Structural basis of ATG3 recognition by the autophagic ubiquitin-like protein ATG12

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The autophagic ubiquitin-like protein (ublp) autophagy-related (ATG) 12 is a component of the ATG12~ATG5~ATG16L1 E3 complex that promotes lipid conjugation of members of the LC3 ublp family. A role of ATG12 in the E3 complex is to recruit the E2 enzyme ATG3. Here we report the identification of the ATG12 binding sequence in the flexible region of human ATG3 and the crystal structure of the minimal E3 complexed with the identified binding fragment of ATG3. The structure shows that 13 residues of the ATG3 fragment form a short β -strand followed by an α -helix on a surface area that is exclusive to ATG12. Mutational analyses of ATG3 confirm that four residues whose side chains make contacts with ATG12 are important for E3 interaction as well as LC3 lipidation. Conservation of these four critical residues is high in metazoan organisms and plants but lower in fungi. A structural comparison reveals that the ATG3 binding surface on ATG12 contains a hydrophobic pocket corresponding to the binding pocket of LC3 that accommodates the leucine of the LC3-interacting region motif. These findings establish the mechanism of ATG3 recruitment by ATG12 in higher eukaryotes and place ATG12 among the members of signaling ublps that bind liner sequences.

autophagy | protein–peptide interaction | X-ray crystallography

Autophagy is a bulk degradation/recycling process essential for quality control in eukaryotic cells (1, 2). During autophagy, portions of cytoplasm are sequestered into membrane-bound vesicles called autophagosomes and transported into lysosomes for degradation. Autophagosome formation is mediated by a concerted action of a number of conserved autophagy-related (ATG) proteins (2, 3). The two classes of ubiquitin-like proteins (ublp) among these are members of the LC3 family in mammals or Atg8 in yeast and the conserved ATG12, which share some sequence similarities (4). LC3 and ATG12 are activated by the same E1 enzyme, ATG7, and then transferred to different E2 enzymes, ATG3 and ATG10, respectively. ATG3 conjugates LC3 to a lipid molecule, phosphatidylethanolamine (PE), on autophagosomal membranes. PE-conjugated LC3 plays crucial roles in control of membrane dynamics during autophagosome formation as well as in recruitment of cargos, such as aberrant proteins and damaged organelles, through binding to receptor proteins carrying an LC3-interacting region (LIR) motif (5). On the other hand, ATG10 attaches ATG12 to a structural protein, ATG5, and the resulting conjugate ATG12~ATG5 acts like an E3 factor by stimulating the transfer of LC3 from ATG3 to PE (6). ATG12~ATG5 exists as a complex with the coiled-coil protein ATG16L1 that localizes on autophagosome precursor membranes, resulting in LC3 lipidation for autophagosome formation (7). We previously showed that E3 activity requires the native covalent linkage between ATG12 and ATG5 and a composite surface patch formed by the residues of both of these proteins, and that the interaction with the E2 ATG3 is mostly mediated by ATG12 (8). However, it remains unclear how E3 communicates with or recruits E2.

Here we describe atomic details of the ATG3–ATG12 interaction based on our crystal structure of a human E2–E3 complex containing this interaction. The results from accompanying

interaction analyses and cellular LC3 lipidation assays support the importance of this interaction in LC3 lipidation and autophagy. We also show that the ATG3–ATG12 and LIR–LC3 interactions share some structural features. This unexpected finding together with our experimental data suggests that ATG12 is a protein recruitment apparatus in autophagy.

Results

Identification of the Region Interacting with ATG12 in ATG3. To better understand the mechanism of ATG3 recruitment by E3, we set out to identify the region of ATG3 interacting with E3. Because ATG3 consists of a canonical ubiquitin conjugation domain (ATG3^{E2core}) and an ~100-residue-long flexible region (ATG3^{FR}) inserted between β 2 and β 3 of ATG3^{E2core} and involved in ATG7 binding (9), we asked whether ATG3^{FR} is also involved in E3 binding. In a size-exclusion chromatography column, the E3 complex consisting of ATG12~ATG5 and the N-terminal fragment of ATG16NL1 (ATG16N) coeluted with ATG3^{FR} but not with ATG3^{E2core}, indicating that E3 binds tightly only to ATG3^{FR} (Fig. 1A). Isothermal titration calorimetry (ITC) experiments confirmed this tight binding with a dissociation constant (K_D) of 41 nM (Fig. 1B). We also obtained a K_D of 94 nM for the interaction between ATG3^{FR} and unconjugated ATG12. These K_D values are close to the K_D of 50 nM for the interaction between full-length ATG3 and

