

Supporting Information

Probing the Cell Delivery of Synthetic Diubiquitin Chains

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1. General reagents

Peptides were prepared by Solid Phase Peptide Synthesis (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), and N, N-diisopropylethylamine (DIEA) were purchased from Sigma. trifluoroacetic acid (TFA) and dichloromethane (DCM) were purchased from Biolab. Resins were purchased from Creosalus, protected amino acids were purchased from GL Biochem and activating reagents N, N, N', N'-Tetramethyl-O-(1H-benzotriazole-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)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) was procured from Novabiochem®. Triisopropylsilane (TIS), ammonium carbonate dithiothreitol (DTT) were purchased from Alfa Aesar. Dimethyl sulfoxide (DMSO), Palladium (II) chloride (PdCl₂) were purchased from Sigma-Aldrich. 5-Carboxytetramethylrhodamine (TAMRA), N-(3-Carboxypropyl) maleimide and 4-Dimethylaminoazobenzene-4'-carboxylic acid (DABCYL) were purchased from Tzamal D-Chem.

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(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Boc-Ala-OH, Fmoc-Lys(Alloc)-OH, Boc-Cys(Trt)-OH, Fmoc-

Cys(Acm)-OH, Fmoc-Nle-OH, Fmoc-Ile-Thr(ψ Me,MePro)-OH, Fmoc-Leu-Thr(ψ Me,MePro)-OH, Fmoc-Leu-Ser(ψ Me,MePro)-OH, Fmoc-Asp(OtBu)-(DMB)Gly-OH. N-methyl-3,4-diaminobenzoic acid (Fmoc-NMeDbz), was prepared using the reported procedure.^{1,2}

3. Fmoc-SPPS general procedure

Fmoc-SPPS was carried out on an automated peptide synthesizer (CS336X, CSBIO) in presence of AA (4 eq.), HCTU (4 eq.), and DIEA (8 eq.) to the initial loading of the resin for 60 min. The dipeptides were coupled manually using 2.5 eq. of AA, 2.5 eq. of HATU, and 5 eq. of DIEA to the initial loading of the resin for 1 h 30 min.

Global deprotection method: To cleave the peptides from the solid support the resin was washed with DMF, DCM X5, and dried over a high vacuum. A cocktail of trifluoroacetic acid (TFA): triisopropyl silane (TIS): water (95:2.5:2.5) was added to the 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 for freeze-drying in the lyophilizer to give the crude peptide.

Alloc deprotection method: The resin was washed with dry DCM to remove all the DMF present. The Alloc deprotection was carried out by using Pd(PPh₃)₄ (0.4 eq.) and phenyl silane (40 eq.) in dry DCM for 1 h followed by washing with DMF and DMF containing 0.5% DIEA.

MeDbz cyclization: The resin was washed with DCM and a solution containing 30 eq. p-nitrophenyl chloroformate (120 mg, for 0.1 mmol scales) in 4 mL DCM was added and shaken for

30 min at room temperature followed by washing with DCM (3X5 mL). This step was repeated three times. The peptide resin was then treated with three cycles of DIEA (0.45 mL) in DMF (4 mL) and was shaken for 10 min for a complete cyclization and washed with DMF (X2).

4. HPLC for peptide analysis and purification

Analytical high-performance liquid chromatography (HPLC) was performed on a Thermo instrument (Dionex Ultimate 3000) using analytical XSelect (waters, CSH C18, 3.5 μm , 4.6 \times 150 mm) and Xbridge (waters, BEH300 C4, 3.5 μm , 4.6 \times 150 mm) columns at a flow rate of 1.2 ml/min. Semi preparative HPLC was performed on a Thermo Scientific instrument (Dionex Ultimate 3000) using Jupiter C18 (Phenomenex, 10 μm , 300 \AA , 250 \times 10 mm) column and Jupiter C4 (Phenomenex, 10 μm , 300 \AA , 250 \times 10 mm) column, at a flow rate of 4 mL/min. Preparative HPLC was performed on a Thermo Scientific instrument (Dionex Ultimate 3000) using XSelect (waters, CSH C18, 10 μm , 19 \times 250 mm), Jupiter C18 (Phenomenex, 5 μm , 300 \AA , 250 \times 21.2 mm) and Jupiter C4 (Phenomenex, 10 μm , 300 \AA , 250 \times 22.4 mm) column, at a flow rate of 15 mL/min. All synthetic products were purified by HPLC and characterized by mass spectrometry using the 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.

