Atg4 proteolytic activity can be inhibited by Atg1 phosphorylation

Jana Sánchez-Wandelmer, Franziska Kriegenburg, Sabrina Rohringer, Martina Schuschnig, Rubén Gómez-Sánchez, Bettina Zens, Susana Abreu, Ralph Hardenberg, David Hollenstein, Jieqiong Gao, Christian Ungermann, Sascha Martens, Claudine Kraft & Fulvio Reggiori

The biogenesis of autophagosomes depends on the conjugation of Atg8-like proteins with phosphatidylethanolamine. Atg8 processing by the cysteine protease Atg4 is required for its covalent linkage to phosphatidylethanolamine, but it is also necessary for Atg8 deconjugation from this lipid to release it from membranes. How these two cleavage steps are coordinated is unknown. Here we show that phosphorylation by Atg1 inhibits Atg4 function, an event that appears to exclusively occur at the site of autophagosome biogenesis. These results are consistent with a model where the Atg8-phosphatidylethanolamine pool essential for autophagosome formation is protected at least in part by Atg4 phosphorylation by Atg1 while newly synthesized cytoplasmic Atg8 remains susceptible to constitutive Atg4 processing.

1 Department of Cell Biology, University of Groningen, University Medical Center Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands. 2 Department of Cell Biology, University Medical Center Utrecht, Heidelberglaan 100, 8564 CX Utrecht, The Netherlands. 3 Max F. Perutz Laboratories, University of Vienna, 1030 Vienna, Austria. 4 University of Osnabrück, Department of Biology/Chemistry, Biochemistry section, Barbarastrasse 13, 49076 Osnabrück, Germany. Sabrina Rohringer, Martina Schuschnig, Bettina Zens, David Hollenstein, Sascha Martens and Claudine Kraft contributed equally to this work. Correspondence and requests for materials should be addressed to F.R. (email: f.m.reggiori@umcg.nl)
Macroautophagy (hereafter autophagy) is highly conserved among eukaryotes, and it is crucial for the maintenance of cellular homeostasis in response to cellular and environmental stresses. This pathway is also essential for a multitude of physiological processes, such as cell differentiation and defense against pathogens, and it is associated with the pathophysiology of several diseases, including cancer and neurodegeneration. During autophagy, double-membrane vesicles called autophagosomes sequester cytoplasmic components and target them to lysosomes/vacuoles for degradation. The resulting metabolites are subsequently recycled back to the cytoplasm and reused for the synthesis of new macromolecules or as a source of energy. In yeast, the orchestrated action of the autophagy-related (Atg) proteins at the phagophore assembly site or pre-autophagosomal structure (PAS) mediates the formation, expansion and sealing of a cistern, known as the phagophore or isolation membrane, to create an autophagosome. The kinase activity of the Atg1 complex, composed by the Atg1 kinase, Atg13, Atg17, Atg29, and Atg31, is a key regulator of this process.

Previous studies suggest that sealed autophagosomes cannot fuse with lysosomes/vacuoles until the Atg proteins get dissociated from autophagosomal membranes, which partially depends on phosphatidylinositol-3-phosphate (PI3P) turnovers. The Ymr1 phosphatase is pivotal in PI3P clearance on autophagosomes, which otherwise accumulate in the cytoplasm in its absence. Although PI3P turnover could be sufficient for autophagosome maturation, it is not expected to be enough for autophagosome expansion and sealing of a cistern, known as the phagophore or isolation membrane, to create an autophagosome.

In this study, we describe a novel regulatory mechanism, in which the Atg1 kinase specifically inhibits the deconjugating activity of Atg4 at the PAS, possibly contributing to the protection of the Atg8–PE pool necessary for autophagosome biogenesis.

Results

Atg1 phosphorylates Atg4 and inhibits autophagy. In our search for regulators of Atg4 activity, we made the assumption that the Atg4 protein is a substrate of Atg1 kinase. The Atg1 kinase is a key player in the autophagy pathway, because it dynamically localizes to the PAS and has been shown to phosphorylate its substrates. Interestingly, the sequence analysis of Atg4 revealed that it contains at least seven putative Atg1 phosphorylation consensus sites. We therefore decided to test whether Atg4 is a substrate of Atg1. Purified Atg1 complexes containing either Atg1 or kinase-dead Atg1 (Atg1D211A) were incubated with [32P]-ATP and recombinant GST-Atg4. GST or the GST-tagged C-terminus of Atg19 (Atg19Cterm), a positive control for Atg1 phosphorylation. The wild-type (WT) Atg1 complex, but not the one containing Atg1D211A, phosphorylated Atg4 and Atg19Cterm but not GST alone, indicating that Atg4 could indeed be a substrate of Atg1 kinase (Fig. 1a and Supplementary Fig. 6a).

