Electronic Supplementary Material (ESI) for Chemical Science.

Supplementary Information

Protein Ubiquitination and Formation of Polyubiquitin Chains Without ATP, E1 and E2 Enzymes

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Supplementary Methods

General Information

Yeast UBE1, Human UbcH5a (E2-616-100), SUMO1, Nedd8 and all ubiquitin mutants were purchased from R&D Systems. Sodium 2-mercaptoethanesulfonate (MESNa) and wildtype Ubiquitin from bovine erythrocytes were purchased from Sigma-Aldrich. Restriction enzymes (NdeI and SapI) and Chitin beads were purchased from New England Biolabs. These purchased proteins and chemicals were used without further purification. All biochemical reactions were performed in the ubiquitination buffer (See the Buffers section below). For the native E1-E2-Rsp5 protein ubiquitination reaction, UBE1 (0.09 μM), UbcH5a (1.0 μM), ubiquitin (150 μM), Rsp5 (1.5 μM), ATP (4mM) and Sic60-GFP (0.5 μM) were used, unless specified otherwise. In-gel fluorescence scanning was performed using the Typhoon 9600 (GE Healthcare). All Coomassie images were obtained with InstantBlue stain (Expedeon). Anti-Ub rabbit antibody, anti-DYKDDDDK tag (FLAG-tag) rabbit antibody and Anti-K48-ubiquitin linkage antibody were purchased from Cell Signaling Technology. Anti-K63-ubiquitin linkage antibody was purchased from Millipore. Goat Anti-Rabbit IgG (H+L)-HRP Conjugate antibody was purchased from Bio-Rad. GST-Rsp5 in pGEX-6p-1 and Cat6-Sic60PY-GFP-6×His in pET3a vectors were gifts from Prof. Andreas Matouschek, and Rsp5ΔWW in pET30 vector was a gift from Prof. Linda Hicke. The original 3×FLAG-6×His-$Ub_{1\text{-}75}$ cloned into PTXB1 vector was a gift from Prof. David O. Morgan, UCSF. Nedd4-1 plasmid was a gift from Prof. Simona Polo. The Wwp1 HECT plasmid was originally cloned in the lab of Prof. Tony Hunter. All protein mutagenesis was performed with the Quickchange II kit (Agilent Technologies). Protein concentrations were assessed by BioSpec-nano (Shimadzu) or Bradford assay (Biorad).

Preparation of Ub-MES

The 3×FLAG-6×His-$Ub_{1\text{-}75}$ region of 3×FLAG-6×His-$Ub_{1\text{-}75}$ cloned into PTXB1 vector was amplified by PCR. The amplified fragment was cloned into the pTYB1 vector by NdeI and SapI restriction digestion of insert and vector. Digested DNA fragments were purified with the standard PCR purification kit (Qiagen), followed by ligation using DNA ligase (New England Biolabs). Using the Quickchange II kit (Agilent Technologies), an extra glycine was inserted to the C-terminus of Gly^{75} of the inserted protein. The nucleotide sequence of the resulting construct was validated. DNA constructs were then transformed into BL21 (DE3) cells. Cells were grown in 1 L of LB media, which was supplemented with 100 μg/mL ampicillin. When the culture reached OD_{600} of 0.6, protein expression was induced with 0.2 mM (final concentration) IPTG for 18 hours at 15 °C. Cells were harvested by centrifugation at 8,000 g, for 20 minutes at 4 °C and resuspended in lysis buffer I containing protease inhibitors (Roche complete protease inhibitor cocktail). Suspended cells were sonicated (5 cycles, 1 min each cycle with 1 min delay in between) on ice. The resulting cell lysates were centrifuged at 18,000 rpm, for 30 minutes at 4 °C, and the resulting supernatants were collected. In parallel, 10 mL of chitin bead slurry (New England Biolabs) were washed with lysis buffer I by preincubating chitin beads with lysis buffer I at 37 °C for 30 minutes. Cell lysates were then incubated with chitin beads for 1 hour at 37 °C with shaking at 150 RPM. Chitin beads were subsequently washed with 5×20 mL of lysis buffer I and incubated with 5 mL of cleavage buffer I with shaking (125 rpm) at 30 °C for at least 12 hours. Eluates were collected and chitin beads were washed with cleavage buffer I (2×5 mL). Combined eluates were concentrated with Amicon Ultra Centrifugal Filter Units (MWCO 3,000, Millipore) to 2 mL total volume. Concentrated Tagged-Ub-MES was further purified using HPLC, using C18 column, 5 μm, 250×21.2 mm (Restek) with solvent B gradient (5% to 95%) for 40 minutes (solvent A is 95:5 H_2O:CH_3CN and 0.1% TFA; solvent B 5:95 H_2O:CH_3CN and 0.1% TFA). Collected fractions were pooled and freeze-dried overnight to obtain white powder. The typical yield of Tagged-Ub-MES is 1-2 mg/L. The dried Tagged-Ub-MES was reconstituted in diluted lysis buffer (HEPES 10 mM, Sodium Acetate 25 mM, NaCl 38 mM, pH 6.5) on ice, aliquoted, snap-frozen and stored at -80 °C. We

have found that the Tagged-Ub-MES could be stored for months without significant decomposition. All other UB-MES variants were prepared using the same experimental procedure as above.

Preparation of Rsp5

BL21(DE3) cells (Novagen) were transformed with GST-Rsp5 cloned into pGEX-6p-1 vector. GST-Rsp5 expression was induced with IPTG (0.5 mM final concentration) at 18 °C overnight. Cells were then harvested by centrifugation, resuspended in PBS (Complete Mini Protease Inhibitor Cocktail, Roche) and lysed by sonication. The glutathione agarose beads (Pierce Biotechnology) were washed with PBS at 37 °C for 1 hour prior to subsequent incubation with cell lysate for 1–2 h at 4°C. Glutathione beads were washed (3×10 mL PBS), and Rsp5 was eluted following cleavage with PreScission Protease in PreScission Protease buffer overnight at 4°C. All Rsp5 mutants were prepared using the same experimental procedure.

Preparation of Rsp5ΔWW

Rsp5ΔWW was transformed into Rosetta (DE3)pLysS (Novagen), and expression was induced with IPTG (0.2 mM final concentration) at 18 °C overnight. Cells were then harvested by centrifugation, resuspended in 6xHis purification Buffer A supplemented with DTT (1 mM), $MgCl_2$ (8 mM), and DNAse I from bovine pancreas (10 μg/ml, Sigma-Aldrich). Following sonication, the lysate was cleared with centrifugation and then incubated with TALON Metal Affinity Resin (Clontech; 2 ml slurry equilibrated with 6xHis purification Buffer A) for 1 hour at 4 °C with rocking. The resin was then washed with 6xHis purification Buffer A (20 ml) and 6xHis purification Buffer E (20 mL) before elution with 6xHis purification Buffer F (1 x 5 ml). The combined eluates were concentrated with Amicon Ultra Centrifugal Filter Units (MWCO 30,000, Millipore) to 2 mL and then buffer exchanged to 400 mM NaCl, 100 mM HEPES 7.0, 1 mM TCEP (Zeba Spin Desalting Columns, Thermo). Rsp5ΔWW used for quantitative analysis (Figures 6E and 6F of the main text) underwent additional purification by gel filtration on Superdex 75 resin (GE Healthcare) equilibrated with the same storage buffer. The purity of gel filtration fractions was assessed with SDS-PAGE before concentrating pure fractions to ~ 40 μM and storing at -80 °C.

Preparation of Nedd4-1 HECT

Nedd4-1 HECT domain (residues 938-1318) in a PGEX6P1 vector plasmid (GST-Nedd4-1 HECT) was transformed into BL21 cells (Novagen). 1L TB media containing 100μg/ml ampicillin was inoculated with 50 mL overnight cell culture and incubated at 37°C until OD reached ~3. Then, IPTG (1.0 mM final concentration) was added to the cell culture media at 18°C, followed by 16 hour incubation at the same temperature. Cells were then harvested and lysed by sonication in phosphate buffered saline (PBS) with protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche). The supernatant was incubated with glutathione agarose beads (Pierce Biotechnology) for 1 hour at 4°C. The beads were washed three times with PBS and incubated with PreScission Protease (GE Healthcare) for 4h at 23°C to elute Nedd4-1 HECT domain (50mM HEPES, 150 mM NaCl, 0.1 mM EDTA 1mM DTT).

Preparation of Nedd4-2 HECT

We obtained a pET28-MHL plasmid containing the Nedd4-2 HECT domain without the last eight C-terminal residues (Addgene #25169). We added these residues by PCR reaction. The Nedd4-2 HECT domain (residues 594-975) was then expressed, purified and stored according to the procedure use for Rsp5ΔWW.

Preparation of Wwp1 HECT

Rosetta (DE3) cells (Novagen) were transformed with a pET28 plasmid containing the Wwp1 HECT domain (residues 546-922). Cells were grown in LB media to OD_{600} = 0.7 before inducing expression with IPTG (1.0 mM final concentration) at 20 °C over six hours. Cells were then harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 2 mM Imidazole, 1% Tween-20, 5% glycerol with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche). Following sonication, the lysate was cleared with centrifugation, and then incubated with TALON Metal Affinity Resin (Clontech; 2 ml slurry equilibrated with the above resuspension buffer) for 1 hour at 4 °C with rocking. The resin was then washed first with 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 2 mM imidazole, 1% Tween-20, 5% glycerol, 1 mM PMSF, 2 mM BME (1 x 10 ml), and then with 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM Imidazole, 1% Tween-20, 5% glycerol, 1 mM PMSF, 2 mM BME (2 x 10 ml). His-tagged ligase was eluted with 50 mM Tris-HCl, 100 mM NaCl, 300 mM imidazole, 5% glycerol, pH 8.0 (2 x 1 ml) and then buffer exchanged to 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5% glycerol (Zeba Spin Desalting Columns, Thermo).

Preparation of Itch HECT

BL21 (DE3) cells (Novagen) were transformed with a pET28-MHL plasmid containing the Itch HECT domain (residues 544-903, Addgene #36197). Cells were grown in TB media to OD_{600} = 3 before inducing expression with IPTG (2.0 mM final concentration) at 15 °C over 16 hours. Cells were then harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5% glycerol, 2 mM imidazole, 0.1 μM PMSF, 10 mM BME with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche). Following sonication, the lysate was cleared with centrifugation, and then incubated with TALON Metal Affinity Resin (Clontech; 2 ml slurry equilibrated with the above resuspension buffer) for 2 hours at 4 °C with rocking. The resin was then washed with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5% glycerol, 10 mM imidazole (4 x 15 ml). His-tagged ligase was eluted with 50 mM Tris-HCl, pH 7.0, 250 mM NaCl, 5% glycerol, 200 mM imidazole (3 x 1.5 ml) and then buffer exchanged to 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5% glycerol, and 0.5 mM TCEP.

Preparation of GST-Parkin and GST-HHARI

BL21 cells (Novagen) were transformed with GST-Parkin (rat, residues 217-465, Addgene #31255) or GST-HHARI (human, residues 177-393, Addgene #31254) in pGEX-4T2. Cells were grown in LB medium supplemented with $ZnCl_2$ (0.2 mM) to OD_{600} = 0.7 before inducing expression with IPTG (0.4 mM final concentration) at 15 °C over 22 hours. Cells were then harvested by centrifugation and resuspended in 50 mM Tris pH 8.0, 50 mM NaCl, 1 mM DTT with DNAse I from bovine pancreas (10 μg/ml, Sigma-Aldrich), and protease inhibitors (Complete Protease Inhibitor Cocktail, Roche). Following sonication, the lysate was cleared with centrifugation, and then incubated with glutathione agarose beads (Pierce Biotechnology, 2 ml slurry equilibrated with 50 mM Tris pH 8.0, 50 mM NaCl, 1 mM DTT) for 2 hours at 4 °C with rocking. The beads were then washed with 50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT (3 x 20 ml) before eluting GST-tagged ligase with 50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, 15 mM GSH (5 x 1 ml). The combined eluates were concentrated with Amicon Ultra Centrifugal Filter Units (MWCO 3,000, Millipore) to 1.5 mL and then buffer exchanged to 50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT (Zeba Spin Desalting Columns, Thermo).

Preparation of His-tagged Sic60-GFP

BL21(DE3)pLysS cells (Novagen) were transformed with Cat6-Sic60PY-GFP-6×His in pET3a vector, and the cell cultures were grown to OD_{600} = 0.6 prior to induction of Sic60-GFP with IPTG (1.0 mM final concentration) at 37 °C for 4 hours. Cells were then harvested, resuspended in 6×His purification Buffer A, which was supplemented with protease inhibitors (Complete Mini Protease Inhibitor Cocktail, EDTA free, Roche). After centrifugation, the resulting supernatant was incubated with HisPur Ni-NTA Resin (Pierce Biotechnology) for

1–2 h at 4 °C. Beads were washed with PBS and 6×His purification Buffer B. Bound proteins were eluted with 6×His purification Buffer C. The eluate was dialyzed against 6×His purification buffer D overnight and stored at -80 °C. All other Sic60-GFP mutants were prepared using the same experimental procedure.

Protein ubiquitination via Rsp5/ByS.

All reactions (30 µL total volume) were performed in Ubiquitination buffer I. Protein ubiquitination was initiated by adding Tagged- or Tagless-Ub-MES to the reaction mixtures. All reactions were performed at room temperature for the indicated amounts of time and were quenched with reducing Laemmli buffer containing 20 mM of hydroxylamine to consume remaining thioester, unless specified otherwise. To analyze thioester adducts of $Rsp5^{806stop}$~Ub, non-reducing Laemmli buffer was used. Sample mixtures (7.0 µL total volume) were resolved by 7.5% SDS-PAGE, unless specified otherwise. The ubiquitination of Sic60-GFP was monitored by in-gel fluorescence scanning. DYKDDDDK FLAG tag antibody (Cell Signaling Technology) and anti-K63-ubiquitin linkage antibody (Millipore) were used according to the manufacturer's protocol.

MALDI-TOF analysis.

Protein bands on SDS-PAGE gels were visualized using Coomassie staining, excised, and in-gel digested with trypsin for 12-16 hours according to the protocol released by the Virginia Tech Center for Genomics.[1] Digested peptides were analyzed by MALDI-TOF (Bruker Autoflex III MALDI, reflectron positive mode, α-Cyano-4-hydroxycinnamic acid as a matrix). The tryptic peptides are predicted using Swiss-prot software. All molecular masses of peptides derived from branching at any lysines on Tagged-Ub-MES, Tagless-Ub-MES, Tagless-Ub(K48R)-MES, or Tagless-Ub(K63R)-MES were calculated as listed (Supplementary Table 2 - 5). In order to acetylate tryptic peptides, 10 µL of digested solution was treated with 25 µL of acetic anhydride in Methanol (1:3 v/v) for one hour, followed by evaporating the solution with Speed-vac, reconstituting in water (0.1 % TFA) and desalting with ZipTip (Millipore) for subsequent MALDI-TOF analysis.

