Autophagy is a catabolic process that contributes to nutrient homeostasis and damage control in eukaryotic cells. During autophagy, cytoplasmatic materials, such as proteins, protein aggregates, damaged organelles and invasive bacteria, are encapsulated in double-membrane-bound vesicles called autophagosomes and transported to lysosomes for degradation. Autophagosomes form de novo in the cytoplasm, which is unusual for a vesicular system, but current descriptions of this process are limited to the morphological level. At the molecular level, many protein factors are known to participate in autophagosome formation. One category of these proteins includes the ubiquitin-like proteins of the LC3 family consisting of seven members in mammals (Atg8 in yeast) and the conserved ATG12 (Atg12 in yeast). Upon induction of autophagy, LC3 becomes conjugated to ATG5, a structural protein consisting of two ubiquitin-like fold domains (UFDs) and an α-helical bundle region (HBR) on which Lys130, the lysine used for conjugation, is located. The conjugation between ATG12 and ATG5 is essential for LC3 lipidation and therefore for autophagosome formation.

LC3 and ATG12 are conjugated to their respective targets by enzymatic cascades that are analogous to ubiquitination and involve the same E1 activation enzyme, ATG7, but different E2 conjugation enzymes, ATG3 and ATG10 for LC3 and ATG12, respectively. Although these cascades lack canonical E3 ligases such as RING domain–containing proteins, a recent biochemical study showed that the yeast Atg12-Atg5 conjugate facilitates Atg8 transfer from Atg3 to phosphatidyethanolamine in vitro. That report, together with the requirement of ATG12-ATG5 for LC3 lipidation in cells, has led to the recognition of ATG12-ATG5 as the E3 factor for LC3 lipidation. However, much less is understood about the physical role of the covalent linkage between ATG12 and ATG5, and about determinants required for E3 activity.

ATG3 has been reported to interact with ATG12 (refs. 20–22). ATG5 binds to ATG16L1 (Atg16 in yeast), the factor that recruits ATG12-ATG5 to sites of autophagosome formation. Because these interactions can occur independently of the conjugation between ATG12 and ATG5, the conjugation provides a simple means of recruiting ATG3 to autophagosomal membranes. This idea is supported by a recent report showing that forced localization of an artificial ATG16L1 construct at the plasma membrane resulted in LC3 lipidation at the plasma membrane. However, given that ATG12 is conjugated to the specific lysine of ATG5, ATG3 recruitment is unlikely to be the sole purpose of conjugation, unless the specific site is required only for formation of, but not for the function of, ATG12-ATG5. Canonical ubiquitin-like proteins modulate the function of target proteins often by conjugation to specific lysine sites. In several cases, structural evidence has suggested that conjugations of ubiquitin-like proteins also cause conformational changes in the target proteins or that the conjugate provides a new surface for the recruitment of other proteins to specific sites of the target protein. In the case of ATG12-ATG5, it remains to be established whether similar mechanisms operate in addition to ATG3 recruitment.

Here we set out to identify the role of the conjugation between ATG12 and ATG5 in LC3 lipidation. We demonstrate that the native conjugation moiety is crucial for E3 activity using artificial covalent linkages. The crystal structure of human ATG12-ATG5 in complex with the N-terminal region of ATG16L1 (referred to as ATG16N) shows that the conjugate forms an integrated architecture through
covalent and noncovalent contacts. Structural and mutational analyses suggest that both ATG12 and ATG5 are directly involved in E3 activity through residues that are assembled into a continuous surface patch upon conjugation. ATG12 also has another surface patch that is responsible for ATG3 binding. These findings establish the structural role of the covalent linkage in building an architecture required for E3 activity and provide insights into how the E2–E3 interaction occurs.

RESULTS
Native conjugation moiety is critical for E3 activity
To examine the importance of the specific conjugation site of ATG12–ATG5 for E3 activity, we tested the activity of a conjugate mimic generated by genetic tethering of ATG12 and ATG5. It has previously been reported that in Atg5 knockout (Atg5<sup>−/−</sup>) mouse embryonic fibroblasts (MEFs) LC3–phosphatidylethanolamine (also referred to as LC3-II in cellular contexts) is not detected<sup>13</sup>. This defect can be restored by expression of wild-type ATG5 but not by expression of the conjugation-incompetent mutant ATG5<sup>K130R</sup>, indicating that ATG12–ATG5 is necessary for LC3-II formation<sup>13</sup>. We obtained the same results with stable expression of 3×Flag–ATG5 using a retrovirus system (Fig. 1a). We then expressed 3×Flag–ATG12–ATG5<sup>K130R</sup> fusion construct, in which the C terminus of ATG12 is fused to the N terminus of ATG5<sup>K130R</sup> (through a 2×Gly–Gly–Ser linker (the K130R mutation prevents conjugation to endogenous ATG12), and assayed LC3-II formation. Despite expression of the expected fusion protein in cells, we did not detect LC3-II (Fig. 1a), even when we induced autophagy by starvation in the presence of chloroquine, a compound that prevents lysosomal degradation of LC3-II and therefore allows more sensitive detection of LC3-II<sup>59</sup>. We also tested the same fusion protein in an in vitro LC3 lipidation assay and confirmed that it was not active (Fig. 1b). These data indicate that the attachment of ATG12 to the N terminus of ATG5 does not generate a functional E3.

