both the LC3 and Gabarap family proteins).

noncanonical function of the autophagy pathway called LC3-associated endocytosis (LANDO), in which LC3 is lipidated on early endosomes. Here, we focus on the molecular machinery and signaling pathways shared between different noncanonical functions of autophagy proteins, and we discuss recent findings that highlight LC3 conjugation on single membranes.

THE INITIAL DISCOVERY OF LANDO IN MICROGLIA

LANDO was initially characterized in microglia cells, in which endocytic uptake of oligomerized amyloid-β (Aβ) peptide leads to conjugation of LC3 to the membrane to rab5-positive, clathrin-positive endosomes (*18*). Ablation of Rubicon or Atg5 in microglial cells had no effect on primary Aβ uptake or lysosomal degradation but led to a marked reduction in the recycling of putative Aβ receptors, including TLR4, TREM2, and CD36. It was only after a secondary exposure that Aβ uptake was diminished in vitro in microglial cells lacking Rubicon or ATG5. Previous results in microglial cells showed a role for Beclin 1 and VPS34 in the recycling of TREM2 and CD36 by the retromer complex, a conserved complex responsible for endosome to Golgi retrograde transport and consisting of VPS26, VPS29, VPS35, and sorting nexin subunits (*19*). Recycling of TLR4 and CD36 was also observed to be dependent on LANDO in macrophages, as was that of CD36 and transferrin receptor in mouse embryonic fibroblasts (MEFs) (*18*).

In vivo, microglial- or myeloid-specific deletion of Rubicon or Atg5 in the 5xFAD genetic mouse model of Alzheimer's disease resulted in an early-onset accumulation of neurotoxic Aβ plaques, microgliosis, and an increase in proinflammatory cytokines within the cortex and the hippocampus (*18*). Similar results were observed in aged mice with whole-body deletion of the WD domain of Atg16L1, which is dispensable for canonical autophagy but essential for LANDO (*20*), notably without expression of any transgene. 5xFAD mice lacking microglial Rubicon or Atg5 or mice lacking the Atg16L1 WD domain display substantial neurodegeneration, including neuronal apoptosis, impaired long-term potentiation, and severe short-term memory impairment in behavioral tests. Reduced levels of Rubicon, Atg16L1, Atg5, and Beclin 1 protein expression were observed in human Alzheimer's disease brains, suggesting that these results may be relevant to human disease.

In a similar manner to LAP, Beclin 1 and Rubicon in the VPS34 complex are required for LANDO, as are the proteins of the LC3 conjugation system, while ULK, FIP200, and the VPS34 complex I adaptor protein ATG14 are dispensable for LANDO and receptor recycling. Indeed, during LAP and LANDO, single-membranes vesicles with extracellular cargo are enriched for PI3P and decorated

with LC3 (Fig. 1). However, LANDO appears to be distinguished from LAP by one main feature; LANDO is not required for lysosomal degradation of the engulfed material in the endosomes, while LAP facilitates such degradation in phagosomes. In microglial cells, phagocytosis of zymosan induces LAP, which promotes lysosomal degradation, while Aβ oligomers induce LANDO, in which lysosomal degradation is unaffected by disruption of LC3 lipidation. This suggests that regulatory pathways differ between LAP and LANDO. Given the crucial roles of receptor recycling in human physiology, it is likely that LANDO will emerge as an important pathway in physiopathology of human diseases. Can LANDO be induced by other stimuli and occur in other cell types? A critical distinction between canonical autophagy and the processes of LAP and LANDO is the regulation of the latter by two key effectors, Rubicon and the WD domain of ATG16L1.

While LANDO is not required for acidification of endocytosed Aβ oligomers (*18*), the LC3 lipidation machinery is required for acidification of endocytosed DNA–anti-DNA antibody complexes (*21*) and activation of the endosomal TLR9 to signal type I interferon responses. Interestingly, LC3 was shown to interact with an LC3-interacting region in IκB kinase α, necessary for signaling in this pathway. It is possible that this interaction functions in signaling events under other conditions of LANDO.

KEY REGULATORS OF LANDO

Rubicon

Rubicon not only is essential for LANDO and LAP but also acts as a negative regulator of autophagy. Multiple functional domains of Rubicon are involved in protein-protein interactions with several binding partners, which dictate its downstream signaling (Fig. 2). Rubicon consists of an N-terminal RUN domain, a middle region that includes its PI3K-binding domain (PIKBD), a coiled-coil domain (CCD), a serine-rich region (S-R), a helix-coil–rich domain (H-C), and a C-terminal Rubicon homology domain (RH). Here, we discuss the mechanisms by which Rubicon may regulate canonical and noncanonical functions of the autophagy pathway, with an emphasis on its role in mammalian physiology and pathophysiology.

