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Crystallographic Characterization of ATG Proteins and Their Interacting Partners

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Abstract

Autophagosome formation and specific substrate recruitment during Autophagy require ligation of the ubiquitin-like protein (UBL) Atg8 to the head group of the lipid phosphatidylethanolamine (PE). Atg8 lipidation is mediated by distinctive UBL cascades involving autophagy-specific E1, E2, and E3 enzymes that differ substantially in sequence from components of other UBL conjugation cascades. Structural studies are important for elucidating the roles of Atg proteins that regulate multiple steps involved in autophagy. This chapter describes methods to prepare and crystallize selected proteins and complexes involved in autophagy UBL conjugation pathways, as a guide for strategies for structural and biochemical characterization of Atg proteins.

Keywords

Autophagy; Crystallization; ATG; Lipidation; Ubiquitin-like proteins

1. Introduction

Autophagy is characterized by the formation of double-membrane structures called autophagosomes, which sequester the cellular components to be degraded (Mizushima, 2007). This process comprises phagophore initiation and vesicle nucleation, vesicle elongation, fusion with lysosome/vacuole, and degradation (Kraft and Martens, 2012; Levine and Klionsky, 2004). Each step is tightly regulated by numerous proteins, including at least 15 distinct autophagy-related (Atg) proteins that are core components of many forms of autophagy (Hurley and Schulman, 2014; Klionsky and Schulman, 2014; Mizushima et al., 2011). Of these, Atg12 and Atg8 are ubiquitin-like proteins (UBLs) required for autophagosome formation and recruitment of specific substrates (e.g., protein aggregates and damaged organelles) to autophagosomes (Geng and Klionsky, 2008). In mammals, 6 subfamilies of Atg8, including LC3, GABARAP, and GATE-16 proteins (for simplicity, referred to as Atg8 hereafter) have been identified (Geng and Klionsky, 2008; Schulman and

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Harper, 2009) (Ohsumi and Mizushima, 2004). Similar to ubiquitin, Atg12 and Atg8 are ligated to their targets via E1–E2–E3 enzyme cascades. Atg12 is unusual in that after activation by the E1 enzyme Atg7, it is transferred to its E2 enzyme Atg10 and is then directly ligated to its protein substrate Atg5 without the involvement of an E3 enzyme (Figure 1A–1C). Unlike Atg12, Atg8 is processed by the cysteine protease Atg4 to expose its C-terminal glycine, activated by the E1 enzyme Atg7, and then transferred to its E2 enzyme Atg3. This results in a transient Atg3~Atg8 intermediate in which the C-terminus of Atg8 is linked by a covalent but reactive thioester bond to the catalytic cysteine of Atg3 (“~” represents a covalent bond). Atg8 is then transferred from Atg3 to the head group of the lipid phosphatidylethanolamine (PE). This reaction is catalyzed by a multi-protein E3 enzyme composed of the product of the Atg12 ligation reaction bound to Atg16 (Atg12~Atg5-Atg16) (Figure 1D–1F) (Geng and Klionsky, 2008; Nakatogawa et al., 2009; Ohsumi and Mizushima, 2004). Mutations of the genes involved in these cascades are observed in human diseases such as ataxia, asthma and Crohn’s disease (Barrett et al., 2008; Hampe et al., 2007; Kim et al., 2016; Martin et al., 2012; Poon et al., 2012; Rioux et al., 2007).

Given the important role of the UBL conjugation system in autophagy and its close association with human health, the modulation of protein function in the Atg8 lipidation pathway through chemical inhibition of the E1–E2–E3 cascade has become an area of significant interest. Several promising autophagy inhibitors have been used in clinical trials for cancer (Jiang and Mizushima, 2014), and a recent study showed that an intestinal tissue-specific knockout of Atg7 prevents tumor initiation (Levy et al., 2015).

Structures of enzymes in this pathway have greatly enhanced our understanding of the molecular mechanisms underlying autophagy, and may facilitate the development of approaches to manipulate autophagy to treat diseases in the future. In this chapter, we present the strategy and detailed methodology for crystallizing several Atg proteins and their interacting partners involved in the Autophagy UBL conjugation pathway.

