Cargo binding to Atg19 unmasks additional Atg8 binding sites to mediate membrane–cargo apposition during selective autophagy

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Autophagy protects cells from harmful substances such as protein aggregates, damaged mitochondria and intracellular pathogens, and has been implicated in a variety of diseases. Selectivity of autophagic processes is mediated by cargo receptors that link cargo to Atg8 family proteins on the developing autophagosomal membrane. To avoid collateral degradation during constitutive autophagic pathways, the autophagic machinery must not only select cargo but also exclude non-cargo material. Here we show that cargo directly activates the cargo receptor Atg19 by exposing multiple Atg8 binding sites. Furthermore, Atg19 mediates tight apposition of the cargo and Atg8-coated membranes in a fully reconstituted system. These properties are essential for the function of Atg19 during selective autophagy in vivo. Our results suggest that cargo receptors contribute to tight membrane bending of the isolation membrane around the cargo.

The yeast cargo receptor Atg19 is a distant relative of the mammalian p62/SQSTM1 and NBR1 (neighbour of BRCA1 gene 1) cargo receptors16. Atg19 mediates the delivery of the precursor aminopeptidase I (prApe1) protease and other substances into the vacuole in a constitutive process called the cytoplasm-to-vacuole targeting (Cvt) pathway17–21. The Cvt pathway is mechanistically equivalent to selective autophagy in mammals3,5,13–16.

As the Cvt pathway is constitutive, it is important that other cellular material is not constantly lost into the vacuole. Consequently, the membrane of the autophagosome-like Cvt vesicles is tightly wrapped around the prApe1 oligomers22. In contrast, when yeast cells starve they respond by the formation of autophagosomes that are larger than Cvt vesicles and contain random cytoplasmic material22. During this process the cargo receptor Atg19 and its relative Atg34 tether cargo to the isolation membrane23,24. They thereby confer some cargo selectivity to autophagosome formation but not exclusivity, because bulk cellular material is not excluded from sequestration within autophagosomes.

Here we find that cargo binding to the Atg19 receptor positively regulates its association with Atg8. Atg8 binding by Atg19 is not only mediated by its known LIR motif but by multiple cryptic Atg8 binding sites. Furthermore, in vitro reconstitution reveals that Atg19 is sufficient for the tight apposition of Atg8 positive membranes with...
Figure 1 Atg19 and Atg34 interaction with Atg8. (a-f) Atg8 was co-incubated with Atg19 or Atg34 (wild type and mutants) at concentrations of 40 μM and 800 μM, respectively, and run on a size exclusion column. Aliquots of individual fractions were run on SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gels and Coomassie stained. The white box indicates the N-terminal domain, the light grey box the coiled-coil domain, the dark grey box the Ams1-binding domain and the purple box the C-terminal domain.

The cargo. Our results suggest that certain cargo receptors contribute to bending of the isolation membrane around the cargo to exclude non-cargo material from sequestration into autophagosomes.

RESULTS

LIR-independent interaction of Atg19 with Atg8

We first studied the interaction between Atg19 and Atg8 in solution using size exclusion chromatography (SEC; Fig. 1). At high (800 μM) but not low (40 μM) concentrations, Atg8 and Atg19 co-eluted (Fig. 1a,b). Consistently, we determined the dissociation constant \( K_D \) to be about 35 μM by isothermal titration calorimetry (Supplementary Fig. 1). This value is about 10–20 times lower than the \( K_D \) for other cargo receptors interacting with Atg8 family proteins\(^\text{12} \). The interaction of cargo receptors with Atg8 family proteins has been shown to occur through so-called LIRs (W\(_{412}\)EEL\(_{415}\) in Atg19; refs 10,12–15). To test the requirement of the known LIR motif for the interaction of Atg19 with Atg8, we mutated Trp 412 to alanine or deleted the entire LIR motif\(^\text{13} \). Unexpectedly, although these mutations weakened the interaction, the Atg19 mutants still bound to Atg8 (Fig. 1c,d). This indicates that the known LIR motif contributes to Atg8 interaction but is not essential. In contrast, the interaction between the Atg34 cargo receptor and Atg8 was entirely dependent on the known LIR motif, because the equivalent mutant (W409A) showed no interaction with Atg8 (Fig. 1e,f).

To identify the Atg8-binding activity independent of the classical LIR motif in Atg19 we investigated the ability of Atg19 fragments containing the W412A mutation to interact with Atg8. Strikingly, the short carboxy-terminal fragment (365–415 W412A) showed a strong...
interaction with Atg8 at 40 μM (Fig. 1g), stronger than that of the wild-type full-length protein (Fig. 1b), and this was maintained when the fragment was further extended by 111 amino acids (254–415 W412A; Fig. 1h). However, when a further extension to include the coiled-coil domain was introduced (amino acids 124–415 W412A) the interaction became undetectable at low concentration (40 μM; Fig. 1i). These results suggest that the C-terminus of Atg19 (but not Atg34) interacts strongly with Atg8 independent of the known LIR motif, and further that the coiled-coil domain inhibits this interaction.

Cargo activates the Atg8–Atg19 interaction

The coiled-coil domain of Atg19 mediates the interaction with the prApe1 cargo. We therefore reasoned that the coiled-coil domain mediated inhibition of the interaction of the Atg19 C-terminus with Atg8 might be relieved on addition of the prApe1 cargo. This was indeed the case, as the presence of the Atg19-binding prApe1 propeptidet greatly stimulated the amount of Atg8 pulled down by Atg19 (Fig. 2a and Supplementary Figs 1). To test if the C-terminus of Atg19 that showed strong LIR-independent interaction with Atg8 is specifically required for the prApe1 cargo-stimulated interaction with Atg8, we constructed an Atg19–Atg34 chimaeric protein by exchanging the C-terminus of Atg19 with the C-terminus of Atg34 (Supplementary Fig. 1). Atg34 showed no interaction with Atg8 independent of the classical LIR motif (Fig. 1e,f). The Atg19–Atg34 chimera efficiently bound the prApe1 propeptide (Fig. 2b and Supplementary Fig. 1) and still interacted with the scaffold protein Atg11 (Fig. 2c; ref. 23) but the propeptide did not stimulate Atg8 binding (Fig. 2d).