Interaction with Beclin 1 in the negative regulation of autophagy

Rubicon was initially discovered as a Beclin 1 binding partner in a VPS34 complex in mouse brains and livers as well as in human epithelial mammary tumor cells overexpressing Beclin 1 (*22*, *23*). The presence of Rubicon in the VPS34 complex reduces its lipid kinase activity and inhibits autophagy induced by starvation, as observed by PI3P generation and LC3 puncta formation, respectively. Knockdown of Rubicon in human A549 carcinoma cells up-regulated autophagosome formation, as observed by ATG16L1 puncta formation. In human embryonic kidney (HEK) cells overexpressing Rubicon, Rubicon directly interacts with the catalytic unit of VPS34 via its RUN domain, which is necessary for autophagy inhibition (*24*). Structural studies also suggest that the C-terminal RH domain may participate in autophagy inhibition (*25*). Using hydrogen-deuterium exchange coupled mass spectrometry, giant unilamellar assays, and cryo–electron microscopy, the Rubicon PIKBD domain was found to bind to the Beclin 1 C-terminal BARA domain (previously known as evolutionary conserved domain). Acetylation of Beclin 1 at multiple sites regulates further Beclin 1-Rubicon interaction and VPS34 kinase activity in HEK cells overexpressing Beclin 1 or in murine hepatocytes (*26*–*28*). In HEK cells coexpressing tagged Rubicon and tagged VPS34, the Rubicon-Beclin 1 protein-protein interaction blocks the recruitment of VPS34 complex to membranes, inhibits VPS34 kinase activity, and leads to an inhibition of autophagosome maturation (*29*). Clearly, this inhibition does not apply to conditions of conjugation of LC3 to single membranes, raising the possibility that Rubicon and ATG14 compete in the formation of VPS34 complexes relevant to autophagy (VPS34 complex I) and single membranes such as in LAP and LANDO (Rubicon-containing VPS34 complex). Studies analyzing PI3P generation, ATG16L1 localization, and LC3 puncta formation are, so far, mainly based on either endogenous protein staining or fluorescent sensors analyzed by classical confocal microscopy. The use of modern approaches such as correlative light electron microscopy, structured illuminated microscopy, photoactivated localization microscopy, and other superresolution techniques will provide a better appreciation of the dynamic nature of ATG-related proteins and their diverse distribution depending on the cell context and mode of stimulation.

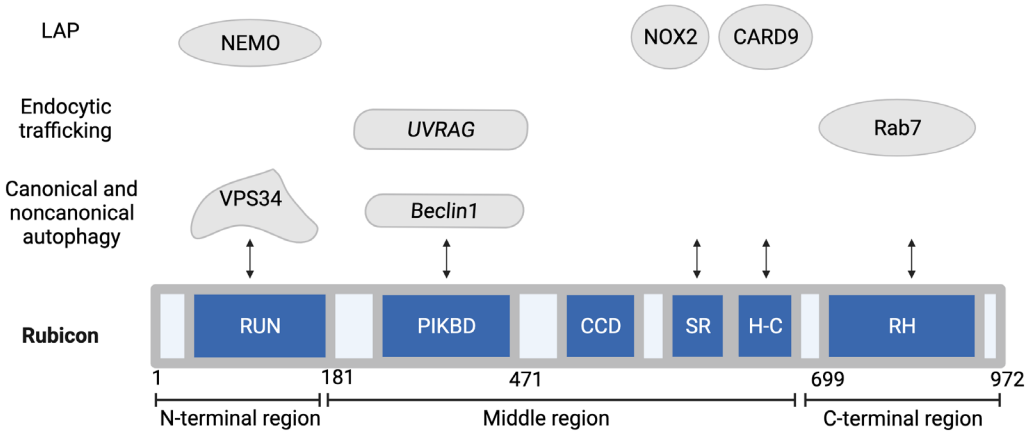

Fig. 2. Schematic Rubicon protein structure, binding partners, and functions in canonical and noncanonical autophagy. Rubicon contains multiple functional domains that interact with several proteins and regulate its downstream signaling. RUN, RUN domain; PIKBD, PI3K-binding domain; CCD, coil-coiled domain; S-R, serine-rich region; H-C, helix-coil–rich domain; RH, Rubicon homology domain.

Interaction with Rab7 and UVRAG in the negative regulation of endocytic trafficking

