Supporting Information

Synthesis and Delivery of a Stable Phosphorylated Ubiquitin Probe to Study Ubiquitin Conjugation in Mitophagy

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1. Materials

1.1. General reagents

Peptides were prepared by SPPS either manually in Teflon filter fitted syringes (purchased from Torviq) or by using an automated peptide synthesizer (CS336X, CSBIO). Analytical grade *N,N*-dimethylformamide (DMF) was purchased from Mercury. Resins were purchased from Chem-impex, protected amino acids were purchased from GL Biochem and activating reagents N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uroniumhexafluorophosphate (HBTU), 1-Hydroxybenzotriazolemonohydrate (HOBt), O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU)] were purchased from Luxembourg Bio Technologies.(7-Azabenzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate (PyAOP Novabiochem®) was purchased from Mercury. Dithiobisnitropyridine (DTNP) and Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were purchased from sigma. {2[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (PEG), 5-Carboxy-tetramethylrhodamine (TAMRA), Dimethylaminoazobenzene-4'-carboxylic acid (DABCYL) and paraformaldehyde were purchased from Tzamal D-Chem. Additional miscellaneous chemicals were purchased from Sigma Aldrich, Strem Chemicals and Alfa Aesar.

1.2. List of the protected amino acids used in peptides synthesis

Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Phe-OH, Fmoc-His(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Thr(^{t}Bu)-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Glu(O^{t}Bu)-OH, Boc-Cys(Trt)-OH, Fmoc-Nle-OH, Boc-Ser(^{t}Bu)-OH, Fmoc-Val-Thr(ψMe,MePro)-OH, Fmoc-Ile-Thr(ψMe,MePro)-OH, Fmoc-Glu(^{t}Bu)-Thr(ψMe,MePro)-OH, Fmoc-Ile-Ser(ψMe,MePro)-OH, Fmoc-Asp(O^{t}Bu)-(DMB)Gly-OH.

1.3. HPLC for peptide analysis and purification

Analytical HPLC was performed on a Thermo instrument (Dionex Ultimate 3000) using analytical column XSelect (Waters, CSH C18, 3.5 µm, 4.6 × 150 mm) at a flow rate of 1.2 mL/min and preparative HPLC was performed on a Thermo instrument (Dionex Ultimate 3000) using preparative column XSelect (Waters, C18, 10 µm, 250 × 19 mm) at a flow rate of 15 mL/min. Semi-preparative HPLC was performed on a Thermo instrument (Dionex Ultimate 3000) using Phenomenex Jupiter C18 10 µm, 300 Å, 250 × 10 mm column, at a flow rate of 4 mL/min. All synthetic products were purified by HPLC and characterized by mass spectrometry using LCQ Fleet Ion Trap (Thermo Scientific). All calculated masses have been reported as an average isotope composition. Buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in acetonitrile.

1.4. Fmoc-SPPS general procedure

2-Chlorotritylchloride (CTC) resin (0.7 mmol/g) was loaded with 0.6 eq. of Fmoc-Gly-OH and 2 eq. of DIEA in dry DCM for 1h. Following resin esterification, the Fmoc-Gly-resin was washed with DCM, methanol, and DMF. The final loading was verified by Fmoc group absorbance after the DBU treatment to be 0.42 mmol/g. After initial resin loading, Fmoc-SPPS was carried on automated peptide synthesizer (CS336X, CSBIO) in presence of 4 eq. of amino acid, 4 eq. of HCTU, and 8 eq. of DIEA to the initial loading of the resin for 50 min. Dipeptides were coupled manually using 2.5 eq. of amino acid, 2.5 eq. of HATU, and 5 eq. of DIEA to the initial loading of the resin for 1 h 30 min. The stable phosphoserine mimetic Fmoc-SPS-OH was coupled manually using 1.5 eq. of amino acid, 1.5 eq. of HATU, and 3 eq. of DIEA to the initial loading of the resin for 2 h. To cleave the peptides from the solid support, the resin was washed with DMF, MeOH, DCM (X5), and dried under high vacuum. A cocktail of TFA: triisopropylsilane (TIS): water (95:2.5:2.5) (for SPS containing peptides TFA: Ethanedithiol (EDT): water (95:2.5:2.5)) was added to resin and the reaction mixture was shaken for 2h at RT. The resin was filtered, and the combined filtrate was added dropwise to a 10-fold volume of cold ether and centrifuged. The precipitate was dissolved in acetonitrile-water and lyophilized to give the crude peptide. The Alloc group deprotection was carried out by using $Pd(PPh_3)_4$ (0.2 eq.) and phenylsilane (20 eq.) in dry DCM for 1 h.

1.5. List of cell culture reagents

U2OS (HTB-96™) were purchased from ATCC®. Dulbecco's modified eagle's medium low glucose (DMEM-LG), phenol free DMEM (Opti-DMEM), Fetal bovine serum (FBS), L-Glu, antibiotics (penicillin/streptomycin), trypsin/EDTA and phosphate buffered saline (PBS) were purchased from biological industries. Hoechst 33342 solution (20 mM), Mitotracker green (L7526), deep red (M22426) and Lysotracker blue (L7525) probes were purchased from Thermo-fisher. 8 well µ-Slide for live cell and 8 well chambered removable immunofluorescence slides for confocal microscopy were purchased from ibidi, 2-((3-chlorophenyl)hydrazono)malononitrile (CCCP) and poly-L-lysine hydro bromide was purchased from sigma.

1.6. List of antibodies for western blotting and immunofluorescence

TOM20 (sc-17764) and α-tubulin (sc-8035) antibodies were obtained from Santacruz Biotechnology. HSP60 (#12165) and Parkin (#4211, #2132) antibodies were purchased from Cell Signaling. Rabbit polyclonal anti β-actin was purchased from Abcam (ab8227). Secondary goat anti rabbit IgG (HRP) antibody were purchased from Abcam. The Fluorochrome conjugated secondary antibodies were obtained from Molecular probes (Invitrogen).

Primary antibodies were kept diluted in Tris stabilizing solution (Candor Bioscience).

2. General procedures

2.1. General cell culture procedure

U2OS cells were cultured in DMEM-LG supplemented with 10% FBS, 0.2 mM L-Glu and antibiotics (penicillin/streptomycin) in a humidified 37°C incubator at 5% CO_2. To detach cells from culture flasks, the media was aspirated and the flask was washed with sterile calcium and magnesium free PBS before cells were treated with 0.25% Trypsin+0.02% EDTA solution and returned to incubation chamber for 5 min. Trypsin was quenched by adding the FBS supplemented media. The cell suspension was collected and the cells were pelleted (2 min at 1,000xg). Media was then aspirated and the cell pellet was re-suspended in fresh media. The cell density was determined using a Countess II automated cells counter. For confocal microscopy, cells were seeded on poly-L-lysine (PLL) treated 8 well chamber slides (Ibidi) with removable silicone chamber in $3 \times 10^4 \frac{cells}{well}$ and were allowed to reach ~90% confluence (24h).

2.2. General procedure for establishing stable parkin over expression

The retroviral plasmid pBMN-Parkin for overexpression of untagged human Parkin was a gift from Michael Lazarou (Addgene plasmid repository #89299). For generation of retroviruses carrying the Parkin gene, the viral packaging HEK293FT cells were transfected with the pBMN-Parkin plasmid, pUMVC-gag-pol plasmid and pMD.G-VSVG plasmid in a ratio of 3:2:1. The media supernatant was collected 48 and 72 hours after transfection. The media was centrifuged to remove cell debris, filtered through 0.25 µm filter and concentrated through 100 Kda cut-off centricon tubes (Milipore) to obtain viral particles, which were then snap frozen and

stored at -80°C. The retroviral particles thus generated were used to infect wild type U2OS cells in presence of 10 µg/ml Polybrene for 16 hours. Next day, the cells were replenished with fresh media. Parkin expression was assessed by western blotting and by immunofluorescence.

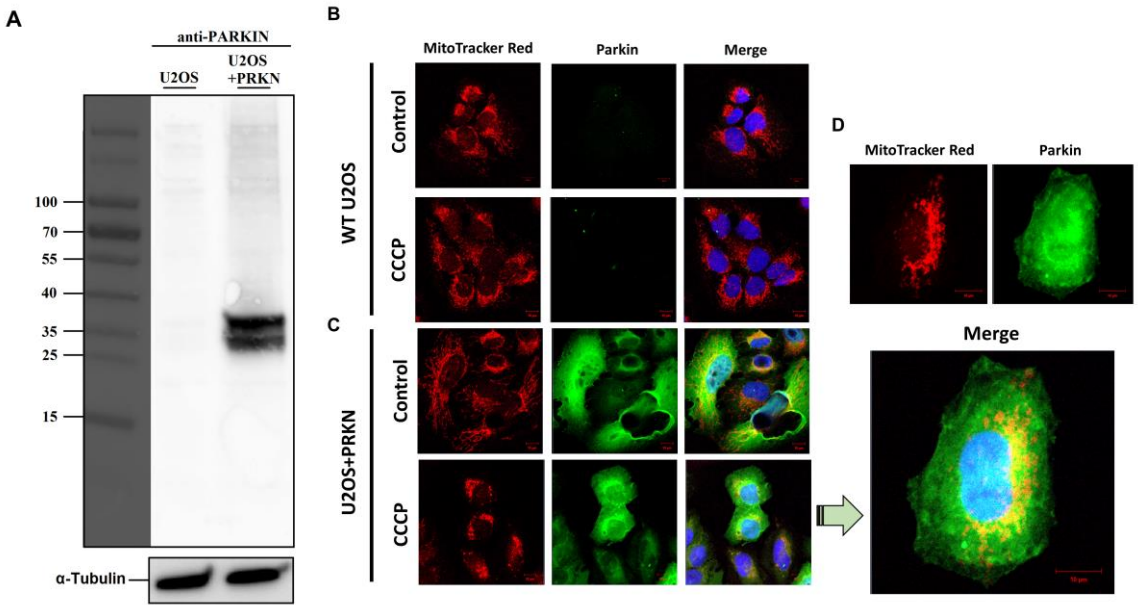


Figure S1. Evaluation of parkin expression in U2OS cells. (A) Whole cell lysates from WT and Parkin overexpressing U2OS cells (U2OS+PRK) was subjected to western blot for the detection of Parkin and α-tubulin as a loading control (B) U2OS (wt) cells with mitotracker red (red) were treated with 0.1% DMSO or CCCP 20 µM for 4 hours, fixed and immunostained against human parkin (green) and DAPI (blue). Scale bars 10 µm. (D) +PRK U2OS cells with mitotracker red (red) were treated with DMSO or CCCP 20 µM for 4 hours, fixed and immunostained against human parkin (green) and DAPI (blue). Scale bars 10 µm. (E) Magnification of a representative CCCP treated cell from D. Scale bars 10 µm.

2.3. General procedure for peptide delivery followed by induction of mitophagy

Cells were cultured in 8 well ibdi removable chamber slides to 90% confluence. The protein constructs were diluted in PBS (from concentrated 1000X DMSO stocks). The protein concentration was determined by Bradford assay and the concentrations were adjusted to a final concentration of 5 µM. The peptide in PBS was then supplemented with DMEM-LG 10X (without serum) to a final concentration of 1X. For protein delivery, the cells were washed X1 with warm PBS followed by addition of 0.3 ml of medium containing Ub analogues with cR10D in 5 µM concentration. The cells were kept with CPP constructs in serum free medium for 1 h and then washed once with PBS containing heparin sulfate to remove excess CPP.[1] Full culture medium with or without 20 µM CCCP was added to the cells followed by incubation for the indicated time to ensure incorporation of synthetic Ub's into PolyUb chains on damaged mitochondria. After completion, the cells were either imaged as live cells (after exchanging the medium to opti-DMEM with 1 µg/ml of Hoechst) or fixed for immunostaining.