Atg19 contains multiple Atg8 binding sites

To identify the Atg8 binding sites independent of the classical LIR motif within the C-terminus of Atg19, we fused a series of shortened versions of the C-terminal domain (residues 365–415) of Atg19 to glutathione S-transferase (GST) and tested its interaction with Atg8 in pulldown experiments (Fig. 3a and Supplementary Fig. 1). Consistent with our SEC results (Fig. 1c,d), the W412A mutation did not abolish the interaction with Atg8 (Fig. 3a and Supplementary Figs 1 and 2). Residues 398–415 containing the W412A mutation showed no interaction with Atg8, suggesting that the interaction of the extreme C-terminal region of Atg19 is dependent on the known LIR motif (Fig. 3a and Supplementary Fig. 1). Extension of the C-terminal domain (residues 386–415, 376–415 and 365–415, respectively) in the context of the W412A mutation enhanced the interaction with Atg8 (Fig. 3a).

In a complementary approach we tested a series of C-terminally truncated fragments of GST–Atg19 for their ability to pull down Atg8 (Fig. 3b and Supplementary Fig. 1). Deletion of residues 375–415 (amino acids, aa1–374) markedly decreased but did not fully abolish its interaction with Atg8 (Fig. 3b). Residues 1–384 showed slightly increased binding to Atg8 when compared with GST–Atg19 aa1–374, whereas GST–Atg19 aa1–396 showed a further enhanced interaction (Fig. 3b). Thus the C-terminus of Atg19 contains multiple Atg8 binding sites: one site localizes to the known LIR motif, one site is close to the Atg11 binding site and at least one site localizes amino-terminally of the Atg11 binding site.

To identify these Atg8 binding sites we aligned the sequence of the C-terminal domain of Atg19 lacking the known C-terminal LIR motif with the LIR-containing regions of other cargo adaptors. Phenylalanine residues Phe 376 and Phe 379 aligned with the two hydrophobic residues of the LIR motif of the human cargo receptor p62 (Tyr 338 and Leu 341; refs 12,15; Fig. 3c). To test if Phe 376 and Phe 379 contribute to Atg8 binding we mutated these residues to alanine. Indeed, the F376A, F379A double mutant showed slightly reduced binding to Atg8 (Fig. 3d and Supplementary Fig. 2). When F376A was co-introduced with W412A the resulting double mutant showed severely reduced binding (Supplementary Fig. 2), as did the F376A, F379A, W412A triple mutant (Fig. 3d and Supplementary Fig. 2). We conclude that one further Atg8 binding site localizes to the region around Phe 376 and Phe 379.

The alignment of Atg19 with p62 also revealed homology around the Atg11-binding site (Fig. 3c). In particular, Pro 385 and Glu 386 are conserved. Interestingly, Pro 385 and Glu 386 are absent from Atg34. As our pulldown experiments suggested that one Atg8 binding site localizes around the Atg11-binding site of Atg19 (Fig. 3a,b), we mutated Pro 385 and Glu 386 to alanine. When P385A, E386A was combined with W412A the resulting triple mutant showed reduced Atg8 binding when compared with the W412A mutant C-terminus (Fig. 3d). Further introduction of F376A, F379A to generate a F376A, F379A, P385A, E386A, W412A mutant resulted in complete loss of Atg8 binding under the tested conditions (Fig. 3d).
Figure 3 The C-terminus of Atg19 contains multiple Atg8 binding sites. (a,b) Anti-Atg8 western blots using the indicated GST fusion proteins shown on the left as bait to pull down Atg8. See Supplementary Fig. 1 for input gels. (c) Alignment of the Atg19 C-terminus lacking the LIR motif with the region around the LIR motif of human p62 (NP_003891.1). The amino acids shown in magenta and green indicate the residues mutated in the experiments shown in d-f. (d) Anti-Atg8 western blots with the indicated GST fusion proteins as bait to pull down Atg8. Quantification of the amount of Atg8 pulled down by the indicated GST fusion protein. The graph is based on three independent experiments (N = 3), one of which is shown below the graph. Shown are the averages and s.d. WT, wild type. (e) Green fluorescent protein (GFP)-trap pulldown experiment using yeast cells with GFP–Atg8 transformed with the indicated Myc–Atg19 constructs.

We corroborated these findings first by carrying out in vivo pulldown experiments (Fig. 3e). These results show that the previously reported LIR motif around Trp 412 is indeed not the only Atg8 site in vivo, whereas the F376A, F379A, W412A triple mutant lost detectable Atg8-binding activity, at least under these conditions.

Using an in vitro experimental set-up that resembles the situation in vivo (Fig. 3f and Supplementary Fig. 2), we found that the W412A mutation and the F376A, F379A double mutation each reduced binding to some extent, but that simultaneous mutation of all three residues severely decreased Atg8 interaction. Additional mutation of P385A, E386A further weakened the interaction with Atg8 (Fig. 3f).

To test if one Atg19 C-terminus molecule can indeed simultaneously interact with multiple Atg8 molecules, we incubated the Atg19 C-terminus with a 10-fold excess of Atg8 and ran the mixture
Figure 4 Reconstitution of Atg8-dependent Atg19 recruitment to the membrane. (a) Giant unilamellar vesicles (GUVs) incubated with mCherry–Atg19. The membrane was marked by incorporation of an Oregon Green labelled lipid. (b) GUV coated with GFP–Atg8 incubated with mCherry–Atg19. (c) Schematic representation showing the proteins and reaction resulting in GFP–Atg8 conjugation to the GUV shown in b. (d) GUVs containing nickel lipids incubated with GFP–Atg8–6×His and wild-type mCherry–Atg19. (e) Set-up of the experiment shown in d. (f) Quantification of membrane binding by the indicated mCherry–Atg19 proteins. The experimental set-up is shown in e. The graph is based on three experiments. Shown are the averages and s.d. of these three experiments (N=3, on the basis of 318 GUVs for Atg19, 171 GUVs for Atg19 W412A, 166 GUVs for Atg19 F376A, F379A, W412A, 188 GUVs for the enhanced GFP (eGFP) control). Scale bars, 5 μm.

Abbreviations: 3, Atg3; 5, Atg5; 7, Atg7; 8, Atg8; 12, Atg12; 16, Atg16; 19, Atg19 redundant. Experiments shown in a, b have been conducted twice and the experiment in d three times.