Pulse-chase Assay

The Ub~UbcH5a was prepared as previously described.[2] E1 enzyme UBA1 (250 nM), UbcH5a (2.0 µM), ubiquitin (5 µM) and ATP (1 mM) were incubated for 40 min at room temperature in the ubiquitination buffer I. The reaction mixture was quenched by adding 5 mM of EDTA, followed by buffer exchange with ubiquitination buffer II using Zeba spin desalting column. 10 µL of the resulting solution was treated with 10 µL of Rsp5ΔWW (3.0 µM) in ubiquitination buffer II to initiate the reaction. Similarly, 10 µL of Tagless-Ub-MES (2.0 µM) in ubiquitination buffer II were treated with the 10 µL of Rsp5ΔWW (3.0 µM) in ubiquitination buffer II to initiate reaction.

Metal affinity purification of ubiquitinated Sic60-GFP

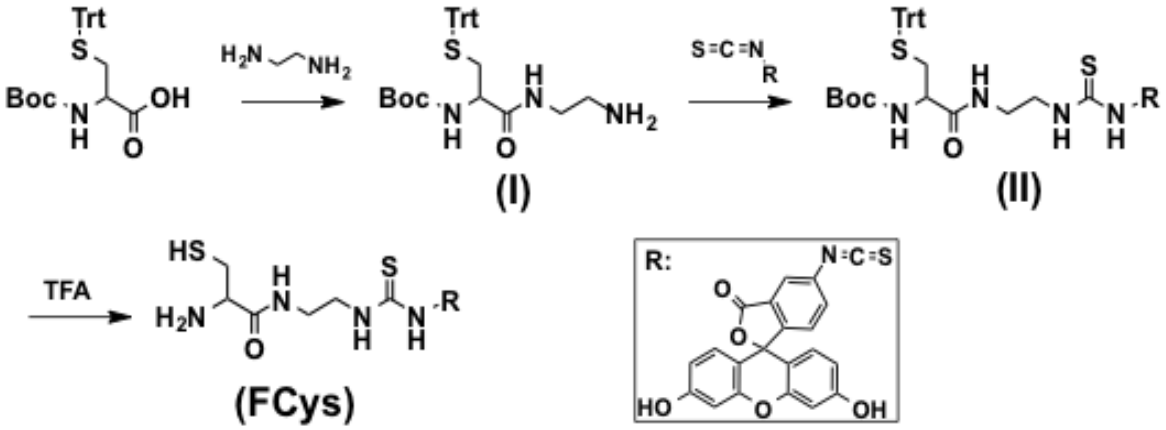
Slurry (100 µL) of Talon Metal Affinity Resin (Clontech) is washed with PBS 2×1 mL. The resin is then resuspended in 100 µL of PBS and 15 µL of this slurry was added to the 60 µL of ByS reaction mixture that contained Rsp5, GFP-Sic60 and Taggless-Ub-MES. After incubation at room temperature for 5 minutes with mild shaking, beads were centrifuged for 2 minutes at 15kG, followed by supernatant removal. Beads were washed with PBS (pH 7.4, 300 µL), wash buffer (25 mM imidazole in PBS, pH 7.4, 300 µL), and bound proteins were eluted with 50 µL of elution buffer (250 mM imidazole in PBS, pH 7.4). Eluted proteins were treated with the 6× reducing Laemmli buffer that contained 20 mM NH_2OH, and resolved by SDS-PAGE.

Kinetic analysis

Triplicate reactions were performed at room temperature and contained Tagless-Ub-MES with Rsp5ΔWW in 25 mM HEPES pH 7.5, 50 mM NaCl, and 4 mM $MgCl_2$. At indicated time points, 5 µL of the reaction mixture

was mixed with 5 µL FCys Stop Buffer. The mixture was then incubated at 37 °C for 16 hours and quenched by adding 2 µL of 6× reducing Laemmli buffer. The mixture was further diluted with 1× reducing Laemmli buffer so that 7 µL of sample loading to each well contained 1 - 2 µg of ubiquitin. 18% SDS-PAGE gels (1.5 mm thick, handcast) were used to maximize the separation between free FCys and labeled ubiquitin that appears at ~10 kDa, followed by in-gel fluorescence scanning. The fluorescence intensity of each band was quantified using ImageJ. The relative intensities against the 0 minute band were multiplied by total nmol of Ub-MES in the reaction mixture. Data sets were fitted to Michaelis-Menten equation using Graphpad Prism 6 to obtain K_{cat}, V_{max} and K_m values. It is important to note that we observed decreased stability of Ub-MES above pH 7.8.

Synthesis of FCys



Scheme S1. Synthetic Scheme of the FCys.

***N*-Boc-*S*-Trityl-*N*-2-aminoethyl-L-cysteinamide (I).** To a DMF solution (10 mL) of *N*-Boc-*S*-trityl-L-cysteine (1.0 g), carbonyl diimidazole (430 mg) was added and stirred for 30 min at room temperature. Ethylene diamine (1.4 mL) was added and stirred for an additional 2 hours at room temperature. Dichloromethane (50 mL) was added to the solution before washing with water (5 × 50 mL) and brine (1 × 50 mL). The solution was further dried with anhydrous magnesium sulfate and concentrated in vacuo until white crystals of compound **I** precipitated. The precipitated material was collected and further dried (750 mg, 68%).
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.25 (6H,d), 7.12 (6H,t), 7.06 (3H,t), 6.20 (1H,t), 4.7 (1H, d), 3.69 (1H, dd), 3.07 (2H, ddd) , 2.58 (3H, m), 2.37 (1H, dd), 1.42 (9H, s); ^{13}C NMR (500 MHz, $CDCl_3$): δ 170.7, 155.4, 144.4, 129.6, 128.0, 126.1, 80.3, 53.7, 46.1, 42.3, 41.3, 33.9, 28.3.

N-Boc-S-Trityl-N-[2-[[[(fluorescein-5-yl)amino]-thioxomethyl]amino]ethyl]-L-cysteinamide (II). Fluorescein isothiocyanate (54.0 mg) was added to a solution of compound **I** (70.13 mg) in DMF (2 mL) and stirred for 16 hours at room temperature. The reaction mixture was diluted with dichloromethane (30 mL), and then washed with water (2× 30 mL) and brine (1× 30 mL). Organic layers were dried over anhydrous magnesium sulfate and then concentrated in vacuo. The residue was purified by silica gel chromatography (ethyl acetate : hexanes = 3 : 1, 3 % MeOH) to afford orange red solid (**II**). ^{1}H NMR (500 MHz, DMSO-d_6), δ 8.17 (1H, s), 8.0 (1H, s), 7.72 (1H, d), 7.35-7.22 (15H,m), 7.17 (1H,d), 6.69 (2H,d), 6.60-6.64 (4H,m), 3.95 (1H,m), 3.56 (2H,m), 3.29 (2H,t), 2.37 (2H,d), 1.31 (9H,s); ^{13}C NMR (500 MHz, CD3OD) δ 181.1, 172.8, 170.9, 169.1, 159.9, 155.2, 152.3, 144.8, 141.6, 129.8, 129.5, 128.7, 128.6, 127.2, 127.1, 126.6, 125.0, 120.0, 113.1, 110.2, 102.7, 83.4, 78.8, 60.2, 53.9, 43.9, 38.3, 34.5, 28.6.

***N*-[2-[[[(Fluorescein-5-yl)amino]thioxomethyl]amino]ethyl]-L-cysteinamide (FCys).** 50 mg of compound II was treated with 1 mL of TFA containing water (2.5 v/v %) and triisopropylsilane (2.5 v/v %) under nitrogen. The reaction mixture was stirred at room temperature for 1 hour before transferring it to 50 mL of cold diethyl ether. The resulting precipitate was centrifuged at 4000 g (10 min). After decanting the ether layer, the orange precipitate was dissolved in MeOH and transferred to a glass scintillation vial and concentrated in vacuo (25 mg, 83%). ^{1}H NMR (500 MHz, CD_3OD), δ 8.13 (1H, s), 7.75 (1H, s), 7.19 (1H, d), 6.78-6.70 (4H,m), 6.58 (2H,d), 4.02 (1H,d), 3.96-3.75 (2H,m), 3.60-3.45 (2H,m), 3.10-2.99 (2H,m); ^{13}C NMR (500 MHz, DMSO-d6) δ 180.02, 168.50, 166.92, 159.51, 158.05, 151.88, 147.45, 140.95, 129.89, 129.89, 129.01, 126.63, 124.21, 117.59, 115.24, 112.59, 109.66, 102.26, 83.09, 54.15, 43.11, 38.08, 25.12.; ESI-MS: Calculated for 552.11, Found m/z 553.14 (MH^+).
Anticipating the need to make FCys Stop Buffer, synthesized FCys can be stored as a DMSO stock solution. After dissolving FCys in DMSO (200 mM), we neutralize residual TFA by slowly adding saturated aqueous sodium bicarbonate until the solution color changes from yellow to a dark orange. This stock solution was stored at -20 °C until use.

Buffers
Ubiquitination buffer I: HEPES (25 mM. pH 7.5), NaCl (50 mM), and $MgCl_2$ (4 mM).
Ubiquitination buffer II: HEPES (25 mM. pH 7.5) and NaCl (50 mM).
Lysis buffer I: HEPES (20 mM, pH 6.5), Sodium Acetate (50 mM) and NaCl (75 mM).
Cleavage buffer I: MESNa (100 mM) in lysis buffer I, pH 6.5.
PreScission Protease Buffer: HEPES (50 mM, pH7.0), NaCl (150 mM) and EDTA (0.1 mM).
6×His purification Buffer A: $NaPO_4$ (50 mM, pH 7) and NaCl (300 mM).
6×His purification Buffer B: $NaPO_4$ (50 mM, pH 7), NaCl (300 mM) and imidazole (10 mM).
6×His purification Buffer C: $NaPO_4$ (50 mM, pH 7), NaCl (300 mM) and imidazole (150 mM).
6×His purification buffer D: HEPES (50 mM, pH 7), NaCl (150 mM) and EDTA (0.1 mM).
6×His purification Buffer E: $NaPO_4$ (50 mM, pH 7), NaCl (300 mM) and imidazole (25 mM).
6×His purification Buffer F: $NaPO_4$ (50 mM, pH 7), NaCl (300 mM) and imidazole (250 mM).
6× reducing laemmli loading buffer: Tris (60 mM, pH 6.8), SDS (120 mg/mL), Glycerol (47 % v/v), Bromophenol blue (0.6 mg/mL) and DTT (600 mM).
FCys Stop Buffer: HEPES (50 mM, pH 7.0), Urea (6 M), FCys (6 mM), TCEP (9 mM)

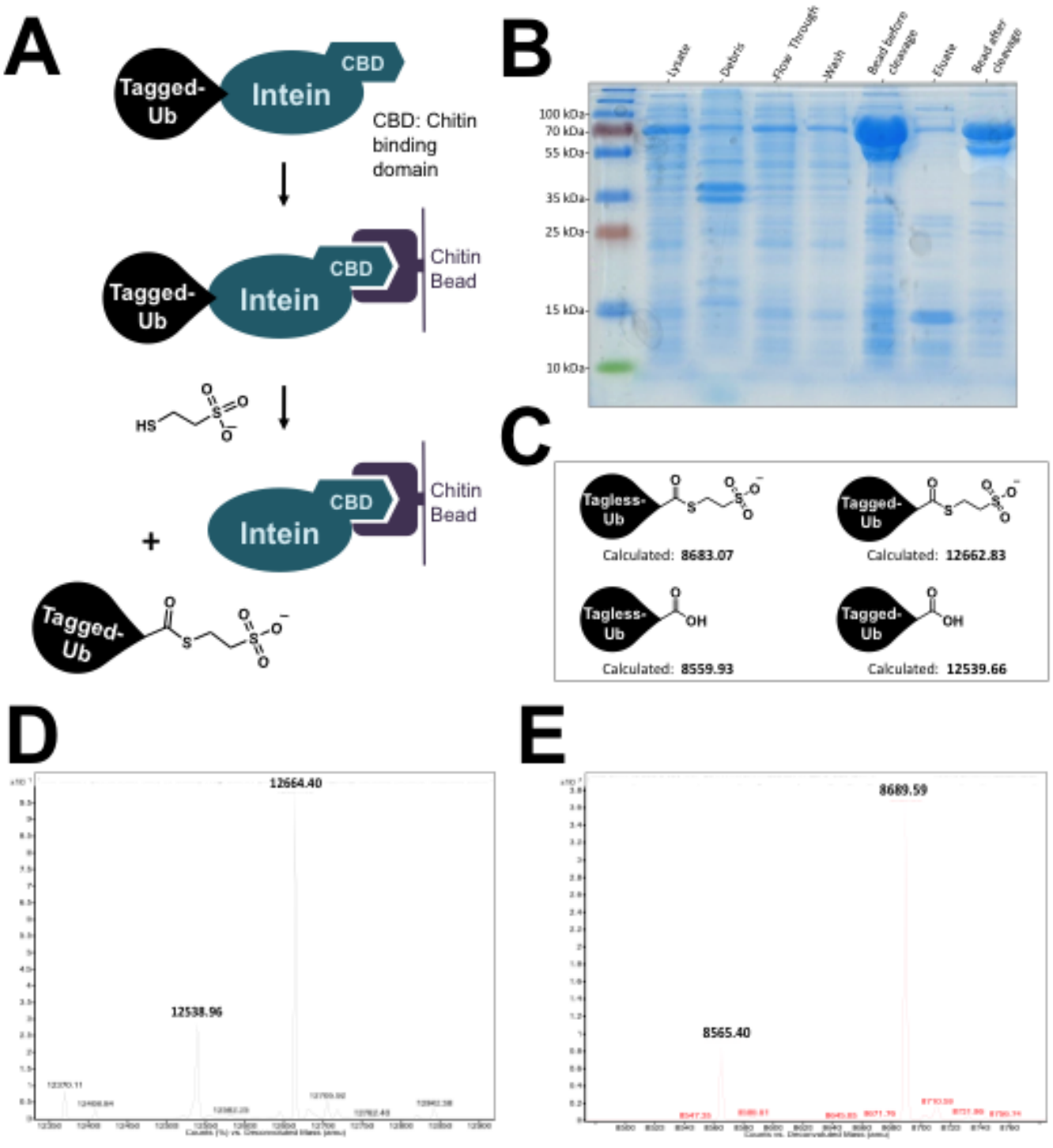


Figure S1. Preparation of the 3×FLAG-6×His-ubiquitin$_{1-76}$-mercaptoethansulfonate (Tagged-Ub-MES). (A) Tagged-Ub is inserted into the pTYB1 vector and expressed with BL21(DE3) cells. The resulting recombinant Tagged-Ub-Intein-CBD fusion protein was isolated using chitin beads. Chitin beads were washed and treated with sodium mercaptoethansulfonate (MESNa) solution to cleave and elute Tagged-Ub-MES. Tagged-Ub-MES is further purified using HPLC and analyzed by ESI-MS. **(B)** Coomassie staining showing all protein fractions. Ub-MES undergoes hydrolysis during the cleavage by MESNa. The usual yield after HPLC purification is ~2 mg from 1 L of cell culture. **(C)** Molecular weight of the Tagged-Ub-MES, Tagless-Ub-MES and Tagged- or Tagless Ub. ESI-MS of purified **(D)** Tagged-Ub-MES and **(E)** Tagless-Ub-MES.