2. Domain structures of Atg proteins in the UBL conjugation pathway

Seven proteins or protein conjugates are involved in the UBL conjugation cascade for autophagy: Atg7 (E1), Atg3 (E2), Atg10 (E2), Atg12~Atg5-Atg16 (E3), Atg12 (UBL), Atg8 (UBL), and Atg4 (de-conjugation enzyme). These E1, E2, and E3 enzymes are “non-canonical,” because they differ in many respects from the “canonical” E1, E2, and E3 enzymes such as those for ubiquitin, SUMO, and NEDD8. Canonical E1 enzymes contain three domains: a pseudosymmetric UBL adenylation domain for Ubl activation; a separate catalytic cysteine-containing domain for Ubl thiolester formation; and a ubiquitin-fold domain that recruits the E2 for transfer of the UBL from the catalytic cysteine on the E1 to that on the E2. In contrast, Atg7 is a symmetric homodimer, also defined by three distinct domains: a C-terminal domain (CTD) that houses both the adenylation domain and the catalytic cysteine; a flexible extreme-CTD (ECTD) extension; and, a unique N-terminal domain (NTD) responsible for recruiting the E2s. Atg7 homodimerization is mediated via its CTD (Figure 2). Atg3 and Atg10 contain unique catalytic residues around the catalytic cysteine, and they have additional secondary structures inserted within the E2 fold (Figure

2). Atg12~Atg5–Atg16 is a multi-protein complex (Figure 2) that functions as an E3 enzyme to ligate Atg8 to the head group of PE. This is in contrast to other canonical E3 enzymes that ligate their UBLs to a lysine residue of a protein substrate. The Atg12~Atg5–Atg16 complex does not share sequence similarity with any known UBL E3 ligases. Given these differences between the autophagy and canonical UBL conjugation systems, it is important to structurally characterize each component in the autophagy UBL conjugation pathway in order to understand the mechanisms that control the ligation of Atg8 to PE (Figure 3).

3. Proteins that have been crystallized in the UBL conjugation system in autophagy

Table 1 lists existing crystal structures for several components and protein complexes in the UBL conjugation system.

4. Crystallization of *S. cerevisiae* Atg7 alone or in complex with Atg3 or Atg10

4.1 Crystallization of *S. cerevisiae* Atg7^{NTD} and Atg7^{CTD}

Atg7 is composed of two domains (NTD, residues 1–289; and CTD, residues 294–630) separated by a short linker. We previously crystallized the NTD and CTD separately (Taherbhoy et al., 2011) (Figure 3). Atg7^{NTD} and Atg7^{CTD} coding sequences were each cloned into the pGEX-4T1 vector (GE Healthcare Life Sciences) and transformed into BL21 Gold (DE3) cells (Novagen).

4.1.1 Expression

1. Inoculate 1-L LB medium supplemented with 100 µg/mL ampicillin with 5-mL LB culture containing Atg7^{NTD} or Atg7^{CTD}. Grow the 1 L culture in a 2-L flask at 37°C until OD_{600nm} reaches 0.6–0.8.
2. Transfer the flask to a 23°C shaker and add 1 mL of 0.6 M isopropyl β-D-1-thiogalactopyranoside to the 1-L culture to induce protein expression, and continue to grow the culture for 16–20 h. Harvest the bacteria by centrifuging at 4°C.

4.1.2 Purification

1. Vortex to fully homogenize the cell paste in ice-cold lysis buffer containing 25 mM Tris pH 7.6, 200 mM NaCl, 10 mM dithiothreitol (DTT), and 2.5 mM PMSF (freshly added).
2. Disrupt the bacteria by using a microfluidizer (Avestin) or by sonication.
3. Centrifuge the lysate at 20,000 *g* for at least 40 min and collect the supernatant.
4. Load the supernatant onto pre-equilibrated Glutathione Sepharose 4B beads (GE Healthcare Life Sciences).

5. Wash the beads with 12 column volumes (CVs) of lysis buffer, and then elute the protein with 5 CVs of elution buffer containing 25 mM Tris pH 7.6, 200 mM NaCl, 5 mM β -mercaptoethanol (BME), and 10 mM glutathione.
6. Collect all 5 elution fractions, and check the purity of each fraction by SDS-PAGE.
7. Pool the fractions containing the pure target protein, and add thrombin to cleave the GST-tag from Atg7^{NTD} or Atg7^{CTD} with a mass ratio of 1:150. Dialyze overnight into buffer containing 25 mM Tris pH 7.6, 200 mM NaCl, and 5 mM BME at 4°C.
8. Check the cleavage efficiency by SDS-PAGE, then pass the cleavage reactions back over pre-equilibrated Glutathione Sepharose 4B beads (GE Healthcare Life Sciences) to remove GST.
9. Collect the flow-through, and use 2 CV of buffer to wash out protein remaining in the column bed volume.
10. Further purify the Atg7^{NTD} or Atg7^{CTD} protein by anion-exchange chromatography (HiTrap Q HP, GE Healthcare Life Sciences) and gel filtration (Superdex 200 10/300 GL, GE Healthcare Life Sciences) in a final buffer containing 25 mM Tris pH 7.6, 150 mM NaCl, and 10 mM DTT; concentrate to 30 mg/mL for Atg7^{NTD} and 15 mg/mL for Atg7^{CTD}; divide into aliquots; flash freeze in liquid nitrogen; and store at -80°C.

