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VIEWPOINT

ATG8ylation of proteins: A way to cope with cell stress?

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The ATG8 family of proteins regulates autophagy in a variety of ways. Recently, ATG8s were demonstrated to conjugate directly to cellular proteins in a process termed "ATG8ylation," which is amplified by mitochondrial damage and antagonized by ATG4 proteases. ATG8s may have an emerging role as small protein modifiers.

ATG8 proteins directly conjugate to cellular proteins

Autophagy describes the capture of intracellular material by autophagosomes and their delivery to lysosomes for destruction (Kaur and Debnath, 2015). This process homeostatically remodels the intracellular environment and is necessary for an organism to overcome starvation (Kaur and Debnath, 2015). The autophagy pathway is coordinated by autophagy-related (ATG) proteins that are controlled by diverse post-translational modifications (e.g., phosphorylation, acetylation, ubiquitination, and lipidation; Ichimura et al., 2000; McEwan and Dikic, 2011). Recently, a previously uncharacterized post-translational modification termed "ATG8ylation" was uncovered (Agrotis et al., 2019; Nguyen et al., 2021). ATG8ylation is the direct covalent attachment of the small ubiquitin-like family of ATG8 proteins to cellular proteins (Agrotis et al., 2019; Nguyen et al., 2021). Until now, the only known instances of ATG8 conjugation to proteins were of a transient nature, as E1- and E2-like intermediates with ATG7 and ATG3, respectively, as a way of ligating ATG8 to the lipid phosphatidylethanolamine during autophagy (Ichimura et al., 2000). Therefore, ATG8ylation may represent an underappreciated regulatory mechanism for many cellular proteins that coordinate pathways such as mitophagy.

ATG8s play many roles in the autophagy pathway

During canonical autophagy, the ATG8 family (comprising LC3A, -B, and -C and GABARAP, -L1, and -L2) undergoes molecular processing that concludes with their attachment to phosphatidylethanolamine, enabling proper construction of autophagosomes and subsequent autophagosome–lysosome fusion (Nguyen et al., 2016). The ATG4 family of cysteine proteases (ATG4A, -B, -C, and -D) cleaves ATG8 proteins immediately after a conserved glycine residue in their C terminus in a process dubbed "priming," which leads to the formation of ATG8-I (Skytte Rasmussen et al., 2017; Tanida et al., 2004). ATG7 then attaches to the exposed glycine residue of ATG8-I via a thioester linkage to form an E1 ubiquitin-like complex that transfers ATG8-I to ATG3 in a similar way to generate an E2-like complex (Ichimura et al., 2000). The ATG5–ATG12–ATG16L1 complex then catalyzes the E3-like transfer of ATG8-I from ATG3 to phosphatidylethanolamine to form ATG8-II, which is the lipidated species that is incorporated into double membrane–bound compartments such as autophagosomes (Hanada et al., 2007). The lipidation of ATG8s and their recruitment to the phagophore are not essential for the formation of autophagosomes but are important for phagophore expansion, the selective capture of autophagic substrates, and autophagosome–lysosome fusion (Kirkin and Rogov, 2019; Nguyen et al., 2016). Intriguingly, ATG8 lipidation is multifaceted, as ATG8s can be alternatively lipidated with phosphatidylserine (instead of phosphatidylethanolamine) to enable their recruitment to single membrane–bound compartments during LC3-associated phagocytosis, influenza infection, and lysosomal dysfunction (Durgan et al., 2021).

The discovery of ATG8ylation

Key insights into ATG8ylation came from the observation that various ATG8s form high-molecular-weight species in cells following the expression of their primed forms that have their C-terminal glycine exposed (for example, LC3B-G), bypassing the need for cleavage by ATG4 (Agrotis et al., 2019; Nguyen et al., 2021). Indeed, on an immunoblot, ATG8$^+$ "smears" resemble that of ubiquitinated proteins (Agrotis et al., 2019; Nguyen et al., 2021). Traditionally, in the autophagy field, ATG8$^+$ smears were thought to arise from poor antibody specificity. However, in light of recent findings, this widely accepted interpretation has been challenged, given that ATG8$^+$ smears are enriched following ATG8 overexpression and disappear in the absence of ATG8s (Agrotis et al., 2019; Nguyen et al., 2021). Smearing has also been detected after

