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SPOTLIGHT

ATG16L strikes again! New findings link lysosome stress and physiology

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Lysosome stress responses are emerging, but their connections to normal physiology are not well understood. In this issue, Duque et al. (https://doi.org/10.1083/jcb.202503166) discover that the autophagy protein ATG16L, a mediator of a stress response called CASM, also regulates normal lysosome function.

Stress responses are typically discovered by imposing harsh conditions on cells and then asking, "How do they respond?" So is the story for a lysosomal stress response called CASM, or conjugation of Atg8s to single membranes, which was found by treating cells with proton ionophores, lysosomotropic drugs, or lysosome-rupturing agents that perturb lysosomal pH and disrupt membrane integrity (1, 2). In return, the autophagy protein ATG16L initiates a stress response that involves the covalent conjugation of six related ubiquitin-like proteins, called ATG8s, onto lipid headgroups at lysosomal membranes. ATG8 conjugation, or "ATG8ylation," sets in motion a set of downstream responses that help to maintain lysosome fitness, for example, by repairing and turning over damaged membranes and by inducing the expression of lysosomal genes (2). Known mutations that predispose to neurodegenerative or inflammatory conditions are increasingly becoming linked to CASM, suggesting this stress response may be critical to staving off lysosome dysfunctions that occur in numerous diseases. But while autophagy proteins are now well known to target the membranes of stressed lysosomes through this mechanism, whether a similar activity might contribute to controlling normal lysosome function in the absence of stress has remained elusive. Now a study in this issue by Duque et al. uncovers a direct link between stress signaling and normal physiology, as they find that ATG16L, which is recruited to stressed lysosomes by binding to the lysosomal vacuolar-type H+-ATPase (v-ATPase), is also, reciprocally, a basal regulator of v-ATPase activity (3).

The v-ATPase is a complex machine composed of 13 protein subunits, organized into a cytosolic eight protein subcomplex called V1, which binds to a membrane-integral five-protein complex called V0 to form the holoenzyme that pumps protons into the lysosome lumen. Cells spend considerable energy using this pump to maintain lysosomes at low pH, creating a specialized environment where the activity of degradative enzymes is sequestered from the rest of the cell. In Duque et al., the authors follow an initial observation that lysosome activity is elevated in cells with knockout of *ATG16L*, a consequence, it turns out, of a "hyperacidification" lysosomal phenotype that results from increased v-ATPase activity (3). The same phenotype occurs in cells with other CASM-regulating gene knockouts (e.g., *ATG5*, *ATG3*, and *ATG7*) but not in cells with canonical autophagy-specific knockouts (*FIP200* and *ATG13*) and is recapitulated in vitro with purified lysosomes, where elevated proton flux can also be rescued by adding purified ATG16L protein.

So how is ATG16L regulating v-ATPase activity? The authors observe no changes in levels of expression of v-ATPase proteins in *ATG16L* knockout cells, excluding effects on transcription or protein abundance, yet they find increased levels of holoenzyme complexes at lysosomal membranes, suggesting that ATG16L can somehow affect v-ATPase assembly. They further identify, through APEX2 labeling, interactions between ATG16L and V1 v-ATPase subcomplex proteins. In rescue experiments with wild-type or mutant *ATG16L* constructs, they show that direct interaction between ATG16L and one particular V1 subcomplex protein, V1H, is required for regulation. This interaction is mediated by the C-terminal WD domain of ATG16L, previously implicated in regulating CASM (4), as well as specific residues in the coiled-coil region that are predicted to bind to V1H and which the authors show are also required. They further study this regulation by using a mouse model with deletion of the *ATG16L* WD domain and show that the V1H-interacting interface is required for control over infection by *Mycobacterium tuberculosis*, a phenotype they speculate could relate to an inability to properly control v-ATPase pumping.

This new study positions the v-ATPase—a fundamental regulator of lysosome physiology—at a critical nexus of health and disease, with regulation informing on both normal lysosome function and stress responses centered on its interaction with ATG16L (Fig. 1). It will be important in future studies to examine if regulation of the v-ATPase is mediated by a unique function of ATG16L, or whether it might instead involve the induction of CASM downstream. That knockouts of other CASM-regulating genes share the same hyperacidification phenotype points toward a CASM-based

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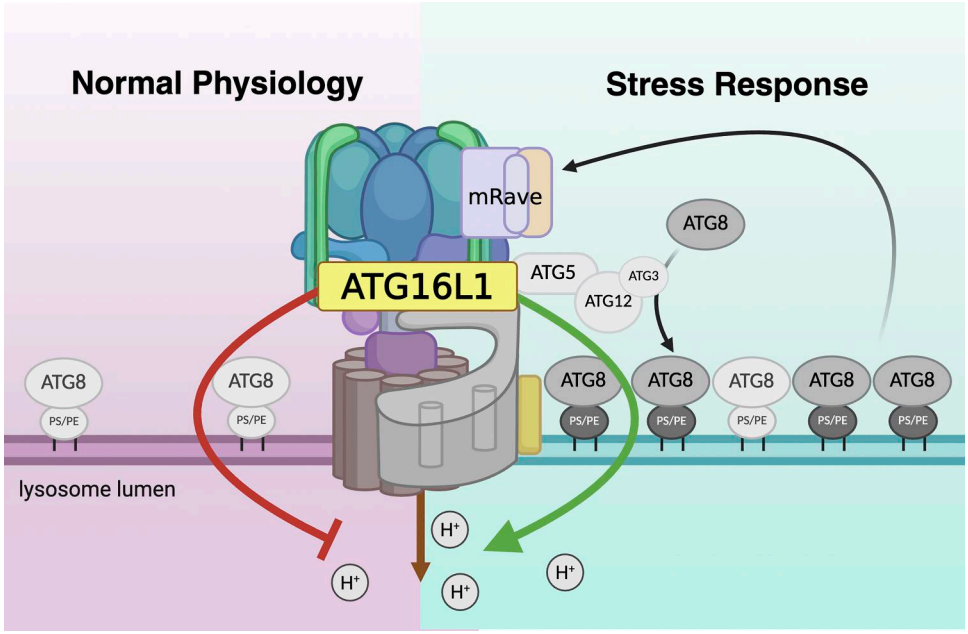