To identify the residues involved in autophagy regulation, we individually mutated the putative phospho-acceptor serines in the different Atg1 phosphorylation consensus sites to alanine or aspartate to generate non-phosphorylatable or phospho-mimicking forms of Atg4, respectively. The resulting Atg4 variants were expressed in an atg4Δ strain carrying Pho8Δ60 and autophagy progression was determined enzymatically. Only cells expressing Atg4S307D exhibited an autophagy block identical to the one in the atg4Δ mutant carrying an empty vector (Fig. 1b). Interestingly, the non-phosphorylatable version of the same serine, i.e., Atg4S307A, could not completely bypass the autophagy impairment in atg4Δ cells (Fig. 1b). These phenotypes were not due to an effect of those mutations on the protein structure because introduction of a cysteine at the same position, did not impact autophagy (Supplementary Fig. 1b). Protein mass spectrometry analysis of Atg4V297R,Q314K-GFP isolated from WT cells revealed that the peptide containing serine 307 (S307) is indeed phosphorylated in vivo (Supplementary Fig. 2a and Supplementary Table 2). Atg4V297R,Q314K is a version of Atg4 where two extra trypsin cleavage sites were introduced to obtain peptides containing the region of interest detectable by protein mass spectrometry (Supplementary Figs. 2b and 7b). Because of the very low abundance of the phospho-peptide of interest and the presence of 3 consecutive serines, however, we could not assign with certainty a phosphorylation to S307. To precisely determine the residue modified by Atg1, we performed an in vitro kinase phosphorylation assay. We confirmed that a peptide containing the Atg1 phosphorylation consensus region around serine 307 (WT) was phosphorylated by Atg1 like the positive control peptide, i.e., GDS (Fig. 1c and Supplementary Fig. 6b). As expected, the mutant peptides S307A and AAA were not phosphorylated (Fig. 1c and Supplementary Fig. 6b). We concluded that the S307 residue can be modified by Atg1.

To further assess the role of S307 in autophagy, we examined by electron microscopy the presence of autophagic bodies (AB) in cells lacking the major vacuolar protease Pep4. The atg4Δ pep4Δ strain expressing Atg4S307D exhibited the same severe decrease in the number of AB as the one carrying protease-dead Atg4 (Atg4PD) or an empty vector (atg4Δ pep4Δ) (Fig. 1d and Supplementary Fig. 3a). The very few AB observed in the vacuole lumen of this mutant have a strongly reduced size (Fig. 1e, d). In contrast, the strain expressing Atg4S307A displayed the same number of AB as the control, i.e. cells carrying Atg4 (Fig. 1e, d). Interestingly, the average diameter of the AB in this mutant was smaller than in the control (Fig. 1f), also explaining its slightly lower autophagy activity (Fig. 1b) and impaired aminopeptidase 1 (Ape1) maturation (Supplementary Fig. 3b).

The similarity in phenotype shared by Atg4S307D and Atg4PD prompted us to analyze the proteolytic activity of the Atg4S307D and Atg4S307A mutants. First, we assessed the post-translational C-terminal cleavage of Atg8 by Atg4 using the Atg8-GFP chimera. This analysis revealed that the Atg4S307D mutant is proteolytically inactive similarly to Atg4PD (Fig. 2a and Supplementary Fig. 7a). Atg4S307A, in contrast, behaved as WT Atg4 and normally cleaved Atg8-GFP (Fig. 2a and Supplementary Fig. 7a).

Expression of GFP-Atg8ΔAR in an atg4Δ background allows the conjugation of Atg8 to PE independently of Atg4, permitting to specifically analyze the deconjugating capacity of Atg4. As reported, PE-anchored GFP-Atg8 failed to be released from the surface of autophagosomes in atg4Δ cells carrying either an empty vector or Atg4PD, and thus the fluorescence signal was mainly detected on the vacuolar limiting membrane highlighted with the specific vacuolar membrane dye FM4–64 (Fig. 2b, c).
Fig. 1 Atg1 phosphorylation of Atg4 inhibits autophagy. a GST, GST-Atg19Cterm, and GST-Atg4 were expressed in E. coli, immobilized on beads and in vitro phosphorylated with soluble Atg1-TAP and Atg1D211A-TAP complexes. The phosphorylation of the substrates was analyzed by autoradiography while their amounts were assessed by Coomassie brilliant blue staining of SDS-PAGE gels. b The atg4Δ (SAY130) strain carrying an empty pRS416 vector (atg4Δ) or plasmids expressing different Atg4 variants (Atg4, Atg4S307A, Atg4S307D, Atg4S354A, or Atg4S354D) was grown in SMD or nitrogen starved SD-N for 3 h before measuring Pho8Δ60 activity in cell lysates. The symbol * indicates statistical significance (p < 0.01) with the cells carrying Atg4 was calculated with the paired two-tailed Student’s t-test and arbitrary units (a.u.).

c GST fusions of the indicated peptides (right panel) were expressed in E. coli and analyzed as in a. The amino acid in position 307 is in bold and mutated amino acids are in red.

d The atg4Δ pep4Δ strain transformed with integration plasmids expressing Atg4-GFP (SAY144), Atg4S307A-GFP (JSY164), or Atg4S354D-GFP (JSY165) was grown in YPD to an early log phase and then nitrogen starved in SD-N for 3 h before processing the samples for EM. Autophagic bodies AB are highlighted in the EM micrographs with asterisks. CW cell wall; ER endoplasmic reticulum; LD lipid droplet; M mitochondrion; N, nucleus; PM plasma membrane; V vacuole. Scale bar, 1 µm.