Reconstitution of Atg8-mediated recruitment of Atg19 to the membrane

In the cell, the concentrations of Atg19 and Atg8 are in the nanomolar range and thus very low\cite{26,27}. However, the Atg8–Atg19 interactions occur at a membrane where the local concentrations are increased. We therefore analysed the effect of Atg8 membrane localization on Atg19 recruitment in a reconstituted system using giant unilamellar vesicles (GUVs; Fig. 4). In the absence of Atg8 on the GUV membrane, no Atg19 recruitment was detectable (Fig. 4a). When we reconstituted Atg8 conjugation using recombinant Atg3, Atg7 and the Atg12–Atg5–Atg16 complex\cite{28}, we detected efficient recruitment of Atg19 to the GUV membrane (Fig. 4b,c). To more accurately control the amount of Atg8 on the membrane we added a C-terminal 6×His-tagged version of Atg8 to GUVs containing nickel lipids. Under these conditions Atg8 detectably recruited Atg19 and Atg34 at low nanomolar concentrations (Supplementary Fig. 3). Thus, the local concentration of Atg8 on the membrane was sufficient for recruitment of Atg19 and Atg34 to the membrane at concentrations that are close to the concentrations found in vivo\cite{26,27}. Consistent with these findings, we observed a markedly increased interaction in pulldown assays when we used GST–Atg8 concentrated on beads to pull down Atg19 (Supplementary Fig. 4; compared with the reverse situation shown in Fig. 3b and Supplementary Figs 1 and 2).

Correlating with reduced Atg8 interaction of the Atg19 W412A and F376A, F379A, W412A mutants in the pulldown assays (Fig. 3 and Supplementary Fig. 2), the recruitment of these
mutant Atg19 proteins to Atg8-harbouring GUVs was markedly reduced (Fig. 4d–f).

Reconstitution of membrane bending during selective autophagy

Next we asked if Atg19 could recruit cargo to Atg8-coated membranes. To this end we added the propeptide of the prApe1 cargo to Atg8-coated GUVs (Fig. 5a). It was indeed the case, because on addition of Atg19 did we detect recruitment of the Ape1 propeptide to the GUV membrane (Fig. 5a). To mimic the in vivo situation where the prApe1 propeptide is concentrated on the surface of the prApe1 oligomer we coated 2 μm diameter polystyrene beads with the prApe1 propeptide (Fig. 5b–e and Supplementary Fig. 3). In the absence of Atg19 only a few beads were found peripherally attached to the Atg8-coated GUV membrane. In contrast, in the presence of Atg19 the beads attached much more frequently and we also noticed that Atg19 bound directly to the prApe1-coated beads (Fig. 5e and Supplementary Fig. 3). Strikingly, in the presence of Atg19 the GUV membrane was closely wrapped around parts of the prApe1 propeptide beads (Fig. 5b–e and Supplementary Fig. 3). Frequently, the beads were almost completely invaginated into the lumen of the GUVs (Fig. 5b).

Atg19-mediated membrane bending positively correlated with the density of Atg8 on the membrane (Fig. 5c). Furthermore, the ability of the Atg19 mutants to bend the membrane around the beads correlated well with their Atg8-binding activity (Figs 5d and 3f). In the absence of Atg19 few membrane bending events were observed (Fig. 5d) and these were also far less pronounced. Thus the tripartite interaction between prApe1, Atg19 and Atg8 is sufficient for close membrane–cargo apposition.
Figure 6 Requirements for the Atg19–Atg8 interaction for cargo delivery during selective and bulk autophagy. (a) Anti-Ape1 western blot of atg19Δ cells transformed with the indicated expression constructs. The lower Ape1 band indicates prApe1 processing and thus its delivery into the vacuole. Rap., rapamycin; Log.,Log. Cvt vesicle Rap. autophagosome Memebrane, cargo, Atg8, Atg19 ΔPRap. anti-Ape1 anti-Ape1 VectorMyc–Atg19 Myc–Atg19–34 VectorMyc–Atg19–34 anti-Myc. (b) Representative electron micrographs of ypl7Δ, atg19Δ yeast cells grown under cytoplasm-to-vacuole targeting (Cvt) conditions expressing the indicated proteins labelled with an anti-Myc antibody. The white arrowheads indicate the isolation membrane. The dashed line indicates the circumference of the prApe1 oligomer. Gold particles, 10 nm. Scale bars, 200 nm. See Supplementary Fig. 5 for full images. (c) Quantification of electron micrographs of yeast cells expressing either Myc–Atg19 or Myc–Atg19–Atg34 co-labelled with anti-Myc (10 nm gold) and anti-prApe1 (5 nm gold). See also Supplementary Fig. 5. Three independent experiments; Myc–Atg19, N = 48, Myc–Atg19–34, N = 24. (d) Anti-Ape1 western blot of atg19Δ cells transformed with the indicated expression constructs. The lower Ape1 band indicates prApe1 processing and thus its delivery into the vacuole. Supplementary Fig. 6 shows the expression of the Myc-tagged proteins and a quantification of the assay. (e) Y2H assay testing for the interaction of Atg11 with Atg19 and the indicated Atg19 mutants. (f) Atg19Δ yeast cells expressing the indicated Atg19 proteins and prApe1–RFP (red fluorescent protein) were labelled with the vacuolar membrane dye MDY-64. Scale bars: 2 μM. The experiments in a–d, f have been conducted three times, the experiment in e twice. Images of uncropped western blots and gels can be found in Supplementary Fig. 7.

Multiple Atg8 interaction sites are required for Atg19-dependent selective autophagy

To test the requirements for the function of Atg19 during selective autophagy in vivo, we monitored prApe1 processing under nutrient rich conditions where only the selective Cvt pathway is active, and in cells treated with rapamycin, where bulk autophagy mediates transport of prApe1 into the vacuole (Fig. 6a; ref. 22). Intriguingly, the Atg19–Atg34 chimaeric protein was unable to support the Cvt pathway (Fig. 6a). Consistent with the ability of the Atg19–Atg34 chimaera to bind the prApe1 propeptide (Fig. 2b), immunoelectron microscopy showed that the protein localized to the prApe1 oligomers (Fig. 6b and Supplementary Fig. 5). However, in contrast to Atg19 the Atg19–Atg34 chimaera did not support the growth of an isolation membrane around the prApe1 particles (Fig. 6b,c). On rapamycin treatment, Atg19–Atg34 was almost fully functional (Fig. 6a), suggesting that multiple Atg8 binding sites are required for transport of the prApe1 cargo during selective (Cvt) but not bulk autophagy. This rapamycin effect was totally dependent on the presence of Atg17,
a scaffold protein essential for autophagosome formation but not the Cvt pathway29 (Supplementary Fig. 6).

