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The UbL protein UBTD1 stably interacts with the UBE2D family of E2 ubiquitin conjugating enzymes

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ABSTRACT

UBTD1 is a previously uncharacterized ubiquitin-like (UbL) domain containing protein with high homology to the mitochondrial Dc-UbP/UBTD2 protein. Here we show that UBTD1 and UBTD2 belong to a family of proteins that is conserved through evolution and found in metazoa, funghi, and plants. To gain further insight into the function of UBTD1, we screened for interacting proteins. In a yeast-2-hybrid (Y2H) screen, we identified several proteins involved in the ubiquitylation pathway, including the UBE2D family of E2 ubiquitin conjugating enzymes. An affinity capture screen for UBTD1 interacting proteins in whole cell extracts also identified members of the UBE2D family. Biochemical characterization of recombinant UBTD1 and UBE2D demonstrated that the two proteins form a stable, stoichiometric complex that can be purified to near homogeneity. We discuss the implications of these findings in light of the ubiquitin proteasome system (UPS).

1. Introduction

Ubiquitylation is an important modification that can tag proteins for proteasomal degradation or alter their properties. The covalent attachment of ubiquitin to target proteins is vital for most cellular processes including for example gene expression, mitosis, protein sorting, and signal transduction. Mitochondria do not contain a ubiquitin proteasome system (UPS), but are nonetheless closely linked to the UPS particularly regarding quality control, dynamics, and apoptosis [1–4]. Ubiquitylation at the outer mitochondrial membrane is a well-established process for which several E3's have been identified including MITOL, MULAN and Parkin [5–7]. There have also been reports of intra-mitochondrial proteins being ubiquitylated [8], though the mechanistic details remain to be clarified.

The ubiquitin like (UbL) proteins contain an integrated ubiquitin domain, but cannot themselves be conjugated to other proteins. The ubiquitin domain containing 2 (UBTD2) protein, also termed dendritic cell-derived ubiquitin (ub)-like protein (DC-UbP), is a short UbL protein that is localized to mitochondria and is implicated in apoptosis [9]. Phylogenic analyses presented here revealed that mammals contain a second homolog, UBTD1, which is hitherto unstudied. To gain further insight into UBTD1 function we screened for interacting proteins. A yeast-2-hybrid (Y2H) and affinity capture screen both identified proteins involved in the ubiquitylation pathway. One group of proteins that was identified in both screens was the UBE2D family of E2 ubiquitin conjugating enzymes. Further assays using recombinant proteins showed that UBTD1 and UBE2D form a stoichiometric complex that is stable after purification over several chromatographic steps. Our studies suggest that UBTD1 belongs to an evolutionarily conserved family of proteins that may regulate the activity and/or specificity of E2 ubiquitin conjugating enzymes belonging to the UBE2D family.

2. Materials and methods

2.1. Sequence and phylogeny analyses

In order to identify homologs to UBTD1 and UBTD2 the N-terminal half of the human proteins (not containing the UbL domain) were used as queries in PSIBLAST [10] searches against the NCBI protein sequence database. These searches identified a number of homologs in metazoa, plants and fungi. These proteins were aligned using ClustalW [11]. The same program was used to create phylogenetic trees using neighbor-joining and 1000 bootstrap replicates.

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2.2. Y2H analysis

Yeast two-hybrid screening was performed by Hybrigenics Services, S.A.S., Paris, France (http://www.hybrigenics-services.com). In brief, a pB28 construct encoding UBTD1 (GenBank accession number gi: 34222339) (aa 49–218) fused at the C-terminus to LexA was used as bait to screen a random-primed human adult and fetal skeletal muscle cDNA library (129 million clones). The growth of yeast transformants on −HIS plates was used to indicate an interaction. To semi-quantify the strength of interactions, 3-Amino-1,2,4-triazole (3-AT), a competitive inhibitor of the yeast His3 protein, was added to −HIS plates at increasing concentrations (5–50 mM).

2.3. Affinity capture screen

A total of 40 μg GST or GST-UBTD1 (residues 1–227) were immobilized on glutathione beads (GE Healthcare) and incubated overnight with pre-cleared HeLa whole cell extract (4 mg) in GST buffer containing 20 mM Tris–Cl pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, and 1× proteinase inhibitors [12]. Bead complexes were then washed before eluting with glutathione (50 mM Tris, 10 mM reduced glutathione, pH 8.0). Eluted material was separated by SDS–PAGE and proteins were visualized by silver staining (Pierce).