We next generated another conjugate mimic by chemically cross-linking ATG12 and ATG5 molecules at the native conjugation site and examined its E3 activity. To this end, we constructed ATG12 and ATG5 mutants whose residues used for native conjugation were replaced by cysteine (ATG12<sup>G140C</sup> and ATG5<sup>K130C</sup>) and native cysteine residues were changed to noncysteine amino acids, and cross-linked these mutant proteins with bismaleimidoethane (BMOE). As a control, we made a mutant conjugate containing the same substitutions for the native cysteine residues. Whereas this cysteine-less mutant exhibited an E3 activity in vitro (Fig. 1b), albeit slightly lower than that of the wild type, the BMOE–cross-linked conjugate mimic was not active (Fig. 1b), indicating that BMOE cross-linking caused a too large structural deviation from the functional form. These data support the importance of the native conjugation moiety and rationalize the need for structural investigation of ATG12–ATG5.

Structure determination of the ATG12–ATG5–ATG16N complex
To gain structural insights into the role of the covalent linkage, we crystallized human ATG12–ATG5 in complex with a 33-residue ATG16N construct. Crystals appeared under two different solution conditions and exhibited space groups, α root mean square (r.m.s.) deviation of all the Cα atoms among the three complex molecules (Supplementary Fig. 1a).

Architecture of the ATG12–ATG5–ATG16N complex
In the crystals, ATG12–ATG5–ATG16N formed a compact structure (Fig. 2a). As previously observed in the structure of Saccharomyces cerevisiae Atg5–Atg16N<sup>17,30</sup>, ATG16N is bound to a surface of ATG5 consisting of two UFDs (referred to as UFD–1 and UFD–2, which correspond to UblA and UblB, respectively, in the previous report<sup>15</sup>) in an α-helical conformation with a slight kink in the middle. ATG12 is located on ATG5 at the side opposite ATG16N, and there are no contacts between ATG12 and ATG16N. The electron density for the C-terminal residue Gly140 of ATG12 and that for Lys130 of ATG5 are clearly connected, validating our structure as the conjugated form (Supplementary Fig. 1b). There are no major conformational changes in ATG12 and ATG5 upon conjugation (Fig. 2b and Supplementary Fig. 1c). Although Arabidopsis thaliana ATG12b in the previous crystal structure formed a domain-swapped dimer<sup>30</sup>, ATG12 in our structure is monomeric. At a local level, there is a small change in ATG12 upon conjugation. In unconjugated A. thaliana ATG12b, the penultimate tryptophan residue in the C-terminal tail makes contacts with the other monomer in the domain-swapped dimer but not with its own UFD<sup>30</sup>. In our structure, the C-terminal tail of ATG12 folds back onto its own UFD through rearranged side chain–side chain interactions that include contacts between the penultimate tryptophan Trp139 and

![Figure 1](https://example.com/f1.png)

**Figure 1** Artificial mimics of ATG12–ATG5 do not promote LC3 lipidation. (a) Western blots of LC3-II formation assays carried out in Atg5<sup>−/−</sup> MEFs stably transduced with the pMX-puro retroviral vectors expressing the indicated 3×Flag-tagged constructs under nonstarvation (−) and starvation (+) conditions. (b) SDS-PAGE of in vitro LC3 lipidation assays carried out with the indicated ATG12–ATG5 conjugate mimics complexed with ATG16N.
Tyr103 of its own UFD (Fig. 2b). Such conformation of the C-terminal tail of ATG12 has not been observed among structures of ubiquitin-like proteins; the C terminus of canonical ubiquitin-like proteins lacks such an aromatic residue and is considered to be flexible.\(^{31}\)

The noncovalent contacts between ATG12 and ATG5 bury a total of 1,300 Å\(^2\) of solvent-accessible surface area (Fig. 2c). The aliphatic atoms of the side chain of Lys130 are buried in this interface, indicating that Lys130 not only provides its ε-amino group for isopeptide bonding to ATG12 but also has a structural role as part of the interface. A patch on ATG12 comprising the turn-loop-turn-α\(^2\) segment (Asn105–Phe123) is associated with the interaction surface on ATG5 that is formed by residues from several regions in its primary structure, including UFD-1 (Gly14–Lys105), the loop connecting UFD-1 and HBR (S106–S117), α4 of HBR (Lys118–His137) and the loop of UFD-2 (Phe198–Leu202). This turn-loop-turn-α\(^2\) segment is the region that was exchanged between the two molecules in the Arabidopsis ATG12b dimer (Fig. 2b and Supplementary Fig. 1d), indicating that ATG12 may be stabilized upon conjugation. This idea is supported by our observation that unconjugated ATG12 formed oligomers slowly during purification and storage in solution (Supplementary Fig. 2).

