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TITLE:

In Vitro Analysis of E3 Ubiquitin Ligase Function

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SUMMARY:

The present study provides detailed *in vitro* ubiquitylation assay protocols for the analysis of E3 ubiquitin ligase catalytic activity. Recombinant proteins were expressed using prokaryotic systems such as *Escherichia coli* culture.

ABSTRACT:

The covalent attachment of ubiquitin (Ub) to internal lysine residue(s) of a substrate protein, a process termed ubiquitylation, represents one of the most important post-translational modifications in eukaryotic organisms. Ubiquitylation is mediated by a sequential cascade of three enzyme classes including ubiquitin-activating enzymes (E1 enzymes), ubiquitin-conjugating enzymes (E2 enzymes), and ubiquitin ligases (E3 enzymes), and sometimes, ubiquitin-chain elongation factors (E4 enzymes). Here, *in vitro* protocols for ubiquitylation assays are provided, which allow the assessment of E3 ubiquitin ligase activity, the cooperation between E2-E3 pairs, and substrate selection. Cooperating E2-E3 pairs can be screened by monitoring the generation of free poly-ubiquitin chains and/or auto-ubiquitylation of the E3 ligase. Substrate ubiquitylation is defined by selective binding of the E3 ligase and can be detected by western blotting of the *in vitro* reaction. Furthermore, an E2~Ub discharge assay is described, which is a useful tool for the direct assessment of functional E2-E3 cooperation. Here, the E3-dependent transfer of ubiquitin is followed from the corresponding E2 enzyme onto free lysine amino acids (mimicking substrate ubiquitylation) or internal lysines of the E3 ligase itself (auto-ubiquitylation). In conclusion, three different *in vitro* protocols are provided that are fast and easy to perform to address E3 ligase catalytic functionality.

INTRODUCTION:

Ubiquitylation is the process by which Ub is covalently linked to a substrate protein[1]. The Ub modification is catalyzed by successive enzymatic reactions involving the action of three different enzyme classes, *i.e.*, Ub-activating enzymes (E1s), Ub-conjugating enzymes (E2s), Ub ligases (E3s), and possibly Ub chain elongation factors (E4s)[2,3,4,5]. After adenosine triphosphate (ATP)- and magnesium (Mg^{2+})-dependent activation of Ub by E1, the active site cysteine of E1 attacks the C-terminal glycine of Ub, forming a thioester complex (Ub~E1). The energy drawn from ATP hydrolysis causes the Ub to attain a high energy transitional state, which is maintained throughout the following enzyme cascade. Next, the E2 enzyme transfers the activated Ub to its internal catalytic cysteine, thereby forming a transient Ub~E2 thioester bond. Subsequently, Ub is transferred to the substrate protein.

This can be done in two ways. Either the E3 ligase may first bind to E2, or the E3 ligase can directly bind Ub. The latter way results in the formation of an E3~Ub intermediate. In either case, Ub is linked to the substrate protein by formation of an isopeptide bond between the C-terminal carboxyl group of Ub and the lysine Ɛ-amino group of the substrate[6]. The human genome encodes two E1s, approximately 40 E2s, and more than 600 putative ubiquitin ligases[7]. Based on the Ub transfer mechanism of the E3, Ub ligases are divided into three categories involving Homologous to E6AP C-Terminus (HECT)-type, Really Interesting New Gene (RING)/U-box-type, and RING between RING-type ligases[8]. In this study, the U-box containing ligase, carboxyl terminus of HSC70-interacting protein (CHIP), is used as a representative E3 enzyme. In contrast to HECT-type E3 enzymes that form Ub~E3 thioesters, the U-box domain of CHIP binds E2~Ub and promotes the subsequent Ub/substrate transfer directly from the E2 enzyme[8,9]. Based on the importance of the U-box for enzymatic function, an inactive CHIP U-box mutant, CHIP(H260Q), is utilized as a control. CHIP(H260Q) fails to bind to its cognate E2s, thus losing its E3 ligase activity[10].

Protein ubiquitylation plays a crucial role in regulating a multitude of cellular events in eukaryotic cells. The diversity of cellular outcomes that are promoted by the reversible attachment of Ub molecules to substrate proteins can be attributed to the molecular characteristics of Ub. As Ub itself contains seven lysine (K) residues for further ubiquitylation, there is rich variety of Ub chain-types with different sizes and/or topologies[11]. For example, substrates can be modified by a single Ub molecule at one (mono-ubiquitylation) or multiple lysines (multi mono-ubiquitylation), and even by Ub chains (poly-ubiquitylation)[11]. Ub chains are either formed homo- or heterotypically via the same or different lysine residues of Ub, which could even result in branched Ub chains[9]. Thus, protein ubiquitylation leads to diverse arrangements of Ub molecules that provide specific information, *e.g.*, for degradation, activation, or localization of conjugated proteins[12,13]. These different Ub signals enable fast reprogramming of cellular signalling pathways, which is an important requirement for the cell's ability to respond to changing environmental needs.

A central aspect of ubiquitylation is related to protein quality control. Misfolded or irreversibly damaged proteins must be degraded and replaced by newly synthesized proteins to maintain protein homeostasis or proteostasis[14]. The quality control E3 ligase, CHIP, collaborates with molecular chaperones in the Ub-dependent degradation of damaged proteins[9,15,16,17]. Apart from that, CHIP regulates the stability of the myosin-directed chaperone, UNC-45B (Unc-45 Homolog B), which is tightly coordinated with muscle function and deviations from the optimal levels lead

to human myopathy[18,19,20,21]. Degradation of UNC-45B by the 26S proteasome is mediated by attachment of a K48-linked poly-Ub chain[9]. In the absence of substrate proteins, CHIP performs auto-ubiquitylation[10,22,23], which is characteristic of RING/U-box E3 ubiquitin ligases[24,25] and considered to regulate ligase activity[26]. Application of the *in vitro* ubiquitylation assay methods described in this paper helped to systematically identify E2 enzymes that team up with CHIP to promote the formation of free poly-Ub chains and/or auto-ubiquitylation of CHIP (protocol section 2). Furthermore, CHIP-dependent ubiquitylation of UNC-45B was observed, which is a known substrate of the E3 ligase[18,19] (protocol section 3). Ultimately, CHIP-dependent transfer of activated Ub from the Ub~E2 thioester was monitored (protocol section 4).

