Intrinsic lipid binding activity of ATG16L1 supports efficient membrane anchoring and autophagy

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Abstract

Membrane targeting of autophagy-related complexes is an important step that regulates their activities and prevents their aberrant engagement on non-autophagic membranes. ATG16L1 is a core autophagy protein implicated at distinct phases of autophagosome biogenesis. In this study, we dissected the recruitment of ATG16L1 to the pre-autophagosomal structure (PAS) and showed that it requires sequences within its coiled-coil domain (CCD) dispensable for homodimerisation. Structural and mutational analyses identified conserved residues within the CCD of ATG16L1 that mediate direct binding to phosphoinositides, including phosphatidylinositol 3-phosphate (PI3P). Mutating putative lipid binding residues abrogated the localisation of ATG16L1 to the PAS and inhibited LC3 lipidation. On the other hand, enhancing lipid binding of ATG16L1 by mutating negatively charged residues adjacent to the lipid binding motif also resulted in autophagy inhibition, suggesting that regulated recruitment of ATG16L1 to the PAS is required for its autophagic activity. Overall, our findings indicate that ATG16L1 harbours an intrinsic ability to bind lipids that plays an essential role during LC3 lipidation and autophagosome maturation.

Keywords: ATG16L1; autophagy; coiled-coil domain; phagophore; PI3P

Introduction

While some organelles, such as the endoplasmic reticulum (ER) or mitochondria, are generated by growing and budding from pre-existing organelles, autophagosome formation is initiated through the de novo nucleation of membranous structures (Joshi et al., 2017). This process requires the activity of distinct protein complexes that act to relay upstream signals in order to facilitate the growth of precursor membranes known as pre-autophagosomal structures (PAS; Lamb et al., 2013). Of these protein complexes, the ATG5 complex, comprised of the ATG12–ATG5 conjugate and ATG16L1, plays a pivotal role in both the nucleation of the PAS and the downstream conjugation of the ATG8 ubiquitin-like family of proteins (such as LC3) to phosphatidylethanolamine (PE; Sakoh-Nakatogawa et al., 2013). The conjugation of LC3 on the PAS facilitates the maturation of autophagosomes and the recruitment of cargo molecules for their subsequent lysosomal degradation.

In order to better understand how autophagy-related complexes are activated and recruited to the growing PAS, recent studies have started to uncover the genetic and temporal hierarchy of these complexes in mammalian cells (Itakura & Mizushima, 2010; Koyama-Honda et al., 2013). Following inhibition of mTORC1 (e.g. by amino acid starvation or small molecule inhibitors), the ULK1 complex is relieved from its inhibitory phosphorylation by mTOR, resulting in its recruitment to the PAS independently of downstream autophagy complexes (Itakura & Mizushima, 2010). The ULK1 kinase can phosphorylate and activate members of the ATG14 complex, containing the class III PI3K kinase Vps34, which facilitates the recruitment of phosphatidylinositol 3-phosphate (PI3P) sensors (such as DFCP1, WIP1 and WIP2; Axe et al., 2008; Matsunaga et al., 2010). FIP200, a component of the ULK1 complex, and WIP1b can both directly interact with ATG16L1, providing a mechanism for the localisation of the ATG5 complex to the PAS during mTORC1 inactivation (Gammoh et al., 2013; Nishimura et al., 2013; Dooley et al., 2014).

Membrane recruitment of most autophagy complexes is pivotal for their role in autophagy. Ectopic recruitment to the plasma membrane of an ATG5-binding fragment of ATG16L1 results in aberrant and constitutive lipidation of LC3 (Fujita et al., 2008). Similar results were also obtained upon plasma membrane tethering of the ATG16L1 binding partner, WIP1b (Dooley et al., 2014). These findings suggest that membrane localisation of ATG16L1 in cells is sufficient to drive the conjugation of LC3 to PE and that its...
regulation could provide means to fine-tune autophagy. Despite the above proposed hierarchy, it remains unknown how autophagy players are recruited during ULK1-independent autophagy (Gammoh et al., 2013). Furthermore, live-cell imaging analyses suggest that both the recruitment and displacement of autophagy proteins to the PAS occurs in an asynchronous manner, indicating that protein–protein interactions are not sufficient to stabilise autophagy complexes on membranes (Koyama-Honda et al., 2013). In addition, sequences that correspond to WIP12b and FIP200 binding sites in mammalian ATG16L1 are absent in yeast Atg16 (Fujioka et al., 2010; Gammoh et al., 2013; Nishimura et al., 2013; Dooley et al., 2014). These observations suggest the existence of previously unknown mechanisms that can mediate the localisation of the ATG5 complex to membranes.

Given the central role of the ATG5 complex during various forms of LC3 conjugation, including both canonical and non-canonical autophagy (Fletcher et al., 2018), we aimed to investigate how the ATG5 complex is recruited to autophagy-related membranes. In this study, we have identified highly conserved sequences within the coiled-coil domain (CCD) of ATG16L1 that mediate its direct interaction with lipids, thereby enhancing its PAS localisation and autophagic activity.

**Results**

**ATG16L1 membrane targeting activity is retained in the absence of ATG5 or WIP12**

To investigate the membrane recruitment of the ATG5 complex, we first addressed the role of either ATG5 or ATG16L1 in PAS targeting. Since deletion of either protein can destabilise the other (Fig 1A, Nishimura et al., 2013), we generated knockout cell lines stably expressing GFP-tagged proteins to avoid any discrepancies resulting from reduction in protein levels or ectopic localisation (Li et al., 2017). As seen in Fig 1B, the recruitment of stably expressed ATG16L1 to punctate structures was not disrupted in the absence of ATG5. On the other hand, ATG5 showed a diffused pattern of staining in the absence of ATG16L1. Biochemical fractionation further confirmed the finding that ATG16L1 accumulates in membrane fractions in the absence of ATG5 (Fig 1C), as well as in the absence of ATG3 (Gammoh et al., 2013). These results suggest a role for ATG16L1 in the membrane targeting of the ATG5 complex. Previous studies show that the PAS localisation of an ATG16L1 mutant lacking both WIP12b and FIP200 binding (ATG16L1ΔWIPD) was markedly reduced but not completely inhibited, suggesting that these interaction partners may act as signalling players that enhance the membrane recruitment of ATG16L1 (Gammoh et al., 2013; Nishimura et al., 2013). This was further confirmed in WIP12−/− cells where ATG16L1 puncta formation was reduced but not fully inhibited (Fig 1D and E). Residual ATG16L1-positive puncta formed in WIP12−/− cells were sensitive to Vps34 inhibition by 3-methyladenine (3’MA) treatment, in agreement with previous data showing the requirement of PI3P for the PAS recruitment of the ATG5 complex (Koyama-Honda et al., 2013). Overall, these findings suggest the existence of additional previously undescribed mechanisms that mediate the recruitment of ATG16L1 to the PAS.