4.1.3 Crystallization

1. Add 1 mL of the reservoir solution in each well of a 24-well VDXm Plate GreasedTM (Hampton Research).
2. To obtain the initial Atg7^{NTD} crystal, mix 1 μ L of 30 mg/mL Atg7^{NTD} in 25 mM Tris pH 7.6, 150 mM NaCl, and 10 mM DTT with 1 μ L of the well solution containing 9.2% isopropanol, 0.1 M potassium thiocyanate, and 0.1 M citrate buffer (pH 4.0) by using hanging-drop vapor diffusion at 4°C.
3. To improve crystal quality, use streak seeding. Set up drops under conditions that contain 60%–80% of the precipitant concentration for a normal condition that produces crystals (in order to avoid the formation of crystal nuclei) and equilibrate the drop overnight at 4°C.
4. To make the seed stock, crush the crystals into microcrystals as best possible within the drop, and then mix the drop containing microcrystals with 100 μ L of well solution and vortex.
5. Dip a clean cat whisker or seeding tool (Hampton) into the seed stock, and run the cat whisker or seeding tool through the pre-equilibrated drops. Use the loaded cat whisker or seeding tool for 3 more drops in order to transfer progressively fewer seeds to subsequent drops.
6. After crystal growth, cryoprotect the crystals in well solution supplemented with 6% glycerol, 6% ethylene glycol, and 6% sucrose.

7. To obtain crystals of Atg7^{CTD}, mix 1 μ L of 15 mg/mL Atg7^{CTD} in 25 mM Tris, pH 7.6, 150 mM NaCl, and 10 mM DTT with 1 μ L of 12% polyethylene glycol 20K (PEG-20k), 0.1 M Tris pH8.5, and 10 mM TCEP and incubate at 4°C. For harvesting, soak crystals in 13% PEG-20k, 0.1 M Tris pH 8.5, 10 mM TCEP, 7.5% ethylene glycol, and 7.5% glycerol before cryoprotection in 13% PEG-20k, 0.1 M Tris pH 8.5, 10 mM TCEP, 15% ethylene glycol, and 15% glycerol.

4.2 Crystallization of *S. cerevisiae* Atg7^{NTD}–Atg3FR^{pep}

To understand the molecular mechanism underlying the recognition of Atg3 by Atg7, we have crystallized Atg7^{NTD} in complex with Atg3FR^{pep} (Figure 3) by the following procedure:

1. Synthesize Atg3FR^{pep} (Atg3 residue 128–144: SIDDIDELIQDMEIKEE) with N-terminal acetylation and an additional NH₂ group at the C terminus.
2. Dissolve Atg3FR^{pep} in 25 mM Tris pH 7.6, 150 mM NaCl, 10 mM DTT. Adjust the pH to 7.6 with NaOH.
3. To prepare the sample for crystallization, mix 30 mg/mL Atg7^{NTD} with an 8-fold molar excess of the Atg3FR^{pep} peptide.
4. Grow crystals of Atg7^{NTD}–Atg3FR^{pep} at room temperature by mixing 1 μ L of the Atg7^{NTD}–Atg3FR^{pep} sample and 1 μ L of the well solution containing 1.8 M ammonium sulfate, 0.1 M Bis-Tris pH 5.5, and 5 mM DTT.
5. After soaking the crystals in the well solution supplemented with 6.25% glycerol and 6.25% ethylene glycol, cryoprotect crystals in the well solution supplemented with 12.5% glycerol and 12.5% ethylene glycol.

