Identification, biochemical characterization and crystallization of the central region of human ATG16L1

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ATG16L1 plays a major role in autophagy. It acts as a molecular scaffold which mediates protein–protein interactions essential for autophagosome formation. The ATG12~ATG5–ATG16L1 complex is one of the key complexes involved in autophagosome formation. Human ATG16L1 comprises 607 amino acids with three functional domains named ATG5BD, CCD and WD40, where the C-terminal WD40 domain represents approximately 50% of the full-length protein. Previously, structures of the C-terminal WD40 domain of human ATG16L1 as well as of human ATG12~ATG5 in complex with the ATG5BD of ATG16L1 have been reported. However, apart from the ATG5BD, no structural information for the N-terminal half, including the CCD, of human ATG16L1 is available. In this study, the authors aimed to structurally characterize the N-terminal half of ATG16L1. ATG16L1_{11–307} in complex with ATG5 has been purified and crystallized in two crystal forms. However, both crystal structures revealed degradation of ATG16L1, resulting in crystals comprising only full-length ATG5 and the ATG5BD of ATG16L1. The structures of ATG5–ATG5BD in two novel crystal forms are presented, further supporting the previously observed dimerization of ATG5–ATG16L1. The reported degradation points towards a high instability at the linker region between the ATG5BD and the CCD in ATG16L1. Based on this observation and further biochemical analysis of ATG16L1, a stable 236-amino-acid subfragment comprising residues 72–307 of the N-terminal half of ATG16L1, covering the residual, so far structurally uncharacterized region of human ATG16L1, was identified. Here, the identification, purification, biochemical characterization and crystallization of the proteolytically stable ATG16L1_{72–307} subfragment are reported.

1. Introduction

Autophagy is a highly conserved eukaryotic degradation process. In classical autophagy, upon nutrient depletion or stress, a double-membrane vesicle referred to as the autophagosome engulfs a section of the cytoplasm and delivers it to the lysosome for degradation (Klionsky, 2005; Mizushima, 2007). Apart from its classical role, autophagy is involved in various aspects of immunity, especially in the capture and clearance of intracellular pathogens (Levine *et al.*, 2011; Yuk *et al.*, 2012). The mechanism of autophagy comprises three major steps: initiation/nucleation, autophagosome formation and autophagolysosome formation/cargo degradation (Periyasamy-Thandavan *et al.*, 2009). To date, more than 34 autophagy-related genes (ATGs) have been reported that encode different ATG proteins. Most of them are conserved from yeast to humans and provide vital information to understand the molecular mechanism of autophagy (Klionsky *et al.*, 2003;

Ohsumi, 1999). Among them, one of the key players in the mechanism of autophagy is the yeast ATG16 protein (the mammalian homologue is ATG16L1), which plays an essential role during nucleation and autophagosome formation. Yeast ATG16 comprises 150 amino acids with an N-terminal ATG5-binding domain (ATG5BD) and a coiled-coil domain (CCD) (Fig. 1*a*). While the CCD mediates homodimerization of ATG16 or ATG16L1 (Parkhouse *et al.*, 2013; Fujioka *et al.*, 2010), the ATG5BD is required for interaction with the ATG12~ATG5 protein conjugate, where the ATG12~ATG5~ATG16 (or, in mammals, ATG12~ATG5~ATG16L1) complex catalyzes the conjugation of ATG8 (LC3 in mammals) to phosphatidylethanolamine (PE) in the pre-autophagosomal structure (Mizushima *et al.*, 2011; Walczak & Martens, 2013). In contrast to yeast ATG16, mammalian ATG16L1 is significantly larger in size and comprises 607 amino acids in the case of the human homologue (Fig. 1*a*). The ATG5BD and CCD, which fulfill a conserved function compared with yeast and are essential and sufficient for functional classical autophagy, are

present in the N-terminal part of ATG16L1 as well, but cover approximately half of the full-length protein, including insertions and length variations that are not present in yeast ATG16 (Fig. 1*a*). In addition to ATG5BD and the CCD, human ATG16L1 also contains a C-terminal WD40 domain with seven WD40 repeats (Bajagic *et al.*, 2017), which has been reported to act as an interaction platform for the recruitment of various proteins (reviewed in Zavodszky *et al.*, 2013).