PROTOCOL:

1. Preparation of buffers and reagents

NOTE: Buffers and reagents that were manually prepared in the laboratory are listed below. All other buffers and reagents used in the protocols were purchased from different sources and used according to the manufacturers' instructions.

1.1. Prepare 10x phosphate-buffered saline (10x PBS). For this purpose, mix 1.37 M sodium chloride (NaCl), 27 mM potassium chloride (KCl), 80 mM of disodium-hydrogen-phosphate dihydrate ($Na_2HPO_4.2\ H_2O$), and 20 mM of potassium-dihydrogen phosphate (KH_2PO_4) in 1 L of double-distilled H_2O (ddH_2O). Autoclave the 10x PBS, and prepare a 1x PBS solution in ddH_2O.

1.1.1. Autoclaving is carried out for 15 min at 121 °C and 98.9 kPa.

NOTE: Autoclaving is carried out under these conditions for all buffers and solutions. If not indicated otherwise, solutions are stored at room temperature (RT).

1.2. Prepare 1 L of PBS-Tween (PBS-T) solution by adding 0.1% Tween to 1 L of 1x PBS.

1.3. Prepare 10 mL of a 0.5 M L-lysine stock solution by dissolving L-lysine in ddH_2O. Aliquot L-lysine in 1 mL portions, and store them at -20 °C until further use.

NOTE: L-lysine can be frozen and thawed repeatedly.

1.4. Prepare a 0.25 M ethylene diamine tetra acetic acid (EDTA) solution by dissolving EDTA in 200 mL of ddH_2O.

1.4.1. Adjust the pH to 8.0 with sodium hydroxide (NaOH).

1.4.2. Adjust the volume to 250 mL with ddH_2O.

1.4.3. Autoclave the solution.

1.5. Prepare 10 mL of a 20 mg/mL bovine serum albumin (BSA) solution by dissolving BSA in ddH_2O. Store at -20 °C in 200 µL aliquots.

NOTE: BSA can be frozen and thawed repeatedly.

1.6. Prepare 1 L of 2-(N-morpholino)ethane sulfonic acid (MES) running buffer by dissolving 50 mM MES, 50 mM Tris base, 3.47 mM sodium dodecyl sulfate (SDS), and 1.03 mM EDTA in 0.9 L of ddH_2O. After mixing properly, adjust the volume to 1 L.

1.6.1. The pH of the 1x buffer is 7.6. Do not adjust the pH with acid or base.

1.7. Prepare 200 mL of blocking solution (5% milk) by dissolving 10 g milk powder in 1x PBS-T. Store the blocking solution at 4 °C for up to one week.

1.8. Prepare 1 L of transfer buffer (see the **Table of Materials**) according to the manufacturers' instruction.

1.9. Prepare 2x SDS sample buffer by dissolving 125 mM Tris base, 4% SDS, 4% glycerol, 0.03% bromophenol blue, and 50 µL/mL of β-mercaptoethanol in 50 mL ddH_2O. Aliquot in 1 mL portions, and store at -20 °C until use.

2. *In vitro* auto-ubiquitylation assay

2.1. Assay preparation and execution

2.1.1. Calculate the volume of each reagent required per reaction based on the given molarities of the proteins and the protein concentrations of the protein solutions. Per auto-ubiquitylation reaction, use 100 nM E1, 1 µM E2, 1 µM E3, and 50 µM Ub. Adjust the volume of the reaction to 20 µL with ddH_2O.

NOTE: ATP solution and ubiquitylation buffer were purchased as 10x stock solutions and used at 1x.

2.1.2. Prepare a pipetting scheme for all reactions. Test nine different E2 enzymes for their ability to function (I) with CHIP, (II) with a catalytically inactive CHIP(H260Q) mutant, and (III) without CHIP in individual ubiquitylation reactions.

2.1.3. To avoid pipetting errors, prepare a master mix on ice that contains the volume required for all reactions plus one extra reaction. Calculate the amount of master mix required per reaction.

NOTE: Master mix components are reagents that are equally required for each reaction, *i.e.*, E1, E3, Ub, ATP, and ubiquitylation buffer.

2.1.4. Add ddH_2O and master mix to the polymerase chain reaction (PCR) tubes. Keep the tubes on ice.

2.1.5. Before starting the ubiquitylation reactions by adding the E2 enzymes, set up a PCR thermal cycler with the following program: 2 h, 37 °C followed by 4 °C, infinitely.

2.1.6. Add 1 µM of the E2 enzymes in the respective tubes, and incubate the samples in the PCR thermal cycler for the indicated period of time.

2.1.7. After 2 h, add SDS sample buffer to each reaction, and mix by pipetting up and down several times.

NOTE: The required volume of the sample buffer depends on the stock concentration of the sample buffer. Here, 20 µL of 2x SDS sample buffer were added to each reaction.

2.1.8. Boil the samples immediately at 95 °C for 5 min, and continue with polyacrylamide gel electrophoresis (PAGE). Store the denatured proteins at -20 °C.

2.2. Gel electrophoresis and western blot

2.2.1. After thawing, spin down the samples for approximately 10 s and load the SDS-PAGE gel. Use a protein ladder as size reference.

NOTE: Here, 4–12% Bis-Tris gradient gels and 3 µL of protein ladder were used. Divide the sample volumes and load two gels equally using 20 µL of each sample.

2.2.2. Run the gel at 160–200 V for approximately 30–45 min so that the sample front reaches the bottom of the gel.

2.2.3. Transfer each gel to a plastic container filled with semidry blotting buffer, and incubate it for 2–5 min at RT. Remove the stacking gel.

2.2.4. Prepare two pieces of gel-sized blotting paper and a gel-sized nitrocellulose membrane per SDS gel and pre-soak in semidry blotting buffer. Assemble the western blot (WB) sandwich in a WB chamber from bottom to top in the following order: blotting paper, nitrocellulose membrane, SDS-PAGE gel, blotting paper.

2.2.5. Remove air bubbles that may have been trapped between the sandwich layers. For this purpose, use a roller to carefully roll over the sandwich a few times.