Rubicon regulates endosome maturation via a mechanism of protein-protein interaction competition with either Rab7 or UVRAG (*30*). Highly enriched in rab5-positive endosomes in human osteosarcoma U2OS cells, Rubicon directly interacts with rab7, the late endosome marker, through its C-terminal RH domain (previously described as FYVE-like domain). The Rubicon PIKBD domain also directly binds the C-terminal BARA2 domain of UVRAG, the VPS34 complex II subunit (*29*). When Rubicon and UVRAG are in the same complex, it prevents UVRAG interaction with the C-VPS/HOPS (homotypic fusion and vacuole protein sorting) complex, a tethering protein multiplex involved in the endolysosomal fusion in Hela cells coexpressing Rubicon and UVRAG or in HCT116 upon epidermal growth factor (EGF) stimulation (*31*, *32*). In the presence of its active guanosine triphosphate (GTP)–bound form, Rab7 competes with UVRAG for Rubicon binding and allows the release of UVRAG, which can now bind to C-VPS/HOPS as a feed-forward mechanism. Nutrient availability regulates this protein-protein interaction cascade. Under nutrient-rich conditions, mTORC1 phosphorylates UVRAG at its Ser^{498} residue, which facilitates Rubicon-UVRAG interactions. Under nutrient-deprived conditions, UVRAG is dephosphorylated, is released from Rubicon, and can interact with C-VPS/HOPS (*33*). In HEK cells depleted of Rubicon, epidermal growth factor receptor (EGFR) uptake is accelerated and transferrin receptor recycling from endosomes to the plasma membrane is reduced, suggesting that Rubicon acts as a negative regulator of endocytic trafficking (*22*, *30*). These observations are consistent with the role of Rubicon in LANDO observed in microglial cells, macrophages, and fibroblasts (*18*). To what extent other domains of Rubicon and protein binding partners have a specific function in LANDO remains to be elucidated.

Interaction with NOX2, CARD9, and NEMO in the regulation of immune responses

Several Rubicon domains are associated with its role in LAP and the clearance of extracellular particles (*34*). Upon zymosan stimulation in Raw264.7 or bone marrow–derived macrophages (BMDMs), Rubicon directly interacts via its serine-rich region (S-R) with two subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, p22phox, the common integral protein subunit, and gp91phox, the catalytic subunit of NOX2. The Rubicon-NOX2 interaction stabilizes the NADPH oxidase complex and facilitates the production of reactive oxygen species (ROS) against microbes (*34*). Hence, depletion of Rubicon in macrophages reduces the production of ROS and inflammatory cytokines, resulting in a defective response toward TLR2 stimuli such as zymosan and an impairment of bacterial killing after infection with *Listeria monocytogenes* or *Mycobacterium bovis* BCG.

Interestingly, Rubicon directly interacts via its helix-coiled region (H-C) with CARD9, an adaptor protein forming the CBM complex (CARD9, BCL10, MALT1), which is critical for signaling between the activation of pathogen pattern recognition receptors, such as Dectin-1 and RIG-I, and the downstream immune response in macrophages (*35*, *36*). Upon fungal, bacterial, or viral infection, Rubicon acts as a negative regulator of the CBM complex. Patients with chronic hepatitis B virus (HBV) or hepatitis C virus show a substantial up-regulation of Rubicon in peripheral blood mononuclear cells and serum at both mRNA and protein expression levels compared to healthy individuals (*37*, *38*). Upon HBV infection of hepatic hepG2 cells, a direct interaction between Rubicon via its RUN domain and NEMO, the nuclear factor κB (NF-κB) essential modulator, leads to an inhibition of type I interferon signaling, as observed by the phosphorylation of TBK1 and IRF3. Similar results are observed in BMDMs infected with several other stimuli such as influenza virus PR8, vesicular stomatitis virus, polyinosinic: polycytidylic acid [poly(I:C)], and double-stranded RNA, emphasizing the role of Rubicon as a negative regulator of the type I interferon (IFN) response (*39*). By dampening the inflammatory response, Rubicon seems to maintain immune homeostasis upon acute viral infection but can also provide a mechanism that favors viral immune evasion and viral replication. While the pathways involved in signaling Rubicon and LAP to an immune response are still largely unknown, *L. monocytogenes* or fungal melanin can escape and inhibit LAP by up-regulating calcium signaling, which increase bacterial and fungal survival (*40*, *41*). Acetylation of Rubicon by acetyl–coenzyme A at the NOX2 interaction decreases LAP formation (*41*).

Together, depending on the stimuli and its binding partners, Rubicon acts as a decision-making hub that either facilitates bacterial clearance in a LAP-dependent manner or dampens the downstream immune response, with a potential for viral escape. Since NOX2, CARD9, and NEMO are also activated in sterile environments such as hypoxia or hyperlipidemia, it will be interesting to understand whether those binding domains in Rubicon play a role in LAP or LANDO under nonpathogenic conditions.

Rubicon in physiology and age-associated disease

Although the factors that specifically determine whether Rubicon is engaged in LAP, LANDO, or canonical autophagy are still under investigation, the cell type and several environmental factors including nutrient availability, inflammatory status, and cell stress may be informative. In retinal pigment epithelial cells (RPE), the clearance of photoreceptor outer segments (POS) engages LAP, which facilitates its retinal reutilization (*42*). In the morning, when POS degradation is at its highest to support vision, Rubicon is also at its highest level of protein expression and colocalizes mainly with phagosomes (*43*). We can speculate that Rubicon protein availability is important for the induction of LAP or LANDO in (patho-)physiological settings. It may be that high levels of Rubicon compete with ATG14 for formation of VPS34 complexes, limiting canonical autophagy and thereby facilitating LAP and LANDO (see above). How promotion of canonical autophagy may limit LAP and LANDO remains unclear.

