Introduction

The degradation of cellular material is an important homeostatic function that enables the removal of redundant, broken and potentially harmful material; whilst simultaneously increasing resource availability in the cell. Two key components of cell degradation pathways are the proteosome and the process of macroautophagy (referred hereafter as autophagy). In autophagy, the formation of a double-membrane autophagosome around cellular targets such as damaged organelles, or invasive microorganisms, facilitates subsequent fusion with lysosomes and the breakdown of the material within the autophagosome. The formation of an autophagosome requires a hierarchical series of interactions between both individual autophagy proteins and preformed protein complexes. ATG16L1 is central to this process, forming part of the ATG12-ATG5-ATG16L1 complex, which is required for the recruitment of LC3 (ATG8 in yeast) to the autophagosome [1]. Removal of ATG16L1 abrogates the ability of cells to form autophagosomes [2].

The N-terminus of ATG16L1, and its yeast ortholog ATG16, is responsible for inclusion of ATG16L1 in the ATG12-ATG5-ATG16L1 complex via interaction with two ubiquitin-like fold domains in ATG5. The molecular basis of this interaction has been determined for both the yeast and human systems and highlights the importance of a helical segment of ATG16L1/ATG16 [3,4]. Two recent reports both identified FIP200 (focal adhesion kinase family interacting protein of 200 kDa), a member of the ULK1 (UNC 51 like kinase 1) autophagy complex along with ULK1, ATG13 and ATG101, as a direct binding partner of ATG16L1 [5,6]. The interaction between FIP200 and ATG16L1 allows recruitment of the ATG12-ATG5-ATG16L1 complex to the ULK1 complex at the site of the assembling autophagosome. The critical involvement of ATG16L1 as a key mediator of essential protein interactions required for autophagy is highlighted by the recruitment of ATG16L1 to the site of bacterial invasion by the pattern recognition receptors NOD1 and NOD2 [7–9]. This interaction requires the CARD of NOD1 and NOD2 and the WD40 repeats of ATG16L1 [10,11]. In the case of NOD2 the interaction involves a newly reported 19-amino-acid ATG16L1-binding motif also found in TLR2 (Toll-like receptor 2), T3JAM (TRAf3 interacting protein 3), DEDD2 (death effector domain containing 2) and transmembrane protein 59 (TMEM59) [10]. Currently it is unclear whether all these proteins play an active role in autophagy. However, at least in the case of TMEM59 the interaction with ATG16L1 mediates the degradation of its own endosomal compartments and enables a protective autophagic response to Staphylococcus aureus infection [10].

In addition to mediating heterotypic protein interactions ATG16L1 also undergoes homotypic interactions via its coiled-coil domain. The structure of the coiled-coil domain of the yeast ortholog, ATG16, revealed the formation of a parallel dimeric coiled-coil. Coiled-coils are found in almost all areas of cell functionality and are common protein interaction surfaces formed...
between extended amphipathic helices. Numerous oligomerisation states have been observed for coiled-coils, with dimers, trimers and tetramers the most common [12]. In addition to yeast ATG16 coiled-coils have also been reported for other autophagy proteins including Beclin-1 [13], TIP200 [14] and ATG11 [15]. In this work we have expressed and characterised the coiled-coil domain of human ATG16L1. We show that it folds as a helical protein and exists as a dimer in solution, consistent with the structural information from the yeast ortholog ATG16. A crucial role for the ATG16L1 coiled-coil in complex formation is supported by an extremely high level of sequence conservation between vertebrate species.

**Results and Discussion**

**Expression of the ATG16L1 coiled-coil domain**

Although functionally similar distinct differences exist in the domain organisation of yeast ATG16 and mammalian ATG16L1 (Figure 1A). Studies of ATG16 from *Saccharomyces cerevisiae* have shown that the protein possesses an ATG5 binding motif at its N-terminus, followed by a coiled-coil domain. Both these domains have previously been successfully crystallised (Figure 1B). The human form, ATG16L1, also contains an N-terminal ATG5 binding motif. However, unlike the yeast protein, this is followed by an extended linker region leading into a coiled-coil, a second linker region, and a series of WD-40 repeats (Figure 1A).