2.2.6. Close the chamber, and allow excess blot buffer to drain out.

2.2.7. Place the chamber in the respective blotting device, and use the following program for semidry blotting: 25 V, 1.0 A, 30 min.

2.2.8. Transfer the blotted membrane to a plastic container filled with blocking solution, and incubate for 30 min at RT.

2.2.9. Prepare the primary antibody solution by adding the antibody to 10 mL of PBS-T containing a blocking reagent (see the **Table of Materials**).

NOTE: The working concentration of the antibody is antibody-specific. In this case, a monoclonal mouse anti-ubiquitin antibody was used at a dilution of 1:5,000. For the second gel, a monoclonal rabbit anti-CHIP antibody was used at 1:5,000 in PBS-T/blocking reagent.

2.2.10. Replace the blocking solution with the primary antibody solution, and incubate overnight at 4 °C on a rocker.

2.2.11. Wash the membrane three times for 10 min with PBS-T.

2.2.12. Prepare the secondary antibody solution by adding the respective antibody to 10 mL of PBS-T.

NOTE: Here, goat anti-mouse and mouse anti-rabbit antibodies are used at a dilution of 1:10,000.

2.2.13. Incubate the membrane with the secondary antibody for 1 h at RT on a rocker.

2.2.14. Wash the membrane three times for 5 min with PBS-T.

2.3. Data analysis

2.3.1. Add western blot detection reagents for horseradish peroxidase (HRP)-conjugated antibodies to the washed membrane according to the manufacturer's instructions, and capture the HRP signal by using X-ray films or a charge-coupled device camera (**Figure 1**).

3. *In vitro* substrate ubiquitylation assay

3.1. Assay preparation and execution

3.1.1. Calculate the volume of each reagent required per reaction, based on the given molarities of the proteins and the protein concentrations of the protein solutions. Per substrate ubiquitylation reaction, use 100 nM E1, 1 µM E2, 1 µM E3, 1 µM substrate, and 50 µM Ub. Use 10x ATP solution and 10x ubiquitylation buffer at 1x. Adjust the volume of the substrate ubiquitylation reaction to 20 µL with ddH_2O.

3.1.2. Prepare a pipetting scheme for all reactions. Include proper control reactions verifying that the substrate ubiquitylation is E3-specific. Use the protein UNC-45B as a representative substrate of CHIP and a catalytically inactive CHIP mutant (H260Q) as a control. Prepare the

following reactions: **E1, E2, Ub mix** (including Ub, ATP, ubiquitylation buffer); **E1, E2, Ub mix, UNC-45B**; **E1, E2, Ub mix, CHIP(H260Q), UNC-45B**; and **E1, E2, Ub mix, CHIP, UNC-45B**.

3.1.3. To avoid pipetting errors, prepare a master mix on ice that contains the volume required for all reactions plus one extra reaction. Calculate the volume of the master mix that is required per reaction.

NOTE: Master mix components for this assay include E1, E2, Ub, ATP, and ubiquitylation buffer.

3.1.4. Add reaction components in the following order: ddH_2O, substrate, E3 ligase.

3.1.5. Before starting the ubiquitylation reaction by addition of the master mix, set up a PCR thermal cycler with the following program: 2 h, 37 °C followed by 4 °C, infinitely.

3.1.6. Add the master mix to each tube, and incubate the samples in the PCR thermal cycler for the indicated period of time.

3.1.7. After 2 h, add 2x SDS sample buffer to each reaction, and mix by pipetting up and down several times.

3.1.8. After the run, boil the samples immediately at 95 °C for 5 min, and continue with PAGE. Alternatively, store the denatured proteins at -20 °C.

3.2. Gel electrophoresis and western blot

3.2.1. Perform gel electrophoresis and western blot as described in section 2.2. Use specific antibodies for the detection of the substrate and the E3 ligase, respectively.

NOTE: Here, UNC-45B is fused to a polypeptide protein tag derived from the *c-myc* gene product allowing for the use of a monoclonal mouse anti-MYC antibody. Anti-MYC is used at 1:10,000.

3.3. Data analysis

3.3.1. Perform data analysis as described in section 2.3 (**Figure 2**).

4. Lysine discharge assay

4.1. Assay preparation and execution

4.1.1. Charging of the E2 enzyme with Ub by E1

4.1.1.1. Calculate the volume of each reagent required per reaction based on the given molarities of the proteins and the protein concentrations of the protein solutions. Per charging reaction, use 2 µM E1, 4 µM E2, and 4 µM lysine-free ubiquitin (Ub K0). Use 10x ATP and 10x

ubiquitylation buffer at 1x. Adjust the volume of the charging reaction to 20 µL with ddH_20.

NOTE: The lysine-free Ub K0 mutant is used to enforce the exclusive production of mono-ubiquitylated E2. Wild-type Ub can also be used; however, this might lead to diverse E2~Ub modifications (*e.g.*, poly-ubiquitylation of E2), which are more difficult to analyze. For first time use or when using a new E2 enzyme, determine the level of Ub charging on E2 that can be achieved by E1. To determine the yield of charging, visualize uncharged vs. charged E2 via Coomassie staining. Here, approximately half of Ub K0 was reliably converted to UBE2D2~Ub when using equimolar ratios of UBE2D2 and Ub K0 (**Supplemental Figure S2A**).

4.1.1.2. Prepare a pipetting scheme for the charging reaction. Adjust the volume of the charging reaction to the subsequent discharge reactions of choice, *e.g.*, use CHIP and CHIP(H260Q) in individual discharge reactions. Thus, prepare double the volume of one charging reaction.

NOTE: For first time use, test different concentrations of the E3 ligase of choice to monitor optimal discharge conditions, and analyze them by western blotting. In an ideal condition, the discharge of Ub from E2 will increase over time until the respective E2~Ub protein band disappears.

4.1.1.3. Incubate the charging reaction for 15 min at 37 °C.

4.1.2. Termination of the charging reaction

4.1.2.1. Stop the charging reaction by addition of apyrase at a final concentration of 1.8 U/mL. Carry out the incubation with apyrase for 5 min at RT, followed by addition of EDTA at a final concentration of 30 mM. Adjust the volume of the charging reaction to 30 µL with ddH_2O.