**Table 1 Data collection, phasing and refinement statistics**

|                          | ATG12–ATG5–ATG16N: crystal I\(^a\) | ATG12–ATG5–ATG16N: crystal II\(^a\) | ATG12–ATG5–ATG16N(I17M L21M I36M L43M)\(^b\) |
|--------------------------|--------------------------------------|--------------------------------------|-----------------------------------------------|
| **Data collection**       |                                      |                                      |                                               |
| Space group               | \(P2_1,2,2_1\)                        | \(C2\)                               |                                               |
| Cell dimensions \(a, b, c\) (Å) | 43.8, 113.2, 210.7                   | 135.1, 58.9, 90.8                   |                                               |
| \(\alpha, \beta, \gamma\) (°) | 90, 90, 90                           | 90, 129.1, 90                        |                                               |
| Wavelength (Å)            | 1.0000                               | 1.0092                               |                                               |
| Resolution (Å)\(^c\)      | 50.0–2.7 (2.80–2.70)                 | 50.0–2.9 (3.00–2.90)                 | 50.0–2.9 (3.00–2.90)                         |
| \(R_{merge}\)             | 10.0 (63.6)                          | 5.6 (54.1)                           | 6.7 (33.5)                                   |
| \(I / \sigma_I\)          | 18.5 (2.7)                           | 22.6 (1.9)                           | 24.0 (5.3)                                   |
| Completeness (%)\(^c\)    | 99.9 (100.0)                         | 98.6 (92.4)                          | 99.9 (99.4)                                  |
| Redundancy\(^c\)          | 6.9 (6.1)                            | 4.4 (3.7)                            | 7.2 (6.6)                                    |
| **Refinement**            |                                      |                                      |                                               |
| Resolution (Å)            | 44.1–2.7                             | 36.2–2.9                             |                                               |
| No. reflections           | 29,598                               | 12,465                               |                                               |
| \(R_{work} / R_{free}\)   | 17.6 / 23.6                          | 21.5 / 25.4                          |                                               |
| No. atoms                 | Protein: 6,469                       | 3,226                                |                                               |
|                          | Ligand/ion: 2                        | 1                                    |                                               |
|                          | Water: 76                            | 0                                    |                                               |
| B-factors                | Protein: 67.5                        | 138.8                                |                                               |
|                          | Ligand/ion: 58.9                     | 121.6                                |                                               |
|                          | Water: 50.1                          | n/a                                  |                                               |
| r.m.s. deviations         | Bond lengths (Å): 0.003               | 0.006                                |                                               |
|                          | Bond angles (°): 0.66                 | 0.51                                 |                                               |

\(^a\)Native. \(^b\)Selenomethionine. \(^c\)Values in parentheses are for the highest-resolution shell. Each data set was collected from one crystal.

**Figure 2** The crystal structure of human ATG12–ATG5–ATG16N. (a) Cartoon representation of the ATG12–ATG5–ATG16N structure. ATG12 and ATG16N are colored yellow and red, respectively. ATG5 is shown in three colors to represent three subdomains: gray, wheat and blue for UFD-1, HBR and UFD-2, respectively. Protein N termini are indicated with the letter N. This view is defined as the front view throughout the rest of the figures. (b) Superimposition of the “monomeric form” of Arabidopsis ATG12b (PDB 1WZ3; turquoise) onto ATG12 (yellow) of the ATG12–ATG5–ATG16N structure. Residues around the C-terminal tail of ATG12 are shown as stick models. Residues in the first turn of the turn-loop-turn-α\(^2\) segment of ATG12 and the corresponding ones of Arabidopsis ATG12b are indicated as spheres at the Cα positions. Ser60 and Ala61 of Arabidopsis ATG12b are not connected because they belong to different chains in the domain-swapped Arabidopsis ATG12b dimer. (c) Enlarged approximately top (left) and bottom (right) views of the interface.
of ATG12, whose corresponding residue in yeast Atg12 has previously been suggested to be important for autophagy,32 is located in the center of the interface patch of ATG12 and makes contacts with Glu131 and Gln114 in α4 of ATG5. There is an intermolecular hydrogen bond between the side chains of this Glu131 and Gln114 in α4 of ATG12 as well as a salt bridge between the side chains of Asp113 of ATG12 and His80 in UFD-1 of ATG5, which are located at a distal site from the conjugation site (Fig. 2c). Evaluation of the properties of this interface using PDBProtein33 revealed that the interface is hydrophobic, favorable for association, and unlikely to be a specific interaction site, as indicated by ∆G (change in the total solvation energy upon complexation) of −6.8 kcal mol⁻¹, a ∆G P value (P-value of the observed solvation free-energy gain) of 0.25 and a complex formation significance score of 0, respectively. Consistent with this result, we did not observe an interaction between unconjugated ATG12 and ATG5 molecules. However, the interface between ATG12 and ATG5 is unlikely to be a crystallographic artifact because essentially the same structure has been observed in two different space groups; the only appreciable difference between the two crystal structures is at the loop between β1 and β2 in UFD-1 of ATG5, which is involved in crystal packing in the C2 but not in the P2₁2₁2₁ crystal and is far away from the ATG12–ATG5 interface (Supplementary Fig. 1a). Based on these analyses and considerations, we propose that the architecture of ATG12–ATG5 is based on the stabilization of weak and less specific but favorable hydrophobic interfaces by the covalent linkage and the fixed conformation of the C-terminal tail of ATG12.