There is limited sequence homology between the coiled-coil regions of human ATG16L1 and yeast ATG16. A search of the NCBI non-redundant protein sequence database with the coiled-coil of *S. cerevisiae* ATG16 failed to return any significant hits when limiting results to proteins from *Homo sapiens*. Despite this limited primary sequence homology Fujioka and colleagues were previously able to align the two proteins on the basis of a pattern of repeating hydrophobic residues in the a and d positions of the helix (Figure 1 in [16]). We used this alignment as a basis for the design of three initial expression constructs containing the human ATG16L1 coiled-coil domain (Figure 1C). These were: full-length ATG16L1 spanning residues M1-Y607 (FL); residues M1-A207 containing the ATG5 binding motif, the first linker region and the coiled-coil (CCD1); and residues M126-A207 encompassing the minimal coiled-coil domain proposed by the alignment with yeast ATG16 (CCD2) (Figure 1C). All constructs were screened for expression with a variety of N-terminal fusion tags: 6-His alone; GST (glutathione S-transferase); 6His-NuSA (N utilisation substance protein A); and 6His-MBP (Maltose binding protein). Each construct also possessed a C-terminal FLAG-6His epitope tag. Full-length protein was entirely insoluble. However, CCD1 and CCD2 expressed with each tag except the 6-His tag alone (Table 1). Expression levels were comparable between fusion partners so the GST fusion constructs were selected for large scale expression and purification as GST is simple and effective to use and has been previously used to successfully purify yeast ATG16 [17].

**Purification of the ATG16L1 coiled-coil domain**

CCD1 and CCD2 were both purified by GST pull down, on column TEV cleavage and anion exchange (Figure 2). SDS-PAGE analysis of both recombinant proteins indicated the presence of truncated, or cleaved, protein products (Figure 2A and 2D). Analysis by mass spectroscopy and N-terminal sequencing indicated that the CCD1 truncation had lost the first 53 amino acids and now began at L56 within the linker region. The truncated CCD2 protein had been cleaved between Q125 and M126. Given the observed cleavage immediately before M126, we designed a new construct (CCD3) spanning residues M126-A207. CCD3 was expressed as a GST-CCD3-FLAG-6His fusion and purified to homogeneity using glutathione sepharose, on column TEV cleavage, and HIC (Figure 2E). CCD3 showed no evidence of truncation indicating the formation of a stable protein and was selected as the final construct for further characterisation.

**The ATG16L1 coiled-coil adopts a helical conformation**

The region of ATG16L1 (M126-A207) encompassed by our minimal CCD3 construct closely correlates with the section

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**Table 1. Summary of human ATG16L1 construct expression in *E. coli* Rosetta™ 2 cells.**

| N-terminal tag | Human ATG16L1 construct | FL (M1-Y607) | CCD1 (M1-A207) | CCD2 (M117-A207) |
|---------------|--------------------------|--------------|----------------|------------------|
| 6His          | Insoluble                | Insoluble    | Insoluble      |                  |
| GST           | Insoluble                | Soluble      | Soluble        |                  |
| 6His-NuSA     | Insoluble                | Soluble      | Soluble        |                  |
| 6His-MBP      | Insoluble                | Soluble      | Soluble        |                  |

Footnote: All constructs possessed a C-terminal FLAG-6His epitope tag.

Abbreviations: 6His – 6× Histidine; GST – Glutathione-S-transferase; 6His-NuSA – 6× Histidine N utilisation substance protein A; 6His-MBP – 6× Histidine Maltose binding protein.

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aligned to the coiled-coil of ATG16 by Fujioka and colleagues [16]. Consistent with this the secondary structure of CCD3 (M126–A207) was predicted to be entirely alpha helical by the PSIPRED server (Figure 3A). The helical nature of CCD3 was confirmed by circular dichroism (Figure 3B). SELCON3 analysis revealed the protein to be approximately 80% helical, 5% turns and 15% disordered. Together these results provide strong indication that the core, stable, and folded portion of the coiled-coil domain of human ATG16L1 is found between residues M126 and A207.