Next we addressed the relevance of the extra regions in Atg19 that contribute to Atg8 binding (Fig. 3). Consistent with the biological relevance of these sites, the W412A mutation in the LIR motif of Atg19 was not essential for prApe1 processing, during either Cvt or autophagy (Fig. 6d and Supplementary Fig. 6). Next, we tested the effect of the F376A and F379A mutations on prApe1 processing. In isolation, the two mutations had weak if any effects on prApe1 processing during the Cvt pathway (Fig. 6d and Supplementary Fig. 6). However, when either of the two single mutations was combined with the W412A mutation, the Cvt pathway was non-functional whereas prApe1 processing was still apparent under rapamycin-induced autophagic conditions (Fig. 6d and Supplementary Fig. 6). We observed an almost identical effect for the F376A, F379A double mutant (Fig. 6d and Supplementary Fig. 6), but when the F376A, F379A double mutant was combined with the W412A mutation prApe1 processing was lost under both Cvt and autophagic conditions (Fig. 6d and Supplementary Fig. 6).

An identical phenomenon was observed for Pro 385 and Glu 386 (Fig. 6d and Supplementary Fig. 6). Introduction of P385A, E386A only slightly decreased prApe1 processing under rich conditions. When combined with W412A or F376A, F379A the P385A, E386A mutation markedly reduced prApe1 processing during the selective Cvt pathway but much less under autophagic conditions (Fig. 6d and Supplementary Fig. 6). None of the mutations affected Atg11 binding (Fig. 6e) or the localization of the prApe1 cargo to the vacuole (Fig. 6f).

**DISCUSSION**

Here we provide a number of fundamental insights into the action of cargo receptors during selective autophagy.

In the absence of cargo, Atg19 binds only weakly to Atg8. Binding of the prApe1 cargo to Atg19 enhances interaction of Atg19 with Atg8. Thus the cargo has an active role during its sequestration into autophagosomes. This is consistent with the requirement of the prApe1 cargo for assembly of the pre-autophagosomal structure for selective autophagy in nutrient rich conditions in yeast30. We thus provide a molecular mechanism for the requirement of the cargo and the Atg19 receptor for pre-autophagosomal structure formation in nutrient rich conditions23,30. The inhibition of cargo receptors in the absence of cargo may be a general feature, as it prevents cargo receptors from associating with the growing isolation membrane in the absence of their respective cargo.

We found that Atg19 contains multiple Atg8 binding sites. One of these sites localizes to two phenylalanine residues (Phe 376 and 379) that align with the LIR motif of human p62. Given the hydrophobic nature and the spacing between these two residues, it is likely that they bind to Atg8 in a manner that is similar to the binding of other LIR motifs to Atg8 family proteins13–15,31. In fact, it is becoming increasingly clear that LIR motifs can deviate from the canonical W/F/YxxL/I/V sequence31–34. In addition, we found that mutation of Pro 385 and Glu 386 to alanine negatively affects Atg8 binding by Atg19, in particular in the context of other Atg8-binding mutations. This site shows no resemblance to previously identified LIR motifs. Interestingly, Pro 385 and Glu 386 are also found in p62 (Fig. 3c). Future studies will have to elucidate how Pro 385 and Glu 386 contribute to Atg8 binding. Atg19 may contain another Atg8 binding site N-terminal of Phe 376 (Fig. 3b). In combination, the multiple Atg8 binding sites result in high avidity interactions with Atg8 family proteins. This in turn favours the interaction with Atg8 family proteins when they are locally concentrated on the isolation membrane.

In the case of Atg19, multiple sites per se are not required for the interaction of Atg19 with Atg8, as the C-terminus of Atg34 that lacks detectable Atg8 binding sites apart from the known C-terminal LIR motif is able to functionally replace the C-terminus of Atg19 during bulk autophagy. Instead, the multiple Atg8 interaction sites in the C-terminus of Atg19 may provide sufficient energy to tightly wrap the membrane around the cargo and therefore enable the exclusion of non-cargo material during selective autophagy (Fig. 7). Consistently, Atg19 was sufficient for tight membrane–cargo apposition in a fully reconstituted system, and this activity correlated with the number of Atg8–Atg19 interactions (Fig. 5).

It has recently been proposed that membrane-localized Atg8 assemblies together with the Atg12–Atg5–Atg16 complex into a membrane scaffold on the isolation membrane, and that the cargo receptor Atg32 disrupts this scaffold owing to its LIR-dependent interaction with Atg8 (ref. 35). Thus tight, LIR-dependent interactions of cargo receptors with Atg8 family proteins on the membrane may more efficiently disrupt this putative scaffold.

The Atg19–Atg34 chimaera was rather effective in the in vitro reconstitution assay (Fig. 5). This may be due the fact that this assay is less stringent than the in vivo situation, where the Cvt particles are around 150 nm in diameter as opposed to 2 μm for the cargo mimetic beads in the reconstituted assay.

Multiple Atg8 binding sites in Atg19 were specifically needed for the selective Cvt pathway but not for Atg19-dependent delivery of prApe1 to the vacuole during bulk autophagy. Consistently, for Cvt vesicles the membrane is tightly apposing the prApe1 particle22. During bulk autophagy, where the prApe1 particle is tethered to the membrane of the larger autophagosome22, the faster growth of the isolation membrane may outpace the membrane bending activity of Atg19. Alternatively, during selective autophagy the isolation membrane may be formed directly around the cargo, whereas during bulk autophagy the cargo may be tethered to a pre-existing isolation membrane, not enabling the receptor to mediate tight membrane–
cargo apposition. Furthermore, a lower density of Atg8 on the membrane during bulk autophagy or posttranslational modification of the Atg19 C-terminus may reduce the membrane binding capacity of Atg19.

Interestingly, the mammalian cargo receptor NBR1 also contains two LIR motifs\(^9\). Furthermore, p62 may utilize oligomerization to increase the density of Atg8 interaction sites to mediate tight apposition of the isolation membrane and the cargo\(^10\). Thus, many autophagic cargo receptors may contribute to the bending of the isolation membrane around cellular cargo to ensure exclusivity of the pathway and thereby prevent loss of non-cargo material into the lysosomal system.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

J.S-M., C.A, J.R., B.Z., I.I. and S.M. carried out experiments, J.S-M., C.A and S.M. planned experiments and S.M. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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and Atg8 were premixed at 40°C. To analyse the complex formation at high concentrations, Atg19 or Atg34 variants with a buffer containing 25 mM HEPES at pH 7.5, 150 mM NaCl and 1 mM DTT.

