Published in final edited form as:

Science. 2017 August 04; 357(6350): 472–475. doi:10.1126/science.aan0178.

UBE2O is a quality control factor for orphans of multi-protein complexes

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Abstract

Many nascent proteins are assembled into multi-protein complexes of defined stoichiometry. Imbalances in the synthesis of individual subunits result in orphans. How orphans are selectively eliminated to maintain protein homeostasis is poorly understood. Here, we found that the conserved ubiquitin-conjugating enzyme UBE2O directly recognized juxtaposed basic and hydrophobic patches on unassembled proteins to mediate ubiquitination without a separate ubiquitin ligase. In reticulocytes, where UBE2O is highly upregulated, unassembled α -globin molecules that failed to assemble with β -globin were selectively ubiquitinated by UBE2O. In non-reticulocytes, ribosomal proteins that did not engage nuclear import factors were targets for UBE2O. Thus, UBE2O is a self-contained quality control factor that contains substrate recognition and ubiquitin transfer activities within a single protein to efficiently target orphans of multi-protein complexes for degradation.

Cells have evolved a wide range of quality control pathways to monitor failures in protein biogenesis and promptly target defective products for degradation (1, 2). Ineffective quality control is implicated in aging, and can cause neurodegeneration (3); conversely, robust quality control may be especially crucial in cancer to support high rates of protein synthesis in the face of an aberrant genome and environmental stresses (4). The suite of protein quality control pathways needed for cellular homeostasis in metazoans is incompletely defined (1).

To identify additional cytosolic quality control pathways, we sought an aberrant protein whose ubiquitination in a cell-free cytosolic extract occurs without engaging known quality control factors. A transmembrane (TM) domain interrupted by three basic residues (hereafter termed 3R; fig. S1A) is not recognized by quality control factors specialized for mis-localized membrane proteins, such as BAG6 or the Ubiquilin family members (5–7); yet, 3R was ubiquitinated similarly to a BAG6 substrate (fig. S1B). Although 3R is an artificial mutant, we reasoned that mechanistic dissection of its ubiquitination pathway might lead us to a quality control pathway and provide tools that could be exploited to identify physiological substrates.

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In vitro translated ^{35}S -labeled 3R immunopurified under native conditions became modified with His-ubiquitin in the presence of recombinant E1, E2 (UBCH5), and ATP (fig. S1C, S1D), indicating that the immunopurified complexes contained a ubiquitin ligase. The primary 3R-associated ubiquitination activity had a native molecular weight of ~150-300 kD (Fig. 1A, 1B). Crosslinking reactions of the active fractions revealed interacting partners of ~200, ~120, and ~100 kD (Fig. 1B). Mass spectrometry identified UBE2O as the ~200 kD product, Importin 5 (IPO5) and Importin 7 (IPO7) as the 120 kD product, and Importin β as the ~100 kD product (fig. S2A). Although none of these are ubiquitin ligases, UBE2O is a ubiquitin conjugating (E2) enzyme with suspected ubiquitin transfer capability (8). Notably, UBE2O is upregulated in cells in response to induced misfolding of a cytosolic protein (9). We thus investigated its potential role in recognition and ubiquitination of 3R and other aberrant proteins.

Immunoblotting of natively immunopurified 3R verified its interaction with UBE2O. By contrast, BAG6 interacted efficiently with an intact TM domain, but poorly with 3R (Fig. 1C), consistent with its preference for long uninterrupted hydrophobic domains (5, 10). Immunoprecipitation of crosslinking reactions verified that the ~200 kD crosslinking partner from Fig. 1B was indeed UBE2O, suggesting a direct interaction with 3R (fig. S2B). Furthermore, the small globular protein KRAS did not interact with UBE2O, while mutants designed to disrupt its folding (termed K1 and K2; see fig. S3A) showed increased ubiquitination (fig. S3B) and UBE2O interaction (fig. S3C). Given that UBE2O was observed prominently in stained gels of affinity purified samples (figs. S2A and S3D), we conclude it is a major and relatively stable interactor with selectivity for aberrant proteins such as 3R and misfolded KRAS.

Experiments with purified recombinant factors demonstrated that UBE2O is sufficient for interaction with and ubiquitination of its clients. We first synthesized 3R in a 'PURE' in vitro translation system reconstituted from recombinant *E. coli* translation factors (11). Nascent 3R normally precipitates in this chaperone-free PURE system, but was prevented from aggregation by including Calmodulin (CaM) during the translation (fig. S4A). CaM acts as a chaperone for hydrophobic domains (12), and can be induced to retain or release 3R using Ca^{2+} or EGTA, respectively. Crosslinking assays verified that purified UBE2O (fig. S4B) engaged 3R released from CaM with EGTA, but not with the CaM-3R complex (Fig. 2A, 2B). In this system, UBE2O (together with E1, ubiquitin, and ATP) was sufficient for 3R ubiquitination (Fig. 2C), while the promiscuous E2 enzyme UBCH5 was ineffective towards 3R on its own (Fig. 2C and fig. S5A).

