The insufficiency of ATG4A in macroautophagy

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Abstract

During autophagy, LC3 and GABARAP proteins become covalently attached to phosphatidylethanolamine (PE) on the growing autophagosome. This attachment is also reversible. Deconjugation (or delipidation) involves the proteolytic cleavage of an isopeptide bond between LC3 or GABARAP and the PE headgroup. This cleavage is carried about by the ATG4 family of proteases (ATG4A, B, C and D). Many studies have established that ATG4B is the most active of these proteases and is sufficient for autophagy progression in simple cells. Here we examine the second most active protease, ATG4A, to map out key regulatory motifs on the protein and to establish its activity in cells. We utilize fully in vitro reconstitution systems where we control the attachment of LC3/GABARAP members and discover a role for a carboxy-terminal (COOH-terminal) LC3-interacting region (LIR) on ATG4A in regulating its access to LC3/GABARAP. We then use a gene-edited cell line in which all four ATG4 proteases have been knocked out to establish that ATG4A is insufficient to support autophagy and is unable to support GABARAP proteins removal from the membrane. As a result, GABARAP proteins accumulate on membranes other than mature autophagosomes. These results suggest that to support efficient production and consumption of autophagosomes, additional factors are essential including possibly ATG4B itself, or one of its proteolytic products in the LC3 family.

Introduction

Macroautophagy is a conserved pathway for intracellular degradation (1–4) and is highly induced under amino acid starvation (1, 5–8). After induction, portions of cytoplasm are captured as cargo into a double-membrane cup-shaped structure called the phagophore, which eventually closes upon itself sequestering the cargo within and becoming the autophagosome. The process culminates in autophagosome-lysosome fusion where degradation of cargo is driven by lysosomal hydrolases (2, 9, 10). Disruption of autophagy is associated with a multitude of diseases related to neurodegeneration, aging, microbial infection, and cancer (11–13).

Atg8 is a peripheral ubiquitin-like protein that is crucial for phagophore expansion, autophagosome completion, cargo targeting, and regulation of autophagosome-lysosome fusion (2, 10, 14–17). Each of these activities depends upon the covalent attachment of Atg8 to the lipid phosphatidylethanolamine (PE) in a process we call lipidation (18–20). In yeast there is only one Atg8 gene, but in mammals there are 6-8 genes spread across two families of ATG8 orthologs: LC3 and GABARAP, including LC3A/B/C, GABARAP, and GABARAPL1/2/3 (9, 21).

Atg8, LC3B, and GABARAP proteins are initially expressed in a pro-form that is not competent for conjugation. Before lipidation can proceed, this pro-form must be cleaved at its COOH-terminus by a cysteine protease called Atg4 (yeast) or ATG4 (mammals) to expose the glycine residue (10, 22–25). This initial cleavage event is known as priming. Lipidation of Atg8/LC3B/GABARAP then proceeds through an enzymatic cascade very similar to ubiquitination, eventually coupling this free glycine to the amine group on PE (14, 26). Atg8/LC3B/GABARAP-PE stays bound to the autophagosome membrane through its formation but must be removed just prior to or concomitantly with fusion of the autophagosome into the lysosome or vacuole. The removal of external Atg8/LC3B/GABARAP from PE is also performed by Atg4/ATG4-mediated proteolysis, in a process known as delipidation (18, 27). Defects in delipidation of Atg8-PE by Atg4 prevents recycling of Atg8 off membranes which both limits the available pool for future rounds of autophagy and prevents fusion of the autophagosome and the lysosome (27, 28).

Thus, ATG4 family proteins act constitutively to prime newly expressed Atg8/LC3B/GABARAP proteins, but also must function under temporal and spatial regulation to release lipid-conjugated forms of Atg8/LC3/GABARAP from fully-formed mature autophagosomes. We and others have begun to study how priming and delipidation are independently regulated to achieve these drastically different kinetics in the cell (i.e. (29, 30)). ATG4B
is an extremely fast priming enzyme \textit{in vitro} \cite{29, 31} and is also the major driver of priming for both the LC3 and GABARAP subfamilies in cells. KO of ATG4B, but not of the other three mammalian ATG4 proteases, eliminates priming of all LC3 proteins and dramatically impairs priming of the GABARAP family proteins \cite{29, 30}. Delipidation is more complicated. Our lab has established that while ATG4A, ATG4C, and ATG4D are very poor at priming, consistent with many previous studies, they are surprisingly just as efficient as ATG4B \textit{in vitro} at delipidation \cite{29}, suggesting the possibility that they play a key role in deciding when and which LC3 or GABARAP proteins are released from the membrane. Thus far however, the study of ATG4A, ATG4C and ATG4D function in cells has largely been confounded by the redundancy of the family. In particular, how and whether ATG4A-mediated priming and delipidation are independently controlled has not been known.

Here, we explore the limits of ATG4A-mediated delipidation in a gene-edited cell line we created where all four mammalian ATG4 genes are missing which allows us to specifically rescue with ATG4A and various engineered mutants. We discover that like ATG4B, the COOH-terminal LC3-interaction region (LIR) of ATG4A is essential to its function, but unlike ATG4B, this motif controls \textit{both} priming and delipidation on ATG4A. Furthermore, we show that ATG4A is not sufficient to support autophagy in our cell lines, and more surprisingly, does not appear to support any delipidation of its substrate, GABARAPL1 (from now on referred to as GL1), in this context. We show that instead, GL1 accumulates on a variety of membranes and remains membrane associated even if the normal cues for activating the lipidation pathway are shut off for as long as 24 hours. This strongly implies that ATG4A does not have access to GL1-PE (also known as GL1-II) under these conditions, suggesting more complex intracellular regulation of its delipidation activity.

**Results**

\textit{Macroautophagic degradation is fully-stalled in cells expressing only ATG4A}

The redundancy of function across the ATG4 family limits the useful interpretation of single knockouts, and thus we have created a gene-edited HEK293 cell line that we call our ATG4 quadruple knockout (ATG4\textsubscript{QKO}). In this cell line, there is no detectable expression of any of the ATG4 proteins by western blot (Fig. S1A and (29)), and we have confirmed genetic alteration at each locus (Fig. S1B). Without any ATG4 expression, these cells are completely incapable of priming the ATG8 protein family and thus all lipidation of the ATG8 proteins is also inhibited \cite{29}. We previously showed that re-expression of ATG4B in this background is sufficient to rescue LC3 and GABARAP protein priming and lipidation, and even to support flux of these proteins in a Bafilomycin A1 (Baf A1) dependent way, suggesting restoration of autophagy \cite{29}. In contrast, re-expression of ATG4A is unable to rescue LC3 processing, consistent with many previous publications, and also leads to anomalous processing of GABARAP proteins. In particular, these cells continue to have a large pool of unprimed pro-GL1 and accumulate fully-lipidated GL1-II to high levels, but do not show any significant levels of free-floating processed GL1 (GL1-I) \cite{29} and Fig. S2). This result suggests that ATG4A can prime a portion of the GABARAP family protein pool, and this entire population of primed GL1-I is utilized for lipidation. Should any delipidation take place, newly released protein is again rapidly lipidated. Thus, we have an opportunity to consider how autophagy progresses when only the GABARAP family is able to become lipid-attached, and to assess how ATG4A contributes to this process.

Recent papers have suggested that the GABARAPs can be fully sufficient for at least some forms of macroautophagy and that even in the absence of all LC3 and GABARAP proteins, some macroautophagy persists \cite{32–34}. Therefore, we first established whether the delivery of cargo is possible in our ATG4\textsubscript{QKO} cells, where no primed LC3 or GABARAP is available to be lipidated. To look at starvation-mediated turnover of bulk cytoplasm, we used a long-lived protein degradation (LLPD) assay in which cells are incubated in media supplemented with \textsuperscript{14}C-valine and then chased with media supplemented with cold valine. These cells were then incubated in...
either normal or nutrient-reduced (EBSS) media for four hours to look for starvation-dependent changes in bulk protein turnover. Consistent with previous results (35, 36), starvation induced an approximately 50% increase in long-lived protein degradation in wild type (WT) cells (Fig. 1A). Critically, when these cells were treated with the PI3K inhibitor 3-MA to block initiation of the macroautophagy pathway, starvation-dependent LLPD is mostly prevented. In contrast to WT cells, ATG4QKO cells were incapable of starvation-dependent LLPD. Thus starvation-dependent flux depends on ATG4 proteins in this cell line.