4.3 Crystallization of *S. cerevisiae* Atg7–Atg3 and Atg7–Atg10

Atg7 is the only E1 enzyme that activates the two UBLs Atg8 and Atg12 and transfers them to two distinct E2 enzymes - Atg3 and Atg10, respectively. Crystallization of the Atg7–Atg3 and Atg7–Atg10 complexes provided insights into how a single E1 enzyme recognizes the two different E2s and how active sites are juxtaposed for these noncanonical E1–E2 complexes to promote autophagy. To visualize the juxtaposition of E1 and E2 catalytic cysteines and to facilitate the crystallization process, cross-linking is performed with the homobifunctional sulfhydryl cross-linker bismaleimidoethane (BMOE) by using the following strategy. Versions of Atg3 and Atg10 containing only a single cysteine at the active site are prepared, BMOE is reacted with each E2, and excess cross-linker is removed by desalting. For Atg3, Atg7 (1–613) is added. For Atg10, a version of Atg7 (1–613) with C39S C195S C375A mutations is added to reduce background cross-linking. By this strategy, we obtain crystals of Atg7–BMOE–Atg3 and Atg7–BMOE–Atg10 (hereafter referred to as Atg7–Atg3 and Atg7–Atg10, respectively) (Figure 3). The detailed methodology is as follows:

4.3.1 Protein preparation

1. Prepare Atg7 (1–613) and Atg7 (1–613) C39S C195S C375A by the same method as that used for Atg7^{NTD} or Atg7^{CTD}.
2. Clone Atg3 C41A C76A C83A (Atg3 C234only) and Atg10 C26S C137S (Atg10 C133only) into the pGEX-4T1 vector (GE Healthcare Life Sciences), and transform into RIL codon-enhanced BL21 (DE3) (Agilent Technologies).
3. For expression, dilute small-scale culture in 1 L of LB medium containing 100 µg/mL ampicillin and 25 mg/mL chloramphenicol in a 2-L flask and grow at 37°C until OD_{600nm} reaches 0.6–0.8.
4. Transfer the flask to shaker equilibrated at 16°C and add 1 mL of 0.6 M IPTG (isopropyl β-D-1-thiogalactopyranoside) to the 1-L culture to induce protein expression. Grow the culture for 16–20 h.
5. Use the same purification procedure as that used for Atg7^{NTD} or Atg7^{CTD}.

4.3.2 Atg7–Atg3 crosslinking

1. Reduce Atg3 C234only and Atg7 (1–613) with 10 mM DTT and purify by using a Superdex 200 10/300 GL column (GE Healthcare Life Sciences) equilibrated with buffer containing 20 mM HEPES pH 7.0 and 150 mM NaCl.
2. Mix approximately 0.5 mM Atg3 C234only with a 5× molar excess of BMOE (Pierce) on ice for 1 hour, followed by desalting on a PD-10 column (GE Healthcare Life Sciences) to remove unreacted cross-linker.
3. Mix Atg3 C234only~BMOE (3 µM) and Atg7 (1–613) (1.5 µM) for 20 s, and quench the cross-linking reaction with 50 mM DTT.
4. Purify Atg7–Atg3 C234only by anion exchange chromatography (5 mL HiTrap Q HP) and gel filtration (Superdex 200 10/300 GL) into a final buffer of 50 mM HEPES pH 7.0, 150 mM NaCl, and 10 mM DTT; concentrate to 12.5 mg/mL; divide into aliquots; flash freeze in liquid nitrogen; and store at –80°C.

4.3.3 Atg7–Atg10 crosslinking—Prepare Atg10 C133only cross-linked to Atg7 (1–613) C39S C195S C375A by the same procedure as that used for Atg7–Atg3 crosslinking, except that a 5× molar excess of BMOE is added to approximately 0.2 mM Atg10 C133only. Perform cross-linking with 1.2 µM Atg7 (1–613) C39S C195S C375A and 2.4 µM Atg10 C133only in the final buffer of 25 mM HEPES pH 7.0, 100 mM NaCl, and 5 mM DTT. Concentrate Atg7–Atg10 to 7 mg/mL.

4.3.4 Crystallization of Atg7–Atg3

1. Grow crystals of Atg7–Atg3 by microseeding at room temperature sitting drops of 2 µL of Atg7 (1–613)~BMOE~Atg3 C234only with 1.4 µL of the well solution of 100 mM sodium citrate pH 5.8, 13.5% PEG 4000, 10% isopropanol, 5% dioxane, and 88 mM ammonium sulfate.

2. To prepare the seed stock, mix drops containing microcrystals with the well solution.
3. After vortexing, serially dilute the seed stock from 10^{-1} to 10^{-6} fold and add 200 nL to 2 μ L of protein and 1.8 μ L of the well solution mixture.
4. Cryoprotect the crystals in the well solution supplemented with 15% glycerol and 15% ethylene glycol before data collection.

