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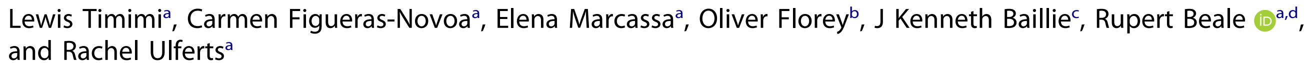
The V-ATPase complex regulates non-canonical Atg8-family protein lipidation through ATG16L1 recruitment



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ABSTRACT
Conjugation of the Atg8 (autophagy related 8) family of ubiquitin-like proteins to phospholipids of the phagophore is a hallmark of macroautophagy/autophagy. Consequently, Atg8 family members, especially LC3B, are commonly used as a marker of autophagosomes. However, the Atg8 family of proteins are not found solely attached to double-membrane autophagosomes. In non-canonical Atg8-family protein lipidation they become conjugated to single membranes. We have shown that this process is triggered by recruitment of ATG16L1 by the vacuolar-type H^+-translocating ATPase (V-ATPase) proton pump, suggesting a role for pH sensing in recruitment of Atg8-family proteins to single membranes.

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Non-canonical lipidation describes the conjugation of Atg8-family proteins to single membranes (CASM). These single-membrane structures are distinct from autophagosomes. This process has been observed in several related contexts, including LC3-associated phagocytosis (LAP), entosis (a live-cell engulfment event seen in tumors), STING1 signaling, ionophore drug treatment, viral infection and bacterial internalization. These processes also reflect a diversity of functions ascribed to non-canonical lipidation, which includes roles in inflammation regulation, antigen presentation, pathogen clearance and virion assembly.

The regulation of non-canonical Atg8-family protein lipidation is distinct from autophagy. The MTOR-ULK signaling axis is dispensable for CASM, whereas ATG16L1 recruitment to single-membrane targets is independent of RB1CC1/FIP200 and WIPI2 but dependent on the ATG16L1 WD40 domain. The core lipidation machinery (comprising ATG3, ATG4, ATG5, ATG7, ATG10, ATG12 and ATG16L1) is required for both canonical and non-canonical lipidation. However, the upstream signaling events responsible for the targeting of this lipidation machinery to single-membrane sites remain poorly understood.

While Atg8-family protein lipidation is commonly employed as a proxy for induction of autophagy and autophagic flux, most standard assays do not discriminate canonical from non-canonical lipidation. This can confound experimental data derived from these assays. Better tools are required to dissect these processes but will ultimately rely on a mechanistic understanding of non-canonical lipidation.

In a recent study [1], we conducted a whole-genome CRISPR-Cas9 knockout screen for regulators of non-canonical LC3B lipidation. Influenza A virus drives non-canonical lipidation through the virally encoded proton channel matrix 2 (M2). Central to our approach was the production of a GFP-LC3B cell line expressing M2 under the control of a doxycycline-inducible promoter. This allowed us to strongly switch on non-canonical LC3B lipidation after CRISPR-Cas9-dependent knockout. We then exploited the resistance of GFP-LC3B-II to washes following permeabilization with a mild detergent to sort cells for an increase or decrease in lipidation. Using a subtractive scoring method for individual guide RNAs versus unpermeabilized control, we successfully identified all six components of the core lipidation machinery as the top hits.

In addition to the lipidation machinery, we identified three novel regulators of M2-induced non-canonical LC3B lipidation. ATG4D, of the Atg4 family of Atg8-family protein proteases, was the only antagonist of this process discovered. Knockout studies confirmed negative regulation by ATG4D, and further kinetic studies pointed to a prominent role for ATG4D in LC3 delipidation. These observations are consistent with recent work describing the ability of ATG4D to delipidate LC3/GABARAP-phosphatidylserine observed specifically during non-canonical lipidation.

RALGAPB, encoding the non-catalytic subunit of the RAL GTPase activating protein (RALGAP) complex, was one of two novel positive regulators identified. Although follow-up studies confirmed the requirement for the intact RALGAP complex in M2-induced lipidation, this effect appears specific for expression and localization of M2 and does not seem to regulate non-canonical LC3 lipidation universally.