NOTE: Apyrase is used to convert ATP molecules to ADP (adenosine diphosphate) molecules. As the activity of the E1 enzyme is ATP-dependent, E1 can no longer charge E2. EDTA is additionally used to ensure that E1 is inhibited by quenching Mg^{2+} ions that are necessary co-factors for E1. The volume of apyrase that is required to stop the reaction can vary among assay conditions. Although apyrase alone already quenches available ATP molecules, a combination of apyrase and EDTA was more effective at stopping the charging reaction for the E1-UBE2D2 pair. As stopping the reaction is a critical factor for assay reproducibility, efficient stopping should be tested for new E1-E2 pairs. For this purpose, set up a charging reaction, stop it, and collect samples at specific time points, *e.g.*, after 2, 5, 10, and 15 min. Non-changing levels of E2~Ub indicate that the reaction has stopped, and that the thioester was stable during that period of time (**Supplemental Figure S2B**).

4.1.3. Discharging of E2~Ub by E3

4.1.3.1. Prepare four tubes corresponding to the different time points (t_0, t_1, t_2, t_3); add non-reducing sample buffer (6.7 µL of 4x lithium dodecyl sulfate (LDS) buffer) to each tube. Collect

samples of 20 µL volume after 5, 30, and 60 min.

NOTE: Do not use reducing agent in the sample buffer.

4.1.3.2. Remove 6 µL from the stopped charging reaction for t_0, and adjust the volume to 20 µL with ddH_2O.

4.1.3.3. Incubate the t_0 sample for 10 min at 70 °C.

NOTE: Do not boil the sample by extending the incubation time as thioesters are labile at higher temperatures.

4.1.3.4. Calculate the volume of each reagent required per discharge reaction. Use the remaining 24 µL of the charged E2, and add 10 mM L-lysine, 1 mg/ml BSA, and 500 nM of the E3 ligase. Use the ubiquitylation buffer at 1x, and adjust the volume to 80 µL with ddH_2O.

4.1.3.5. Prepare a pipetting scheme for all discharge reactions, and use CHIP(H260Q) as control in addition to CHIP(WT).

4.1.3.6. Set up discharge reactions on ice by adding the required components in the following order: ddH_2O, ubiquitylation buffer, BSA, lysine, E3 ligase, and the charged E2 to start the reaction.

4.1.3.7. Incubate the discharge reaction at RT.

4.1.3.8. Take samples after 5, 30, and 60 min by transferring 20 µL of the discharge reaction into the respective sample tubes.

NOTE: Suitable time points that allow for proper monitoring of the discharge can vary between E2-E3 pairs.

4.1.3.9. Vortex the sample immediately, and incubate it at 70 °C for 10 min.

4.1.3.10. Directly proceed with PAGE.

NOTE: E2~Ub thioesters can be labile even after denaturing in sample buffer. Thus, gel electrophoresis should be performed directly after assay execution.

4.2. Gel electrophoresis and western blot

4.2.1. Perform the gel electrophoresis and the western blotting as described in section 2.2. Use a Ub-specific antibody and, if available, also use an E3-specific antibody that was raised in a different species, allowing for simultaneous detection of the Ub and E3 ligase signal.

4.3. Data analysis

4.3.1. Perform data analysis as described in section 2.3 (**Figure 3**).

REPRESENTATIVE RESULTS:

To identify E2 enzymes that cooperate with the ubiquitin ligase CHIP, a set of E2 candidates was tested in individual *in vitro* ubiquitylation reactions. Cooperating E2-E3 pairs were monitored by the formation of E3-dependent ubiquitylation products, *i.e*., auto-ubiquitylation of the E3 ligase and the formation of free Ub polymers. The ubiquitylation products were analyzed by western blotting. Data interpretation is based on the size comparison of the resulting protein bands with molecular weight markers. Protein ubiquitylation leads to the formation of specific band patterns characterized by the appearance of double bands or multiple iterative bands with a respective size difference of 8.6 kDa (size of a single Ub molecule).

Here, the ability of nine E2s to promote the formation of ubiquitylation products was tested in the presence of wild-type CHIP (**Figure 1A**), the inactive U-box mutant CHIP(H260Q) (**Figure 1B**), or without CHIP (**Supplemental Figure S1**). E3-independent ubiquitin products were formed in the presence of inactive CHIP and in the absence of CHIP (**Figure 1B** and **Supplemental Figure S1**). Inactive CHIP was not auto-ubiquitylated (**Figure 1B**). In contrast, wild-type CHIP was auto-ubiquitylated when combined with members of the UBE2D family (D1–D3) and members of the UBE2E family (E1, E3), respectively (**Figure 1A**, lanes 3, 4, and 5). Whereas free poly-Ub chains were produced in cooperation with UBE2D1–3, this was not detected for UBE2E1 or UBE2E3, respectively (**Figure 1A**, lanes 6 and 7).

The ability of the UBE2D family to promote both the formation of free Ub polymers and the auto-ubiquitylation of CHIP has been attributed to the presence of a non-covalent ubiquitin binding site on the backside of the E2[27,28]. Similarly, the exclusive formation of free Ub chains by UBE2N/V1 (**Figure 1A**, lane 9) has been attributed to the binding of Ub by a specific UBE2V1 subunit (Uev subunit), directing the formation of K63-linked Ub chains[29]. No ubiquitylation products were formed in the presence of UBE2C1 and UBE2H (**Figure 1A**, lanes 2 and 8). In conclusion, CHIP can collaborate with several E2 enzymes *in vitro*; however, its auto-ubiquitylation is E2 enzyme-specific.

Next, the UBE2D2-CHIP pair was used to investigate the ubiquitylation of the myosin-directed chaperone, UNC-45B, by CHIP ligase activity (**Figure 2**). Substrate ubiquitylation was analyzed via western blotting. Non-ubiquitylated UNC-45B has a molecular weight of 103 kDa (**Figure 2**, lane 4). The inactive U-box mutant of CHIP performed neither ubiquitylation of UNC-45B nor auto-ubiquitylation (**Figure 2**, lane 3). In contrast, ubiquitylation of UNC-45B and auto-ubiquitylation of CHIP was detected upon incubation with wild-type CHIP (**Figure 2**, lane 6). Thus, CHIP can ubiquitylate UNC-45B *in vitro*, suggesting that UNC-45B is a conserved substrate of CHIP[18].