Figure 3 Identification of potential functional patches on ATG12–ATG5 by conservation mapping. Surface representations of ATG12–ATG5 (left top and right top images for the front and approximately back views, respectively) as well as ATG12 (left bottom) and ATG5 (right bottom) that are isolated from the ATG12–ATG5 structure are shown with colors according to the conservation scores obtained from the Consurf server.44 Analysis was performed using 50 and 45 sequences of ATG12 and ATG5, respectively (Supplementary Fig. 5). On the surfaces of the isolated ATG12 and ATG5, the interfaces in ATG12–ATG5 are indicated by black borders. Highly conserved residues as well as less conserved residues that are isolated from the ATG12–ATG5 structure are shown with colors according to the conservation scores obtained from the ConSurf server.44

Figure 4 Conserved residues of ATG12–ATG5 are important for E3 activity. (a) SDS-PAGE of an in vitro kinetic LC3 lipidation assay carried out with wild-type ATG12–ATG5–ATG16N. (b,c) The quantified data of the in vitro kinetic LC3 lipidation assays carried out with ATG12–ATG5–ATG16N containing mutations in ATG5 (b) or ATG12 (c). Raw data for b,c are shown in Supplementary Figure 3. (d) Mapping of the results of the in vitro mutagenesis data onto the structure. The residues that have been affected by mutation are shown in the same color as indicated on the right of the description for each mutation in b and c.
Perturbation of the ATG12–ATG5 interface affects E3 activity

Having revealed the architecture of ATG12–ATG5, we sought to establish its functional importance. Conservation mapping on the surface of each molecule revealed that the ATG12–ATG5 interface contains highly conserved residues, including Phe108 and Asp113 of ATG12, and His80, Leu113, Ser127, Glu131, Arg188 and Gln200 of ATG5 (Fig. 3); this high level of conservation at the interface suggests that there is evolutionary pressure on the ATG12–ATG5 architecture. To confirm the importance of the interface, we mutated these conserved residues and some additional less conserved peripheral residues and tested E3 activity of the mutants in vitro (Fig. 4 and Supplementary Fig. 3). We included amino acids that are bulkier or have opposite chemical properties compared to the native ones because we predicted that such mutations would interfere with the packing at the interface and thus sterically disrupt the architecture more than alanine would. The results agree well with this prediction: the mutations D113V and C122W in ATG12, and H80L, S127L and E131A in ATG5 severely impaired E3 activity, whereas the effects of the mutations to alanine such as H80A and L113A in ATG5 were moderate, and E131A in ATG5 had no effect. In addition to those mutations, we attempted to test F108A and F108D in ATG12, and E131F in ATG5 but could not do so because these mutations impaired the production of the conjugate in *Escherichia coli*.

We extended the mutagenesis study to the cellular context to confirm our in vitro results. To test ATG5 mutations in cells, we expressed 3×Flag-ATG5 mutants in *Atg5<sup>−/−</sup>* MEFs. Mutations at the interface, such as H80A, H80L, S127L, A134E, L135R and Q200W decreased LC3-II formation, and some combinations of these single mutations had more severe effects (Fig. 5a). The decreased amounts of some ATG12–3×Flag-ATG5 mutants, such as of H80L and of a few double mutants, are unlikely to affect the interpretation because it has previously been demonstrated that amounts of ATG12–ATG5 as low as the detection limit of western blotting are sufficient for LC3-II formation. The mutations E131A and E131G showed little effect in MEFs. Overall, these data are consistent with the in vitro results described above. Single mutations L113D and E131F, and double mutation H80A L135R abolished the formation of ATG12–3×Flag-ATG5. We presume that these mutations destabilize the interface in a way that is incompatible with the formation of ATG12–ATG5 or the protein folds.