Analysis of the multimeric nature of the ATG16L1 coiled-coil

Yeast ATG16 was originally reported to homo-oligomerise in a process dependent on the coiled-coil domain [18]. Analysis of the complex formed between yeast ATG16, ATG5 and ATG12 suggested a molecular weight of approximately 350 kDa, for which a tetrameric assembly was postulated [19]. The murine ortholog, which, like the human protein, contains WD40 repeats, was suggested to exist in an octomeric assembly with murine ATG5–ATG12 conjugates following detection of an approximately 800 kDa complex of ATG16L1–ATG5–ATG12 from murine cells [20]. In the isolated form yeast ATG16 exists as a dimer both in the crystal structure and in solution as determined by analytical ultracentrifugation [16].

To investigate the oligomeric state of human ATG16L1 CCD3 in solution, we first analysed the recombinant protein using two standard techniques, size exclusion chromatography (SEC) and Native-PAGE. Based on its amino acid sequence the calculated molecular weight of CCD3 is 11.3 kDa. SEC produced a single symmetrical peak (Figure 4A) with an estimated molecular mass of approximately 70 kDa, suggesting that human ATG16L1 is a hexamer in solution. However, Native-PAGE produced a dominant band just above the 20 kDa marker, indicative of a dimeric protein (Figure 4B). Weak bands were visible for higher molecular weight species at sizes broadly consistent with tetrameric and octomeric protein. The relative proportions of oligomeric CCD3 was unaffected by storage at −80°C and subsequent thawing suggesting that the minimal coiled-coil motif is stable (Figure 4B). The difference in predicted mass observed with these techniques likely results from the influence of molecular charge in the Native-PAGE. CCD3 is 22.7% acidic, negatively charged, residues, but only 13.4% basic, positively charged, residues. Consequently its migration through the gel matrix will be increased.

AUC and crystallography show that yeast ATG16 is a dimer [16]. As SEC can overestimate the molecular weight of non-globular proteins [21], and because the results of Native-PAGE
may be overly affected by molecular charge, we sought alternative confirmation of the oligomeric status of CCD3. We used nanospray Electrospray Ionisation Mass Spectroscopy (ESI-MS), a well established reliable technique for the study of the stoichiometry and interactions of non-covalent complexes in the gas phase [22–26]; and also AUC, the gold-standard for determination of protein molecular weight independently of protein shape and charge. Both approaches indicated that the human ATG16L1 was a dimer in solution. ESI-MS identified a single species with a measured molecular weight of 22,687 ± 66 Daltons (Figure 4C); whilst AUC measured the molecular weight to be approximately 23.7 kDa with an rmsd of 0.006 (Figure 4D). Hence, just like the yeast ATG16 coiled-coil, the isolated human ATG16L1 coiled-coil exists as a dimer and is likely to be involved in homomeric interactions during the creation of the multiprotein complexes involved in autophagosome formation. This would then allow the ATG5 binding motif and WD40 motifs to recruit the appropriate interaction partners to facilitate multiprotein complex formation.

The coiled-coil domain of ATG16L1 is highly conserved across vertebrate evolution

Cross-species comparisons of protein sequences can provide insight into the functional and structural importance of particular regions of the protein. We used human ATG16L1 to perform a BLASTp search of the non-redundant protein database. From the resulting hits we extracted a broad range of vertebrate orthologs of ATG16L1 including representatives of fish, reptiles, amphibians,
Method

Plasmids and Gateway® cloning

Potential domain boundaries of the human ATG16L1 coiled-coil region were identified using the sequence alignments of Fujioka and colleagues [16]. Full-length ATG16L1 and fragments corresponding to residues 1–207 (CCD1) and 126–207 (CCD2) were PCR amplified, using Gateway® compatible primers, from pCMV-FLAG-ATG16L1 (a kind gift from Dr Duncan Massey, Cambridge Institute for Medical Research). Forward primers contained an N-terminal TEV cleavage site; reverse primers a Flag-6xHis tag. Invitrogen Gateway® cloning technology enabled insertion of each PCR product into the destination vectors pDest15 (GST), pDEST HisMBP, pDEST 544 (HisNusA).