5. Cell culture reagents

U2OS (HTB-96TM) were purchased from ATCC®. Dulbecco's modified eagle's medium (low glucose, DMEM), Fetal bovine serum (FBS), L-Glutamine, antibiotics (penicillin/streptomycin), trypsin/EDTA and phosphate-buffered saline (PBS) were purchased from biological industries.

Hoechst 33342 solution (20 mM) was purchased from Thermo-fisher. μ -Slide 8 well for live-cell confocal microscopy was purchased from ibidi.

6. Cell culture procedure

U2OS (HTB-96TM) cells were cultured in DMEM (low glucose) supplemented with 10% FBS, 0.2 mM L-Gln and antibiotics (penicillin/streptomycin) in a humidified 37°C incubator at 5% CO₂. 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 the incubation chamber for 5 min. Trypsin was quenched by adding the supplemented media. The cell suspension was collected, and the cells were pelleted (2 min at 1,000xg). Then the media has aspired, and the cell pellet was resuspended in fresh media. The cell density was determined using an automated cell counter (Countess II, Invitrogen) and seeded accordingly.

For confocal microscopy and image stream analysis U2OS cells were seeded on ibidi poly-L-lysine (PLL) 8 well μ -slides in $[[3.5 \times 10]]^4$ cells/well, were allowed to reach ~85-90% confluency.

7. Protein delivery

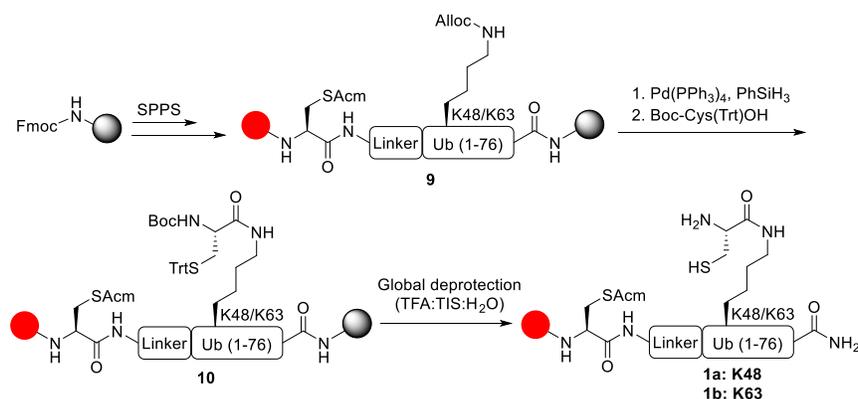
Cells in ~85-90% confluency were washed twice with warm PBS followed by incubation for 1h with warm PBS containing CPP-fused proteins. Cells were then washed three times with warm PBS followed by adding 0.1 mg/ml heparin sulfate in PBS and incubating for 5min. After this step, cells were washed again with PBS three times and once with a full culture medium and allowed to stay in full medium for at least 1h before imaging. Cells were then stained using manufacturer standard protocols with Hoechst (2 μ g/ml) and imaged by CLSM.

8. Confocal microscopy and image analysis

The distribution of fluorescent proteins in live cells was analyzed using a confocal laser scanning microscope (Confocal Zeiss LSM 710) equipped with a 40X NA 1.2 water immersion objective lens having a 1-1.5 AU pinhole resolution limit. Two lasers were used for the different tags: UV laser (Hoechst) – 405 nm (10 mW), and green laser (TAMRA) – 543 nm (10 mW). During CLSM analysis the samples were kept at room temperature.

Image analysis was performed on all images collected using FiJi software. Relative fluorescence intensities were determined by first identifying individual cells and the cytosolic compartment (nucleus) by applying a cell masking algorithm based on Hoechst nuclear staining. The values presented are the averaged nuclear intensity of TAMRA.