To test ATG12 mutations in cells, we used wild-type MEFs. When we stably expressed 3×Flag-ATG12 in wild-type MEFs, we detected 3×Flag-ATG12–ATG5 and additional 3×Flag-ATG12–containing bands as well as LC3-II–containing bands (Fig. 5b). This observation is consistent with a previous report in which stable expressions of ATG12 in mammalian cells using a retrovirus system similarly generated multiple ATG12-containing species but did not affect autophagy. However, we found that the expression of 3×Flag-ATG12 resulted in ~75% reduction in the amount of LC3-II compared to the controls (Fig. 5b). This reduced amount of LC3-II was likely due to the presence of unconjugated 3×Flag-ATG12 in the cells because expression of a conjugation-incompetent form, 3×Flag-ATG12<sup>M145D</sup>, also showed a reduction similar to that for the wild type. This interpretation...
is consistent with a previous report that transient overexpression of ATG12 in mammalian cells had abolished LC3-II formation. While performing this experiment, we noticed that expression of ATG12 caused a downregulation of endogenous ATG12–ATG5 to a level undetectable in western blots, an effect not discussed in literature previously. These observations suggested that despite the smaller dynamic range described above, the effect of a mutation in ATG12 on LC3-II formation could be examined in wild-type MEFs, as long as the expressed 3xFlag-ATG12 mutant conjugates with the endogenous ATG5 efficiently enough to knock down the native ATG12–ATG5 as observed with wild-type ATG12 expression. Mutation of most of residues at the interface, such as Phe108, Asp113 and Phe123 in ATG12, did not knock down endogenous ATG12–ATG5 (Fig. 5b), presumably owing to inefficient conjugation to ATG5; as a result, we could not obtain information on the effects of these mutations on LC3-II production. However, the C122W mutant efficiently conjugated to ATG5 and abolished the endogenous ATG12–ATG5. LC3-II formation was severely inhibited by C122W, consistent with the in vitro data described above. Unconjugated mutants F108A, F108D, F108R and F123D were not detectable (Fig. 5b), indicating that these mutations destabilized the protein. In contrast, the F108R mutant was detectable and conjugated to ATG5. Thus, the hydrophobic nature of Phe108 seems to be necessary for protein stability and conjugation to ATG5. But the conjugation was not efficient enough to knock down the endogenous ATG12–ATG5. These observations contrast to ATG5. But the conjugation was not efficient enough to knock down endogenous ATG12–ATG5. LC3-II formation could be examined in wild-type MEFs, whereas other single mutations affected LC3-II formation less severely affected the activity. The mutation of these residues, as well as of Trp73, located adjacent to these two residues on the surface, severely affected E3 activity only slightly (Fig. 4b). In MEFs, the mutations G63D and Q106A severely impaired LC3-II formation, whereas other single mutations affected LC3-II formation less severely (Fig. 5a). Double mutants based on these less effective mutations abolished LC3-II formation both in vitro and in MEFs (Figs. 4c,d and 5b,c); therefore, these residues make up another functional patch.

ATG12 is responsible for high-affinity binding to ATG3

Understanding how ATG12–ATG5 interacts with ATG3 is essential for a mechanistic description of the E3 activity of the conjugate.
Previous data suggest that both ATG12–ATG5 and the unconjugated ATG12 can bind to ATG3 (refs. 19–22). However, how these interactions are related to the E3 activity of ATG12–ATG5 and how ATG5 is involved in this interaction are unclear. To probe the role of ATG5 in the interaction between ATG12–ATG5 and ATG3, we examined the binding abilities of conjugated and unconjugated proteins using isothermal titration calorimetry (ITC). ATG12–ATG5–ATG16N binds tightly to ATG3 with a $K_d$ of 51 nM, whereas unconjugated ATG12 binds to ATG3 with a slightly higher $K_d$ of 117 nM (Fig. 6a,b). However, under our experimental conditions, unconjugated ATG5–ATG16N did not show detectable binding to ATG3 (Fig. 6c). These results establish that the direct contribution of ATG5 to ATG3 binding is very small, if such a contribution exists at all. To identify the high-affinity interaction site, we assayed the binding of ATG3 to ATG12–ATG5–ATG16N complexes containing mutations in ATG12. The results show that mutations in the surface patch exclusively on ATG12 affected the binding markedly; the K54D and K72D mutations resulted in greater than 100-fold increases in $K_d$ values, and the W73A mutant showed no detectable binding (Fig. 6d–f). In contrast, ATG12 mutations in the interface to ATG5 (D1113V, C122W and A134E) or in the continuous patch (V62R, G63D, S107W, A138R and W139Y) resulted in only minor (less than 2.5-fold) differences in $K_d$ values (Supplementary Fig. 4). We also examined some of the ATG5 mutations that had severely impaired the E3 activity both in vitro and in MEFs (H80L, K138D, M145D, S127L A134E and K138A Q146A) and confirmed that these ATG5 mutations did not affect the binding (Supplementary Fig. 4).