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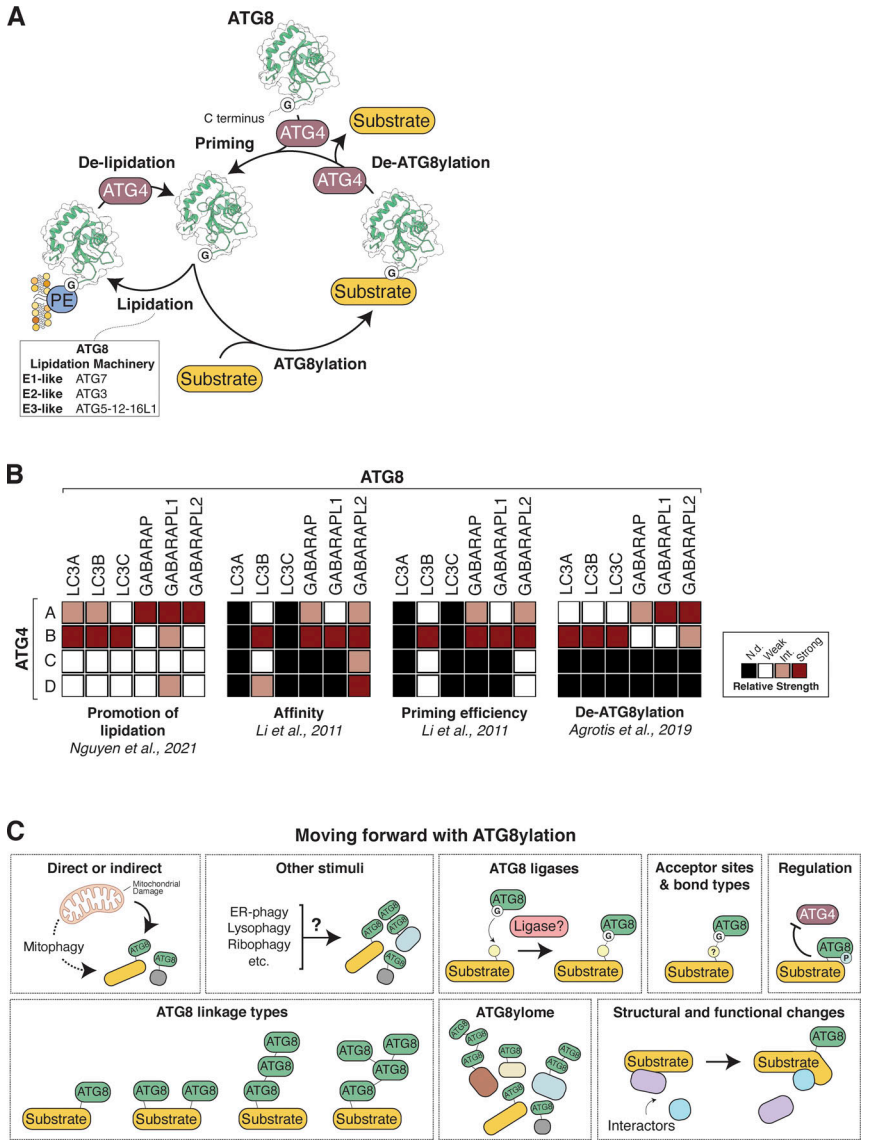

Figure 1. **The many roles of ATG4 in ATG8 processing. (A)** Molecular processing of ATG8 proteins by ATG4 illustrating its roles in priming, de-lipidation, and de-ATG8ylation. The structure of LC3B (Protein Data Bank accession no. 1V49) was used to denote ATG8 (G, glycine; PE, phosphatidylethanolamine). **(B)** Heatmap summarizing relationships between ATG4 isoforms and ATG8 family members. Data were summarized for qualitative interpretation (Agrotis et al., 2019; Li et al., 2011; Nguyen et al., 2021). Int., intermediate; N.d., not determined. **(C)** Graphical summary of questions moving forward with ATG8ylation (P, phosphorylation).

immunoprecipitation of epitope-tagged ATG8s from cell extracts under denaturing conditions, ruling out noncovalent interactions accounting for this upshift (Agrotis et al., 2019; Nguyen et al., 2021). Further, smearing is not abolished by deubiquitinase treatment, arguing strongly against ATG8 ubiquitination as the cause (Nguyen et al., 2021). Everything considered, the most plausible explanation is that ATG8 itself undergoes covalent linkage to cellular proteins, akin to ubiquitin and NEDD8 modifiers, which are structurally similar to ATG8s. Remarkably, the protease ATG4 antagonizes the ATG8ylation state of many proteins (Agrotis et al., 2019; Nguyen et al., 2021).

ATG4 displays isoform-specific proteolytic cleavage of ATG8

ATG4 is required for the formation of autophagosomes, but its protease activity is not (Nguyen et al., 2021). The protease activity of ATG4 is, however, required for ATG8 processing, such as priming ahead of lipidation and de-lipidation, which removes excess ATG8 from autophagosomes and other membranes (Nguyen et al., 2021; Tanida et al., 2004; Fig. 1 A). Apart from these functions, ATG4 regulates the deubiquitinase-like removal of ATG8 from cellular proteins (de-ATG8ylation; Agrotis et al., 2019; Nguyen et al., 2021; Fig. 1 A). Consistent with this role, deletion of all four ATG4 isoforms (A, B, C, and D) increases the abundance of ATG8ylated proteins (Nguyen et al., 2021). In contrast, overexpression of ATG4B has the opposite effect, but only if its protease activity is intact (Agrotis et al., 2019). As such, ATG4 inhibits the ATG8ylation state of many proteins, which is likely to modulate their downstream functions.

