Supporting information

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1. General information.

1.1 Materials.

All reagents were obtained directly from commercial sources and used as specified.[1,2,3] 2-Cl-(Trt)-Cl resin (0.69 mmol/g) was purchased from Tianjin Nankai HECHENG S&T Co., Ltd (Tianjin, China). Fmoc-amino acids were purchased from GL Biochem (Shanghai, China). N,N'-diisopropylcarbodiimide (DIC), Ethyl cyanoglyoxylate-2-oxime (Oxyma), Tris (2-carboxyethyl) phosphine (TCEP) and guanidine hydrochloride (Gn-HCl) were purchased from Adamas-beta (Shanghai, China). 5(6)-carboxytetramethylrhodamine (TAMRA) and 2,2'-dithiodipyridine (DPDS) were purchased from J&K Scientific (Beijing). LB Broth Powder, NaCl, Isopropyl-D-thiogalactopyranoside (IPTG), HEPES, Tris-base, Ampicillin, Kanamycin, $CaCl_2$, Ni-NTA resin, Acetic acid glacial (CH_3COOH), MESNA, propargylamine (PA) and NH_2NH_2-HCl were purchased from Energy Chemical. BL21 (DE3) cells were purchased from TransGen Biotech (Beijing, China). Express Plus™ PAGE Gels (4-20% gradient) was purchased from GenScript. $NaNO_2$ was purchased from Beijing Chemical Works Co., Ltd. $HClO_4$ was purchased from Sinopharm Chemical Reagent. Protein marker (26616) was purchased from Thermo Scientific. Acetonitrile (CH_3CN, HPLC grade), trifluoroacetic acid (TFA, HPLC grade), Triisopropylsilane (TIPS), Dichloromethane (DCM), N, N-Dimethylformamide (DMF) and phenylsilane were purchased from J. T. Baker.

1.2 RP-HPLC, FPLC and Mass Spectrometry.

The instrument used for analysis and purification was the SHIMADZU Prominence HPLC, specifically the LC-20AT equipped with a SPD-20A detector. Analytical RP-HPLC was performed using a Welch XB-C18 column (4.6 × 250 mm, 5 μm, 120 Å, flow rate of 1.0 mL/min). For purification, semi-preparative HPLC was utilized with a semi-preparative Welch XB-C18 column (250 × 10 mm, 5 μm, 120 Å, at a flow rate of 4.0 mL/min). Monitoring for analysis and separation was conducted at wavelengths of 214 nm and 254 nm. Analysis and separation were performed using a linear gradient of 20-70% acetonitrile in water over 30 min. The buffers used for RP-HPLC were 0.08% TFA in acetonitrile and 0.1% TFA in water.

FPLC was conducted using an AKTA purifier (GE Healthcare Life Sciences) with a Superdex™ 75 Increase 10/300 GL column. Each injection was monitored at a wavelength of 280 nm. ESI mass spectra were acquired using a LC/MS-2020 system from Shimadzu.

1.3 Molecular biology, SDS-PAGE, immunoblotting and Antibodies

Bacterial cells were grown in LB agar medium (Sigma) or LB medium (Sangon Biotech). For SDS-PAGE, all samples were loaded onto a 4-20% gradient gel in Tris-MES Buffer, electrophoresed at 90 V for 5 min, and then electrophoresed at 120 V for 60 min. All pictures of protein gels were taken on ChemiDocXRS+ (Bio-Rad). For immunoblotting, protein samples were transferred to polyvinylidene difluoride membranes (PVDF, Bio-Rad) at 300 mA for 1.5 hours. Next, a solution of 3% (m/v) BSA in TBS buffer (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) was incubated on PVDF overnight at 4 °C. Then anti-biotin antibody in TBS buffer was incubated on PVDF for 2 h at 35°C. PVDF was washed with TBST (20 mM Tris-HCl, 137 mM NaCl, 0.1% tween-20, pH 7.6), and then HRP (horseradish peroxidase)-conjugated goat anti-rabbit IgG was added and incubated at 35 °C for 2 h. Chemiluminescence solution (ECL, Sangon Biotech) was used for protein detection.