Expression and purification of human ATG16L1 constructs

Human ATG16L1 expression was screened in E. coli Rosetta™ 2 cells (Merck Millipore) induced with 1 mM Isopropyl β-D-thiogalactopyranoside (IPTG) at 20°C for 14 hours in 2 ml Luria Broth (LB; 10 g NaCl, 10 g Tryptone and 5 g Yeast Extract per Litre deionised water) supplemented with 100 μg/ml of Carbendazil and 34 μg/ml Chloramphenicol. Cells were pelleted by centrifugation (16,300 × g, 5 min); and lysed with 100 μl of BugBuster (Novagen). Soluble and insoluble fractions were separated by centrifugation (16,300 × g, 5 min), mixed with SDS-PAGE sample buffer and analysed using 10% SDS-PAGE.

Large-scale protein expression was performed in 1 litre LB as described above. Following centrifugation (4,000 × g, 20 min) pellets were resuspended in lysis buffer [1× phosphate buffered saline (PBS), 0.137 M, NaCl, 2.7 mM KCl, 4.0 mM Na2HPO4, 10 mM Dibasic potassium phosphate (DTT), 30 mg/ml Lysozyme and 1× Protein Inhibitor Cocktail V (Calbiochem)], sonicated on ice and centrifuged (48,384 × g, 30 minutes). Soluble fusion protein was recovered using glutathione sepharose beads (GE Healthcare) at 4°C for 2 hours with rolling. Beads were placed in a gravity flow column and washed 5 times with (then resuspended in) 20 ml 1× PBS, 1 mM DTT, 1× Protein Inhibitor Cocktail V. One tenth volume of Tobacco Etch Virus (TEV) protease was added and incubated for fourteen hours at 4°C. Cleaved protein was eluted in 2 ml fractions. Eluates were pooled and purified further using either anion exchange or hydrophobic interaction chromatography (HIC). For anion exchange pooled eluates were diluted 10-fold in IE diluent buffer (25 mM Tris pH 7.0, 1 mM DTT), then applied to a Resource™ Q anion exchange column (GE Healthcare) pre-equilibrated in IE diluent buffer. Protein was eluted over 30 column volumes with a 0–50% gradient of 25 mM Tris pH 7.0, 1 M NaCl and 1 mM DTT. For HIC elutions from the glutathione resin were diluted 20-fold in HIC binding buffer (2 M Ammonium sulphate, 25 mM Tris pH 8.0) and applied to a 5 ml HiTrap™ Butyl FF column (GE Healthcare) pre-equilibrated with HIC binding buffer. Loosely bound protein was removed with a 0–40% gradient of elution buffer (25 mM Tris pH 8.0) before purified recombinant protein was recovered over 18 column volumes with a 40–100% gradient of elution buffer. Purified CCD3 was buffer exchanged into 25 mM Tris pH 7.0, 100 mM NaCl and 1 mM DTT through a HiPrep™ 26/10 Desalting column (GE Healthcare) before concentration and further analysis.

TEV Protease Expression and Purification

E. coli Rosetta™ 2 cells were transformed with TEV expression plasmid (a kind gift from Prof N Gay, University of Cambridge). Expression of recombinant protein was induced with 1 mM IPTG for 14 hours at 20°C. Cells were pelleted (4,000 × g, 30 min); resuspended in 50 ml of TEV lysis buffer (300 mM NaCl, 25 mM Sodium phosphate, 20 mM Imidazole, 0.1% Triton (v/v) and 5 mM β-mercaptoethanol); lysed by sonication; centrifuged (48,384 × g, 30 min); and the soluble extract incubated with HisSelect™ Nickel Affinity Gel (Sigma Aldrich) for 3 hours. Nickel Affinity Gel was applied to a gravity flow column, washed five times with 20 ml of TEV lysis buffer, and recombinant TEV eluted using TEV lysis buffer supplemented with 200 mM Imidazole and 5% glycerol (v/v). TEV was further purified using a HiLoad™ 16/60 Superdex™ 75 column (GE Healthcare) equilibrated with 300 mM NaCl, 25 mM Sodium phosphate and 5% glycerol (v/v). Purified protein was collected in 2 ml elutions.