4.3.5 Crystallization of Atg7–Atg10

1. Grow crystals of Atg7–Atg10 by at room temperature microseeding sitting drops containing 400 nL of Atg7 (1–613) C39S C195S C375A~BMOE~Atg10 C133only in 25 mM HEPES pH 7.0, 100 mM NaCl, and 5 mM DTT mixed with 400 nL of a well solution containing 76.5 mM Na/K phosphate pH 6.5, 9 mM Tris pH 8.5, 153 mM NaCl, 100 mM glycine, 72 mM Na/K tartrate, 19.125% PEG 1000, 0.045% PEG 5000MME, and 4.5% dioxane.
2. To prepare the seeding solution, mix drops containing microcrystals with the well solution.
3. After vortexing, serially dilute the seeding solution from 10^{-1} to 10^{-6} fold, and add a 20 nL of seed stock dilution to 500 nL of protein and 500 nL of the well solution mixture.
4. Cryoprotect the crystals in well solution supplemented with 30% glycerol before data collection.

5. Crystallization of human ATG5–ATG16L1 (1–69) E122D disease-associated mutant

5.1 Baculovirus/insect cell expression system

To obtain wild-type or mutant version of the ATG5–ATG16L1 (1–69) complex, a baculovirus/insect cell expression system is used. Specifically, ATG5 wild type or the ATG5 E122D mutant is cloned into the pFastBac-HTb vector, which contains an N-terminal His tag fusion with ampicillin resistance (Life Technologies). ATG16L1 (1–69) is cloned into the pFastBac-hisMBP vector, which is modified by insertion of MBP between the His-tag and the TEV cleavage site from pFastBac-HTb. After amplification by DH5alpha cells, the ATG5 containing pFastBac-HTb plasmid and ATG16L1 (1–69) containing plasmid are transformed into DH10-EMBacY cells. DH10-EMBacY cells are DH10B *E. coli* containing a plasmid encoding the transposase and tetracycline resistance and the bacmid encoding the baculovirus genome, yellow fluorescent protein, kanamycin resistance, and ampicillin resistance. At this step, the *ATG5* gene or *ATG16L1* (1–69) gene, as well as a gentamycin marker, are transferred from the pFastBac-HTb plasmid into a bacmid encoding the baculovirus genome via transposase-mediated recombination, or transposition, in *E. coli*. When the *ATG5* or *ATG16L1* (1–69) gene is transposed into the bacmid, it gets inserted into the *lacZa* gene. The *lacZa* gene provides β -galactosidase complementation and turns colonies blue on plates containing X-gal. Therefore, the desired recombinant bacmids

produce white colonies, whereas undesirable transformants that contain non-recombinant bacmids are blue when grown on plates containing X-gal. To verify blue/white selection, white colonies need to be re-streaked on selective plates with X-gal before proceeding to the next steps. The detailed methodology is as follows:

5.2 Transposition

1. Thaw competent DH10-EmBacY cells on ice. Spread 40 μ L of 40 mg/mL X-gal (dissolved in dimethylformamide) and 40 μ L of 100 mM IPTG on plates containing 50 μ g/mL kanamycin, 7 μ g/mL gentamicin, 10 μ g/mL tetracycline, and 100 μ g/mL ampicillin.
2. Add 1 μ g of ATG5 containing pFastBac-HTb plasmid to DH10-EmBacY-competent cells. Incubate on ice for 30 min.
3. Heat-shock the competent cells at 42°C for 30 sec. Then, allow cells to recover on ice for 2 min.
4. Add 900 μ L of the SOC medium, transfer to a culture tube, and incubate at 37°C with shaking for 4–8 h.
5. Spread 500 μ L of cells on LB-agar plates containing the 4 antibiotics and incubate at 37°C until blue color develops. This step takes at least 24 h.
6. Re-streak several white colonies on a fresh plate with X-gal, IPTG, and the 4 antibiotics. Include a blue colony as a control for color development. Incubate overnight at 37°C until blue color develops. This step usually takes less than 24 h.