While autophagy activity and lysosomal function decline during aging in most cell types, recent evidence demonstrates an increase in Rubicon protein expression in flies and in mouse kidneys during aging (*44*). Whole-body and specific genetic depletion of Rubicon in neurons substantially extends worm and fly life span, suggesting that autophagy inhibition might represent a major function of Rubicon in neurons. Further beneficial effects of genetic depletion of Rubicon in mice against hepatic steatosis induced by high-fat diet (*45*), kidney fibrosis, α-synuclein accumulation in the brain (*44*), lipopolysaccharide-induced stroke (*46*), doxorubicin-induced cardiotoxicity (*47*), cardiac pressure overload (*48*), or cardiac ischemia/reperfusion injury (*49*) identify Rubicon depletion as a potential therapeutic target against age-associated phenotypes. However, the mechanisms by which aging dampens autophagy activity and up-regulates Rubicon are largely unknown. Intriguingly, in contrast with other tissues, in aged murine white adipose tissue, autophagy activity is up-regulated, and Rubicon expression is down-regulated at both protein and mRNA levels. Specific genetic ablation of Rubicon in adipose tissue leads to fat atrophy and hepatic lipid accumulation

(*50*), emphasizing the critical need to better understand in which tissue, cell type, physiological, and pathological context the functions of Rubicon in canonical autophagy inhibition versus LAP or LANDO induction are most prominent.

ATG16L1

ATG16L1 is a protein at the core of ATG12-ATG5-ATG16L E3 ligase that leads to LC3 lipidation of phagophores, endosomes, and phagosomes (*51*, *52*). ATG16L1 consists of an N-terminal domain necessary for ATG5 binding, a middle region containing the CCD, and a C-terminal domain with seven WD repeats (Fig. 3). This C-terminal WD domain is lacking in some species, including *Saccharomyces cerevisiae* (in which canonical autophagy was originally described). While the mechanisms are still poorly understood, the WD domain of ATG16L1 is required for the recruitment of ATG16L1 complex and the lipidation of LC3 at single membranes including phagosomes and endosomes (*53*). Several protein-lipid and protein-protein interactions with ATG16L1 domains are crucial for its downstream signaling (*54*).

Interaction with ATG5 and dimerization

In higher eukaryotes, ATG16L1 is not observed in its monomeric form but in large homodimer complexes consisting of ATG12-ATG5-ATG16L1 dimerized through the ATG16L1 CCD. ATG7, an E1 ligase, and ATG10, an E2 ligase, catalyze the conjugation of ATG12 to ATG5. Once conjugated to ATG12, ATG5 interacts with ATG16L1 through its N-terminal helix 1 region (*55*). In canonical autophagy, ATG12-ATG5-ATG16L1 complexes exert E3 ligase activity that catalyzes LC3 recruitment to double-membrane phagophores and activates the E2 ligase, ATG3, mediating LC3 conjugation to phosphatidylethanolamine (PE) (Fig. 4). In contrast, during LAP and LANDO, ATG7, ATG3, and ATG12-ATG5-ATG16L1 conjugate LC3 to both PE and phosphatidylserine (PS) (*56*).

Interaction with FIP200, WIPI2, PI3P, or Rab33b, inducers of autophagy

Ablation of adjacent residues of the ATG16L1 middle region, which are involved in the interaction with FIP200, the ULK1 adaptor protein, or with WIPI2, a PI3P-binding molecule, suppresses ATG16L1 recruitment to the phagophore and dampens canonical autophagy induced by amino acid starvation in knockout cell lines stably expressing tagged proteins (*57*–*60*). The middle region of ATG16L1 as well as residues within the CCD (I171, K179, and R193) mediate direct binding to PI3P that allows ATG16L1 complex to be targeted at the phagophore membrane. Sustaining the lipid binding of ATG16L1 to the phagophore by mutating the CCD residues to negatively charged ones also suppresses canonical autophagy, emphasizing the need to unravel the mechanisms underlying the removal of ATG16L1 complex from the phagophore as well as the delipidation of LC3 (*61*).

Interestingly, in murine embryonic fibroblast NIH3T3 cells, under both nutrient-rich and amino acid starvation conditions, ATG16L1 interacts through its CCD domain with Rab33b, a Golgi resident small GTPase, known to be involved in the retrograde transport between Golgi and ER (*62*, *63*). Although the function of the ATG16L1-Rab33 interaction remains poorly understood, overexpression of Rab33b or its binding partner OATL1 suppresses autophagosome formation and maturation as observed by LC3 puncta and colocalization of LC3 with LAMP1-positive lysosome under amino acid starvation (*62*, *64*). Recent findings in Hela cells and MEF cells show that depletion of Rab33b binding sites in ATG16L1 disrupts the recruitment of the ATG12-ATG5-ATG16L1 complex to the phagophore and the transfer of Rab33b from the Golgi to the phagophore under starvation, suggesting that Rab33b$^+$ Golgi vesicles may provide a source of membrane for autophagosome formation (*65*, *66*). While in rat adrenal gland PC12 cells, the Rab33b binding domain of ATG16L1 is involved in neuropeptide Y sequestration in dense core vesicles and its secretion in an autophagy-independent manner, to what extent the Rab33b-ATG16L1 interaction participates in endocytosis and LANDO is not yet known (*65*).