Anti-Biotin antibody (ab1227) and Anti-OTUB1 (ab175200) were purchased from Abcam. Anti-USP15 antibody was purchased from Santa Cruz Biotechnology Inc. Anti-β-Actin antibody was purchased from Cell Signaling Technology (4970). The secondary goat anti rabbit IgG (HRP) antibody (D111018) and the secondary goat anti mouse IgG (HRP) antibody (D110087) were purchased from sangon Biotech.

2. Construction of protein expression plasmids

GGGS-Ub(G76C): GGGS-Ub(G76C) gene was codon optimized and synthesized by Genscript (Nanjing) and cloned into pET22b vector with *Nde1* and *Xho1* as restriction site.

Amino Acid sequence:

GGGSMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSD YNIQKESTLHLVLRLRG**C**

UCHL1: UCHL1 gene was codon optimized and synthesized by Genscript (Nanjing) and cloned into pET-28a vector with *Nde*I and *Xho*I as restriction site.

Amino Acid sequence:

MQLKPMEINPEMLNKVLSRLGVAGQWRFVDVLGLEEESLGSVPAPACALLLLFPLTAQHE
NFRKKQIEELKGQEVSPKVYFMKQTIGNSCGTIGLIHAVANNQDKLGFEDGSVLKQFLSET
EKMSPEDRAKCFEKNEAIQAAHDAVAQEGQCRVDDKVNFHFILFNNVDGHLYELDGRMP
FPVNHGASSEDTLLKDAAKVCREFTEREQGEVRFSAVALCKAA

USP7 catalytic domain: USP7catalytic domain (208-560) gene was codon optimized and synthesized by Genscript (Nanjing) and cloned into pET-28a vector with *Nde*I and *Xho*I as restriction site.

Amino Acid sequence:

KKHTGYVGLKNQGATCYMNSLLQTLFFTNQLRKAVYMMPTEGDDSSKSVPLALQRVFY
ELQHSDKPVGTKKLTKSFGWETLDSFMQHDVQELCRVLLDNVENKMKGTCVEGTIPKLF
RGKMVSYIQCKEVDYRSDRREDYYDIQLSIKGKKNIFESFVDYVAVEQLDGDNKYDAGE
HGLQEAEKGVKFLTLPPVLHLQLMRFMYDPQTDQNIKINDRFEFPEQLPLDEFLQKTDPK
DPANYILHAVLVHSGDNHGGHYVVYLNPKGDGKWCKFDDDVVSRCTKEEAIEHNYGGH
DDDLSVRHCTNAYMLVYIRESKLSEVLQAVTDHDIPQQLVERLQEEKRIEAQKRKERQE

Sortase A: The plasmid of evolved sortase A pentamutant with improved kinetics and activity is available through Addgene.

Amino acid sequence:

MQAKPQIPKDKSKVAGYIEIPDADIKEPVYPGPATREQLNRGVSFAEENESLDD
QNISIAGHTFIDRPNYQFTNLKAAKKGSMVYFKVGNETRKYKMTSIRNVKPTA
VEVLDEQKGKDKQLTLITCDDYNEETGVWETRKIFVATEVKLEHHHHHH

3. General Procedure for Protein Expression and Purification.

3.1 Expression and purification of GGGS-Ub(G76C).

The ubiquitin mutant with three glycines and one serine at its N-terminus (GGGS-Ub(G76C)) was sub-cloned into a pET22b derivative vector. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells. The transformed cells were cultured in LB medium supplemented with 0.1 mg/mL ampicillin at 37°C. When the OD600 reached 0.6-0.8, protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. The culture was further incubated at 37°C for 12 hours. After centrifugation at 4,500 rpm for 5 min, the cell pellet was resuspended in lysis buffer (25 mM Tris-base, 150 mM NaCl, pH 7.5) and lysed by sonication for 60 min in an ice bath. Subsequently, 70% perchloric acid was slowly added to the lysate to a final concentration of 1% (v/v) with vigorous stirring. After centrifugation at 12,000 rpm for 30 min, the precipitate containing unwanted proteins was removed, and the supernatant was dialyzed against distilled water containing 0.1% trifluoroacetic acid (TFA) for 24 h at 4°C using a dialysis membrane with a molecular weight cutoff of 3.5 kDa. The ubiquitin mutant GGGS-Ub(G76C) in the supernatant was concentrated to a final protein concentration of 10 mg/mL and stored at -80°C for subsequent ammonolysis.