Lentiviral-mediated rescue of individual ATG4A, B, C, or D genes in the ATG4QKO restored ATG8 protein family processing to varying degrees (29). We observed in these rescues that high expression of ATG4A drove significant lipidation of GL1 while re-expression of ATG4C or ATG4D resulted in more modest levels of lipidated GABARAP family proteins and only ATG4B re-expression restored any LC3 processing (Fig. S2). To establish whether the ATG4A-, ATG4C-, or ATG4D-mediated processing of GABARAP proteins alone can drive full autophagy, we looked at LLPD in these lines. Surprisingly, only ATG4B re-expression was sufficient to support a starvation-mediated LLPD and did so at essentially WT levels. Cells expressing only ATG4A, ATG4C or ATG4D behaved exactly like the ATG4QKO (Fig. 1A). Thus, bulk cargo delivery depends absolutely on the presence of ATG4B, consistent with other reports (36–40).

To look at how a specific autophagic cargo is handled, we followed levels of the adaptor proteins p62/SQSTM1 and NBR1 and a potential autophagic cargo LDH across various cellular stress conditions. Neither NBR1 nor LDH levels depended upon ATG4 expression and neither changed in response to small molecule treatments in WT HEK293 cells (Fig. S3) and so were not further examined. We also could not detect changes in p62 levels in WT cells following starvation, even when the lysosomal inhibitor Baf A1 was included to accumulate lysosome-directed proteins (Fig. 1B and S4). Note that the levels of LC3B-II did increase in both Baf A1 and starvation conditions, indicating that only p62 flux in these cells was undetectable. As expected, there was also no flux of p62 (or of pro-LC3) in our ATG4QKO cells. Interestingly however, overall p62 levels were elevated ~2x higher in ATG4QKO cells relative to WT cells (Fig. 1B), indicating that loss of the ATG4 family alters the overall homeostasis of p62 (29). Re-expression of ATG4B in the ATG4QKO but not re-expression of ATG4A, C, or D restored p62 to levels similar to WT (Fig. 1C, S2, and S4). Thus, ATG4B is able to restore essentially normal processing of the LC3 and GABARAP families, fully support LLPD, and maintain normal p62 homeostasis, while expression of any other ATG4 alone can prime GABARAP protein families (to varying levels) but cannot otherwise support autophagy.

In cells expressing only ATG4A, GL1-II does not appear to undergo delipidation

Some studies have observed that in cells depleted of LC3, the GABARAP family is sufficient to drive autophagosome formation and even cargo delivery (32, 41). Thus, in principle, the ATG4QKO-ATG4A cell line which primes GABARAP, GL1, and GL2 and accumulates lipidated forms of all three proteins might also support macroautophagy. However, the absence of both starvation-mediated LLPD and normal p62 homeostasis indicates that instead there are unexpected detriments to autophagy.

To that end, we suspected that the strong accumulation of GL1-II in cells expressing only ATG4A (Fig S2) reflected a breakdown in the normal GL1 cycle in cells which might underlie a larger problem with autophagosome progression (Fig. 2A cartoon).

In particular, the complete absence of GL1-I could arise because the total pool of primed LC3 and GABARAP proteins is very small and thus all free GL1-I formed from either priming or from delipidation becomes immediately relipidated. Alternatively, GL1-I depletion could indicate a general failure of ATG4A to support delipidation in these cells.

To distinguish between these two possibilities, we reasoned that by blocking autophagosome biogenesis signals which generally drive lipidation, we might uncouple the two
processes and measure delipidation rates. We treated cells with Wortmannin, a potent phosphatidylinositol 3-kinase inhibitor that blocks autophagy initiation. In the absence of PI3P, the activation of autophagy in general and of the lipidation machinery in particular is largely inhibited. For example, while WT cells incubated in Baf A1 accumulate significant LC3-II and less but detectable GL1-II, two hours of treatment with Wortmannin blocks this accumulation entirely (Fig. 2B and (42)). However, GL1-II levels in ATG4QKO-ATG4A cells stay the same whether these cells were treated or not with Wortmannin and GL1-I remains undetectable (Fig. 2B). Even prolonged Wortmannin treatment of up to 24 hours, did not result in detectable GL1-I (Fig. 2C). Likewise, treatment with another PI3K inhibitor (3-MA) also did not reduce GL1-II levels or allow for any detection of GL1-I (Fig. 2D). These results strongly suggest that the pool of GL1-II in ATG4QKO-ATG4A cells is not dynamic.

As the GABARAPs and LC3 proteins are thought to be only partially redundant, and may specialize in different aspects of autophagosome formation (43), we considered that perhaps GL1-II is accumulating because both LC3B-II and GL1-II are needed on autophagic membranes for autophagy to complete. To test whether lipidated LC3B would support the flux and/or delipidation of GL1-II, we transduced a FLAG-tagged LC3B protein in which the terminal glycine is already exposed (LC3BG120) into our ATG4QKO-ATG4A cells. We have previously shown that FLAG-tagged Atg8 family members undergo ordinary flux in WT cells (29), and confirmed that our FLAG-tagged LC3B construct also undergoes ordinary flux (Fig. S5A). Critically, LC3BG120 can be lipidated without the need of priming from ATG4B. We observed about half of the FLAG-tagged LC3BG120 population became lipidated in our ATG4QKO-ATG4A cells while all of the endogenous LC3B molecules remained in the pro-form as expected (Fig. S5, A and B). However, the FLAG-tagged LC3B molecules did not exhibit any EBSS starvation or Baf A1 dependent flux, suggesting LC3B-associated autophagosomes in these cells did not reach the lysosome. Endogenous GL1 did not exhibit any flux and there was no evidence of GL1-I. Furthermore, LLPD results showed starvation-mediated bulk autophagy was still absent (Fig. S5C). Collectively, these results imply that ATG4A alone is unable to support significant delipidation in these cells and is unable to support macroautophagy even when supplemented with primed LC3B-I.

The impact of kinase inhibition on GL1-II accumulation

Both mammalian ATG4B and yeast Atg4 can be regulated by kinases. Thus, one explanation for our results could be a specific down-regulation of ATG4A delipidation activity, but whether and how this protein is regulated is largely unknown. Furthermore, even where phosphorylation-dependent regulation of ATG4B is established, the regulation is generally inferred by following changes in LC3B proteins, but there is much less understanding of how and whether these kinases also impact the pool of GL1.

We next used small molecule inhibitors to manipulate the activity of three kinases previously implicated in either inhibiting or activating ATG4B to test whether they impact GL1-II accumulation in either WT or ATG4QKO-ATG4A cells. In WT cells, inhibition of ULK1 was largely without effect (Fig. 2E). As ULK1 has been shown to inhibit ATG4B, it normally functions to increase LC3B-II levels. In our cells where LC3B-II/GL1-II are already at very low levels in the basal condition, it is unsurprising that further inhibition of ULK1 did not lead to a significant change. Inhibition of AKT1/2 in WT cells leads to significant increases of both LC3B-II and GL1-II, consistent with work showing that AKT2-mediated phosphorylation of ATG4B broadly activates this enzyme (44). In contrast, the inhibition of MST4 leads to a significant increase in LC3B-II but not GL1-II (Fig. 2E). MST4 was previously shown to limit the accumulation of LC3B-II (45), though whether that was a direct consequence of phosphorylation on ATG4B or resulted from a less direct intervention was not clear. In the next section, we consider how the MST4 locus on ATG4B influences protease activity in vitro to explore this result.

In contrast to WT cells, in ATG4QKO-ATG4A cells we were not able to detect significant changes following treatment with any of the small molecule inhibitors (Fig. 2E). GL1-II levels did not appreciably change and there was no detectable GL1-I. We noted a modest change in pro-GL1 levels, particularly in the ULK1 inhibition, but
again, this did not correlate with a change in the levels of either processing product. Thus, in cells expressing only ATG4A, these three kinases do not play a significant role in maintaining the GL1-I/GL-II ratio, and again, delipidation appears to be completely inhibited.

**Regulation of ATG4A priming and delipidation by its COOH-terminal LIR**

Delipidation by both Atg4 and ATG4B depends on motifs outside the catalytic pocket, which engage LC3 (LC3-interacting regions or LIRs) or Atg8 (Atg8-interacting motifs or AIMS) (29, 45–47). In particular, at the COOH-terminus of ATG4B there is an LIR motif which is involved in regulating protease activity (29, 45, 47). The COOH-termini of ATG4A and ATG4B are very similar; the last 9 amino acids are either identical or have the same side-chain properties, and the core of the ATG4B COOH-terminal LIR (FEIL) is fully conserved in ATG4A (47). Importantly, in an *in vitro* reconstitution assay where we can follow proteolysis over time, we previously showed that removal of the COOH-LIR dramatically slowed ATG4B-mediated delipidation but had no effect on the speed of priming (29). This surprising result implies that ATG4B on membranes can be regulated differently from its priming activity in solution and may offer an explanation for how ATG4A could support priming but not delipidation in our cells.