9. Synthesis of proximal ubiquitin(s) (1a/1b)



Scheme S1. Synthesis of fluorescently labeled Ub monomer **1a** and **1b**

The synthesis of proximal Ub was performed on a preswollen Knorr Resin (0.26 mmol/g loading, 0.2 mmol scales) using an automated peptide synthesizer. Standard Fmoc-SPPS protocol was applied to complete the sequence as described in section 3. Pseudoproline dipeptides Fmoc-Lue-Thr(ψ Me, MePro)-OH, Fmoc-Ile-Thr(ψ Me, MePro)-OH, Fmoc-Asp(OtBu)-(DMB)Gly-OH and

Fmoc-Leu-Ser(ψ Me, MePro)-OH were coupled manually at positions Ile₁₃-Thr₁₄, Lue₈-Thr₉, Asp₅₂-Gly₅₃ and Leu₅₆ -Ser₅₇ using of the dipeptide (2.5 eq.), HATU (2.5 eq.) and DIEA (5 eq.) for 1.5 h. Either at position 63 or 48, Fmoc-Lys(Alloc)-OH was coupled using standard Fmoc-SPPS protocol. The linker [2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid was coupled manually using linker (2 eq.), HATU (2 eq.), and DIEA (4 eq.) for 1.5 h to afford **9**. After completion of the sequence, the peptide resin was washed with DMF (3 \times 5 mL) and TAMRA was coupled to the freed *N*-terminus using TAMRA (1.5 eq.), HATU (1.5 eq.), and DIEA (3 eq.) for 2 h. Upon dye coupling, the Lys48/ Lys 63 Alloc protection was removed using Pd(Ph₃)₄ (0.4 eq) and PhSiH₃ (40 eq) dissolved in dry DCM for 1h. Upon Alloc removal and subsequent washing with DMF (repeated 5 times) was done, followed by 10 min washing of the resin with 0.5% DIEA/DMF (repeated 3 times). On the Lys48/Lys63 side chain Boc-Cys(trt)-OH was coupled using Boc-AA (4 eq.), HATU (4 eq.), and DIEA (8 eq.). The crude peptide **10** was purified by preparative HPLC using a C4 column with a gradient of 0-60% buffer B over 30 min to afford the corresponding peptide TAMRA-Cys(Acm)-UbLys48/Lys63Cys (**1a/1b**) in ~19% or 10% for **1a** or **1b**, respectively.

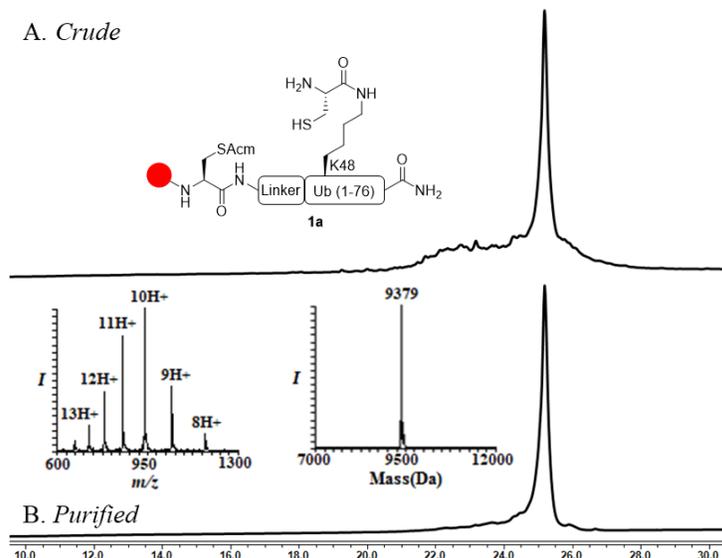


Figure S1: Synthesis of TAMRA-Cys(Acm)-Ub(Lys48Cys)-CONH₂ (**1a**): (A) Analytical HPLC of crude **1a**. (B) Analytical HPLC and mass analysis of purified **1a** with the observed mass 9378 ± 1 Da, calcd 9379 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