2.4. Western blot

Proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline (TBS)-0.1% Tween 20 containing 5% dry milk and incubated with anti-UBE2D3 primary antibody (AbCam). Blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare) for 1 h at room temperature. Immunocomplexes were visualized using the ECL Western Blotting Substrate (Pierce) and exposed to X-ray film (FujiFilm).

2.5. Cloning and purification of the UBTD1-UBE2D3 complex

Codon-optimized (DNA 2.0) DNA constructs corresponding to the mature form of human UBTD1 and full length UBE2D3 were cloned into a pCDFDuet-1 vector (Novagen). UBE2D3 incorporates a TEV-cleavable 6× His fusion tag at the N-terminus. The UBTD1-UBE2D3 complex was purified following the procedure for the MTERF4-NSUN4 complex as previously described [13] with an additional Superose 12 gel filtration step in buffer H-0.5 (25 mM Tris–HCl [pH 7.8], 0.5 mM EDTA, 1 mM dithiothreitol, 500 mM NaCl).

2.6. Mass spectrometry

For protein digestion and peptide extraction, we used a previously described method for in-gel protein digestion [14] with some minor modifications [15]. Briefly, the gel pieces were destained by washing three times in 25 mM NH_4HCO_3 in 50% CH_3CN and one time in 25 mM NH_4HCO_3 in 50% CH_3OH. Gel pieces were dried in a vacuum centrifuge and incubated with digestion buffer (50 mM NH_4HCO_3, 10 ng/μl trypsin) at 37 °C overnight. Peptides were extracted in 50% CH_3CN/1% CH_3COOH and the supernatant was evaporated to dryness in a vacuum centrifuge. Prior to MS analysis, the peptides were reconstituted in 0.2% HCOOH. For nanoflow LC-MS/MS FT/ICR, two-microliter sample injections were made with an HTC-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) connected to an Agilent 1100 binary pump (Agilent Technologies, Palo Alto, CA, USA). The peptides were trapped on a precolumn (45 × 0.075 mm i.d.) and separated on a reversed phase column, 200 × 0.050 mm. Both columns are packed in-house with 3 μm Reprosil-Pur C_{18}-AQ particles. The flow through the analytical column was reduced by a split to approximately 100 nl/min. A 40 min gradient 10–50% CH_3CN in 0.2% COOH was used for separation of the peptides. The nanoflow LC-MS/MS were performed on a hybrid linear ion trap-FTICR mass spectrometer equipped with a 7T ICR magnet (LTQ-FT, Thermo Electron, Bremen, Germany). The spectrometer was operated in data-dependent mode, automatically switching to MS/MS mode. MS-spectra were acquired in the FTICR, while MS/MS-spectra were acquired in the LTQ-trap. For each scan of FTICR, the three most intense, doubly or triply charged, ions were sequentially fragmented in the linear trap by collision induced dissociation. All the tandem mass spectra were searched by MASCOT (Matrix Science, London) against the NCBI database The search parameters were set to: All species, MS accuracy 5 ppm, MS/MS accuracy 0.5 Da, one missed cleavage by trypsin allowed and variable modification of propionamide modification of cysteine and oxidized methionine. For protein identification the minimum criteria were two tryptic peptide matched at or above the 95% level of confidence.

3. Results

3.1. Sequence alignment and phylogeny of UBTD1

Human UBTD2 is a 190-residue mitochondrial protein that contains a C-terminal UbL domain and an N-terminal extension, which acts as a low affinity ubiquitin binding domain [16]. We show here that a second protein of 227 amino acids, UBTD1 (Fig. 1A), is 83% identical to UBTD2 in the amino-terminus (aa 14–141) and also contains a UbL domain (aa 129–234; 58% identical, 79% similar to UBTD1; 34% identity and 55% similarity to ubiquitin) in the carboxy-terminus. No other paralogs to UBTD2 were identified in human cells.

Protein similarity searches demonstrated that UBTD1 and UBTD2 belong to a family of proteins highly conserved in evolution. Homologs to the two proteins were observed both in metazoa and plants (Fig. 1B). Amphibia and teleosts (an infraclass of ray-finned fishes) seem to have an additional paralog to UBTD1 (here referred to as UBTD1A and UBTD1B) as shown in Fig 1. We also observed homologs in plants, but these do not contain the UbL domain characteristic of metazoan UBTD proteins. It is therefore possible that in plants the N- and C-terminal domains of UBTD proteins exist as separate proteins. Furthermore, a more distant homolog to UBTD was identified in funghi, but not in *Saccharomyces cerevisiae*. It would seem that these homologs are more distant than the plant proteins, and it is therefore not clear how the evolutionary history of the fungal proteins is related to that of the metazoa/plant group of proteins.