e Quantification of the average number of AB per vacuole section in the samples of d. Significant differences (p < 0.0001) between the various Atg4 mutants and the WT are indicated with the symbol * and were calculated with the paired two-tailed Student’s t-test.

f Determination of the average diameter of the AB in WT, Atg4S307A, and Atg4S354D samples of d (n = 50). Significant differences (p < 0.01) with the WT are indicated with the symbol * and were calculated with the paired two-tailed Student’s t-test.
Complementation of the atg4Δ mutant with WT Atg4 led to normal Atg8 recycling and delivery of autophagosomes into the vacuolar lumen (Fig. 2b, c). The same was observed in cells expressing Atg4S307A, indicating that GFP-Atg8–PE was normally processed upon autophagosome completion. In contrast, the GFP signal was mainly localized to the vacuolar rim in cells harboring Atg4S307D, revealing that this mutant is unable to recycle GFP-Atg8–PE, as observed in the Atg4PD mutant (Fig. 2b, c). The fluorescence microscopy observations about the distribution of the GFP signal were confirmed by western blot analysis, in which higher amounts of free GFP were observed in WT Atg4 and Atg4S307A strains, in which GFP signal was localized inside the vacuole, indicating normal autophagy flux (Supplementary Fig. 3b, c). To acquire more information about the dynamics of autophagosomes in presence of the different Atg4 variants, the same strains, which express GFP-Atg8–AR and thus allow to bypass the post-translational C-terminal cleavage defect of cells expressing Atg4S307D (Fig. 2a), were examined by time-lapse microscopy. As shown in Supplementary Fig. 4 and Supplementary Movie 1, the average life time of autophagosomes in the strain carrying WT Atg4 was slightly longer than the one reported, i.e., 11.73 ± 0.96 min vs. 5–8 min, probably because cells expressing the same GFP-Atg8ΔR construct generate bigger autophagosomes. The rate of formation and/or fusion of autophagosomes in cells expressing Atg4S307A was longer (20.01 ± 1.08 min) and these vesicles appeared to have more fusogenic properties. These alterations possibly lead to a reduction in the autophagic flux providing a possible explanation for its slightly lower autophagic activity (Fig. 1b) and impaired Ape1 maturation (Supplementary Fig. 3b) of the Atg4S307A strains. The autophagosome life time in the Atg4S307D mutant (8.59 ± 2.14 min) did not significantly differ from that in the WT (Supplementary Fig. 4 and Supplementary Movie 3). However, the strongly reduced size of these carriers (Fig. 1d, e) implies that the time of autophagosome formation in Atg4S307D-expressing cells is a fraction of the one in the WT strain resulting in a marked reduction of the fusion events.

To further demonstrate that phosphorylation of S307 modulates Atg4-mediated deconjugation of Atg8 from PE,
we examined the functionality of the different Atg4 mutants in vitro. Atg8–PE was completely deconjugated from small unilamellar vesicles (SUVs) 10 min after the addition of Atg4 (Fig. 2d). The deconjugation of Atg8 from PE by Atg4S307A was slightly delayed compared to WT Atg4. In agreement with this, a perivacuolar punctate BiFC signal became evident in an atg2Δ mutant in which Atg1 associates more pronouncedly to the PAS17. These BiFC puncta co-localized with mCherryV5-Atg8, supporting that Atg4 interacts with Atg1 at this specific location (Fig. 4a). Additional deletion of ATG13 in this background prevents recruitment of Atg1 to the PAS17 and led to the disappearance of BiFC signals confirming that the interaction between Atg1 and Atg4 specifically takes place on autophagosomal membranes (Fig. 4a).

**Discussion**

The recruitment of Atg proteins to the PAS and their dissociation from the surface of complete autophagosomes must be subjected to spatial and temporal regulation to avoid the formation of aberrant intermediate structures. It has been shown that Atg4 constitutively deconjugates Atg8 from PE on all membranes except the PAS, suggesting the existence of regulatory elements protecting Atg8–PE from cleavage by Atg4 at this site6. Here, we found that active Atg1 inhibits Atg4 action at the PAS. In contrast to the rest of the Atg proteins17, Atg4 does not localize at this PAS17. This hypothetical model is indirectly supported by the observation that the human homologue of Atg13, very likely in complex with Atg1/ULK1, is one of the first Atg proteins dissociating from autophagosomes when those separate from omegasomes, a step that possibly takes place at their sealing26.