**PAS targeting activity of ATG16L1 lies within its CCD**

To identify the region within ATG16L1 required for its localisation to the PAS, we examined ATG16L1 puncta formation in U2OS cells expressing a series of ATG16L1 truncation mutants (depicted in Fig 2A). As seen in Fig 2B, the deletion of N-terminal sequences containing the ATG5 binding (fragment Δ1, residues 39–623) or the further downstream linker region (fragment Δ2, residues 120–623) did not affect puncta formation when compared to wild-type ATG16L1 (ATG16L1WT). On the other hand, subsequent fragments lacking the CCD of ATG16L1 (fragments Δ3 and A4, residues 206–623 and 336–623, respectively) were diffused in cells, suggesting that PAS targeting requires either the dimerisation of ATG16L1 or additional unknown activities within the CCD. To distinguish these two possibilities, we aimed to further delete sequences within the CCD that were predicted to be dispensable for dimerisation. Based on ATG16L1 structural predictions and comparisons to yeast Atg16 (Fujioka et al., 2010), we analysed conserved regions within the ATG16L1 CCD that were predicted to not contribute to the dimer-dimer interface. A combined deletion of amino acids 182–205 within the context of the Δ2 fragment (ATG16L1Δ2Δ182–205) resulted in a diffused pattern of staining, indicating the requirement of these residues of ATG16L1 for puncta formation (Fig 2C). We further confirmed that deleting residues 182–205 within the context of the full-length protein (ATG16L1Δ182–205) did not interfere with the ability of ATG16L1 to interact with FIP200 and ATG5 or homodimerise (Fig 2D and E), whereas a truncation mutant lacking the CCD, but not the WD40 domain, was unable to homodimerise (Fig 2F). When expressed in ATG5−/− cells, the deletion mutant, ATG16L1Δ182–205, exhibited a diffused pattern of staining (Fig 2G), whereas ATG16L1WT formed punctate structures. These findings confirm that sequences within the CCD are required for PAS targeting but dispensable for previously identified functions of ATG16L1, including dimerisation and binding to FIP200 and ATG5.

**ATG16L1 binds liposomes through CCD sequences**

Having shown that the localisation of ATG16L1 to punctate structures requires sequences within its CCD of unknown function, we further investigated the relevance of these sequences in recruiting ATG16L1 to the PAS. Further analyses of the CCD domain region indicated the presence of a hydrophobic region and positively charged residues that could mediate direct lipid binding of ATG16L1 to membranes (Fig 3A). To address this possibility, we used a microscopy-based technique to test the recruitment of rhodamine-labelled small unilamellar vesicles (SUVs) to beads coated with ATG16L1-GFP (Fracchiolla et al., 2016), a sensitive approach to detect protein-lipid binding activities. As seen in Fig 3B and C, there was no significant recruitment of liposomes to ATG16L1-GFP-bound beads when using liposome preparations that contained phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS) suggesting an inability of ATG16L1 to bind these phospholipids. Consistent with the hypothesis that ATG16L1 can directly bind to autophagy-related membranes, liposome recruitment to ATG16L1-GFP beads was enhanced when incubated with PI3P-containing liposomes, an essential lipid for the biogenesis of autophagosomes (Axe et al.,
2008). Moreover, liposome preparations containing phosphatidylinositol 4-phosphate (PI4P) or phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) also resulted in enhanced recruitment to ATG16L1-GFP containing beads, indicating that these membrane phospholipids can also bind ATG16L1. The binding of ATG16L1 to liposomes was independent of ATG5 or its WD40 domain as a deletion mutant of ATG16L1 lacking its C-terminal half was able to bind liposomes independent of ATG5 or its WD40 domain as a deletion mutant of ATG16L1. The binding of ATG16L1 to liposomes was confirmed the ability of wild-type ATG16L1 to bind PI3P in an independent assay using lipid-coated beads, where we also detected its binding to phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2) (Fig EV1C). In contrast, ATG16L1 did not significantly bind to phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) when compared to control beads, suggesting a degree of specificity for phosphoinositides. Interestingly, this assay also revealed that ATG16L1 has low binding affinity for PE and PS, while also exhibiting an affinity for PA, although this was found to be non-significant compared to the control beads.

The ability of ATG16L1 to bind PI3P suggests that positively charged residues, potentially located within or juxtaposed to amino acids 182–205 of its CCD, may mediate this interaction. Although previous studies have identified interactions between coiled-coil domains and phospholipids (Horikoshi et al., 2011; Zheng et al., 2014), the structural bases underlying these have not yet been directly elucidated. To explore the potential structural mechanism of ATG16L1-PI3P interaction, we performed structural prediction analyses of a short region of mouse ATG16L1 CCD and modelled its interaction with PI3P embedded in a lipid bilayer. These analyses predict that ATG16L1 CCD could potentially interact with the negatively charged headgroup of PI3P by lying flat on the membrane surface (Fig 3D). Molecular dynamics simulation of the protein in association with PI3P in a model lipid bilayer indicated that this...
association was stable over the course of the trajectory. Interestingly, helices and coiled-coil domains have also been previously shown to interact with lipid bilayers by lying flat on the membrane surface, although adopting a different mechanism than that predicted for ATG16L1 (utilising primarily hydrophobic rather than electrostatic interactions; Pluhackova et al., 2015; Woo & Lee, 2016).

Our homology modelling highlights three residues, including K179, R193 and the further upstream residue I171, which line the outer faces of the coiled-coil and are solvent-exposed, thereby free to interact with the phosphate groups of PI3P or PI(3,4)P2. These residues are conserved in yeast Aig16 but are missing from ATG16L2, a protein closely related to ATG16L1 that does not localise to the PAS.
**Figure 3.** ATG16L1 binds liposomes through CCD sequences.