Thus, to better understand the relative specific activities of priming and delipidation on both ATG4A and ATG4B, here we have modified our *in vitro* reconstitution system to measure the extent of proteolysis at a single endpoint and titrated ATG4 proteins over several logs of concentration (Fig. 3 and Fig. 4; cartoon model in Fig. S6A). In this assay, priming is measured as the release of a GFP from a soluble LC3B-GFP or GL1-GFP construct, while delipidation is measured as the release of LC3B or GL1 from PE-conjugated forms anchored in proteoliposomes.

ATG4B-mediated delipidation of GL1-II requires about 8 times as much enzyme as priming of a soluble pro-form of GL1-GFP (Fig. 3A and Fig. S6B), confirming our published results that ATG4B is naturally much better at priming (29). Deletion of the last 9 amino acids including the COOH-LIR (ATG4B ΔLIR) does not appreciably change the efficiency of priming, but markedly increases the concentration of ATG4B needed to support delipidation (Fig. 3A and Fig. S7). Thus, on ATG4B, the COOH-terminus is a delipidation-specific regulatory motif.

To test whether the ATG4A COOH-terminus impacts proteolysis, we made a deletion mutant of ATG4A also removing the last 9 amino acids (ATG4A ΔLIR) (Fig. 4), and then subjected both the WT and deletion mutant protein to the same *in vitro* proteolysis assays (Fig. 4A, 4B, and Fig. S8). Similarly to our previous kinetic measurements, we found that ATG4A priming and delipidation occurred with roughly equivalent efficiencies (half-maximal cleavage at 300 nM and 150 nM ATG4A, respectively). Like ATG4B, removal of the LIR dramatically impaired delipidation, requiring more than 2mM protein to cleave half of the GL1-II. Unlike ATG4B, loss of the LIR also dramatically impaired ATG4A-mediated priming, reducing its specific activity to 1/10th of WT protein. Thus, *in vitro*, the ATG4A LIR is an essential component of the protease efficiency against all substrates.

To test the role of this motif *in vivo*, we rescued our ATG4QKO cell line with ATG4A ΔLIR (ATG4QKO-ATG4A ΔLIR; Fig. 4C and S9). Despite being expressed at a much higher level than ATG4A in ATG4QKO-ATG4A cells, ATG4A ΔLIR is unable to support any detectable priming of pro-GL1. These results strongly imply that the LIR motif on ATG4A is intimately associated with substrate capture for both priming and delipidation.

The interaction of LIR motifs with LC3 or GABARAP proteins can often be modulated by phosphorylation on immediately adjacent serine or threonine amino acids (45, 46). In both ATG4A and ATG4B, the COOH-LIR is adjacent to serines and the serines at positions 383 and 392 are known sites of phosphorylation in ATG4B (29, 45, 46). Furthermore, MST4-mediated control of ATG4B delipidation is thought to occur through phosphorylation at S383 (45), though whether this phosphorylation directly modulates ATG4B activity or interferes with some other interaction in the cell is not known. Our *in vitro* reconstitution is
an ideal system to test directly whether modifying these amino acids impacts protease activity and to explore whether ATG4A might also be subject to phosphorylation-dependent regulation at the LIR.

We expressed and purified recombinant phosphomimetic versions of ATG4A and ATG4B with the relevant serines mutated to aspartic acid (D) and tested these proteins in our in vitro assays. Mutation of one or both serines abutting the ATG4B LIR had no effect on either priming or delipidation in our assay (Fig. 3B and Fig. S10). In parallel, rescue of ATG4KO cells with various ATG4B phosphomimetic mutants restored LLPD (Fig. 3C) and maintained p62 homeostasis levels (Fig. 3D, S11, and S12A) similar to wildtype ATG4B. These results suggest that any regulation via this motif is not due to a direct impact on protease-substrate interaction.

In contrast, mutation of the ATG4A S397 to D modestly activated delipidation but not priming in vitro, leading to a 5-fold increase in delipidation efficiency (Fig. 4B). Finally, to test whether aberrant phospho-regulation at this site might explain the accumulation of GL1-II in our ATG4A rescue, we created stable rescues of our ATG4KO cells with either ATG4A S397D (phosphomimetic) or ATG4A S397A (phosphonull). The phosphomimetic had a slight but consistent increase in total pro-GL1 levels, but there was no apparent impact of either mutation on delipidation and no accumulation of any GL1-I (Fig. 4C and Fig. S9). In addition, none of the ATG4A mutants that we tested could support LLPD (Fig. 4D) or restore normal p62 homeostasis (Fig. 4E, S9, and S12B). Thus, ATG4A utilizes its COOH-LIR to support substrate proteolysis and is potentially subject to local regulation, but even with an activated phosphomimetic form, ATG4A alone is unable to support delipidation of the GABARAP proteins in ATG4KO cells.

GL1-II accumulates on both incomplete autophagosomes and non-autophagic membrane structures in ATG4A-only rescues

GL1-II protein accumulates in our ATG4A rescues, but autophagy is inhibited. To establish whether classic autophagosomes are even forming, we next looked at which membranes become GL1-II positive. To image GL1 distributions directly, we expressed a FLAG-tagged version of GL1 with a COOH-terminal deletion to expose the reactive glycine (FLAG-GL1G116), thus bypassing the already inefficient priming step in the ATG4A rescue. Like the endogenous protein, FLAG-GL1G116 is almost entirely GL1-I in WT cells and GL1-II in ATG4KO-ATG4A (Fig. 5A). FLAG-GL1G116 accumulates in discrete puncta in both wildtype and ATG4KO-ATG4A cells (Fig. 5 and Fig. S13-16), but is diffuse across the cytoplasm in full ATG4 knockouts (29), suggesting the puncta represent accumulations of GL1-II. In WT cells, the numbers of puncta increase in response to starvation and Bafilomycin. Furthermore, GL1-II puncta largely colocalize with the autophagosome marker WIP1-2 in the absence of Bafilomycin, and the lysosome marker LAMP1 in the presence of Bafilomycin (Fig. S13 and S15), consistent with its association to autophagosomes throughout their maturation. In contrast, GL1 puncta in ATG4KO-ATG4A cells are apparent even in the absence of stress (Fig. S14 and S16). Some of these puncta are WIP1-2 positive indicating that GL1 can be recruited into sites of autophagosome biogenesis, but much of the observable FLAG-GL1G116 is found in other WIP12-negative structures (Fig. 5B and S14) and only a small fraction of the FLAG-GL1G116 colocalize with LAMP1 (Fig. 5B and S16).

We next assessed whether GL1-II becomes protected within a closed structure, as would be expected if half of the protein on the limiting membrane becomes encapsulated within an autophagosome. Cells were incubated in full media with Baf A1 for 4 hours to accumulate as many closed autophagic structures as possible and then total membranes were collected and fractionated by multiple rounds of varying speed centrifugation (48, 49) with fractions identified as “high speed pellet” containing larger membranes including lysosomes, mitochondria etc. and “low speed pellet” containing small vesicles. Each isolate was then subjected to incubation with or without Proteinase K to proteolyze accessible outward facing proteins.

About 50% of GL1-II molecules are protected in WT pellets consistent with their recruitment to maturing autophagosomes (Fig. 6, A and B, black). In contrast, in membranes isolated from ATG4KO-ATG4A cells, most GL1-II molecules were digested (Fig. 6, A and B, red) and
Thus not incorporated into fully closed mature autophagosomes. The autophagic cargo p62 exhibited similar trends, although the absolute changes in protease protection fell below statistical significance.

To better establish the identity of these GL1-II-positive membranes in ATG4A-only rescues, we used a more comprehensive membrane fractionation protocol, which has been well-established to segregate autophagosomes from other compartments (50). In WT cells, GL1-II is strongly enriched in the autophagosome fraction along with p62 and membrane-associated GAPDH (Fig. 6, C and D). In addition, all three of these proteins are largely resistant to added Proteinase K, consistent with their accumulation inside mature closed autophagosomes. In contrast, in ATG4 KO-ATG4A cells much less p62, GAPDH or GL1-II is associated with the autophagosome fraction. Instead, GL1-II and p62 are found in both the ER and AV (Fig. 6C), and when we account for the fact that there is far more ER in our sample than AV, it is apparent that the vast majority of these proteins are found in membranes copurifying with endoplasmic reticulum (Fig. 6D). Furthermore, proteins accumulating with the ER or mitochondrial fractions are completely digested by Proteinase K as expected if these are not autophagosomal membranes.