2.4. General procedure for cell fixation and immunostaining

After the indicated treatments, the cells in ibdi 8 well chamber slides were gently washed twice with warm PBS followed by incubation with warm 4% Paraformaldehyde (w/v in PBS) for 20 minutes in dark at room temperature (RT). The cells were again washed thrice with PBS and then permeabilized with 1% Triton X-100 for 5 minutes in dark at RT. Blocking was done with 5% BSA (w/v in PBS) for 1 h in dark at RT. The cells then were incubated with specific primary

antibodies at manufacturer recommended dilutions for either 1 h at RT or overnight at 4°C, followed by 3 washes with PBST (PBS with 0.02% Tween-20) and then incubated with appropriate fluorophore-conjugated secondary antibodies (Molecular probes, Invitrogen) for 1 h at RT in dark. The cells were again washed thrice with PBST. Following the completion of treatments, the silicone chamber was removed and the slides were mounted with fluorescence anti-quenching agent Fluoromount G (Invitrogen) either with or without DAPI and sealed with CoverGrip (Invetrogen). The slides were stored at -20°C until acquisition.

2.5. General method for confocal imaging and image analysis

Distribution of fluorescent peptides in live cells was analyzed using a confocal laser scanning microscope (Confocal Zeiss LSM 710) equipped with X40 NA 1.2 water immersion objective for live cell imaging and a X63 Plan-Apochromat NA 1.4 oil immersion lens for fixed samples. All channels were adjusted to 0.6 AU pinhole settings with 1.5 µs pixel dwell. Four lasers were used for the different tags; UV laser (Hoechst, DAPI, Lysotracker blue, AF405) – 405 nm (10 mW), Argon multi-line laser (Mitotracker green, Lysotracker green, AF488) – 454, 488 and 514 nm (30 mW), Green laser (TAMRA) – 543 nm (10 mW) – Red laser (Cy5, AF637, AF647) – 639 nm (5 mW). During live cell CLSM analysis the samples were maintained at 37 °C in a humidified chamber. Fixed cells were imaged at room temperature.

Image analysis of Z-stacked images was performed using Imaris software. Fluorescence quantification was performed on all images collected (> 100 cells per experimental condition, ~10 images per condition) using FiJi software.

Relative fluorescence intensities for live cell delivery were determined by first identifying individual nuclei by applying a nuclear dye marking algorithm based on Hoechst staining followed by calculating the average intensity under the nuclear mask.

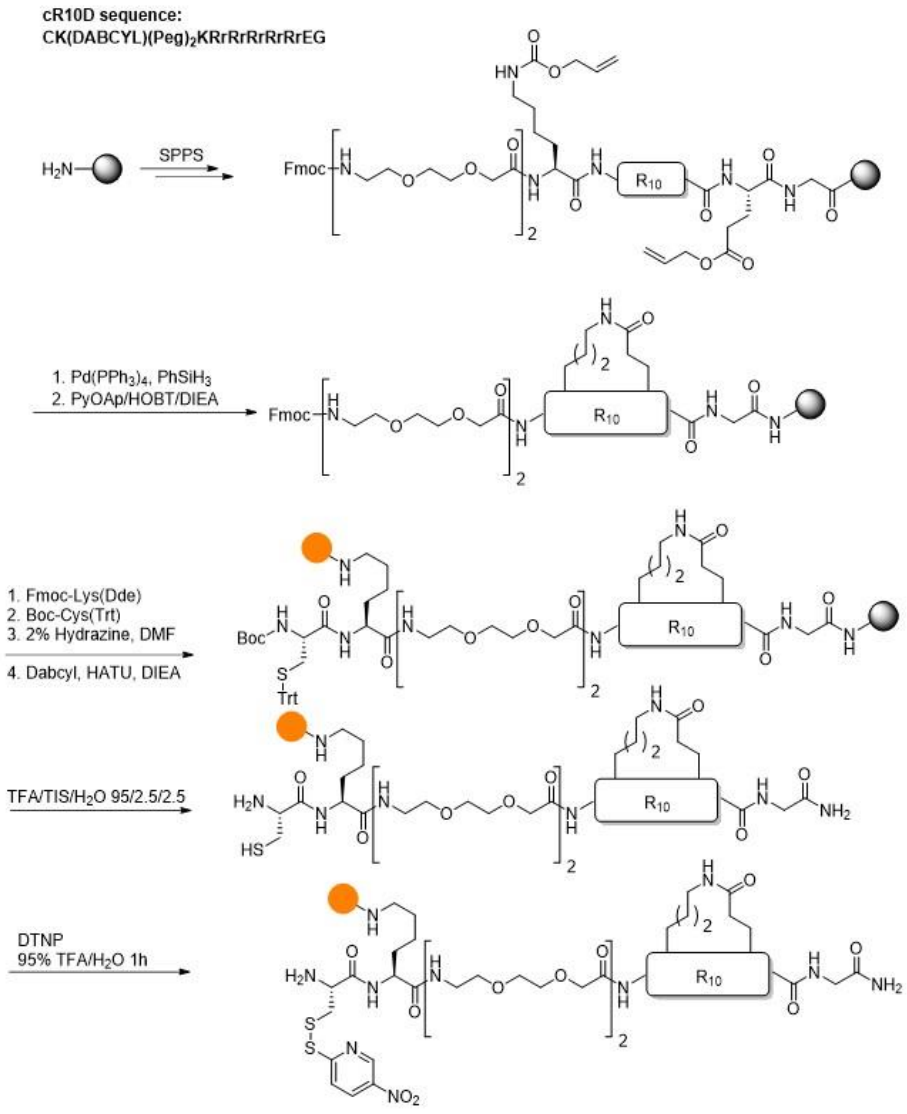
Co-localization analysis was performed on all images collected (> 100 cells per experimental condition in each independent experiment) using the FiJi coloc plug-in with Otsu dark auto threshold under the relevant masks (TAMRA fluorescence was not used for masking as it can be affected by differences in peptide delivery). The resulting Pearson's coefficients for each of the channel pairs in each treatment are displayed as average of averages from three independent experiments (unless stated otherwise) with error bars representing the standard deviation.

P values for averages of averaged experiments were directly calculated using an unpaired t-test based on the standard deviation of averaged values from each experiment by GraphPad Prism 8 software.

3. Synthesis of ubiquitin analogues

3.1. Synthesis of TNP-cR10D

The synthesis was carried out according to Scheme S1:



Scheme S1: Synthesis of TNP-cR10D.

The cR10D peptide was prepared as previously reported.[2] All amino acids were manually double coupled on pre-swollen rink amide resin (0.2 mmol) using

standard Fmoc-SPPS. After coupling of two PEG units (without removing the Fmoc group), Alloc and OAllyl protecting groups were simultaneously removed and cyclization was performed on the solid support using 5 eq. of PyAOp, 5 eq. HOBT and 10 eq. of DIEA in DMF (12 ml for 0.2 mmol) for 2h. After completion of the sequence, the Dde protecting group was removed by three cycles of 2% hydrazine hydrate in DMF for 30 min each cycle. Then, DABCYL was coupled using 4 eq. of DABCYL carboxylic acid, 4 eq. HATU and 8 eq. DIEA for 2 h. The peptide was then cleaved as mentioned in the general procedure and purified by preparative HPLC using C18 column with a gradient of 0-60% buffer B over 30 min (15ml/min flow) to afford the corresponding cR10D peptide in ~18 % yield. Final product cR10D is with the observed mass 2648.4 ± 0.4 Da, calcd 2648 Da (average isotopes) as reported. Next, the pure cR10D peptide was dissolved with 10 eq. of DTNP in 95% TFA 5% H_2O and stirred for 1 h at 37^{o}C followed by confirmation of TNP activation by HPLC analysis via a strong increase in 330 nm absorbance of the peptide peak. The product was then precipitated by addition of cold diethyl ether and centrifugation followed by washing (X2) of the peptide pellet with cold DCM to remove excess DTNP. The remaining peptide was dissolved in H_2O with 0.01% TFA, lyophilized resulting in ~95% yield of TNP-cR10D that was used for the next stages without further purification.

3.2. Synthesis of TAMRA-Ub-COOH, (1)

The synthesis was carried out according to Figure S2:

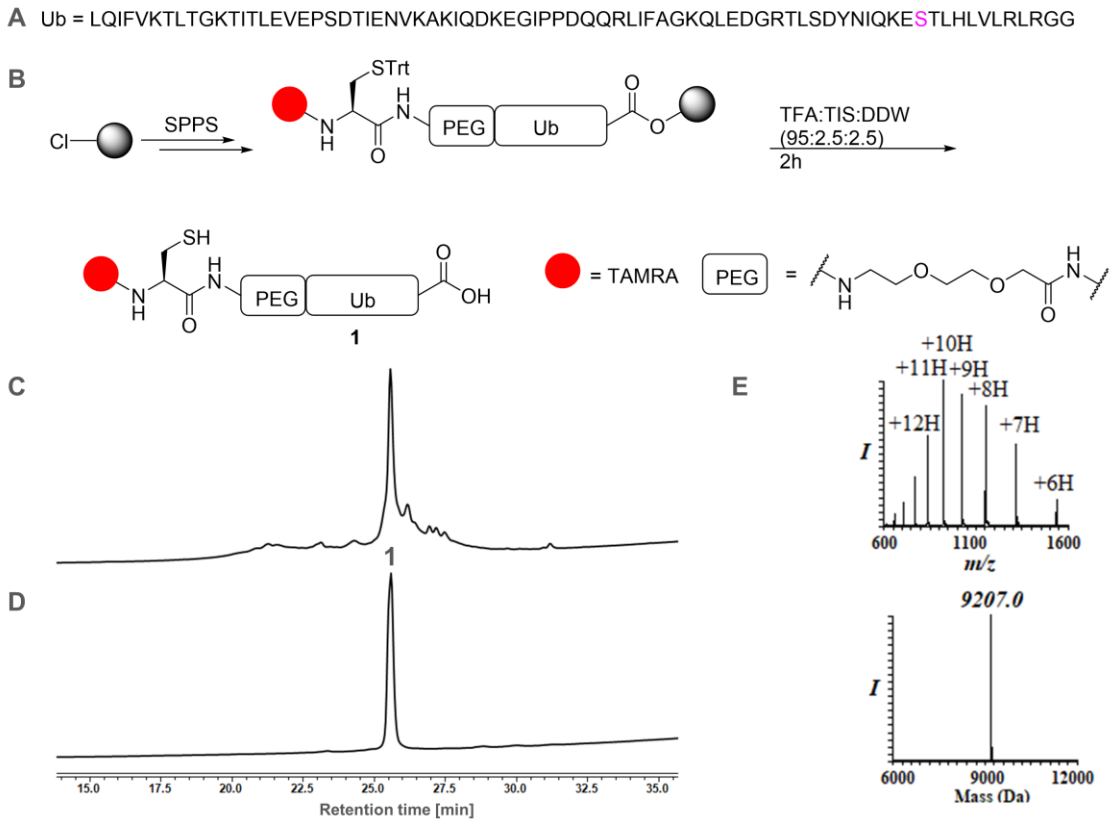


Figure S2: Synthesis of TAMRA-Ub-COOH, **1**. (A) Sequence of Ub with S65 in magenta. (B) Schematic representation of the synthetic route for **1**. (C) HPLC chromatogram of crude **1** (detection at 214 nm). (D) HPLC chromatogram of purified **1** (detection at 214 nm). (E) Mass analysis of **1** with the observed mass 9207.0 ± 0.6 Da, calcd 9207.0 Da (average isotopes).