**A** Sequence alignment of ATG16L1 CCD segment from various species and ATG16L2 (Sc: Saccharomyces cerevisiae; Xt: Xenopus (Silurana) tropicalis; Hs: Homo sapiens; Mm: Mus musculus). Residues that mediate WIP12b and FIP200 binding are highlighted in orange and magenta, respectively. Cyan-shaded residues (I171, K179 and R193) are exposed conserved residues predicted to not contribute to the dimer-dimer interface and are mutated in this study. Cherry-shaded residues are mutated in Figs 6 and 7.

**B** Microscopy-based protein-liposome binding assay. ATG16L1-GFP immobilised on beads incubated in the presence of rhodamine-labelled liposome preparations containing the indicated phosphoinositides. Scale bar: 50 μm.

**C** Quantification of relative liposome binding in (B).

**D** Structural modelling of ATG16L1 residues 160–205 (magenta helix) in the presence of lipid bilayer (green lines). Highlighted residues include I171, K179 and R193 as sticks, which are mutated in this study. A PIP3 molecule is shown as a yellow stick embedded in the lipid bilayer and interacting with the highlighted positively charged residues of ATG16L1.

**E** Microscopy-based protein–liposome binding assay as in (B). ATG16L1WT- and ATG16L1LD-GFP immobilised on beads were incubated with rhodamine-labelled, PIP3-positive liposome preparations. Scale bar: 50 μm.

**F** Protein–protein interaction assay in 293T cells transiently transfected with the indicated S-tagged ATG16L1 constructs. S tag pull-down was performed and protein complexes were analysed by immunoblotting using the indicated antibodies.

**G** Dimerisation assay in 293T cells transiently transfected with ATG16L1-GFP and the indicated S-tagged ATG16L1 constructs and analysed as in (F).

Data information: Quantifications depict means and error bars (SEM) from at least three independent experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 (pairwise unpaired Student’s t-test).
To examine the functional impact of disrupted PAS localisation in ATG16L1, we named this mutant ATG16L1LD for lipid binding-deficient ATG16L1 mutant. Given that these residues are adjacent to WIP2b and FIP200 binding sites, we further confirmed that ATG16L1LD did not interfere with the ability of ATG16L1 to bind these two interactors nor interfere with ATG16L1 homodimer formation (Fig 3F and G). Overall, these data suggest that ATG16L1 contains residues within its CCD that mediate interactions to negatively charged lipids but are dispensable for its dimerisation and binding to WIP2 and FIP200.

Binding of ATG16L1 to lipids is required for PAS recruitment

To dissect the functional relevance of ATG16L1 lipid binding in cells, we further analysed the localisation of ATG16L1WT and ATG16L1LD, stably expressed in ATG16L1−/− cells. When compared to ATG16L1WT, we observed a strong inhibition of ATG16L1LD recruitment to puncta positive for ATG5 and WIP2 during amino acid starvation (Fig 4A and B), suggesting that ATG16L1 lipid binding is required for its efficient recruitment to the PAS. Additionally, the overall intensity of ATG16L1LD puncta was significantly reduced compared to that of ATG16L1WT, implying that ATG16L1 is recruited less efficiently to these sites (Fig 4C). Furthermore, enhanced formation of WIP2-positive puncta, as observed by reconstituting ATG16L1−/− cells with ATG16L1WT, was impaired in ATG16L1LD-expressing cells, indicating that ATG16L1 lipid binding may influence early autophagic events (Fig 4B). Residual ATG16L1LD punctate structures could potentially be due to its ability to bind upstream autophagy players, including WIP2b and FIP200, which have been proposed to facilitate its recruitment to the PAS (Gammoh et al, 2013; Nishimura et al, 2013; Dooley et al, 2014). To test this possibility, we stably expressed ATG16L1LD in WIP2−/− cells and observed a further reduction in puncta formation of this mutant to levels comparable to background levels in mock infected cells (Fig 4D). Overall, these data suggest that the efficient recruitment of ATG16L1 to the PAS relies on its ability to interact with both PI3P through CCD sequences and protein binding partners, such as WIP2b.

Binding of ATG16L1 to lipids is required for autophagy

To examine the functional impact of disrupted PAS localisation in the lipid binding-deficient mutant, ATG16L1LD, we further assessed its ability to mediate LC3 lipidation in cells. When compared to ATG16L1−/− cells reconstituted with ATG16L1WT, ATG16L1LD-expressing cells exhibited a strong inhibition of LC3 lipidation during amino acid starvation (Fig 5A). To confirm that autophagy was inhibited, we measured the levels of p62, an adaptor protein that is degraded during autophagy, following amino acid starvation. Consistent with the LC3 lipidation results, p62 degradation was impaired in ATG16L1LD-expressing cells, suggesting that autophagic flux was also inhibited (Fig 5A). Furthermore, we tested LC3 lipidation induced by carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 μM) treatment, shown to induce the selective degradation of mitochondria (Narendra et al, 2008), and similarly observed defective LC3 lipidation in ATG16L1LD-expressing cells (Fig 5B). Similar results were obtained during autophagy induced by glucose starvation, suggesting that the lipid binding domain of ATG16L1 is also required for autophagy induced in the absence of mTORC1 inhibition and ULK1-complex activation (Fig 5C). Given that non-canonical LC3 lipidation can occur on single membranes and requires the ATG5 complex but not additional upstream autophagy machinery, such as WIP2 or the ULK1 complex, we further examined whether ATG16L1LD could support LC3 lipidation during treatment with monensin, ammonium chloride (NH4Cl) or CCCP (100 μM), known to act as ionophores and/or lysosomotropic agents (Jacquin et al, 2017). Consistent with previous results, these treatments required the activity of ATG16L1WT to support LC3 lipidation (Fig 5D and E; Fletcher et al, 2018), whereas LC3 lipidation was strongly diminished in ATG16L1LD-expressing cells. Collectively, these data suggest that the lipid binding activity of ATG16L1 is required to facilitate both LC3 lipidation induced by various stimuli and the efficient degradation of autophagic cargo.