To follow the Atg19-Atg8 interaction in solution at low concentrations, the constructs were transformed into Rosetta pLysS. The cultures were incubated grown at 37°C in terrific broth to an OD₆₀₀ of 0.6, induced with 0.1 mM isopropyl-β-D-1-thiogalactoside and incubated at 37°C for 4 h. Cells were then spun down, resuspended in a buffer containing 50 mM HEPES at pH 7.5, 300 mM NaCl, 10 mM imidazole, 2 mM MgCl₂, 2 mM β-mercaptoethanol, complete protease inhibitor (Roche) and DNase I (Sigma) and disrupted by freeze–thawing followed by short sonication. The cell lysate was centrifuged at 140,000 g (Beckman Ti45 rotor) for 40 min at 4°C and the cleared supernatant was incubated with glutathione beads (GE Healthcare) for 1 h at 4°C. The beads were washed. The protein was then either cleaved off from the GST tag by incubation with thiolase protease (SERVA) overnight at 4°C or eluted with 50 mM HEPES at pH 7.5, 300 mM NaCl, 1 mM dithiothreitol (DTT) and 20 mM reduced glutathione overnight at 4°C. The supernatant containing the protein was concentrated and applied to a Superdex 200 or Superdex 75 column (160/60 prep grade, GE Healthcare) and eluted with a buffer containing 25 mM HEPES at pH 7.5, 150 mM NaCl and 1 mM DTT. Fractions containing pure protein were pooled, concentrated and stored at −80°C.

The mCherry–Atg19 variants containing an N-terminal His tag followed by a tobacco etch virus (TEV) protease recognition site was expressed from the pETDuet-1–His–TEV–mCherry–Atg19 vector in the E. coli Rosetta pLysS strain. Cells were grown at 37°C in terrific broth to an OD₆₀₀ of 0.8, induced with 0.5 mM isopropylthiogalactoside and grown for a further 5 h at 25°C. Harvested cells were resuspended in a buffer containing 50 mM HEPES at pH 7.5, 300 mM NaCl, 10 mM imidazole, 2 mM MgCl₂, 2 mM β-mercaptoethanol, complete protease inhibitor (Roche) and DNase I (Sigma) and disrupted by freeze–thawing followed by brief sonication. After ultra centrifugation of the cell lysate (140,000 g) the supernatant containing the protein was either cleaved off from the GST tag by incubation with thiolase protease (SERVA) overnight at 4°C or eluted with 50 mM HEPES at pH 7.5, 300 mM NaCl, 1 mM dithiothreitol (DTT) and 20 mM reduced glutathione overnight at 4°C. The supernatant containing the protein was concentrated and applied to a Superdex 200 or Superdex 75 column (160/60 prep grade, GE Healthcare) and eluted with a buffer containing 25 mM HEPES at pH 7.5, 150 mM NaCl and 1 mM DTT. Fractions containing pure protein were pooled, concentrated and stored at −80°C.

The mCherry–Atg19 was diluted to reach a final imidazole concentration of 40 mM and incubated with nickel beads. The supernatant containing mCherry–Atg19 was concentrated and applied onto a Superdex 200 column (16/60, GE Healthcare) and eluted with a buffer containing 25 mM HEPES at pH 7.5, 150 mM NaCl and 1 mM DTT. Fractions containing pure mCherry–Atg9 fusion proteins were pooled, concentrated and stored at −80°C.

Atg8 lacking the C-terminal arginine was expressed as N-terminal His-tagged protein from pETDuet-1–His–TEV–Atg8 and purified as described in8.

Atg8–His and GFP–Atg8–His were purified by adding a 6× His tag to the Atg8 C-terminus by PCR followed by insertion into pETDuet-1. For protein expression, the constructs were transformed into Rosetta pLysS. The cultures were incubated in isopropyl-β-D-1-thiogalactoside-containing medium for 4 h to OD₆₀₀ of 0.8. Cells were induced with 0.5 mM isopropylthiogalactoside and incubated at 37°C for 4 h. Cells were then spun down, resuspended in 50 mM HEPES, 300 mM NaCl, 1 mM MgCl₂, 2 mM β-mercaptoethanol, 10 mM imidazole, complete protease inhibitors and DNase and frozen in liquid nitrogen.

For protein purification, pellets were thawed, sonicated and centrifuged at 140,000 g (Beckman Ti45 rotor) for 40 min. The protein was purified by HisTrap and gel filtration (16/60 75S column).

All proteins required for Atg8PE (phosphatidylethanolamine) conjugation (Atg3, Atg7, Atg10, Atg8, mEGFP–Atg8, Atg5–Atg12–Atg16) were expressed and frozen in liquid nitrogen.

To carry out the Atg8-PE conjugation the following lipid mix was used: 40% 1,2-dioleoyl-sn-glycero-3-phosphocholine, 35% 1,2-dioleoyl-sn-glycero-3-phosphoserine, 20% 1,2-dioleoyl-sn-glycero-3-phosphothanolamine, and 5% phosphatidylglycerol. To bind Atg8–His to Ni-NTA-containing beads, the following lipid mix was used: 85% 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-ylidylinositol and 15% 1,2-dioleoyl-sn-glycero-3-[N-(5-amino-1-carboxypentyl)-iminodiacetic acid]succinyl (nickel salt) (DGS–NiNTA). For the experiment shown in Fig. 5c, DGS–NiNTA lipids were titrated into 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. Electroformation was conducted at 35°C. When necessary to visualize the membrane, either 0.1% rhodamine–PE or 1% Oregon Green–PE was used. All lipids were purchased from Avanti Polar Lipids.

Coating microspheres with fluorescently labelled propeptide. To mimic the cargo in GUV assays cargo modified UltraClean high-activity polystyrene microspheres (Invitrogen) with a 2 μm diameter were used. Fluorescently labelled prApe1 propeptide was covalently coupled to the bead’s surface by use of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimine according to the manufacturer’s protocol. 50 μl of 100 μM mCherry–propeptide or mEGFP–propeptide in 50 mM MES at pH 6.0 were mixed with 50 μl of a 2% aqueous microsphere suspension and incubated at room temperature for 15 min. 0.8 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimine was added to the mix and incubated further for 2 h at room temperature on an orbital shaker. To quench the coupling reaction glycine was added to a final concentration of 100 mM and incubation continued for 30 min at room temperature. To remove uncoupled protein the microspheres were washed three times with PBS by gentle vortexing followed by a centrifugation step at 13,000 rpm for 10 min. After the final wash the microspheres were resuspended in 10 μl final buffer containing 1% BSA in 15 mM HEPES at pH 7.5 and 135 mM NaCl and stored in 4°C. Before each use the microspheres were gently sonicated in a sonication bath to disperse occurring agglomeration.