Fmoc protected amino acids were coupled on pre-loaded 2-CTC resin (0.2 mmol scale) using an automated peptide synthesizer. Next, TAMRA was coupled to the free *N*-terminus using 2 eq. TAMRA, 2 eq. HATU and 4 eq. DIEA for 90 min. The final peptide was cleaved from the resin as described above and lyophilized. TARMAR-wt-Ub-COOH peptide was purified by preparative HPLC (C18 column) using a gradient of 0-60% B over 30 min to afford TAMRA-Ub-COOH (1) in ~19 % yield.

3.3. Synthesis of TAMRA-Ub(S65A)-COOH (2)

The synthesis was carried out according to Figure S3:

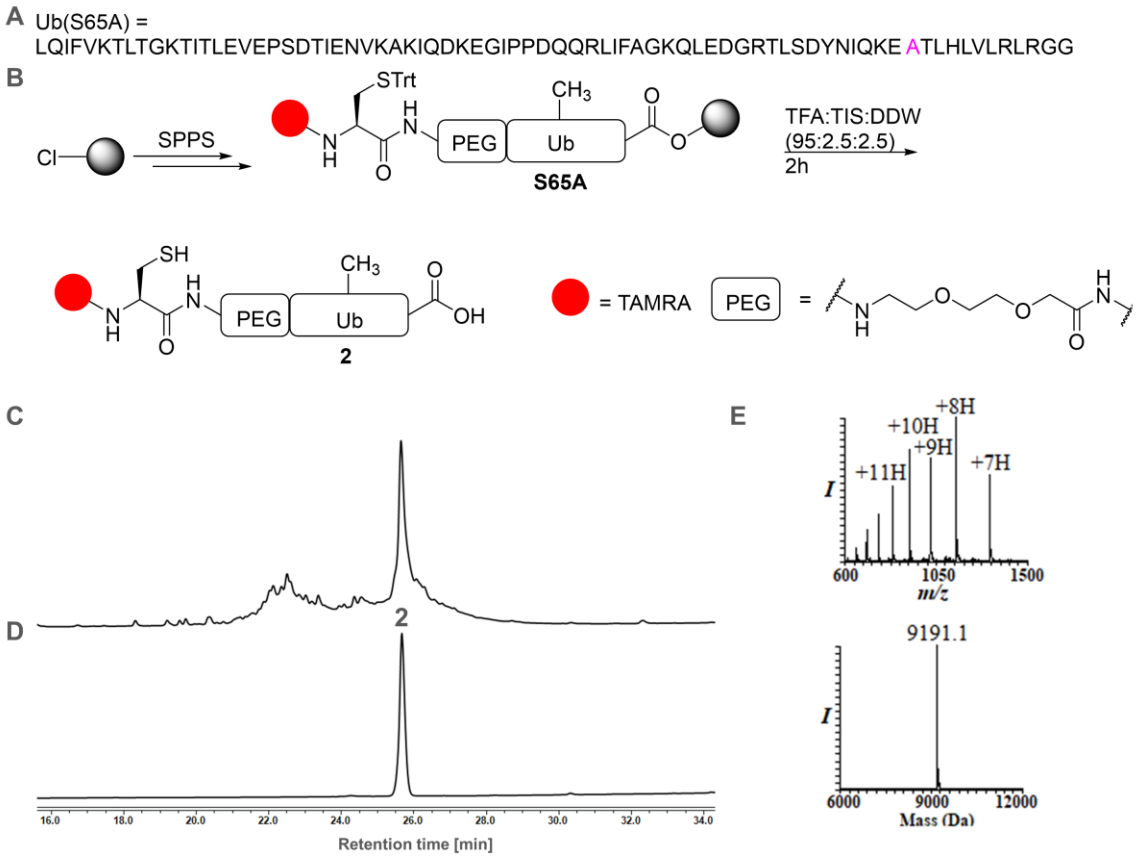


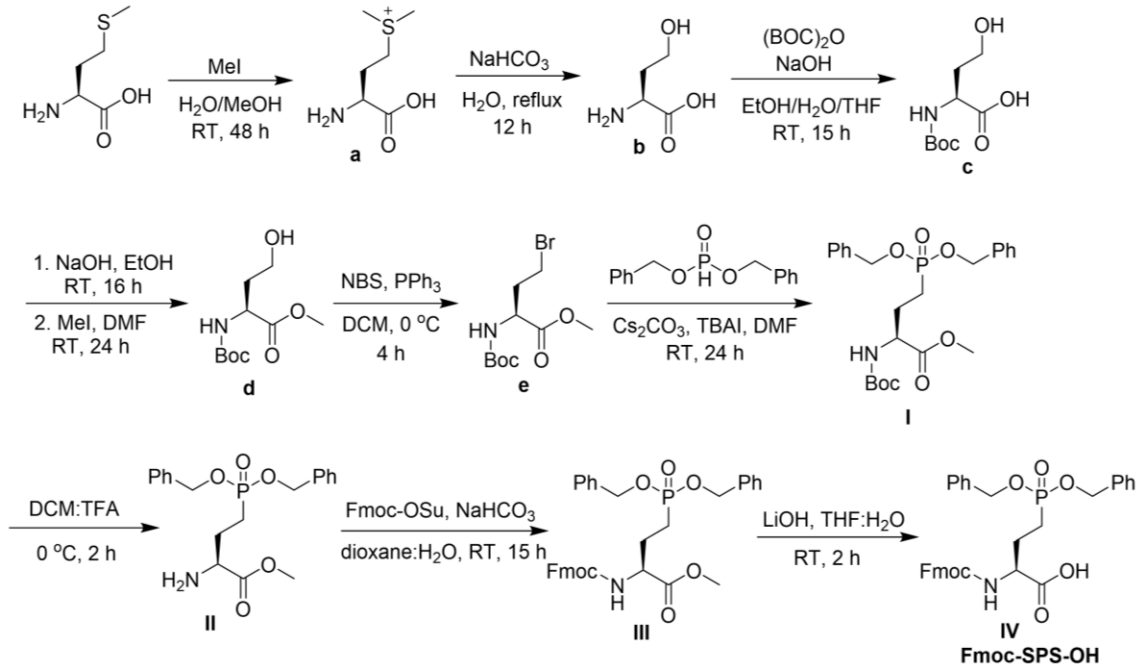
Figure S3: Synthesis of TAMRA-Ub(S65A)-COOH, **2**. (A) Sequence of UbS65A with S65A mutation in magenta. (B) Schematic representation of the synthetic route for **2**. (C) HPLC chromatogram of crude **2** (detection at 214 nm). (D) HPLC chromatogram of purified **2** (detection at 214 nm). (E) Mass analysis of **2** with the observed mass 9191.1 ± 1.4 Da, calcd 9190.2 Da (average isotopes).

Fmoc protected amino acids were coupled on pre-loaded 2-CTC resin (0.2 mmol scale) using an automated peptide synthesizer as for the previous constructs with S65 replaces with Ala. Next, TAMRA was coupled to the free *N*-terminus using 2 eq. TAMRA, 2 eq. HATU and 4 eq. DIEA for 90 min. The final was cleaved from resin as described above and lyophilized. TAMAR-Ub(S65A)-COOH peptide was

purified by preparative HPLC (C18 column) using a gradient of 0-60% B over 30 min to afford purified TAMRA-Ub(S65A)-COOH (**2**) in ~19 % yield.

3.4. Synthesis of FmocSPS-OBn (i) and TAMRA-Ub(SPS65)-COOH (3)

Fmoc-SPS-OH building block was prepared according to the following scheme S2:



Scheme S2. Scheme for Fmoc-SPS-OH building block synthesis.

The compounds **a-e** and **I** were synthesized according to the reported procedures.[3,4]

Compound II: To a solution of **I** (1.0 g, 2.09 mmol) in dichloromethane (10 mL) was added trifluoroacetic acid (10 mL) slowly and stirred the reaction for 2 h at 0 °C. The reaction mixture was distilled under reduced pressure and further co-distilled with DCM (X3) and kept under high vacuum for overnight. The reaction mixture was used further step without need of any purification.

Compound III: To a solution of **II** (0.8 g, 2.1 mmol) in dioxane:H_2O (15 mL, 1:1), 10% $NaHCO_3$ followed by Fmoc-OSu (0.78 g, 2.31 mmol) was added at room temperature and stirred for overnight and was monitored by TLC. Upon

completion, the reaction mixture was distilled under reduced pressure and diluted with ethyl acetate and water. The organic layer was separated, dried over anhydrous Na_2SO_4, and distilled under reduced pressure. The reaction mixture was passed through a silica loaded column chromatography to give the purified product **h** in 73% yield.

Fmoc-SPS-OH (IV): To a solution of **III** (0.5 g, 0.83 mmol) in THF-water (4:1) was added LiOH (0.105 g, 2.49 mmol) at 0 ^oC in portion wise and stirred the reaction for another 2 h and was monitored by TLC. Upon completion, the reaction mixture was adjusted to pH 3-4 by adding 10% citric acid solution and extracted with ethyl acetate. The organic layer was separated, dried over anhydrous Na_2SO_4 and distilled under reduced pressure. The crude was passed through a silica loaded column chromatography to obtain the pure product **i** in 76% yield (0.37 g).

^{1}H NMR (400 MHz, DMSO): δ 7.87 (d, J = 8.0 Hz, 2H), 7.68 (q, J = 4.0 Hz, 2H), 7.40-7.27 (m, 14H), 5.02-4.91 (m, 4H), 4.32-4.20 (m, 3H), 3.96 (s, 1H), 3.50-3.31 (m, 1H), 2.52 (d, J = 16.0 Hz, 1H), 1.99-1.81 (m, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 154.3, 142.1, 141.8, 139.4, 134.0, 134.0, 126.7, 126.6, 126.1, 125.8, 125.2, 123.2, 118.0, 65.9, 65.1, 45.2, 23.5, 20.6, 19.1.

HRMS (+ESI): calcd. for $C_{33}H_{32}NNaO_7P$ = 608.1809; found 608.1802.