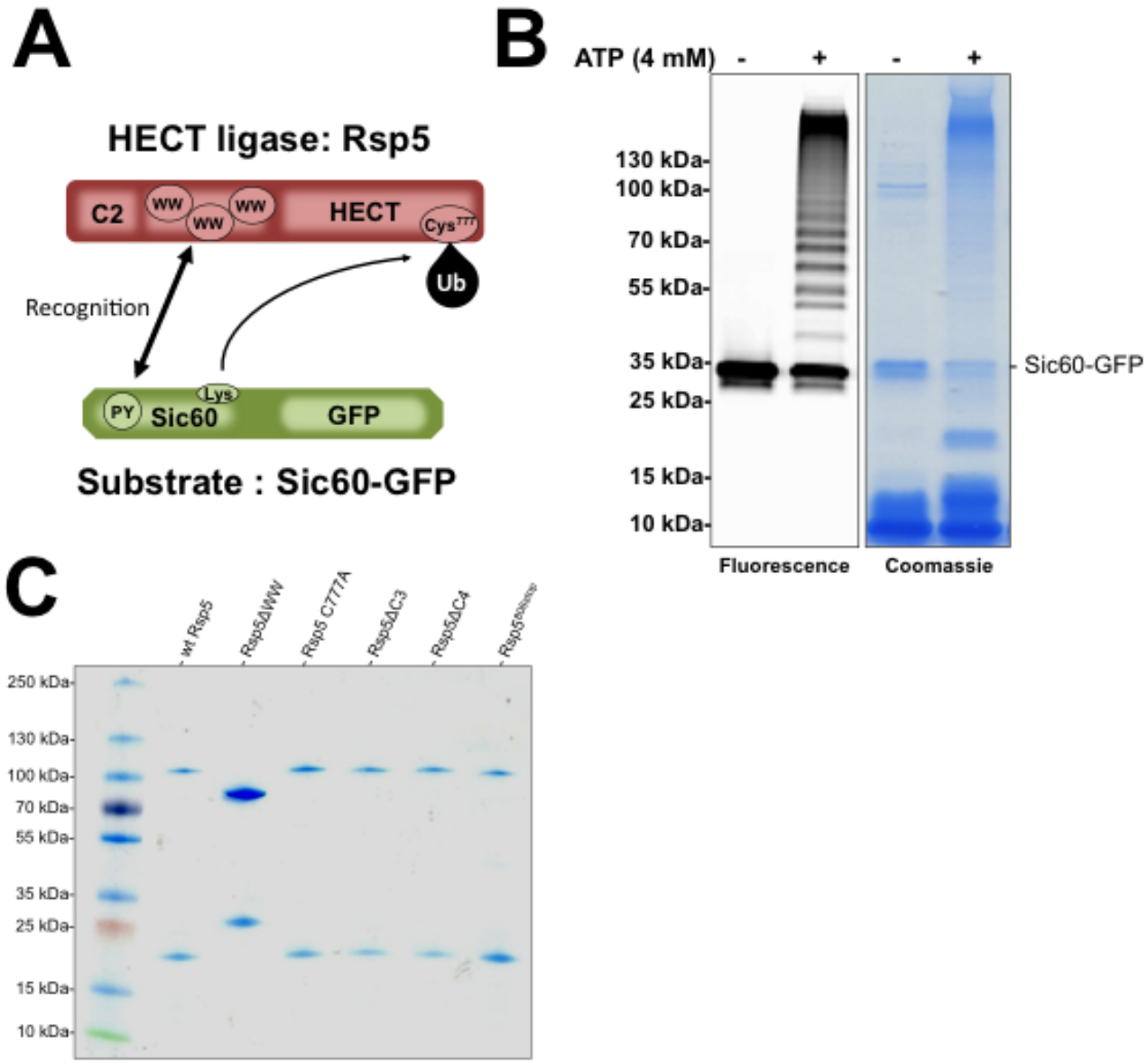


Figure S2. Preparation of the model system. (A) The domain structure of HECT E3 ligase Rsp5 and fluorescent substrate (Sic60-GFP) are shown. Rsp5 contains a characteristic set of protein domains shared by the Nedd4 subfamily: a C2 domain, three WW domains and the catalytic HECT domain that harbors the catalytic cysteine (Cys^{777}). The three WW domains of Rsp5 interact with the proline rich PPPY motif in Sic60-GFP, which facilitates the transfer of ubiquitin onto the lysines of the protein substrate. **(B)** Ubiquitination of Sic60-GFP by the native E1-E2-Rsp5 cascade can be detected by in-gel fluorescence scanning and Coomassie staining. Reaction mixtures containing UBE1 (0.24 µg, 60 nM), UbcH5a (1.4 µg, 2.7 µM), ubiquitin (40 µg, 155 µM), Rsp5 (1.0 µg, 0.4 µM) and Sic60-GFP (1.2 µg, 1.2 µM) with or without ATP (4.0 mM) in ubiquitination buffer I were incubated at room temperature for 1 hour, followed by addition of 6× reducing Laemmli loading buffer, resolving protein bands by SDS-PAGE (12 %), and then in-gel fluorescence scanning or Coomassie staining. **(C)** Coomassie stained gel of expressed wild type full length Rsp5 or its mutants. Rsp5 and its mutants have lower MW impurities (~20 or ~25 kDa)

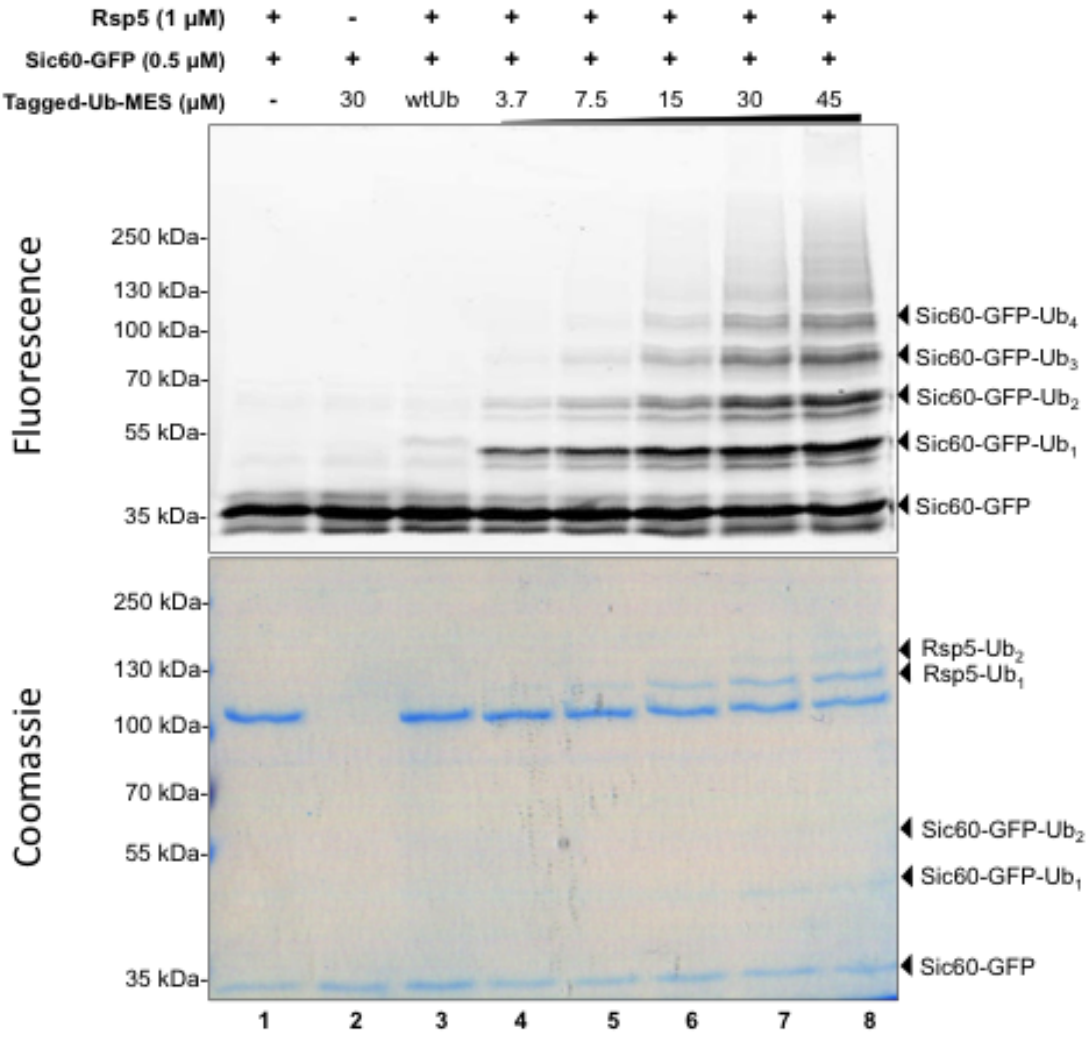


Figure S3. The ubiquitination of Sic60-GFP is dependent on the concentration of Tagged-Ub-MES. Rsp5 and Sic60-GFP were treated with increasing amounts of Tagged-Ub-MES in ubiquitination buffer I. The reaction was incubated for 2 hours at room temperature, followed by quenching with 6× reducing Laemmli buffer containing hydroxylamine (20 mM, final concentration), resolved by 7.5% SDS-PAGE gel and analyzed by in-gel fluorescence scanning and Coomassie staining. The fluorescence scanning (upper) and Coomassie staining (lower) of SDS-PAGE gel showed higher molecular weight bands that correspond to the combined mass of Sic60-GFP-Ub_x and Rsp5-Ub_x.

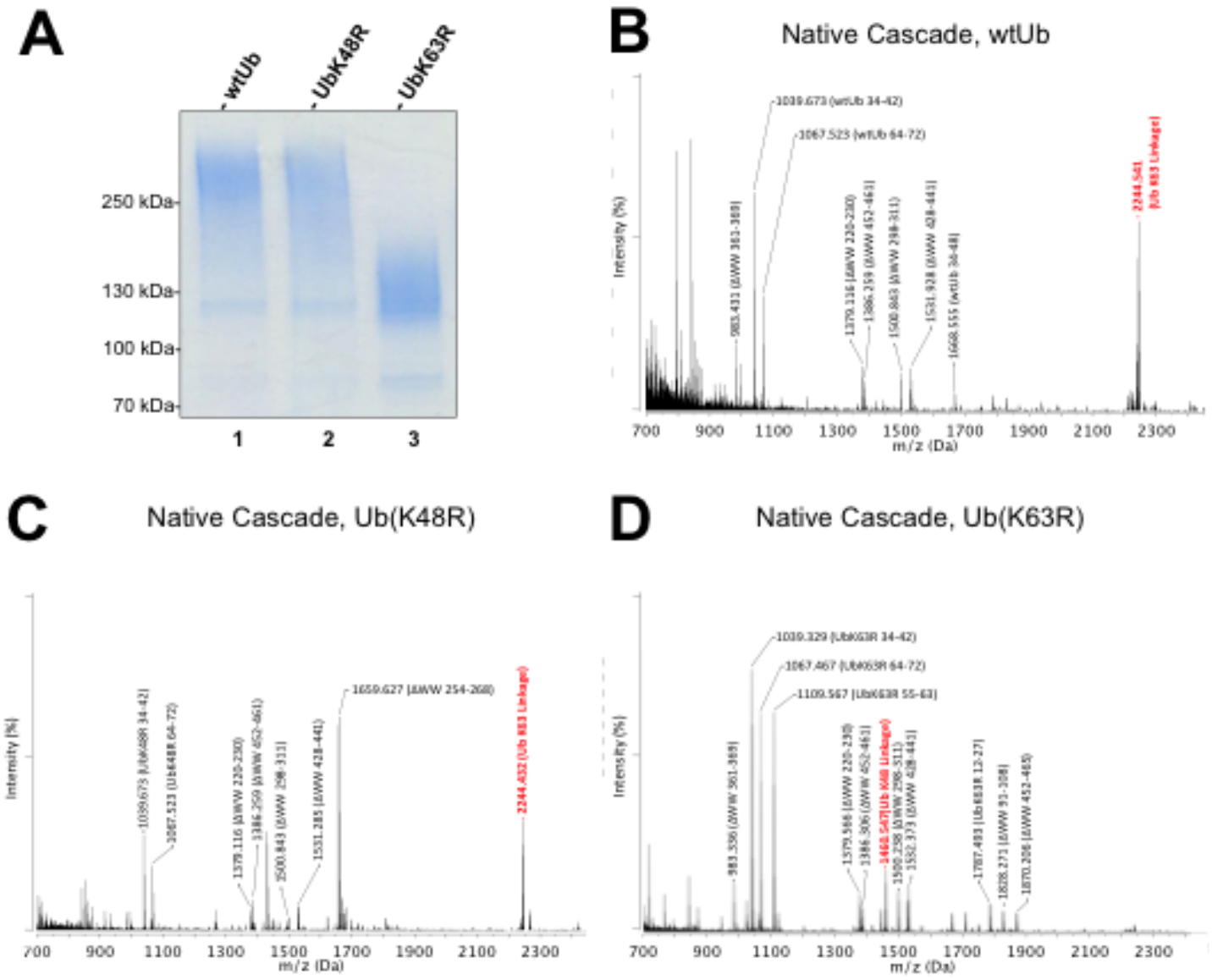


Figure S4. MALDI-TOF analysis of polyubiquitin chain formed by Rsp5ΔWW via native cascade. (A) The ubiquitination reaction was performed with UBE1 (0.09 μM), UbcH5a (1.0 μM), Rsp5ΔWW (1.5 μM), ATP (4 mM) and Sic60-GFP (0.5 μM) with wildtype ubiquitin (wtUb), ubiquitin K48R (Ub (K48R)), and ubiquitin K63R (Ub(K63R)) in ubiquitination buffer I. The reaction was quenched by 6× reducing buffer containing NH_2OH (20mM, final concentration), resolved by SDS-PAGE and imaged by Coomassie staining. The region between MW 130~250 kDa was isolated and digested with trypsin. The MALDI-TOF analysis of polyubiquitin chains formed with **(B)** wtUb, **(C)** Ub(K48R) and **(D)** Ub(K63R) is shown. Any peak corresponding to calculated polyubiquitin linkage signal (Table S2-S4) is marked with red color.