3.2 Expression and purification of DUBs and Sortase A.

Deubiquitinating enzymes (DUBs, including UCHL1 and USP7-CD) and Sortase A were subcloned into a pET28a derivative vector. The recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) cells. Transformed cells were cultured in LB medium supplemented with 0.1 mg/mL kanamycin at 37°C. When the optical density at 600 nm (OD600) reached 0.6-0.8, protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. The culture was incubated at 16°C for 12 hours. After centrifugation at 4,500 rpm for 5 min, the cell pellet was resuspended in lysis buffer (UCHL1 and USP7-CD: 25 mM Tris-HCl, 150 mM NaCl, pH 7.5; Sortase A: 50 mM HEPES, 100 mM NaCl, pH 7.0) and lysed by sonication for 60 min in an ice bath. All DUBs and Sortase A were fused with an N-terminal His_6 tags and purified using a Ni-NTA affinity column. The eluted proteins were further purified by fast protein liquid chromatography (FPLC) using a Superdex^{TM} 75 Increase 10/300 GL column.

4. Synthesis of GGGS-Ub-PA

Approximately 30 mg of the ubiquitin mutant GGGS-Ub(G76C) was obtained from 1 L of LB medium. The supernatant containing GGGS-Ub(G76C) (**1**) was concentrated to a protein concentration of 10 mg/mL (3 mL). To convert the C-terminal cysteine to the hydrazide, **1** was treated with a hydrazinolysis reagent (100 mg/mL MESNA, 50 mg/mL NH_2NH_2-HCl, 5 mg/mL

TCEP, pH 7.0, prepared in H_2O under N_2 protection) at 50°C for 72 hours to GGGS-Ub-$NHNH_2$ (**2**).[4,5] Subsequently, **2** was purified by semi-preparative HPLC using a C4 column with a gradient of 20-70% acetonitrile in water over 30 min, and the collected fractions were freeze-dried to obtain a dry powder (isolated yield 38%). The purified **2** was then dissolved in 3 mL of 6 M guanidine hydrochloride (Gn-HCl) buffer (pH 3.0, prepared in distilled water), and 2.36 mg of sodium nitrite ($NaNO_2$, 10 equiv.) was added.[6,7] The reaction mixture was incubated at -15°C for 20 min to convert the peptide hydrazide to the corresponding acyl azide. Next, 21.8 µL of propargylamine (100 equiv.) was added to the reaction mixture, which was stirred at room temperature for 1 hour to obtain GGGS-Ub-PA (**3**). Finally, **3** was purified by semi-preparative HPLC using a C18 column with a gradient of 20-70% acetonitrile in water over 30 min (isolated yield 43%).

5. Supplementary chemically synthesized methods

5.1 General procedure for solid phase peptide synthesis

Rink Amide AM resin (0.1 mmol) was swelled in 10 mL of a DMF/DCM mixture (1:1, v/v) for 30 min. The Fmoc-protected amino acid (10 equiv.) was dissolved in 5 mL of DMF with coupling reagents (Oxyma, 10 equiv.; DIC, 20 equiv.) and pre-activated for 30 seconds. Each coupling reaction was carried out at 75°C for 20 min. The Fmoc protecting group was removed by treatment with 20% piperidine in DMF for 5 min, followed by washing the resin with DMF (3 times), DCM (3 times), and DMF (3 times). After peptide assembly, the resin was washed with DCM and treated with 10 mL of a cleavage cocktail (TFA: H_2O: phenol: thioanisole: 1,2-ethanedithiol = 82.5:5:5:5:2.5, v/v; 2-3 mL per 100 mg of resin) for 3 hours. The TFA was removed under a stream of N_2, and the peptides were precipitated by the addition of ice-cold diethyl ether. The crude peptides were purified by semi-preparative HPLC using a C18 column with a gradient of 20-70% acetonitrile in water over 30 min and analyzed by ESI-MS on a Shimadzu LC/MS-2020 system.