Analytical Gel Filtration

6 mg of purified protein was loaded onto a Superdex™ 200 10/300 GL column (GE Healthcare) equilibrated with 25 mM Tris pH 7.0, 150 mM NaCl and 1 mM DTT. The following molecular standards were used for estimation of molecular weight: Aprotinin (6.5 kDa), Cytochrome C (12.4 kDa), Carbonic Anhydrase (29 kDa), Albumin (66 kDa), Alcohol dehydrogenase (150 kDa), β-amylose (200 kDa) and Blue Dextran (2,000 kDa; all from Sigma Aldrich).

Native-PAGE

The native conformation of CCD3 was analysed using Novex® 4–20% Tris-glycine native gels (Life Technologies). Samples were
mixed with Novex® native Tris-glycine 2x sample buffer (Life Technologies) and 1 μg of protein was loaded per lane. Gels were run at 125 V for 1.5–2 hours using Novex® Tris-glycine native running buffer (Life Technologies). Nativemark protein size standards specific for native gels were run in parallel.

Mass spectroscopy

CCD3 was buffer exchanged using Biospin 6 micro-spin columns (Biorad) into 200 mM ammonium acetate, pH 7. Analysis of the oligomeric nature of CCD3 was performed using previously described protocols [22]. In summary, sample ionisation was achieved by nano-electrospray ionisation using in-house prepared gold-coated glass capillaries. A Synapt-HDMS mass spectrometer (Waters) fitted with a 32K quadrupole and set in positive ion mode was used to acquire mass spectra. Nitrogen was used in the IMS T-wave cell and argon in the trap/transfer T-wave region. Raising the collision energy in the trap T-wave region led to collision-induced dissociation and ion activation. Calibration was achieved with CsnIn+ clusters. Data processing and analysis used Masslynx.

Analytical Ultracentrifugation (AUC)

Sedimentation velocity experiments were conducted with an Optima XL-I (Beckman Coulter) using an An60 Ti four-hole rotor. Standard double-sector Epon centerpieces equipped with sapphire windows contained 400-μL of CCD3FH at 1.5 and 0.5 mg/ml. Interference data were acquired in the continuous mode without averaging and with radial increments of 0.003 cm. The density and viscosity of the buffer and the partial specific volume of CCD3 were determined.

Figure 5. The coiled-coil of ATG16L1 is highly conserved across vertebrates. (A) Cross-species alignment of the coiled-coil (human residues M126 – A207) of ATG16L1 reveals levels of sequence identity with the human sequence of between 73% and 100%. The percentage identity listed is in comparison with the human sequence. The common names and database identifiers for each sequence are listed in Materials and Methods. (B) The syntenic position of Atg16L1 is well conserved across species. The three adjacent upstream and downstream genes to Atg16L1 are displayed. Genes are denoted by individual blocks; yellow indicates a position on the forward strand and green a position on the reverse strand. Gene identities are as follows: ATG16L1 – autophagy related protein 16 isoform 1; SAG – S-antigen, retina and pineal gland; DGKD – diacylglycerol kinase delta; USP40 – ubiquitin specific peptidase 40; INPP5D – inositol polyphosphate-5-phosphatase; NEU2 – sialidase 2; NGEF – neuronal guanine exchange factor; TRPV2 – transient receptor potential cation channel, subfamily V, member 2; UBB – ubiquitin B; AC106876.2 – uncharacterised; 00570 (ENSMODG00000000570) – uncharacterised; 23152 (ENSMODG00000023152) – uncharacterised. BLASTp indicates a possible ortholog of glyceraldehydes 3-phosphate dehydrogenase.