Ultimately, the catalytic activity of CHIP was analyzed in the absence of any substrate protein. To this end, a lysine discharge assay was performed in which free lysine amino acids can serve as Ub acceptor in the absence of E3 substrates (**Figure 3**, **Supplemental Figure S2C**). The discharge assay consists of a charging step in which Ub is loaded onto the E2 by E1, a stop step to prevent

further loading of Ub onto E2, and a discharge step where Ub is transferred from the transient Ub~E2 thioester onto free lysine amino acids and/or onto lysine residues of the E3 ligase. Western blot analysis was performed to visualize both the Ub-modified proteins and the E3 ligase CHIP. The uncharged UBE2D2 enzyme has a molecular weight of 17 kDa (**Supplemental Figure S2C**).

When charged with a single Ub molecule—ensured by the use of lysine-free Ub (Ub K0)—the molecular weight of UBE2D2~Ub shifts upwards to approximately 26 kDa. The time point zero (t_0) represents the overall yield of charged E2 (**Figure 3**). In the presence of inactive CHIP (35 kDa), a faint E3 ligase-independent discharge of UBE2D2[30,31], but no auto-ubiquitylation of CHIP, was detected. In contrast, in the presence of wild-type CHIP (35 kDa), a faster discharge of UBE2D2 was detected, yielding a complete discharge within 60 min. Simultaneously, auto-ubiquitylation of CHIP was observed, indicating that CHIP promoted the transfer of Ub onto its own lysine residues.

FIGURE AND TABLE LEGENDS:

Figure 1: Western blot analysis for E2-E3 enzyme collaboration *in vitro*. *In vitro* auto-ubiquitylation reactions with different human E2 enzymes (from left to right: empty, UBE2-D1, -D2, -D3, -E1, -E3, -H, and -N/V1) were performed as indicated using (**A**) wild-type CHIP or (**B**) the inactive CHIP U-box mutant, CHIP(H260Q), as E3 ubiquitin ligase. The samples were divided equally, run on separate polyacrylamide gels, and immunoblotted with anti-CHIP and anti-Ub antibodies to visualize reaction products. Abbreviations: Ub = ubiquitin; CHIP = carboxyl terminus of HSC70-interacting protein; ATP = adenosine triphosphate.

Figure 2: Western blot analysis monitoring substrate ubiquitylation. *In vitro* substrate ubiquitylation reactions were performed as indicated to detect ubiquitylation of human UNC-45B by CHIP wild-type or the inactive CHIP U-box mutant, CHIP(H260Q). Human UBE2D2 was used as E2 enzyme. Abbreviations: WT = wild-type; Ub = ubiquitin; CHIP = carboxyl terminus of HSC70-interacting protein.

Figure 3: Western blot analysis monitoring the CHIP-dependent Ub transfer from UBE2D2~Ub thioester. Charging of human UBE2D2 by lysine-free Ub (Ub K0), stopping of the charging reaction, and discharging of UBE2D2~Ub was performed as described. For the discharge reactions, wild-type CHIP or the inactive CHIP U-box mutant, CHIP(H260Q), were used as ubiquitin ligases and 10 mM L-lysine was supplied as the potential Ub acceptor. Samples were collected after the indicated time periods, run on a polyacrylamide gel, and immunoblotted with an anti-Ub/anti-CHIP antibody mixture. Abbreviations: Ub = ubiquitin; CHIP = carboxyl terminus of HSC70-interacting protein; ATP = adenosine triphosphate.

Supplemental Figure S1: Representative western blot monitoring E2 enzyme activities in the absence of E3. *In vitro* ubiquitylation reactions with different human E2 enzymes (from left to right: empty, UBE2-D1, -D2, -D3, -E1, -E3, -H, and -N/V1) were performed as indicated in the absence of an E3 ligase to screen E3-independent reaction products. The samples were run on a

polyacrylamide gel and immunoblotted with anti-Ub. Abbreviations: Ub = ubiquitin; ATP = adenosine triphosphate.

Supplemental Figure S2: Analysis of the charging yield and stability of the UBE2D2~Ub K0 thioester. (**A**) The yield of UBE2D2 charging achieved by E1 was tested under various conditions. The first charging reaction (I) consisted of 5 µM E1, 5 µM E2, and 50 µM Ub K0. The second charging reaction (II) consisted of 2 µM E1, 4 µM E2, and 4 µM Ub K0. The charging reactions were performed as described for 30 min at 37 °C. Samples were collected after 15 and 30 min. The reactions were stopped by addition of 4x LDS sample buffer and incubated at 70 °C for 10 min prior to gel electrophoresis and Coomassie staining. Uncharged UBE2D2 was used as control. Approximately half of the UBE2D2 was converted to UBE2D2~Ub. No additional charging was detected after 15 min. Moreover, neither the increase in E1 nor the increase in free ubiquitin altered the charging yield of UBE2D2~Ub. Thus, charging was subsequently performed for 15 min at 37 °C by using 2 µM E1, 4 µM E2, and 4 µM Ub K0. (**B**) After charging, the reaction was stopped by addition of 1.8 U/mL apyrase and 30 mM EDTA and incubated at RT. Samples were collected after 2, 5, 8, 10, and 15 min (lanes 2–6), and uncharged UBE2D2 was used as control (lane 1). 4x LDS sample buffer was added, and samples were incubated at 70 °C for 10 min prior to gel electrophoresis and Coomassie staining. Stopping was efficient as measured by the constant band intensity of the UBE2D2~Ub protein, also indicating that the thioester was stable during the indicated time period. (**C**) Charging of human UBE2D2 by lysine-free Ub (Ub K0), stopping of the charging reaction, and discharging of UBE2D2~Ub was performed as described. For the discharge reactions, wild-type CHIP or the inactive CHIP U-box mutant, CHIP(H260Q) were used as ubiquitin ligases, and 10 mM L-lysine was supplied as the potential Ub acceptor. Samples were collected after the indicated time periods, run on separate polyacrylamide gels, and Coomassie-stained.