Ubiquitination required the TM-3R region and was markedly impaired if release from CaM was inhibited with excess Ca^{2+} (Fig. 2C). Other hydrophobic regions could also be directly recognized and ubiquitinated by UBE2O in this purified system (fig. S5B), even though they would be recognized by other factors (such as BAG6) in a complete cytosol (5, 7, 12). The maximal number of ubiquitins added to the client corresponded to the number of available primary amines, and was not affected appreciably by using methyl-ubiquitin incapable of forming polyubiquitin chains (fig. S5C). C1040 in the E2 domain of UBE2O was essential for ubiquitination activity (Fig. 2D), but not for interaction with client. Of the conserved regions (CR) of UBE2O (13), deletion of CR2, and to a lesser extent CR1, reduced client

ubiquitination without affecting UBE2O activity as judged by auto-ubiquitination (Fig. 2D). Correspondingly, recombinant CR2, and to a lesser extent CR1, could directly interact with in vitro translated 3R (fig. S6). Thus, the CR regions of UBE2O directly recognize exposed hydrophobic patches on 3R and other clients to mediate E2 domain-dependent multi-mono-ubiquitination.

In considering potential physiologic quality control clients of UBE2O, we were struck by its marked upregulation during erythrocyte differentiation (14), hinting that the massive increase in hemoglobin synthesis in pre-erythrocytes might necessitate greater quality control. During adult hemoglobin assembly (fig. S7A, S7B), α -globin (also called HBA1) uses alpha-hemoglobin stabilizing protein (AHSP) to temporarily shield the interface that is eventually occupied by β -globin (15). Several human mutations in α -globin impair interaction with AHSP and cause a variant thalassemia-like disease (16). Two such mutants were ubiquitinated at elevated levels compared to wild type α -globin when translated in a reticulocyte lysate (Fig. 3A). These α -globin mutants interacted directly and specifically with UBE2O as judged by co-fractionation (fig. S7C) and crosslinking (Fig. 3B). AHSP is abundant in reticulocyte lysate (15) (the native context for hemoglobin assembly), explaining why nascent wild type α -globin was not ubiquitinated in this experiment. By contrast, UBE2O ubiquitinated wild type α -globin in the chaperone-free PURE translation system (Fig. 3C). Recombinant AHSP prevented UBE2O-mediated ubiquitination of wild type α -globin, but was less effective for the mutant (Fig. 3D). Thus, UBE2O appears to recognize unassembled α -globin via the region normally covered by AHSP. Consistent with this, lower levels of ubiquitinated α -globin are observed in reticulocytes from *UBE2O*-null mice (17).

While unassembled α -globin represents a physiologically important UBE2O client, a more general client range is suggested by UBE2O's deep conservation across eukaryotes and broad tissue expression in mammals. We reasoned that as with the α -globin-AHSP system, UBE2O may identify other clients via elements that normally should have engaged a protein biosynthesis factor. Major factors engaged by 3R included IPO5, IPO7, and Importin β (fig. S2A), all involved in nuclear import of ribosomal proteins (18). Of note, changing 3R to 3D strongly impaired interaction with both UBE2O and IPO7 (fig. S8A), supporting the idea they recognize juxtaposed basic and hydrophobic residues, a feature also seen on the surface of α -globin recognized by UBE2O (fig. S8B). We thus investigated whether nascent ribosomal proteins, many of which would contain exposed basic and hydrophobic surfaces, might be UBE2O clients.

We found that several (e.g., RPL3, RPL8, and RPL24) but not all (e.g., RPS10) ribosomal proteins were highly ubiquitinated after synthesis in reticulocyte lysate, and the ubiquitinated products co-fractionate with UBE2O (Fig. 4A and fig. S9). Affinity purification of these nascent ribosomal proteins showed that UBE2O was a major interactor of each protein that was ubiquitinated, but not of RPS10 (Fig. 4B). Reconstitution experiments in the PURE system showed that RPL8 was efficiently ubiquitinated by UBE2O, and that its ubiquitination required the catalytic cysteine of the E2 domain and the CR2 region for efficient recognition (Fig. 4C).