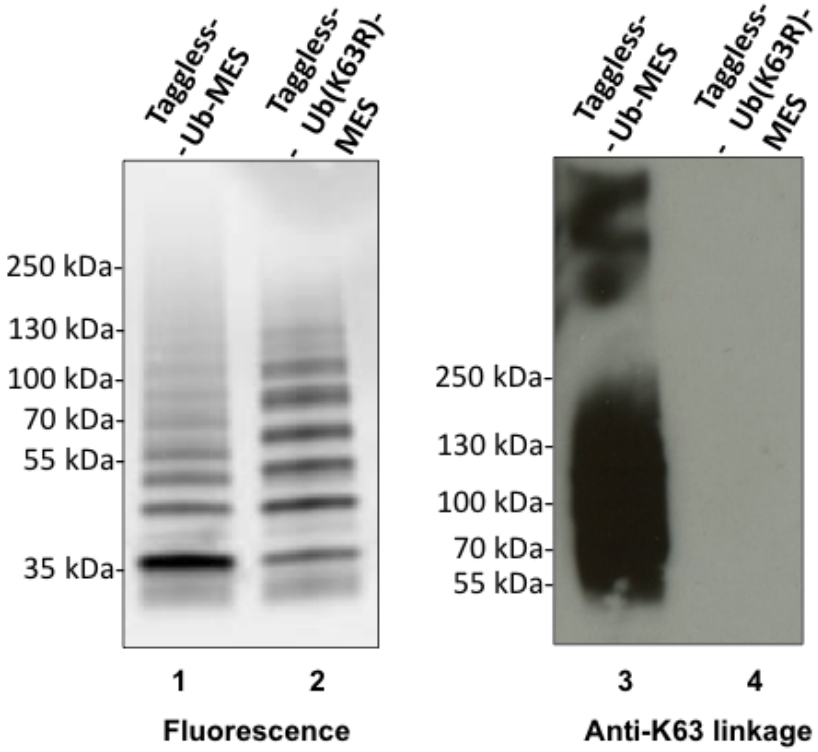


Figure S5. Rsp5/ByS catalyzes K63-specific polyubiquitin chain synthesis on the Sic60-GFP substrate. Full length Rsp5 (5 µM), Sic60-GFP (10 µM) and Tagged- or Taggless-Ub-MES (40 µM) in ubiquitination buffer I were incubated 6 hours at room temperature. The ubiquitinated Sic60-GFP that has C-terminal 6×His tag was immobilized on nickel beads and eluted by imidazole (250 mM). The eluate was resolved by SDS-PAGE (4-20%) and the amount of K-63 linked polyubiquitin chains on Sic60-GFP was probed using K63-linkage specific antibodies.

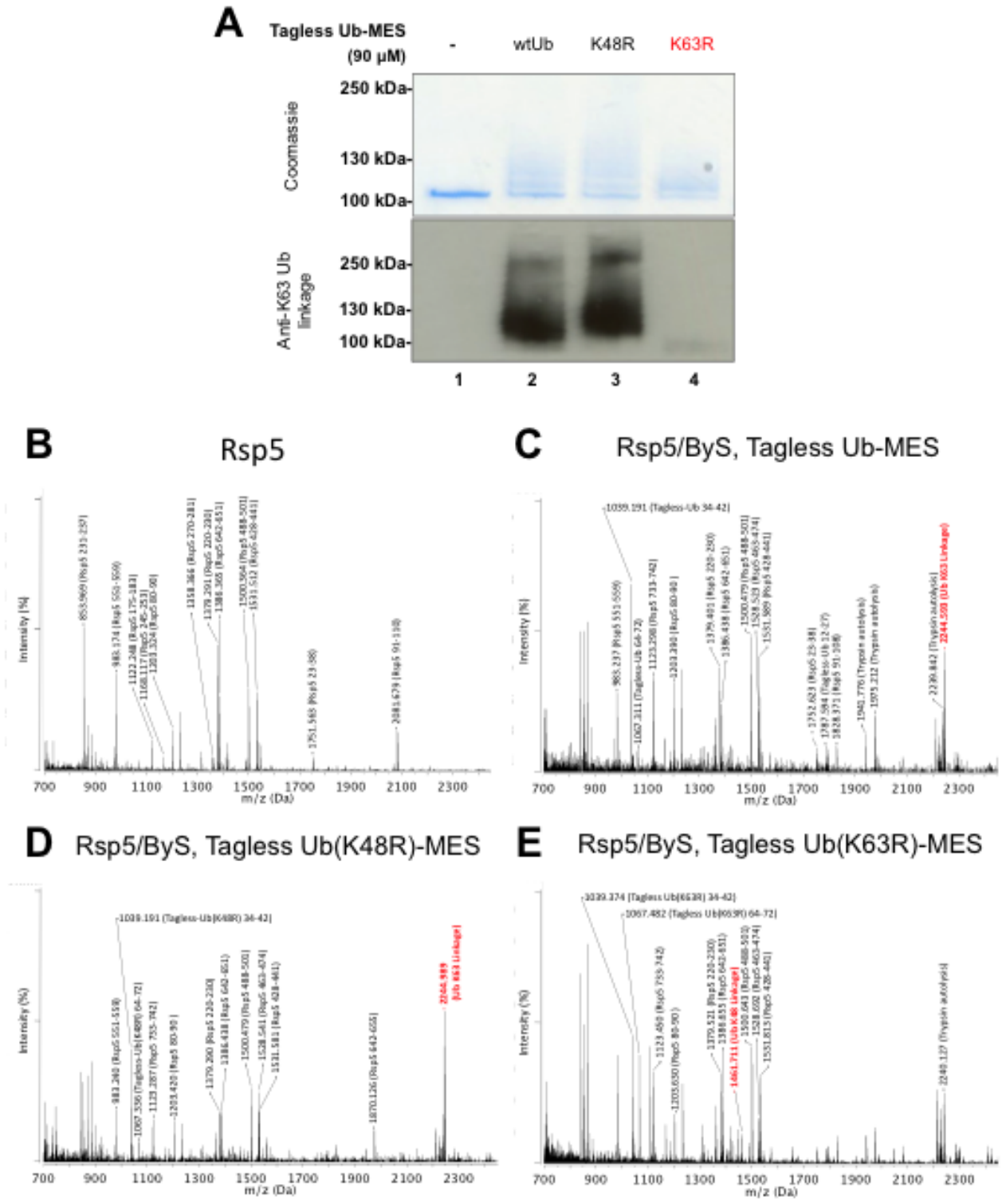


Figure S6. Western blotting and MALDI-TOF analysis of polyubiquitin chain formed in autoubiquitination of the full length Rsp5 via BySS. (A) The presence of K63-linkage in polyubiquitin chains formed by full length Rsp5 and Tagless-Ub-MES (autoubiquitination) was confirmed by Western-blotting with anti-K63-linkage specific antibody. **(B)** MALDI-TOF analysis of full length Rsp5 (lane 1). The band at ~100 kDa from lane 1 was excised, digested by trypsin and analyzed by MALDI-TOF. The same MALDI-TOF analysis was performed for the polyubiquitin chains formed by full length Rsp5 autoubiquitination with **(C)** Tagless Ub-MES (lane 2), **(D)**

Tagless-Ub(K48R)-MES (lane 3), and **(E)** Tagless-Ub(K63R)-MES (lane 4). The region 100 kDa – 250 kDa from lane 2, 3 and 4 were excised, in-gel digested by trypsin and analyzed by MALDI-TOF to confirm the presence of K63-linkage signal. Any peak corresponding to a calculated polyubiquitin linkage signal (Table S2-S4) is marked with red color. There are three tryptic peptides of full length Rsp5 that appears at similar m/z regions: (1) ^{733}LLQFTTGTSR^{742} (m/z = 1123.6106), (2) ^{403}VYFVDHNTK^{411} (m/z=1122.5578) and (3) ^{347}AYFVDHNTR^{355} (m/z = 1122.5327).

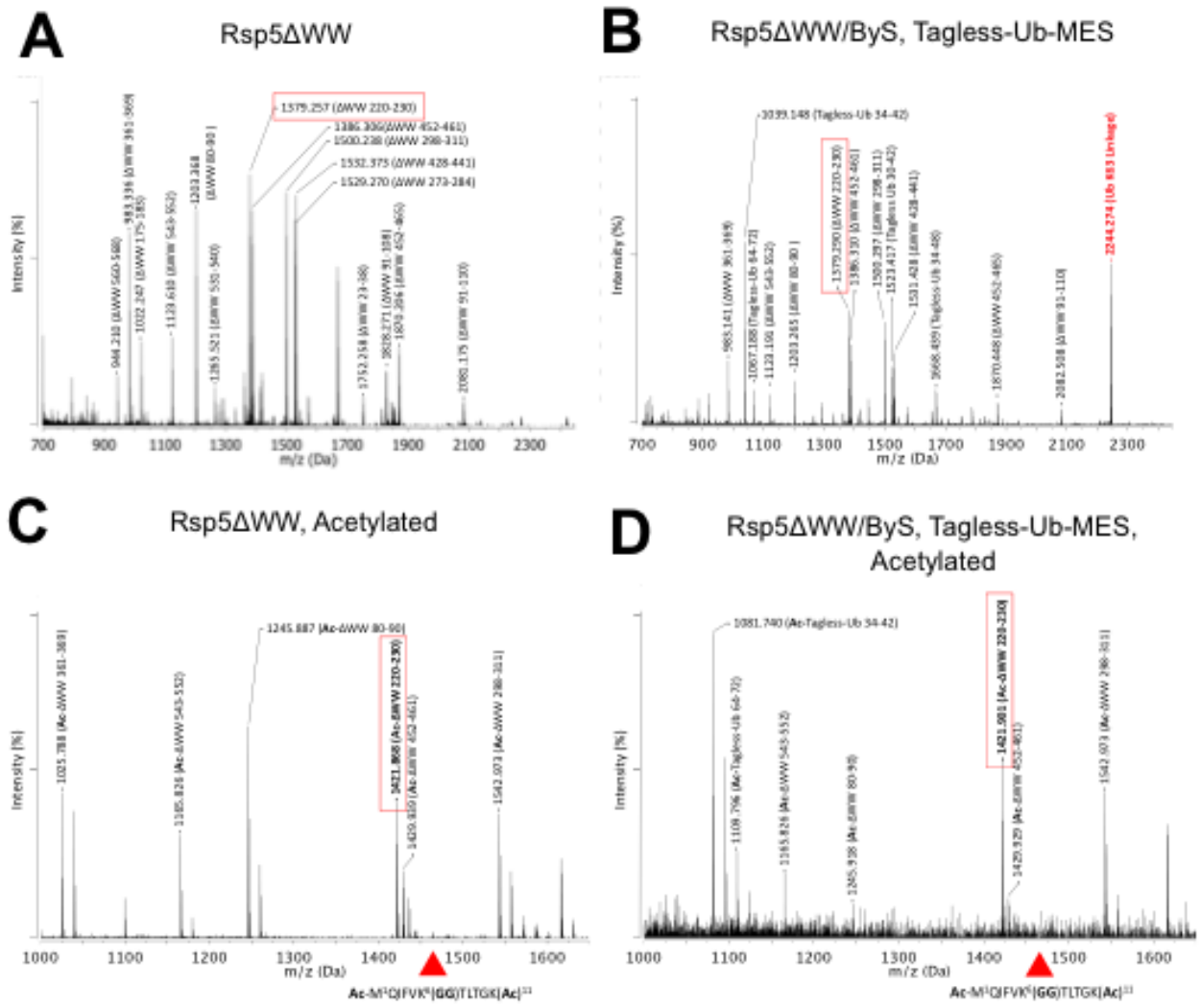


Figure S7. MALDI-TOF analysis of acetylated tryptic peptides of Rsp5ΔWW and Rsp5ΔWW/ByS autoubiquitination. (A) The band at ~ 80 kDa of lane 1 of Figure 4A was excised, in-gel digested by trypsin and analyzed by MALDI-TOF. Representative m/z signal corresponding to ^{220}QYSSFEDQYGR^{230} Rsp5 peptide (calculated average m/z = 1379.402) is marked with the red rectangle. **(B)** The same ^{220}QYSSFEDQYGR^{230} signal was detected in the 100 -250 kDa region from lane 4 of Figure 4A. However, the calculated m/z value for the K6-polyubiquitin linkage signal (^{1}MQIFVK^{6}(**GG**)TLTGK^{11}, m/z = 1379.6765) is close to the m/z 1379.402 value of Rsp5 peptide, which precludes the clear distinction between these peptides. To test if the signal from K6-polyubiquitin linkage is present, tryptic peptides from both reactions were treated with the acetic anhydride (Table S5). The tryptic peptide from Rsp5ΔWW has only one free amine at the N-terminus of the peptide (^{220}QYSSFEDQYGR^{230}), whereas that of K6-linked polyubiquitin chain has two (^{1}MQIFVK^{6}(**GG**)TLTGK^{11}). Therefore, the expected acetylation pattern is different for the two peptides: **Ac**-^{220}QYSSFEDQYGR^{230} (m/z = 1421.424) and **Ac**-^{1}MQIFVK^{6}(**GG**)TLTGK(**Ac**)^{11} (m/z = 1463.8086), which can be distinguished. Indeed, MALDI-TOF analysis of acetylated tryptic peptides of both **(C)** Rsp5ΔWW and **(D)** polyubiquitin chain formed by Rsp5/ByS with Tagless-Ub-MES showed the signal that corresponds to singly acetylated peptide of Rsp5ΔWW. We did not detect the signal for the doubly acetylated K6-linkage Ub peptide, which suggest that Rsp5ΔWW is not likely to form K6-linked polyubiquitin chains.