5.2 General steps of cR10-S-S-PYS

Briefly, cR10 was synthesized using a previously reported method. The thiol group of cR10 was activated by reacting with 2 equiv. of 2,2'-dithiodipyridine (DPDS) in 6 M guanidine hydrochloride (Gn-HCl) buffer pH 7.0 at room temperature for 30 min to form cR10-PYS. The reaction progress was monitored by RP-HPLC using a C18 column with a gradient of 20-70% acetonitrile in water over 30 min. The product (**7**) was identified by ESI-MS on a Shimadzu LC/MS-2020 system (isolated yield 63%)

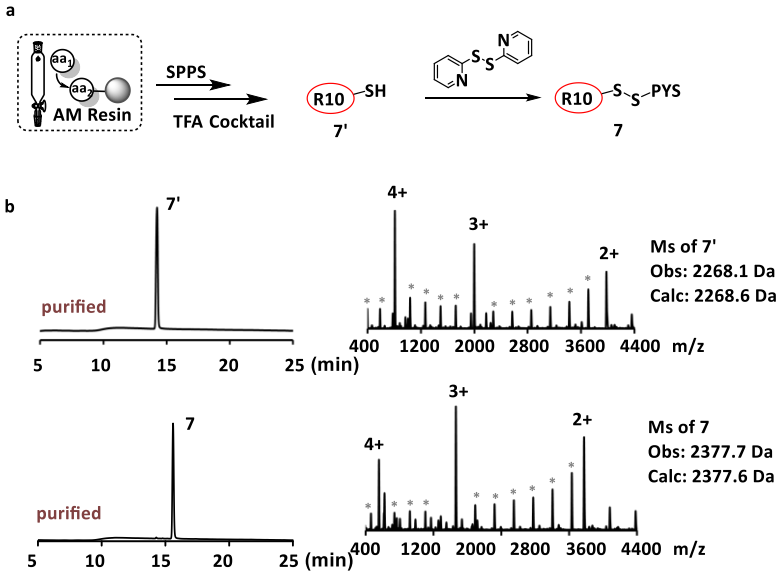

Figure S1. Chemical synthesis of peptide **7** (cR10-S-S-PYS). (a) Synthetic route for **7**. (b) Analytical HPLC chromayogram (214 nm) and ESI-MS analysis of purified peptide **7'**. Analytical HPLC chromayogram (214 nm) and ESI-MS analysis of the purified peptide **7**. '*' represents TFA adducts.

5.3 Sotase-A-Mediated Ligation of GGGS-Ub-PA and TAMRA-$AEEA_2$-Cys-$AEEA_2$-LPETG

GGGS-Ub-PA (**3**) (100 μM) was mixed with TAMRA-AEEA$_2$-Cys-AEEA$_2$-LPETG (**4**) (300 μM) in reaction buffer (50 mM HEPES, 100 mM NaCl, 1 mM $CaCl_2$, pH 7.0). After the addition of Sortase A (2 μM), the reaction mixture was incubated in a 25ºC water bath and allowed to react for 1 hour.[8] The ligation reaction was monitored by RP-HPLC and the products were identified by ESI-MS (isolated yield 12%).

5.4 Sotase-A-Mediated Ligation of GGGS-Ub-PA and Biotin-AEEA$_2$-Cys-AEEA$_2$-LPETG

Like above, GGGS-Ub-PA (**3**) (100 μM) was mixed with Biotin-AEEA$_2$-Cys-AEEA$_2$-LPETG (**5**) (300 μM) in reaction buffer (50 mM HEPES, 100 mM NaCl, 1 mM $CaCl_2$, pH 7.0). After Sortase A (2 μM) was added, the reaction mixture was incubated in a 25ºC water bath and allowed to react for 1 hour. The ligation reaction was monitored by RP-HPLC and the products were identified by ESI-MS (isolated yield 13%).