Atg4 phosphorylation affects its interaction with Atg8. Atg1 phosphorylation at S307 could inhibit Atg4 functionality by either allosterically altering the catalytic site or by interfering with the substrate binding. We first tested the Atg4–Atg8 interaction using the yeast two-hybrid (Y2H) assay. As shown in Fig. 3a, cells exclusively expressing Atg8 did not grow on selective medium whereas those expressing both Atg4 and Atg8 displayed cell growth, confirming the interaction between these two proteins (Fig. 3a)24. Noteworthy, the interaction of Atg4S307A and Atg4PD with Atg8 was stronger than the one detected between Atg4 and Atg8. In contrast, cells carrying Atg4S307D and Atg8 did not grow on the selective medium implying that this Atg4 mutant is unable to interact with Atg8 (Fig. 3a). The Y2H assay, however, cannot distinguish between Atg4 interaction with Atg8 or Atg8–PE. To overcome this limitation and test the binding of Atg4 variants to Atg8–PE, we performed a pull-down experiment in cells expressing GFP-Atg8ΔR. In line with the Y2H results, Atg4PD and Atg4S307A interaction with Atg8–PE was considerably stronger than the one observed with WT Atg4 (Fig. 3b and Supplementary Fig. 8). Atg4S307D showed scarce binding to Atg8–PE indicating that this mutant lost almost completely its ability to interact with both Atg8 and Atg8–PE (Fig. 3b and Supplementary Fig. 8). Taken together, these data suggest that phosphorylation at S307 modulates the binding of Atg4 to Atg8.

**Atg1 and Atg4 interact on autophagosomal membranes.** Atg4 phosphorylation at S307 blocks Atg8 proteolytic processing and recycling. Hence, Atg4 must be spatially regulated to allow the initial cleavage of Atg8 essential for its conjugation to PE and also avoid a premature Atg8–PE deconjugation until autophagosome biogenesis is completed. With Atg1 being a key regulator at the PAS and Atg4 one of its substrates (Fig. 1a, c), their interaction on autophagosomal membranes would provide a local regulation of Atg4 function. To address this notion, we performed a bimolecular fluorescence complementation (BiFC) assay25. In strains expressing only the N (YN) or the C-terminal (VC) fragment of Venus fused with Atg1 and Atg4, respectively, no fluorescence signal was detected (Supplementary Fig. 3d). BiFC signal was also absent in the strain carrying both Atg4–VC and Atg1–YN (Fig. 4a), probably due to the transient location of Atg proteins at the PAS. In agreement with this, a perivacuolar punctate BiFC signal became evident in an atg2Δ mutant in which Atg1 associates more pronouncedly to the PAS17. These BiFC puncta co-localized with mCherryV5-Atg8, supporting that Atg4 interacts with Atg1 at this specific location (Fig. 4a). Additional deletion of ATG13 in this background prevents recruitment of Atg1 to the PAS17 and led to the disappearance of BiFC signals confirming that the interaction between Atg1 and Atg4 specifically takes place on autophagosomal membranes (Fig. 4a).
Atg8 (Fig. 3). Nonetheless, based on the predicted Atg4 structure (Supplementary Fig. 5a), S307 is close to amino acids D322 and H324, which correspond to D278 and H280 in ATG4B’s catalytic site. In particular, S307 faces H324 and when phosphorylated, its interaction with the positively charged H324 could induce a conformational change that alters the predicted Atg8-binding region (Supplementary Fig. 5a). The interaction between phosphorylated S307 and H324, however, could also lead to a closure of the catalytic pocket. Our multiple approaches to quantify the degree of phosphorylation of S307 in vivo have been unsuccessful.

Fig. 4 Atg1 and Atg4 interact at the PAS. a Atg1–Atg4 interaction at the PAS was visualized by BiFC. WT (JSY185), atg2Δ (JSY190), and atg2Δ atg13Δ (JSY215) cells expressing both endogenous Atg1-VN and Atg4-VC, and carrying a pCumCherryV5ATG8 plasmid were grown in SMD before being nitrogen starved in SD-N medium for 1 h. Fluorescence images were taken before and after nitrogen starvation. Differential interference contrast (DIC). Scale bar, 5 µm. b Mechanistic model for the regulation of Atg4 during autophagy. Newly synthesized Atg8 is constitutively processed by the cysteine protease Atg4 in the cytoplasm, where it is not inhibited by Atg1. After the cleavage of the C-terminal arginine, a glycine residue is exposed allowing Atg8 to be conjugated to the PE on autophagosomal membranes at the PAS. During the phagophore expansion, the Atg4 adjacent or coming in proximity of autophagosomal membranes, is locally inhibited by the action of the Atg1 kinase complex. Upon autophagosome completion, the release of Atg1 form autophagosomal membranes and/or its local inactivation allows Atg4 to act on Atg8-PE and release Atg8 from the PE anchor. This molecular mechanism, possibly together with other unknown regulatory events, would drive the dissociation of other Atg proteins from the surface of autophagosomes allowing their subsequent fusion with vacuoles.
probably due to the very low amounts of this modification, which make it not always detectable. This suggests that only a very small subpopulation of S307 is modified and/or is a very transient phosphorylation of S307. One possibility is that Atg4 approaching the Atg8–PE on autophagosomal membranes gets phosphorylated and, it is rapidly dephosphorylated after immediate release into the cytoplasm. Another possible scenario emerges from few recent publications. A structural study describing the mechanism of Legionella RavZ-mediated LC3–PE C-terminal processing reached the conclusion that the N-terminal and C-terminal LIR motifs in RavZ are essential to bind the substrate simultaneously and open the catalytic groove to allow the access to the bond that has to be cleaved. LIR motifs, plus a newly identified domain, are important for the recognition and deconjugation of lipidated Atg8/LC3 proteins by Atg4 proteases in yeast and mammals. It is thus plausible that regulation of LIR motif-mediated recruitment to autophagosomal membrane is the major mechanism for Atg8–PE processing, and Atg1 kinase inhibition represents an extra control mechanism. This latter would operate when the catalytic groove opens and therefore S307 could be of easily accessible for Atg1.