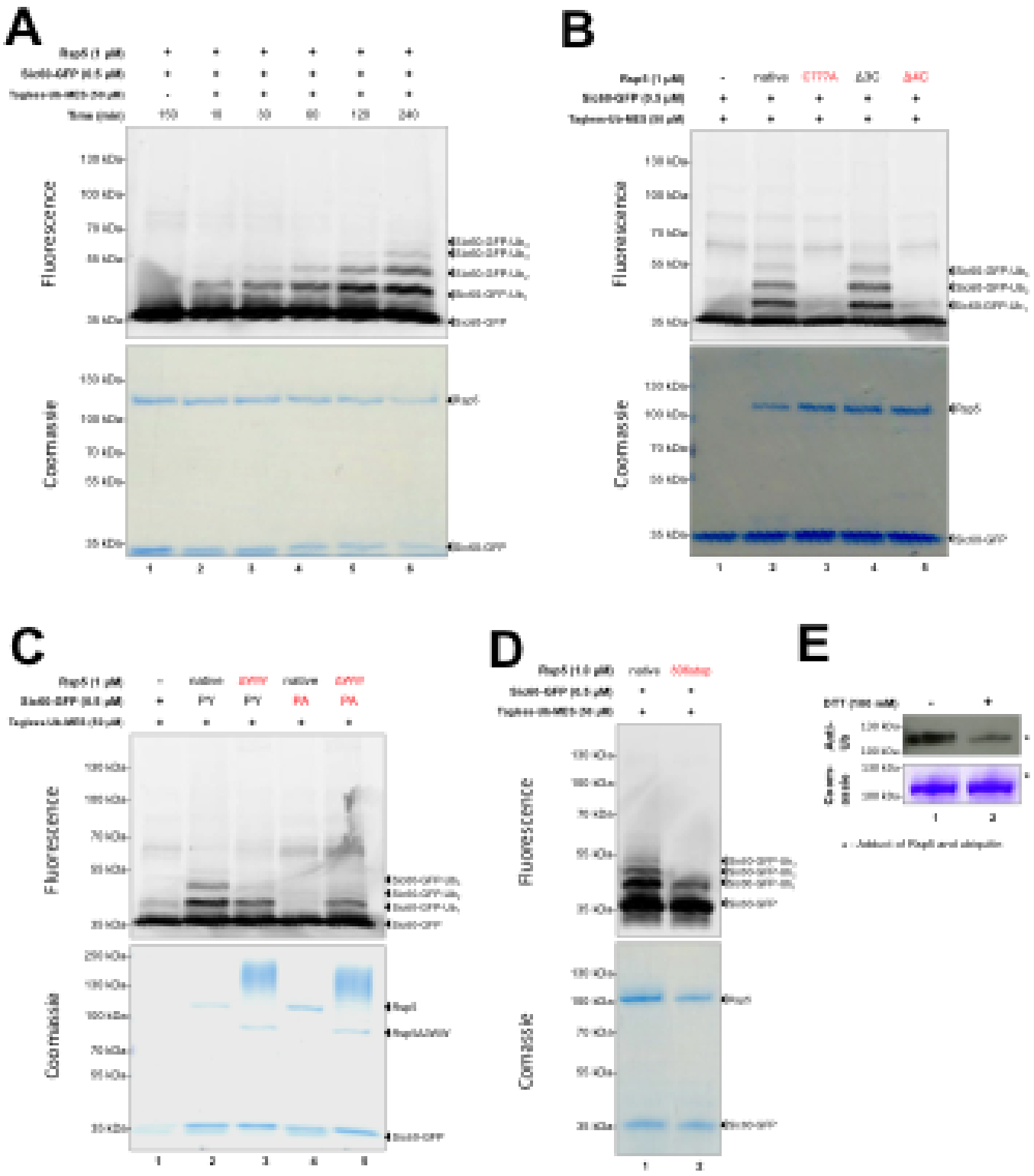


Figure S8. Protein ubiquitination via Rsp5/ByS with Tagless-Ub-MES. Reaction mixtures containing indicated proteins in ubiquitination buffer I were treated with Tagless-Ub-MES to initiate the reaction. Reaction mixtures were incubated at room temperature for indicated times. Reactions were terminated with 6× Laemmli buffer, resolved by 7.5% SDS-PAGE gel and imaged by in-gel fluorescence scanning. **(A)** Time dependence of Sic60-GFP ubiquitination by Rsp5 and Tagless-Ub-MES. The reaction mixtures were incubated at room temperature for indicated times, quenched with 6× reducing Laemmli buffer (NH_2OH, 20 mM final),

resolved by SDS-PAGE (7.5% gel) and imaged by in-gel fluorescence scanning and Coomassie staining. **(B)** Protein ubiquitination via Rsp5/Bys with Tagless-Ub-MES depends on the catalytic cysteine of Rsp5. The ubiquitination of Sic60-GFP by Rsp5 or Rsp5 cysteine to alanine mutants in the presence of Tagless-Ub-MES was analyzed by in-gel fluorescence scanning. Rsp5 mutants without the catalytic cysteine (Cys^{777}) are marked with red color. Reactions were incubated for 4 hours at room temperature, quenched with 6× reducing Laemmli buffer (NH_2OH, 20 mM final), resolved by SDS-PAGE (7.5% gel) and imaged by in-gel fluorescence scanning and Coomassie staining. **(C)** Protein ubiquitination via Rsp5/Bys with Tagless-Ub-MES is dependent on the enzyme-substrate binding interaction. Sic60-GFP and its mutants were incubated with Rsp5 or Rsp5 mutants under ByS reaction conditions for 4h, quenched with 6× reducing Laemmli buffer (NH_2OH, 20 mM final), resolved by SDS-PAGE (7.5% gel) and imaged by in-gel fluorescence scanning and Coomassie staining. Mutants of Rsp5 and substrate, with recognition motif deleted (ΔWW and PA, respectively) are marked with red color. **(D)** Protein ubiquitination via Rsp5/Bys with Tagless-Ub-MES is dependent on the last four amino acids of Rsp5. Ubiquitination of Sic60-GFP via $Rsp5^{806stop}$/ByS is analyzed by in-gel fluorescence scanning and Coomassie staining. The reaction mixture was incubated for 4 hours at room temperature, quenched with 6× reducing Laemmli buffer (NH_2OH, 20 mM final), resolved by SDS-PAGE (7.5% gel) and imaged by in-gel fluorescence scanning and Coomassie staining. **(E)** $Rsp5^{806stop}$ forms a thioester adduct with Tagless-Ub-MES ($Rsp5^{806stop}$~Tagged-Ub). The formation of $Rsp5^{806stop}$~Tagless-Ub was confirmed by Western-blotting with Anti-Ub antibody. The reaction mixtures of $Rsp5^{806stop}$ (1.0 μM) and Tagless-Ub (50 μM) were incubated for 45 minutes at room temperature and quenched with either 6× non-reducing Laemmli buffer or 6× reducing Laemmli buffer without NH_2OH. For this experiment, we did not use NH_2OH to exclude the possibility that NH_2OH could cleave the stable isopeptide adduct between $Rsp5^{806stop}$ and Ub. The presence or absence of NH_2OH, however, did not affect our conclusions. The band corresponding to adduct of $Rsp5^{806stop}$ and Tagless-Ub is marked with "*".

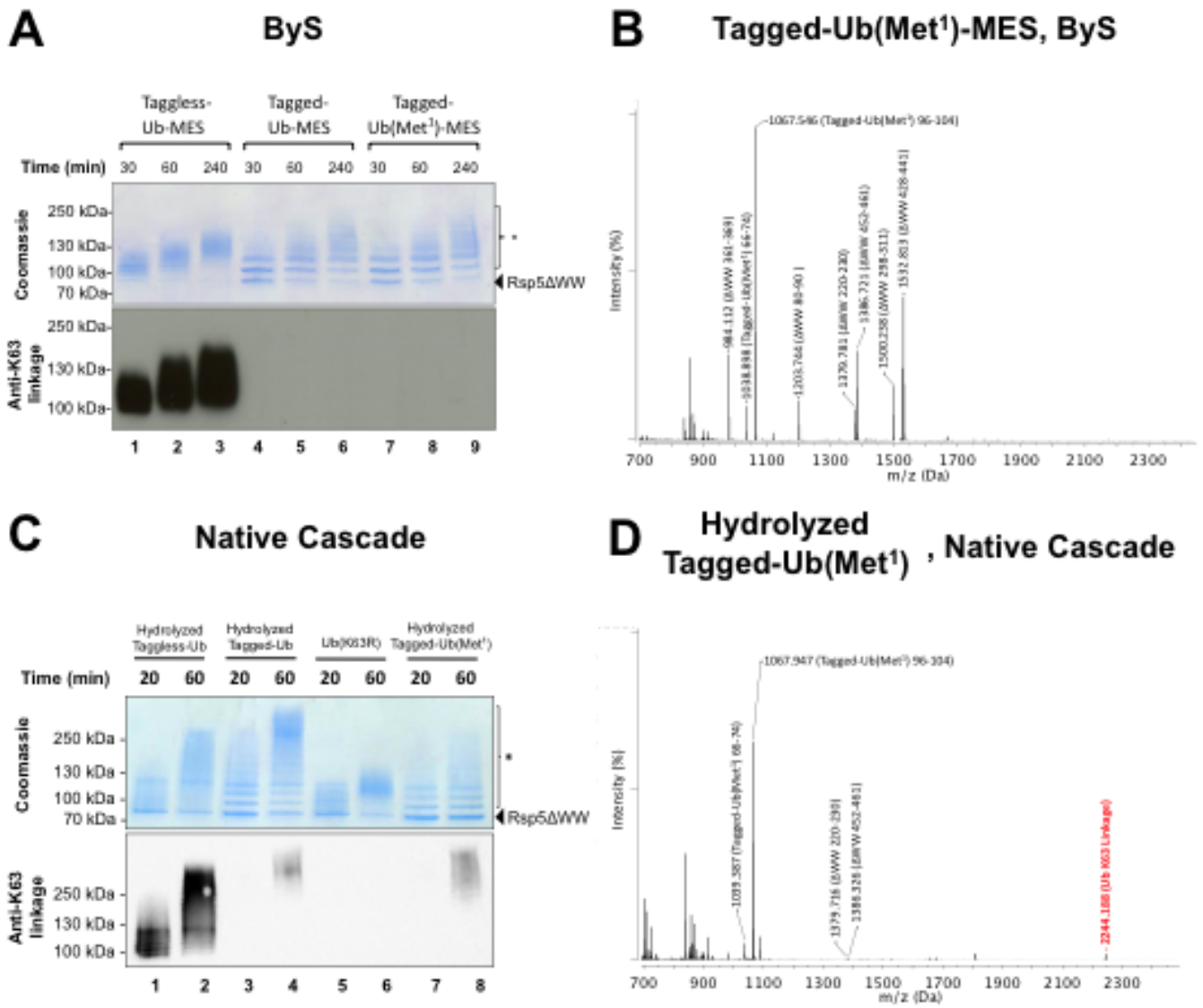


Figure S9. Tagged-Ub(Met^1)-MES forms lower amount of K-63 linked polyubiquitin chains. Polyubiquitinated Rsp5ΔWW was prepared as in Figure 5. **(A)** For ByS reactions, Rsp5ΔWW (1.8 μM) was incubated with 90 μM of each ubiquitin-MES mutant in ubiquitination buffer I. **(B)** 100 – 250 kDa region from lane 9 was excised, in-gel digested by trypsin and analyzed by MALDI-TOF **(C)** For native cascade reactions, the hydrolyzed Ub-MES mutants (100 μM) were mixed with UBE1 (0.09 μM), UbcH5a (1.0 μM), Rsp5 (1.5 μM) and ATP (4mM) at room temperature for indicated times. **(D)** The 100 – 250 kDa region of lane 8 was excised, in-gel digested by trypsin and analyzed by MADLI-TOF. All reactions were incubated for indicated times at room temperature, and quenched by 6× reducing Laemmli buffer containing NH_2OH (20 mM, final). Reactions were resolved by 4-20% SDS-PAGE gel (Biorad), and the amounts of polyubiquitinated proteins were detected with Coomassie staining or Western blotting with anti-K63 linkage antibody.

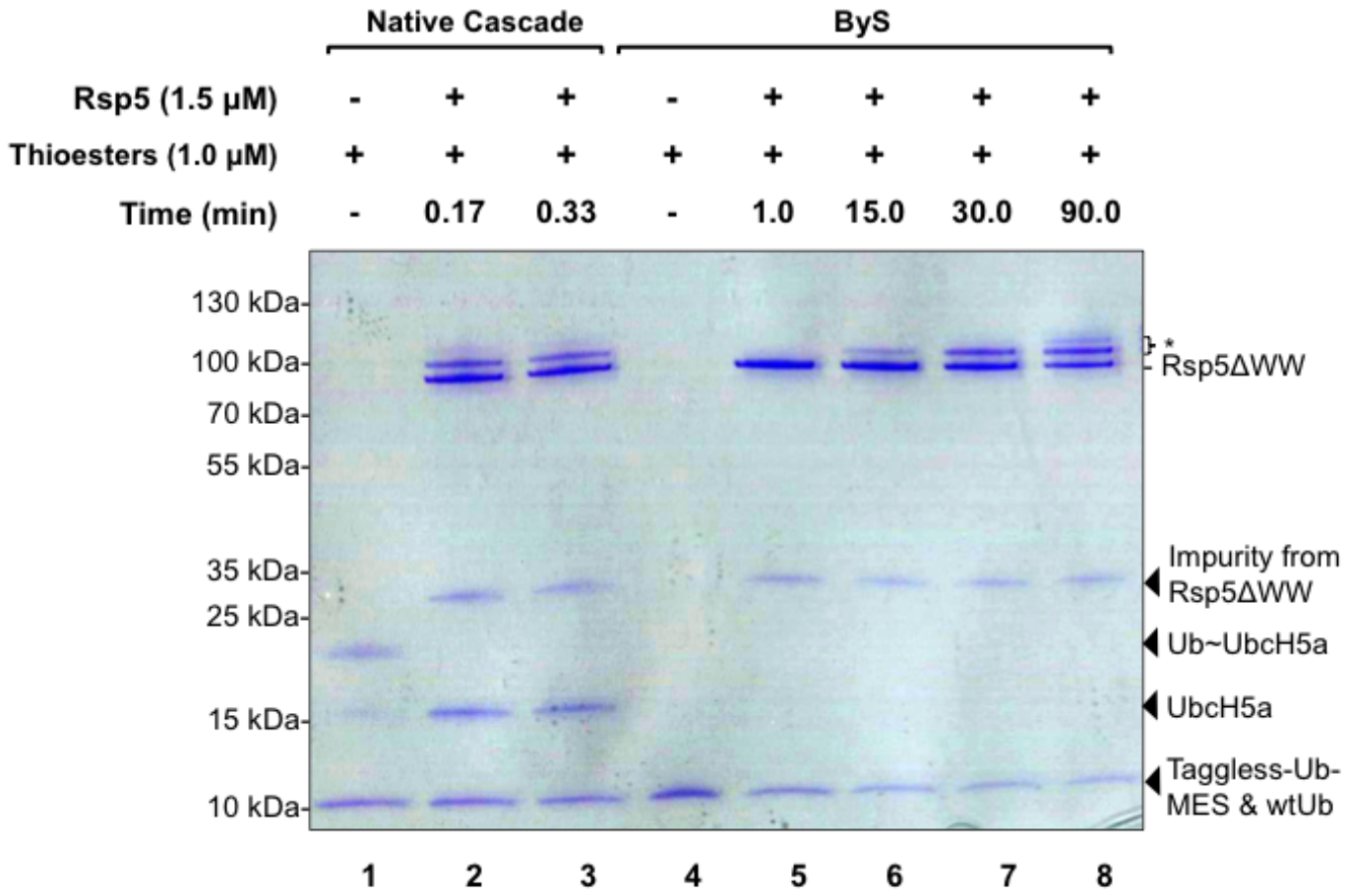


Figure S10. Comparison of the ubiquitination efficiency in ByS and native systems using pulse-chase experiments. Ub~UbcH5a or Tagless-Ub-MES in ubiquitination buffer II were treated with Rsp5ΔWW to initiate ubiquitination reactions, and incubated at room temperature for indicated times, followed by quenching with 6× non-reducing Laemmli loading buffer. Reaction mixtures were resolved by SDS-PAGE (4-20 % gel) and the amount of ubiquitinated proteins as well as unreacted thioesters were visualized with Coomassie staining. Procedure: 10 μL of solution of UbcH5a~Ub (2.0 μM) or Tagless-Ub-MES (2.0 μM) in ubiquitination buffer II was treated with 10 μL of Rsp5ΔWW (3.0 μM) in ubiquitination buffer II to initiate reaction. Bands corresponding to ubiquitinated Rsp5ΔWW are marked with "*". UbcH5a~Ub preparation is described in the methods section.