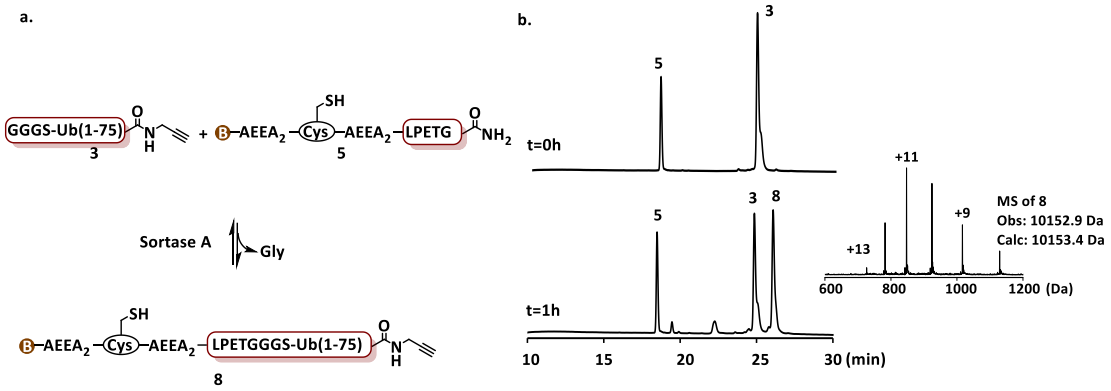

Figure S2. Chemical synthesis of peptide **8** (Biotin-AEEA$_2$-Cys-AEEA$_2$-LPETGGGS-Ub-PA). (a) Synthetic route for peptide **8**. (b) Analytical HPLC chromayogram (214 nm) and ESI-MS analysis of peptide **8**.

5.5 Synthesis of TAMRA-AEEA$_2$-Cys(cR10)-AEEA$_2$-LPETGGGS-Ub-PA (Probe 1)

The TAMRA-AEEA$_2$-Cys-AEEA$_2$-LPETGGGS-Ub-PA (**6**) was obtained by Sortase-A-Mediated ligation. cR10-S-S-PYS (**7**) (2 equiv.) was mixed with **6** in 6 M Gn-HCl buffer (pH 7.0) containing 10% DMSO for 30min at room temperature. The ligation reaction was monitored by RP-HPLC and the product (**Probe 1**) was identified by ESI-MS (isolated yield 51%).

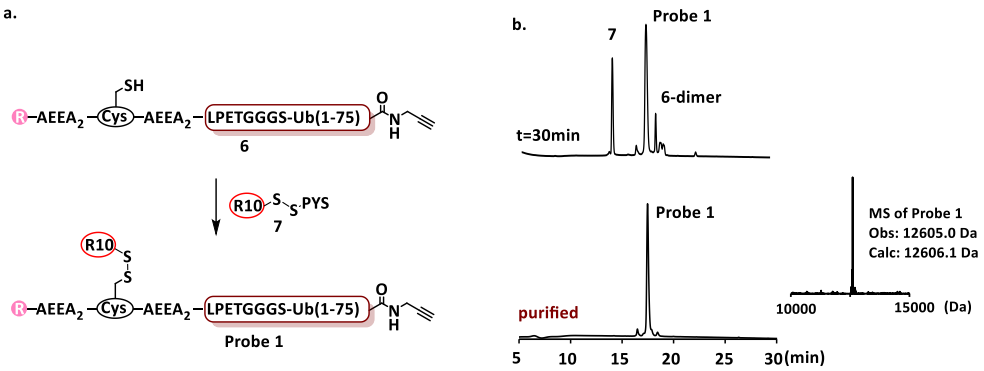

Figure S3. Chemical synthesis of **Probe 1** (TAMRA-AEEA$_2$-Cys(cR10)-AEEA$_2$-LPETGGGS-Ub-PA). (a) Synthetic route for **Probe 1**. (b) Analytical HPLC (214 nm) and ESI-MS analysis of **Probe 1**.