The central region of ATG16L1, which comprises the CCD and is located between the N-terminal ATG5BD and the C-terminal WD40 domain, has been reported to mediate interactions with diverse proteins. ATG16L1 interacts with the PI3P effector protein WD-repeat domain phosphoinositide-interacting protein 2 isoform b (WIPI2b) through its CCD and thereby facilitates the recruitment of ATG12~ATG5~ATG16L1 to the PI3P-positive phagophore assembly site (PAS) (Proikas-Cezanne *et al.*, 2015; Müller & Proikas-Cezanne, 2015). The central region of ATG16L1 additionally interacts with focal adhesion kinase family-interacting protein

Figure 1

(*a*) Domain organization of full-length yeast ATG16 and human ATG16L1. ATG5BD, ATG5-binding domain; I., insert; CCD, coiled-coil domain. The striped region (amino acids 126–207) within the predicted CCD (amino acids 78–230) corresponds to the previously identified minimal CCD region. (*b*) ATG5–ATG16L1_{11–307} complex elution profile from a Superdex 200 26/600 column. The Coomassie-stained SDS–PAGE gel shows the final purity of the purified complex. (*c*) Optimized crystals from a drop containing pure ATG5–ATG16L1_{11–307}. (*d*) Overlay of both structures of ATG5 in complex with the ATG5BD of ATG16L1. The structures obtained from crystal 1 and crystal 2 are shown in combinations of orange/green and yellow/blue, respectively. The observed boundaries of the ATG5BD helices in the structures are annotated.

Table 1
Macromolecule-production information.

Protein	ATG5	ATG16L1 _{11–307}	ATG16L1 _{72–307}
Source organism	<i>Homo sapiens</i>	<i>Homo sapiens</i>	<i>Homo sapiens</i>
DNA source	DNASU	Open Biosystems	Open Biosystems
Forward primer	5′-GGAATTCATATGACAGATGACAAAG ATGTGCTTC-3′	5′-AAGAATGCGGCCCGCCCGCTGGAAG CGCCACATCTCG3′	5′-AAGAATGCGGCCCGGACATGATGGC ACATGGAATGAC-3′
Reverse primer	5′-GGGTACCTCAATCTGTGGCTGTGG GATG-3′	5′-GGGTACCTTATTCTTACCAGAACCA GGATG-3′	5′-GGGTACCTTATTCTTACCAGAACCA AGGATG-3′
Cloning vector	Modified pET-15b	Modified pCOLA Duet	Modified pCOLA Duet
Expression host	<i>E. coli</i> Rosetta2 DE3	<i>E. coli</i> Rosetta2 DE3	<i>E. coli</i> Rosetta2 DE3
Complete amino-acid sequence of the protein constructs	MTDDKDVLRDVWFGRIPCTCFTLYQDEITE REAEPPYLLPRVSYLTLDVKVKKHF QKVMRQEDISEIWFYEGTPLKWHYPI GLLFDLLASSALPWNITVHFKSFPEK DLLHCPSKDAIEAHFMSCKEADALKH KSQVINEMQKKDHKLWMGLQNDRFQ FWAINRKLMEYPAEENGFRYIPFRIYQ TTERPFIQKLFPRVAADGQLHTLGD LKEVCPSAIDPEDGEKKNQVMHIGIEP MLETPQLWSEHLSYPDNFLHISIIPIQ PTD	PRWKRHISEQLRRLRLRQAFEEIILQY NKLEKSDLHSLVAQLQAEKHDVNP HEISPGHDGTWNDNLQEMAQLRIKHQ EELTELHKRGELALVIDLNNQMQRK DREMOMNEAKIAECLQTSIDLETECLD LRTKLCDLERANQTLKDEYDALQITFT ALEGKLRKTTEENQELVTRWMAEKAQE ANRLNAENKDSRRRQARLQKELAEAA KEPLPVEQDDDDIEVIDETS DHTEETS PVRAISRATKRLSQPAGGLDSITNI FGRRSVSSFPVPQDNVDTHPGSGKE	GHDTWNDNLQEMAQLRIKHQEELTELH KKRGELALVIDLNNQMQRKDREMOMN EAKIAECLQTSIDLETECLDLRTKLCD LERANQTLKDEYDALQITFTALEGKLR KTTEENQELVTRWMAEKAQEANRLNAE NEKDSRRRQARLQKELAEAAKEPLPVE QDDDDIEVIDETS DHTEETSPVRAISR AATKRLSQPAGGLDSITNIFGRRSVS SFPVPQDNVDTHPGSGKE