3.2. UBTD1 Y2H screen identifies proteins of the ubiquitylation pathway

We first performed a UBTD1 Y2H screen against a human adult/fetal skeletal muscle cDNA library. A total of nine protein interactions were identified (Table 1). Dozens of clones were found for members of the UBE2D (UbcH5) family of E2 ubiquitin conjugating enzymes. Together with E1 activators and E3 ligases, E2 conjugating enzymes are needed for ubiquitylation of target proteins [17]. A one-by-one Y2H assay between UBTD1 and UBE2D3 is shown (Fig. 2A), confirming the results of the screen. The interaction was resistant to high concentrations of 3-AT (Fig. 2B). In addition to this family of proteins several other proteins implicated in the ubiquitylation pathway were identified, including ring finger pro-

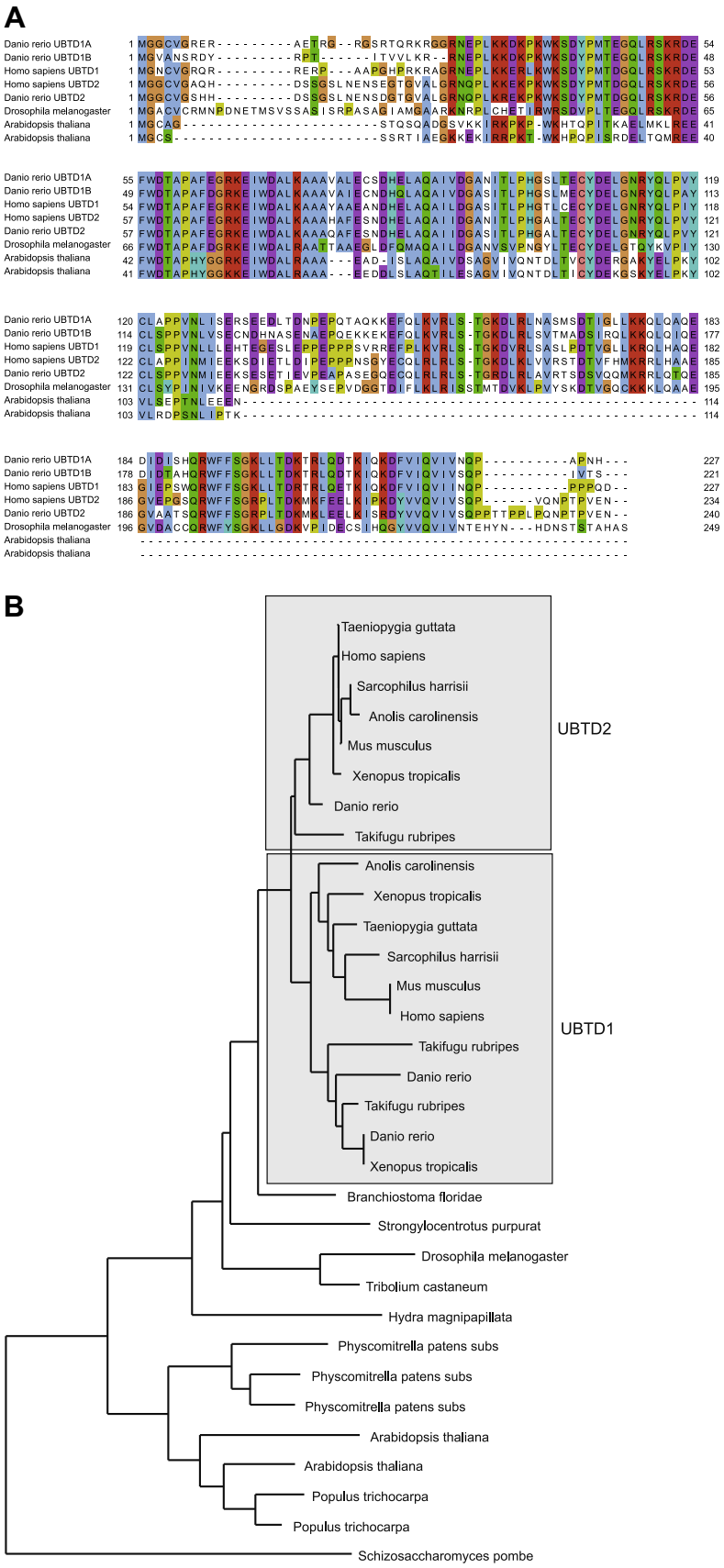

Fig. 1. UBTD1 is a conserved protein. (A) Sequence alignment of UBTD1 and UBTD2 homologs. The C-terminal portion of the alignment where sequence is missing in the Arabidopsis proteins corresponds to the ubiquitin-like domain characteristic of the metazoan UBTD proteins. (B) Phylogenetic tree based on neighbor-joining showing relationship of selected UBTD-related proteins from metazoa, plants and fungi.