Collectively, our results show that ATG4A alone is not sufficient to support autophagy in these cells and this failure likely derives from two primary defects. First, ATG4A-mediated priming is inefficient. In WT cells, unprimed pro-forms of the GABARAP and LC3 proteins are undetectable as priming occurs fast and constitutively (driven largely by ATG4B), however in ATG4A-only rescues, about half of the GL1 pool remains as pro-GL1. Second, in cells expressing only ATG4A there is a general inability to delipidate GL1 proteins which end up mistargeted to non-autophagic sites in the cell. These two defects combine to strongly limit the pool of soluble GL1-I protein needed to support nascent autophagosome formation.

**Discussion**

Discussion

ATG4A is relatively understudied despite being the second most catalytically active mammalian ATG4 protein, after ATG4B (51, 52). As ATG4B knockouts in mice are viable (53), and as GABARAP proteins, in the absence of LC3 proteins, are sufficient to support autophagy in cells (32), it seemed likely that ATG4A would play a significant and largely sufficient role in supporting autophagy in simple cellular systems. However, several earlier studies, including our own, have suggested the opposite: that cells lacking ATG4B were largely incapable of completing autophagy (21, 29, 47). Thus, here we explored the limits of ATG4A activity. Our primary discovery is that the turnover of lipid-attached GABARAP proteins is incredibly slow or non-existent in cells expressing only ATG4A, despite the fact that intrinsic ATG4A delipidation activity measured in vitro is roughly equal to ATG4B driven delipidation (Fig. 3, 4, and 29). As a result, essentially all of the primed GABARAP family protein accumulates in a lipid-attached form in ATG4A-only cells. A small fraction of this protein co-purifies at densities equivalent to autophagosomes and is protease protected, suggesting at least some closed autophagosomes are produced. The vast majority of the GL1-II however, is found in our ER fraction and is not protease-protected. Thus, these cells may face two major challenges in completing autophagy; 1) the presence of GABARAP proteins on the outside of the mature autophagosome may hinder the delivery of these organelles into the lysosome as this step is thought to require removal of all LC3/GABARAP (54), but 2) equally problematic is that most of the available GABARAP protein in the cell accumulates at what may be non-autophagy sites and is unable to be recycled from these locales, limiting the available pool of GABARAP proteins for new autophagosome biogenesis (28).

**Modulation of priming and delipidation by the COOH-termini of ATG4A and ATG4B**

Many groups have shown previously that ATG4A primes GL1 and other GABARAP proteins very slowly relative to ATG4B (29, 51, 52), however the two proteases can remove GL1 and other GABARAPs from membranes in vitro with essentially equal kinetics (29). Further, an LIR in the COOH-terminus of ATG4B (29, 47) is a critical determinant of its rate of delipidation, but is
dispensable for its priming (29). As the C-termini LIR motifs of ATG4A and ATG4B are highly conserved it is curious that this study shows they appear to regulate GLI1 processing differently, with essentially all of ATG4A’s protease activities requiring an intact LIR (Fig. 4). We had previously speculated that a “delipidation-only” role for the LIR on ATG4B strongly implied a membrane-dependent activation of the enzyme perhaps through a simple model where ATG4B recognizes one substrate at its active site and simultaneously recognizes a second LC3/GABARAP at its LIR, something that would be highly promoted at membranes where multiple lipitated substrates would be present. However, our results here that ATG4A requires this same motif for priming, argue against that model and perhaps favors a conformational change that occurs when substrate first engages the LIR before moving into the active site. Details of this possible model will require further study.

There is mounting interest in understanding how the proteolytic activities of ATG4 proteins are regulated as a possible therapeutic node in diseases like cancer (55, 56). To that end, it is interesting that these COOH-terminal LIR sequences are flanked by serines where phosphorylation might regulate their substrate interactions as is commonly the case at other LIRs. Indeed, phosphorylation of S383 and S392 in ATG4B were previously shown to affect autophagic flux in cells through mechanisms interpreted as a change in delipidation (45, 46). Moreover, phosphorylation of S383 by MST4 enhances autophagic activity (measured as an increased conversion of LC3-I to LC3-II and more p62 degradation) and correlates with increased glioblastoma tumorigenicity (45). However, our results suggest the mechanism by which MST4 activates autophagy requires more investigation. First, when we tested the activity of ATG4B harboring phosphomimetic mutations, we did not observe a clear change in activity in vitro, and like a previous study by Rasmussen et al. (47), we also did not detect a significant difference between WT or phosphomimetic ATG4B in our knock-out/rescue system. Thus, it appears unlikely that these serines directly impact the catalytic activity of ATG4B. In addition, small molecule inhibition of MST4 only appeared to impact LC3B-II levels, but not GL1-II, perhaps suggesting it works through a more precise effector of LC3 than ATG4B.

Considerably less is known about whether ATG4A is subject to kinase regulation, but our in vitro experiments with ATG4A phosphomimetics (S397D) were somewhat promising as we could detect a roughly 5-fold increase in specific activity. Whether this is meaningful in vivo is currently unclear, as we cannot detect delipidation at all in our KO-rescue.

Why is ATG4A alone insufficient when ATG4B KO mice are viable?

Knockout studies with mouse models revealed that while ATG3-/- (57), ATG5-/- (58), ATG7-/- (59) mice are neonatal lethal, ATG4B-/- (53) and ATG4C-/- (60) mice are viable with relatively mild phenotypes (including balance problems in ATG4B-/- and elevated vulnerability to fibrosarcoma in ATG4C-/-). Furthermore, LC3B and GABARAP KO mice are viable with no abnormalities (61). Thus, there is likely tremendous redundancy across the ATG4 and ATG8 protein families. In this background, it is somewhat surprising that ATG4A is not sufficient to support autophagy. Our long-lived protein degradation assay is a good measure of bulk starvation-dependent autophagy which is completely absent in these ATG4A-only cells, but we did not search for autophagosomes by other criteria, such as electron microscopy and thus we may have missed ATG5/ATG7-independent autophagy (33) and ATG8-independent autophagy (32) previously shown to function in mammalian cells.

An obvious limitation of removing ATG4B, ATG4C, and ATG4D from these cells is a loss of LC3 family processing, however, we could not rescue autophagy simply by adding LC3B back into the mix (Fig. 2E). Likewise, in cells still expressing ATG4C and ATG4D which can facilitate some LC3 family processing, we previously did not detect any autophagic flux at the level of GABARAP/LC3 turnover when ATG4B was missing (29). Thus, we conclude that ATG4A is insufficient on its own, and we suggest that the ATG4B-/- mouse is viable due to some compensation, perhaps through additional activation/expression of ATG4C and ATG4D.
The most surprising aspect of our study is the complete absence of detectable GL1 delipidation in cells expressing only ATG4A. Delipidation in cells is notoriously difficult to assess because LC3/GABARAP proteins are almost always in a steady-state of priming, lipidation, and delipidation, and thus changes in the relative amounts of form I and form II proteins can result from fluctuations in any of these parameters. In our case however, there is no flux at all. All of the GL1, even when massively overexpressed as in our FLAG-tag experiments, is completely in the lipidated form. Furthermore, the lipidated protein appears to be collecting at sites other than the autophagosome, including on membranes that co-purify with ER. This is the same phenotype observed in yeast when primed Atg8 protein is introduced into Atg4 KO cells (28, 54), and suggests that perhaps ATG4A is simply not competent to recycle GL1 from off-target sites, an activity that is considered to be essential to support LC3/GABARAP-dependent macroautophagy.