of 200 kDa (FIP200) and participates in ULK1 complex-dependent activation of autophagy (Gammoh *et al.*, 2013). Furthermore, it mediates interaction with the small GTPase Rab33b, a protein involved in the regulation of autophagy and membrane trafficking (Itoh *et al.*, 2008).

Therefore, the central region of ATG16L1, encompassing approximately 260 amino acids, plays an important role in recruiting the ATG12~ATG5~ATG16L1 complex to its site of action and in regulating its general function in the context of autophagy. Despite its important function, previous structural studies mainly focused on structure determination of the N-terminal ATG5-binding domain of human ATG16L1 (Kim *et al.*, 2015; Otomo *et al.*, 2013) and we have recently reported the structure determination of the C-terminal WD40 domain (Bajagic *et al.*, 2017). Thus, apart from the N-terminal ATG5BD, covering residues 11–48, and the C-terminal WD40 domain, encompassing residues 307–607, no structural information is available for the central region consisting of approximately 260 residues between the ATG5BD and the WD40 domain. In a previously reported study, an 81-amino-acid fragment ATG16L1_{126–207} from the central region was defined and biochemically characterized as the minimal stable fragment of the CCD, but remained structurally uncharacterized (Parkhouse *et al.*, 2013). While that work focused on the identification of the minimal stable fragment of the CCD, we instead aimed at obtaining a stable fragment of the complete central region for further structural characterization. Here, we describe the identification, characterization and crystallization of a stable construct ATG16L1_{72–307}, covering 236 of the approximately 260 residues of the central region of human ATG16L1, which corresponds to a construct that is almost three times longer than the previously reported minimal CCD fragment. The biochemical and structural characterization of ATG16L1_{72–307} will thus provide important information regarding the various protein-interaction interfaces and lead to a better understanding of the assembly/dimerization of ATG16L1.