Significance

Autophagy-related (ATG)12 is a ubiquitin-like protein essential to autophagy. It has been known for years that ATG12 is conjugated to the structural protein ATG5 and that the resulting protein–protein conjugate acts like an E3 enzyme that facilitates the attachment of the LC3 ubiquitin-like protein to a lipid molecule, phosphatidylethanolamine. However, the exact role of ATG12 in the E3 complex and the significance of ATG12 being a ubiquitin-like protein have remained elusive. We show that ATG12 binds to a short peptide region of the E2 enzyme ATG3 and describe the structural details of this interaction. This work establishes ATG12 as the ATG3 recruitment factor and explains how ATG12 uses its ubiquitin-like fold for binding to the ATG3 peptide.

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Fig. 1. Identification of the E3-interacting region in ATG3^{FR}. (A) Size-exclusion chromatography profiles of the mixtures of 10 μM each ATG12~ATG5~ATG16N and ATG3^{FR} (green) or ATG3^{E2core} (black), and ATG12~ATG5~ATG16N alone (blue) and ATG3^{FR} alone (red). (Lower) SDS/PAGE for confirming the protein contents in each fraction. (B) ITC data obtained by injecting the indicated ATG3 constructs into a solution of ~7 μM ATG12~ATG5~ATG16N or unconjugated ATG12. (C) Overlay of ¹H-¹⁵N HSQC NMR spectra of 0.3 mM ¹⁵N-labeled ATG3^{FR} in the absence (black) and presence (red) of 0.15 mM ATG12~ATG5~ATG16N. (D) Mapping of the peak-broadening effect for each residue in C. Normalized ratios of ¹H-¹⁵N HSQC ATG3^{FR} peak intensities in the presence of ATG12~ATG5~ATG16N to ¹H-¹⁵N HSQC ATG3^{FR} peak intensities alone are plotted against the sequence of human ATG3. Residues whose cross-peaks are not assigned (residues 89, 113, and 145–150) or are overlapping with other peaks have an intensity ratio of 0 in the plot. Metazoan and plant Atg3 sequences were aligned by the Clustal Omega program (27). The flexible regions of fungus Atg3 sequences do not align well with metazoan organisms and plants, and are shown in Fig. S1.

ATG12~ATG5~ATG16N, establishing that the major source of binding energy in this E2–E3 interaction is provided by ATG3^{FR} and ATG12.

We then used NMR spectroscopy to identify the residues in ATG3^{FR} that are interacting with ATG12~ATG5~ATG16N. The narrow chemical shift dispersion in the ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) spectrum of ¹⁵N-labeled ATG3^{FR} confirms that this flexible region of human ATG3 is not folded (Fig. 1C), which was observed similarly for ScAtg3 (9). When we added unlabeled ATG12~ATG5~ATG16N, many peaks of ATG3^{FR} broadened, indicating interactions. The plot of changes of peak intensities over the primary sequence revealed that the broadening occurred in a single continuous region from about residues 140–170 (Fig. 1D). Although we did not perform these NMR experiments with unconjugated ATG12 due to its aggregation-prone character (8), our ITC and crystallographic data described above and below, respectively, show that this region of ATG3 interacts with ATG12. Hereafter this minimal ATG3 region interacting with ATG12 is referred to as RIA12. As shown, RIA12 contains many acidic and some hydrophobic amino acids, some of which are conserved among metazoan organisms and plants. However, its conservation in fungi appears to be less clear (Fig. S1).

Crystal Structure of the ATG3^{RIA12}–ATG12~ATG5~ATG16N Complex. Having identified RIA12 of ATG3 in solution, we next determined the crystal structure of the ATG3^{RIA12}–ATG12~ATG5~ATG16N complex at 2.2 Å resolution (Table S1). The overall structure of ATG12~ATG5~ATG16N is essentially the same as the previously determined structure of the free form (Fig. 2A) (8).