In addition to binding FIP200 and WIP2, ATG16L1 has also been shown to bind Rab33B through sequences within its middle region (Itoh et al, 2008). The relevance of Rab33B binding remains to be further explored as published studies suggest that shRNA-mediated inhibition of Rab33B expression did not affect LC3 lipidation while overexpression of a GTP-hydrolysis-deficient (constitutively active) mutant of Rab33B enhanced LC3 lipidation (Itoh et al, 2008, 2011). We further tested whether mutating lipid binding residues within the CCD of ATG16L1 affected the interaction between ATG16L1 and Rab33B. Surprisingly, ATG16L1LD exhibited a strongly diminished affinity to GFP-Rab33B compared to ATG16L1WT (Fig EV2A). To assess whether genetic inhibition of Rab33B influenced autophagy in a manner that mimicked mutating the residues involved in ATG16L1 lipid binding, we generated Rab33B−/− cells using CRISPR/Cas9-mediated gene editing in MEF cells. Rab33B−/− cells did not exhibit reduced LC3 lipidation (Fig EV2B) or disrupt ATG16L1 puncta formation (Fig EV2C) in a manner that resembled ATG16L1LD-reconstituted cells (Figs 4A and 5A). Altogether, these data suggest that the functional relevance of ATG16L1 binding to Rab33B is distinct from its binding to PI3P (Ishibashi et al, 2011; Fujita et al, 2013).
Figure 4. Binding of ATG16L1 to lipids is required for PAS recruitment.

A ATG16L1<sup>−/−</sup> stably expressing the indicated Flag-S-ATG16L1 constructs were amino acid starved for 2 h prior to immunofluorescence analyses using antibodies against Flag tag (green) and ATG5 (red). Scale bar: 9 μm. Right panels show quantifications of average number of Flag- and ATG5-positive dots per cell.

B Cells, as in (A), were immunostained using antibodies against S tag (to detect ATG16L1, red) and WIPI2 (green). Scale bar: 9 μm. Lower panel shows quantification of average number of WIPI2-positive dots per cell.

C Average intensities of individual Flag-ATG16L1 dots in (A). Underlying grey circles represent individual data points. Dots were quantified (n = 1,225 for ATG16L1<sup>WT</sup>; n = 334 for ATG16L1<sup>LD</sup>).

D WIPI2<sup>−/−</sup> cells reconstituted with the indicated Flag-S-ATG16L1 constructs were amino acid starved for 2 h prior to immunofluorescence analyses using antibodies against Flag tag (green) and FIP200 (red). Scale bar: 9 μm. Lower panel shows quantification of average number of Flag-positive dots per cell.

Data information: Quantifications depict means and error bars (SEM) from at least three independent experiments. *P ≤ 0.05, **P ≤ 0.001, ***P ≤ 0.0001 (pairwise unpaired Student's t-test).
and E165A) and termed the resulting mutant ATG16L1LE for its potentially lipid binding-enhancing ability. Furthermore, mutating residues D164 and E165 retained the ability of ATG16L1 to bind Rab33B (Fig EV3A). To test lipid binding activity, we generated recombinant ATG16L1 protein spanning residues 72–307, which lacks both the ATG5 binding domain and the C-terminal WD40 domain, in order to enhance protein expression as shown previously (Fig SB, Archna & Scrima, 2017). We next tested the lipid binding activity of the ATG16L1LE mutant using liposome co-sedimentation assays and SUVs prepared by sonication. Similar to previously published data (Romanov et al, 2012), we observed a weak interaction between ATG16L1WT and liposomes under these conditions (Fig 6C). Importantly, this fragment of ATG16L1 (ATG16L172–307WT) was able to bind liposomes in the imaging-based experiment, suggesting that its weak pelleting in the liposome co-sedimentation assay is not due to the lack of ATG5 binding or WD40 domains but due to differences in the experimental approach (Fig EV3B). Interestingly, ATG16L1LE exhibited enhanced lipid binding, suggesting that residues D164 and E165 can modulate the affinity of ATG16L1 for lipids (Fig 6C). Similar to ATG16L1WT protein tested using the GFP-coated beads setting (Fig 3B), the enhanced liposome binding ability of ATG16L1LE was also dependent on PI3P (Fig 6D). Importantly, mutations in residues D164 and E165 and lipid binding residues (I171, K179 and R193) in the combined mutant ATG16L1LELD (D164A, E165A, I171D, R179D and K193D) abolished the lipid binding activity of ATG16L1 (Fig 6C), suggesting that ATG16L1LE enhances the lipid binding activity of CCD residues. In the context of our model (Fig 3D), we conclude that the loss of negative charges on the exposed surface of this CCD region enhances the affinity of ATG16L1 for negatively charged lipid headgroups, such as PI3P.

**Persistent localisation of ATG16L1 to PAS inhibits autophagy**

To assess the consequence of persistent lipid binding in cells, we tested the recruitment of ATG16L1LE to the PAS. When ATG16L1WT and ATG16L1LE were expressed in ATG16L1−/− cells, similar numbers of cells were positive for ATG16L1 puncta following amino acid starvation (Fig 7A and C). Furthermore, these puncta showed comparable colocalisation with WIPI2 (Fig 7A). Importantly, puncta formed by ATG16L1LE were sensitive to Vps34 inhibition by 3MA treatment suggesting the relevance of PI3P in its PAS localisation (Fig 7B and C). Interestingly, ATG16L1LE localised to PAS structures that were positive for WIPI2 and responsive to Vps34 inhibition under unstarved conditions or upon replenishment with full growth media following amino acid starvation (Figs 7D and EV4A–C). Importantly, these observations indicate that ATG16L1LE persistently localises to autophagic structures. To test whether persistent localisation of ATG16L1 to the PAS resulted in enhanced or deleterious effects on autophagy, we tested LC3 lipidation in ATG16L1−/− cells expressing either ATG16L1WT or ATG16L1LE. As can be seen in Fig 7E, LC3 lipidation was significantly inhibited in ATG16L1LE-expressing cells. Punctate structures formed by ATG16L1LE under unstarved conditions could be due to
inhibited phagophore maturation which has been shown to accumulate the ATG5 complex on the PAS (Sou et al, 2008; Gammoh et al, 2013). To test whether ATG16L1<sup>LE</sup> affects autophagy in cells that contain endogenous ATG16L1, we expressed ATG16L1 constructs in WIPI2<sup>−/−</sup> cells where autophagic vesicle formation is impaired but not fully inhibited (Bakula et al, 2017). Expression of ATG16L1<sup>LE</sup>, but not ATG16L1<sup>WT</sup>, resulted in inhibited LC3 lipidation in a dominant negative manner, thereby suggesting that enhanced lipid binding can have deleterious effects on phagophore maturation during both basal and amino acid starvation-induced autophagy (Fig 7F). We did not observe similar inhibition of LC3 lipidation upon ATG16L1<sup>LE</sup> expression in wild-type MEFs (data not shown). Since wild-type ATG16L1 puncta formation was markedly reduced in WIPI2<sup>−/−</sup> cells (Fig 1E), the dominant negative effects of ATG16L1<sup>LE</sup> are likely to require higher expression levels in the context of wild-type cells in order to compete with endogenous ATG16L1<sup>WT</sup>. Overall, these results suggest that regulated localisation of ATG16L1 to the PAS is required for autophagy.