2. Materials and methods

2.1. Protein expression and purification

Genes for full-length human ATG5 and human ATG16L1_{11–307} constructs were separately cloned in modified pET-15b and pCOLA Duet vectors, respectively (Table 1). *Escherichia coli* strain Rosetta2 DE3 was co-transformed with both recombinant plasmids. Afterwards, *E. coli* cells harbouring the expression plasmids for both proteins were cultured in 11 Terrific Broth medium containing 50 or 100 µg ml^{−1} ampicillin, 50 µg ml^{−1} kanamycin and 34 µg ml^{−1} chloramphenicol at 37°C. When the OD₆₀₀ reached approximately 0.8, 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Carbosynth) was added and the culture was maintained at 20°C for 16 h. The cells were harvested by centrifugation at 5000 rev min^{−1} for 15 min and resuspended in 20 ml buffer [50 mM HEPES pH 7.0, 5% glycerol, 300 mM NaCl, 5 mM β-mercaptoethanol (BME), 5 mM MgCl₂, 50 µg DNase (F. Hoffmann-La Roche AG) and 5 mg lysozyme (Sigma–Aldrich) per litre of culture]. The cells were lysed by homogenization in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) and the cell lysate was further centrifuged for 1 h at 16 000 rev min^{−1}. ATG5 and ATG16L1_{11–307} were expressed as N-terminal His₆ and Strep fusion proteins, respectively. The protein complex was first isolated from lysate using Strep-affinity chromatography on an 8 ml Strep-Tactin Superflow high-capacity column. Following loading of the clear lysate onto the column, unbound protein was washed off with wash buffer (50 mM HEPES pH 7.0, 5% glycerol, 300 mM NaCl, 5 mM BME) and the pure complex was eluted using elution buffer (50 mM HEPES pH 7.0, 5% glycerol, 300 mM NaCl, 20 mM desthiobiotin, 5 mM BME). The tags of both proteins were removed by digestion with 2% (w/w) TEV protease. Finally, the protein complex was purified using a Superdex 200 16/600 column (GE Healthcare) in 50 mM HEPES pH 7.0, 5% glycerol, 300 mM NaCl, 5 mM DTT (Fig. 1b).

Table 2
Crystallization information.

PDB code/protein	5npv	5npw	ATG16L1 _{72–307}
Method	Sitting-drop vapour diffusion	Sitting-drop vapour diffusion	Sitting-drop vapour diffusion
Plate type	96-well Intelli-Plate	96-well Intelli-Plate	96-well Intelli-Plate
Temperature (K)	293	293	293
Protein concentration (mg ml ⁻¹)	10	10	15
Buffer composition of protein solution	20 mM HEPES pH 7.0, 300 mM NaCl, 5% glycerol, 5 mM DTT	20 mM HEPES pH 7.0, 300 mM NaCl, 5% glycerol, 5 mM DTT	20 mM HEPES pH 7.0, 300 mM NaCl, 5% glycerol, 5 mM DTT
Buffer composition of reservoir solution	0.1 M MES pH 5.76, 0.378 M KCl, 21.7% PEG 3350	0.4 M KCl, 18% PEG 3350	Crystal 3: 0.1 M Tris base pH 7.0, 20% (v/v) Sokalan CP 42 Crystal 4: 0.1 M Tris base pH 7.0, 10% (v/v) Sokalan CP 42, 1 M sodium malonate
Volume and ration of drop (nl)	200, 1:1	200, 1:1	200, 1:1
Volume of reservoir (μl)	70	70	70

For the heterologous expression of ATG16L1_{72–307}, the gene for this construct was cloned into a modified pCOLA Duet vector (Table 1). *E. coli* strain Rosetta2 DE3 was transformed with the plasmid and the cell culture was grown in TB medium containing 50 μg ml⁻¹ kanamycin and 34 μg ml⁻¹ chloramphenicol. The purification protocol for Strep-tagged ATG16_{72–307} was similar to that for the ATG5–ATG16L1_{11–307} complex from protein expression to Strep-affinity chromatography. After Strep-affinity chromatography, the Strep tag on the protein was removed by incubation with 2% (w/w) TEV protease. Further purification was achieved by anion-exchange chromatography using a Source 15Q column with a shallow salt gradient (a gradient of 30–500 mM NaCl in 50 mM HEPES pH 7.0, 5% glycerol, 5 mM BME over 20 column volumes). Finally, the protein was purified using a Superdex 200 16/600 size-exclusion column in 20 mM HEPES pH 7.0, 5% glycerol, 300 mM NaCl, 5 mM DTT (Fig. 3*b*).