ATG4 is an important "gatekeeper" for ATG8 conjugation events. ATG4 primes ATG8s to expose their C-terminal glycine, which is required for conjugation to proteins or lipids; however, ATG4 also catalyzes de-ATG8ylation and de-lipidation events, respectively (Agrotis et al., 2019; Nguyen et al., 2021; Tanida et al., 2004). Because the C-terminal glycine of a single ATG8 is occupied when conjugated to a protein or lipid, it is unlikely that ATG8ylated proteins directly engage with phagophore membranes in the same way as ATG8-II. Indeed, protease protection assays with recombinant ATG4B reveal that de-ATG8ylation of cell lysates remains unchanged with or without organellar membrane disruption, suggesting that ATG8ylated proteins are largely cytoplasmic facing rather than intraluminal (Agrotis et al., 2019). Paradoxically, however, ATG8ylation is enhanced by lysosomal V-type ATPase inhibition, which blocks the degradation of lysosomal contents, indicating that ATG8ylated substrates may undergo lysosome-dependent turnover (Agrotis et al., 2019; Nguyen et al., 2021). One explanation for these differences may be that the process of ATG8ylation is itself sensitive to lysosomal dysfunction.

Functional relationships between ATG4s and ATG8s

Isoforms of ATG4 show clear preferences for proteolytically processing ATG8 subfamilies (i.e., LC3s and GABARAPs) for de-ATG8ylation and priming upstream of phosphatidylethanolamine ligation (Agrotis et al., 2019; Li et al., 2011; Nguyen et al., 2021; Fig. 1 B). ATG4A strongly reduces the abundance of proteins that have been ATG8ylated with the GABARAP family while

promoting ligation of GABARAPs to phosphatidylethanolamine (Agrotis et al., 2019; Nguyen et al., 2021; Fig. 1 B). In contrast, ATG4B strongly reduces the abundance of proteins that have been ATG8ylated with LC3 proteins while promoting ligation of LC3s to phosphatidylethanolamine (Agrotis et al., 2019; Nguyen et al., 2021; Fig. 1 B). In comparison, ATG4C and -D lack obvious de-ATG8ylation activity, although the latter weakly promotes phosphatidylethanolamine ligation to GABARAPL1 only (Nguyen et al., 2021). These functional similarities between ATG4 isoforms are consistent with both their sequence and structural homology (i.e., ATG4A and -B are most similar; Maruyama and Noda, 2018; Satoo et al., 2009). Structurally, ATG4B adopts an auto-inhibited conformation with its regulatory loop and N-terminal tail blocking substrate entry to its proteolytic core (Maruyama and Noda, 2018). LC3B induces conformational rearrangements in ATG4B that involve displacement of its regulatory loop and its N-terminal tail, with the latter achieved by an interaction between the ATG8-interacting region in its N-terminal tail with a second copy of LC3B that functions allosterically (Maruyama and Noda, 2018; Satoo et al., 2009). These rearrangements permit entry of LC3B into the proteolytic core of ATG4B, where cleavage of LC3B following its C-terminal glycine occurs (Li et al., 2011; Maruyama and Noda, 2018). ATG4B^{L232} is directly involved in LC3B binding and its selectivity for LC3s (Satoo et al., 2009). This residue corresponds to ATG4A^{I233} and, when substituted for leucine, gives ATG4A^{I233L} the ability to efficiently process LC3 proteins, whereas without this mutation it preferentially processes GABARAPs (Satoo et al., 2009). Moreover, the ATG8–ATG4 interaction is necessary for the de-ATG8ylation of cellular proteins, as an LC3B-G^{Q116P} mutant that cannot bind to ATG4 leads to widespread ATG8ylation (Agrotis et al., 2019). Altogether, these observations hint toward a common mechanism of ATG8 cleavage that regulates priming, de-lipidation, and de-ATG8ylation.

