AUTOPHAGY
2020, VOL. 16, NO. 7, 1169–1171
https://doi.org/10.1080/15548627.2020.1760057

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EDITOR'S CORNER

The LC3-conjugation machinery specifies cargo loading and secretion of extracellular vesicles

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ABSTRACT

Classical macroautophagy/autophagy functions to maintain cell health during stressful conditions by targeting cytosolic components for degradation and recycling through the lysosomal pathway. In contrast, nondegradative secretory autophagy functions as an alternative autophagy mechanism to mediate extracellular secretion. A recent study published in *Nature Cell Biology* from the laboratory of Jayanta Debnath has identified components of the LC3-conjugation machinery as mediators in the selection of cargo for nonclassical secretion. Termed LC3-dependent extracellular vesicle loading and secretion (LDELS), this mechanism provides an additional link between secretory autophagy and the release of extracellular vesicles.

Abbreviations: ATG, autophagy-related; BioID, proximity-dependent biotinylation; CM, conditioned medium; EV, extracellular vesicle; HNRNPK, heterogeneous nuclear ribonuclear protein K; ILVs, intralumenal vesicles; LDELS, LC3-dependent EV loading and secretion; LIR, LC3-interacting region; MAP1LC3/LC3, microtubule associated protein 1 light chain 3; MS, mass spectrometry; MVBs, multivesicular bodies; ncRNA, non-coding RNA; NSMAF/FAN, neutral sphingomyelinase activation associated factor; P-bodies, processing bodies; PE, phosphatidylethanolamine; RB1CC1/FIP200, RB1 inducible coiled-coil 1; RBP, RNA-binding protein; RNA-seq, RNA sequencing; SAFB, scaffold-attachment factor B; SILAC, stable isotope labeling of amino acids in cell culture; SMPD3/nSMase2, sphingomyelin phosphodiesterase 3; TEM, transmission electron microscopy; TMT, tandem mass tagging

ARTICLE HISTORY
Received 17 April 2020
Revised 17 April 2020
Accepted 17 April 2020

KEYWORDS
Autophagy; LC3-II; macroautophagy; multivesicular bodies; secretory autophagy

Extracellular vesicles (EVs) are a heterogenous population of cell-derived, membrane-bound structures that range in size from ~50-1000 nm [1,2]. EVs are secreted under both physiological and pathological conditions [1,2], and are produced by almost all organisms and cell types [3]. EVs have been isolated from various biofluid sources, including serum, plasma, breast milk, semen, urine, and amniotic and synovial fluids [2,4–7]. Various cargoes have been identified as being housed within EVs, including proteins, lipids, and nucleic acids [2,3]. These cargoes may be transferred from one cell type to another via EVs, functioning as modulators of intercellular communication [8]. However, how cells select and load unique cargo, such as nucleic acids and proteins, for secretion within extracellular vesicles has remained an intriguing question.

Although canonically characterized as a degradative mechanism, increasing evidence has indicated a role for autophagy in extracellular secretion [9–11]. Secretory autophagy engages components of the autophagy machinery to selectively transport and secrete cargo outside of the cell (for review, see [12,13]). Nonetheless, many questions remain as to exactly how secretory autophagy is mechanistically orchestrated and how a unique repertoire of autophagy-related (ATG) factors may be involved. Here, we highlight recent work from the laboratory of Jayanta Debnath demonstrating a role for the LC3-conjugation machinery in cargo selection and secretion of EVs; this mechanism has been termed LC3-dependent extracellular vesicle loading and secretion (LDELS) [14].

The Debnath lab applied multiple proteomic approaches to identify components involved in the autophagy-dependent secretome [14]. The authors hypothesized that intermediates targeted for extracellular secretion by an autophagy-dependent pathway would be captured by MAP1LC3B/LC3 [14]. LC3 is a mammalian ortholog of the highly conserved Atg8-family proteins, which functions to mediate substrate selection [15] and associates with both the nascent phagophore and mature autophagosome [16]. Using proximity-dependent biotinylation (BioID), Leidal *et al.* identified 200 potential targets of LC3-dependent secretion [14]. To perform these studies, the authors fused a mutant *Escherichia coli* biotin ligase (BirA*) to LC3 to label intracellular substrates [14]. Next, in order to identify the BirA*-LC3-associated secretome, Leidal and colleagues performed analyses to determine which substrates are secreted by examining their presence in conditioned medium (CM) in combination with stable isotope labeling of amino acids in cell culture (SILAC) and mass spectrometry (MS). The authors determined that the BirA*-LC3-associated secretome is enriched in RNA-binding proteins (RBPs) and EV-associated proteins.