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Circular Dichroism

Circular dichroism spectra were measured using a 25 nm sodium phosphate buffer (pH 7.0, 100 mM sodium fluoride, 1 mM TCEP; centrifuged at 16,000 x g, 10 minutes, 4°C; and 400 µl loaded into a 1.0 mm quartz cuvette at an OD200 reading of ~0.4). CD analysis was performed using an Aviv model 4.0 CD spectrophotometer. Dichroweb [29] was used to convert machine units to mean residue ellipticity [9] prior to graphical presentation with GraphPad Prism 5.

Bioinformatics, database sequences and secondary structure prediction

Protein sequences for database searching and bioinformatics were extracted from the NCBI depositories for human ATG16L1 isoform 1 (NP_110430.3) and Saccharomyces cerevisiae ATG16 (NP_013882.1). Protein data bank accession codes used in figure preparation and homology modelling were as follows: 3A70 – S. cerevisiae coiled-coil domain 16; 4DK – human ATG16L1 ATG5 binding domain 4; 2DYO – S. cerevisiae ATG16 ATG5 binding domain 3. The secondary structure of human ATG16L1 residues 126–207, corresponding to the CCD3 construct, was predicted using PSIPRED [30]. Protein alignments were performed using MUSCLE and manually refined; Clustal W2 was used to calculate pairwise sequence identity. The sequences used in the cross-species alignment were: Homo sapiens (Human, NP_110430.5), Pongo abelii (Sumatra orangutan, NP_01125757.1), Nomascus leucogenys (Northern white-cheeked gibbon, XP_003278806.1), Pan troglodytes (Chimpanzee, XP_001150112.1), Macaca mulatta (Rhesus macaque, AFE78970.1), Callithrix jacchus (Common Marmoset, XP_002749988.2), Sus scrofa (Pig, NP_001177201.1), Cricetulus griseus (Chinese Hamster, EGW02492), Orctylosmus cuniculus, (European rabbit, XP_002714811), Cavia porcellus (Guinea Pig, XP_003474455.1), Alluropodala melanoleuca (Giant Panda, XP_002918010.1), Canis lupus familiaris (Dog, XP_850664.1), Rattus norvegicus (Brown Rat, NP_001102979.2), Mus musculus (Mouse, BAC55091.1), Bos taurus (Cow, DAA30946.1), Equus caballus (Horse, XP_001915181.7), Loxodonta africana (African Bush Elephant, XP_003418005.1), Meleagris gallopavo (Wild Turkey, XP_063209019.1), Gallus gallus (Chicken, XP_422568.2), Taeniopygia guttata (Zebrafinch, XP_002197297.1), Anolis carolinensis (Carolina Anole Lizard, XP_003218430.1), Monodelphis domestica (Grey short-tailed opossum, XP_001373496.2), Xenopus tropicalis (Western clawed frog, CAFJ21691.1), Ornithorhynchus anatinus (Platypus, XP_001520939.2), Xenopus laevis (African clawed frog, NP_0011091437.1), Danio rerio (Zebrafish, NP_001017854.1), Oreochromis niloticus (Nile Tilapia, XP_003457670.1), Tetraodon nigroviridis (Green-spotted pufferfish, CAG02123.1).

Conclusion

There is strong functional and evolutionary evidence for the importance of autophagy. Here we have shown that the coiled-coil region of vertebrate ATG16L1 is highly conserved and just like its yeast ortholog likely to exist as a homodimer to facilitate ATG16L1 self-self interactions. ATG16L1 is a crucial component of the inter-protein scaffold required for formation of the autophagosome. This work lays the foundation for future structural studies to enhance our understanding of how ATG16L1 interacts with itself and other proteins, which in turn could help the modulation of autophagy for therapeutic purposes.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: TP I-OE CVR TPM. Performed the experiments: TP I-OE TPM. Analyzed the data: TP I-OE CVR TPM. Wrote the paper: TPM.

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