The synthesis TAMRA-Ub(SPS65)-COOH, **3** was carried out according to Figure S4:

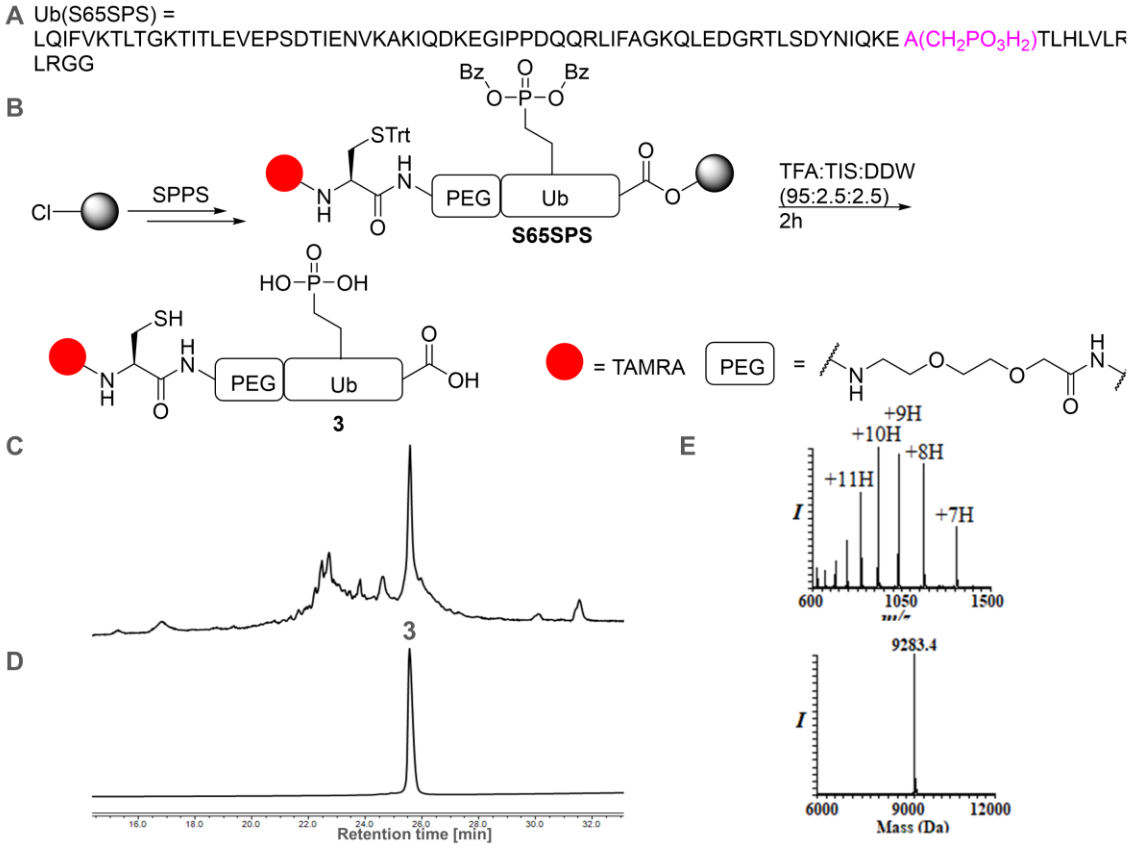


Figure S4: Synthesis of TAMRA-Ub(SPS65)-COOH, **3**. (A) Sequence of SPSUb with SPS65 highlighted in magenta. (B) schematic representation of the synthetic route for **3**. (C) HPLC chromatogram of crude **3** (detection at 214 nm). (D) HPLC chromatogram of purified **3** (detection at 214 nm). (E) Mass analysis of **3** with the observed mass 9283.4 ± 1.6Da, calcd 9284.7 Da (average isotopes).

Fmoc protected amino acids were coupled on pre-loaded 2-CTC resin (0.2 mmol scale) using an automated peptide synthesizer. Fmoc-SPS-OH (1.5 eq.) was manually coupled at position 65 using 1.5 eq. HATU and 3 eq. DIEA in DMF for 2 h following completion of the sequence by automated peptide synthesizer with no further variation from the previous constructs. Next, TAMRA was coupled to the free *N*-terminus using 2 eq. TAMRA, 2 eq. HATU and 4 eq. DIEA for 90 min. The final peptide was cleaved from resin using TFA:ethanedithiol(EDT):water

(95:2.5:2.5) cocktail for 2 h and lyophilized as described above. TAMRA-Ub(SPS65)-COOH peptide was purified by preparative HPLC (C18 column) using a gradient of 0-60% B over 30 min to afford TAMAR-Ub(SPS65)-COOH, **3** in ~18 % yield.

3.5. Synthesis TAMRA-Ub(1-75)-COOH (UbΔG76, 4)

The synthesis was carried out according to Figure S5:

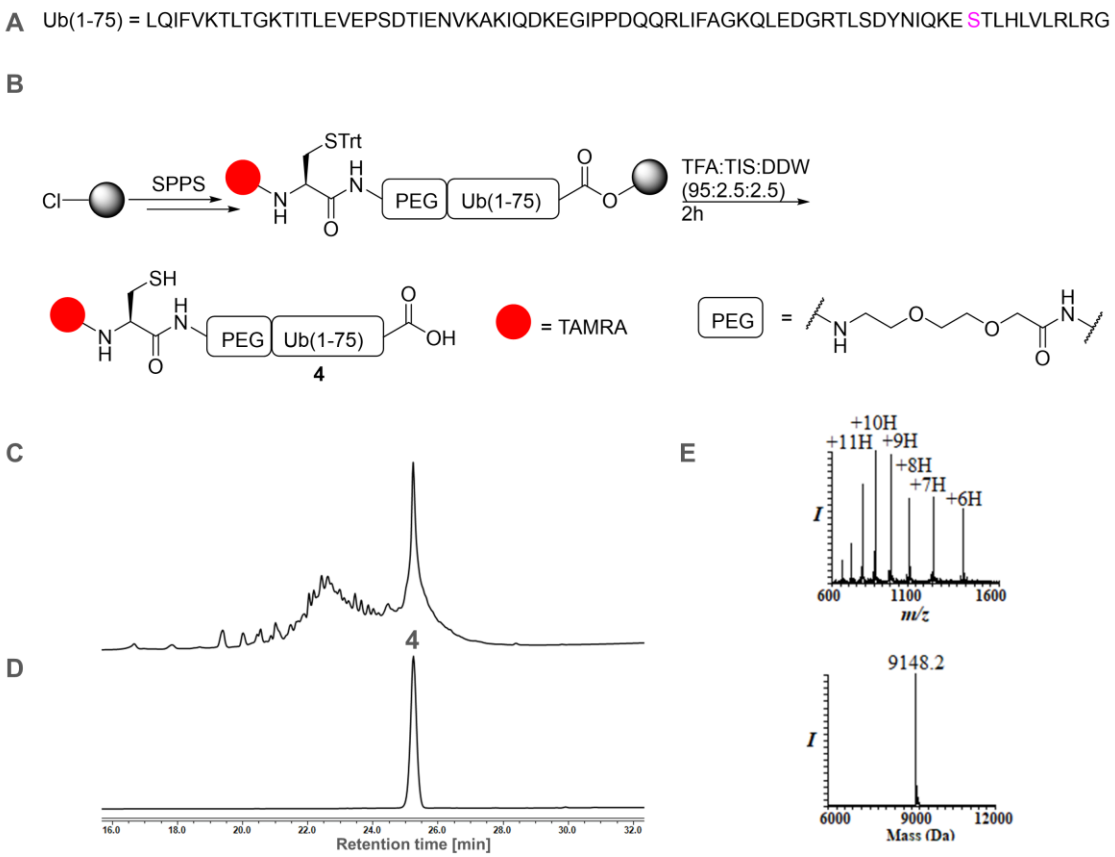


Figure S5: Synthesis of TAMRA-UbΔG76-COOH, **4**. (A) Sequence of Ub(1-75) with S65 in magenta. (B) Schematic representation of the synthetic route for **4**. (C) HPLC analysis of crude **4** (HPLC detection at 214 nm). (D) HPLC of purified **4** (detection at 214 nm). (E) Mass analysis of **4** with the observed mass 9148.2 ± 1.2 Da, calcd 9148.9 Da (average isotopes).

Fmoc protected amino acids were coupled on pre-loaded 2-CTC resin (0.2 mmol scale) using an automated peptide synthesizer. Next, TAMRA was coupled to the free *N*-terminus using 2 eq. TAMRA, 2 eq. HATU and 4 eq. DIEA for 90 min. The final peptide was cleaved from the resin as described above and lyophilized. TAMRA-Ub(1-75)-COOH peptide was purified by preparative HPLC (C18 column) using a gradient of 0-60% B over 30 min to afford TAMRA-Ub(1-75)-COOH, **4** in ~17 % yield.

3.6. Synthesis of CPP conjugated TAMRA-ubiquitin analogues (1a-4a)

In a conventional reaction, 0.4 mmol of ubiquitin analogue was dissolved in N_2 flushed 6M Gnd·HCl (pH 7.3) to 1 mM concentration. TNP-cR10D 0.6 mmol (1.5 eq. to ubiquitin analogue) was dissolved in identical volume of N_2 flushed 6M Gnd·HCl (pH 7.3) to 1 mM concentration and the two peptide solutions were mixed and kept at 37^{o} C for 5 min followed directly (without prolonged incubation) by semi preparative HPLC purification (C18 column) using a gradient of 0-60% B over 30 min to afford cR10D-SS-TAMRA-Ub-COOH (**1a-4a**) in 50-65% yields for all the corresponding analogues.

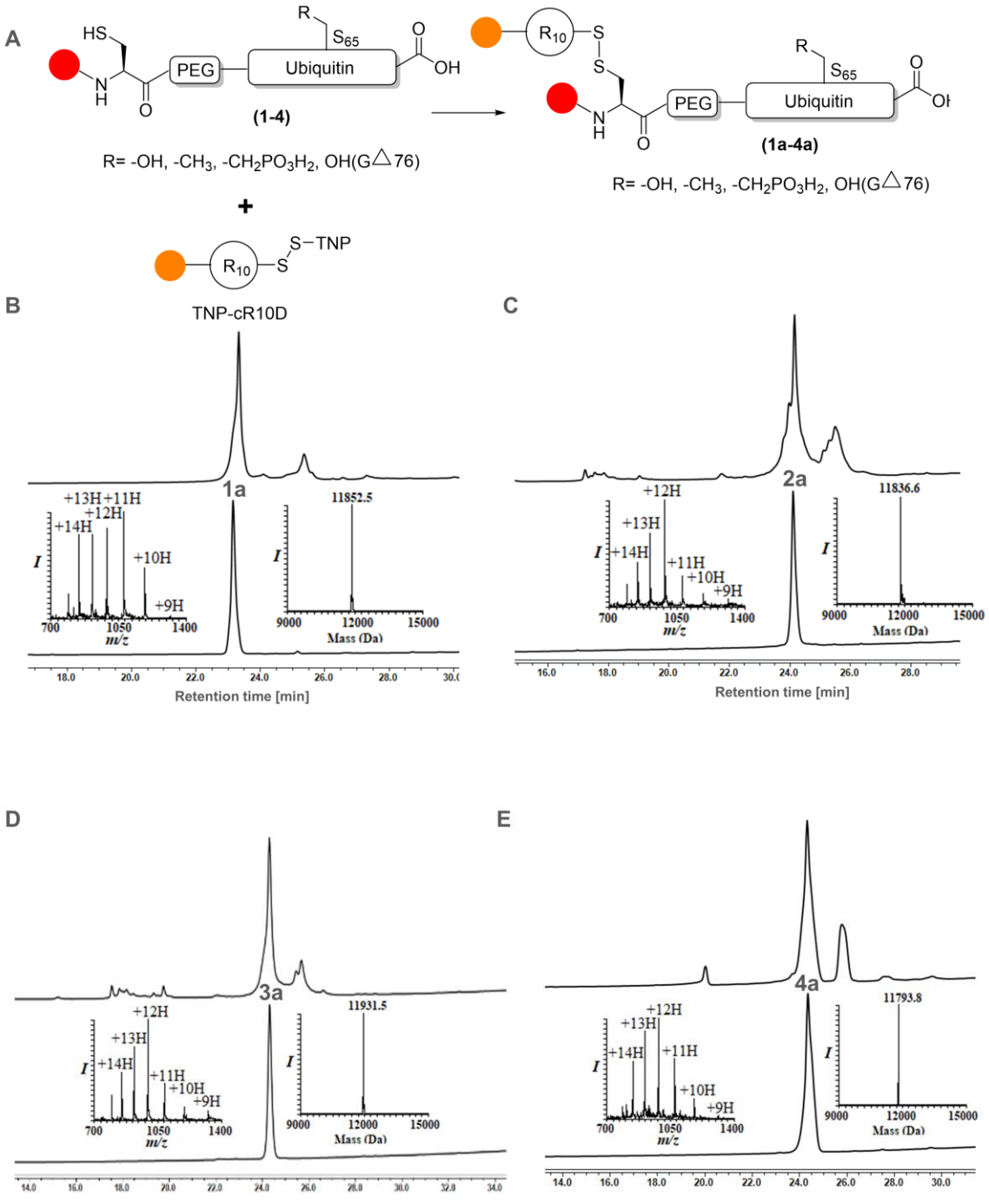


Figure S6: A symmetric disulfide formation between cR10D and Ub analogues. (A) Schematic representation of the reaction. (B) HPLC chromatogram of the conjugation between **1** and cR10D(TNP) at t=5 min. Main peak corresponds to the conjugation product **1a** with observed mass 11852.5 ± 1.2 Da, calcd 11851.4527 Da (average isotopes). (C) HPLC chromatogram of the conjugation between **2** and cR10D(TNP) at t=5 min. Main peak corresponds to the conjugation product **2a** with observed mass 11836.6 ± 1.8 Da, calcd 11835.5 Da (average isotopes). (D) HPLC chromatogram of the conjugation between **3** and cR10D(TNP) at t=5 min. Main peak corresponds to the conjugation product

3a with observed mass 11931.5 ± 2.0 Da, calcd 11930.5 Da (average isotopes). (E) HPLC chromatogram of the conjugation between **4** and cR10D(TNP) at t=5 min. Main peak corresponds to the conjugation product **4a** with observed mass 11793.8 ± 1.0 Da, calcd 11794.4 Da (average isotopes). Detection of HPLC chromatograms at 214 nm.