Alignment of the amino acid sequence of Atg4 protein family members from different organisms shows that Atg4 proteins have either a serine or an alanine in the position corresponding to that of yeast S307 (Supplementary Fig. 5b). The phylogenetic analysis reveals an evolutionary relation within Atg4 proteins with serines and within those with alanines (Supplementary Fig. 5c). The higher Atg8–PE deconjugating activity of the Atg4 isoforms with an alanine compared to those with a serine, reveals a major defect in autophagy progression when mutated to aspartate or alanine (Fig. 1b). Interestingly, the residue equivalent to S307 of yeast Atg4 is substituted by an alanine in ATG4B (Supplementary Table 1). The corresponding serine in yeast, i.e., S354, however, shows no major defect in autophagy progression when mutated to aspartate or alanine (Fig. 1b). Interestingly, the residue equivalent to S307 of yeast Atg4 is substituted by an alanine in ATG4B (Supplementary Table 1). The corresponding serine in yeast, i.e., S354, however, shows no major defect in autophagy progression when mutated to aspartate or alanine (Fig. 1b).

Alignment of the amino acid sequence of Atg4 protein family members from different organisms shows that Atg4 proteins have either a serine or an alanine in the position corresponding to that of yeast S307 (Supplementary Fig. 5b). The phylogenetic analysis reveals an evolutionary relation within Atg4 proteins with serines and within those with alanines (Supplementary Fig. 5c). The higher Atg8–PE deconjugating activity of the Atg4 isoforms with an alanine compared to those with a serine, reveals a major defect in autophagy progression when mutated to aspartate or alanine (Fig. 1b). Interestingly, the residue equivalent to S307 of yeast Atg4 is substituted by an alanine in ATG4B (Supplementary Table 1). The corresponding serine in yeast, i.e., S354, however, shows no major defect in autophagy progression when mutated to aspartate or alanine (Fig. 1b).

Methods

**Plasmids.** The pATG4GFP(416) plasmid was generated by PCR amplification of ATG4 promoter and the ATG4-GFP fusion from the MNN006 strain genome, and another cloning into the pRS416 vector as a promoter and the 3xmyc tag from the pFA6a-3xmycTRP1 plasmid35. The sequence coding for Atg4V297R,Q314K was inserted into the pATG4GFP(406), a strategy that allows to generate a peptide of the region of interest detectable by protein mass spectrometry analysis.

**Plasmids.** The pATG4GFP(416) plasmid was generated by PCR amplification of ATG4 promoter and the ATG4-GFP fusion from the MNN006 strain genome, and another cloning into the pRS416 vector as a promoter and the 3xmyc tag from the pFA6a-3xmycTRP1 plasmid35. The sequence coding for Atg4V297R,Q314K was inserted into the pATG4GFP(406), a strategy that allows to generate a peptide of the region of interest detectable by protein mass spectrometry analysis.

**Plasmids.** The pATG4GFP(416) plasmid was generated by PCR amplification of ATG4 promoter and the ATG4-GFP fusion from the MNN006 strain genome, and another cloning into the pRS416 vector as a promoter and the 3xmyc tag from the pFA6a-3xmycTRP1 plasmid35. The sequence coding for Atg4V297R,Q314K was inserted into the pATG4GFP(406), a strategy that allows to generate a peptide of the region of interest detectable by protein mass spectrometry analysis.

**Plasmids.** The pATG4GFP(416) plasmid was generated by PCR amplification of ATG4 promoter and the ATG4-GFP fusion from the MNN006 strain genome, and another cloning into the pRS416 vector as a promoter and the 3xmyc tag from the pFA6a-3xmycTRP1 plasmid35. The sequence coding for Atg4V297R,Q314K was inserted into the pATG4GFP(406), a strategy that allows to generate a peptide of the region of interest detectable by protein mass spectrometry analysis.

**Plasmids.** The pATG4GFP(416) plasmid was generated by PCR amplification of ATG4 promoter and the ATG4-GFP fusion from the MNN006 strain genome, and another cloning into the pRS416 vector as a promoter and the 3xmyc tag from the pFA6a-3xmycTRP1 plasmid35. The sequence coding for Atg4V297R,Q314K was inserted into the pATG4GFP(406), a strategy that allows to generate a peptide of the region of interest detectable by protein mass spectrometry analysis.