DISCUSSION:

This paper describes basic *in vitro* ubiquitylation methods for the analysis of E3 ligase function. When performing *in vitro* ubiquitylation assays, it should be considered that some E2 enzymes can perform auto-ubiquitylation owing to the attack of their active cysteine on their own lysine residues that are located in close proximity to the active site[30]. To circumvent this problem, the use of an E2 mutant is recommended in which the respective lysine residue is exchanged for arginine, which results in a catalytically active E2 enzyme that is resistant to auto-ubiquitylation[32]. This is of particular importance when monitoring the Ub transfer via lysine discharge assays to ensure reproducibility of the assay. Another critical factor is the transient nature of the E2~Ub thioester bond. In case the thioester stability limits possible in *vitro* applications, the active cysteine residue of the E2 could be exchanged for a serine to generate a more stable E2~Ub oxyester[33]. For first time use or when using a new E2 enzyme, determine the level of Ub charging on E2 that can be achieved by E1. The charging efficiency can be affected by several factors including the species origin of E1, the temperature of the charging reaction, and the molar ratio of Ub to E2.

To determine the yield of charging, visualize uncharged *vs*. charged E2 via Coomassie staining. Altogether, these potential restrictions highlight the importance of the empirical optimization of *in vitro* assay conditions, such as E2-E3 pair, E2 charging and discharging kinetics, molarity and

activity of the recombinant proteins, especially for the lysine discharge assay, to obtain reproducible results. Given the diverse cellular functions of the covalent attachment of Ub to substrate proteins, the analysis of protein ubiquitylation is a popular research field. However, the analysis of ubiquitylation events can be difficult, especially *in vivo*. This difficulty arises from multiple factors including the transient nature of the E3-substrate interaction[34], redundancy of many E3 ligases, as well as promiscuity of E3 ligases for several substrates[35]. Furthermore, the characterization of specific ubiquitylation events *in vivo* is hindered by the existence of additional contributing factors such as ubiquitin chain elongation factors and deubiquitylation enzymes that modulate ubiquitin chain topology[36]. These constraints underline the importance of robust *in vitro* techniques that help to characterize enzymatic activities of E3 ubiquitin ligases in a defined recombinant system.

Apart from the described applications, *in vitro* ubiquitylation assays can also be used to detect the Ub-linkage type by using linkage-type specific antibodies. As an additional more detailed approach, the respective protein band of the Ub-modified substrate can be extracted from the polyacrylamide gel and analyzed via mass spectrometry. The identification of the linkage type supports the understanding of the physiological role of the E3-substrate interaction, which is of particular importance for the characterization of disease-associated substrate degradation pathways[37,38]. Similarly, the lysine discharge assay can be used as an effective tool to unravel catalytic differences between E3 ubiquitin ligases or E3 ligase families[30]. In conclusion, the *in vitro* ubiquitylation methods described herein are effective tools to analyze different aspects of E3 ligase function. Besides the relatively simple execution of the described methods, a major advantage is the general applicability as proteins from all eukaryotic sources could be recombinantly expressed and studied *in vitro* without the need for special equipment.

ACKNOWLEDGMENTS:

We thank the members of our laboratory for critical discussion and helpful advice on the manuscript. We apologize for not having cited valuable contributions due to size limitation. This work is supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – SFB 1218 – Projektnumber 269925409 and Cluster of Excellence EXC 229/ CECAD to TH. This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany´s Excellence Strategy – EXC 2030 – 390661388 and – SFB 1218 – Projektnumber 269925409 to T.H. Diese Arbeit wurde von der Deutschen Forschungsgemeinschaft (DFG) im Rahmen der deutschen Exzellenzstrategie – EXC 2030 – 390661388 und – SFB 1218 – Projektnummer: 269925409 an T.H. gefördert.

DISCLOSURES:

The authors have no conflicts of interest.