Final constructs were dissolved as concentrated DMSO stocks (~2 mM) and kept as aliquots at -20^0 C (completely stable for at least 8 months by HPLC analysis). For SDS-PAGE analysis each of the stocks was diluted in PBS followed by centrifugation (10000RPM 10min). Peptide concentration was estimated by Bradford. Protein concentrations were adjusted 5 µM and supplemented with reducing/no-reducing X6 laemmli sample buffer, boiled at 95^o C for 5 min and separated by 10% tris-tricin gel[5]. The gel was imaged for TAMRA fluorescence (Figure S7)

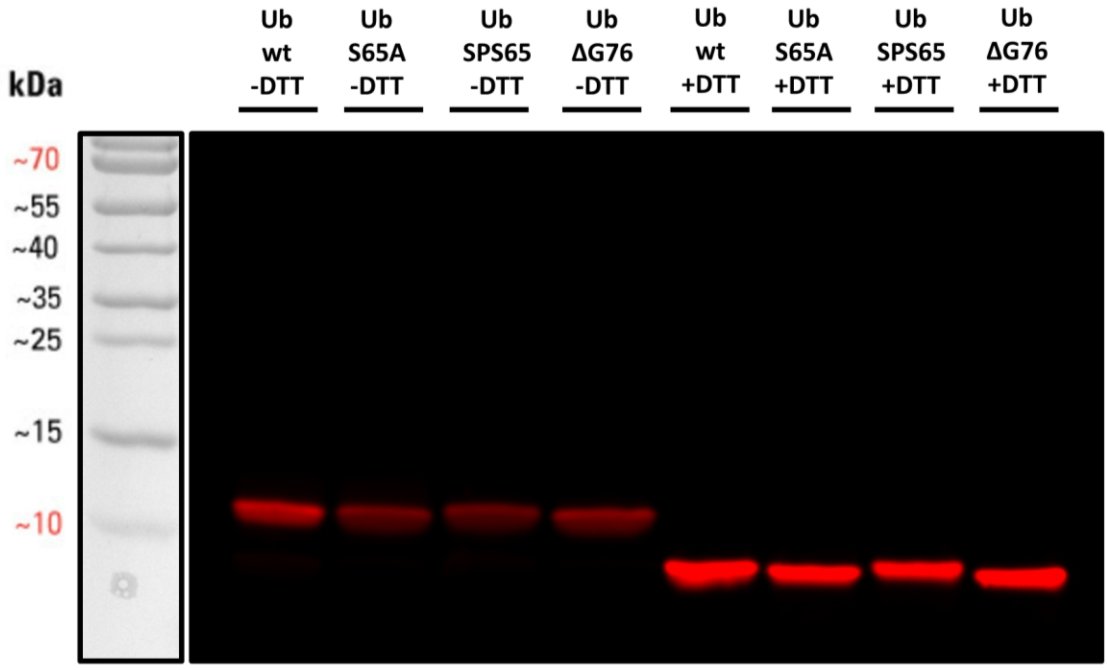


Figure S7: Fluorescent gel analysis of cR10D conjugated TAMRA-Ub-COOH analogues **1a-4a** with and without reducing conditions.

4. Biological experiments for analysis of synthetic construct in mitophagy

4.1. Confirmation of live cells delivery of S65A Ub (2) and SPS Ub (3) analogues by live cell imaging

Initially our attempts to deliver Ub analogues **2** and **3** linked to reported vector cR10 resulted in mainly endosomal entrapment (red and blue merged images Figure S8).

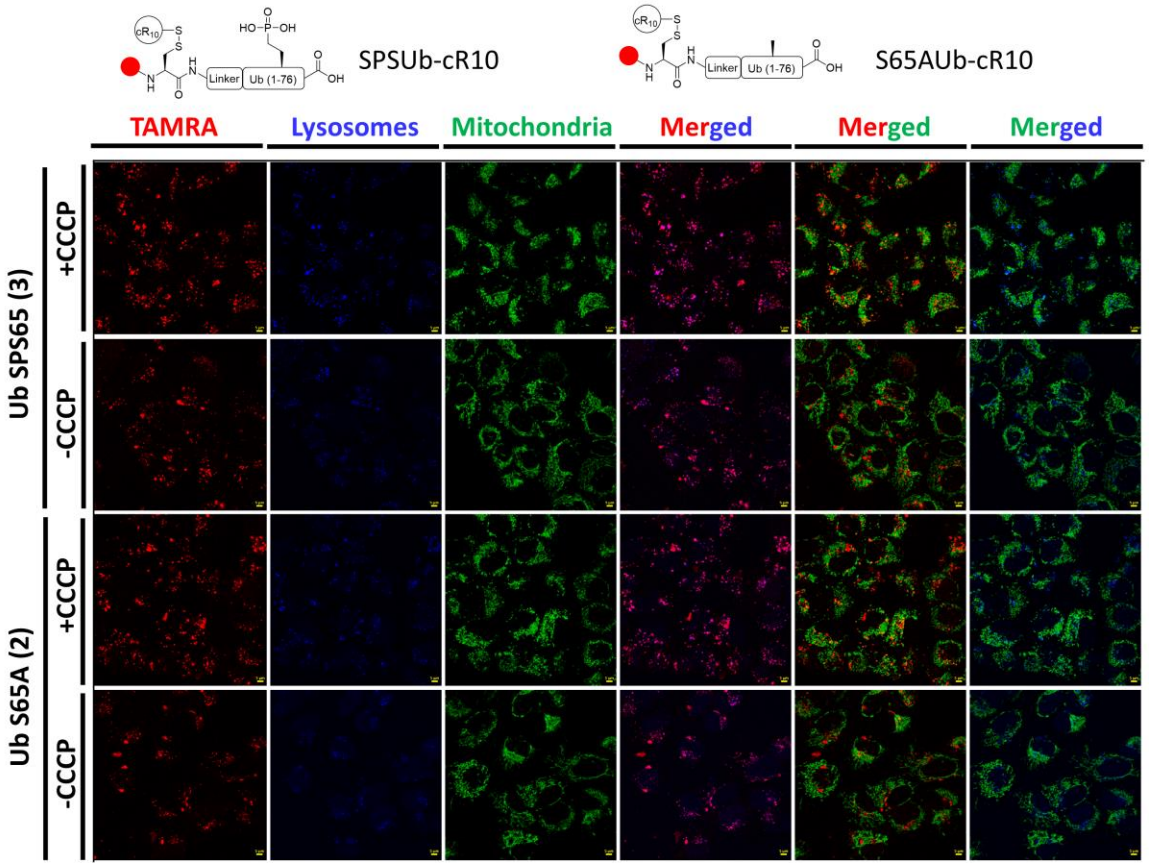
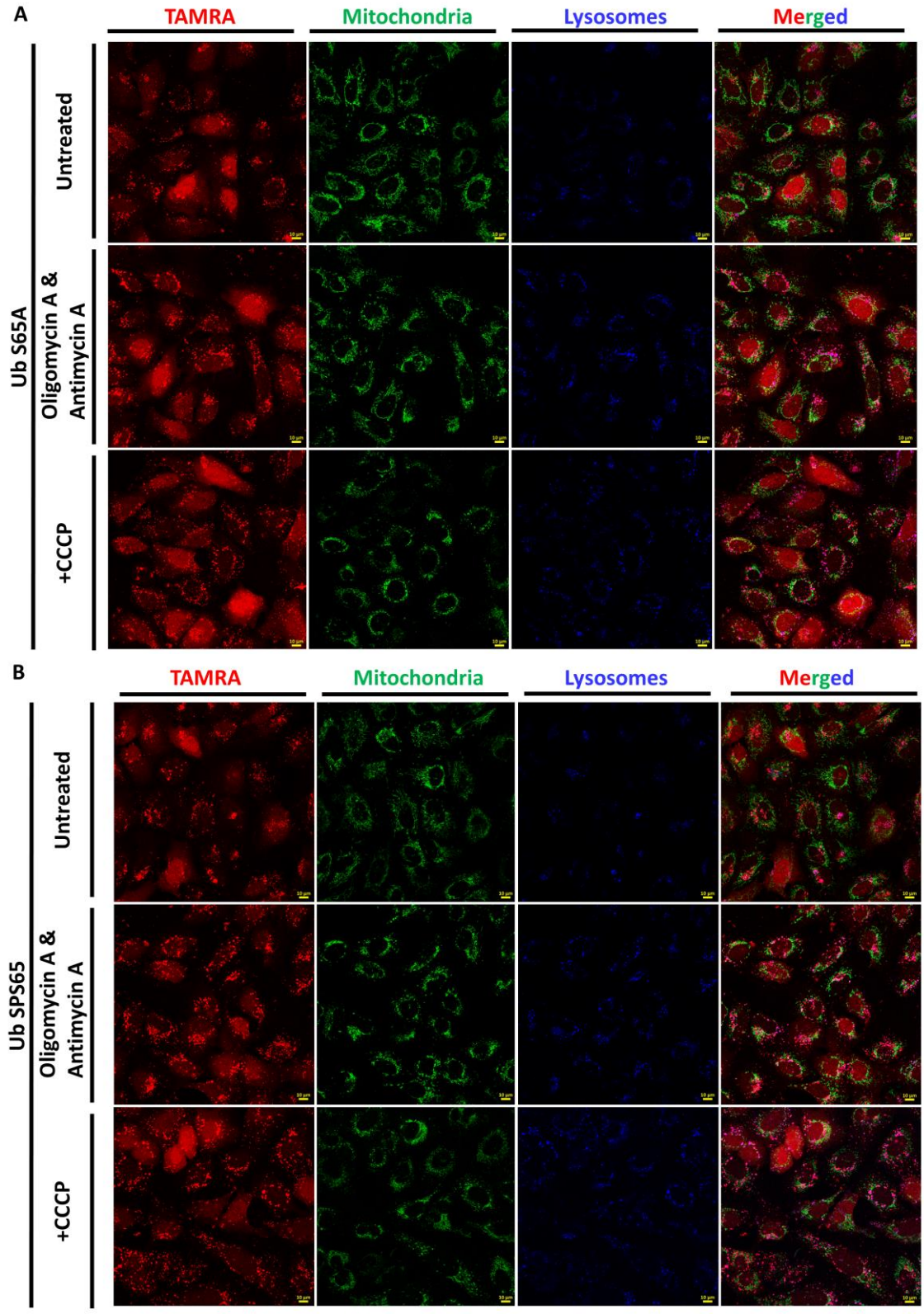


Figure S8. Live cell delivery in induction of mitophagy of **2** and **3** linked to cR10 without DABCYL results in mainly endosomal entrapment (TAMRA – Red, Mitotracker – green, Lysotracker – Blue). Scale bars 5 µm.