Thirteen residues in ATG3^{RIA12} are visible in the electron density map (Fig. S2) and are bound to ATG12 in a short extended conformation at the N terminus (residues 153–157), followed by an α-helical conformation at the C terminus (residues 157–165) (Fig. 2B). There are no apparent contacts between ATG3^{RIA12} and ATG5, consistent with the ITC data described above. Remarkably, the ATG3^{RIA12} binding surface of ATG12 matches the previously identified surface region for ATG3 interaction, which includes Lys54, Lys72, and Trp73 (8). This interface buries a solvent-accessible surface area of 500 Å² and consists of both nonpolar and polar contacts. It appears that a shape complementarity is provided by Met157 of ATG3, which is at the boundary between the extended and helical regions of ATG3^{RIA12} and is inserting its side chain into a small cavity surrounded by a few hydrophobic residues on α1 and β2 of ATG12 including Trp73 (Fig. 2B and C). The aromatic ring of Tyr160 on ATG3^{RIA12}'s α-helix makes contacts with a solvent-exposed hydrophobic surface patch consisting of Trp73, Phe87, and Phe91 of ATG12, suggesting its contribution to the binding. Acidic residues Asp156 and Glu158 on the extended and helical regions, respectively, of ATG3^{RIA12} interact with Lys54 of ATG12, of which we previously showed strong contribution to the high-affinity interaction to ATG3 (8). Glu161 on ATG3^{RIA12}'s α-helix interacts with Arg79 of ATG12, adding another electrostatic contribution to the interaction. Backbone atoms of residues 155–157 in the extended region of ATG3^{RIA12} make a few hydrogen bonds with those of β2 of ATG12, forming a short intermolecular β-sheet. Glu153, the most N-terminal residue in the visible region of ATG3^{RIA12}, is located adjacent to Lys72 of ATG12, another important residue

Fig. 2. Crystal structure of the human ATG3^{RIA12}-ATG12-ATG5-ATG16N complex. (A) Cartoon representation of the overall structure. Protein N termini are indicated with the letter N. (B) A blown-up view around the ATG3^{RIA12}-ATG12 contacts. (C) Solvent-accessible surface of ATG12-ATG5 colored according to electrostatic potential shown from two angles with a tube and stick representation of ATG3^{RIA12}.

for ATG3 interaction. It is of note that the invisible N-terminal region (residues 140–152) contains a stretch of eight negatively charged residues (Fig. 1E). Based on the direction of the succeeding β -strand, these negatively charged residues are likely positioned on a highly basic surface area of ATG12 that has many lysine residues, such as 60, 69, 71, 72, and 128, suggesting a contribution of electrostatic interactions to the overall binding (Fig. 2C).

Identification of RIA12 Residues Important for E3 Binding. To probe the energetic contribution of each residue of RIA12 to E3 interaction, we generated alanine mutants of full-length ATG3 and measured their affinities to ATG12-ATG5-ATG16N using ITC (Fig. 3). Mutations of the residues that make contacts with ATG12 and show their electron densities of side-chain atoms, such as D156A, M157A, Y160A, and E161A, increased the K_D by 18- to 330-fold. Combining the two most severe mutations, D156A and M157A, weakened the interaction to a level undetectable at the concentration of ATG3 used for this assay ($\sim 7 \mu\text{M}$). Mutations of the residues that may make contacts with ATG12 but show weaker electron densities, such as E153A, E158A, and L165A, increased the K_D only by up to 2.4-fold. In contrast, a simultaneous mutation of two glutamic acid residues located on the α -helix at the side opposite the interface to ATG12 to alanine (E159A/E162A) showed only a minor effect on the K_D . We also examined the contribution of the eight consecutive negatively charged residues (residues 144–151) of ATG3. Replacing all eight residues with alanine simultaneously (termed 8 $\times$ Ala) increased the K_D by 38-fold, confirming their importance for the high-affinity interaction. The four critical

residues Asp156, Met157, Tyr160, and Glu161 as well as the cluster of negatively charged residues are conserved among other metazoan organisms and plant species. Although these four residues show some variations, such as Leu or Ile, Phe or Leu, and Asp for the positions corresponding to Met157, Tyr160, and Glu161, respectively, of human ATG3 (Fig. 1D), these substitutions are to amino acids similar to the original ones, and therefore the same interaction would likely occur in other higher eukaryotes.