Interaction with Rip2, TMEM59, and vATPase, inducers of LAP and LANDO in innate immunity

In contrast to the FIP200 domain, the WD repeat–containing C-terminal domain of ATG16L1 is completely dispensable for canonical autophagy (*53*, *57*). In ATG16L1-deficient HCT116 cells, the reconstitution

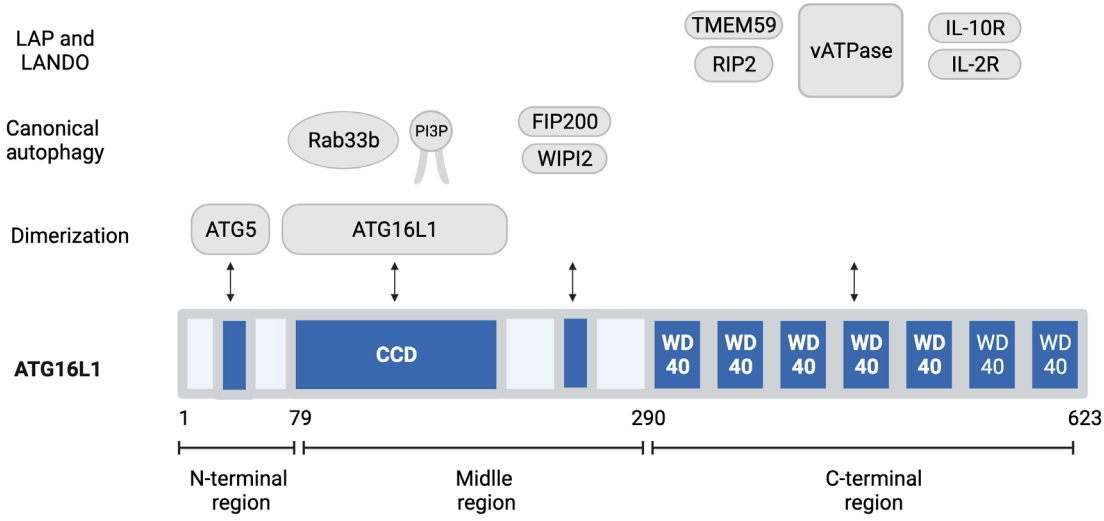

Fig. 3. Schematic ATG16L1 protein structure, binding partners, and functions in canonical and noncanonical autophagy. ATG16L1 contains multiple functional domains that interact with several proteins or phospholipids and regulate its downstream signaling. WD-40, β-transducin repeat containing approximately 40 amino acids and often terminated with tryptophan (W) and acid aspartic (D).