Experimental Procedures

Materials:

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE; 850725C), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; 850457C), L-a-phosphatidylinositol (bi-PI, Bovine Liver/PtdIns; 840042C) were purchased from Avanti Polar Lipids. Nycodenz (1002424) was purchased from Accurate Chemical and Scientific Corp. pCMV-VSV-G (8454) and psPAX2 (12260) were purchased from Addgene. BL21-Gold (DE3) Competent Cells (230132) were purchased from Agilent Technologies. ATP (AB00162), Dithiothreitol (DTT; AB00490), IPTG (AB00841), HEPES (AB00892), Sodium Chloride (NaCl; AB01915), Sodium Dodecyl Sulfate – SDS (AB01920), Sucrose (AB01900), Tris-HCl (AB02005), Triton X-100 (AB02025), Tween 20 (AB02038), were purchased from AmericanBio. Protein Assay Dye Reagent (5000006) was purchased from Bio-Rad. Glass Bottom Dishes - 35 mm Dish (D35-20-1.5-N) was purchased from Cellvis. Lenti-X Concentrator (631232) was purchased from Clontech. 16% Parafomaldehyde (15710) was purchased from Electron Microscopy Sciences. MAP/MST4/AMPK inhibitor (375680) was purchased from EMD Millipore. Bafilomycin A1 (BML-CM110) was purchased from Enzo. DMSO (D12345), Lipofectamine 3000 (L3000015), NuPAGE™ MOPS SDS Running Buffer 20X (NP0001) were purchased from Invitrogen. 100% Glycerol, Anhydrous (2136), Calcium Chloride (CaCl2; 1332), EDTA (8993), Magnesium Chloride (MgCl2; 2444) were purchased from J.T.Baker. Immobilon-FL PVDF membranes (IPFL00010) were purchased from MilliporeSigma™. L-[U-14C]-Valine (NEC291EU050UC) was purchased from PerkinElmer. 3-Methyladenine (3MA; M9281), Akt1/2 inhibitor (A6730), BSA (A9647), Carbenicillin (C1389), Casein (C7078), cOmplete, EDTA-free Protease Inhibitor Tablets (1187358001), D-Mannitol (M4125), Glutathione Beads (G4501), Imidazole (I5513), Octyl-gluconoside (O8001), OptiPrep™ Density Gradient Medium (D1556), Percoll® (P4937), Proteinase K (3115887001), Saponin (47036), Sodium Azide (NaN3; S2002), Thrombin (T6884), ULK1/2 inhibitor (SML1540), Whatman® Nuclepore™ Track-Etched Polycarbonate Membranes (10417104), Wortmannin (W1628) were purchased from Sigma-Aldrich. 0.05% Trypsin-EDTA 1X (25300-054), 10% FBS (10438062), Coomassie Blue Stain (Imperial™ Protein Stain), CyQUANT™ Cell Lysis Buffer 20X Concentrate (C7027), CyQUANT™ NF Cell Proliferation Assay Kit (C35006), DMEM (11965092), DPBS 10X (14200075), Imperial™ Protein Stain (24615), NuPAGE™ 12% Bis-Tris Gels (NP0341BOX, NP0349BOX), NuPAGE® Transfer Buffer 20X (NP0006-1), ProLong™ Gold antifade reagent (P36935), ScintiVerse™ BD Cocktail (SX18-4), SeeBlue Plus2 Pre-stained
Protein Standard (LC5925), SuperSignal™ West Femto Substrate (34096), TCEP (T2556), were purchased from Thermo Fisher Scientific. Borosilicate Glass 10x75mm (47729-568) was purchased from VWR.

**Antibodies used in this manuscript:**

For immunoblotting:
- Primary-
  - ATG4A: 7613S, Cell Signaling Technology
  - ATG4B: 13507S, Cell Signaling Technology
  - ATG4C: 5262S, Abcam
  - ATG4D: MA5-18110, Thermo Fisher Scientific
  - FLAG: F1804, Sigma-Aldrich
  - GABARAPL1 (GL1): 26632S, Cell Signaling Technology
  - GAPDH: ab9484, Abcam
  - LC3B: 3868S, Cell Signaling Technology
  - p62: 610832, BD Biosciences
  - Lactate Dehydrogenase (LDH): ab47010, Abcam.
  - NBR1: ab126175, Abcam.
- Secondary-
  - ECL Rabbit IgG, HRP-linked: NA934V, GE Life Sciences
  - IRDye® 680RD Donkey anti-Mouse IgG: 925–68072, LICOR
  - IRDye® 800CW Donkey anti-Rabbit IgG: 925–32213, LICOR

For immunofluorescence imaging:
- Primary-
  - FLAG: F1804, Sigma-Aldrich
  - FLAG: 14793S, Cell Signaling Technology
  - LAMP1: 9091S, Cell Signaling Technology
  - WIPI2: MABC91, EMD Millipore
- Secondary-
  - Alexa Fluor™ 488 Donkey anti-Rabbit IgG: A11008, Thermo Fisher Scientific
  - Alexa Fluor™ 594 Donkey anti-Mouse IgG: A21203, Thermo Fisher Scientific

**Mutagenesis of ATG4s:**

The human ATG4A and ATG4B/C/D constructs were cloned in the pGEX-4T and pGEX-2T GST backbone vectors respectively as described (29). ATG4A and ATG4B were mutagenized to generate mutants using the Quick Change II Site-Directed Mutagenesis Kit (Agilent Technologies) and verified by sequencing.

**Recombinant protein expression and purification:**

Human ATG4, mouse ATG3, and the mammalian homologues of ATG8 (human GABARAPL1 (GL1) and human LC3B) were expressed and purified as previously described (29, 62). In summary, human GL1/LC3B were cloned in the PGEX-2T GST vector, mouse ATG3 was cloned into PGEX-6p GST vector. GL1/LC3B/ATG3/ATG4s were expressed in BL21-Gold (DE3) Competent Cells. Cells were cultured in 4L Luria Bertani Broth (LB) media with 1:1000 carbenicillin (50 mg/mL) and induced with 0.5 mM final concentration IPTG. Bacterial pellets were treated with EDTA-free protease inhibitor cocktail tablets in either thrombin buffer (20 mM Tris pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 2 mM CaCl₂, 1 mM DTT) for GL1/LC3B/ATG4s or precision protease buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) for ATG3. Cells were broken in a cell disrupter and the lysate was incubated with glutathione beads for 3 hours at 4°C. Beads were washed several times and then incubated with GL1/LC3B/ATG4s cutting buffer (10 μL thrombin + 500 μL thrombin buffer + 0.5 μL DTT + 500 μL beads) or ATG3 cutting buffer (25 μL precision protease + 500 μL precision protease buffer + 0.5 μL DTT + 500 μL beads) to cut the proteins from GST tags overnight. Purified proteins were stored in 20% glycerol at -80°C. GL1/LC3B were expressed in both a truncated form ending in the reactive COOH-terminal glycine (such that no ATG4-mediated pre-processing was needed for lipidation process), and a form with YFP (for GL1) or tag (for LC3B) to determine whether ATG4 priming activity of ATG8 took place.

**Protein expression & purification for human ATG7:**

Human ATG7 was expressed and purified as previously described (35). In summary, the ATG7-
containing plasmid was transformed into Bacmid DNA. 2 µg DNA was used to infect 8x10^5 SF9 cells by using Cellfectin II two times to increase viral titer to 5 x 10^6 pfu/ml. 1 x 10^8 pfu/mL of SF9 cells were infected with virus and they grew for 72 hours. Cells were treated with EDTA-free protease inhibitor cocktail tablets in the lysis buffer (20 mM Tris pH 8, 500 mM NaCl, 20 mM Imidazole, 1 mM DTT, 10% glycerol), sonicated with the Virsonic 600 (VirTis) microtip sonicator on for 3 minutes (30 sec on, 30 sec off. Speed 3.5) and centrifuged at 18000 rpm for 1 hour. The lysate was incubated with 1 mL Nickel resin (Ni-NTA Agarose) for 2 hours at 4°C. The beads were washed with the wash buffer (20 mM Tris pH 8, 300 mM NaCl, 20 mM Imidazole, 1 mM DTT) three times and eluted with the elution buffer (20 mM Tris pH 7.5, 300 mM NaCl, 500 mM Imidazole, 1 mM DTT). Purified proteins were stored in 20% glycerol at -80°C.

Liposome and Proteoliposome preparation:

Liposomes and proteoliposomes were prepared as previously described (29). Composition of liposomes was 55% DOPE, 35% POPC, and 10% bi-PI. Liposomes were extruded 21 times through polycarbonate membranes to 400 nm with the LipSoFast-Basic extruder (Avestin) and then were sonicated with the Virsonic 600 (VirTis) microtip sonicator at a size of 50 nm immediately prior to the lipidation reaction. Next, GL1 and LC3B underwent the lipidation process to be coupled to liposomes. In short, GL1/LC3B proteins (final concentration 15 µM), ATG3 (2 µM), ATG7 (2 µM), and sonicated liposomes (3 mM) were mixed with DTT (1 mM) in SNH buffer (20 mM Tris at pH 8, 100 mM NaCl, and 5 mM MgCl2). Lipidation was initiated by adding ATP (1 mM) and reactions were incubated at 37°C for 90 minutes.

Gel visualization and quantification:

15 µL samples were mixed with 4 µL 4x LDS loading buffer and boiled at 90°C for 5 minutes. Electrophoreses of samples were run in 12% Bis-Tris gel with 1X MOPS Buffer for 55 minutes at 200V using the Hoefer EPS 2A200 Power Supply C. Samples were visualized with Coomassie Blue stain. Band intensities of samples were quantified with ImageJ software as described for densitometry (29). Gels were imaged with the VersaDoc Imaging System (Bio-Rad) and analysed for densitometry using the ImageJ software.