ATG3^{RIA12}-ATG12 Interaction Is Essential for LC3-II Production. To examine the functional relevance of the ATG3^{RIA12}-ATG12 interaction, we stably expressed wild-type or mutant ATG3-T7 constructs in ATG3^{-/-} mouse embryonic fibroblasts (MEFs) and examined LC3 lipidation upon starvation. Quantification of the PE-conjugated form of LC3 (LC3-II) using Western blotting revealed good correlations between the affinities obtained from the ITC analyses and the amounts of LC3-II produced with the expression of ATG3 mutants (Fig. 4A). The point mutations D156A and M157A, which markedly lowered the affinity, severely impaired LC3-II formation, and LC3-II was not detectable with the D156A/M157A double mutant. Furthermore, the 8 $\times$ Ala mutation also caused a severe reduction in the amount of LC3-II, supporting the importance of these negatively charged residues in cells.

To confirm that these mutations impair the interaction with endogenous ATG12-ATG5, we subjected lysates of MEFs stably expressing ATG3 mutants to immunoprecipitation using an anti-ATG3 antibody. A Western blot analysis of the immunocomplexes showed that ATG12-ATG5 precipitated together with wild-type ATG3 as well as ATG3 with an alanine mutation of the catalytic cysteine (C264A), but not with control or D156A/M157A mutant ATG3, revealing that the E2-E3 interaction at an endogenous level requires ATG3^{RIA12}-ATG12 interaction but not the formation of the thioester conjugate with LC3 (Fig. 4B). We also examined whether the double mutation had effects on the E1-E2 interaction and thus LC3 loading on E2, because Atg3^{FR} has been previously shown to be critical for Atg7 interaction in yeast and plant systems (9–13). We detected endogenous ATG7 in the immunocomplexes with all three ATG3 constructs tested in the immunoprecipitation experiment described above, indicating that the D156A/M157A double mutation does not affect ATG7 binding (Fig. 4B). The amount of ATG7 that was coprecipitated with C264A ATG3 was less than the other two ATG3 constructs carrying native Cys264, suggesting that C264A ATG3 binds to ATG7 more weakly than wild-type ATG3. This observation may indicate that the LC3-ATG3 intermediate, which requires Cys264 to form, has a higher affinity for ATG7 than uncharged ATG3, although E3 binding was not affected by the C264 mutation, as shown above (Fig. 4B). Finally, we examined the effect of the D156A/M157A double mutation on the formation of the LC3-ATG3 intermediate. To this end, we expressed C264S ATG3, which forms a more stable oxyester-linked LC3-ATG3 than the native thioester linkage, in MEFs and performed Western blotting of the lysates (Fig. 4C). Consistent with a previous report (7), wild-type ATG3-GFP failed to show the thioester intermediate in the Western blot, whereas LC3-loaded C264S ATG3-GFP was detectable. Similarly, we detected the intermediate with the C264S/D156A/M157A mutation, indicating that the D156A/M157A mutation does not affect the formation of the LC3-ATG3 intermediate. These data allowed us to conclude that the loss of LC3 lipidation by the D156A/M157A mutation in ATG3 was due to the impaired interaction with the ATG12-ATG5-ATG16L1 E3 complex, establishing that the ATG3^{RIA12}-ATG12 interaction is essential for LC3 lipidation in cells.

Structural Comparison Between ATG12 and LC3 Systems. The members of the LC3 family interact with LIRs (also called LRS

Fig. 3. Identification of the residues in ATG3 that are energetically important for E3 binding. ITC data obtained by injecting the indicated ATG3 constructs into a solution of $\sim 7 \mu\text{M}$ ATG12~ATG5~ATG16N are shown. The dissociation constants of the binding (K_D) and stoichiometry values (N) were determined by curve fitting except for ATG3 D156A and ATG3 M157A, whose data were fit with a fixed stoichiometry of 1.