**Binding of ATG16L1 to lipids is required for ferroptosis**

Ferroptosis is a recently described cell death mechanism dependent on iron availability (Gao et al, 2015) and the induction of autophagy (Gao et al, 2016). Metabolic stress induced by amino acid starvation in the presence of serum has been shown to induce ferroptosis which can be suppressed by the genetic inhibition of autophagy or use of dialysed serum to deplete glutamine. To test whether the lipid binding mutants of ATG16L1 can mediate the role of autophagy in supporting ferroptosis, we cultured cells in amino acid free media in the presence of 10% FBS (to induce ferroptosis) or 10% dialysed FBS (dFBS, as a control). As previously shown, cell death measured by propidium iodide (PI) staining was robustly induced in cells expressing ATG16L1<sup>LD</sup> or ATG16L1<sup>LE</sup>, emphasising the important role of regulated ATG16L1-lipid binding for autophagy and its functional activities.

**Discussion**

Our study shows for the first time that ATG16L1 harbours an intrinsic ability to bind autophagy-related membranes through direct interaction with PI3P. This interaction requires conserved sequences within the CCD of ATG16L1 that, when mutated, result in inhibited recruitment to the PAS and LC3 lipidation. Importantly, enhancing lipid binding of ATG16L1 by mutating negatively
Figure 7.

A

AA starve

ATG16L1\textsuperscript{WT}  WIPI2  merge

ATG16L1\textsuperscript{LE}  WIPI2  merge

B

AA starve + 3'MA

ATG16L1\textsuperscript{WT}  WIPI2  merge

ATG16L1\textsuperscript{LE}  WIPI2  merge

C

% cells positive for ATG16L1 puncta

|       | ns | AA starve | AA starve + 3'MA |
|-------|----|-----------|------------------|
| WT    |    |           |                  |
| LE    |    |           |                  |

D

Untreated  AA starve  AA starve \rightarrow refeed

ATG16L1\textsuperscript{WT}

ATG16L1\textsuperscript{LE}

E

ATG16L1\textsuperscript{Δ/Δ}

AA starve:  -  +  +  -  +  +  -  +  +
BafA1:  -  +  +  -  +  +  -  +  +

LC3-I  LC3-II  p62  ATG16L1  Actin

F

WIP12\textsuperscript{Δ/Δ}

AA starve:  -  +  +  -  +  +  -  +  +
BafA1:  +  +  +  +  +  +  +  +  +

LC3-I  LC3-II  ATG16L1  Actin

G

% cells positive for PI

|       | ATG16L1\textsuperscript{WT} | ATG16L1\textsuperscript{LE} | ATG16L1\textsuperscript{Δ/Δ} |
|-------|----------------------------|-----------------------------|------------------------------|
| Untreated | -                          | +                           | +                           |
| AA starve  + FBS | +                          | -                           | -                           |
| AA starve  + dIFBS | -                          | +                           | -                           |

Figure 7.

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charged residues also disrupted LC3 lipidation, suggesting that the tightly regulated recruitment of ATG16L1 to the PAS is important to permit the proper maturation of the phagophore. This is reflected by the weak lipid binding activity of ATG16L1 in the lipidosome co-sedimentation assay, which indicates that additional factors, such as interactions with other autophagy complexes and/or post-translational modifications on ATG16L1, may enhance its recruitment to the PAS in cells.

Given that inhibiting the catalysis of PI3P impairs autophagy (Blommaart et al., 1997), the PI3P dependency of ATG16L1 lipid binding implies its role in directly binding to sites of autophagosome biogenesis. It remains unclear whether the affinity of ATG16L1 for PI3P or other phosphoinositide species provides a mechanism for its targeting to additional membrane compartments including the plasma membrane and endocytic vesicles (Ravikumar et al., 2010; Puri et al., 2013). Interestingly, a recent study shows that the recruitment of WIPI2- to PI3P-positive recycling endosomes requires both its lipid and protein interactions with PI3P and RAB11A, respectively (Puri et al., 2018). In a parallel mechanism, the recruitment of ATG16L1 to the PAS, as well as other membrane compartments, could require both lipid and protein interactions. Furthermore, ATG16L1-dependent lipidation of LC3 on single membranes, for example during treatment with ionophores and lysosomotropic agents, has been shown to occur independently of FIP200, WIPI2 and PI3P (Florey et al., 2015; Fletcher et al., 2018). The ability of ATG16L1 to bind other phosphoinositides as well as potentially unidentified protein interactors may be required to mediate the role of ATG16L1 during non-canonical LC3 lipidation. Additionally, potential binding of ATG16L1 to other phospholipids, such as PA, may be required for unknown functions of ATG16L1 that are independent of PAS recruitment.

The middle region of ATG16L1 has also been shown to bind Rab33B with the specific residues that mediate this interaction not previously identified (Itoh et al., 2008; Ishibashi et al., 2011). Intriguingly, we find that mutating residues I171D, K179D and R193D in the lipid-deficient mutant, ATG16L1LD, strongly reduced its binding to Rab33B. It is therefore possible that the proper recruitment of ATG16L1 to the PAS, mediated through its lipid binding, is required for Rab33B binding. It is also possible that the binding of the CCD of ATG16L1 to lipids and Rab33B occur in a mutually exclusive manner, thereby regulating the function of ATG16L1 during various stages of autophagy. Indeed, previous work has shown that a RabGAP for Rab33B, OATL1, can influence the autophagosome–lysosome fusion, thereby suggesting that Rab33B may also act at later stages of autophagosome maturation (Itoh et al., 2011). Future studies addressing these questions will help further understand the regulation of ATG16L1 activities.