Delivery with cR10D linked conjugates **2a** and **3a** resulted in successful delivery to the cytosol with substantial amount of background signal from material trapped in endosomes from the protein delivery process (Figure S9A&B - red and blue merged). We were able to induce mitochondrial damage and mitophagy (evident from the perinuclear mitochondrial aggregates) by using treatment with either combination of oligomycin A/Antimycin A or CCCP (Figure S9 A&B).


A
TAMRA
Mitochondria
Lysosomes
Merged
Ub S65A
Untreated
Oligomycin A & Antimycin A
+CCCP
B
TAMRA
Mitochondria
Lysosomes
Merged
Ub SPS65
Untreated
Oligomycin A & Antimycin A
+CCCP

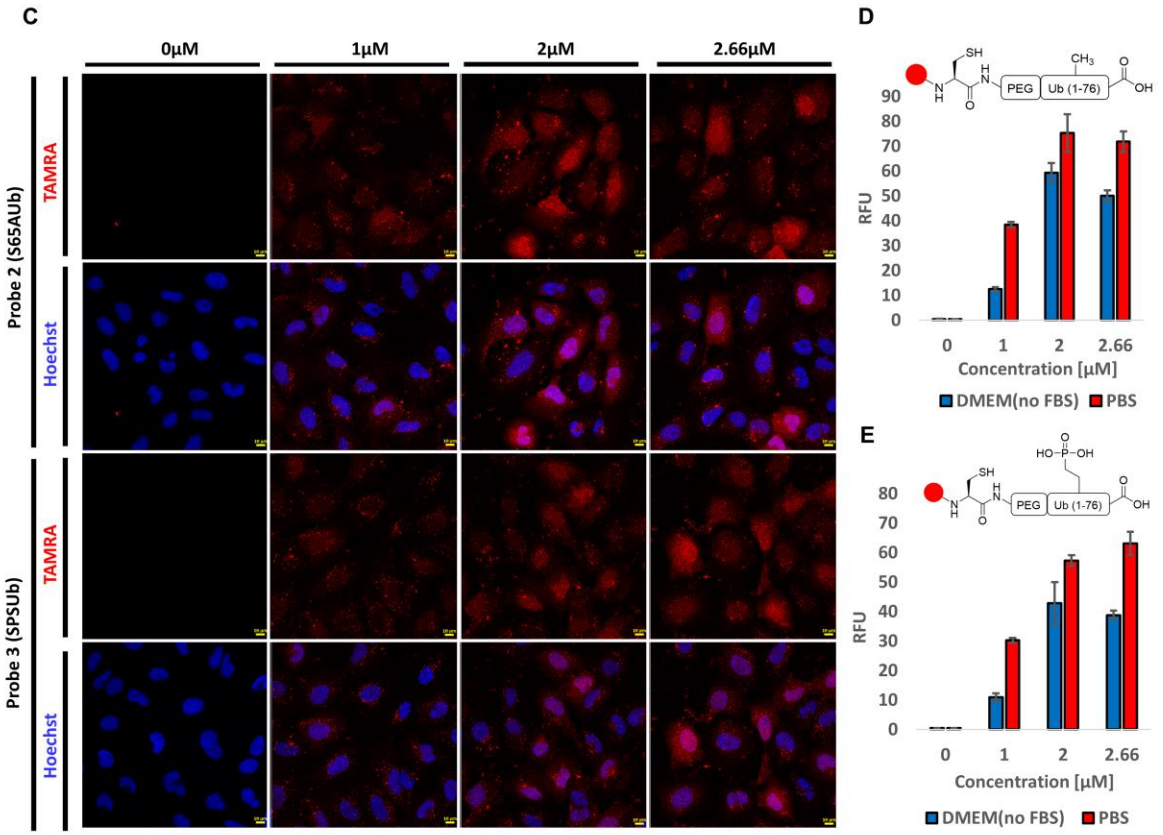


Figure S9. CLSM images of live +PRK U2OS without treatment, treated with oligomycin A and Antimycin A and treated with CCCP for 4 hours (A) S65AUb **2**. (B) SPSUb **3**. (LT-B – blue, MT-G – green, TAMRA – red). Scale bars 10 μm. (C) Representative images of **2**&**3** delivery in live U2OS cells. (D) Quantification of nuclear TAMRA fluorescence of **3** (E) Quantification of nuclear TAMRA fluorescence of **2**. Both experiments in C&B were performed in PBS and serum free medium (over 100 cells each condition). TAMRA – red, Hoechst – blue, scale bars 10μm.

In a separate experiment we delivered **2** and **3** to +PRK U2OS cells followed by replacement of the medium to opti-DMEM containing 1 μg/ml of Hoechst stain and imaged the cells Z-stack. Image analysis with Imaris confirmed TAMRA fluorescence mainly in the nucleus but also in the cytosol of the delivered cells (Figure S10 A&B).

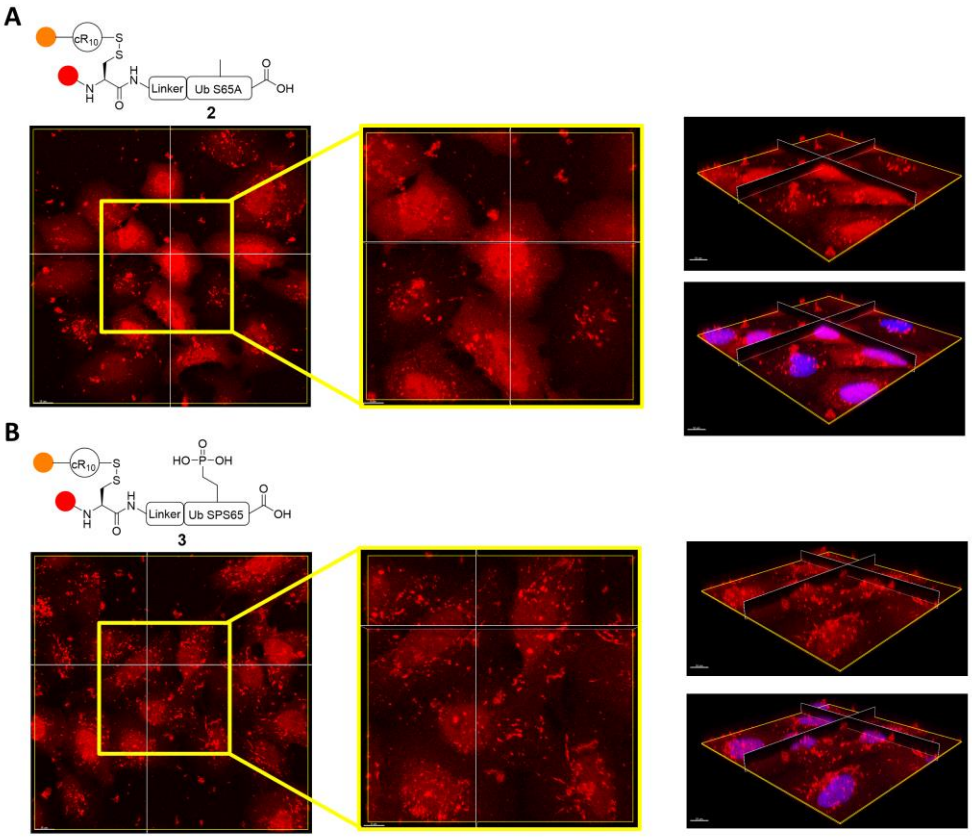


Figure S10. Z-stack images of (A) **2** & (B) **3** in live U2OS cells (TAMRA – red, Hoechst – blue).

4.2. Live cells delivery of S65A Ub (2) and SPS Ub (3) analogues induction of Mitophagy in live U2OS cells followed by fluorescent gel and westen blot analysis

For SDS-PAGE analysis of mitophagy induction and conjugation of Ub analogues, we lysed U2OS+PRK cells that were delivered with **2** and **3** followed by 4 h incubation with or without CCCP as described above. The cells were washed with PBS and trypsin-EDTA to digest external proteins remaining from the delivery. The cells were lysed with extraction buffer[6] containing protease inhibitor cocktail set III (Mercury). Tris-tricine SDS-PAGE was prepared as previously described.[1,5] The

fluorescent gels and western blot analyses were imaged by Fusion FX6 ECL and fluorescent detection system (Figure S11).

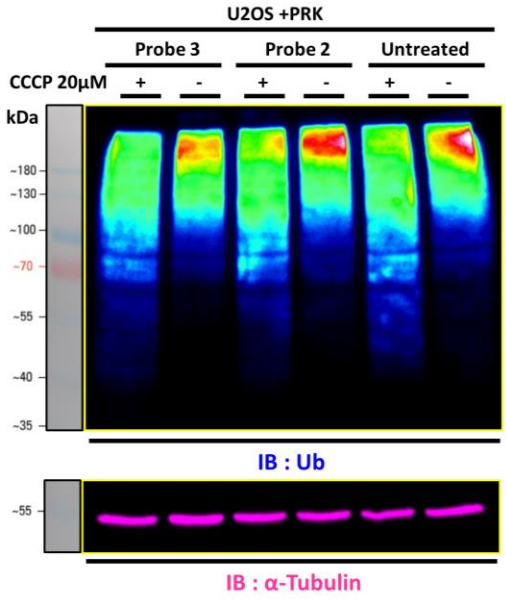
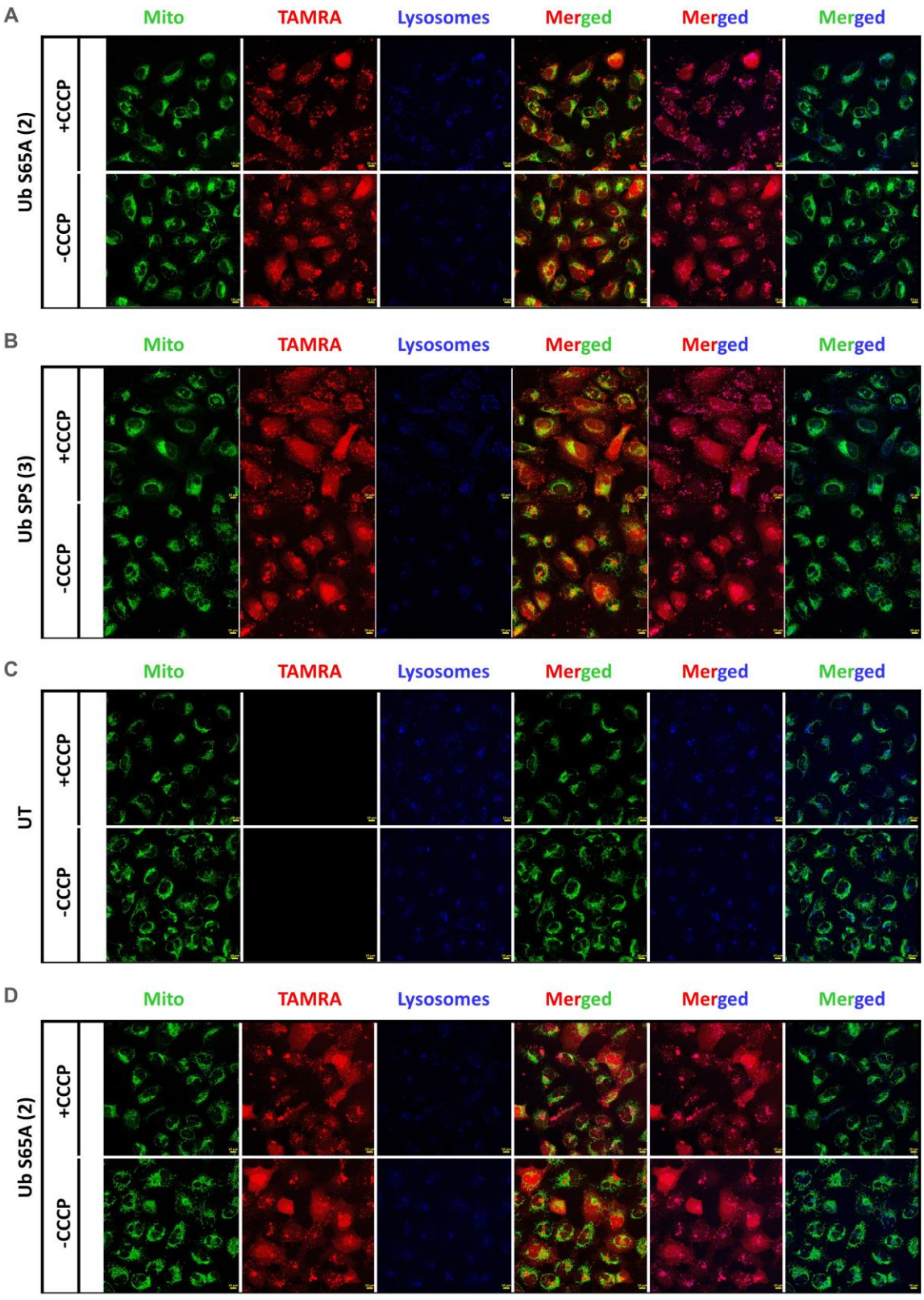