or AIM), which contain a Trp–Xaa–Xaa–Leu tetrapeptide motif, by forming an intermolecular β -sheet with the $\beta 2$ of LC3 proteins (5, 14, 15). The side chains of the Trp and Leu residues in LIRs stick into hydrophobic pockets called W and L sites, respectively, on the flanking surface area of the $\beta 2$ of LC3 proteins. Because the ATG3^{RIA12}–ATG12 interaction also uses the $\beta 2$ of ATG12 as described above, we compared the ATG3^{RIA12}–ATG12 part of our structure with the p62^{LIR}–LC3B complex (14, 16). Strikingly, the structural alignment shows that the backbone residues of the tetrad of p62^{LIR} can be superposed on those of the β -strand region of ATG3^{RIA12} (residues 154–157), and the location of the pocket on ATG12 accommodating ATG3 Met157 (M pocket) corresponds to the L site on LC3 (Fig. 5). In addition to this similarity, the electrostatic interaction between the acidic residues that are N-terminal to the tetrads and basic patches on ATG12 or LC3 contributes to the affinity in both cases, although the structural details appear to be different between these electrostatic interactions. However, there are features in the ATG3^{RIA12}–ATG12 interaction that make this interaction distinct from the LIR–LC3 interaction. First, the M pocket on ATG12 is much narrower than the cavity at the L site on LC3. The narrow cavity is likely optimal for fitting the nonbranched side chain of the methionine residue. Second, there is no hydrophobic cavity on the surface of ATG12 at the site corresponding to the W site on LC3, which is composed of residues of not only the ubiquitin-like fold but also the N-terminal α -helices that are unique to the members of the LC3 family among ubiquitin-like proteins. Accordingly, position 1 of the tetrad of ATG3^{RIA12} is alanine, not tryptophan. LIRs would not bind to ATG12 because of the lack of W site on ATG12. Third, the region succeeding the tetrad in ATG3^{RIA12} forms an α -helix

on which side chains make contacts with the ATG12 surface, and importantly those contacts contribute to the affinity as shown above. Known LIRs do not possess such an α -helix at their C termini. Taken together, we conclude that ATG3^{RIA12} is a unique linear sequence that binds to ATG12.

Discussion

The flexible regions of fungus Atg3 are overall very diverse and do not possess sequences that satisfy all of the four critical positions of RIA12 described above (Fig. S1). However, the C-terminal regions in the flexible regions in fungus Atg3 are generally acidic and contain some of the four residues. Because the RIA12 binding patch of ATG12 contains some less conserved residues including Trp73, which is cysteine in *Saccharomyces cerevisiae* (8), differences of the surface residues around the RIA12 binding area may account for the poorer conservation of RIA12 in fungi. Experimental investigations will be required to clarify the conservation of RIA12 in fungi in the future.

Previously, it was suggested that a highly conserved phenylalanine residue of Atg12 (Phe108 in humans) was critical for ATG3 interaction based on mutational analyses of a plant system (17). In the two crystal structures of yeast and human ATG12~ATG5 conjugates, the conserved phenylalanine residues are completely buried within the interface between ATG12 and ATG5 (8, 17). To rationalize the results of the mutational analyses, Noda et al. proposed a conformational change in which ATG12 and ATG5 no longer make noncovalent contacts between each other and thus the phenylalanine becomes solvent-exposed and accessible to Atg3 (17). However, in our previous work, we showed that mutations of Phe108 in human ATG12 cause aggregation of the mutant conjugate proteins and identified residues important for

Fig. 4. Residues of ATG3 important for E3 binding are also important for LC3 lipidation. (A) A Western blot of an LC3-II formation assay in ATG3^{-/-} MEFs stably expressing ATG3-T7 constructs is shown. The ratios of LC3-II to α -tubulin normalized against that of wild type are shown at the bottom. (B) An immunoblot (IB) of an anti-ATG3 immunoprecipitation (IP) assay carried out with lysates of ATG3^{-/-} MEFs stably expressing the indicated ATG3-T7 constructs is shown. (C) A Western blot for detecting the LC3~ATG3 intermediate is shown. ATG3-GFP constructs with the indicated mutations were stably expressed in ATG3^{-/-} MEFs and are detected with the indicated antibodies.

ATG3 binding, such as Lys54, Lys72, and Trp73, at the side opposite from Phe108 (8). Our work here shows how these residues interact with ATG3^{RIA12}, which is the major source of binding energy in full-length ATG3, and that the architecture of ATG12~ATG5 does not change upon binding to ATG3^{RIA12}. Thus, our results do not suggest that the interaction with ATG3 requires the kind of a conformational change proposed by Noda et al. Whether the ATG12~ATG5 conjugate changes its conformation while exhibiting E3 activity, namely binding to the LC3~ATG3 thioester-bonded conjugate, is an important question that remains open.