There are discrepancies regarding the proposed hierarchies in which autophagy players are recruited to the growing autophagosome (Koyama-Honda et al., 2013; Dooley et al., 2014). The recruitment of these complexes could occur independently of each other given the individual intrinsic membrane-binding abilities of the ULK1 complex (Karanasios et al., 2013), WIPI proteins (Baskaran et al., 2012), and ATG5 complex (this study). However, the efficiency of their recruitment appears to be interdependent. For instance, ATG16L1 and WIPI2 puncta are reduced in WIPI2−/− and ATG16L1−/− cells, respectively (this study and Dooley et al., 2014). Furthermore, multiple ATG-related proteins can recognise PI3P at the phagophore, many of which harbour non-canonical PI3P binding motifs (including WIPI2b and ATG16L1). The question remains as to whether these PI3P effectors can simultaneously bind to PI3P or whether their binding occurs consecutively. If the latter is true, this may represent a mechanism whereby the ATGs can regulate the recruitment and displacement of one another.

Consistent with our findings using the lipid binding-enhanced mutant ATG16L1LE, membrane tethering of an N-terminal half of ATG16L1 disrupted LC3 lipidation in cells in a manner dependent on CCD sequences (Park et al., 2016). It remains to be addressed how persistent localisation of ATG16L1 to the PAS can inhibit downstream lipidation of LC3. These inhibitory effects suggest that the displacement of ATG16L1, and potentially upstream autophagy machinery, is required for autophagosome growth. The mechanism through which this occurs remains to be elucidated. It is possible that the overall growth and curvature of the autophagosome may induce the release of autophagy players. Alternatively, it is also possible that additional factors, for instance local changes in calcium levels at the ER (Engedal et al., 2013) or post-translational modifications, may aid in neutralising acidic residues located on the lipid-exposed face of the CCD (including D164, E165, E178, E185 or E186). Such regulatory modifications may enhance the electrostatic interactions with the negatively charged lipids and modulate ATG16L1 PAS recruitment. Our data suggest that such fine-tuning of this localisation is essential for proper maturation of autophagosomes.
Materials and Methods

Cell culture

Wild-type mouse embryonic fibroblasts (MEFs), ATG3 knockout MEFs (~/-, kind gift from Dr Masaaki Komatsu), ATG16L1~/-, ATG5~/-, and WIPI2~/-, U2OS and 293T cells were cultured in DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (10 units/ml) and streptomycin (0.1 mg/ml). Stable overexpression of GFP-LC3, GFP-ATG or F-S-ATG proteins was obtained by retroviral infection using pBabe expression plasmids followed by blastocidin or puromycin selection. For transient expression, lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s instructions. WIPI2, ATG16L1 and Rab33B knockout cells were generated by CRISPR/Cas9-mediated gene editing in wild-type MEFs and transiently expressing Cas9 and gRNA constructs followed by single clone selection for deleted lines. The following gRNA sequences were used to target WIPI2 or ATG16L1: 5’ GCTCTCA CATACACAACATC or 5’ AAAGCATGACATGCCAAAT, respectively. For Rab33B knockout, a combination of the following gRNA sequences was used: 5’ GACCTCCAGAGCCGAGCC, 5’ CCGCTCTCCA TCAATATCCA and 5’ ACCGCACGGAGCCAGCAT.

Antibodies, reagents and treatments

The following antibodies were used: anti-LC3 (Sigma, #L7543); anti-Actin (Sigma, #A53516); anti-p62/SQSTM1 (Cell Signalling, #5114); anti-ATG16L1 (MBL, #A0731); anti-FIP200 (Abcam, #ab176816); anti-ATG3 (MBL, M133-3); anti-FLAG (Sigma, M2); anti-ATG15 (Sigma, #A0761); mouse anti-ATG16L1 (kind gift from Dr Masaaki Komatsu), ATG16L1Δ1–Δ4 and Δ1–Δ4 (3’ WD40) were previously described (Gammoh et al, 2013). ATG16L1 mutants were obtained by two-step PCR. Mutant ΔΔ1Δ182-205 was generated using the following forward primer: 5’ AAGGAGCTTGCAAGAGGACAATTGTGGATA GACCTCA. To generated ATG16L1Δ1–Δ4 (I171D K179D R193D), consecutive mutations of each residue were performed using the following forward primers: 1171D 5’ TATAGCCGCGTCGAGCAC AACTTCCA TGGCCTAGAAGAG; K179D 5’ GAGAGGACCTGAGAAACT CTGAG; R193D 5’ GAATGGTCACAGTGATGCTGAG. For ATG16L1Δ1–Δ4, the following forward primer was used: 5’ AACCA GACCTCCAGGGTCCTGATGACCCCTGCCAG. The second PCR step of the above mutants was performed using the following common forward primer and reverse primers: 5’ GCAGCAGTCACTGTCCGTGGGCCTGCGG and 5’ GCAGCACAATTTGGACGACCATGATGTTGCTGGAAT and 5’ CCGAAGTCACTAACTTATTACACAGAAC CAGTAT, respectively. The final PCR products were cloned into pBabe-F-S- using Sall and MfeI sites. C-terminal tagged ATG16L1Δ1–Δ4-GFP was previously described (Gammoh et al, 2013). ATG16L1Δ1–Δ4-GFP was subcloned from pBabe-F-S- vector using Xhol and EcoRI sites within ATG16L1 sequences. ATG16L1Δ1–Δ4ΔDNA-GFP was cloned by PCR using MfeI and Sall sites and pBabe-F-S-ATG16L1Δ1–Δ4 as a template. For recombinant protein expression, ATG16L1 fragments spanning residues 72–207 were cloned into pET28a plasmid using Sall and Xhol restriction sites and the following forward and reverse primers: 5′ GCAGCACAATTGGACGACCATGAT GTTGCTGGAAT and 5′ CCGAAGTCACTAACTTTACACAGAAC CAGTAT, respectively. For protein purification from insect cells, wild-type ATG16L1 was cloned by PCR into a pFastBac plasmid containing His-Flag tags and using Sall and Xhol restriction sites and the following forward and reverse primers: 5′ GCAGCAGTCACTGTCCGTGGGCCTGCGG and 5′ GCAGCAGTCACTGTCCGTGGGCCTGCGG CCAGCAG, respectively. GFP-Rab33B was generated by PCR amplification of human Rab33B cDNA (BC036064.1) and cloned into pBabe-GFP vector using Sall and EcoRI restriction sites and the following forward and reverse primers 5′ GCCAAGTCACTACTCAATTTACACAGAAC CAGTAT and 5′ GCCAAGTCACTACTCAATTTACACAGAAC CAGTAT, respectively.