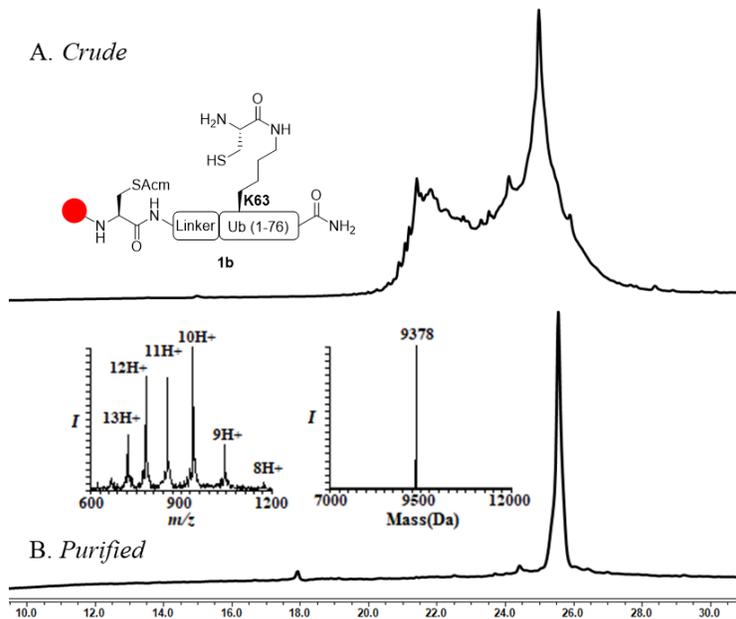
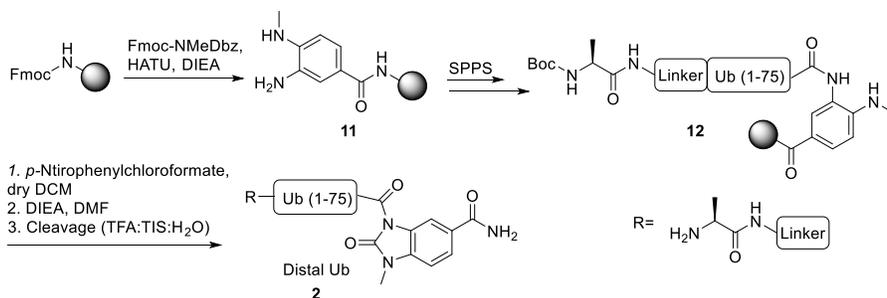


Figure S2: Synthesis of TAMRA-Cys(Acm)-Ub(Lys63Cys)-CONH₂ (**1b**): (A) Analytical HPLC of crude **1b**. (B) Analytical HPLC and mass analysis of purified **1b** with the observed mass 9378 ± 1 Da, calcd 9379 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

10. Synthesis of distal Ub (2)



Scheme S2. Synthetic scheme for **2**.

R-Ub (1-75)-MeNbz was prepared on a pre-swollen Rink amide resin (0.1 mmol) with a MeDbz linker to get **11**.^{2,3} Fmoc amino acids were coupled on an automated peptide synthesizer using amino acids (4 eq.), HCTU (4 eq.), and DIEA (8 eq.). Pseudoproline dipeptides Fmoc-Lue-Thr(ψ Me, MePro)-OH, Fmoc-Ile-Thr(ψ Me, MePro)-OH, Fmoc-Asp(OtBu)-(DMB)Gly-OH and Fmoc-Leu-Ser(ψ Me, MePro)-OH were coupled manually at positions Ile₁₃-Thr₁₄, Lue₈-Thr₉, Asp₅₂-Gly₅₃ and Leu₅₆-Ser₅₇ using the dipeptide (2.5 eq.), HATU (2.5 eq.) and DIEA (5 eq.) for 1.5 h. The resin containing MeDbz linker was then washed in dry DCM and cyclized by treatment with three cycles of *p*-Nitrochloroformate (30 eq. for 30 min in dry DCM) followed by three cycles of DIEA treatment (30 eq. 10 min in DMF) to produce the MeNbz peptide on the solid support to give **12**. Following cyclization, global deprotection was performed using TFA:TIS:water (95:2.5:2.5) cocktail (9 mL for 0.025 mmol peptide resin) for 2 h to give final crude **2**. The cleavage mixture was filtered dropwise to a 10-fold volume of cold ether and centrifuged. The precipitated crude peptide was dissolved in acetonitrile water (1:1) and lyophilized. The crude peptide was purified by preparative HPLC using a C4 column with a gradient of 0-60% buffer B over 30 min to afford the corresponding peptide **2** in ~8 % isolated yield (Figure S2).