LC3-II is the lipidated, phosphatidylethanolamine (PE)-conjugated membrane-associated species of LC3, which

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undergoes cleavage and lipidation following autophagy induction. The authors found that LC3-II is associated with immunopurified EVs [14], and is located within the lumen of purified EVs as evidenced by a protease-protection assay. Furthermore, a subset of EVs are derived from ILVs (intralumenal vesicles) formed from the inward budding of the multivesicular body (MVB) membrane, giving rise to the released EVs [2,17]. To determine whether the LC3-II-associated EVs arose from MVBs, the authors performed transmission electron microscopy (TEM) using the APEX-LC3 probe [18]. TEM analysis revealed LC3-positive ILVs within MVBs [14], supporting the hypothesis that the EVs housing LC3-II arise from MVBs.

Leidal *et al.* used the constitutively active $RAB5^{Q79L}$ mutant, which reduces endosomal trafficking and forms enlarged early endosomes characterized by intralumenal budding [14,19,20] to investigate whether LC3 is delivered directly to the lumen of MVBs. In WT cells, LC3 was found at the MVB limiting membrane and in ILVs [14]. However, this was not the case for cells lacking ATG7; ATG7 functions as an E1-like enzyme [21], and is a component of the machinery that facilitates LC3 conjugation to PE [22]. However, LC3 continues to localize to MVBs even in the absence of ATG14, which functions independently of the LC3 conjugation system to promote autophagy [23], suggesting that components specifically in the LC3-conjugation machinery are involved in the localization of LC3 within ILVs [14]. LC3 also colocalizes with CD63, a marker denoting MVBS and EVs [3].

To further test whether autophagy components – specifically those involved in LC3 conjugation – are required for cargo loading and EV secretion, the authors performed tandem mass tagging (TMT) quantitative proteomics to delineate differences in WT cells and those lacking LC3-conjugation system factors ATG7 and ATG12. ATG12 functions in the heterotrimeric ATG12–ATG5-ATG16L1 complex as an E3-like enzyme to facilitate LC3 conjugation (reviewed in [24]). Leidal *et al.* found significant overlap between the BirA*-LC3B-labeled secretome and the global EV proteome, indicating that EV release is largely dependent on autophagy-mediated unconventional protein secretion [14]. Significant among the identified secretome are components of RNA stress granules, processing bodies (P-bodies), and RBPs such as SAFB (scaffold attachment factor B) and HNRNPK (heterogeneous nuclear ribonuclear protein K) [14].

A long-standing question within the EV research field concerns the mechanism involved in the loading of cargo into the vesicles. How do cells actively select unique RNAs, proteins, and lipids for packaging into EVs for extracellular delivery? As LC3 mediates cargo selection during degradative autophagy, the authors hypothesized that LC3-II may capture proteins at the limiting membrane of MVBs for secretion within ILVs, and, later, EVs [14]. To examine this further, the authors focused on the two RPBs – SAFB and HNRNPK – which were identified by both the BirA*-LC3B secretome and the LC3B-dependent EV proteome studies. SAFB and HNRNPK meet all criteria to suggest that they may be selected and secreted through an autophagy-dependent secretory process, including co-fractionation with membrane-associated LC3-II during EV purification, co-immunoprecipitation with LC3B, and colocalization within mCherry-$RAB5^{Q79L}$ endosomes. The authors also noted that there is no significant loss of either SAFB or HNRNPK expression during starvation, suggesting these RBPs are not degraded during classical autophagy.