Cell lysis, fractionation and Western blotting

For whole cell lysis, cells were washed twice with ice-cold PBS followed by direct scraping in cell lysis buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 1% Triton X-100, 1 mM β-ME, 0.5% sodium deoxycholate and 10% glycerol) supplemented with protease inhibitor cocktail V (Fisher Scientific UK). Lysates were cleared by spinning at 20,000 g for 10 min at 4°C. Cytosolic and membrane fractions were obtained by sequential incubation in a detergent-free buffer (150 mM NaCl, 25 mM HEPES pH 7.5 and 1.5 mM β-ME) supplemented with digitonin and NP-40, respectively. Lysates were analysed by SDS-PAGE and transferred onto nitrocellulose membranes or in the case of LC3 blotting to PVDF membranes (Bio-Rad). Membranes were blocked in 5% milk-TBST for 30 min or overnight followed by immunoblotting with the indicated antibodies. Membranes were developed under UV light using Clarity™ Western ECL substrate (Bio-Rad, 1705061).

Pull-down assays

Cell lysates were obtained from 293T cells grown in 10-cm plates and transfected with the indicated plasmids by direct lysing in
NP-40 buffer (150 mM NaCl, 25 mM HEPES pH 7.5, 1.5 mM MgCl$_2$, 1 mM EDTA, 1.5 mM β-ME and 0.5% NP-40) supplemented with protease inhibitor cocktail V (Fisher Scientific UK) and proteasome inhibitor MG132 (Sigma). Cell lysates were cleared by spinning at 20,000 g for 10 min at 4°C and incubated with S-protein agarose (Novagen) for 5 h or overnight at 4°C. Beads were then washed 3 times with NP-40 buffer, and bound proteins were analysed by SDS–PAGE and Western blotting.

**Recombinant proteins purification**

T7-His-tagged ATG16L1$_{72-307}$ and mutants were expressed from pET28a plasmid in Escherichia coli strain BL21 (DE3, Novagen) and grown overnight at 37°C in 50 ml of lysogeny broth (LB) media, containing 50 μg/ml kanamycin. After the initial incubation, the cell culture was diluted into 800 ml LB media with the selection agent and incubated at 37°C until OD600 reached 0.8. To overexpress the protein, host cells were then induced by 0.3 mM IPTG (Sigma) and the culture was maintained for 5 h at 37°C. The cells were centrifuged at 3,830 g for 5 min, supernatant discarded and pellets stored at −80°C. Cell lysis was performed as previously described (Archna & Scrima, 2017). Briefly, cell pellets were resuspended in 20 ml buffer containing 50 mM HEPES pH 7.0, 5% glycerol, 300 mM NaCl, 5 mM mercaptoethanol (β-ME), 5 mM MgCl$_2$, 5 μg DNase (Roche) and 5 mg lysozyme (Sigma) per litre of culture. Resuspended cells were homogenised using a syringe-based homogenisation method in the presence of 1 mM phenylmethylsulphonyl fluoride (PMSF), and the cell lysate was centrifuged for 30 min at 21,130 g. Cell lysate supernatant was applied on an equilibrated 1 ml Ni-NTA resin (Novagen) column. Following loading of the lysate, the column was washed to remove unbound proteins using 50 ml of buffer (20 mM Tris pH 7.5 300 mM NaCl, 2 mM β-ME, 20 mM imidazole). Protein elution was performed using increasing concentrations of imidazole, ranging from 20 to 250 mM, and collected as 1 ml fractions. Eluted fractions were dialysed for 2 h in buffer containing 150 mM NaCl, 50 mM HEPES pH 7.5 and 1 mM DTT.

Purification of wild-type ATG16L1 from insect cells was performed by the MRC Protein Phosphorylation and Ubiquitylation Unit Reagents and Services, Dundee—UK. Of note, this protein was not used in the liposome co-sedimentation assay as it was prone to pelleting in the absence of liposomes at high centrifugation speeds.

**Microscopy**

For fluorescence analyses, cells were grown on glass coverslips in a 6-well plate. 24 h later, cells were either left untreated or treated as indicated. Coverslips were then fixed and permeabilised by incubating with 0.1% Triton X-100 in PBS for 5 min or cells were fixed and permeabilised by incubating with −20°C methanol on ice for 5 min. Slides were then incubated in primary antibodies in blocking buffer (PBS supplemented with 1% BSA) at 37°C for 2–3 h followed by incubation with Alexa Fluor secondary antibodies (Invitrogen) for 30 min at room temperature. DAPI (Sigma, D9542) was then used to stain nucleic acids. Following extensive washes, coverslips were mounted on microscope slides and images were acquired using a Leica SP5 microscope.

**Liposome preparation and co-sedimentation assay**

All lipids were purchased from Avanti unless otherwise indicated. Small unilamellar vesicles (SUVs) were prepared by drying chloroform-dissolved lipids under nitrogen air followed by desiccation under vacuum for 0.5 h. A buffer containing 150 mM NaCl, 50 mM HEPES pH 7.5 and 1 mM DTT was added to rehydrate the dried lipid layer and produce a final lipid concentration of 1 mg/ml. Following 30-min incubation, liposomes were either sonicated in a water bath for 5–10 min or extruded using 100-nm filters and Avanti mini extruder.

For liposome co-sedimentation assays, 50 ng of His-T7-tagged recombinant ATG16L1 was mixed with 30 μl of 1 mg/ml liposomes (35% DOPC, 35% DOPS, 25% DOPE, 5% PI3P) along with BSA to a final concentration 0.5 mg/ml and incubated for 30 min at room temperature. Samples were then transferred to Thickman Polypolypropylene tubes (Beckman Coulter, 347287) containing liposome binding buffer to yield a final volume of 500 μl and ultracentrifuged for 180,000 g and 22°C (Optima Max Beckman Coulter Ultracentrifuge, TLA 120.2 rotor). Equal proportions of the pellet and supernatant were analysed by SDS–PAGE and Western blotting using anti-T7-HRP antibodies.