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Figure 1

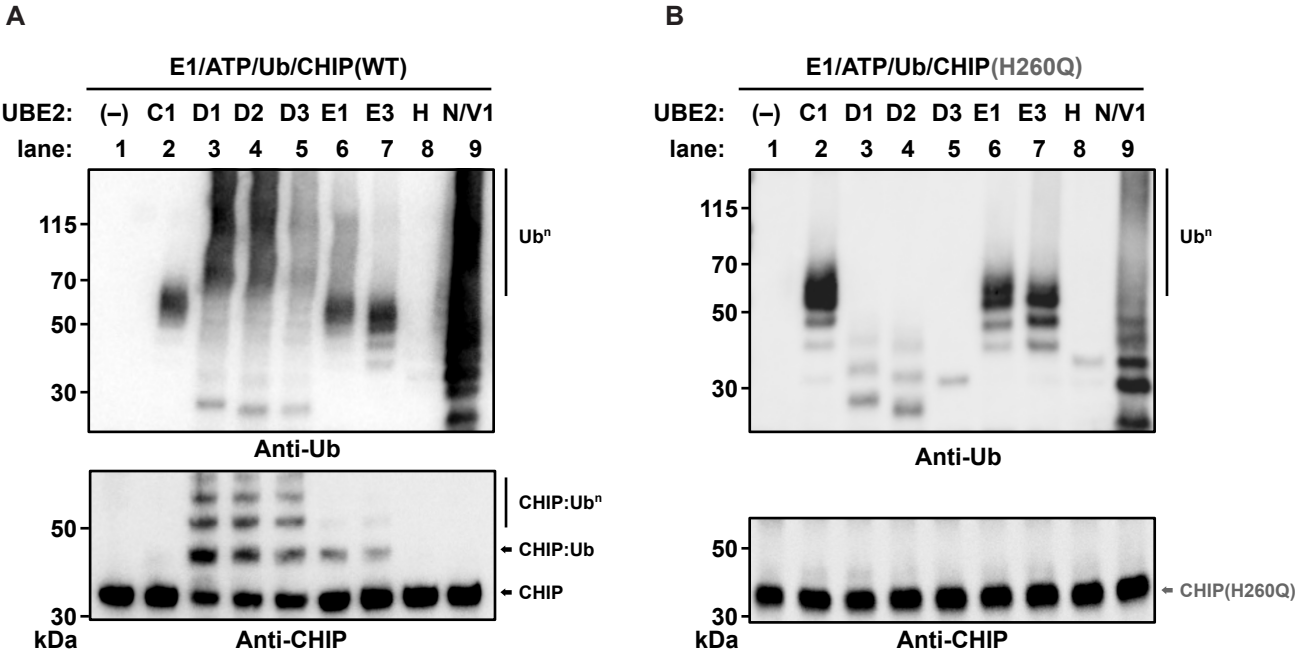


Figure 2

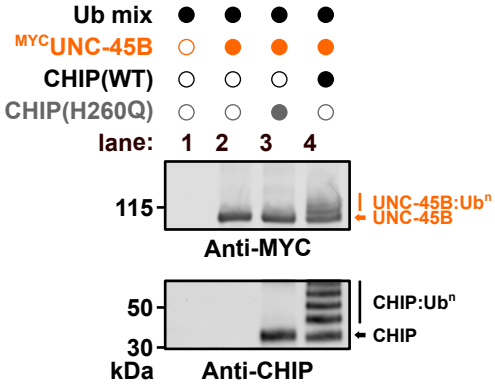
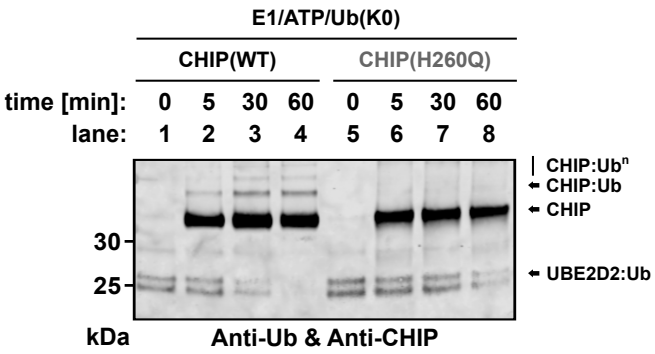


Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Amershan Protran 0.1 µm NC	GE Healthcare	10600000	nitrocellulose membrane
Anti-CHIP	Cell Signaling	2080	Monoclonal rabbit anti-CHIP antibody, clone C3B6
Anti-MYC	Roche	OP10	Monoclonal mouse anti-MYC antibody, clone 9E10
Anti-ubiquitin	Upstate	05-944	Monoclonal mouse anti-Ub antibody, clone P4D1-A11
Apyrase	Sigma	A6535-100UN	
ATP (10x)	Enzo	12091903	
BSA	Sigma	A6003-10G	
EDTA	Roth	8043.2	
KCl	Roth	6781.1	
K2HPO4	Roth	P749.2	
KH2PO4	Roth	3904.1	
LDS sample buffer (4x)	novex	B0007	
L-Lysine	Sigma	L5501-5G	
MES	Roth	4256.4	
MeOH	VWR Chemicals	20,847,307	100%
Milchpulver	Roth	T145.3	
NaCl	Roth	P029.3	
NuPAGE Antioxidant	invitrogen	NP0005	
NuPAGE Transfer buffer (20x)	novex	NP0006-1	
Page ruler plus	Thermo Fisher	26619	Protein ladder
RotiBlock	Roth	A151.1	Blocking reagent
SDS (20%)	Roth	1057.1	
S1000 Thermal Cycler	Bio Rad	1852196	
Trans-Blot Turbo	Bio Rad	1704150EDU	Transfer system
Tris base	Roth	4855.3	
Tween 20	Roth	9127.2	
UbcH Enzyme Set	BostonBiochem	K-980B	E2 enzymes
Ubiquitin	BostonBiochem	U-100H	
Ubiquitin-activating enzyme E1	Enzo	BML-UW941U-0050	
Ubiquitylation buffer (10x)	Enzo	BML-KW9885-001	

Whatman blotting paper	Bio Rad	1703969	Extra thick filter paper

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2021/02/22

We thank the reviewers for their critical assessment of our work. We are pleased to read that the reviewers recognized the manuscript as “well written” and that it “deserves to be published in a journal such as Jove”. We have considered the editorial comments and reviewer’s suggestions and are happy to submit the final version of the manuscript.

Please find the detailed answers to the editorial and peer review comments below.

Thank you very much.

With best regards,
Thorsten Hoppe

Editorial comments:

Point 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
Reply: *Spelling and grammar issues are revised. Abbreviations are defined at first use.*

Point 2: Please revise the following lines to avoid overlap with previously published work: 38-41; 41-43; 46-49; 91-99.
Reply: *The indicated lines were revised.*

Point 3: Please provide an email address for each author.
Reply: *We added the following e-mail addresses from all authors to the manuscript:*
Müller, Leonie: lmuel111@uni-koeln.de
Kutzner, Carl Elias: ckutzner@uni-koeln.de
Balaji, Vishnu: vbalaji1@uni-koeln.de

Point 4: Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Reply: *We removed all personal pronouns from the manuscript.*

Point 5: Please number all steps in the order of section 1, step 1.1. (sub-steps 1.1.1, 1.1.1.1 etc), 1.2 etc. Add one-line spacing between all steps and notes.
Reply: *All steps were numbered in the order of section 1, 1.1, etc. and a one-line spacing was added between all steps and notes.*

Point 6: After including a one-line space between each protocol step, highlight not more than 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed. Notes need not be highlighted, and shorter steps can be combined so that there are maximum of 4 sentences per step, and each step contains 2-3 actions.
Reply: *We highlighted the respective pages.*

Point 7: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Reply: *We removed the first paragraph of the introduction which summarizes the main results of the assay protocols. Now, we explicitly cover the suggested discussion aspects.*

Point 8: Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (italics). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate journal names.
Reply: *We changed the formatting of the references.*

Reviewer #1:

Point 1: In the Protocol, I am completely missing how to prepare the ubiquitination buffer and how to prepare, and store, the ATP. Both crucial component for the in vitro Ub assay. Please add to the text.
Reply: *We agree with the reviewer on this important point. However, ATP solution and ubiquitylation buffer, as well as all other reagents that are not listed in paragraph 1, were purchased and are thus listed in the JoVE material list. Purchased reagents were used and stored as defined by the manufacturers' instructions.*