Figure S11: Western blot analysis of lysates from U2OS+PRK cell with S65AUb **2** and SPS65Ub **3** with and without 4 h CCCP treatment using anti-Ub antibody (total ubiquitin). Loading control is α-tubulin. (Representative result of two independent experiments).

4.3. Live cells delivery of S65A Ub (2) and SPS Ub (3) analogues and imaging of Mitophagy in live U2OS cells with and without parkin

To measure the involvement of Ub analogues in mitophagy in live cells we delivered Ub analogues to cell with or without parkin expression and induced mitophagy using 20μM CCCP treatment for 4 h (Figure S12). These results demonstrate that the strong background of lysosomal Ub limits the analysis of mitophagy to early stages (before fusion with lysosomes) and requires colocalization analysis with more selective staining then mitotracker in lysotracker (Figure S12 A-G).


A
Mito
TAMRA
Lysosomes
Merged
Merged
Merged
Ub S65A (2)
+CCCP
-CCCP
B
Mito
TAMRA
Lysosomes
Merged
Merged
Merged
Ub SPS (3)
+CCCP
-CCCP
C
Mito
TAMRA
Lysosomes
Merged
Merged
Merged
UT
+CCCP
-CCCP
D
Mito
TAMRA
Lysosomes
Merged
Merged
Merged
Ub S65A (2)
+CCCP
-CCCP

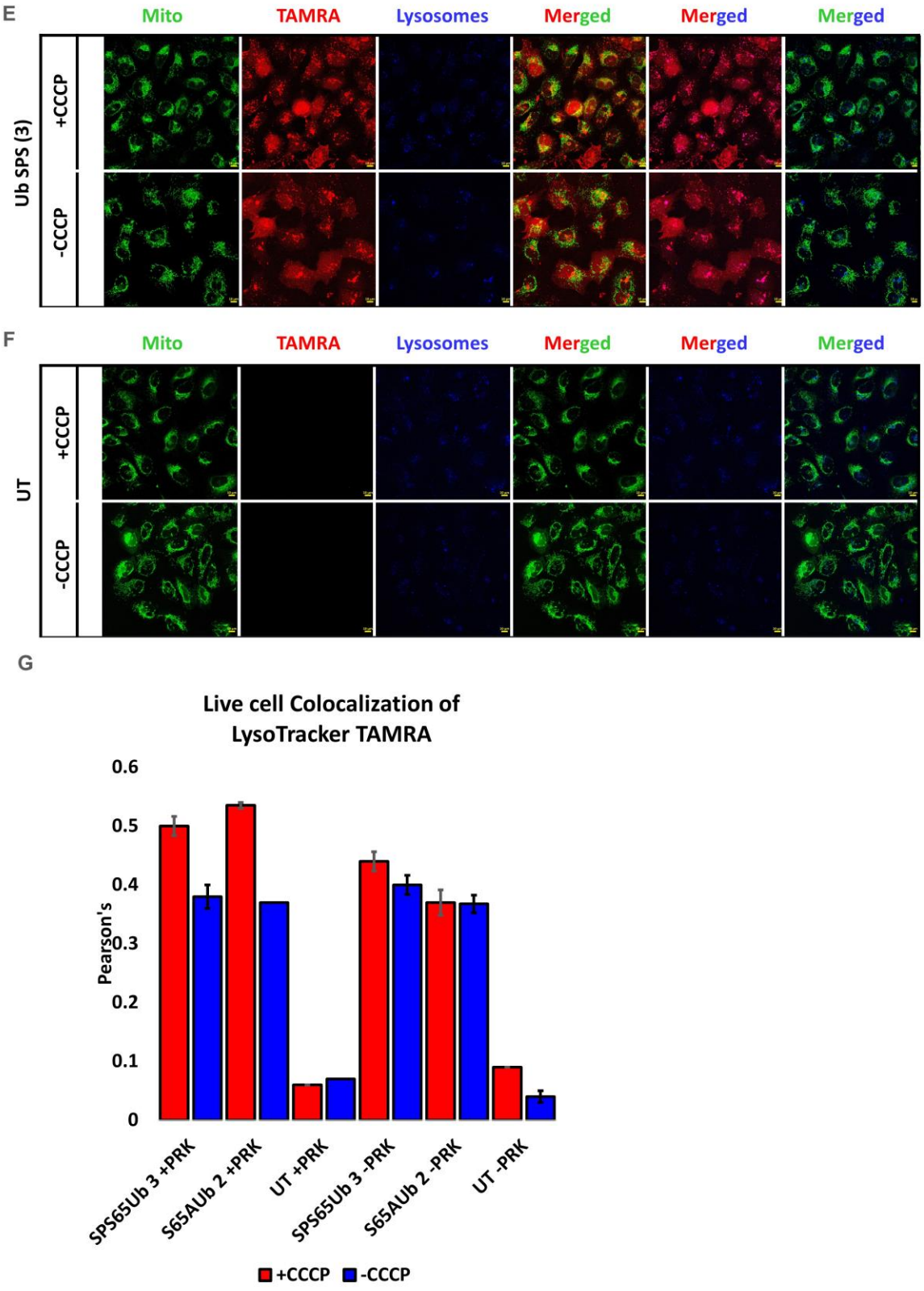
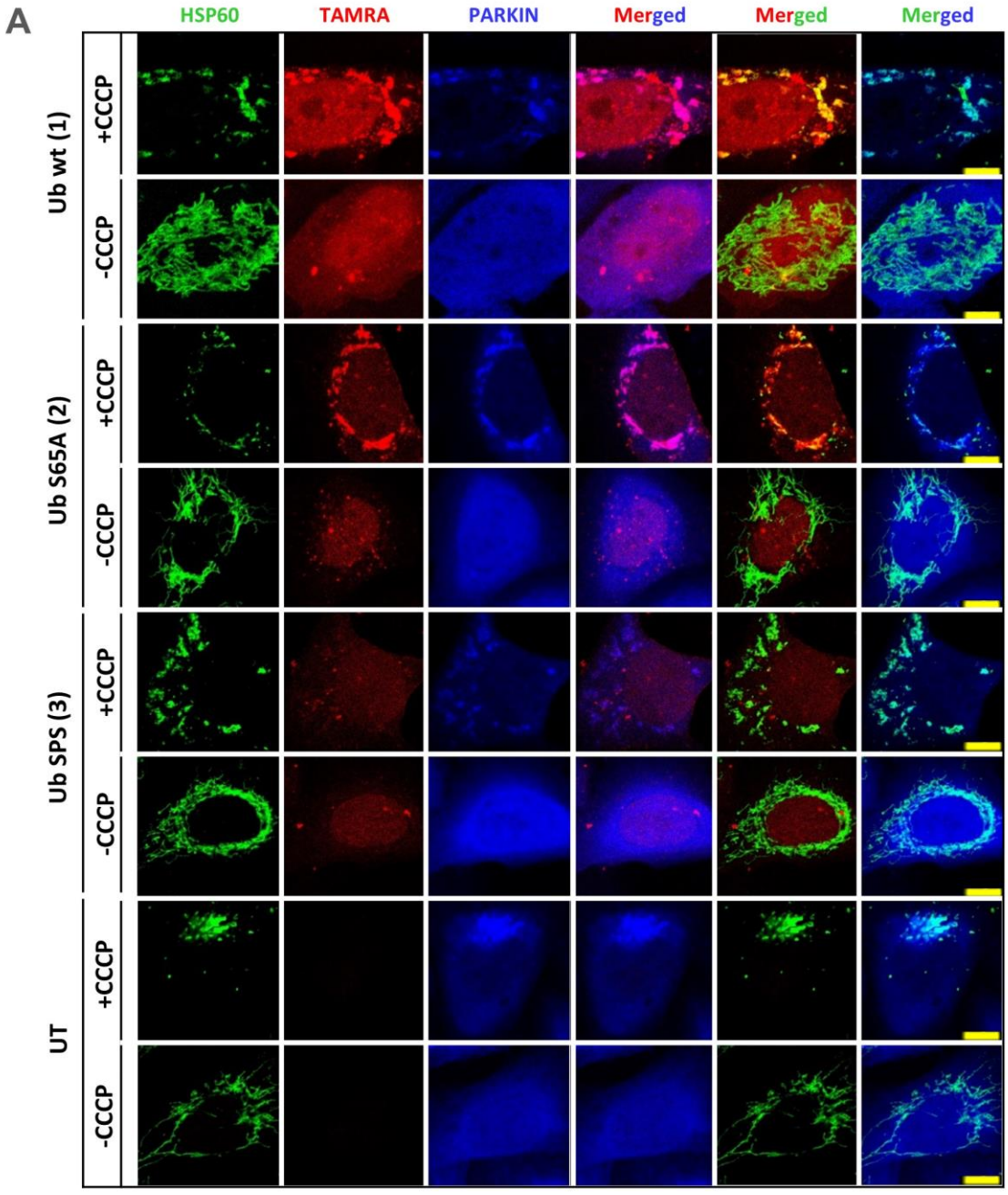


Figure S12. Representative CLSM images of live U2OS treated ±CCCP for four hours (A) S65AUb **2** in +PRK cells. (B) SPSUb **3** +PRK cells. (C) +PRK cells without Ub probe

treatment (UT). (D) S65AUb **2** in wt U2OS cells. (E) SPSUb **3** wt U2OS cells. (F) wt U2OS cells without Ub probe treatment (UT). (LT-B – blue, MT-G – green, TAMRA – red). Scale bars 10 µm. (G) Colocalization analysis of TAMRA and lysosomes in live U2OS cells with and without parkin delivered with **2** and **3** ±CCCP treatment (from two independent experiments each with ~80 cells).