We verified the effects of the mutations in ATG12 on ATG3 interaction in cells using immunoprecipitation. We immunoprecipitated ATG12 mutant proteins in lysates of the MEFs described above using anti-Flag antibody. For this assay, we choose the mutants with mutated sites located on the conjugate surface and that exhibited impaired LC3-II formation in cells. Both 3×Flag-ATG12–ATG5 and 3×Flag-ATG12 were detected in the immunoprecipitates (Fig. 7). Endogenous ATG3 precipitated with wild-type ATG12 but not with the control, demonstrating the interaction between these proteins. The immunoprecipitates containing the mutant K54D, K72D or W73A, had markedly less ATG3, whereas a similar amount of ATG3 as for wild type was observed for G63D. The quantification of these immunocomplexes indicates that ATG3 interaction was severely weakened by the former three mutations but not much by G63D. These data agree well with the in vitro binding data described above. Thus, we conclude that the ATG12-exclusive patch including Lys54, Lys72 and Trp73 is the high-affinity binding site for ATG3, and the patch across ATG12 and ATG5 has a different role in the E3 function.

**DISCUSSION**

Our structural and mutational analyses of ATG12–ATG5–ATG16N revealed that the conjugation generates a patch across ATG12 and ATG5 required for E3 activity. This finding explains why the ATG12–ATG5 fusion construct was not functional; the ATG12 in the fusion that was attached to the N terminus of ATG5 was too far away from its original position in ATG12–ATG5 to form such a functional patch even with a flexible 2×Gly-Gly-Ser linker inserted between ATG12 and ATG5. The BMOE–cross-linked conjugate mimic was inactive perhaps because it could not form the native ATG12–ATG5 architecture owing to the increased number of the chemical bonds in the covalent linkage (eight bonds more than the native isopeptide bond). Alternatively, the extra atoms of BMOE may have caused steric hindrance for a binding partner during the E3 function, as the native conjugation site is located in the continuous patch. Our mutational analyses also suggested that the interface between ATG12 and ATG5 is important for E3 activity. Although mutations in the interface that exhibited negative effects are likely to have destabilized the ATG12–ATG5 architecture and thus disrupted the continuous patch, we do not know the precise mechanisms underlying the effects by each mutation. Investigations on these mutants as well as the BMOE conjugate would provide additional insights into the mechanism of the E3 activity of ATG12–ATG5.

We identified a surface location on ATG12 at the side opposite from the interface to ATG5 as the hotspot for ATG3 interaction and demonstrated that the residues in the hotspot are important for E3 activity. These data, however, challenge a previous report in which Phe108 of ATG12 was shown to be necessary for ATG3 interaction. We identified a surface location on ATG12 at the side opposite from the interface to ATG5 as the hotspot for ATG3 interaction and demonstrated that the residues in the hotspot are important for E3 activity. These data, however, challenge a previous report in which Phe108 of ATG12 was shown to be necessary for ATG3 interaction. This previous conclusion was drawn from the result of an immunoprecipitation experiment using HEK293A cells overexpressing Phe108 mutants of ATG12. As described above, in our structure Phe108 is located at the interface with ATG5. In principle, it is possible that the mutations of Phe108 allosterically affect ATG3 binding, which occurs at the side opposite Phe108. However, given the aggregation-prone nature of the Phe108 mutants, we suggest that the previous observation might have resulted from aggregation or destabilization of the expressed mutant proteins in cells. Ultimately, these contradictory data should be clarified by careful characterizations of the Phe108 mutants.

The next question regarding the E3 function of ATG12–ATG5 is exactly how this conjugate interacts with ATG3 or LC3-loaded ATG3 and facilitates the transfer of LC3 to phosphatidylethanolamine. Unlike E3 ubiquitin-ligases, ATG12–ATG5 does not appear to contribute specificity in substrate recognition; ATG3 alone can conjugate LC3 to phosphatidylethanolamine in vitro, albeit at much slower rates than in the presence of ATG12–ATG5 (refs. 19,36–38). Thus, ATG12–ATG5 may function as an E2-stimulating machine analogously to RING domains in ubiquitin ligases. Although ATG12 alone binds tightly to ATG3, ATG12 alone is not sufficient for E3 activity, suggesting that the high-affinity binding site for ATG3 does not stimulate ATG3. Rather, we suggest the possibility that the patch across ATG12 and ATG5 serves this purpose. Our data showing that the affinity of ATG12–ATG5 for ATG3 is only slightly higher than that of ATG12 alone (Fig. 6) indicate that the continuous patch does not contribute much to the affinity for ATG3. However, such low affinity of the patch may be consistent with the typically observed low affinities ($K_d$ values of micromolar to millimolar)
between RING domains and E2 enzymes for ubiquitinination\textsuperscript{39}. Alternatively, the continuous patch may function in stabilizing a conformation of LC3-charged ATG3 in an optimal orientation to enhance conjugation, as previously proposed for SUMO and ubiquitin systems\textsuperscript{40–43}. Answers to these questions will be provided by future structural and biochemical studies on larger complexes.

**METHODS**

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

**Accession codes.** The structures of human ATG12–ATG5–ATG16N have been deposited to the Protein Data Bank under the accession codes 4G DK and 4GDL.