CRISPR/Cas9 knockout and rescue with lentiviral transfection:

CRISPR/Cas9 knockout of ATG4A/B/C/D genes in HEK293T was described in the previous paper (29). 3xFLAG-tag GL1/LC3B and ATG4A constructs were cloned into high-expression pLVX-Puro vector. ATG4B constructs were cloned into low-expression pLenti-III-PGK vector. Lentiviral
transfection to make stable cell lines was done as described previously (29). In short, HEK293FT cells were seeded for 24 hours at 37°C with 5% CO2 in DMEM (containing 10% FBS) until confluency reached 50%. Next, cells were transfected with psPAX2, pCMV-VSV-G, and target plasmids using Lipofectamine 3000. After transfection, media was collected every 24 hours for 3 days and stored at 4°C. The media was then filtered with a 0.45-mm filter, then Lenti-X Concentrator was added at a ratio of 1:3, and mixed before incubation at 4°C for 4 hours. The mixture was centrifuged at 2700 rpm for 45 minutes at 4°C. The pellet was gently resuspended in 1/250 of the original volume using complete DMEM and stored at -80°C. ATG4 QKO HEK293T cells were transduced with 30 µL of the virus and 8 µL polybrene at 50% confluency for 48 hours. Puromycin was used to a final concentration of 5 µg/mL for selection of successfully transduced cells every 24 hours for 3 days.

CRISPR/cas9 guides for making the ATG4QKO:
ATG4A: CCCAACCAGCATCTGATGAA
ATG4B: CTAGACTTTGGTTTACATAC
ATG4C: AATTCTCCTGTATTATTGCT
ATG4D: ACCGTACTTGACGTTGTTCC

Primers for making the ATG4A/B constructs to rescue ATG4QKO cells:
FP = forward primer, RP = reverse primer.

ATG4A S397A FP: GCC - GACCTTTGAAAATCTGTGeeCTTTGAGCGGCGCTG
ATG4B S397A RP: GCC - CAGCGGCCGCTCAAAGgtgcCAGGATTTCGAGATCT
ATG4B S392D FP: GAC - GACCTTTGAAAATCTGTGacicCTTTGAGCGGCGCTG
ATG4B S392D RP: GTG - CAGCGGCCGCTCAAAGgtgcCAGGATTTCGAGATCT

Immunoblotting/Western blot protocol:

Cells were treated with DMEM or EBSS media with or without 0.1 µM Bafilomycin A1. Cell lysates were collected after reaching 80-90% confluency in 1X PBS, and centrifuged at 2000 rpm for 5 minutes. The supernatant was aspirated and replaced in 250 µL lysis buffer (1X PBS with protease inhibitor cocktail tablet) and resuspended on ice for 5 minutes. The solution was centrifuged at 4300 rpm for 10 minutes at 4°C, and the supernatant was collected and stored at -80°C. Protein concentration was determined with Bio-Rad protein assay. 45 µg of lysates were electrophoresed on 12% Bis-Tris gels for 70 minutes at 180V. Transfer membranes were placed on gels in transfer buffer (5% NuPAGE transfer buffer, 10% methanol) and electrophoresed for 70 minutes at 30V. For GABARAPL1, LDH, and NBR1, membranes were washed with PBST (10% DPBS 10X, 0.05% Tween in 1X PBS) and blocked with 1% BSA in PBST for 1 hour. Then membranes were washed 3x in PBST and placed in primary antibodies (1:500 dilution in 5% BSA) overnight. Next, membranes were washed 3x in PBST and placed in secondary antibodies (1:5000 dilution of HRP antibody in 1% BSA) for 1 hour. Membranes were washed 3x in PBST and incubated with SuperSignal™ West Femto substrate before imaging with VersaDoc Imaging System (Bio-Rad). For other antibodies, membranes were dried for 40 minutes and washed with methanol and PBST before incubation in LICOR blocking buffer (10% DPBS 10X, 0.5% Casein, 0.1% NaNS) for 1 hour. Membranes were washed in PBST and placed in primary antibodies overnight. Next, membranes were washed in PBST and placed in secondary antibodies (1:10,000 dilution of antibody in LICOR secondary antibody solution (LICOR blocking buffer with 0.25% Tween 20 and 0.1% SDS)) for 1 hour. Membranes were washed and incubated in

ATG4B S392A FP: GCC - GACCTTTGAAAATCTGTGeeCTTTGAGCGGCGCTG
ATG4B S392A RP: GCC - CAGCGGCCGCTCAAAGgtgcCAGGATTTCGAGATCT
ATG4B S392D FP: GAC - GACCTTTGAAAATCTGTGacicCTTTGAGCGGCGCTG
ATG4B S392D RP: GTG - CAGCGGCCGCTCAAAGgtgcCAGGATTTCGAGATCT

ATG4B S397A FP: GCC - GACCTTTGAAAATCTGTGeeCTTTGAGCGGCGCTG
ATG4B S397A RP: GCC - CAGCGGCCGCTCAAAGgtgcCAGGATTTCGAGATCT
ATG4B S392D FP: GAC - GACCTTTGAAAATCTGTGacicCTTTGAGCGGCGCTG
ATG4B S392D RP: GTG - CAGCGGCCGCTCAAAGgtgcCAGGATTTCGAGATCT

ATG4B S397A FP: GCC - GACCTTTGAAAATCTGTGeeCTTTGAGCGGCGCTG
ATG4B S397A RP: GCC - CAGCGGCCGCTCAAAGgtgcCAGGATTTCGAGATCT
ATG4B S392D FP: GAC - GACCTTTGAAAATCTGTGacicCTTTGAGCGGCGCTG
ATG4B S392D RP: GTG - CAGCGGCCGCTCAAAGgtgcCAGGATTTCGAGATCT
substrate before imaging with LICOR Odyssey system. Densitometry quantifications of p62 and ATG8 proteins levels were done as described previously. Quantifications of p62 levels in a blot were done normalizing to the highest densitometry value by using the ImageJ software. Statistical significance is assessed with two-sample Student’s t-Test.

Long-lived protein degradation assay:

The experimental plan was adapted from previously published methods (36, 38, 40, 63). In summary, cells were seeded to be grown overnight in DMEM so in 24 hours the confluency is 80-90%. After 24 hours, DMEM supplemented with L-[U-14C]-valine (final concentration of 0.5 µCi mL⁻¹) was added to cells and maintained for another 24 hours. After this 24 hours period, cells were washed with fresh DMEM and maintained for 4 hours in DMEM supplemented with cold valine to remove the degradation products of short-lived proteins. Next, cells were washed and treated with DMEM or EBSS media along without or with 10 mM 3-Methyladenine for another 4 hours. Then, supernatants and lysates of cells were treated separately the in scintillation liquid and measured with the scintillation counter to determine acid-soluble (counts per minute (cpm) released) and acid-precipitate radioactivity (cell cpm). Protein degradation assessment calculation is cpm released over total cpm X 100. Statistical significance is assessed with two-sample Student’s t-Test.

Cell viability assays:

Cells were plated to 80-90% confluence before being incubated with Trypsin for 3 minutes. Cell suspension was spun down and cells were resuspended in 50 µL of fresh DMEM. 10 µL was mixed with 10 µL Tryphan blue and this mixture was used to count total number of viable cells with the hetocytometer. Cells were also plated in 96-well plates with DMEM before being treated with the CyQuant Cell Proliferation fluorescence assay. Next, the SpectraMax M5 (Molecular Devices) was used to read fluorescence intensity (Excitation: 485 nm. Emission 530 nm). Cells were plated on glass bottom dishes and visualized with light microscopy from DeltaVision Imaging System (GE Applied Precision) to observe for viability and morphology. Statistical significance is assessed with two-sample Student’s t-Test.

Cell fractionation and protease protection assay:

The experimental plan was adapted from previously published (49) for experiments in Figure 6A and 6B. In summary for cell fractionation, cells were seeded to 80-90% confluency in DMEM and incubated with 0.1 µM Bafilomycin A1 for 4 hours. Cells were washed with PBS, centrifuged at 1995 rpm for 5 minutes at 4°C, resuspended in 400 µl EDTA-free protease inhibitor-containing homogenization buffer (10 mM HEPES, 0.22 M mannitol, and 0.07 M sucrose, pH = 7.5) cocktail, and lysed with 2-mL Dounce homogenizer. Lysates were centrifuged 1700 rpm for 5 minutes at 4°C to get the nucleus pellets (NP). Post-nuclear supernatants were spun at 8500 rpm for 5 minutes to get the low-speed pellets (LSP), while supernatants were further centrifuged in a Beckman TLA-100.3 rotor at 43000 rpm for 30 minutes to collect the high-speed pellets (HSP) and high speed supernatants. NP, LSP, and HSP were resuspended in 40 µl homogenization buffer. For the protease protection assay, 45 µg of each sample was incubated with 100 μg/ml Proteinase K, or with 100 μg/ml Proteinase K and 0.5% Triton X-100 on ice for 30 minutes. Next, samples were boiled for 10 minutes to stop the reactions then subjected to immunoblotting protocol. For each cell line, finding the percentage of protection entails measuring the densitometry of each protected sample then dividing that over the densitometry of each control sample. Statistical significance is assessed with two-sample Student’s t-Test.