Finally, we identified ATP6V0A1, a subunit of the V-ATPase. This hit was particularly provocative as non-canonical lipidation seems to follow proton gradient disturbances and is sensitive to V-ATPase inhibitors. ATG16L1 has been described to interact with the V-ATPase during bacterial

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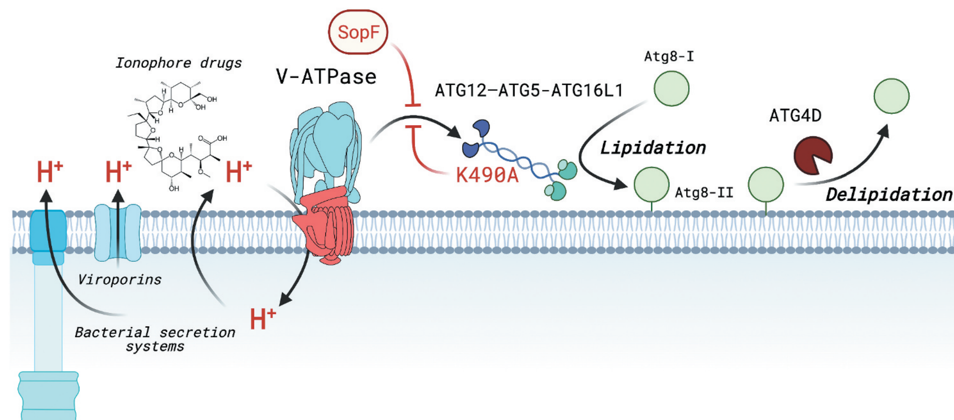

Figure 1. A model of non-canonical Atg8-family protein lipidation (CASM) driven by V-ATPase-mediated ATG12–ATG5-ATG16L1 recruitment. This interaction is driven by a variety of stimuli that affect intracellular pH gradients. Downstream of this, Atg8-family proteins can be delipidated by ATG4D. SopF can selectively inhibit this pathway through the disruption of ATG16L1-V-ATPase complex formation. This interaction is also disrupted by the K490A point mutation in the WD40 domain of ATG16L1. Created with BioRender.com.

infection, although this observation was not interpreted in the context of non-canonical lipidation. We confirmed that ATG16L1 interacts with V-ATPase subunits following M2 expression, IAV infection and ionophore treatment. Moreover, this interaction can be disrupted by the K490A point mutation in the WD40 domain of ATG16L1, accounting for the requirement of this domain in single-membrane lipidation. Finally, the bacterial effector SopF, which disrupts ATG16L1-V-ATPase binding, is able to inhibit non-canonical lipidation in all studied contexts, demonstrating the importance of this interaction for downstream activity.

Collectively, our work leads us to a model of non-canonical Atg8-family protein lipidation in which the V-ATPase complex is central to the recruitment of the ATG16L1 complex to single-membrane sites, via the N453, F467, K490 pocket of ATG16L1 (Figure 1). Lipidated LC3 at the single membrane is subsequently turned over in part through ATG4D-mediated delipidation. Notably, this mechanism provides a potential basis for the selective modulation of non-canonical lipidation – a longstanding challenge within the field. Through targeting the ATG16L1-V-ATPase interaction, SopF and the K490A point mutation in the ATG16L1 WD40 domain provide powerful tools to identify non-canonical Atg8-family protein lipidation.

Despite these advances, the cell biological purpose of non-canonical Atg8-family protein lipidation remains uncertain. It coincides with a loss of proton gradient integrity in many contexts, and we propose that it represents a conserved response to pH-gradient disturbance. This hypothesis is supported by the role of a ubiquitous proton pump, the V-ATPase, in the response to distinct stimuli. The V-ATPase complex is proposed to couple its activity to lumenal pH, but how this is linked to ATG16L1 binding remains to be determined. Further mechanistic insight will help to define the cellular consequences of V-ATPase-dependent Atg8-family protein lipidation, as well as shed light on the longstanding problem of how compartment specific lumenal pH is maintained.

Disclosure statement

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Reference

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