Table 1
Proteins identified in UBTD1 Y2H screen.

Identified protein	Name	UniProt Accession	Biological process
UBE2D2	Ubiquitin-conjugating enzyme E2 D2	P62837	UbL conjugation pathway
UBE2D3	Ubiquitin-conjugating enzyme E2 D3	P61077	UbL conjugation pathway
UBE2D4	Ubiquitin-conjugating enzyme E2 D4 (putative)	Q9Y2X8	UbL conjugation pathway
RNF10	RING finger protein 10	Q8N5U6	Transcription, transcription regulation
TEAD1	Transcriptional enhancer factor TEF-1	P28347	Transcription, transcription regulation
RANBP2	E3 SUMO-protein ligase RANBP2	P49792	Protein transport, translocation transport, mRNA transport, UbL conjugation pathway
KIAA2026	Uncharacterized protein KIAA2026	Q5HYC2	Unknown
PFKM	6-Phosphofructokinase, muscle type	P08237	Glycolysis
BCOR	BCL-6 corepressor	Q6W2J9	Transcription, transcription regulation

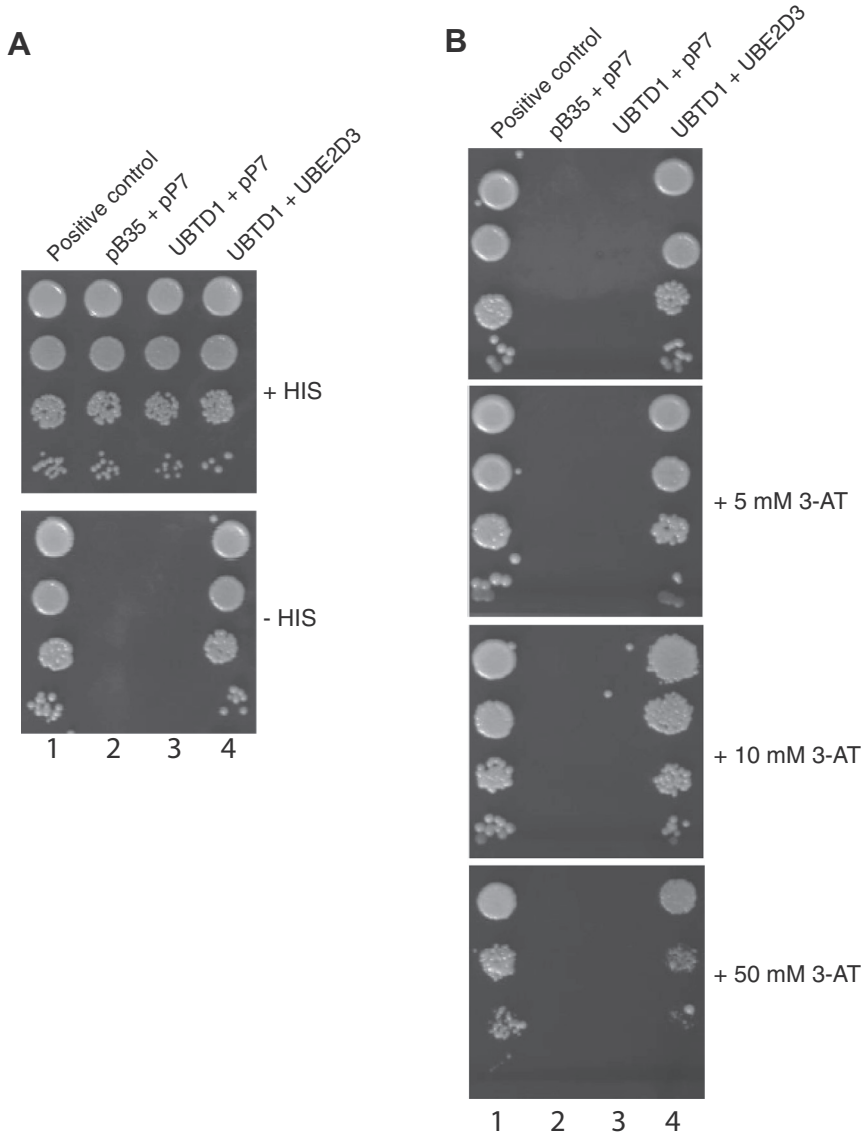

Fig. 2. Y2H between UBTD1 and UBE2D3. (A) Columns 1–3 are controls. Plasmids pB35 and pP7 are empty vectors without UBTD1 and UBE2D3 inserts respectively. Growth on −HIS plates indicates an interaction. (B) As for panel A, except that transformants were plated on increasing concentrations of 3-AT to test the strength of the interaction.

tein 10 (RNF10), which has also been shown to interact with the UBE2D family [18]. Another protein identified in the screen, BCOR, is known to interact with the E3 ligase SKP1-CUL1-FBOX complex [19]. Finally, Ran-binding protein 2 (RANBP2) is a nuclear pore complex protein that is a small ubiquitin-related modifier (SUMO) E3 ligase [20,21].

3.3. Affinity capture screen shows interaction between UBTD1 and UBE2D proteins

Since Y2H screens may generate false positive results, we decided to also use an alternative approach for identification of UBTD1 interacting proteins. To this end, we employed GST or GST-UBTD1 coupled resins as bait in an affinity capture screen to pull down proteins from HeLa cell extracts. Two proteins of about 16 kDa and 27 kDa were identified that were unique to the GST-UBTD1 sample (Fig. 3A). The 27 kDa band contained the actin-binding protein SHROOM3, which we had not observed in the Y2H screen. We also identified a 16 kDa band that contained peptides matching members of the UBE2D family of E2 enzymes, which was confirmed by immunoblot analysis against UBE2D3 (Fig. 3B). Together, the Y2H and affinity capture screens strongly implicate that UBTD1 directly interacts with the UBE2D family of E2 enzymes and thus participates in the ubiquitylation pathway. To further substantiate this interpretation we analyzed interactions between recombinant UBTD1 and UBE2D3 *in vitro*. The two proteins were heterologously coexpressed in *Escherichia coli* and purified to homogeneity over three different columns. During all purification steps, the two proteins co-migrated as a single complex, and when subjected to gel filtration, UBTD1 and UBE2D3 co-eluted, indicating that they interact directly and form a tight complex (Fig. 3C). The identities of the protein bands were verified by mass spectrometric analysis (data not shown).