Using a GFP-tagged RPL24 (fig. S10A) that cannot assemble into ribosomes for steric reasons, and is degraded in a proteasome-dependent manner (fig. S10B), we verified its interaction with both UBE2O and IPO7 in cultured cells (fig. S10C). Immunoprecipitation of UBE2O recovered unmodified and mono-ubiquitinated GFP-RPL24, but not IPO7, suggesting that they do not form a ternary complex (fig. S10D). Catalytically inactive UBE2O(C1040S) also recovered endogenous and exogenous RPL24 (but no detectable ubiquitinated forms), while UBE2O(CR2) interacted poorly with RPL24 (fig. S10D). Thus, in vitro and in cells, nascent unassembled ribosomal proteins engage either nuclear import factors or UBE2O. The absence of a ternary complex with IPO7, and UBE2O's capacity to ubiquitinate bound clients, suggests that it recognizes the population of nascent ribosomal proteins that fail nuclear import or assembly into the ribosome and targets it for proteasomal degradation.

Using a quantitative flow cytometry assay for GFP-RPL24 degradation (in which RFP serves as an expression control; fig. S10A), we found that three independent siRNAs that reduce UBE2O by ~90% stabilize GFP-RPL24 (Fig 4D and fig. S10E). In contrast, overexpression of UBE2O stimulated GFP-RPL24 degradation, while over-expression of a catalytically inactive mutant (C1040S) acted as a dominant-negative to increase GFP-RPL24 (Fig. 4E). Knockdown of IPO7 to reduce GFP-RPL24 nuclear import resulted in its increased degradation, suggesting that cytosolic degradation is more efficient than a recently described nuclear quality control pathway for ribosomal proteins (19). Cytosolic degradation of GFP-RPL24 was attributable to UBE2O because GFP-RPL24 was stabilized by additionally knocking down UBE2O (Fig. 4F). Thus, UBE2O targets for degradation excess ribosomal proteins in the cytosol by recognizing elements that are ordinarily bound by nuclear import factors or shielded in assembled ribosomes. Whether the multi-monoubiquitination mediated by UBE2O is sufficient for proteasomal degradation, or requires another ligase to build poly-ubiquitin chains remains to be determined.

We conclude that UBE2O represents a widely expressed, conserved, and inducible quality control factor that can recognize juxtaposed basic and hydrophobic patches on misfolded, unassembled, and mislocalized proteins. Direct client recognition by UBE2O is analogous to San1p, an unrelated quality control ligase in fungi that also engages clients without a chaperone intermediary (20). Whether they have converged on similar mechanisms of client selection remains to be seen (fig. S11). The generic recognition features of UBE2O indicate a broad client range, a property that is apparently exploited for degradation of normal cellular proteins in some contexts (21, 22). When expressed at very high levels, as during erythrocyte differentiation, UBE2O can even drive wide-scale proteome remodeling that includes elimination of intact ribosomes (17). In most cells under normal conditions, orphan proteins probably represent the predominant clients for quality control given the large number of abundant multi-protein complexes (23, 24), the challenges of precisely matching expression of different subunits (25), and inherent inefficiencies in complex assembly (1). Indeed, proteomic analyses indicate that ~10% of all newly made proteins may be orphans that are rapidly degraded (26). It is likely that many pathways exist to recognize orphans (27) because no single biochemical feature would universally define them. Considering that subunits of many complexes are distributed between different chromosomes, aneuploidy increases the cellular burden of orphans (28, 29), perhaps explaining why the 17q25

genomic region containing Ube2O is amplified in several human cancers, and UBE2O-deficient mice are resistant to multiple models of cancer in mice (21, 22).

Supplementary Materials

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are grateful to M. Skehel and his team for mass spectrometry analysis, V. Bittl, M. Daly, and E. Zavodszky for initial help with flow cytometry assays, M. Rodrigo-Brenni for useful discussions about ubiquitination, S. Shao for useful discussions about preparing complexes in the PURE system, S. Elsasser for bioinformatics advice, and D. Finley for sharing results prior to publication. This work was supported by the UK Medical Research Council (MC_UP_A022_1007 to RSH). K.Y. was supported by an Overseas Research fellowship from the Japan Society for the Promotion of Science, a fellowship from The Uehara Memorial Foundation, and an MRC Postdoctoral Fellowship. Additional data can be found in the supplementary materials.

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One Sentence Summary

Excess subunits of multi-protein complexes, such as hemoglobin and ribosomes, are selectively recognized and targeted for degradation by a hybrid E2-E3 enzyme.

Fig. 1. UBE2O associates with an aberrant nascent protein in the cytosol.