The entire ATG3^{FR} is disordered in solution as shown here and previously by others for human and yeast Atg3 (9), respectively. The α -helical conformation of the C-terminal residues of ATG3^{RIA12} is likely induced and stabilized upon binding to ATG12. Because residues Glu159 and Glu162 on the solvent-exposed side of RIA12's α -helix are conserved in higher eukaryotes (Fig. 1D), it seemed reasonable to consider that the newly

generated composite surface containing these glutamic residues may play a role in stimulating the LC3 transfer from ATG3^{E2core} to PE. However, our results with the E159A/E162A double mutant indicate that these residues are not essential for LC3-II lipidation, eliminating such a possibility. Thus, we conclude that the ATG3^{RIA12}-ATG12 interaction is responsible for ATG3 recruitment. This function of RIA12 is analogous to the role of Atg3^{FR} in E1-E2 interaction. The recent structures of the Atg7-Atg3 complex show that α -helix C in ATG3^{FR} binds to the N-terminal domain of Atg7, resulting in placement of Atg3^{E2core} at a position distant from α -helix C of ATG3^{FR} (10, 11). This positioning allows the catalytic cysteine of Atg3^{E2core} to face that of Atg7 in its C-terminal domain, facilitating the transfer of Atg3 to Atg7. We previously showed that the conjugation of ATG12 to ATG5 creates a composite surface patch that is essential for the E3 function of the conjugate but does not contribute to the high-affinity binding to ATG3 (8). Considering these data, the high-affinity interaction between ATG3^{RIA12} and ATG12 may allow positioning LC3-loaded ATG3^{E2core} on the composite surface for stimulating LC3 transfer. Structural investigation of a larger E2-E3 complex containing full-length ATG3 would provide key information for the complete understanding of the molecular mechanisms of LC3 lipidation.

In contrast to members of the LC3 family, which have the well-established function of cargo recruitment through LIR motif recognition, the function of ATG12 and its significance being a ubiquitin-like protein have remained unclear. The work presented here now uncovers that ATG12 binds to a linear sequence similarly to ubiquitin and other ublps including LC3 (18). Moreover, we found that ATG12 uses a binding pocket that is similar to the L site of LC3 for binding to RIA12. This unexpected similarity between ATG12 and LC3, together with previously

Fig. 5. Comparison between the ATG3^{RIA12}-ATG12 and p62^{LIR}-LC3B structures. (A) The superposition of the ATG3^{RIA12}-ATG12 and p62^{LIR}-LC3B structures is shown in a cartoon model. Positions 1-4 in the tetrads of ATG3^{RIA12} and p62^{LIR} are labeled and shown with side-chain atoms. The two N-terminal α -helices of LC3B are indicated by α N1 and α N2. (B and C) ATG12 (B) and LC3B (C) are shown as white surface models. The residues within 6 Å of the side-chain atoms of position 4 of the tetrad (ATG3 Met157 and p62 Leu343) and those of the tryptophan at position 1 of p62 are colored yellow and light red, respectively.

recognized but underappreciated sequence similarities between these autophagic ublps (4), implies a common evolutionary root for these proteins.

Materials and Methods

Protein Preparation. Human full-length ATG3 and ATG12~ATG5~ATG16N were prepared as described previously (8). The DNA sequences coding ATG3_{88–192} or ATG3_{140–170} were cloned into the NdeI-XhoI site of a modified pET-Duet-1 vector (Novagen); the resulting vectors express the 6×His-maltose binding protein (MBP)-tobacco etch virus (TEV) protease cleavage sequence (TEVs)-ATG3^{FR} fusion proteins. *Escherichia coli* BL21(ΔDE3) cells transformed with each of these vectors were grown in Luria broth media or M9 minimal media for stable isotope labeling or selenomethionine incorporation. Protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside at an OD₆₀₀ of 0.8, after which the cells were grown at 37 °C for 3 h and then harvested. The 6×His-MBP-TEVs-ATG3^{FR} fusion was purified by nickel-affinity chromatography (Qiagen) followed by TEV cleavage and Source 15Q anion-exchange chromatography (GE Healthcare). To isolate the ATG3^{FR}-ATG12~ATG5~ATG16N complex for crystallization, ATG12~ATG5~ATG16N was mixed with ATG3^{FR} constructs and passed through a Superdex 200 size-exclusion column (GE Healthcare).