Membrane recruitment—GUV assays. Electroformed GUVs were diluted 1:2 or 1:4 in 15 mM HEPES at pH 7.5, 135 mM NaCl buffer and transferred to a 96-well glass-bottom microplate (Greiner Bio-One). To conjugate Atg8 to the PE-containing membrane, a conjugation reaction was carried out as described in ref. 28. To attach Atg8 to the Ni-NTA-containing membrane, recombinant Atg8–His or GFP–Atg8–His was added to a final concentration of 400 nM and incubated at room temperature for 20 min. To follow the Atg8 recruitment, mCherry–Atg8 was added to a final concentration of 400 nM and incubated at room temperature for a further 20 min. To examine the propeptide–Atg8 interaction and concomitant membrane recruitment, mEGFP–propeptide was incubated with mCherry–Atg8 in 1:1 ratio for 15 min and added to Atg8-coated GUVs to a final concentration of 400 nM. To observe membrane attachment of propeptide-coated microspheres, 4 μl of 10 diluted microsphere stock suspension was premixed with 4 μl of untagged Atg8 or mCherry–Atg8 in a volume of 8 μl, incubated for 15 min at 4°C, spun down, resuspended in the same volume of buffer and added to the well containing 32 μg Atg8-labelled GUVs.

For the mCherry–Atg8 and mCherry–Atg34 titration experiments, proteins were used at the following final concentrations: GFP–Atg8–His, 200 nM; mCherry–Atg8 and mCherry–Atg34, 25–1.5 nM; GFP–His, 200 nM. GFP–Atg8–His and His–GFP were briefly incubated in 15 μl with the GUVs before addition of mCherry–Atg8 or mCherry–Atg34. GUVs were prepared by the electroformation method as described in ref. 28, containing 95% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 5% DGS–NTA (mole fraction).
To compare the recruitment of Atg19 and different mutants to Atg8 on membranes the following concentrations of proteins were used: GFP–Atg8–His, 200 nM; GFP–His, 400 nM; mCherry–Atg19 versions, 100 nM. GUV preparation and incubation procedures were as described for the titration experiments.

All microscopic images were acquired using a confocal spinning disc microscope (Visiunot) and processed with ImageJ software.

GST pulldown binding assays. The purified GST-fused proteins (15 μM or 20 μM) and purified GST-free proteins (15 μM or 20 μM) as well as gluthathione–Sepharose 4B beads (GE Healthcare) were simultaneously incubated for 1 h at 4 °C. After washing the beads three times with 25 mM HEPES at pH 7.5, 150 mM NaCl and 1 mM DTT, the glutathione beads together with bound proteins were subjected to SDS–PAGE and then the protein bands were detected with Coomassie Brilliant Blue staining. To detect Atg8, immunoblotting was carried out with an anti-Atg8 antiserum used as a primary antibody.

Antibodies. The rabbit polyclonal anti-Ape1 antibody29 (diluted 1:20,000) and the rabbit polyclonal anti-Atg8 antibody30 (raised against GST–Atg8, diluted 1:1000) were a kind gift from C. Kraft (University of Vienna). The anti-Myc tag antibody (clone 4A6, diluted 1:1000) is available from Millipore (catalogue number 05-724). The anti-HA antibody (clone 12CA5, diluted 1:5000) is available from Abcam (catalogue number ab16918). Anti-Pgk1 (diluted 1:10000) was purchased from Invitrogen (catalogue number 49250). The mouse anti-GFP antibody (clones 7.1 and 13.1, diluted 1:5000) is available from Roche (catalogue number 11 814 460 001). For antibodies immunoelectron microscopy please refer to the corresponding paragraph (Cryo-sectioning and immunolabelling of yeast).

Statistical tests. For the statistical tests shown Fig. 5c,d a two-tailed, equal variance Student t-test was used. No data were excluded from the analysis.

Yeast strains. The yeast strains used here can be found in Supplementary Table 1. The genotype of the Saccharomyces cerevisiae S288C genetic background used here is his3Δ1 leu2Δ0 met15Δ0 ura3Δ0: knockout strains were used as diploids from EUROSCARF and haploid spores were selected.

Yeast expression constructs. Plasmids for yeast expression were produced as follows: the Atg19 promoter (800 base pairs upstream of the ATG start codon) was amplified by PCR and inserted into pRS316 using NotI and Xmal, then a fragment encompassing 6× Myc tags was amplified by PCR and inserted using Xmal and SalI. Finally, a fragment encompassing the open reading frame of Atg19 or the Atg19–Atg34 chimaera (below) as well as the terminator (300 base pairs downstream of the stop codon) was amplified by PCR and inserted using SalI and Xhol.

To generate the Atg19–Atg34 chimaeric expression construct, first a fragment encompassing the promoter as well as the 5′ region of the Atg19 open reading frame encoding for amino acids 1–361 was ligated with a fragment that codes for amino acids 348–412 as well as the Atg34 terminator using Xmal. The Xmal site was added by PCR. The resulting chimaeric fragment was cloned into pRS316 followed by deletion of the Xmal site.

Y2H: 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) assay. DNA constructs were cloned into the Y2H interaction plasmids plexA-N and pGAD-HA and transformed into the Y2H reporter yeast strain L40. Single colonies were grown overnight in 2 ml synthetic defined minimal medium (1.7 g l−1 yeast nitrogen base without amino acids and ammonium sulphate (ForMedium), 5 g l−1 ammonium sulphate, 20 g l−1 glucose, 25 g l−1 agar), supplemented with the appropriate amino acid drop-out mix (CSM; ForMedium) according to the lithium acetate, single-stranded DNA, polyethylene glycol transformation protocol. As controls, the empty vector pRS316 was transformed into SMy155 and pRS316–6×Myc–Atg19 into SMy59. All Atg19 variants were expressed under their endogenous promoter.

Transformed yeast strains were grown in pre-cultures in synthetic defined minimal medium (SD), supplemented with the appropriate amino acid drop-out mix (CSM; ForMedium) overnight and then used to inoculate YPD (yeast extract peptone dextrose) cultures for harvest.

Lysates were prepared from cultures in log phase by centrifugation of 150 OD600 un at 3000 g at room temperature for 10 min. The pellets were then washed once with PBS with 2% glucose and centrifuged again as described above and frozen in liquid nitrogen.