Figure 1. **ATG16L inhibits the lysosomal v-ATPase under normal conditions but leads to its activation in response to stress.** Left: ATG16L binds to V1H of the v-ATPase under normal conditions, reducing lysosome acidification (red arrow). Inhibition by ATG16L may or may not involve CASM. Right: Under stress or damage, ATG16L-dependent CASM activation occurs with increased v-ATPase assembly through mRAVE (DMXL1/2, WDR7, and ROGDI). Increased acidification may necessitate ATG16L disengagement. Alternatively, different ATG8 effectors could mediate different effects on v-ATPase activity, potentially linked to conjugation of different ATG8 orthologs (depicted in grayscale), and attachment to either phosphatidylserine or phosphatidylethanolamine (PS/PE). Note: mRAVE is depicted on a v-ATPase holoenzyme but may disengage after assembly, and ATG16L and mRAVE binding to the v-ATPase may be mutually exclusive. Figure created on https://Biorender.com.

mechanism. On the other hand, a CASM-independent mechanism is favored by results from the in vitro assay, where recombinant ATG16L can control acidification at purified lysosomes, and in cells where overexpressed ATG16L can regulate lysosome acidification even in *ATG5* knockouts that are CASM deficient.

Intriguingly, CASM induced by lysosomal stress was recently shown to enhance v-ATPase activity by increasing holoenzyme assembly (5). Here, lipid-conjugated ATG8s were found to lead to the recruitment of DMXL1/2 proteins and their binding partners WDR7 and ROGDI, forming a complex recently coined mRAVE (6), which promotes v-ATPase assembly to restore proper pH gradients as lysosomes recover from stress. mRAVE may also control the increased holoenzyme assembly that first recruits ATG16L to activate CASM (6). This then begs the question: How can ATG16L function to restore pH gradients in response to stress, while at the same time inhibit acidification in the absence of stress, through the same V1H interface?

If a CASM-based mechanism is involved in basal regulation, then opposing effects on v-ATPase activity could result from differential recruitment of different ATG8 effectors to lysosomal membranes. Curiously, among six different ATG8 orthologs in mammalian cells, one subfamily of three proteins called "GABARAP," has emerged as a critical mediator of stress responses, in some cases with a unique ability to bind to specific effectors compared with the "LC3" subfamily (7). It is conceivable then that different conjugations of ATG8 proteins in non-stressed versus stressed conditions could specify unique effector recruitments. Similarly, ATG8s can also be conjugated onto two different phospholipids at lysosomes (phosphatidylethanolamine and phosphatidylserine) (8), suggesting an additional layer of complexity. It is finally important to consider that the amount of ATG8ylation at lysosomal membranes under non-stressed conditions is predicted to be low, so relative amounts of ATG8 conjugation could also be a contributing factor to v-ATPase activation.

ATG16L could also regulate activity of the v-ATPase in the absence of CASM. It has previously been shown that ATG16L binds to V1H in assembled holoenzymes and that this binding is reduced or unavailable during v-ATPase pumping activity induced by the addition of ATP (4). This has suggested that the presence of inactive holoenzymes recruits ATG16L to lysosomes to initiate a CASM response. The new findings from Duque et al. may add to this model by predicting that ATG16L not only binds to inactive holoenzymes but also functions to keep them inactive, perhaps acting as a brake to inhibit pumping until lysosomal membranes can be repaired (3). One could imagine that ATG16L might then need to disengage to initiate pumping and that lipid-conjugated ATG8s might also need to persist long enough on lysosomal membranes after ATG16L disengagement to recruit mRAVE and drive further v-ATPase assembly.

Beyond a stress response, the new findings from Duque et al. focus attention on the ability of ATG16L to inhibit the v-ATPase under non-stressed conditions (3). This is reminiscent of regulation by the mTORC1 kinase, which has also been shown to limit lysosome function by inhibiting v-ATPase holoenzyme assembly, a mechanism that is relieved upon nutrient starvation when lysosome activity is upregulated (9). Cells seem to exert considerable effort to constrain v-ATPase activity in a way that it is

also poised to respond rapidly to stress, but the question remains, why hold lysosome activity at sub-maximal levels in the first place? In the case of *ATG16L* knockout cells, the authors observe increased AMP levels and lowered energy charge when lysosomes are hyperacidified, suggesting that elevating v-ATPase activity in excess of cellular demand comes with a significant cost. Keeping lysosomes at sub-maximal activity may not only conserve ATP but would also slow the degradation of complex substrates, potentially allowing lysosomes to fine-tune control over metabolite flux or storage.

Finally, it may be important to consider that most cells contain many individual lysosomes, from one to several hundred, and v-ATPase holoenzymes are assembled across lysosome networks in an uneven distribution, and therefore with corresponding effects on pH that are also distributed unevenly (10). So, whether constitutive or stress-induced regulations target lysosomes indiscriminately, or whether they can instead modify fractions of lysosome networks, to be, for example, more or less degradative, is an open question whose answers may provide deeper insights into the interplay between lysosome physiology, stress responses, and disease.

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