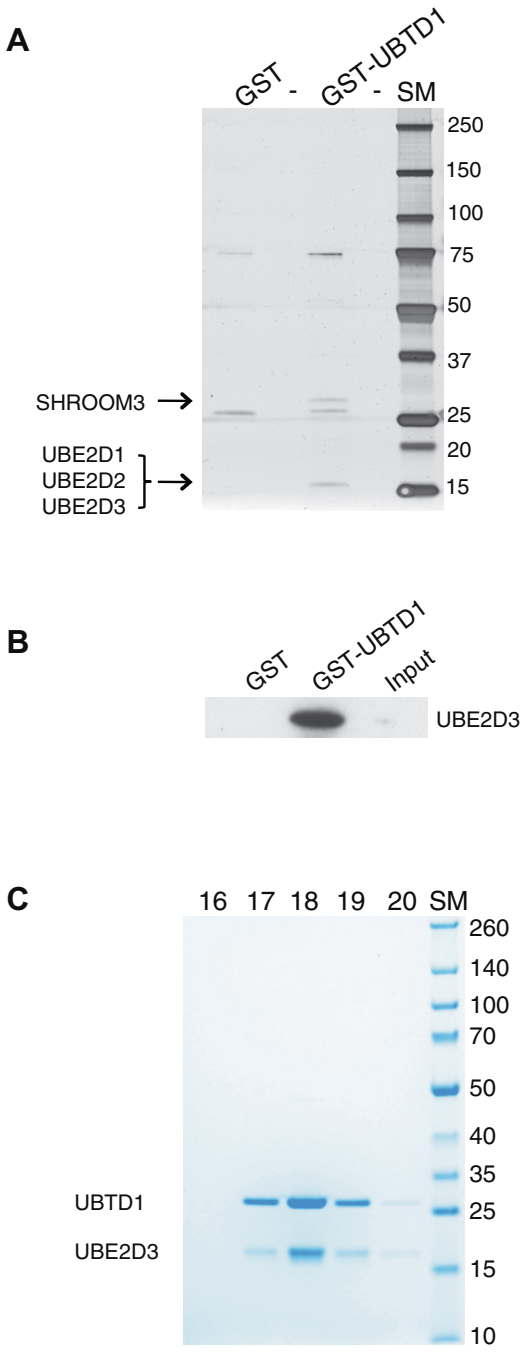

Fig. 3. UBE2D proteins interact with UBTD1 in a biochemical screen. (A) Affinity capture screen of UBTD1 interacting proteins from HeLa cell extracts using GST or GST-UBTD1 coupled resin as bait. The arrows indicate the identified proteins. (B) Immunoblot verification of identified UBE2D proteins. (C) Purified UBTD1 and UBE2D3 form a complex. The UBTD1–UBE2D3 complex was subjected to gel filtration and peak fractions were separated on a SDS–PAGE gel and identified with MALDI-TOF mass spectrometry. Identified proteins are indicated on the left, fraction number on top and molecular masses according to standard on the right.

4. Discussion

Many UbL proteins have direct links to the UPS, but some are also linked to other cellular processes, making functional predictions difficult [22–24]. Our findings here directly link UBTD1 to the UPS and identify processes that may be influenced by this protein. UBTD1 is similar in sequence to UBTD2, which has been structurally characterized. The C-terminal region of the UBTD2 protein contains a UbL domain that has been crystallized and shown to assume a ubiquitin fold [9,16]. Moreover, the N-terminus of UBTD2 (aa 14–141) forms a novel structural fold that acts as a low affinity ubiquitin binding domain [25]. One group of proteins which contain both a UbL and ubiquitin-binding domain are proteasome delivery factors, which are able to bind both polyubiquitylated substrates and the proteasome [26].

In light of this, our finding that UBTD1 interacts with ubiquitin conjugating enzymes is intriguing. Using two different screening approaches, we have demonstrated that UBTD1 interacts with the ubiquitin conjugating E2D family proteins. Using purified recombinant proteins and gel filtration chromatography we further show that the two proteins interact directly with each other in a complex. Mammals have around 30–40 different types of E2 enzymes that transfer activated ubiquitin from E1s to either E3s, or directly to target proteins in conjunction with E3s [27,28].