**Plasmids.** The pATG4GFP(416) plasmid was generated by PCR amplification of ATG4 promoter and the ATG4-GFP fusion from the MNN006 strain genome, and another cloning into the pRS416 vector as a promoter and the 3xmyc tag from the pFA6a-3xmycTRP1 plasmid35. The sequence coding for Atg4V297R,Q314K was inserted into the pATG4GFP(406), a strategy that allows to generate a peptide of the region of interest detectable by protein mass spectrometry analysis.
Electron microscopy. Fifteen OD_{600} unit equivalents of cells were resuspended in 1 ml of freshly prepared ice-cold 1.5% KmoO₄ (Sigma) and transferred into a 1.5-ml Eppendorf tube. For freezing, the samples were plunged into liquid N₂ before being stored at −80°C. Subsequently, the samples were thawed at room temperature. This operation was repeated once more before washing the pellets five times with 1 ml of distilled water. Cells were then dehydrated in increasing amounts of acetone (10, 30, 50, 70, 90, 95, and three times 100%) by incubation on a rotary wheel for at least 20 min at room temperature, each step followed by centrifugation at 3000 rpm for 5 min at room temperature. After centrifugation, the supernatant was removed, and the pellets were resuspended in 33% Spurr’s resin in acetone and mixed on the same device for 1 h at room temperature. This operation was repeated twice overnight and successively during all the day in 100% Spurr’s resin. The Spurr’s resin mixture was prepared by mixing 1:2:1 proportions of Spurr resin (Polysciences, Warrington, PA, USA), polyvinylpyrrolidone epoxy resin 736, 26 g of (2-nonen-1-yl)succinic anhydride, and 0.4 g of N,N-diethyltoluenediamine (all from Sigma). Incubating the preparations overnight at 70 °C polymerized the Spurr’s resin. Section of about 65–80 nm were then cut using an Ultracut E ultramicrotome (Leica Microsystems) and transferred on Formvar carbon-coated copper grids. Sections where then stained with uranyl acetate for 30 min at room temperature and then with a lead-citrate solution (0.004% uranyl acetate and 0.003% lead citrate) for 3 min at room temperature. The sections were negatively stained with 2% sodium phosphotungstate (pH 7.1) for 2 min before being viewed.

To determine the number of AB per vacuole and their diameter, three grids with sections obtained from the same preparation were evaluated. For every grid, the number and diameter of AB in 50 cells with apparent vacuoles were determined. Error bars represent the standard deviation from the counting of the three grids.

Protein expression and purification. Atg3, Atg4, Atg4 mutants, Atg7, Atg8, and the Atg12-Atg5 complex were expressed and purified as described.

Full length Atg8 and Atg19Cterm were expressed as N-terminal GST fusion proteins from pGEX4T1. Cells were grown at 37 °C to an OD_{600} of 0.8, induced with 1 mM IPTG and grown for another 4 h at 37 °C. Collected cells were resuspended in the resuspension buffer (300 mM NaCl, 5 mM HEPES, pH 7.5, 10 mM imidazole, 2.5 mM Pefabloc (Roht), 1 mM MgCl₂, 2 mM β-mercaptoethanol, and DNAse I (Sigma), and disrupted by freeze-thaw method and sonication. The cleared lysate was applied to a HisTrap column (GE Healthcare) and the proteins were eluted by a step-wise imidazole gradient. The Atg8 fraction of the 12 elute was concentrated using Amicon Ultra centrifugal filter (MW cut-off 30 kDa) and further purified using a 16/60 S200 size exclusion column (GE Healthcare). The protein complex was eluted from the column with 130 mM NaCl, 50 mM HEPES, pH 7.5, and 1 mM DTT. Atg8 and its mutants were expressed as an N-terminal GST fusion protein. The GST-Atg8 were grown in the presence of 160 mM NaCl. Protein expression was induced by addition of IPTG to a final concentration of 0.1 mM and the protein was expressed at 18 °C overnight. The cell pellets were resuspended in 50 mM HEPES/KOH, pH 7.5, 300 mM NaCl, 1 mM DTT, 1 mM MgCl₂, DNAse I, Complete protease inhibitors (Roche), and PEFABLOC. Cells were lysed by freeze-thawing and the lysate was centrifuged at 200000 × g for 40 min at 4 °C and the supernatant was incubated with equilibrated glutathione beads (GE Healthcare) for 2 h at 4 °C. The beads were washed five times with 50 mM HEPES/KOH, pH 7.5, 300 mM NaCl, 1 mM DTT, and finally two times with 50 mM HEPES, pH 7.5, 300 mM NaCl, 1 mM DTT. The protein was eluted with 50 mM HEPES, pH 7.5, 300 mM NaCl, and 1 mM DTT supplemented with 20 mM L-glutathione and with thrombin. The cleaved protein was run on a Superdex 200 16/60 column (GE Healthcare) and the peak fractions containing Atg8 were pooled, concentrated, and flash frozen in liquid nitrogen.