5.6 Synthesis of Biotin-AEEA$_2$-Cys(cR10)-AEEA$_2$-LPETGGGS-Ub-PA (Probe 2)

Like above, the Biotin-AEEA$_2$-Cys-AEEA$_2$-LPETGGGS-Ub-PA (**8**) was obtained by Sortase-A-Mediated ligation. cR10-S-S-PYS (**7**) (2 equiv) was mixed with **8** in 6 M Gn-HCl buffer (pH 7.0) containing 10% DMSO for 30min at temperature. The ligation reaction was monitored by RP-HPLC and the product (**Probe 2**) was identified by ESI-MS (isolated yield 48%).

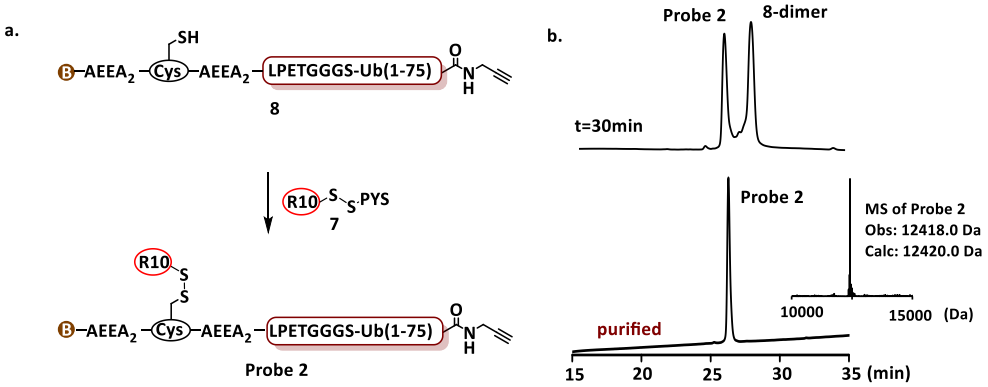

Figure S4. Chemical synthesis of **Probe 2** (Biotin-AEEA$_2$-Cys(cR10)-AEEA$_2$-LPETGGGS-Ub-PA). (a) Synthetic route for **Probe 2**. (b) Analytical HPLC (214 nm) and ESI-MS analysis of **Probe 2**.

6. Probes refolding

In brief, 1 mg of the probe was dissolved in 1 mL of refolding buffer (6 M Gn-HCl, 0.2 M Na_2HPO_4, pH 7.0). This solution was then subjected to buffer exchange into PBS buffer (2 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) four times at 4ºC, with each exchange taking 4 hours and resulting in an 80% yield.

7. MTT assay for evaluation of cell viability

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HeLa cells were seeded into 96-well plates at a density of 5000 cells per well and cultured in 100 μL of DMEM supplemented with 10% fetal bovine serum (FBS) for 24 hours. Each experimental group included five replicate wells. A positive control with 1% phenol and a negative control with untreated cells were established. The cells were exposed to 10 μM **Probe 2** for 1 hour to ensure adequate cellular internalization. Following this, the cells were washed three times with cold PBS and then reincubated in fresh DMEM containing 10% FBS for an additional 24 hours. Subsequently, 20 μL of a 5 mg/mL MTT solution was added to each well. The cells were further incubated for 4 hours to enable the reduction of MTT to formazan crystals. The formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm using a Bio-Rad 680 microplate reader to quantify cell viability.

8. Labeling of DUBs with Probe 1 and Probe 2.

8.1 Labeling of DUBs (UCHL1 and USP7-CD) with Probe 1 and Probe 2.

5 μM DUBs were mixed with 10 μM probes in PBS buffer at 37ºC for 30 min. The reactions were terminated by adding an equal volume of 2× SDS loading buffer and then subjected to SDS-PAGE analysis.

8.2 Labeling of DUBs with Probe 2 in living cells.

Briefly, HeLa cells were incubated with 10 μM of **Probe 2** for 4 hours at 37ºC. Afterward, the probe-containing medium was removed, and the cells were washed with PBS. Subsequently, 0.25% trypsin and 0.1% EDTA were applied to the cells and incubated for about 10s at room temperature. Then trypsin solution was removed, and the cells were incubated at 37ºC for 60 seconds before being collected and rinsed with PBS buffer. Subsequently, the cells were suspended in RIPA Lysis Buffer (with 1mM iodoacetamide) and 2 mM PMSF, incubated on ice for 30 min, and then centrifuged at 11,000 rpm at 4ºC for 10 min to remove cellular debris. The total protein concentration was measured using the BCA Protein Assay from Sangon Biotech. Cell lysates containing 100 μg of protein were then used directly for immunoblotting with antibodies against Biotin, OTUB1, and USP15.