Leidal and colleagues further focused on these RBPs to demonstrate their association with LC3 and their secretion within LC3-II-enriched EVs [14]. The authors determined that the secretion of SAFB and HNRNPK is dependent upon ATG3 (encoding an E2-like enzyme that facilitates LC3 conjugation [22]), ATG7, and ATG12. EVs derived from the corresponding null cells are devoid of LC3-II, SAFB, and HNRNPK. However, these same EVs are characterized by classical markers, including PDCD6IP/ALIX, TSG101, and CD9, supporting the idea that components of the autophagy conjugation machinery (including LC3-II, ATG3, ATG7, and ATG12) are involved in selection and secretion of these RBPs into EVs. In contrast, cells lacking either ATG14 or RB1CC1/FIP200 (which functions independently of LC3 conjugation [25]) exhibit no defects in EV secretion or packaging of RBPs [14]. Thus, components of the LC3-conjugation machinery are required for cargo loading of EVs and secretion of RBPs.

LC3 and other Atg8-family proteins mediate interactions with autophagy receptors for cargo selection via LC3-interacting regions (LIRs). The canonical LIR consists of a consensus motif [W/F/Y]-X-X-[L/I/V], where X is any amino acid [26] (reviewed in [27]). Considering that LC3-dependent cargo selection may function as a form of selective autophagy for capturing desired targets, Leidal *et al.* examined whether the selection of SAFB and HNRNPK for secretion were due to the presence of LIR motifs [14]. Analysis for these regions revealed that amino acids 199–202 of SAFB (F_{199}TIL) form a canonical LIR, whereas HNRNPK contains only a minimal consensus. The authors then mutated the LIR in SAFB, and found that $SAFB^{F199A}$ exhibits both reduced interaction with LC3 and impaired secretion within EVs.

EVs transfer nucleic acids, including various small non-coding RNAs (ncRNAs) between EVs and recipient cells [8]. Subsequently, in addition to RBPs, Leidal and colleagues investigated whether LDELS is involved in the selection and secretion of another cargo, ncRNAs, into EVs [14]. Quantitative RNA sequencing (RNA-seq) of both cells and EVs isolated from either wild-type, *ATG7*- or *ATG12*-deficient cells revealed that the expression of intracellular small RNA species were largely similar. However, EVs derived from LDELS-deficient (*ATG7* or *ATG12* null) cells show significantly less enrichment of small nucleolar RNAs/snoRNAs and microRNAs/miRNAs. Thus, the selection and packaging of RBPs and small RNA species is dependent on LC3-conjugation components, including ATG7 and ATG12. Loss of the LC3-conjugation machinery, including *ATG7* and *ATG12*, is sufficient to alter the profile of extracellular small ncRNAs in EVs.

The authors further examined whether cell trafficking pathways involved in ILV formation from the MVB limiting membrane contribute to LDELS [14]. Although they did not find any evidence for the endosomal sorting complex required for transport/ESCRT machinery, Leidal *et. al* also analyzed whether an alternate mechanism – the SMPD3/neutral sphingomyelinase 2 (sphingomyelin phosphodiesterase 3) pathway, which produces ceramide to promote vesicle curvature and

inward budding from MVBs to promote EV release, is involved [14,20]. Through bioinformatics and biochemical approaches, the authors demonstrated the involvement of SMPD3 and the downstream factor NSMAF/FAN (neutral sphingomyelinase activation associated factor) [14]. Indeed, NSMAF harbors a LIR (F_{602}EDL), which mediates its interaction with LC3 and the selection of LC3-interacting RBPs for packaging and secretion within EVs.

The roles of LC3 and the associated conjugation-machinery are distinct between canonical autophagy and LDELS described by Leidal *et al.* [14]. Rapamycin treatment (which strongly induces classical autophagy) appears to reduce LDELS, including the secretion of LC3-II and LC3-binding RBPs. These results highlight an important distinction in the regulation of the classical degradative versus the secretory functions of autophagy. What are the mechanisms directing the shunting of cellular resources toward secretory autophagy rather than toward degradation? Furthermore, could dysregulation of this "switch" contribute to an enhancement (or reduction) of degradation, thus tilting the balance in the opposite direction toward reduced (or increased) secretion, and contribute as a previously unrecognized factor in disease pathogenesis? Certainly, the role of classical autophagy as an underlying mechanism in human disease is well established [28]. Undoubtedly, further investigation into the emerging roles of, and mechanisms regulating, secretory autophagy will be critical to understanding the full spectrum of autophagy in cell physiology and human disease.

Acknowledgments

The authors apologize to those whose work was not included here due to space limitations.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Institute of General Medical Sciences [GM131919].

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