*Note: Supplementary information is available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

C.O. performed protein preparation, crystallization and cell biology, and assisted with biochemistry; Z.M. assisted with structure refinement, biochemical analyses and manuscript preparation; G.T. managed cell biological assays; T.O. conceived the project and performed structure determination, in vitro biochemistry and ITC analyses, and wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Preparation of the ATG12–ATG5–ATG16N complex. All proteins used in this work are from human. The ATG16L1 Δ1–140 (ATG16N)-coding DNA sequence was cloned into the NdeI-XhoI site of a modified pET-Duet-1 vector (Novagen); the resulting vector expresses the 6×His-maltose binding protein (MBP)-tobacco etch virus (TEV) protease cleavage sequence (TEV)-ATG16N fusion. E. coli BL21 (DE3) cells transformed with this vector were grown in Luria broth medium, and protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD60 at 0.8, after which the cells were grown at 37 °C for 3 h and then collected. The 6×His-MBP-TEVs-ATG16N fusion was purified by nickel affinity chromatography (Qiagen) followed by Source 15S cation-exchange chromatography (GE Healthcare). The purified protein was stored at −80 °C until use. The full-length ATG7 and ATG10-coding DNA sequences were cloned into the Ncol-Sall and Ndel-Xhol sites, respectively, of a modified pCDF-Duet-1 vector (Novagen); the resulting vector expresses glutathione S-transferase–fused ATG7 and ATG10. The ATG12Δ2–140 and full-length ATG5–coding DNA sequences were cloned into the BamHI-Sall and Ndel-Xhol sites, respectively, of a modified pACYC Duet-1 vector (Novagen); the resulting vector expresses the MBP-6×His-TEVs-ATG12Δ2–140 fusion and ATG5. BL21 (DE3) cells co-transformed with these two plasmids were grown in Terrific broth medium, and protein expression was induced with 0.2 mM IPTG at an OD60 of 2.4. Then, the cells were grown at 18 °C and collected after 18 h. The MBP-6×His-TEVs-ATG12–ATG5 fusion was purified by nickel affinity chromatography and Superdex 200 (SD200) size-exclusion chromatography (GE Healthcare), treated with TEV protease and separated by Source 15S cation-exchange chromatography. Then, a 1.2-fold molar excess of 6×His-MBP-TEVs-ATG16N was mixed with ATG12–ATG5 and treated with TEV protease. Lastly, the cleaved protein was purified on Source 15S and SD200 columns. Mutations in ATG12 or ATG5 were incorporated into the coding sequences using a two-step PCR method, and mutant complexes were generated similarly to the way wild-type ones were. None of the purified mutant complexes showed aggregation in this work are described in the Supplementary Note.

Crystalization of the ATG12–ATG5–ATG16N complex. Crystals of the native human ATG12–ATG5–ATG16N complex were grown at 20 °C using the sitting-drop vapor-diffusion method from drops containing 1 μl of 10 mg ml⁻¹ protein in 10 mM Hapes, pH 7.0, 300 mM NaCl and 1 mM DTT; and 1 μl of reservoir solution: 0.1 M Hapes, pH 7.5, 0.2 M lithium citrate and 20% (v/v) PEG 3350 for crystal I and 0.1 M BICINE, pH 9.0, 0.2 M sodium phosphate dibasic, and 18% (v/v) PEG 3350 for crystal II. Under these conditions, the crystals typically grew to 80–120 μm × 50 μm × 10–20 μm in 5 d. The crystals were cryoprotected with 0.1 M Hapes, pH 7.5, 0.2 M lithium citrate, 21.5% (v/v) PEG 3350, 0.1 M NaCl and 25% (v/v) ethylene glycol for crystal I and 0.1 M MES, pH 6.5, 0.2 M sodium phosphate dibasic, 19.5% (v/v) PEG 3350, and 20% (v/v) ethylene glycol for crystal II and flash-cooled in liquid nitrogen. The crystals I and II contained two and one complexes in the asymmetric unit, respectively. Crystals of the native ATG12–ATG5 in complex with the selenomethionine-labeled ATG16NΔ51–173Δ1721–121MΔ136Δ1438 mutant were grown at 4 °C from drops containing 1 μl of 9 mg ml⁻¹ protein in 10 mM Hapes, pH 7.0, 300 mM NaCl and 5 mM DTT and 1 μl of reservoir solution (0.1 M Hapes, pH 7.0, 0.2 M lithium citrate and 18% (v/v) PEG 3350) and cryoprotected with 0.1 M Hapes, pH 7.0, 0.2 M lithium citrate, 20% (v/v) PEG 3350, 0.1 M NaCl and 25% (v/v) ethylene glycol, and flash-cooled in liquid nitrogen.