2.2. Crystallization

Purified ATG5–ATG16L1_{11–307} complex (10 mg ml⁻¹) was used for crystallization trials in 96-well Intelli-Plates (Art Robbins Instruments). Initial crystals were obtained after 15 d at 293 K in a condition from the JCSG Core II Suite consisting of 0.2 M KCl, 20% PEG 3350 using the sitting-drop vapour-diffusion method. The crystallization condition for these

crystals was further optimized by using grid screening and random screening around the original condition. Two different conditions were obtained (Table 2) and under these conditions larger crystals were grown in 21 d at 293 K (Fig. 1*c*). Similarly, crystallization of ATG16L1_{72–307} was performed with 15 mg ml⁻¹ protein in 96-well Intelli-Plates using the sitting-drop vapour-diffusion method. Initial crystals of ATG16L1_{72–307} were observed at 293 K in two different conditions: condition 1 [0.1 M disodium malonate, 0.1 M MES pH 5.5, 25% (w/v) Jeffamine SD-2001] and condition 2 (0.1 M Tris pH 8.0, 5% methanol, 20% Sokalan CP-42) of the MIDAS screen after 3 d. Crystal conditions were further optimized using manually designed Grid and Additive Screens (Hampton Research). Optimization of condition 2 resulted in two improved conditions (Table 2) with similar crystal forms (crystals 3 and 4 in Fig. 4*a*).

2.3. Data collection and structure determination of the ATG5–ATG16L1 complex

The crystals were cryoprotected with 0.1 M MES pH 5.76, 0.378 M KCl, 21.7% PEG 3350, 25% glycerol (for crystal 1) or 0.4 M KCl, 18% PEG 3350, 25% glycerol (for crystal 2) and flash-cooled in liquid nitrogen. Crystal 1 and crystal 2 both diffracted to 3.1 Å resolution and data sets were collected in-house and at the Swiss Light Source, respectively

Figure 2

Time-dependent stability test of the ATG5–ATG16L1_{11–307} complex. Coomassie-stained SDS-PAGE gel showing the status of all four samples collected on day 0, day 2, day 5, day 8 and day 13. (PI, protease inhibitor.)

(Supplementary Table S1). All X-ray diffraction data for both crystals were indexed and integrated using *XDS* (Kabsch, 2010) and scaling was performed using *XSCALE* (Kabsch, 2010). The structures were solved by the molecular-replacement method using *Phaser-MR* (Adams *et al.*, 2010). The published

ATG12~ATG5-ATG16L1₁₁₋₄₃ structure (PDB entry 4gdk; Otomo *et al.*, 2013) was used as a search model to solve the structures. Structures were refined by iterative model building in *Coot* (Emsley & Cowtan, 2004) and refinement using *phenix_refine* (Terwilliger *et al.*, 2008). All structure figures were generated using *PyMOL*. Data-collection and processing statistics for both of the structures are listed in Supplementary Table S1.

2.4. Stability test of ATG16L1 in the ATG5-ATG16L1₁₁₋₃₀₇ complex

In this assay, 2 mg ml⁻¹ of the ATG5-ATG16L1₁₁₋₃₀₇ protein complex was split into four aliquots of 50 µl each; one

aliquot was incubated at 4°C and the others were incubated at 20°C and were supplemented with 1 mM protease inhibitors (Sigma-Aldrich), supplemented with protease inhibitors and 2 mM EDTA or no further chemicals were added. 5 µl of protein from each aliquot was collected at the indicated time points followed by mixing with 2× DTT loading buffer and analysis using 15% SDS-PAGE.

2.5. Circular-dichroism (CD) spectroscopy

ATG16L1₇₂₋₃₀₇ was dialysed against CD buffer (25 mM NaH₂PO₄ pH 7.5, 100 mM NaF, 5 mM DTT) overnight. A buffer blank was measured which was subsequently subtracted from the protein spectra for baseline correction. The protein concentration was adjusted to 1 mM amide bond and the sample was placed in a cuvette with a 1 mm light path. Spectra were recorded at 260–190 nm at 20°C using a J-815 CD spectrometer (Jasco). Raw data (ellipticity in mdeg) were