4.4. Live cells delivery of Ub analogues (1-3) induction of Mitophagy in live U2OS cells followed by analysis in fixed cells

Analysis of TAMRA and HSP60 colocalization allowed us to directly observe the mitophagy signals in fixed cells (Figure S13 A). HSP60 intensity was increased under PARKIN mask after CCCP treatment demonstrating parkin recruitment to HSP60 (mitochondria) (Figure S13 B).



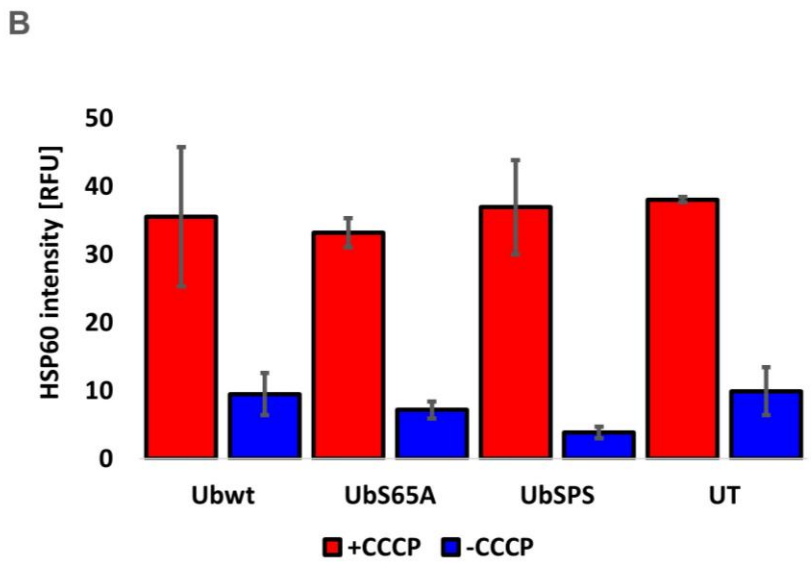
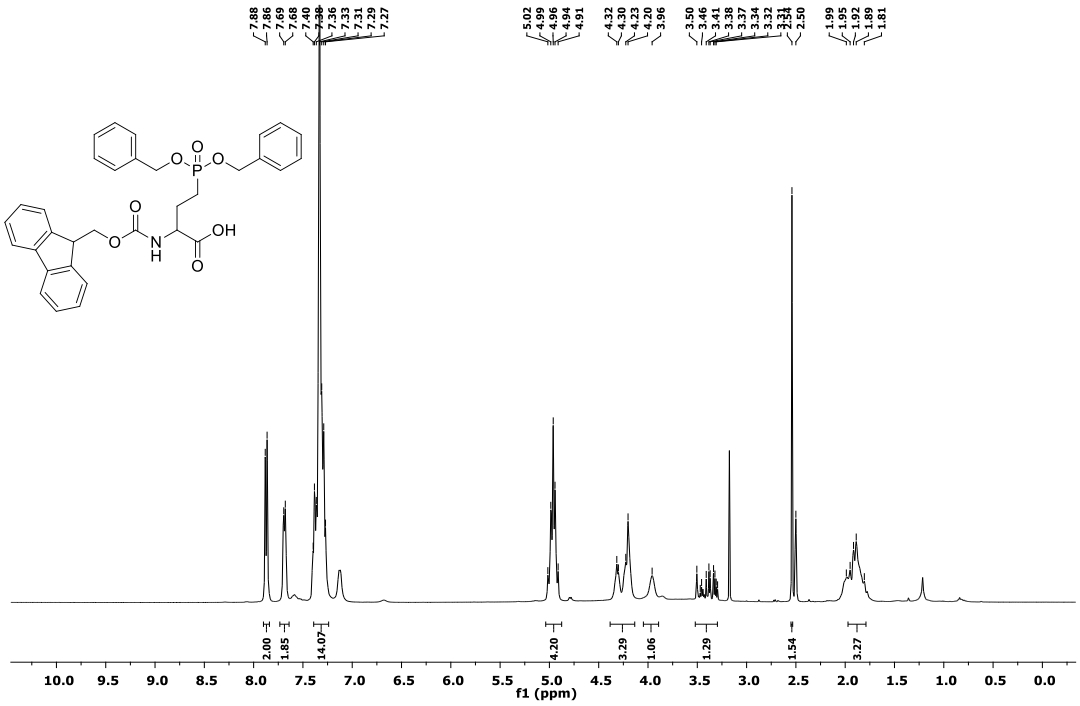
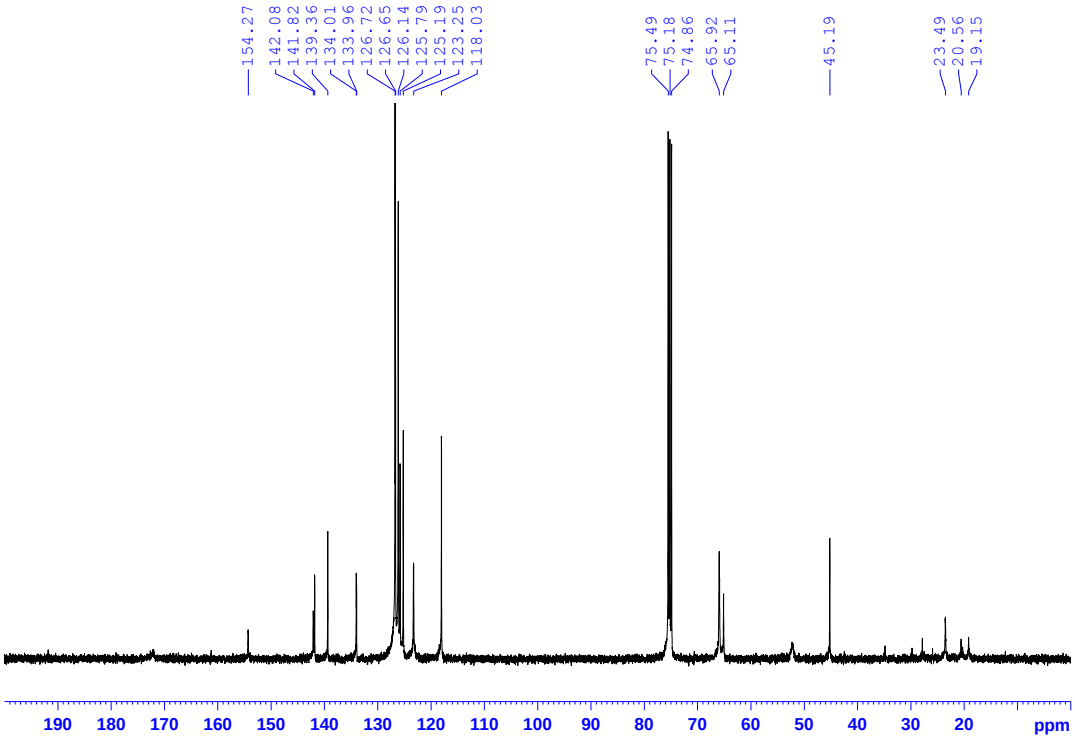


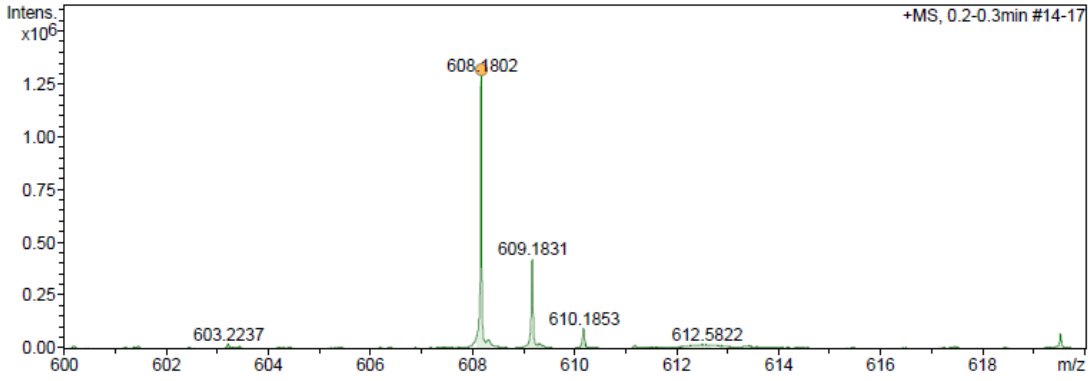
Figure S13. Delivery of Ub analogues do not affect mitophagy induction. (A) Representative Images of live +PRK U2OS delivered with Ub analogues **1**, **2** and **3** and treated ±CCCP for four hours. Scale bars 10 µm. (B) Quantification of HSP60 intensity in the different treatments under parkin mask (results from two independent experiments each with ~100 cells).

5. Analysis of Fmoc-SPS-OH synthesis by NMR and HRMS





+MS, 0.2-0.3min #14-17



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e¯ Conf	N-Rule	|err| [mDa]
608.1802	1	C33H32NNaO7P	608.1809	1.1	19.1	1	100.00	18.5	even	ok	0.7

6. References

(1) Mann, G.; Satish, G.; Meledin, R.; Vamisetti, G. B.; Brik, A. Palladium-Mediated Cleavage of Proteins with Thiazolidine-Modified Backbone in Live Cells. *Angew. Chemie - Int. Ed.* **2019**, *58* (38), 13540–13549. https://doi.org/10.1002/anie.201906545.

(2) Herce, H. D.; Schumacher, D.; Schneider, A. F. L.; Ludwig, A. K.; Mann, F. A.; Fillies, M.; Kasper, M. A.; Reinke, S.; Krause, E.; Leonhardt, H.; Cardoso, M. C.; Hackenberger, C. P. R. Cell-Permeable Nanobodies for Targeted Immunolabelling and Antigen Manipulation in Living Cells. *Nat. Chem.* **2017**, *9* (8), 762–771. https://doi.org/10.1038/NCHEM.2811.

(3) Kang, J.; Chen, H. X.; Huang, S. Q.; Zhang, Y. L.; Chang, R.; Li, F. Y.; Li, Y. M.; Chen, Y. X. Facile Synthesis of Fmoc-Protected Phosphonate PSer Mimetic and Its Application in Assembling a Substrate Peptide of 14-3-3 ζ. *Tetrahedron Lett.* **2017**, *58* (26), 2551–2553. https://doi.org/10.1016/j.tetlet.2017.05.037.

(4) Prasad, V. P.; Wagner, S.; Keul, P.; Hermann, S.; Levkau, B.; Schäfers, M.; Haufe, G. Synthesis of Fluorinated Analogues of Sphingosine-1-Phosphate Antagonists as Potential Radiotracers for Molecular Imaging Using Positron Emission Tomography. *Bioorganic Med. Chem.* **2014**, *22* (19), 5168–5181. https://doi.org/10.1016/j.bmc.2014.08.009.

(5) Schägger, H. Tricine-SDS-PAGE. *Nat. Protoc.* **2006**, *1* (1), 16–22. https://doi.org/10.1038/nprot.2006.4.

(6) Swatek, K. N.; Usher, J. L.; Kueck, A. F.; Gladkova, C.; Mevissen, T. E. T.; Pruneda, J. N.; Skern, T.; Komander, D. Insights into Ubiquitin Chain Architecture Using Ub-Clipping. *Nature* **2019**, *572* (7770), 533–537. https://doi.org/10.1038/s41586-019-1482-y.