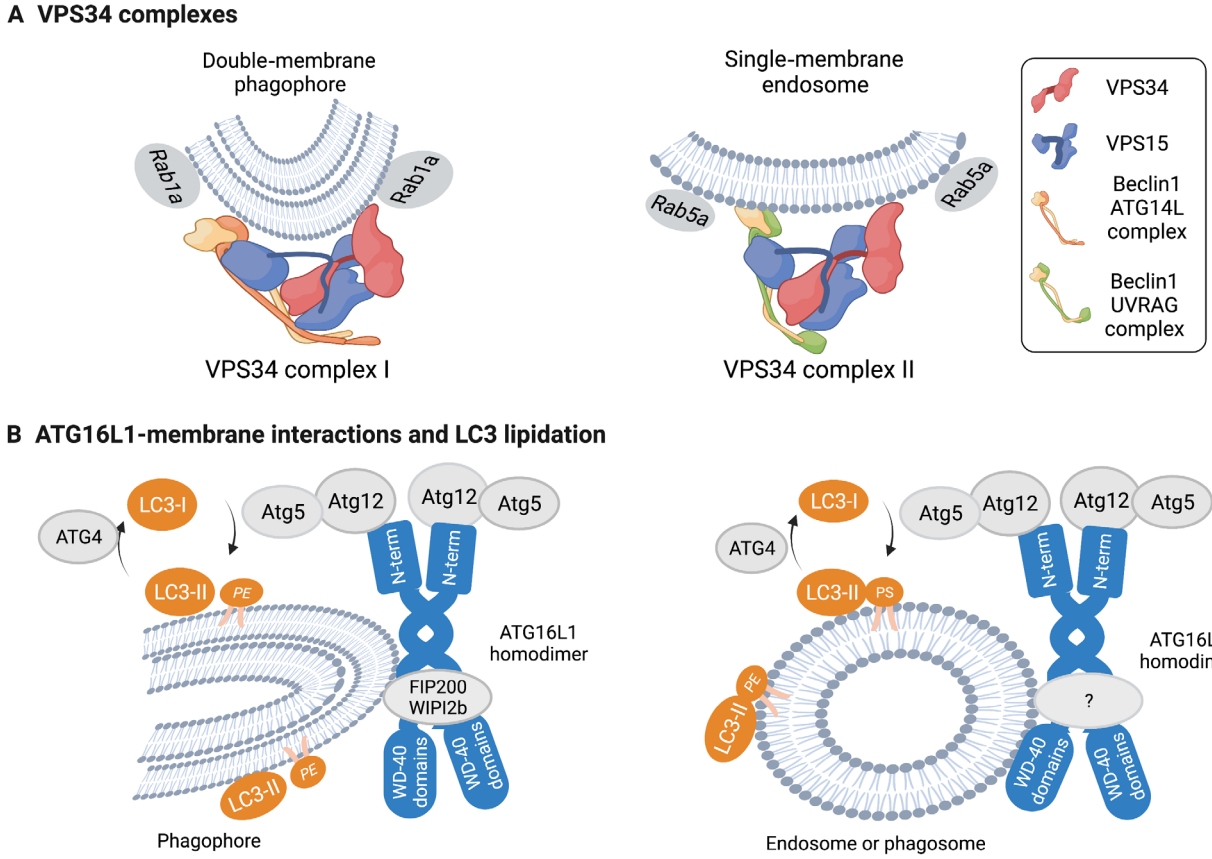

Fig. 4. Emerging models for selecting single or double membranes. Known mechanisms for selecting double-membrane phagophore and single-membrane endosome or phagosome. (**A**) Lipid-protein interactions between VPS34 complexes and membranes. Motifs of ATG14L BATS domain present in VPS34 complex I sense highly unsaturated lipids, curved double-membrane phagophores that contain Rab1a. Motifs of Beclin 1 BARA domain in VPS34 complex II sense negatively charged single-membrane endosomes that contain Rab5. (**B**) LC3 lipidation and lipid interaction between ATG16L1 domains and membranes. Interactions between the middle region of ATG16L1 complex with FIP200 and WIPI2 and PI3P allow its recruitment on double-membrane phagophores. Interaction between ATG16L1 WD domains and single membrane allows its recruitment on endosome or phagosome. While LC3 is conjugated only to PE on double-membrane phagophore during canonical autophagy, LC3 conjugation with PE or PS is observed on single-membrane endosomes or phagosomes. ATG4D catalyzes delipidation of LC3-PS.

of ATG16L1 depleted of its WD repeat domain (ΔWD) allows the recruitment of LC3 on double-membrane phagophores induced by starvation but dampens LAP induced by the engulfment of apoptotic cells or zymosan (*53*), indicating a specific role for the ATG16L1 WD domain in noncanonical autophagy. Determination of the ATG16L1 C-terminal WD domain crystal structure reveals several known and putative binding interaction partners, all involved in regulation of inflammatory responses and xenophagy (*67*).

During *Salmonella* infection in Hela cells, the ATG16L1 WD domain interacts directly with ubiquitin-decorated endosomes containing bacteria (*68*). Furthermore, cells expressing ATG16L1 lacking the C-terminal region show an increased inflammatory response upon *Listeria* or *Shigella* infection in an autophagy-independent manner (*69*). Mechanistically, a direct interaction between ATG16L1 and Rip2, an adaptor protein of intracellular pattern recognition molecules Nod1 and Nod2, may partially explain the function of ATG16L1 C-terminal domain in dampening the cytokine response during bacterial infection. These studies are consistent with the association between a coding variant of human ATG16L1 (T300A) and an increased risk for developing intestinal inflammation in Crohn's disease (*70*–*72*). Interestingly, the T300A variant of ATG16L1 suppresses the interaction between the ATG16L1 WD domain and a transmembrane protein, TMEM59, and further impairs xenophagy induced by *Staphylococcus aureus* (*73*). In addition, after being endocytosed, *Chlamydia trachomatis* secretes a bacterial effector, Taip, which prevents the interaction between the ATG16L1 WD domain and TMEM59 and diverts the bacteria-containing endosome toward Rab6-positive vesicles, known to be involved in retrograde trafficking, trans-Golgi network, and recycling to the plasma membrane (*74*). This latter result highlights the idea that disruption of interactions with the ATG16L1 WD domain can provide a mechanism that facilitates bacterial and viral immune evasion and virulence.

On the basis of a CRISPR screen comparing Hela cells depleted in either FIP200 or ATG5, coupled with mass spectrometry analysis, a recent study found that the interaction between the ATG16L1 WD domain and vacuolar adenosine triphosphatase (vATPase) is essential for initiating xenophagy and LC3 recruitment to single-membrane vacuoles containing *Salmonella* (*75*). vATPase is a multisubunit proton pump that plays a crucial role in organelle acidification (*76*). SopF is a robust bacterial effector that disrupts the ATG16L1 WD domain–vATPase interaction and inhibits LC3 lipidation on single membranes upon *Salmonella* infection (*75*, *77*), detection of

foreign double-stranded DNA inducing the cGAS-STING pathway (*78*), and induction of the viral M2 proton channel of influenza A virus (*79*). Further studies are needed to understand whether the ATG16L1 WD domain and its binding partners can sense pathogens when they are at the plasma membrane surface or already engulfed in endosomes or phagosomes.