ER and Autophagosome (ER/AV) separation and protease protection assay:

The cell fractionation and ER/AV separation assays were adapted from previously published methods (50) for experiments in Figure 6C and 6D. In summary, cells were seeded to 80-90% confluency in DMEM and incubated with 0.1 µM Bafilomycin A1 for 4 hours. Cells were resuspended in 1 ml EDTA-free protease inhibitor-containing buffer (50
mM Tris, 150 mM NaCl, 1X PIC, and 10% sucrose, pH = 7.5) cocktail, and lysed with 2-mL Dounce homogenizer. Lysates were centrifuged 4000 rpm for 2 minutes at 4°C to get the nucleus pellets. Post-nuclear supernatants (PNS) were loaded onto a Nycodenz gradient (105 µL of 22% Nycodenz at bottom, 270 µL of 9.5% Nycodenz in middle, and 225 µL PNS on top) in an ultracentrifuge tube (Beckman 355090), and centrifuged at 38,600 with the Beckman SW55 rotor for 1 hour at 4°C to get these fractions: top of PNS layer, middle of PNS layer (Cytosol), interface between PNS and 9.5% Nycodenz layers, interface between 9.5% and 22.5% Nycodenz layers (ER/AV), and bottom of tube (Mitochondria & Peroxisomes). ER/AV fraction was loaded on a Percoll/Nycodenz gradient (110 µL of 22.5% Nycodenz at bottom, 330 µL of 33% Percoll in middle, and 160 µL ER/AV on top) and centrifuged at 27,600 rpm for 30 minutes at 4°C to get these fractions: interface between ER/AV and 33% Percoll layers (ER), and interface between 33% Percoll and 22.5% Nycodenz layers (AV/Percoll). AV/Percoll fraction was mixed with 168 µL 60% (w/v) OptiPrep in water, loaded onto an OptiPrep gradient (408 µL AV/Percoll/OptiPrep at bottom, 72 µL 30% OptiPrep in middle, and 120 µL buffer on top), and centrifuged at 27,300 rpm for 30 minutes at 4°C to get the AV fraction between the sucrose and 30% OptiPrep layers. All samples derived from cells were resuspended in buffer. For the protease protection assay, 3 µg of each sample was incubated with 100 μg/ml Proteinase K, or with 100 μg/ml Proteinase K and 0.5% Triton X-100 on ice for 30 minutes. Next, samples were boiled for 10 minutes to stop the reactions then subjected to immunoblotting protocol. To calculate the total proteins in each fraction, the densitometry of 3 μg from each sample was measured then normalized to the total volume collected for each respective fraction. Statistical significance is assessed with two-sample Student’s t-Test.

**Immunofluorescence and Confocal Microscopy:**

Cells were plated on coverslips in a 24-well plate at a density of 10,000 cells per well and were grown overnight at 37°C. The next day, media were aspirated from each well, then 250 µL 4% paraformaldehyde were added and incubated for 20 minutes. Coverslips were washed 3x with 1 mL 1X PBS for 5 minutes each. Coverslips were blocked with 500 µL blocking solution (3% BSA/0.1% saponin) diluted in 1X PBS at RT for 15 minutes. Then 40 µL of primary antibodies (1:500 dilution in blocking solution) was applied to coverslips and left overnight at 4°C. The next day, coverslips were placed back into a 24-well plate and washed 3x with 1 mL blocking solution for 5 minutes each. 250 µL of secondary antibodies (1:600 dilution in blocking solution) was applied to the coverslips and incubated for 1 hour in the dark. After 1 hour, coverslips were washed 3x with 1 mL blocking solution. Next, coverslips were mounted with one drop of ProLong™ Gold per coverslip. Coverslips were dried in the dark overnight at 4°C. Images were taken with the ZEISS LSM 880 microscope and analyzed with ImageJ.

**Data availability**

All raw data is available at reasonable request by contacting Dr. Thomas Melia (thomas.melia@yale.edu) from Yale University, all remaining data are contained within the article.

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**Author contributions**

NN, JJ, and TJM conceptualization; NN, TJO, and AM data curation; TJM funding acquisition; NN, TJO, and AM investigation; NN, TJO, AM, and TJM methodology; NN and TJM project administration; NN, JJ, SY, LL, SN, KJK, and TJM resources; NN and TJM supervision; NN, TJO, and
AM validation; NN, TJO, and AM visualization; NN writing—original draft; NN, TJO, and TJM writing—review & editing.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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**A**

Long-lived protein degradation (%)

| EBSS | 3-MA | WT | ATG4<sub>QKO</sub> | ATG4<sub>QKO</sub>-ATG4A | ATG4<sub>QKO</sub>-ATG4B | ATG4<sub>QKO</sub>-ATG4C | ATG4<sub>QKO</sub>-ATG4D |
|------|------|----|------------------|------------------|------------------|------------------|------------------|
| -    | -    | -  | -                | -                | -                | -                | -                |
| +    | +    | +  | +                | +                | +                | +                | +                |

**B**

WB analysis of p62 levels

| Condition | WT | ATG4<sub>QKO</sub> |
|-----------|----|------------------|
| EBSS      |    | Pro-LC3B         |
| Baf       |    | LC3B-II          |

**C**

Normalized p62 levels

| ATG4<sub>QKO</sub> | ATG4<sub>QKO</sub>-ATG4A | ATG4<sub>QKO</sub>-ATG4B | ATG4<sub>QKO</sub>-ATG4C | ATG4<sub>QKO</sub>-ATG4D |
|---------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| EBSS                |                           |                           |                           |                           |
| Baf                 |                           |                           |                           |                           |
Figure 1. Both starvation-induced bulk autophagy and p62 homeostasis are impaired in ATG4\textsubscript{QKO} cells and are rescued only by ATG4B expression. A. Long-lived protein degradation assay reveals ATG4\textsubscript{QKO} cells are unable to degrade long-lived proteins under starvation conditions. Cells were maintained under DMEM or incubated in starvation medium (EBSS) and treated without or with 10 mM 3-Methyladenine (3-MA) for 4 hours. B. Immunoblotting of cells after DMEM or EBSS without or with 0.1 \( \mu \)M Bafilomycin A1 for 2 hours revealed p62 levels are elevated in ATG4\textsubscript{QKO} cells. C. Under the same conditions as in (B), ATG4B expression was able to restore p62 levels back to WT in the ATG4\textsubscript{QKO} background, while expression of ATG4A, ATG4C, or ATG4D could not. Quantifications for p62 levels of ATG4\textsubscript{QKO} cells with single ATG4 isoform rescues are done by dividing p62 density over GAPDH density and normalizing all values to the highest value in the experiment. Raw data for p62 quantification are in Fig. S4. Statistical significance is assessed with two-sample Student’s t-Test. *, \( p < 0.05 \). **, \( p < 0.01 \). ***, \( p < 0.001 \).
Delipidation and lipidation rates are balanced; subtle changes during autophagy induction can drive the formation of GL1-II. Priming is very slow. In addition, reduction in delipidation rates or increases in lipidation (or both) eliminate the free GL1-I pool.
Figure 2. Delipidation of GL1 is not detectable in ATG4QKO-ATG4A rescue cells. A. Cartoon of GL1 states in WT and ATG4QKO-ATG4A rescue cells with a model suggesting how the lipidation/delipidation cycle might work to set those states. B. Suppression of autophagy initiation by wortmannin does not reduce GL1-II levels in ATG4QKO-ATG4A rescue cells. Immunoblot of LC3B and GL1 lipidation in cells incubated in EBSS and treated with/without 0.1 µM Wortmannin and with/without 0.1 µM Bafilomycin A1 for 2 hours. Quantification of GL1-II as a percentage of total GL1 signals in WT and ATG4QKO-ATG4A rescue cells. C. GL1 lipidation is unchanged in ATG4QKO-ATG4A cells exposed to 0.1 µM Wortmannin for up to 24 hours. Quantification of GL1-II was done for 3, 6, 12, and 24 hour time points. D. Conditions as in (B), except treatment with 10 mM 3-MA instead of Wortmannin for 2 hours also had no effect on delipidation extent of ATG4QKO-ATG4A rescue cells. E. Kinases which alter LC3B-II or GL1-II levels in WT cells do not alter GL1-II levels in ATG4QKO-ATG4A rescue cells. 10 µM of Akt/MST4/ULK1 inhibitors were used to treat cells for 12 hours and had no effect on delipidation kinetics in ATG4QKO-ATG4A rescue cells. On all blots, protein molecular weights shown in kilodaltons on left. Statistical significance is assessed with two-sample Student’s t-Test. *, p < 0.05. **, p < 0.01.
A