For lysis the pellets were thawed on ice and resuspended in lysis buffer (20 mM PIPES pH 6.8, 0.5% Triton, 50 mM KCl, 100 mM KCH3CO3, 10 mM MgSO4, 10 mM ZnSO4, 1 mM phenylmethyl sulphonyl fluoride, 1 mM NaF, 1 mM Na3VO4, 20 mM β-glycerophosphate, complete protease inhibitors).

Subsequently, the samples were subjected to bead beating and the lysate was separated from the beads and centrifuged at 16000 g at 4 °C until clear. The concentration of the lysates was measured and all samples were adjusted to an equal volume and a concentration of 20 μg ml−1.

For the co-immunoprecipitation, samples were incubated with GFP-Trap_A beads (ChromoTek) (and empty Sepharose 4B beads (Sigma)), rotating at 4 °C for 1 h. The samples were then centrifuged at 500 g at 4 °C for 4 min and left to settle on ice for another 4 min before the unbound fraction was taken off. The beads were washed five times in this manner with lysis buffer and finally resuspended in urea, SDS loading buffer. To detect Atg19 the GFP-Trap_A beads together with bound proteins were subjected to SDS–PAGE and then immunoblotting. Anti-GFP antibody and anti-Myc antibody were used as primary antibodies. Signals were detected using Super Signal West Pico (Pierce, TPP).

Cryo-sectioning and immunolabelling of yeast. Yeast strain atg19Δ arp7Δ was transformed with pRS316–6×Myc–Atg19 or pRS316–6×Myc–Atg19–34. Logarithmically growing cultures were treated and cryo-sectioned following the Tokuyasu method as previously described37. Sections on copper grids were stored at 4 °C. For immunolabelling, grids were placed on plates with solidified 2% gelatine. Plates were then warmed up to 38 °C for 30 min and transferred to droplets of 100 mM glycine (pH 7.4). Blocking was done in normal goat serum dissolved 1:30 in 0.1% BSA-C (Aurion), PBS (pH 7.4) for 40 min in a wet chamber. For labelling, 6×Myc-tagged Atg19 or Atg19–34 grids were incubated with anti-Myc diluted 1:200 in 0.1% BSA-C, PBS for 80 min in a wet chamber, washed in 0.1% BSA fraction V, PBS (pH 7.4) and transferred to goat anti-mouse IgG 10 nm gold (BBI Solutions) diluted 1:25 in 0.1% BSA-C, PBS for 1 h. Further washes were done with 0.1% BSA-FV, PBS, followed by PBS and H2O.

For double-labelling of 6×Myc tagged proteins and endogenous Ape1, blocked grids were incubated with purified anti-Ape1 1:100 in 0.1% BSA-C, PBS at 4 °C overnight and then transferred to anti-Myc 1:200 in 0.1% BSA-C, PBS for 60 min at room temperature. Grids were washed in 10% BSA-FV, PBS, transferred to goat anti-rabbit IgG 5 nm gold (BBI Solutions) diluted 1:25 in 0.1% BSA-C, PBS for 1 h. Further washes were done with 0.1% BSA-FV, PBS, followed by PBS and H2O.

For staining, grids were transferred to 2% neutral uranyl acetate for 5 min and then to 0.2% uranyl acetate in 2% methylcellulose for 5 min. The excess liquid was removed with a filter paper and grids were left to dry before being stored.

Electron microscopy. Images were acquired with an FEI Morgagni 268D operated at 80 kV and equipped with an 11 megapixel CCD (charge-coupled device) camera.

Localization of Ape1 oligomers in vivo. Yeast strain atg19A was co-transformed with pRS316–6×Myc–Ape1wt, respective variants or empty vector, and pRS313–Ape1–RFP. Logarithmically growing cultures were harvested by centrifugation and vacuoles were stained with MDY-64 (Invitrogen) according to the manufacturer's protocol. A wild-type strain transformed with pRS316 vector and pRS313–Ape1–RFP served as control for normal conditions.

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Supplementary Figure 1 Interaction of Atg19 with Atg8 is weak but stimulated by cargo and depends on multiple interaction sites. (a) Profile of an isothermal titration calorimetry experiment with Atg8 as ligand injected into the cell containing Atg19. The calculated $K_D$ is 35µM. (b) Anti-Atg8 western blot of a pull down experiment with the indicated proteins. (c) Alignment of the C-termini of *S. cerevisiae* Atg19 and Atg34. The position of the Atg11 binding sites and the LIR motifs is indicated. (d) Coomassie stained gel showing the amounts of the indicated proteins used for the pull down experiment shown in Fig. 2d. (e) Coomassie stained gel showing the amounts of the indicated proteins used for the pull down experiment shown in Fig. 3a. (f) Coomassie stained gel showing the amounts of the indicated proteins used for the pull down experiment shown in Fig. 3b. (g) Anti-Atg8 western blot (upper part) using the indicated GST fusion proteins shown on the left as bait to pull down Atg8. The lower part shows the amount of Atg8 in the inputs on a Coomassie stained gel. Abbreviations: In: input, B: bound. The experiments shown in (a, b, g) have been conducted 2 times.
Supplementary Figure 2 The C-terminus of Atg19 interacts in a cargo-dependent manner with Atg8 via multiple sites. (a, b) Anti-Atg8 western blots with the indicated GST-fusion proteins as bait to pull down Atg8 are shown on the left. On the right the Coomassie-stained gels of the respective inputs are shown. (c) Coomassie stained gel showing the amounts of the indicated proteins used for the pull down experiment shown in Fig. 3f. Abbreviations: In: input, B: bound. The experiments shown in (a, b) have been conducted two times.
Supplementary Figure 3 Recruitment of Atg19 and Atg34 to Atg8 coated GUV and reconstitution of membrane bending during selective autophagy. (a, b) GUVs containing 5% Nickel lipids were incubated with Atg8-His or GFP-His. Subsequently, different concentrations of mCherry-Atg19 or mCherry-Atg34 were added to the GUVs. For better visibility the mCherry signal is shown in pseudo-colour. Scale bars: 20µm. (c) Atg19 dependent envelopment of prApe1-coated beads with Atg8 positive membranes Scale bars: 5µM. The experimental setup for the experiment shown in (c) is shown in (d). Abbreviations: 3: Atg3; 5: Atg5; 7: Atg7; 8: Atg8; 12: Atg12; 16: Atg16; 19: Atg19. The experiments shown in (a, b) have been conducted two times; the experiment in (c) two times.
Supplementary Figure 4 Efficient pull down of Atg19 by GST-Atg8. (a) Coomassie stained gel of a pull down experiment using GST-Atg8 as bait to pull down the indicated Atg19 proteins. (b) Coomassie stained gel of a pull down experiment using GST-Atg8 as bait to pull down the indicated Atg19 proteins. The quantification above the gel is based on 3 independent experiments (N = 3), one of which is shown below the graph. Shown are the averages and standard deviations. The experiment shown in (a) has been conducted two times, the experiment in (b) three times.
Supplementary Figure 5 The Atg19-34 chimera localizes to prApe1 oligomers. (a, b) Representative electron micrographs of Ypt7Δ, Atg19Δ yeast cells grown under Cvt conditions expressing the indicated proteins labelled with an anti-Myc primary antibody and a secondary anti-mouse antibody coupled to 10nm gold particles. The boxes indicate the cropped regions shown in Fig. 6b. (c, d) Electron micrographs of Ypt7Δ, Atg19Δ yeast cells grown under Cvt conditions expressing the indicated proteins co-labelling with a mouse anti-Myc antibody and a rabbit anti-prApe1 antiserum followed by incubation with secondary anti-mouse antibody coupled to 10nm gold particles (black arrow) and anti-rabbit antibody coupled to 5nm gold particles (white arrow). Scale bars: 200nm. All experiments have conducted three times.
Supplementary Figure 6 Expression of Atg19 variants in yeast cells and function of Atg19 mutants during selective and bulk autophagy. (a) Anti-Myc and anti-Pgk1 western blots of the same lysates shown in Fig. 6d. (b) Quantification of the percent of processed prApe1 compared to total prApe1. The graphs are based on 3 independent experiments (N = 3), one of which is shown in Fig. 6d. Shown are averages and standard deviations. (c) Anti-Ape1, anti-Myc and anti-Pgk1 western blots of atg17Δ, atg19Δ cells transformed with the indicated expression constructs. The lower mApe1 band indicates prApe1 processing and thus its delivery into the vacuole. The experiment in (a) has been conducted 2 times, the experiment in (c) two times.
Supplementary Figure 7 Uncropped gels and western blots. The red boxes indicate the cropped regions.
**Supplementary Table 1** Yeast strains used in this study