(A) Experimental strategy to identify quality control factors by cross-correlation of native size, physical interactors, and ubiquitination activity. (B) ³⁵S-labeled Sec61β(3R) was translated reticulocyte lysate (RRL) for 15 min, which is just long enough to synthesize the proteins but before appreciable downstream ubiquitination. The reaction was separated by native size on a sucrose gradient, and each fraction analysed for ubiquitination competence (middle panel) or physical interactions (bottom panel). Red asterisks indicate fractions with peak ubiquitination activity, and red arrowheads indicate crosslinked proteins with a peak in these same fractions. (C) Sec61β (WT) or mutants lacking the transmembrane domain (ΔTM) or containing 3R were translated in RRL, immunoprecipitated under native conditions, and analysed by immunoblotting for either BAG6 or UBE2O. BAG6 interacts

with the intact TM domain, while UBE2O preferentially interacts with the 3R-disrupted TM domain.

Fig. 2. Reconstitution of UBE2O-mediated client ubiquitination with purified factors.

(A) Experimental strategy to test UBE2O interaction and ubiquitination in a defined system.

(B) ³⁵S-labeled 3R in complex with Calmodulin (CaM) was treated with either Ca²⁺ or EGTA in the presence or absence of UBE2O and subjected to the amine-reactive crosslinker DSS. Positions of the starting 3R substrate and its crosslinks to CaM (xCaM) and UBE2O (xUBE2O) are indicated. (C) ³⁵S-labeled 3R in complex with CaM was mixed with E1, ATP, ubiquitin, and the indicated E2 (UBCH5 or UBE2O), then treated with EGTA to induce substrate release from CaM. One sample was incubated with Ca²⁺ instead of EGTA.

(D) Ubiquitination reactions as in panel c with the indicated UBE2O variants. The purified UBE2O proteins before and after the ubiquitination reaction are also shown to indicate auto-ubiquitination of UBE2O in all reactions except with the C1040S mutant.

Fig. 3. Unassembled α -globin is a target for UBE2O ubiquitination.

(A) Wild type α -globin (HBA1) or two assembly mutants (H104R and F118S) were translated in RRL supplemented with His-tagged Ubiquitin and either analysed directly (bottom panel; total) or after ubiquitin-pulldown (Ub-PD) via the His tag (top panel). (B) HA-tagged HBA1 or the H104R mutant were translated in RRL, subjected to sulhydryl-reactive crosslinking with BMH, and affinity purified via the HA tag. The purified products were denatured and analysed directly (input) or further immunoprecipitated with antibodies to either UBE2O or GFP. The positions of ubiquitinated H104R and its crosslink to UBE2O

are indicated. (C) ^{35}S -labeled HBA1 synthesized in the PURE translation system was incubated with E1, ATP, Ubiquitin, and either UBCH5 or UBE2O, and analysed for ubiquitination. (D) ^{35}S -labeled wild type (WT) or H104R mutant HBA1 synthesized in the PURE system was incubated with or without UBE2O and AHSP as indicated. The positions of unmodified and ubiquitinated HBA1 are indicated.

Fig. 4. UBE2O facilitates degradation of cytosolic orphan ribosomal proteins.

(A) Wild type KRAS or RPL8 was translated in RRL supplemented with His-tagged Ubiquitin and either analysed directly (total) or after ubiquitin-pulldown (Ub-PD) via the His tag. (B) The indicated FLAG-tagged proteins were translated in RRL, affinity purified under native conditions, and analysed for co-purifying proteins by SYPRO Ruby stain. The asterisks indicate the primary translation product, and the arrowhead indicates UBE2O (verified by immunoblotting and mass spectrometry). (C) ^{35}S -labeled RPL8 was synthesized in the PURE system and incubated with recombinant E1, ATP, ubiquitin, and UBE2O. Aliquots of the reaction after different times of incubation at 37°C are shown in the left panel. In the right panel, RPL8 was incubated for 60 min at 32°C with E1, ATP, ubiquitin, and the indicated UBE2O variant. (D) HEK293 cells stably expressing GFP-RPL24 were treated with three different siRNAs against UBE2O and analysed by flow cytometry. The relative GFP-RPL24 level, normalized to an internal expression control (see fig. S10D), is plotted as a histogram. The grey histogram is from cells treated with a control siRNA. (E) GFP-RPL24 cells were transiently transfected with plasmids encoding UBE2O, a catalytically inactive mutant (C1040S), or BFP (a negative control) and analysed for GFP-RPL24 levels by flow cytometry. (F) GFP-RPL24 cells were treated with the indicated siRNAs and analysed for GFP-RPL24 levels by flow cytometry.