5.3 Bacmid preparation

1. Select 4–8 colonies and grow the culture in a 37°C shaking incubator overnight.
2. Transfer 1.5 mL of bacterial culture to a 1.5-mL microcentrifuge tube and centrifuge at 6800 *g* for 3 min.
3. Remove the supernatant by vacuum aspiration or by pouring, and resuspend the cell pellet in 0.3 mL of P1. Gently vortex or pipet up and down to resuspend.
4. Add 0.3 mL of P2 and gently mix. Incubate at room temperature for 5 min.
5. Slowly add 0.3 mL of 3 M potassium acetate, pH 5.5 (also known as P3), mixing gently during addition. A thick white precipitate of protein and *E. coli* genomic DNA will form. Place the sample on ice for 5–10 min.
6. Centrifuge for 10 min at 16,000 $\times$ *g* at room temperature.
7. Gently transfer the supernatant to a microcentrifuge tube containing 0.8 mL of isopropanol. Do not transfer any white precipitate. Invert the tube a few times to mix and place on dry ice for 15 min.
8. Centrifuge the sample for 20 min at 16,000 $\times$ *g* at room temperature.
9. Carefully remove the supernatant, taking care not to disturb the pellet.

10. Add 0.5 mL of 70% ethanol to the pellet. When adding the ethanol, first cover the pellet and then continue adding ethanol around the edges of the tube to get as much isopropanol as possible. Invert the tube carefully to remove isopropanol completely. Centrifuge for 5–10 min at $16,000 \times g$, remove most of the ethanol gently with a pipette and then aspirate the remaining ethanol carefully, taking care not to disturb the pellet.
11. Air-dry the pellet for 5–10 min at room temperature.
12. Dissolve the DNA pellet in 40 μ L of 1x TE buffer, pH 8.0. To avoid shearing, do not mechanically resuspend the DNA. Allow the solution to sit in the tube, with occasional gentle tapping of the bottom of the tube.
13. Store the DNA at 4°C.
Note: We do not recommend storing the purified bacmid DNA at -20°C , because repeated freezing and thawing might shear the DNA.
14. Perform PCR screening of the recombinant bacmids for the insert gene.

5.4 Transfection of insect cells (P1 production)

The initial virus (P1) is made by transfecting SF9 cells in plates with FuGENE HD (Promega) and harvesting the supernatant 3–5 days later, as described below:

1. Measure cell density of the SF9 culture with a hemocytometer or other cell counter.
2. Seed 5×10^6 cells into the well of a 6-well plate (Corning Costar 3516, Sigma-Aldrich). Allow cells to attach to the plate by placing in an incubator at 27°C for approximately 30 min.
3. Verify cell attachment by using a microscope. Cells should appear very dense.
4. Dilute 2 μ g of bacmid DNA into 100 μ L of medium warmed to 27°C in a sterile, polystyrene tube.
5. Add 10 μ L of FuGENE HD and incubate at 27°C for 30 min.
6. Aspirate the medium from the cells in the plate.
7. Add 0.9 mL of medium to the DNA–FuGENE mixture and transfer to cells in the plate.
8. Incubate cells for 5–6 h at 27°C with occasional rocking.
9. Add another 1 mL of medium to the cells, and incubate at 27°C for 4 days.
10. Pass the medium (containing the P1 virus) through a 0.22- μ m syringe filter to remove detached cells and debris.
11. Store the virus in the dark at 4°C.

5.5 P2/P3 amplification

Later-generation viruses (P2 and P3) are produced in the same manner by infecting a plate of SF9 cells and harvesting the medium 3 days later as follows:

1. Measure cell density of the SF9 culture with a hemocytometer or other cell counter.
2. Seed $25\text{--}30 \times 10^6$ cells in a 150-mm plate (Corning 430599) and allow cells to attach by incubating at 27°C for approximately 30 min.
3. Verify cell attachment with a microscope. Cells should appear 75%–90% confluent.
4. Adjust the volume of medium on the cells to 10 mL by aspirating or adding additional medium, as needed.
5. Add 0.25 mL of virus to the cells and gently rock the plate, mixing thoroughly. Incubate at 27°C for 1 h.
6. Add 12 mL of medium to the plate and incubate for 3–4 days at 27°C, gently rocking or shaking the plate each day to agitate the medium.
7. Remove the medium (containing the virus) from the cells. To remove detached cells and debris, centrifuge the medium in a sterile 50-mL conical tube at $500 \times g$ for 10 min. Store in the dark at 4°C.

5.6 Infection of suspension Hi5 insect cells for protein expression

Hi5 cells need to be maintained in HyQ-SFX serum-free medium, and the culture should be in the mid-log phase.