ATG16L1 WD domain and role of LANDO in physiology and disease

While mice with whole-body depletion of the ATG16L1 WD domain do not show obvious defects in postnatal growth, fertility, or tissue homeostasis (*80*), an increase sensitivity to influenza A virus was associated with lung inflammation and a decrease in survival rate (*81*). Further studies of mice lacking the ATG16L1 WD domain in different tissues and under several conditions of xenophagy or sterile inflammation will be instrumental in our understanding of the functions of LANDO and LAP in mammalian physiology and pathophysiology.

Using a peptide microarray strategy to evaluate the amino acid motifs that preferentially interact with a human recombinant glutathione *S*-transferase (GST)–hemagglutinin (HA) tagged ATG16L WD domain, a recent study identified two intracellular domains of cytokine receptors, interleukin-10 receptor (IL-10R) and IL-2Rγ. Upon cytokine stimulation, the cytokine receptors bound to its substrate and interacted with the ATG16L1 WD repeat C-terminal domain. Early colocalization of IL-10–IL-10R with EEA1-positive endosome was reduced by the depletion of the ATG16L1 WD domain in HEK cells, THP1 cells, and BMDMs (*82*). These results are consistent with a reduced major histocompatibility complex class II (MHCII) antigen presentation (*83*) and a lower production of tumor necrosis factor–α (TNF-α) and IL-1β (*84*) in bone marrow–derived dendritic cells isolated from mice lacking the ATG16L1 WD domain upon *S. cerevisiae* stimulation. We can only speculate at this point that LANDO-dependent recycling of ligated, stimulatory receptors (such as TLRs) may act to limit the engagement of inflammatory pathways. Since the activation of the NLRP3 inflammasome has been associated with a defect in canonical autophagy proteins in microglial cells (*85*), it will be valuable to know whether this effect is due to defective autophagy or defective LANDO (or both). Indeed, neuroinflammation observed in LANDO-deficient mice lacking the WD domain of ATG16L1 is reversed by inhibition of NLRP3 in vivo (*20*). It is also intriguing that IκB kinase α (IKKα) contains LC3-interacting domains that bind to lipidated LC3 (*21*) and promote interferon signaling in ligated TLR9-containing endosomes. Whether this plays any role in LANDO-dependent effects on inflammatory signaling in other contexts is unknown.

Various mechanisms including leukocyte infiltration, PECAM1 expression at endothelial junctions following ischemia-reperfusion injury (*86*), and primary cilium formation in MEF cells (*87*) have been linked to a depletion in ATG16L1 WD domain, suggesting that, in such settings, defective LANDO may contribute to the effects.

EMERGING MODELS FOR SELECTING DOUBLE MEMBRANES VERSUS SINGLE MEMBRANES

Beyond the key role of ATG16L1 and Rubicon, several layers of regulatory pathways differ between canonical autophagy, LAP, or LANDO. Here, we highlight major evidence ranging from the physicochemical properties of single and double membranes, LC3 conjugation, to liquid-liquid phase separation interface.

Lipid-protein interaction between membranes and VPS34 complexes

Although PI3P generation is essential for canonical autophagy, LANDO, and LAP, it is remarkable that, thus far, VPS34 complex I is only observed on double-membrane phagophore and the Rubicon-containing VPS34 complex is localized only on single membrane early endosome or phagosome (*15*). Membrane-protein interaction represents key mechanisms by which VPS34 complexes become activated on specific membranes (Fig. 4A). Recent in vitro studies of both human recombinant VPS34 complex I and the Rubicon-containing complex in giant unilamellar assays, combined with hydrogen-deuterium exchange coupled mass spectrometry, mutagenesis, and lipid kinase assay, demonstrated that the three physicochemical properties of membranes as defined by lipid saturation, membrane curvature, and electrostatic charge can differentially regulate VPS34 kinase activity and critically affect the selection of the complexes (*28*, *88*). Indeed, the amphipathic lipid packing sensor motif of ATG14L BATS domain present in VPS34 complex I is essential in sensing highly unsaturated lipids, curved double membranes such as ER (*88*, *89*). In contrast, the aromatic fingers (AF1/2) and the hydrophobic loop motifs of Beclin 1 BARA domain in VPS34 complex II are key in detecting single membranes high in negatively charged PS and containing Rab5 such as early endosomes (*88*). Including Rubicon in the VPS34 complex II in similar in vitro studies of single membranes and using different stimuli will be necessary to further understand the precise mechanisms that dictate LAP or LANDO activity. In addition, while Rab5a activates VPS34 complex II on early endosomes, Rab1a is an exclusive activator of VPS34 complex I on phagophore, suggesting that protein-protein interactions between specific small GTPases and VPS34 complexes represent additional regulatory mechanisms of membrane selection and canonical autophagy, LANDO, and LAP activity (*90*).