Preparation of small unilamellar vesicles. SUVs used for the conjugation and deconjugation assays were composed of 65% DOPC, 30% DOPE and 5% PI (all purchased from Avanti Polar Lipids). 100 µl of the lipid stock (10 mg/ml) were transferred into a glass vial and dried under an argon stream. The dried lipids were dissolved with ethanol for 1 h in a desiccator. The dried lipids were subsequently incubated with SUV buffer (25 mM HEPES, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1 mM DTT) for 15 min. The lipids were resuspended by tapping and gently sonicated for 2 min in a water bath sonicator. The resuspended SUVs were then extruded through a 0.4 µm membrane followed by extrusion through a 0.1 µm membrane (Whatman Nuclepore, St Louis, MO) using the Mini Extrak (Avanti Polar Lipids). The final SUVs suspension has a concentration of 1 mg lipids/ml buffer.

Atg8 conjugation and deconjugation assay using SUVs. The conjugation and deconjugation reactions were performed at 30 °C and all buffers, solutions and the SUVs with the exception of the proteins were pre-warmed to this temperature. Atg3 and Atg8 were used at a final concentration of 1 µM, whereas Atg12–Atg5 and Atg8–AR were used at a final concentration of 0.5 µM and 5 µM, respectively. ATP was added to a final concentration of 50 µM while MgCl₂ was used at 1 mM. Conjugation reactions were stopped by the addition of 1000 units of calf intestinal phosphatase (New England Biolabs). For the deconjugation reaction, Atg4 was used at a final concentration of 25 nM. The reactions were then stopped by the addition of SDS-PAGE loading buffer and samples separated on 11% SDS-PAGE gels containing 4.5 M urea in the separating part.

Protein mass spectrometry analysis. 700 µl of late stationary growth atgΔ cells (SAY084) transformed with the pTEFATG4V297R,Q314K-GFP(416) plasmid and nitrogen starved in SD-N medium for 1 h were lysed by cryogenic grinding in 45 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Tween-20 supplemented with 1 mM PMSF, Complete protease inhibitors (Roche), 10 mM β-glycerophosphate, 10 mM NaF, and 1 mM NaVO₄ buffer. Lysates were then cleared by centrifugation and incubated with 100 µl G-800 spheroidase beads. Immuno-isolated Atg8 V297R,Q314K-GFP was eluted in sample buffer and separated by SDS-PAGE before cutting the gel band containing the fusion protein. After in-gel trypsinization, the resulting peptides were separated and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS) on a Q-Exactive plus MS instrument and a mass spectrometry database for identification of proteins and modifications, performed with the PEAKS software version 7.5 (Bioinformatics Solutions).

In vitro Atg1 phosphorylation. Atg1-TAP and Atg12DI11A-TAP, and the associated proteins, were immunoprecipitated from yeast grown in 2.1 of YPD medium to an OD_{600} of 2 and treated with 220 mM rapamycin for 1 h, harvested by centrifugation, and washed in PBS, 2% glucose. Cells were then resuspended in the lysis buffer (PBS, 10% glycerol, 0.5% Tween-20, 1 mM NaF, 1 mM phenylmethylsulfonylfluoride, 1 mM NaVO₄, 20 mM β-glycerophosphate, protease inhibitor cocktail (Roche)) and frozen in droplets in liquid nitrogen. After cell lysis in a freezer and thawing, the extracts were cleared by centrifugation. The cleared extracts were then incubated with 160 µl of IgG-coupled magnetic beads (Dynabeads, Invitrogen) for 1 h at 4 °C with

8 NATURE COMMUNICATIONS | DOI: 10.1038/s41467-017-00302-3 | www.nature.com/naturecommunications
1. Choi, A. M., Ryter, S. W. & Levine, B. Autophagy in human health and disease.

2. References

3. Western blot analyses. Western blot analyses were conducted as previously described. Briefly, 2.5 OD\textsubscript{600} equivalents of cells were collected by centrifugation at 13,000×g for 1 min and resuspended in 400 l of iced-acetone (20 mM PIPES, pH 6.8, 0.5% Triton X-100, 50 mM KCl, 100 mM potassium acetate, 10 mM MgCl\textsubscript{2}, 10 μM ZnSO\textsubscript{4}, 2 mM PMFS) by vortexing in presence of 100 l of glass beads (0.4–0.6 mm in diameter) for 3–5 min at 4 °C. Lysates were centrifuged at 13,000×g for 5 min at 4 °C. Then 100 l of supernatant were mixed with 400 l of ALP reaction buffer (250 mM Tris-HCl, pH 8.5, 0.4% Triton X-100, 10 mM MgCl\textsubscript{2}, 10 mM ZnSO\textsubscript{4}, 1.25 mM p-nitrophenol, phosphatase inhibitor cocktail) and boiled for 3 min before adding 500 l of 1 M glycine, pH 11.0. After centrifuge at 13,000×g for 2 min, the absorbance of the supernatant was measured at 400 nm. Enzymatic activity was calculated with the following formula 1000×OD\textsubscript{405}/time×protein concentration in μg/ml and expressed in arbitrary units.

4. Statistical analyses. Statistical analyses were done using the paired two-tailed Student’s t-test.

5. Accession numbers. All proteins used were from Saccharomyces cerevisiae. Atg3: NP_014408; Atg5: NP_015176; Atg7: NP_0120411; Atg8: NP_0094751; Atg10: NP_0130581; Atg12: NP_0097761; Atg16: NP_0138821.