Mitochondrial damage promotes ATG8ylation

ATG8ylation of cellular proteins appears to be enhanced by mitochondrial depolarization and inhibition of the lysosomal V-type ATPase (Agrotis et al., 2019; Nguyen et al., 2021). This may be the consequence of acute ATG4A and -B inhibition, given that cells lacking all ATG4 isoforms display an increased abundance of ATG8ylated proteins and are insensitive to further increase by mitochondrial depolarization or lysosomal V-type ATPase inhibition (Agrotis et al., 2019; Nguyen et al., 2021). Indeed, mitochondrial depolarization leads to activation of ULK1, which phosphorylates ATG4B^{S316} to inhibit its protease activity (Pengo et al., 2017). Similarly, mitochondrial depolarization stimulates TBK1 activation, which prevents de-lipidation of ATG8s by blocking the ATG8–ATG4 interaction through phosphorylation of LC3C$^{S93/S96}$ and GABARAP-L2$^{S87/S88}$ (Herhaus et al., 2020; Richter et al., 2016). As such, ATG8 phosphorylation may render ATG8ylated substrates more resistant to de-ATG8ylation by ATG4s. This may be analogous to how chains of phosphorylated ubiquitin^{S65} are more resistant to hydrolysis by deubiquitinating enzymes than unphosphorylated ones (Wauer et al., 2015). Moreover, ATG8ylation is insensitive to nutrient deprivation and pharmacological inhibition of mTOR, which rules out a functional contribution of this process to starvation-induced autophagy (Agrotis et al., 2019). Therefore, ATG8ylation may be a unique aspect of mitophagy (and perhaps also other forms of selective autophagy) given that depolarization potently activates Parkin-dependent mitophagy (Agrotis et al., 2019; Nguyen et al., 2021).

Substrates of ATG8ylation

Based on ATG8^{+} smearing, ATG4 regulates the de-ATG8ylation of numerous proteins (Agrotis et al., 2019; Nguyen et al., 2021). For the majority, their identity, induced structural and functional changes, and the cellular contexts during which these modifications occur await exploration. Considering that the ATG8 interactome is well characterized, it is likely that at least some ATG8ylated proteins have been mistaken for ATG8-binding partners (Behrends et al., 2010). Given their E2- and E3-like roles in ATG8 lipidation, it is remarkable that ATG3 and ATG16L1 are themselves modified by ATG8ylation (Agrotis et al., 2019; Hanada et al., 2007; Ichimura et al., 2000; Nguyen et al., 2021). Lysine mutagenesis indicates that ATG3^{K243} is the "acceptor" site for ATG8ylation (Agrotis et al., 2019). ATG3^{K243} is essential for its conjugation to either LC3B or ATG12 and is required for autophagosomes to form around damaged mitochondria (Agrotis et al., 2019; Radoshevich et al., 2010). This also raises the possibility that key functions originally attributed to ATG3–ATG12 conjugation may be, at least in part, due to ATG3–ATG8 conjugation. Because multiple high-molecular-weight species of ATG3 are enriched following immunoprecipitation of primed LC3B-G from cells lacking ATG4B, it is likely that ATG3 is either mono-ATG8ylated at several sites or poly-ATG8ylated (Agrotis et al., 2019). ATG8ylation of ATG3 may also reflect the stabilization of its E2-like intermediate (Ichimura et al., 2000). ATG8ylation of ATG16L1 may regulate whether canonical or noncanonical autophagy pathways are activated (Durgan et al., 2021; Nguyen et al., 2021). In line with this possibility, the WD40 domain mutant of ATG16L1^{K490A} prevents lipidation of ATG8s with phosphatidylserine (i.e., during noncanonical autophagy pathways) but not phosphatidylethanolamine (i.e., during canonical autophagy; Durgan et al., 2021). Moreover, given that ATG8ylation of protein targets correlates with the activation of mitophagy, it is tempting to speculate that it may stimulate the E2-/E3-like activity of the ATG8 conjugation machinery to amplify mitochondrial capture and destruction.

Concluding remarks

The finding that numerous cellular proteins are modified by ATG8ylation poses several questions about how signaling networks are coordinated during selective autophagy (i.e., mitophagy). Whether ATG8ylation is augmented by mitochondrial injury per se or is the consequence of mitophagy activation is yet to be determined, as is whether this phenomenon occurs during other types of selective autophagy (e.g., ER-phagy, ribophagy, and lysophagy; Kirkin and Rogov, 2019; Fig. 1 C). While the in vivo relevance of ATG8ylation is not yet understood, it is plausible that this process could be altered in diseases with defective mitophagy (e.g., Parkinson's disease and atherosclerosis). Exploring the mechanistic aspects of ATG8ylation (e.g., ATG8 ligases and regulatory proteins, linkage types, acceptor sites, etc.) and de-ATG8ylation by ATG4 will improve our understanding about how this modifier alters the structure and biological function of

cellular proteins (Fig. 1 C). By identifying ATG8ylated substrates, or the ATG8ylome, insights into whether ATG8ylation is a ubiquitous epiphenomenon or a post-translational modification that is selective to proteins of distinct biological function(s) will become clearer (Fig. 1 C). Considering the similarity of ATG8s with bona fide modifier proteins (e.g., ubiquitin and ubiquitin-like proteins) and the diversity of their substrates (e.g., lipid species and proteins), only now are we beginning to understand the functional complexities of the ATG8 protein family.

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