We found that UBTD1 interacts exclusively with the UBE2D family of E2 enzymes for which several different E3 ligases and targets have been identified (e.g.: [29,30]). The functional significance of UBTD1 forming a complex with UBE2D is still an open question. Some insights might be gained from a crystal structure of the UBTD1–UBE2D complex, which we are currently pursuing. For example, UBTD1 may bind to UBE2D via its UbL domain, thereby forming a possible ubiquitin-binding block. Alternatively, UBTD1 may bind via its UbL domain to UBE2D residues in the beta-sheet opposite to the active site where it has been shown that ubiquitin can bind non-covalently and enhance the processivity of ubiquitylation [31]. To study the latter possibility, we need to identify a physiological ubiquitylation target that is regulated by UBTD1 in combination with UBE2D. For instance, the UBE2D family of ligases had been shown to function in ubiquitylation of the tumor-suppressor protein p53 [32].

Some E2s have been shown to bind to co-factors that can alter the activity or subcellular localization of the recruited E2 [28]. One example is the CUE1 co-factor which recruits the cytoplasmic UBC7 (UBE2G2) E2 enzyme to the endoplasmic reticulum (ER) where it participates in the degradation of misfolded or regulated proteins in the ER [33]. It has been proposed that similar to ER-associated degradation (ERAD), mitochondrial proteins can also

be degraded by the UPS in a process termed mitochondrial associated degradation (MAD).

Others have demonstrated that UBTD2 is a mitochondrial protein and in preliminary studies we have made similar observations for UBTD1 (J.U. unpublished observations). In light of a possible mitochondrial localization, it may be relevant that UBTD1 also interacts with SHROOM3, an actin-associated protein and central regulator of cell shape during organogenesis. There are a number of reports demonstrating that the cytoskeleton is linked to mitochondria at the organelle, nucleoid, and mtDNA maintenance level [34–36]. Interestingly, UBTD1 also interacts with the nucleoporin/E3 ligase RANBP2, which is implicated in microtubule regulation [37] and mitochondrial transport [38,39]. Further studies are clearly required to elucidate the *in vivo* function of UBTD1 and its functional role as a regulator of the UBE2D family of E2 ubiquitin conjugating enzymes.

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