Figure 3

(a) Overview of the optimization steps towards obtaining a stable ATG16L1₇₂₋₃₀₇ construct. In comparison to the previously identified minimal CCD (residues 126–207; Parkhouse *et al.*, 2013), the ATG16L1₇₂₋₃₀₇ construct identified here is almost three times larger in size. (b) ATG16L1₇₂₋₃₀₇ elution profile (Superdex 200 16/600 column) and Coomassie-stained SDS-PAGE gel of analysed elution fractions. The calculated molecular weight of ~27 kDa in (b) and (d) corresponds to the calculated molecular weight of monomeric ATG16L1₇₂₋₃₀₇. (c) CD spectrum of ATG16L1₇₂₋₃₀₇. Molar ellipticity plotted against wavelength indicates a helical protein. (d) SEC-MALS profile of ATG16L1₇₂₋₃₀₇. The molar mass is plotted against the elution time of the protein.

exported as a *Microsoft Excel* file and converted into molar ellipticity in $\text{deg cm}^{-2} \text{dmol}^{-1}$. Finally, the CD spectrum was plotted as molar ellipticity *versus* wavelength in *Microsoft Excel*. The *K2D2* program (Perez-Iratxeta & Andrade-Navarro, 2008) was used to estimate the secondary-structure composition.

2.6. Size-exclusion chromatography with multi-angle light scattering (SEC-MALS)

To determine the exact molecular mass of ATG16L1_{72–307}, we performed inline size-exclusion chromatography (SEC; ÄKTA pure, GE Healthcare) coupled with a multi-angle light-scattering (MALS) detector (miniDAWN TREOS, Wyatt Technology) and a differential refractometer (RI; Optilab T-rEX, Wyatt Technology). Owing to the instability of the RI curve for this sample, the UV detector signal was used to calculate the effective complex concentration by using a standard protein refractive-index increment value of 0.185 ml g^{-1} . ATG16L1_{72–307} was used in the final purification buffer: 20 mM HEPES pH 7.0, 300 mM NaCl, 5% glycerol, 5 mM DTT. Protein separation was achieved using a Superdex 200 10/300 column with a flow rate of 0.5 ml min^{-1} . To obtain the molecular weight of the eluted proteins, UV and MALS signals were recorded at three angles and analysed using the *ASTRA 6* software (Wyatt Technology).

3. Results and discussion

For the structural characterization of ATG16L1, we expressed various C-terminally truncated constructs that cover the central region. Of these, ATG16L1_{1–307} could be expressed and purified from *E. coli*, but turned out to be partially degraded (Supplementary Fig. S1). In order to stabilize the ATG16L1 protein, we co-expressed it with its interaction partner ATG5 and also removed the first ten amino acids, which were predicted to be highly flexible by *PSIPRED* and *DISOPRED* (Supplementary Fig. S2; Ward *et al.*, 2004; Buchan *et al.*, 2013). Co-expressed ATG5–ATG16L1_{11–307} formed a stable complex in solution (Fig. 1*b*), and purified ATG5–ATG16L1_{11–307} complex was used for crystallization, yielding two different crystal forms (Fig. 1*c*). However, determination of the structure in both crystal forms, *I4* and *C2*, revealed that only a short ATG16L1 peptide fragment, corresponding to 38 amino acids of the ATG5BD, was present in the ATG5–ATG16L1 complex (Fig. 1*d*), suggesting degradation of ATG16L1 during the crystallization process. Crystal 1 and crystal 2 contained two and four complexes in the asymmetric unit, respectively. Our two structures both show a heterotetrameric arrangement composed of two ATG5 subunits and two ATG16L1 subunits, thus forming a dimer of ATG5–ATG16L1 complexes. The structures reported here were determined in two novel crystal forms, adding additional structural information, which further confirms the previously

Figure 4
Crystallization of ATG16L1_{72–307}. (a) Crystals of ATG16L1_{72–307} with a similar appearance were obtained in two different conditions. (b) Analysis of crystal content on a Coomassie-stained SDS-PAGE gel. Sample 1 is purified ATG16L1_{72–307}, which was used for crystallization. Sample 2 corresponds to dissolved crystals from condition 3. The protein from the dissolved crystals was further confirmed to be ATG16L1 by mass spectrometry (see Supplementary Fig S4). The black arrow indicates the band for ATG16L1_{72–307}.

observed dimerization of ATG5–ATG16L1 *via* the ATG5BD (Supplementary Fig. S3).