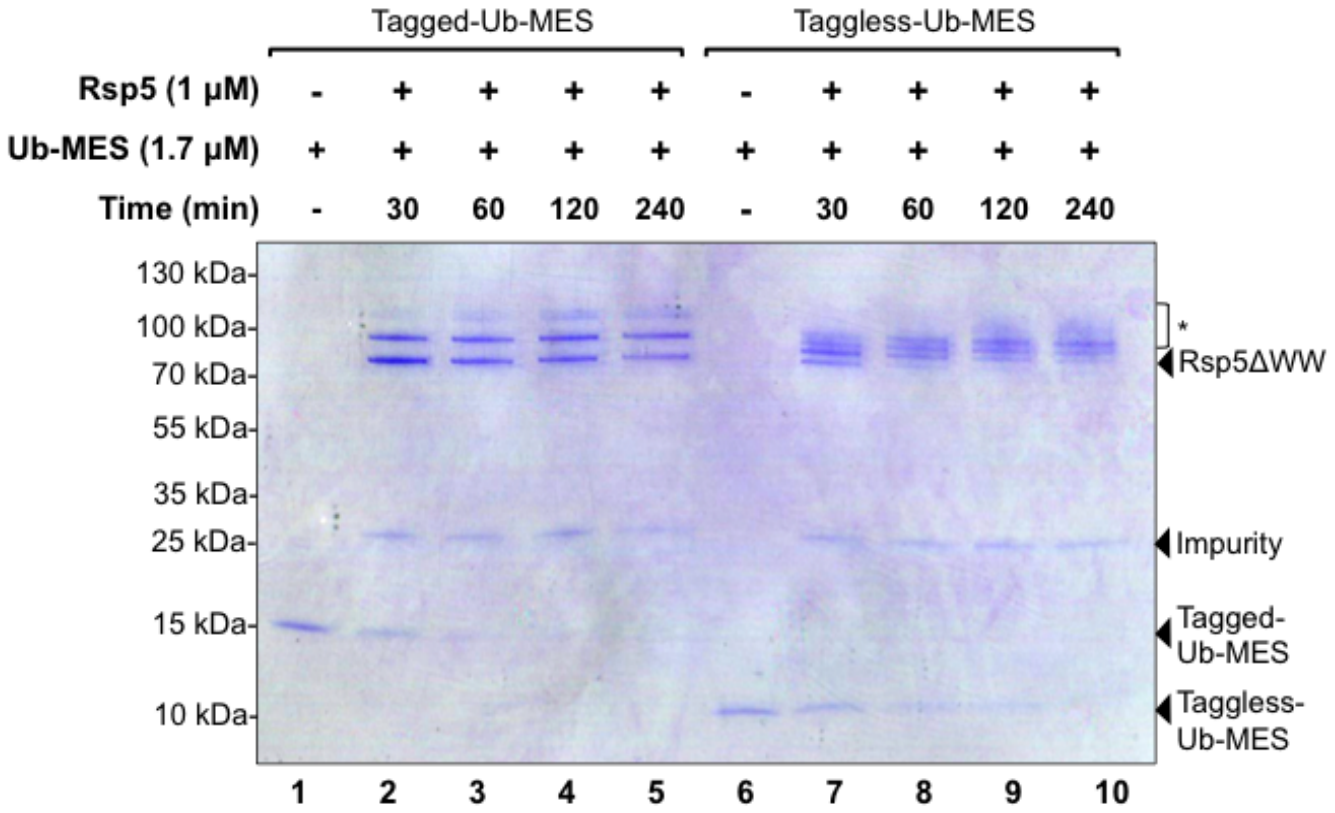


Figure S11. Rsp5 autoubiquitination with Tagged- and Tagless-Ub-MES. The solution of Tagged- and Tagless-Ub-MES was treated with Rsp5ΔWW to initiate the reaction. The reaction mixture was incubated at room temperature for indicated times, quenched with 6× non-reducing Laemmli buffer, resolved by SDS-PAGE (4-20 % gel) and the amount of ubiquitinated proteins along with unreacted thioesters imaged by Coomassie staining. The band corresponding to ubiquitinated Rsp5ΔWW is marked with "*".

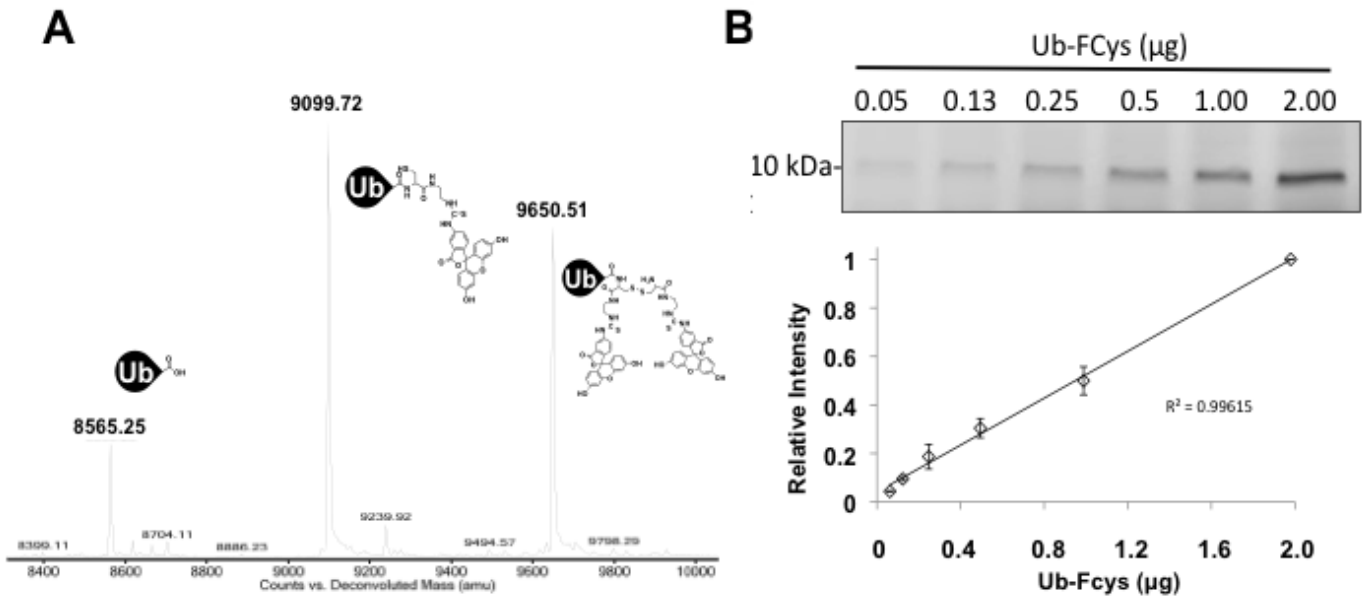


Figure S12. (A) MS analysis of ligation product between Tagless-Ub-MES and FCys. The Tagless-Ub-MES (100 µM) was incubated with FCys Stop Buffer for 16 hours at 37 °C. The reaction was desalted using a Zeba spin column and the eluate was analyzed by ESI-MS. Although we observed the disulfide adduct at m/z 9650.51, the reducing SDS loading buffer contains DTT, which cleaves disulfide bond. (B) The loading amount of Ub-FCys linearly correlates in the range of 50 ng – 2.0 ug. The plot represents mean values ± s.d. for three independent experiments.

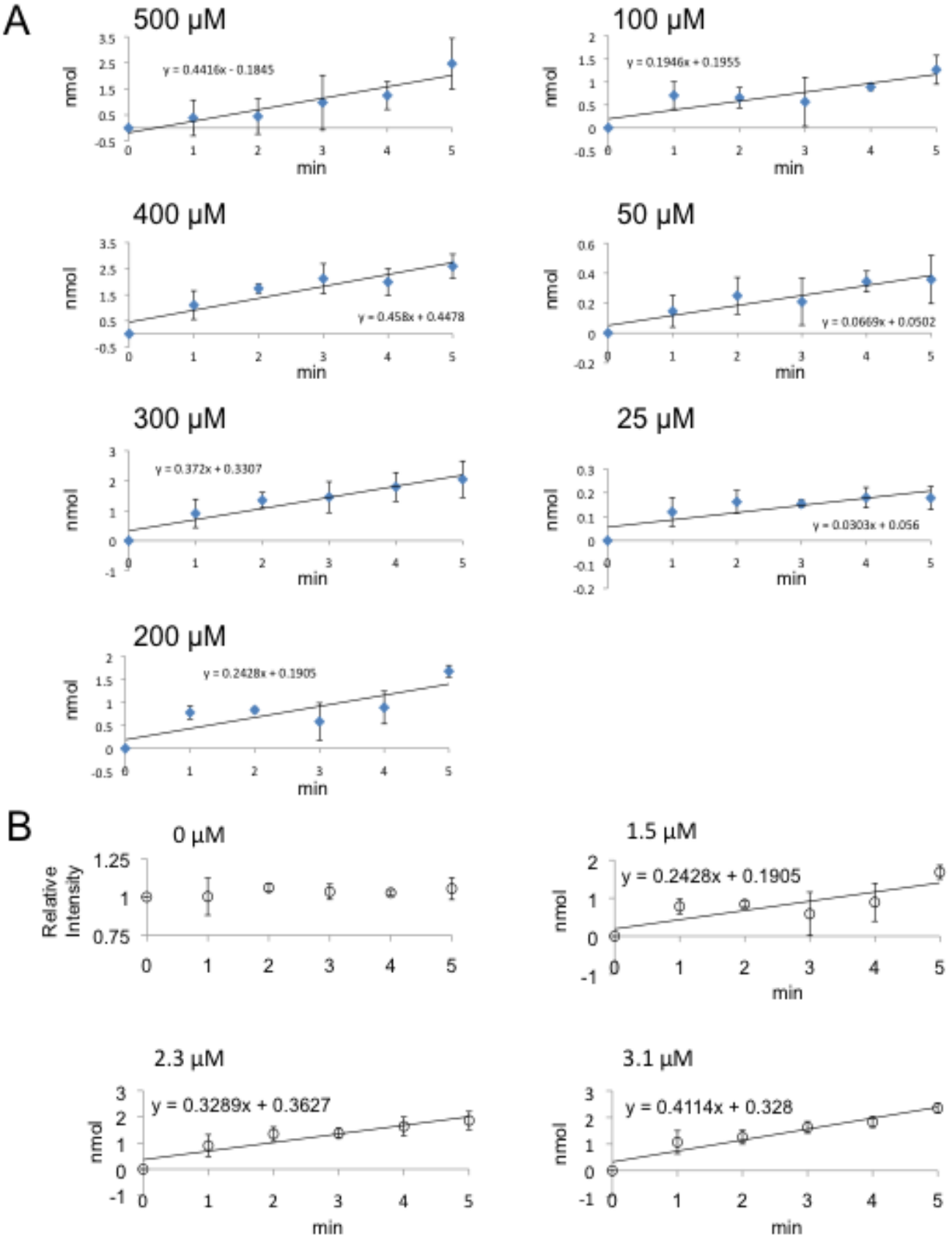

A
500 μM
y = 0.4416x - 0.1845
nmol
min
100 μM
y = 0.1946x + 0.1955
400 μM
y = 0.458x + 0.4478
50 μM
y = 0.0669x + 0.0502
300 μM
y = 0.372x + 0.3307
25 μM
y = 0.0303x + 0.056
200 μM
y = 0.2428x + 0.1905
B
0 μM
Relative Intensity
1.5 μM
y = 0.2428x + 0.1905
2.3 μM
y = 0.3289x + 0.3627
3.1 μM
y = 0.4114x + 0.328

Figure S13. Initial rates of reactions at different concentration of Ub-MES and Rsp5ΔWW. (A) Initial reaction rates (0 to 5 minutes) at different concentrations of Tagless-Ub-MES. Total nmol of Ub-MES consumed by Rsp5 (1.5 μM in 30 μL reaction, 0.045 nmol) was plotted against time. Three sets of experiments were fitted to the Michaelis-Menten equation using Prism 6.0 (Figure 6D). (B) The reaction rate depends on the concentration of Rsp5. The reaction was performed at a fixed concentration of Ub-MES (200 μM). When Rsp5 was not added, the amount of Ub-MES did not change. The plot represents mean values ± s.d. for three independent experiments.