6. Data availability. All data generated or analyzed during this study are included in this published article and its Supplementary Information.

7. Received: 20 July 2016 Accepted: 19 June 2017

8. Published online: 18 August 2017

9. References

10. Choi, A. M., Ryter, S. W. & Levine, B. Autophagy in human health and disease. N. Engl. J. Med. 368, 1845–1846 (2013).

11. Reggiori, F. & Klionsky, D. J. Autophagic processes in yeast: mechanism, machinery and regulation. Genetics 194, 341–361 (2013).

12. Noda, N. N. & Fujioka, Y. Atg1 family kinases in autophagy initiation. Cell. Mol. Life Sci. 72, 3083–3096 (2015).

13. Ganeley, J. I. Autophagosome maturation and lysosomal fusion. Essays Biochem. 55, 65–78 (2013).

14. Cebollero, E. et al. Phosphatidylinositol 3-phosphate clearance plays a key role in autophagosome completion. Curr. Biol. 22, 1545–1553 (2012).

15. Cheng, J. et al. Yeast and mammalian autophagosomes exhibit distinct phosphatidylinositol 3-phosphate asymmetries. Nat. Commun. 5, 3207 (2014).

16. Nair, U., Cao, Y., Xie, Z. & Klionsky, D. J. Roles of the lipid-binding motifs of Atg18 and Atg21 in the cytoplasm to vacuole targeting pathway and autophagy. J. Biol. Chem. 285, 11476–11488 (2010).

17. Kim, J., Huang, W. P. & Klionsky, D. J. Membrane recruitment of Aut7p in the autophagy and cytoplasm to vacuole targeting pathways requires Aut1p, Aut2p, and Atg6. J. Biol. Chem. 279, 36268–36276 (2004).

18. Nakatogawa, H., Ishii, J., Asai, E. & Ohsumi, Y. Atg4 recycles inappropriately lipidated Atg8 to promote autophagosome biogenesis. Autophagy 8, 177–186 (2012).
localization of Atg8 during uptake of aminopeptidase I by selective autophagy. 

*Mol. Biol. Cell* **15**, 3553–3566 (2004).

38. Mari, M. et al. An Atg9-containing compartment that functions in the early steps of autophagosome biogenesis. *J. Cell. Biol.* **190**, 1005–1022 (2010).

39. Gueldener, U., Heinisch, J., Koehler, G. J., Voss, D. & Hegemann, J. H. A second set ofloxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res.* **30**, e23 (2002).

40. Janke, C. et al. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* **21**, 947–962 (2004).

41. Vida, T. A. & Emr, S. D. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell. Biol.* **128**, 779–792 (1995).

42. Griffith, J., Mari, M., De Maziere, A. & Reggiori, F. A cryosectioning procedure for the ultrastructural analysis and the immunogold labelling of yeast *Saccharomyces cerevisiae*. *Traffic* **9**, 1060–1072 (2008).

43. Romanov, J. et al. Mechanism and functions of membrane binding by the Atg5-Atg12/Atg16 complex during autophagosome formation. *EMBO J.* **31**, 4304–4317 (2012).

**Acknowledgements**

We thank Daniel Klionsky for reagents. We also thank Hjalmar Permentier, Marcel de Vries and Margot Jeronimus-Stratingh of the Interfaculty Mass Spectrometry Center (University of Groningen/University Medical Center Groningen) for the mass spectrometry analyses, and Klaas Stijlemans of the UMCG Microscopy & Imaging Center. F.R. is supported by ALW Open Program (822.02.014), DFG-NWO cooperation (DN82-303), SNF Sinergia (CRSII3_154421) and ZonMW VICI (016.130.606) grants. S.M. is supported by (FP7/2007-2013)/ERC grant agreement No. 260304, the FWF Austrian Science Fund (grant number P25546-B20) and the EMBO Young Investigator Program. C.K. is supported by a grant from the Vienna Science and Technology Fund (WWTF, VRG10-001) the Austrian Science Fund (grant number P25546-B20) and the EMBO Young Investigator Program. C.U. is supported by the DFG (UN1117/7-2) and the Hans-Mühlhoff Foundation. R.G.-S. is supported by a Marie Skłodowska-Curie Individual Fellowship (IF-EF) from the European Commission. S.A. is a recipient of the FCT grant SFH1/BD/95013/2013.

**Author contributions**

F.R. and J.S.-W. conceived the study. F.R., J.S.-W. F.K., C.U., C.K. and S.M. designed the experiments. J.S.-W., F.K., B.Z., R.G.-S., M.S., J.G., S.A. and S.R. generated the reagents and performed the experiments. F.R. and J.S.-W. wrote the manuscript, which was corrected by all the authors.

**Additional information**

*Supplementary Information* accompanies this paper at doi:10.1038/s41467-017-00302-3.

**Competing interests:** The authors declare no competing financial interests.

**Reprints and permission** information is available online at http://npg.nature.com/reprintsandpermissions/

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.