1. Count the cells (ideal cell count 1×10^6 – 2×10^6 cells/mL), and resuspend the expression cultures at a final density of 2×10^6 cells/mL. For expression in 1-L scale, 2×10^9 cells are needed.
2. Spin the cells down at $500 \times g$ for 5 min.
3. Mix 10 mL each of the P3 virus for ATG5 and ATG16L1 (1–69).
4. After the cells have been centrifuged, aspirate and discard the supernatant. Resuspend the cells at a density of 1×10^7 cells/mL (200 mL in total). Return the cells to the growth flasks. Add the virus mixture and incubate for 1–2 h at 27°C, with shaking at 75–100 rpm.
5. After 1–2 h, add medium to bring the cells to a density of 2×10^6 cells/mL and incubate at 27°C, with shaking at 155 rpm.
6. After 3 days of expression, harvest the cells by centrifugation and isolate the protein.

5.7 Purification of the ATG5–ATG16L1 (1–69) complex

1. Resuspend the cell pellets in 80 mL (per liter culture) of the lysis buffer containing 25 mM Tris pH 8.0, 500 mM NaCl, 5 mM BME, 20 mg/L aprotinin,

and 10 mg/L leupeptin. Add PMSF after resuspension to a final concentration of 2.5 mM.

2. Transfer the suspension into Falcon tubes with 15–20 mL in each tube. Sonicate each tube 2 times for 15 sec each for insect cells.
3. After sonication, transfer the lysate into cold Oakridge tubes for clarification. Centrifuge for 40 min at 32,000× g.
4. Equilibrate 8 mL of Ni NTA beads (Sigma) with the lysis buffer.
5. Load the supernatant onto the pre-equilibrated NTA beads slowly at 4°C.
6. Wash the beads twice with 10 CV of wash buffer containing 25 mM Tris pH 8.0, 500 mM NaCl, and 5 mM BME.
7. Elute the protein with 6 CV of elution buffer containing 25 mM Tris pH 8.0, 500 mM NaCl, 200 mM imidazole, and 5 mM BME. Collect elution fractions for every CV, and check the purity of each fraction by SDS-PAGE.
8. Pool the fractions containing target protein and add TEV to cleave the His-tag from ATG5 and HisMBP-tag from ATG16L1 (1–69) with a mass ratio of 1:50. Then, dialyze overnight into buffer containing 25 mM Tris pH 8.0, 200 mM NaCl, 5 mM BME at 4°C.
9. Check cleavage efficiency by SDS-PAGE. The cleaved protein should be further purified by anion-exchange chromatography (5 mL HiTrap Q HP, GE Healthcare Life Sciences) and gel filtration (Superdex 200 10/300 GL, GE Healthcare Life Sciences) into a final buffer of 20 mM Tris, pH 8.5, 50 mM NaCl, and 10 mM DTT.
10. Concentrate the complex to 18.5 mg/mL, divide into aliquots, flash-freeze, and store at –80°C until further use.

5.8 Crystallization of the ATG5–ATG16L1 (1–69) complex

1. Crystals of a wild-type complex of ATG5–ATG16L1 were reported elsewhere (Kim et al., 2015). To crystallize the version with the ATG5 E122D patient-derived mutations (Kim et al., 2016), add 1 mL of the well solution containing 37.5 mM MES pH 5.2–5.8, 0.2 M sodium tartrate, and 11%–13% PEG3350 to each well of the 24-well plate.
2. To obtain the initial ATG5 E122D –ATG16L1 (1–69) crystal, mix 1 µL of 18.5 mg/mL ATG5 E122D –ATG16L1 (1–69) complex in buffer containing 20 mM Tris pH 8.5, 50 mM NaCl, 10 mM DTT with 1 µL of the well solution by using hanging drop vapor diffusion at 4°C.
3. To improve crystal quality, use microseeding. Mix drops containing microcrystals by using the well solution as the seeding solution.
4. After vortexing, serially dilute the seeding solution from 10^{–1} to 10^{–6} fold, and add 500 nL to the pre-equilibrated mixture of 1 µL of ATG5 E122D –ATG16L1

(1–69) and 1 μ L of the well solution of 40 mM MES pH 5.5, 0.2 M sodium tartrate, and 8.5% PEG3350.

5. Cryoprotect the crystals in the well solution supplemented with 25% xylitol and flash freeze in liquid nitrogen before data collection.

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Figure 1.

Schematic view of Atg12 and Atg8 conjugation cascades. **A**, Atg7 (E1) mediated Atg12 (Ubl) activation; **B**, *Trans*-thiolation reaction transfers Atg12 (Ubl) from Atg7 (E1) to Atg10 (E2); **C**, Atg12 (Ubl) ligation to its substrate Atg5 from Atg10 (E2); **D**, Atg7 (E1) mediated Atg8 (Ubl) activation; **E**, *Trans*-thiolation reaction transfers Atg8 (Ubl) from Atg7 (E1) to Atg3 (E2); **F**, Atg8 (Ubl) ligation to its substrate lipid PE from Atg3 (E2) catalyzed by Atg12~Atg5-Atg16 (E3).