9. Live-cell Imaging Confocal Microscopy.

The experimental protocols followed standard procedures. Briefly, HeLa cells were plated on sterile 35 mm glass-bottom dishes (Biosharp, BS0-15-GJM) and cultured to allow adherence for 24 hours at 37°C and 5% CO_2 in DMEM (Gibco, C11965500BT) supplemented with 10% fetal bovine serum (FBS, Lonsera, S711-001S), 100 units/mL penicillin, and 0.1 mg/mL streptomycin (HyClone, SV30010). The cells were then treated with DMEM containing **Probe 1** for 1 hour at 37°C and 5% CO_2. After incubation, the cells were washed three times with PBS. The cells were visualized using an Argon laser at 543 nm to detect TAMRA (red emission). Imaging was performed using a Plan-Apochromat 60X/1.4 Oil DIC M27 objective on a Zeiss LSM 880 AxioObserver confocal laser scanning microscope equipped with ZEN software. Laser power and pixel resolution were optimized to minimize photobleaching and ensure high-quality image acquisition.

10. Images for Western bloting and SDS-PAGE.

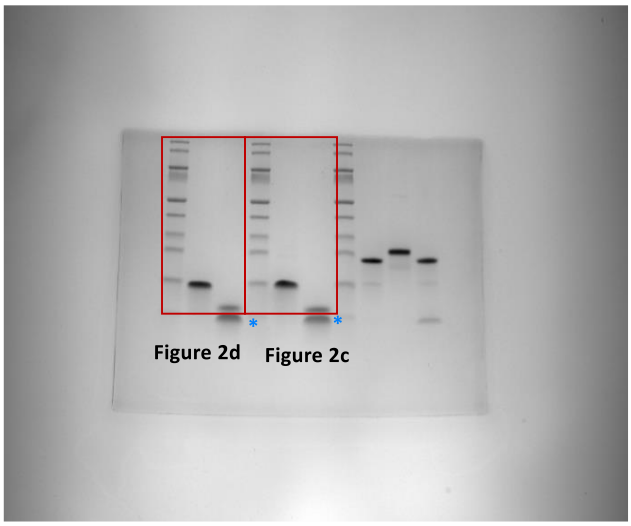

Figure S5. The raw images for electrophoresis data of Figure 2d and Figure 2c. '*' represents the cR10 formed by the breakage of the disulphide bond upon the addition of DTT.

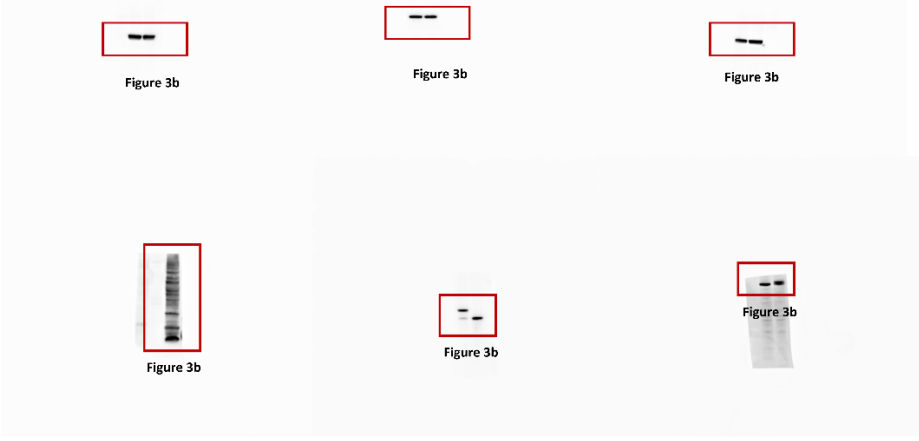

Figure S6. The raw image for Western blot of Figure 3b.

11. References

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