Point 2: I am missing the reference to the supporting figures in the Protocol description, for instance, in the paragraph 3 "in vitro substrate ubiquitylation assay" that is related to Figure 2. On a related note, it would be great to have cross-reference between the Protocol section and the Representative Results.
Reply: *The figure references were added to the end of the respective method descriptions.*

Point 3: In Fig.1A I do not see auto-ubiquitination of CHIP using Ube2D3. I do agree with a slight generation of free Ub chains. Please correct.
Reply: *The "E2 screening" (see 2.1, Protocol A) was repeated. Now, auto-ubiquitylation of CHIP in cooperation with UBE2D3 can be observed in lane 5, Figure 1 A.*

Point 4: I do not see the result showed in Fig.3A strong enough. I am not convinced by the "shift" of Ube2D2-Ub upon E3-dependent discharging of Ube2D2. It would help to add a control, e.g., Coomassie staining on the experiment.
Reply: *We agree that the effect of the discharge shown in Figure 3 A could be more dominant. Therefore, we adjusted the conditions of the lysine discharge assay (Protocol C) and changed the manuscript accordingly. To optimize the result, we lowered the discharge reaction temperature from 37 °C to room*

temperature. In parallel, we increased the E3 ligase concentration from 100 nM to 500 nM. Applying these conditions, we performed the discharge for 60 min and removed samples after 5, 30, and 60 min. We then performed SDS-PAGE splitting the sample volumes to obtain two equally loaded gels that can be used for a) western blotting (Figure 3 A) and Coomassie staining (Figure S2 C). We hope you can appreciate that ubiquitin is predominantly discharged from E2 by the representative E3 ligase CHIP within 60 min (Figure 3 A). To underline this, we probed the respective western blot membrane with an antibody mixture including mouse anti-Ub (as before) and rabbit anti-CHIP. It is visible that CHIP was auto-ubiquitylated with increasing incubation time and decreasing UBE2D2~Ub fraction, suggesting that CHIP promotes the transfer of ubiquitin onto its own lysine residues.

Point 5: In the Introduction I suggest in row 50 to use a more recent reference to describe better the actual number of putative E3 ligases discovered so far (e.g., Michael J. Clague et al., Trends in Cell Biology, 2015) or to add a more recent one.

Reply: *As suggested, we exchanged the reference (reference number 7) to Michael J. Clague et al.*

Point 6: In the Introduction I suggest adding (around row 69) a part about the importance of the Ubiquitin Binding Domains (UBD) in recognizing and decoding the Ub signal.

Reply: *We agree that the Ubiquitin Binding Domain is an important aspect of recognizing and decoding the ubiquitin signal. However, we think that this particular aspect is not necessarily required in this method paper. Therefore, we did not include a part about the Ubiquitin Binding Domain.*

Point 7: The authors may want to add either in the protocol description or in the representative results section why it is better, for the purpose of this experiment, to use an Ub K0 mutant.

Reply: *We agree with the reviewer on this important point. We included the explanation for the use of Ub K0 in the manuscript. Using lysine-free ubiquitin (Ub K0), mono-ubiquitylation of the E2 enzyme is enforced resulting in a single band for ubiquitin modified proteins in the western blot (= E2~Ub). When wild-type ubiquitin is used, other ubiquitin modified species (e.g. polyubiquitylated E2 = E2~Ub^n) might appear after the addition of the E3 ligase. For the purpose of following the decrease of the charged E2 fraction, we found it easier to follow a single band.*

Reviewer #2:

Point 1: Please put the lane numbers below the gels and blots.
Reply: *Lane numbers were added below all gels and blots.*

Point 2: It would have been informative to use a 15 % gel for figure 1 to better appreciate ubiquitin chains.
Reply: *We agree and will consider this suggestion for future work.*

Point 3: Figure 3 can be improved by extending incubation time.
Reply: *We agree with the reviewer on this point and adjusted the lysine discharge assay conditions (Protocol C) in order to obtain a more dominant discharge effect (also see reviewer 1, point 4).*

Point 4: In figure 1 and Figure S1 (UBE2-D1, -D2, -D3, -E1, -E3, -H, and -N), the authors mentioned UBE2E3 but in the text is E2. Also Fix N) versus N/V.
Reply: *We thank the reviewer for noticing this. The figure legends of Figure 1 and Figure S1 were not correct. In the experiment, UBE2E3 was used, not UBE2E2. Figure legends were revised. The issue of UBE2N/V1 vs. UBE2N was clarified.*

Point 5: The authors need to explain from the first mention in the text why Ubiquitin K0, why Apyrase.
Reply: *We agree with the reviewer on this important point. We included the explanation for the use of Ub K0 and apyrase (and EDTA) in the manuscript (also see Reviewer 1, point 7). Apyrase (and EDTA) was used to quench available ATP molecules in the in vitro system, so that the charging reaction is stopped and E1 cannot further charge E2.*

Point 6: The authors could complete the Comments/Description for all reagents.
Reply: *All buffers and solutions that were prepared in the laboratory are listed in paragraph 1. All other reagents were purchased from diverse companies and are listed in the JoVE material file. To clarify this issue in the manuscript, we added a short statement in paragraph 1.*

Point 7: Figure S2 could be more informative if the kinetics show progressive charging.
Reply: *In general, we agree that Fig S2 A could be more informative if kinetics show a progressive charging. However, the information that S2 A is intended to clarify is whether prolonged incubation of the charging reaction results in an increased yield of E2~Ub. The incubation for more than 15 min does not further increase the E2~Ub yield. Therefore, we did not change or improve Figure S2 A.*

Figure S1

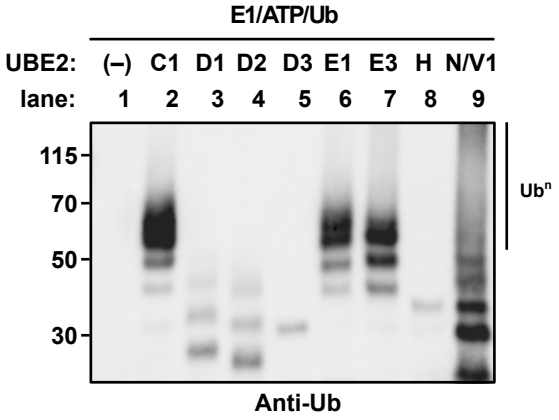

Figure S2