Figure 2.
Scheme showing domain structures of ATG proteins involved in the UBL conjugation pathway in autophagy. NTD: N-terminal domain; AD: adenylation domain; ECTD: extreme C-terminal domain; CTD: C-terminal domain; FR: flexible region; HR: handle region; β : β -hairpin.

Figure 3.
Representative structures of ATG proteins in the UBL conjugation pathway in autophagy.

Table 1

ATG proteins crystallized in the UBL conjugation pathway in autophagy

Protein or protein complex	Organism	PDB ID	Reference
Atg7 (1-595)	<i>Saccharomyces cerevisiae</i>	3VH1	(Noda et al., 2011)
Atg7 (1-613)	<i>Saccharomyces cerevisiae</i>	3VH2	(Noda et al., 2011)
Atg7 NTD	<i>Kluyveromyces marxianus</i>	3VX6	(Yamaguchi et al., 2012)
Atg7 NTD	<i>Saccharomyces cerevisiae</i>	3RUJ, 3T7F	(Hong et al., 2011; Taherbhoy et al., 2011)
Atg7 CTD	<i>Saccharomyces cerevisiae</i>	3T7E	(Taherbhoy et al., 2011)
Atg7 NTD and Atg3FR complex	<i>Saccharomyces cerevisiae</i>	3T7G	(Taherbhoy et al., 2011)
Atg7 NTD and Atg3 complex	<i>Arabidopsis thaliana</i>	3VX8	(Yamaguchi et al., 2012)
Atg7 NTD and Atg10 complex	<i>Kluyveromyces marxianus</i>	3VX7	(Yamaguchi et al., 2012)
Atg7 CTD and Atg8 complex	<i>Saccharomyces cerevisiae</i>	3VH3, 3VH4, 3RUI	(Hong et al., 2011; Noda et al., 2011)
Atg7 (1-613) and Atg3 complex	<i>Saccharomyces cerevisiae</i>	4GSL	(Kaiser et al., 2012)
Atg7 (1-613) and Atg10 complex	<i>Saccharomyces cerevisiae</i>	4GSK	(Kaiser et al., 2012)
Atg3	<i>Saccharomyces cerevisiae</i>	2DYT	(Yamada et al., 2007)
Atg10	<i>Saccharomyces cerevisiae</i>	4EBR	(Hong et al., 2012)
ATG12~ATG5-ATG16L1	<i>Homo sapiens</i>	4GDK, 4GDL	(Otomo et al., 2013)
Atg12~Atg5-Atg16	<i>Saccharomyces cerevisiae</i>	3W1S	(Noda et al., 2013)
ATG12~ATG5-ATG16L1 and ATG3FR complex	<i>Homo sapiens</i>	4NAW	(Metlagel et al., 2013)
Atg12	<i>Arabidopsis thaliana</i>	1WZ3	(Suzuki et al., 2005)
Atg8	<i>Bombyx mori</i>	3M95	(Hu et al., 2010)
Atg8	<i>Trypanosoma brucei</i>	3H9D	(Koopmann et al., 2009)
LC3	<i>Homo sapiens</i>	1UGM	(Sugawara et al., 2004)
LC3A	<i>Homo sapiens</i>	3WAL	(Suzuki et al., 2014)
LC3B	<i>Homo sapiens</i>	3VTU	(Rogov et al., 2013)
LC3C	<i>Homo sapiens</i>	3WAM	(Suzuki et al., 2014)
GABARAP	<i>Homo sapiens</i>	1KJT, 1GNU	(Bavro et al., 2002), (Knight et al., 2002)
GABARAPL1	<i>Homo sapiens</i>	2R2Q	
GABARAPL2	<i>Homo sapiens</i>	1EO6, 4CO7	(Ma et al., 2015)
Atg8 and Atg3 peptide complex	<i>Plasmodium falciparum</i>	4EOY	(Hain et al., 2012)
ATG4A	<i>Homo sapiens</i>	2P82	
ATG4B	<i>Homo sapiens</i>	2CY7, 2D1L	(Kumanomidou et al., 2006; Sugawara et al., 2005)
ATG4B and LC3 complex	<i>Homo sapiens</i>	2Z0D, 2Z0E	(Satoo et al., 2009)

Abbreviation: PDB, Protein Data Bank identifier.