Notably, in a VPS34-independent manner, PI3P generation by PI3K class II (PI3K-C2a) has also been involved in recycling endocytosis in MEF cells (*91*, *92*) and canonical autophagy in human kidney cells HK2 when stimulated with shear stress (*93*). Further investigation of canonical and noncanonical autophagy activation by other PI3K isoforms in different cell types and physiological context may reveal additional mechanisms involved in selection of single membranes versus double membranes.

LC3 lipidation into PE or PS

Beyond the role of ATG16L1 WD domains, alternative LC3 lipid conjugation into PS on single-membrane endosomes or phagosomes represents an important molecular signature of noncanonical autophagy processes LAP and LANDO (*56*). In canonical autophagy, only LC3 lipidation to PE is observed (Fig. 4B). Although the mechanisms are still not clear, ATG4B and ATG4D, two isoforms of ATG4, the enzyme that catalyzes LC3 proteolytic priming and delipidation, might specifically contribute to the delipidation of LC3-PS in LAP and LANDO (*56*). Developing pharmacological or genetical tools that modulate LC3 lipidation or delipidation into PS or PE will provide attractive approaches for regulating noncanonical autophagy.

Liquid-liquid phase separation

Emerging evidence reported that liquid-liquid phase separation that is defined by the compartmentalization of macromolecules and cellular materials into membraneless condensates or droplet-like structures regulates at a very early stage both canonical and noncanonical

autophagy [reviewed in (*94*, *95*)]. Phase separation was initially observed in the selection of cargos during canonical autophagy. In yeast *S. cerevisiae*, the formation of gel-like condensates of aminopeptidase 1 (Ape1) is essential for the selective autophagy-like cytoplasm to vacuole pathway (*96*). In *C. elegans*, P-granule proteins are first assembled in condensates before their canonical autophagic degradation during embryogenesis (*97*). In mammals, phase separation of p62-polyubiquitiniated protein aggregates induces droplet deformation by adhesion at the submillimeter scale, also known as wetting, which supports double-membrane phagophore biogenesis (*96*, *98*, *99*). In *C. elegans* and Hela cells, the inositol polyphosphate multikinase (IMPK) has shown to directly inhibit the phase separation of TFEB, a transcription factor that controls gene expression involved in canonical autophagy and lysosome biogenesis (*97*). Changes in the physical properties of TFEB condensates, such as tension, viscosity, and elasticity, seem to regulate canonical autophagy (*100*). Interestingly, Ede1, the yeast homolog of mammalian Esp15 that usually serves as an early-acting scaffold protein for clathrin-mediated endocytosis, can oligomerize and condense by liquid-liquid phase separation. This leads to selective canonical autophagic degradation of this protein, thus acting as a quality control mechanism of endocytosis (*101*, *102*). Several conditions of adaptive stress response observed during aging and neurodegenerative diseases have been associated with alterations in droplet properties (*103*, *104*). Posttranslational modifications of amyloid-like structures observed in Alzheimer's disease appear to facilitate liquid-liquid phase separation of tau repeats (*105*). New techniques and tools are urgently necessary to probe and quantify the liquid-liquid separation interface in cells and the interactions of condensates with single- and double-membrane organelles.

CONCLUSION AND PERSPECTIVES

During the past 5 years, LANDO has emerged as a noncanonical process characterized by Rab5-positive, single-membrane endosomes interacting with VPS34 complex II and ATG16L1 WD domains and decorated with LC3-PS and LC3-PE. While specific domains in Rubicon, VPS34 complexes, and ATG16L1 are critical for LAP or LANDO activity, the elucidation of the key genes essential only for LANDO is still necessary. In vitro studies of the functions of LANDO in different stimulatory conditions in nonprofessional phagocytes such as endothelial cells and epithelial cells, where we expect a more marginal role for LAP, will be invaluable. Thus far, the generation of animals lacking Rubicon and the Atg16L1 WD domain supports a crucial role for LANDO in endocytic MHCII, cytokine receptors, and Aβ receptor recycling and human diseases such as neurodegeneration, inflammatory disorders, and metabolic diseases. Recent data reported a role for Rubicon in LC3-associated macropinocytosis, another noncanonical autophagy process in which damaged plasma membranes restore membrane integrity with the help of large endocytic vesicles that contain Rab5 (*106*). Further investigation of knockout mammals and model organisms such as *C. elegans* and *Drosophila* with an emphasis on cell-specific approaches will extend our knowledge of LANDO functions in physiological and pathological contexts.

The progress in delineating the role of LANDO relies on the development of methods for monitoring LANDO activity in vitro and in vivo. Last, drug-screening approaches that will define specific inducers and inhibitors for LANDO compared to LAP or canonical autophagy will be required for further progress in this emerging area (*107*).

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