Since we aimed at gaining structural information for the central region of ATG16L1 and crystallization of ATG5–ATG16L1_{11–307} including the central region was not successful, we analyzed the time-dependent degradation in a stability assay at 20°C (Fig. 2). Degradation of ATG16L1_{11–307} was observed between 2 and 5 d even in the presence of protease inhibitors. Addition of EDTA or decreasing the temperature to 4°C delayed, but did not completely inhibit, degradation. Nonetheless, degradation resulted in a stable subfragment which lacks the N-terminal ATG5BD as determined by tryptic digestion and mass spectrometry (data not shown). According to the predicted secondary structure and disorder (Supplementary Fig. S2), the region of ATG16L1 between residues 60 and 70 is highly flexible/disordered. Based on the degradation observed during purification of ATG16L1_{1–307} and crystallization trials of the ATG5–ATG16L1_{11–307} complex, we suspect that the linker region between ATG5BD and CCD in ATG16L1 is rather flexible and might inhibit the crystallization of ATG5–ATG16L1_{11–307} before degradation takes place. Therefore, we aimed to crystallize the stable degradation fragment of the central region of ATG16L1 in isolation to obtain detailed structural information on the so far structurally uncharacterized central region of ATG16L1.

We thus generated the novel expression construct ATG16_{72–307}, for which we chose amino acid 72 in the flexible region as the N-terminal boundary (Fig. 3a). The protein was successfully expressed and purified from *E. coli* (Fig. 3b). However, based on the elution volume from size-exclusion chromatography (Fig. 3b), ATG16L1_{72–307} (with a calculated monomeric molecular weight of ~27 kDa) appeared to form a hexamer with an apparent molecular weight of ~160 kDa in solution. Therefore, the correct oligomeric state of the ATG16L1_{72–307} protein was investigated by SEC-MALS, which confirmed that ATG16L1_{72–307} is present as a dimer in solution with a molecular weight of ~51.3 kDa (Fig. 3d). Additionally, the ATG16L1_{72–307} construct was also analyzed by CD spectroscopy, revealing that it is mostly α -helical with ~87% α -helical secondary structure, which is consistent with the results of secondary-structure prediction (Fig. 3c). These data further show that a helical secondary structure extends beyond the previously determined minimal CCD region encompassing residues 126–207 (Parkhouse *et al.*, 2013). We successfully crystallized ATG16L1_{72–307} in two different conditions (Fig. 4a); to confirm the identity of these crystals, crystals from condition 3 were dissolved and analyzed by SDS-PAGE (Fig. 4b) and MALDI-MS. A band of the same size as the protein used for crystallization was observed, which was further identified as the ATG16L1 protein by MALDI-MS analysis, confirming the successful crystallization of ATG16L1_{72–307} (Supplementary Fig. S4). To date, the ATG16L1_{72–307} crystals only diffracted to ~10 Å resolution using synchrotron radiation, hampering structure determination. We are therefore currently in the process of optimizing the quality of the crystals in order to obtain higher resolution diffraction data for structure determination of the central

region of human ATG16L1. The structure determination of the crystallized ATG16L1_{72–307} should provide the missing structural data for the region linking the N-terminal ATG5BD and the C-terminal WD-40 domain. Its structure would thus contribute to our understanding of the various protein–protein interactions mediated by the central region and lead to a better understanding of the assembly/dimerization of ATG16L1 and its complexes in the context of autophagy.

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