ITC Experiments. All titrations were carried out at 25 °C in a VP-ITC isothermal titration calorimeter (MicroCal). Approximately 6.5 μM ATG12 or ATG12~ATG5~ATG16N in 10 mM Hepes (pH 7.0), 200 mM NaCl, and 2 mM Tris(2-carboxyethyl)phosphine (TCEP) was placed in the cell. Data were fit with the model for one set of binding sites in Origin (OriginLab).

NMR Spectroscopy. NMR experiments were carried out at 25 °C on a Varian INOVA 600 MHz spectrometer with protein samples dissolved in 20 mM sodium phosphate (pH 6.8), 150 mM NaCl, 2 mM DTT, and 10% (vol/vol) D₂O. ¹H-¹⁵N HSQC 2D spectra of 0.3 mM ¹⁵N-labeled ATG3_{88–192} were recorded with or without 0.15 mM unlabeled ATG12~ATG5~ATG16N to monitor the binding between these two molecules. Standard 3D triple-resonance experiments were acquired on 1 mM ¹⁵/13C-labeled ATG3_{88–192} for resonance assignments of backbone atoms. Spectra were processed and analyzed using NMRPipe (19) and NMR View (20), respectively. Peak intensities shown in Fig. 2 were obtained using NMRDraw (19).

Crystallization of the ATG3_{140–170}-ATG12~ATG5~ATG16N Complex. Crystals of the native human ATG3_{140–170}-ATG12~ATG5~ATG16N complex were grown at 4 °C using the sitting-drop vapor-diffusion method from drops containing 1 μL of 10 mg/mL protein in 10 mM Hepes (pH 7.0), 150 mM NaCl, and 1 mM DTT and 1 μL of reservoir solution [0.1 M MES, pH 6.0–6.75, 0.2 M ammonium sulfate, and 10–16% (wt/vol) PEG 5000 monomethyl ether (MME)]. The crystals

typically grew to 80–120 μm × 50 μm × 10–20 μm in 2 wk. The crystals were cryoprotected with 0.1 M MES (pH 6.5), 0.2 M ammonium sulfate, 10–16% (wt/vol) PEG 5000 MME, 0.1 M NaCl, and 26% (vol/vol) ethylene glycol and flash-cooled in liquid nitrogen. Crystals of the selenomethionine-labeled complex were grown similarly.

X-Ray Diffraction Data Collection and Structure Determination. The X-ray diffraction data of the native and selenomethionine crystals were collected at 100 K at Advanced Light Source beamline 8.2.1. All X-ray diffraction data were indexed, integrated, and scaled using the HKL-2000 package (21). The single-wavelength anomalous diffraction dataset was solved using AutoSol of the PHENIX package (22). The structure was refined by iterative model building in Coot (23) and the PHENIX refinement module. The qualities of the final models were assessed with MolProbity (24); 97.6% of all ϕ and ψ geometries were in the most favored region, with no outliers in the Ramachandran statistics. An electrostatic potential map was calculated using ABPS (25). All structure figures were generated using PyMOL (Schrödinger).

LC3-II Formation Assay in MEFs. MEFs stably expressing ATG3-T7 and mutant proteins were prepared using the pBABE-puro vector and Plat-E packaging cells, as described previously (26). Cells were starved in Hanks' balanced salt solution (Sigma-Aldrich) containing 20 μM chloroquine for 90 min, and cell lysates were subjected to Western blot analyses as described previously (8) using the following primary antibodies: anti-LC3 (MBL International; PM036), anti-ATG12 (Cell Signaling; 4180), anti-ATG3 (MBL International; M133-3), anti-T7 (Novagen; 69522), and anti-α-tubulin (Cell Signaling; 3873). Immunoreactive bands were visualized using a LI-COR Odyssey infrared imager and quantified using Image Studio 2.1 (LI-COR).

Immunoprecipitation Assay. Immunoprecipitation was performed as previously described using an anti-ATG3 antibody and MEFs stably expressing ATG3 constructs (8). Anti-ATG7 (Cell Signaling; D12B11) and antibodies described above were used for Western blot analyses.

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