| Yeast strains | Strain | ID   | Background | Genotype                      | Source       |
|---------------|--------|------|------------|-------------------------------|--------------|
| wild type     |        | yCK66| S288C      | MATa                          | Euroscarf    |
| *atg19Δ*      |        | yCK797| S288C      | MATa, *atg19Δ::kanMX6*        |              |
| L40           |        | yCK580| S288C      | Matα                          | OriGene      |
| *atg19Δ, ypt7Δ* |      | SMY80 | S288C      | MATα, *atg19Δ::kanMX6; ypt7Δ::natMX6* |              |
| *atg17Δ, ypt19Δ* |    | SMY107| S288C      | MATα, *atg17Δ::kanMX6; atg19Δ::His3* |              |
| *atg19Δ, atg34Δ* |    | SMY59 | S288C      | MATα, *atg19Δ::kanMX6; atg34Δ::natMX6* |              |
| *atg19Δ, atg34Δ, sfGFP-atg8* |   | SMY155| S288C      | MATα, *atg8Δ::sfGFP-Atg8 atg19Δ::kanMX6; atg34Δ::natNT2* |              |
## Supplementary Table 2 Mutagenesis primers used in this study

| Construct | Primer Forward | Primer Reverse | Primer ID | Primer ID (DMR) Forward | Primer ID (DMR) Reverse |
|-----------|----------------|---------------|-----------|-------------------------|-------------------------|
| Atg19 124-415 | CCCCCCCCGAATTCAGCTTATGTAGAGAAGACGC | CCCCCCCCCGTCGACCTAGAGTTCTTCCCAAGTCAG | 326 | 320 |
| Atg19 254-415 | CCCCCCCCGAATTCGTGGAACCCCCAAATGAGC | CCCCCCCCCGTCGACCTAGAGTTCTTCCCAAGTCAG | 327 | 321 |
| Atg19 365-415 | CCCCCCCCGAATTCTCTGCAGAAAGTTTACAGGC | CCCCCCCCCGTCGACCTAGAGTTCTTCCCAAGTCAG | 328 | 320 |
| Atg19 376-415 | W412A CCCCCCCGAATTCTTTTACTCCTTTCAAATCGATAC | CCCCCCCGTCGACCTAGAGTTCTTCCGAAGTCAGGGCTTTTTCATTGTCGTCACCGTCATAAGAGAGTGAGAATTCGGGGG | 612 | 609 |
| Atg19 1-407 | CCCCCCCCCGAATTCATGAACAACTCAAAGACTAACC | CCCCCCGTCGACCTATTCATTGTCGTCACCGTCATA | 319 | 466 |
| Atg19 1-396 | CCCCCCCCCGAATTCATGAACAACTCAAAGACTAACC | CCCCCCCCCGTCGACCTAGGATGTACTGATGATACTAGAGTC | 319 | 601 |
| Atg19 1-384 | CCCCCCCCCGAATTCATGAACAACTCAAAGACTAACC | CCCCCCCCCGTCGACCTATAACGTATCGATTTGAAAGGA | 319 | 600 |
| Atg19 1-374 | CCCCCCCCCGAATTCATGAACAACTCAAAGACTAACC | CCCCCCCCCGTCGACCTACTCTTGGGATGCCTGTAA | 319 | 599 |
| Atg19 W412A | GAAAAAGCCCTGACTGCGGAAGAACTCTAGGTC GACCTAGAGTTCTTCCGCAGTCAGGGCTTTTTC | 330 | 330 |
| Atg19 F376A | GCATCCCAAGAGCCAGCTTACTCCTTTCAAATCGATACGTTA | TAACGTATCGATTTGAGCGGAGTAAAATGGCTCT | 759 | 760 |
| Atg19 F379A | AGAGCCATTTTACTCCGCTCAAATCGATACGTTAT | TAACGTATCGATTTGAAAGG | 761 | 762 |
| Atg19 P385A | E386A CCTTTCAAATCGATACGTTAGCAGCACTGGATGACTCTAGTATC | GATACTAGAGTCATCCAGTGCTGCTAACGTATCGAT 786 | 787 |
| Atg19 P385A | E386A ATCGATACGTTACCAGCACTGGATGACTCTAGT | ACTAGAGTCATCCAGTGCTGGTAACGTATCGAT | 788 | 789 |

* this primer was a universal primer used to mutate W → A in case W412A wasn’t present in the primary cloning.