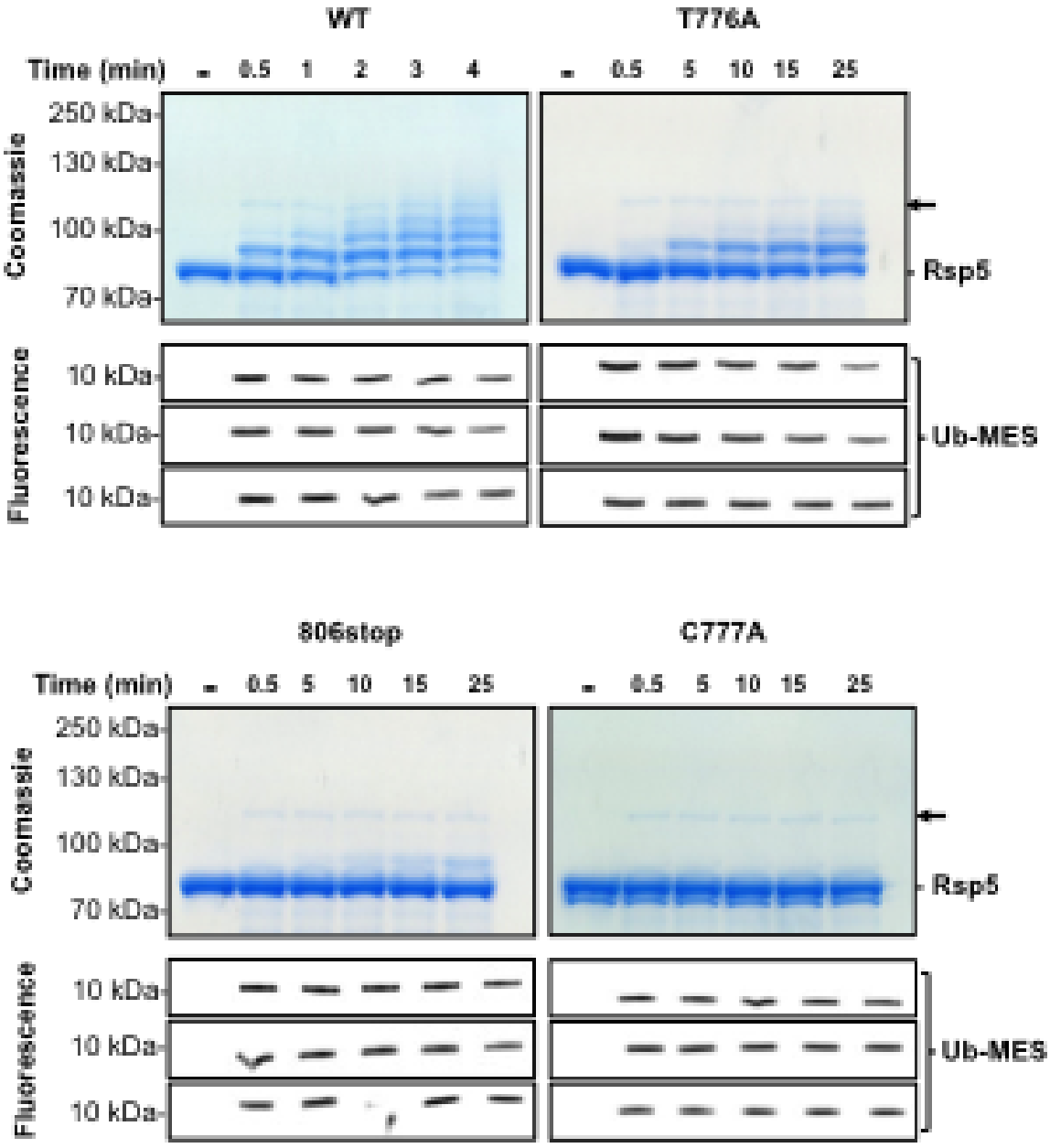


Figure S14. BγS/FCys protocol measures catalytic defects of Rsp5ΔWW mutants. The Rsp5ΔWW mutants as well as wtRsp5ΔWW (5.0 μM) were incubated with Tagless-Ub-MES (250 μM) in 25 mM HEPES pH 7.5, 50 mM NaCl, and 4 mM $MgCl_2$ for the indicated times. The reactions were quenched with reducing Laemmli buffer (coomassie) or FCys Stop Buffer (Fluorescence), resolved by SDS-PAGE and analyzed by coomassie staining or fluorescence scanning as described above. The data of three replicates were used to obtain the initial rates shown in Figures 6E and 6F (mean values ± s.d.). To generate the linear fits of Figure 6E, the relative intensities against the 30 second time point were plotted and fit with a linear equation. We then extrapolated the wtRsp5ΔWW curve to the y-axis to estimate 0 nmol at time = 0. The y-intercepts for fits of all mutant enzymes were then adjusted to match this point at time = 0. The arrow indicates an impurity from Ub-MES.

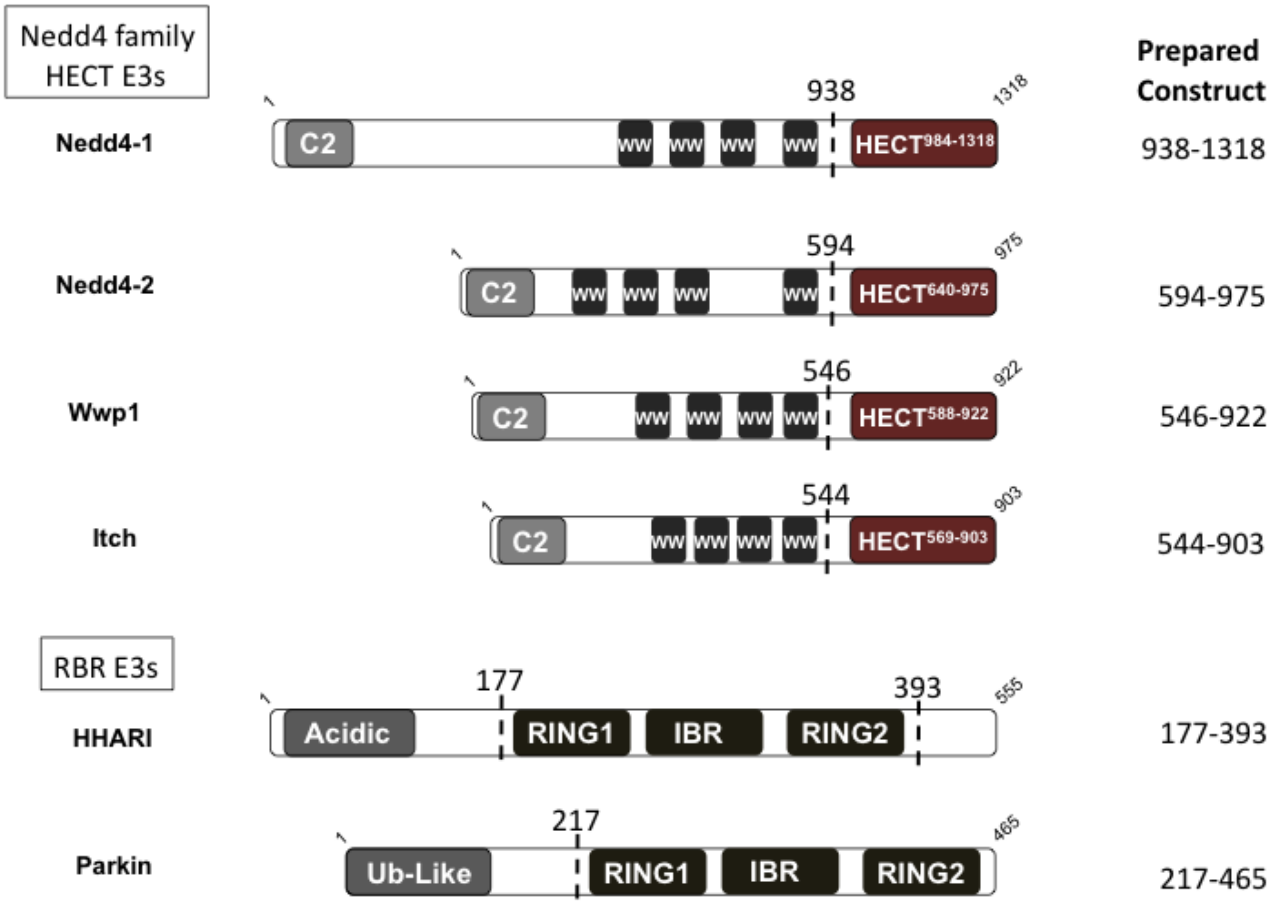


Figure S15. Domain structures of HECT E3s and RBR E3s that were used for enzymatic assays. Schematic view of domain structures of full-length proteins and truncated protein prepared for this research based on Uniprot. Removing the N-terminal auto-regulatory domain of an RBR E3 renders the ligase more active.

Table S1.

(A) Full amino acid sequences for proteins used in this study

Amino acid sequence of hydrolyzed Tagged-Ub-MES (tag is marked with red color)

```
          10        20        30        40        50        60
MYEFDYKDHD GDYKDHDIDY KDDDDKHHHH HHQIFVKTLT GKTITLEVEP SDTIENVKAK
          70        80        90       100       107
IQDKEGIPPD QQRLIFAGKQ LEDGRTLSDY NIQKESTLHL VLRLRGG
```

Amino acid sequence of hydrolyzed Tagged-Ub(Met^1)-MES (Met^1 is inserted after the tag)

```
          10        20        30        40        50        60
MYEFDYKDHD GDYKDHDIDY KDDDDKHHHH HHM1QIFVKTL TGKTITLEVE PSDTIENVKA
          70        80        90       100       108
KIQDKEGIPP DQQRLIFAGK QLEDGRTLSD YNIQKESTLH LVLRLRGG
```

Amino acid sequence of hydrolyzed Tagless-Ub-MES (wtUb)

```
          10        20        30        40        50        60
MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN
          70        76
IQKESTLHLV LRLRGG
```

Amino acid sequence of hydrolyzed Tagless-Ub(K48R)-MES

```
          10        20        30        40        50        60
MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN
          70        76
IQKESTLHLV LRLRGG
```

Amino acid sequence of hydrolyzed Tagless-Ub(K63R)-MES

```
          10        20        30        40        50        60
MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN
          70        76
IQKESTLHLV LRLRGG
```

Amino acid sequence of full length Rsp5 (three WW domains are marked with red color as annotated by UniProt P39940)

```
          10        20        30        40        50        60
MPSSISVKLV AAESLYKRDV FRSPDPFAVL TIDGYQTKST SAAKKTLNPY WNETFKFDDI
          70        80        90       100       110       120
NENSILTIQV FDQKKFKKKD QGFLGVVNVR VGDVLGHLDE DTATSSGRPR EETITRDLKK
         130       140       150       160       170       180
SNDGMAVSGR LIVVLSKLPS SSPHSQAPSG HTASSSTNTS STTRTNGHST SSTRNHSTSH
         190       200       210       220       230       240
PSRGTAQAVE STLQSGTTAA TNTATTSHRS TNSTSSATRQ YSSFEDQYGR LPPGWERRTD
         250       260       270       280       290       300
NFGRTYYVDH NTRTTTWKRP TLDQTEAERG NQLNANTELE RRQHRGRTLP GGSSDNSSVT
```

```
          310        320        330        340        350        360
VQVGGGSNIP PVNGAAAAAF AATGGTTSGL GELPSGWEQR FTPEGRAYFV DHNTRTTTWV
          370        380        390        400        410        420
DPRRQQYIRT YGPTNTTIQQ QPVSQLGPLP SGWEMRLTNT ARVYFVDHNT KTTTWDDPRL
          430        440        450        460        470        480
PSSLDQNVPQ YKRDFRRKVI YFRSQPALRI LPGQCHIKVR RKNIFEDAYQ EIMRQTPEDL
          490        500        510        520        530        540
KKRLMIKFDG EEGLDYGGVS REFFFLLSHE MFNPFYCLFE YSAYDNYTIQ INPNSGINPE
          550        560        570        580        590        600
HLNYFKFIGR VVGLGVFHRR FLDAFFVGAL YKMMLRKKVV LQDMEGVDAE VYNSLNWMLE
          610        620        630        640        650        660
NSIDGVLDLT FSADDERFGE VVTVDLKPDG RNIEVTDGNK KEYVELYTQW RIVDRVQEQF
          670        680        690        700        710        720
KAFMDGFNEL IPEDLVTVFD ERELELLIGG IAEIDIEDWK KHTDYRGYQE SDEVIQWFWK
          730        740        750        760        770        780
CVSEWDNEQR ARLLQFTTGT SRIPVNGFKD LQGSDGPRRF TIEKAGEVQQ LPKSHTCFNR
          790        800        809
VDLPQYVDYD SMKQKLTLAV EETIGFGQE
```

Amino acid sequence of Rsp5ΔWW (amino acids 231-420 of full length Rsp5 were deleted to remove all three WW domains. When we received the plasmid, we noticed that two additional residues were mutated. They are marked with red color. They correspond to N463D and F512S in full length Rsp5. To the best of our knowledge, they are not known to affect the Rsp5 catalytic mechanism.

```
          10         20         30         40         50         60
MPSSISVKLV AAESLYKRDV FRSPDPFAVL TIDGYQTKST SAAKKTLNPY WNETFKFDDI
          70         80         90        100        110        120
NENSILTIQV FDQKKFKKKD QGFLGVVNVR VGDVLGHLDE DTATSSGRPR EETITRDLKK
         130        140        150        160        170        180
SNDGMAVSGR LIVVLSKLPS SSPHSQAPSG HTASSSTNTS STTRTNGHST SSTRNHSTSH
         190        200        210        220        230        240
PSRGTAQAVE STLQSGTTAA TNTATTSHRS TNSTSSATRQ YSSFEDQYGR PSSLDQNVPQ
         250        260        270        280        290        300
YKRDFRRKVI YFRSQPALRI LPGQCHIKVR RKDIFEDAYQ EIMRQTPEDL KKRLMIKFDG
         310        320        330        340        350        360
EEGLDYGGVS REFFFLLSHE MSNPFYCLFE YSAYDNYTIQ INPNSGINPE HLNYFKFIGR
         370        380        390        400        410        420
VVGLGVFHRR FLDAFFVGAL YKMMLRKKVV LQDMEGVDAE VYNSLNWMLE NSIDGVLDLT
         430        440        450        460        470        480
FSADDERFGE VVTVDLKPDG RNIEVTDGNK KEYVELYTQW RIVDRVQEQF KAFMDGFNEL
         490        500        510        520        530        540
IPEDLVTVFD ERELELLIGG IAEIDIEDWK KHTDYRGYQE SDEVIQWFWK CVSEWDNEQR
         550        560        570        580        590        600
ARLLQFTTGT SRIPVNGFKD LQGSDGPRRF TIEKAGEVQQ LPKSHTCFNR VDLPQYVDYD
         610        619
SMKQKLTLAV EETIGFGQE
```

(B) Calculated average MW for tryptic peptides of full length Rsp5

#	Average MW	Position	Peptide Sequence
1	5516.5383	502-546	EFFFLLSHEMFNPFYCLFEYSAYDNYTIQINPNSGINPEHLNYFK
2	4955.4041	288-340	TLPGGSSDNSSVTVQVGGGSNIPPVNGAAAAAFAATGGTTSGLGELPSGWEQR
3	4402.0377	579-617	VVLQDMEGVDAEVYNSLNWMLENSIDGVLDLTFSADDER
4	2986.4727	370-396	TYGPTNTTIQQQPVSQLGPLPSGWEMR