**Microscopy-based protein–liposome interaction assay**

For Fig 3B, Hap1 cells were grown in suspension at 37°C in a 3 l Wheaton spinner flask for 5 days, harvested by centrifugation at 1,300 g for 15 min at 4°C and washed three times with PBS. Pellets were flash-frozen in liquid nitrogen, resuspended in ice-cold liposome binding buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1 mM DTT supplemented with complete protease inhibitors EDTA-free cocktail, Roche Diagnostics) and cleared by centrifugation with 13,000 g at 4°C for 15 min. For purification of ATG16L1, 50 μl of StreptTaxis Sepharose High performance beads (GE Healthcare) was added to 4 mg total protein in the supernatant and incubated for 2 h at 4°C. Beads were washed four times with liposome binding buffer. For microscopy-based protein–liposome interaction assay (Fracchiolla et al., 2016), 1 μl of StreptTaxis beads covered with ATG16L1 was incubated with 15 μl of liposomes (1 mg/ml) prepared by extrusion (100 nm) and contained the following composition 39.5% DOPC, 35% DOPS, 20% DOPE, 5% phosphoinositides (as indicated in the figure) and 0.5% rhodamine-phosphoethanolamine (L1392, Thermo Scientific). The binding reaction was incubated for 15 min at room temperature.

For Figs 3E and EV1A, mouse ATG16L1-GFP and mutants were expressed by transient transfection in 293T cells. 24–48 h later, cells were lysed in cell lysis buffer (as above) and subjected to pull down using GFP-Trap beads (Chromotek) for 2 h at 4°C. Beads were then washed three times in lysis buffer followed by three washes in liposome binding buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1 mM DTT). For Fig EV3B, 15 ng of T7-His-tagged ATG16L1$_{72-307}^{WT}$ was incubated with Ni-NTA resin for 1 h at 4°C. Beads were then washed 3 times in liposome binding buffer. Beads were then mixed with 1 μl of 1 mg/ml extruded liposomes containing 35% DOPC, 35% DOPS, 23% DOPE, 5% PI3P and 2% rhodamine-DOPE as well as 0.25 mg/ml BSA in a final volume of 40 μl and incubated at room temperature for 10 min.

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All samples were imaged immediately by loading onto a 96 glass bottom plate and using a confocal microscope equipped with a 20× objective. Relative liposome binding to beads was quantified in ImageJ by taking maximum brightness along a straight line drawn on the beads and subtracting the values to adjacent empty regions of the image (background fluorescence). Liposome binding of ATG16L1 LD in Fig 3E was normalised to the GFP signal of ATG16L1 WT.

Lipid beads binding assay

The following lipid beads were purchased from Echelon Biosciences: PI3P beads (P-B003a), PI(3,4)P2 beads (P-B034a), PI (3,4,5)P3 beads (P-B345a), phosphatidylserine beads (PS, P-B0PS), phosphatidic acid beads (PA, P-B0PA), phosphatidylethanolamine beads (PE, P-B0PE) and PIP control beads (P-B000). For pull-down assays, 20 ng of recombinant wild-type ATG16L1 purified from insect cells was incubated with 5 μl beads and 0.1 mg/ml BSA diluted in liposome binding buffer supplemented with 0.5% Igepal. After 1-h incubation at 4°C with rotation, beads were washed three times in Igepal-supplemented liposome binding buffer and bound protein was eluted in SDS loading buffer and analysed by Western blotting.

Structural modelling

The i-TASSER webserver (Zhang, 2008) was used to thread the sequence of mouse ATG16L1 onto the structure of the Saccharomyces cerevisiae Atg16 (PDB code 3AT7). This produced a helical monomer. PyMol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.) was used to create a coiled-coil dimer by aligning to the yeast structure. A NAMD topology of the dimer was generated using the psfgen plugin of VMD 1.9.3 (Humphrey et al., 1996). To generate the lipid bilayer model, the Membrane plugin of VMD was used to build a rectangular matrix of PI3P embedded in phosphatidylcholine onto which the dimer was orientated. The VMD script combine.tcl was used to merge the protein and membrane models and remove sterically clashing lipids. It was then solvated and neutralised by the addition of TIP3P water molecules (Jorgensen et al., 1983) and Na+ and Cl− ions (to a concentration of 150 mM) to form a 125.1 Å × 123.7 Å × 87.4 Å simulation box. The full system comprised 123,533 atoms. NAMD 2.12 (Phillips et al., 2005) was used for simulating this system, utilising the CHARMM36 force field (Huang et al., 2017) in a Langevin temperature and pressure controlled (NPT @ 310K) ensemble with periodic boundary conditions and particle-mesh Ewald electrostatics. Following 10,000 energy minimisation steps (2 fs/step) to remove van der Waals clashes within the system, a production run of 10 ns was performed. The Cα RMSD of residues 164–198 between starting structure and final frame of the simulation was 2.05 Å, indicating that protein association with lipid was stable over this timeframe.

Ferroptosis assay

Induction of ferroptotic cell death was performed as previously described (Gao et al., 2015). Briefly, ATG16L1ΔC cells stably expressing F-S-ATG16L1 constructs were seeded in a 12-well dish for 24 h followed by treatment in DMEM lacking amino acids and supplemented with 10% FBS (to induce ferroptosis) or 10% dialysed FBS (dFBS) as a control. 24 h later, cells were stained with propidium iodide (PI) and imaged using a Leica DM IL LED microscope.

Graphs and statistical analyses

Graph-making and statistical analyses were performed on Prism 7 (GraphPad). All quantifications were performed on a minimum of three independent experiments. Statistical significance was measured by performing unpaired Student’s t-tests on no more than two data sets per analysis. Quantifications of immunofluorescence dot numbers and intensities were conducted in ImageJ. For the latter, a suitable threshold was set in ImageJ and the “Analyze particles” feature was used to record the mean grey values of dots. LC3-II/LC3-I ratio densitometry analysis was performed in ImageLab.

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Author contributions

LjD, AGC, ANM, TM and NG performed the experiments and analysed the data. JAM and DRH performed structural modelling and analyses. MS and SM optimised, performed and analysed the microscopy-based protein–liposome interaction assay. XJ provided crucial input. LjD and NG wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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