GL1-YFP cleaved

ATG4B
ATG4B ΔLIR

ATG4B
ATG4B ΔLIR

ATG4B
ATG4B ΔLIR

ATG4B
ATG4B ΔLIR

% GL1-YFP cleaved vs [ATG4] (nM)

% GL1-II cleaved vs [ATG4] (nM)

% LC3B-I cleaved vs [ATG4] (nM)

% LC3B-II cleaved vs [ATG4] (nM)

B

ATG4B S383A
ATG4B S383D

ATG4B S383A
ATG4B S383D

ATG4B S383A
ATG4B S383D

ATG4B S383A
ATG4B S383D

ATG4B S383A
ATG4B S383D

ATG4B S383A
ATG4B S383D

ATG4B S383A
ATG4B S383D

ATG4B S383A
ATG4B S383D

% GL1-YFP cleaved vs [ATG4] (nM)

% GL1-II cleaved vs [ATG4] (nM)

% LC3B-I cleaved vs [ATG4] (nM)

% LC3B-II cleaved vs [ATG4] (nM)

C

LIPD (%)

EBSS
3-MA
ATG4B
ATG4B B383A
ATG4B B383D

ATG4B B383A
ATG4B B383D

ATG4B B383A
ATG4B B383D

ATG4B B383A
ATG4B B383D

ATG4B B383A
ATG4B B383D

ATG4B B383A
ATG4B B383D

ATG4B B383A
ATG4B B383D

ATG4B B383A
ATG4B B383D

D

Normalized p62 levels

ATG4 KO rescued with indicated ATG4B variants

EBSS
3-MA
Baf
Figure 3. ATG4B C-terminus is needed to support delipidation. A. In vitro reconstitution of GL1/LC3B priming and delipidation. For priming, recombinant purified GL1-YFP or LC3B-YFP was mixed with recombinant purified ATG4B or ATG4B ΔLIR for 16 minutes at 37°C, and the extent of YFP release was measured by running samples on an SDS-PAGE gel. For delipidation, GL1 or LC3B was first coupled to PE on liposomes in an in vitro lipidation reaction, and these proteo-liposomes were isolated on a density gradient. Then these samples were mixed with ATG4B or ATG4B ΔLIR for 16 minutes at 37°C. Delipidation was assessed as a band shift of LC3B or GL1 on SDS-PAGE gels. B. Phosphomimetic versions of ATG4B were tested in GL1 priming and delipidation assays as in (A). C. Rescue of ATG4QKO cells with phosphomimetic versions of ATG4B were tested for long-lived protein degradation (as in Fig. 1A). D. Rescue of ATG4QKO cells with phosphomimetic versions of ATG4B were tested for p62 homeostasis (as in Fig. 1B/C). Raw data for p62 quantification are in Fig. S12A. Statistical significance is assessed with two-sample Student’s t-Test. *, p < 0.05. **, p < 0.01.
ATG4QKO rescued with indicated ATG4A variants

ATG4A | ATG4A\textsubscript{S397A} | ATG4A\textsubscript{S397D} | ATG4A ΔLIR
--- | --- | --- | ---
DLEEDFEILSV | DLEEDFEILAV | DLEEDFEILDV | DL

% GL1-YFP cleaved

[ATG4] (nM)

% GL1-II cleaved

[ATG4] (nM)

ATG4\textsubscript{QKO} rescued with indicated ATG4A variants

ATG4QKO-ATG4A | ATG4QKO-ATG4A\textsubscript{S397A} | ATG4QKO-ATG4A\textsubscript{S397D} | ATG4QKO-ATG4A ΔLIR
--- | --- | --- | ---

Normalized p62 levels

EBSS

3-MA

WT | ATG4A | A\textsubscript{S397A} | A\textsubscript{S397D} | A ΔLIR
--- | --- | --- | --- | ---

Normalized p62 levels

EBSS

Baf

- - | ++ | -- | ++ | -- | ++ | ++
Figure 4. ATG4A C-terminus modulates GL1 priming and delipidation. A, B. In vitro reconstitution of GL1 priming (A) and delipidation (B) with various ATG4A constructs carried out as in Fig. 3A, reveals that the COOH-terminal LIR on ATG4A is essential to both processes. C. Rescue of ATG4QKO cells with expression of ATG4A LIR mutants reveals that the LIR is also essential to priming in cells and is potentially modulated by alteration at S397. Immunoblot of cells incubated in DMEM or EBSS, without or with 0.1 μM Bafilomycin A1 for 2 hours. To quantify processing, densitometry was used to calculate the amount of pro-GL1 as a fraction of the total GL1 signal detected on the blot. Note that for values less than 100%, this represents a lower limit, as the antibody recognizes the GL1-II form more efficiently than the soluble proteins. D. Rescue of ATG4QKO cells with COOH-terminal mutants of ATG4A were tested for long-lived protein degradation (as in Fig. 1A). E. Rescue of ATG4QKO cells with COOH-terminal mutants of ATG4A were tested for p62 homeostasis (as in Fig. 1B/C). Raw data for p62 quantification are in Fig. S12B. Statistical significance is assessed with two-sample Student’s t-Test. *, p < 0.05. **, p < 0.01. ***, p < 0.001. ****, p < 0.0001.
Immunofluorescence of ATG4QKO-ATG4A FLAG-GL1G116 cells

A

Western blot analysis showing protein expression levels under different conditions.

B

Immunofluorescence images of WIPI2 and LAMP1 in ATG4QKO-ATG4A FLAG-GL1G116 cells.
Figure 5. GL1-II decorated membranes colocalize with WIPI2- and LAMP1- positive puncta. In order to image GL1 distributions in cells, a FLAG-tagged form of GL1 truncated at position 116 (FLAG-GL1$_{116}$) was expressed in wildtype (WT) or ATG4$_{QKO}$-ATG4A cells. **A.** Immunoblot of cells grown in DMEM or EBSS without or with 0.1 µM Bafilomycin A1 for 4 hours reveals that FLAG-GL1$_{116}$ behaves the same as endogenous GL1, and accumulates as GL1-I form in WT cells or GL1-II form in ATG4$_{QKO}$-ATG4A cells. **B.** Immunofluorescence microscopy of cells in (A) reveals that FLAG-GL1$_{116}$ forms puncta in ATG4$_{QKO}$-ATG4A. B-top: many WIPI2 puncta colocalize with FLAG-GL1$_{116}$ (white arrows) although some WIPI2 sites appear to be without FLAG-GL1$_{116}$ (yellow arrows). B-bottom: FLAG-GL1$_{116}$ frequently colocalizes with the lysosome-related marker LAMP1 (white arrows) although some FLAG-GL1$_{116}$ remains separate (yellow arrows). Scale bar: 10 µm.
Figure 6. GL1-II decorated membranes in ATG4\textsubscript{QKO}-ATG4A rescue cells are mostly unclosed structures localized to the ER. A. Cell fractionation and protease protection assay of cell lysates. Indicated cells were grown in DMEM and treated with 0.1 µM Bafilomycin A1 for 4 hours before being subjected to multiple rounds of centrifugation as in (61). Isolated fractions were treated with 100 µg/ml Proteinase K for 30 minutes and the protection of proteins was evaluated by immunoblot. B. Quantifications of (A) were conducted by dividing GL1-II density (or p62 density) of samples with Proteinase K treatment over density of control samples. Statistical significance was assessed with two-sample Student’s t-Test. *, p < 0.05. C. Cell fractionation and ER/AV separation were performed by multiple density gradient isolations (cartoon and (62)) from cells grown in DMEM and treated with 0.1 µM Bafilomycin A1 for 4 hours. To detect organelle enrichment in each final isolate (labeled in color in cartoon), 3 ug of protein were run on SDS-PAGE gels and imaged by immunoblot. D. To determine the total amount of GL1-II or p62 localized to each organelle isolate, the intensity of the bands in (C) were normalized to the total mass collected of each fraction. Statistical significance is assessed with two-sample Student’s t-Test. *, p < 0.05. **, p < 0.01. ***, p < 0.001.