X-ray diffraction data collection and structure determination. The X-ray diffraction data of the native crystals I and II, and the selenomethionine crystal were collected at 100 K at the beamlines APS 22-BM, ALS 5.0.2 and ALS 8.2.1, respectively. All X-ray diffraction data were indexed, integrated and scaled using the HKL-2000 package. The multilayered wavelength anomalous diffraction (MAD) data set was solved and an initial model was built using the AutoSol and AutoBuild modules of the PHENIX package, respectively. Because the selenomethionine crystal was not isomorphous with respect to the native crystals, the initial model from the MAD data was used for molecular replacement of the native data sets using AutoMR in PHENIX. The structures were refined by iterative model building in Coot and refinement using the PHENIX refinement module. The qualities of the final models were assessed with MolProbity, 95.3–97.0% of all φ and ψ geometries were in the most favored region, with less than 0.3% outliers in the Ramachandran statistics. All structure figures were generated using PyMOL (Schrödinger).

In vitro LC3 lipidation assay. Synthetic 1,2-dioleoyl-sn-glycerol-3-phosphocholine (PC) and 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (PE), and bovine liver 1-α-phosphatidylinositol (pIP) were purchased from Avanti Polar Lipids. Lipids were mixed with a molar ratio of 40% PC, 40% PE and 20% pIP, and were dried under a nitrogen gas stream and under vacuum for 2 h. Lipids were hydrated in 20 mM Hapes, pH 7.5, and 100 mM NaCl for 1 h at room temperature, freeze-thawed three times using dry ice and 42 °C water bath, and extruded through a 100 μm polycarbonate filter using a mini extruder (Avanti Polar Lipids). Liposomes were used within 2 d after extrusion. For the assay, 0.5 µM ATG7, 1 µM ATG3, 1 µM ATG12–ATG5–ATG16N and 10 µM LC3B1–120 (matured form) were mixed with 1 mM liposomes in 50 mM Hapes, pH 7.5, 100 mM NaCl, 0.3 mM DTT and 1 mM MgCl₂. Reactions were initiated by adding 1 mM ATP and were performed at 37 °C. The reactions were quenched at various time points by adding SDS-PAGE sample buffer (2×). Proteins were separated by 6 M urea SDS-PAGE and stained with GelCode Blue (Thermo Pierce). The quarter, half or same volume of the time zero sample as other time points were loaded into three separate lanes to obtain a calibration curve for the quantification of LC3B in each gel. Gels were scanned on a LI-COR Odyssey infrared imager, and the bands corresponding to LC3B–phosphatidylethanolamine were quantified using the calibration curve obtained for the unconjugated LC3B from each gel using Image Studio 2.0 (LI-COR).

LC3-II formation assay in MEFs. MEFs stably expressing 3×Flag-ATG5 (full-length), 3×Flag-ATG12 (full-length) and mutant proteins were prepared using the pMXs-puro vector and Plat-E packaging cells, as described previously. Atg5/−/− MEFs were used as wild-type in this work. Cells were cultured at 37 °C with 5% CO₂ in a humidified incubator. For starvation, the cell medium was changed from Dulbecco’s Modified Eagle’s medium (DMEM; Life Technologies) to Hank’s balanced salt solution (Sigma-Aldrich) containing 20 mM choline. After 90 min, the cells were washed with phosphate-buffered saline on ice and lysed by adding the lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 2% (v/v) Triton X-100) containing Halt Protease and Phosphatase Inhibitor cocktail (Thermo Scientific). Lysates were centrifuged at 28,500 for 15 min at 4 °C. Total protein concentrations of the supernatants were determined using the BCA assay (Pierce). SDS-PAGE sample buffer (2×) was added to the supernatants, and the samples were boiled at 100 °C for 5 min. Twenty micrograms of total protein was separated on 4–20% (w/v) acrylamide gradient gels and subjected to western blot analyses. The antibodies used were anti-LC3 (MBL International, PM036), anti-ATG12 (Cell Signaling, 4180), anti–Flag M2 (Sigma-Aldrich, F1804) and anti–α-tubulin (Cell Signaling, 8378). Immunoreactive bands corresponding to LC3-II and α-tubulin were quantified.

Immunoprecipitation assay. MEFs stably expressing ATG12 constructs were cultured in DMEM, and cell lysates were prepared as described above. Total 500 μg protein in 300 μl of the lysis buffer was incubated with anti–Flag M2 antibody-loaded Dynabeads protein G (Life Technologies) for 20 min at room temperature. Beads were washed four times with 400 μl of the wash buffer (20 mM Tris pH 7.5, 200 mM NaCl, 2 mM EDTA and 0.1% (v/v) Triton X-100). Input and final beads samples were subjected to SDS-PAGE followed by western blot analyses using anti-ATG12 and anti-ATG3 (MBL International, M133-3) antibodies. The bands were quantified in Image Studio 2.1.

Isothermal titration calorimetry experiments. All protein samples for ITC experiments were dialyzed against buffer containing 10 mM Hapes, pH 7.0, 200 mM NaCl and 2 mM TCEP overnight. All titrations were carried out at 25 °C in a VP-ITC isothermal titration calorimeter (Microcal). Approximately 6.5 μM ATG12, ATG5–ATG16N or ATG12–ATG5–ATG16N was placed in the cell. Data were fit with the model for one set of binding sites in Origin (OriginLab).
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