5	2670.2350	138-164	LPSSSPHSQAPSGHTASSSTNTSSTTR
6	2562.2390	184-209	GTAQAVESTLQSGTTAATNTATTSHR
7	2457.1642	662-682	AFMDGFNELIPEDLVTVFDE R
8	2139.0604	57-74	FDDINENSILTIQVFDQK
9	2082.0257	91-110	VGDVLGHLDEDTATSSGRPR
10	2056.0848	683-700	ELELLIGGIAEIDIEDWK
11	1869.9512	642-655	EYVELYTQWRIVDR
12	1828.8671	91-108	VGDVLGHLDEDTATSSGR
13	1814.8384	707-720	GYQESDEVIQWFWK
14	1751.8850	23-38	SPDPFAVLTIDGYQTK
15	1572.7250	781-793	VDLPQYVDYDSMK
16	1531.8123	428-441	FGEVVTVVDLKPDGR
17	1528.7100	463-474	NIFEDAYQEIMR
18	1506.7686	796-809	LTLAVEETIGFGQE
19	1500.6601	488-501	FDGEEGLDYGGVSR
20	1488.7693	420-432	LPSSLDQNVPQYK
21	1412.6845	46-56	TLNPYWNETFK
22	1390.7405	561-572	FLDAFFVGALYK
23	1386.6688	642-651	EYVELYTQWR
24	1379.5862	220-230	QYSSFEDQYGR
25	1358.6659	270-281	GNQLNANTELER
26	1265.5215	721-730	CVSEWDNEQR
27	1203.6480	80-90	DQGFLGVVNVR
28	1168.5381	245-253	TYYVDHNTR
29	1159.5589	260-269	PTLDQTEAER
30	1123.6106	733-742	LLQFTTGTSR
31	1122.5578	403-411	VYFVDHNTK
32	1122.5327	347-355	AYFVDHNTR
33	1106.6092	618-627	FGEVVTVDLK
34	1047.4814	165-174	TNGHSTSSTR
35	1022.4762	175-183	NHSTSHPSR
36	1011.4701	210-219	STNSTSSATR

37	1008.5659	450-458	ILPGQCHIK
38	993.5615	9-17	LVAAESLYK
39	993.4418	121-130	SNDGMAVSGR
40	991.4479	412-419	TTTWDDPR
41	989.4898	632-640	NIEVTDGNK
42	983.5785	551-559	VVGLGVFHR
43	975.4894	356-363	TTTWVDPR
44	969.5363	765-773	AGEVQQLPK
45	944.4432	750-758	DLQGSDGPR
46	864.3781	774-780	SHTCFNR
47	854.4519	231-237	LPPGWER
48	848.4546	1-8	MPSSISVK
49	830.4254	475-481	QTPEDLK
50	778.4094	656-661	VQEQFK
51	774.4508	743-749	IPVNGFK
52	771.5338	131-137	LIVVLSK
53	748.3835	111-116	EETITR
54	709.3264	239-244	TDNFGR
55	707.3835	365-369	QQYIR
56	706.3518	341-346	FTPEGR
57	697.4031	439-443	VIYFR
58	691.3158	702-706	HTDYR
59	675.3784	397-402	LTNTAR
60	671.3835	444-449	SQPALR
61	637.3555	760-764	FTIEK
62	636.3351	254-258	TTTWK
63	564.2987	39-44	STSAAK
64	550.2840	573-576	MMLR
65	536.2827	19-22	DVFR
66	504.3214	484-487	LMIK
67	502.2983	652-655	IVDR

*Calculated with ExPASy PeptideMass

(C) Calculated average MW for tryptic peptides of Rsp5ΔWW

#	Average MW	Position	Peptide Sequence
1	5456.5019	312-356	EFFFLLSHEMSNPFYCLFEYSAYDNYTIQINPNSGINPEHLNYFK
2	4402.0377	389-427	VVLQDMEGVDAEVYNSLNWMLENSIDGVLDLTFSADDER
3	2670.2350	138-164	LPSSSPHSQAPSGHTASSSTNTSSTTR
4	2562.2390	184-209	GTAQAVESTLQSGTTAATNTATTSHR
5	2457.1642	472-492	AFMDGFNELIPEDLVTVFDER
6	2139.0604	57-74	FDDINENSILTIQVFDQK
7	2082.0201	91-110	VGDVLGHLDEDTATSSGRPR
8	2056.0848	493-510	ELELLIGGIAEIDIEDWK
9	1869.9512	452-465	EYVELYTQWRIVDR
10	1828.8671	91-108	VGDVLGHLDEDTATSSGR
11	1814.8384	517-530	GYQESDEVIQWFWK
12	1751.8850	23-38	SPDPFAVLTIDGYQTK
13	1660.937	254-268	SQPALRILPGQCHIK
14	1572.7250	591-603	VDLPQYVDYDSMK
15	1531.8182	428-441	FGEVVTVDLKPDGR
16	1529.6940	273-284	DIFEDAYQEIMR
17	1506.7686	606-619	LTLAVEETIGFGQE
18	1500.6601	298-311	FDGEEGLDYGGVSR
19	1412.6845	46-56	TLNPYWNETFK
20	1390.7405	371-382	FLDAFFVGALYK
21	1386.6688	452-461	EYVELYTQWR
22	1379.5862	220-230	QYSSFEDQYGR
23	1375.6852	231-242	PSSLDQNVPQYK
24	1265.5215	531-540	CVSEWDNEQR
25	1203.6480	80-90	DQGFLGVVNVR
26	1123.6106	543-552	LLQFTTGTSR
27	1106.6092	428-437	FGEVVTVDLK
28	1047.4814	165-174	TNGHSTSSTR
29	1022.4762	175-183	NHSTSHPSR
30	1011.4701	210-219	STNSTSSATR

31	1008.5659	260-268	ILPGQCHIK
32	993.5615	9-17	LVAAESLYK
33	993.4418	121-130	SNDGMAVSGR
34	989.4898	442-450	NIEVTDGNK
35	983.5785	361-369	VVGLGVFHR
36	969.5363	575-583	AGEVQQLPK
37	944.4432	560-568	DLQGSDGPR
38	864.3781	584-590	SHTCFNR
39	848.4546	1-8	MPSSISVK
40	830.4254	285-291	QTPEDLK
41	778.4094	466-471	VQEQFK
42	774.4508	553-559	IPVNGFK
43	771.5338	131-137	LIVVLSK
44	748.3835	111-116	EETITR
45	697.4031	249-253	VIYFR
46	691.3158	512-516	HTDYR
47	671.3835	254-259	SQPALR
48	637.3555	570-574	FTIEK
49	564.2987	39-44	STSAAK
50	550.2840	383-386	MMLR
51	536.2827	19-22	DVFR
52	504.3214	294-297	LMIK
53	502.2983	462-465	IVDR

*Calculated with ExPASy PeptideMass

Table S2. Calculated tryptic peptide and polyubiquitin-linkage peptides of Tagless-Ub-MES and wtUb

(A) Calculated Average MW for tryptic peptides of Tagless-Ub-MES and wtUb

#	Average MW	Peptide Sequence
1	1787.9273	^{12}TITLEVEPSDTIENVK^{27}
2	1668.9126	^{34}EGIPPDQQRLIFAGK^{48}
3	1523.7812	^{30}IQDKEGIPPDQQR^{42}
4	1081.5524	^{55}TLSDYNIQK^{63}

5	1067.6207	^{64}ESTLHLVLR^{72}
6	1039.5167	^{34}EGIPPDQQR^{42}
7	765.4327	^{1}MQIFVK^{6}
8	717.3526	^{49}QLEDGR^{54}
9	648.4079	^{43}LIFAGK^{48}
10	519.3137	^{7}TLTGK^{11}
11	503.2824	^{30}IQDK^{33}

*Calculated with ExPASy PeptideMass

(B) Calculated average MW for polyubiquitin-linkage peptides of Tagless-Ub-MES and wtUb

Linkage	Average MW	Peptide Sequence
K6	1379.6765	^{1}MQIFVK(GG)TLTGK^{11}
K11	2402.6741	^{7}TLTGK(GG)TITLEVEPSDTIENVK^{27}
K27	2101.3325	^{12}TITLEVEPSDTIENVK(GG)AK^{29}
K29	815.9224	^{28}AK(GG)IQDK^{33}
K33	1637.7653	^{30}IQDK(GG)EGIPPDQQR^{42}
K48	1460.6488	^{43}LIFAGK(GG)QLEDGR^{54}
K63	2244.5259	^{55}TLSDYNIQK(GG)ESTLHLVLR^{72}

*Calculated with Scripps protein calculator v3.4

Table S3. Calculated tryptic peptide and polyubiquitin-linkage peptides of Tagless-Ub(K48R)-MES and Ub(K48R).

(A) Calculated MW for tryptic peptides of Tagless-Ub(K48R)-MES and Ub(K48R)

#	Average MW	Peptide Sequence
1	1787.9273	^{12}TITLEVEPSDTIENVK^{27}
2	1081.5524	^{55}TLSDYNIQK^{63}
3	1067.6207	^{64}ESTLHLVLR^{72}
4	1039.5167	^{34}EGIPPDQQR^{42}
5	765.4327	^{1}MQIFVK^{6}
6	717.3526	^{49}QLEDGR^{54}
7	676.4140	^{43}LIFAGR^{48}
8	519.3137	^{7}TLTGK^{11}
9	503.2824	^{30}IQDK^{33}

*Calculated with ExPASy PeptideMass

(B) Calculated MW for polyubiquitin-linkage peptides of Tagless-Ub(K48R)-MES and Ub(K48R)

Linkage	Average MW	Peptide Sequence

K6	1379.6765	^{1}MQIFVK(GG)TLTGK^{11}
K11	2402.6741	^{7}TLTGK(GG)TITLEVEPSDTIENVK^{27}
K27	2101.3325	^{12}TITLEVEPSDTIENVK(GG)AK^{29}
K29	815.9224	^{28}AK(GG)IQDK^{33}
K33	1637.7653	^{30}IQDK(GG)EGIPPDQQR^{42}
K63	2244.5259	^{55}TLSDYNIQK(GG)ESTLHLVLR^{72}

*Calculated with Scripps protein calculator v3.4

Table S4. Calculated tryptic peptide and polyubiquitin-linkage peptides of Tagless-Ub(K63R)-MES and Ub(K63R).

(A) Calculated MW for tryptic peptides of Tagless-Ub(K63R)-MES and Ub(K63R)

#	Average MW	Peptide Sequence
1	1787.9273	^{12}TITLEVEPSDTIENVK^{27}
2	1668.9126	^{34}EGIPPDQQRLIFAGK^{48}
3	1109.5585	^{55}TLSDYNIQR^{63}
4	1067.6207	^{64}ESTLHLVLR^{72}
5	1039.5167	^{34}EGIPPDQQR^{42}
6	765.4327	^{1}MQIFVK^{6}
7	717.3526	^{49}QLEDGR^{54}
8	648.4079	^{43}LIFAGK^{48}
9	519.3137	^{7}TLTGK^{11}
10	503.2824	^{30}IQDK^{33}

*Calculated with ExPASy PeptideMass

(B) Calculated MW for polyubiquitin-linkage peptides of Tagless-Ub(K63R)-MES and Ub(K63R)

Linkage	Average MW	Peptide Sequence
K6	1379.6765	^{1}MQIFVK(GG)TLTGK^{11}
K11	2402.6741	^{7}TLTGK(GG)TITLEVEPSDTIENVK^{27}
K27	2101.3325	^{12}TITLEVEPSDTIENVK(GG)AK^{29}
K29	815.9224	^{28}AK(GG)IQDK^{33}
K33	1637.7653	^{30}IQDK(GG)EGIPPDQQR^{42}
K48	1460.6488	^{43}LIFAGK(GG)QLEDGR^{54}

*Calculated with Scripps protein calculator v3.4

Table S5. Calculated MW for Acetylated peptides.

#	Average MW w/o Acetylation	Average MW w/ Acetylation	Protein	Peptide Sequence
1	983.5785	1026.6235	Rsp5ΔWW	^{361}VVGLGVFHR^{369}

2	1123.6106	1166.6556	Rsp5ΔWW	^{543}TLTGK(GG)TITLEVEPSDTIENVK^{552}
3	1203.6480	1246.693	Rsp5ΔWW	^{80}DQGFLGVVNVR^{90}
4	1379.5862	1422.6312	Rsp5ΔWW	^{220}QYSSFEDQYGR^{230}
5	1386.6688	1429.7138	Rsp5ΔWW	^{452}EYVELYTQWR^{461}
6	1500.6601	1543.7051	Rsp5ΔWW	^{298}FDGEEGLDYGGVSR^{311}
7	1039.5167	1081.5617	Tagless-Ub	^{34}EGIPPDQQR^{42}
8	1067.6207	1109.6657	Tagless-Ub	^{64}ESTLHLVLR^{72}
9	1379.677	1463.809	K6-linkage of Tagless-Ub	**Ac**-^{1}MQIFVK^{6}(**GG**)TLTGK(**Ac**)^{11}

*Calculated with ExPASy PeptideMass

Table S6. Calculated tryptic peptides and polyubiquitin-linkage peptides of Tagged-Ub-MES

(A) Calculated tryptic peptides of Tagged-Ub-MES

#	Average MW	Peptide Sequence
1	2044.9484	^{22}DDDDKHHHHHHQIFVK^{37}
2	1787.9273	^{43}TITLEVEPSDTIENVK^{58}
3	1735.7194	^{8}DHDGDYKDHDIDYK^{21}
4	1668.9126	^{65}EGIPPDQQRLIFAGK^{79}
5	1456.7457	^{27}HHHHHHQIFVK^{37}
6	1081.5524	^{86}TLSDYNIQK^{94}
7	1067.6207	^{95}ESTLHLVLR^{103}
8	1039.5167	^{65}EGIPPDQQR^{73}
9	995.4179	^{1}MYEFDYK^{7}
10	905.3999	^{15}DHDIDYK^{21}
11	849.3373	^{8}DHDGDYK^{14}
12	717.3526	^{80}QLEDGR^{85}
13	648.4079	^{74}LIFAGK^{79}
14	607.2205	^{22}DDDDK^{26}
15	519.3137	^{38}TLTGK^{42}
16	503.2824	^{61}IQDK^{64}

*Calculated with ExPASy PeptideMass

(B) Calculated MW for polyubiquitin-linkage peptides of Tagged-Ub-MES

Linkage	Average MW	Peptide Sequence
K6	2071.3289	^{27}HHHHHHQIFVK(GG)TLTGK^{42}
K11	2402.6741	^{86}TLTGK(GG)TITLEVEPSDTIENVK^{103}
K27	2101.3325	^{43}TITLEVEPSDTIENVK(GG)AK^{60}
K29	815.9224	^{59}AK(GG)IQDK^{64}

K33	1637.7653	^{30}IQDK(GG)EGIPPDQQR^{42}
K48	1460.6488	^{43}LIFAGK(GG)QLEDGR^{54}
K63	2244.5259	^{55}TLSDYNIQK(GG)ESTLHLVLR^{72}
Tag 1	1940.0227	^{1}MYEFDYK(GG)DHDGDYK^{14}
Tag 2	1849.8401	^{8}DHDGDYK(GG)DHDIDYK^{21}
Tag 3	1607.5603	^{15}DHDIDYK(GG)DDDDK^{26}
Tag 4	2159.2620	^{22}DDDDK(GG)HHHHHHQIFVK^{37}

*Calculated with Scripps protein calculator v3.4

Supplementary References

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