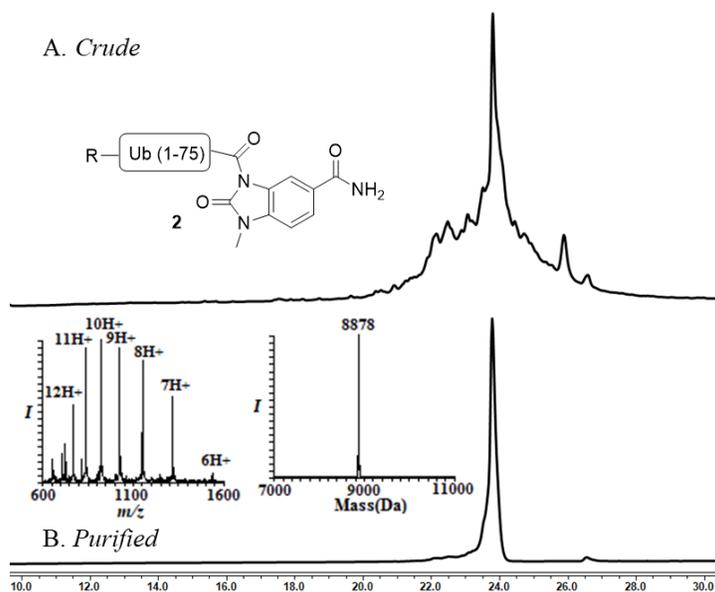
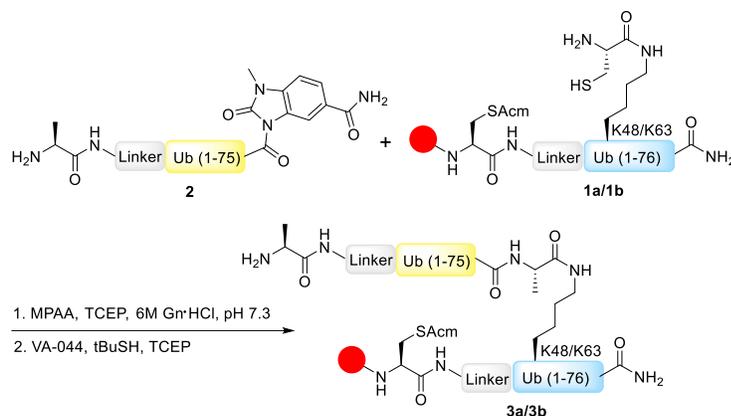


Figure S3: Synthesis of R-Ub(1-75)-MeNbz (**2**): (A) Analytical HPLC of crude **2**. (B) Analytical HPLC and mass analysis of purified **2** with the observed mass 8878 ± 1 Da, calcd 8879 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

11. Synthesis of diUb analogues **3a** & **3b** employing one-pot ligation and desulfurization



Scheme S3: Synthetic scheme for fluorescently labeled diUbs **3a** & **3b**.

In a typical ligation, R-Ub(1-75)-MeNbz (**2**, 6.25 mg, 0.704 μ mol; R= Ala-linker) and TAMRA-Cys(Acm)UbLys48Ub (**1a**) or TAMRA-Cys(Acm)UbLys48Ub (**1b**) 5.08 mg, 0.5416 μ mol) were dissolved in nitrogen-purged 6 M Gn·HCl, 200 mM phosphate buffer (271 μ L, 2 mM) containing 40 eq. MPAA and 20 eq. of TCEP (2.2 mg) at pH 7. The reaction mixture was incubated at 37 $^{\circ}$ C overnight. Progress of the reaction was monitored by analytical HPLC using a C4 column with a gradient of 0-60% buffer B over 30 min. To allow one-pot ligation and desulfurization, dialysis of the ligation mixture was performed against the Gn·HCl buffer to remove the thiol additive MPAA. The reaction mixture was subsequently desulfurized by addition of TCEP (22 mg) to a final concentration of 0.25 M, 40 eq. of VA-044 (7 mg) and 10% volume of t-BuSH (\sim 30 μ L) were added, and the reaction was incubated at 42 $^{\circ}$ C overnight to give **3a** (or **3b**). Progress of the reaction was monitored by analytical HPLC using a C4 column with a gradient of 0-60% buffer B over 30

min. For semi-preparative HPLC, the same gradient was used to isolate **3a** (or **3b**) in ~46% (~30%) isolated yield.

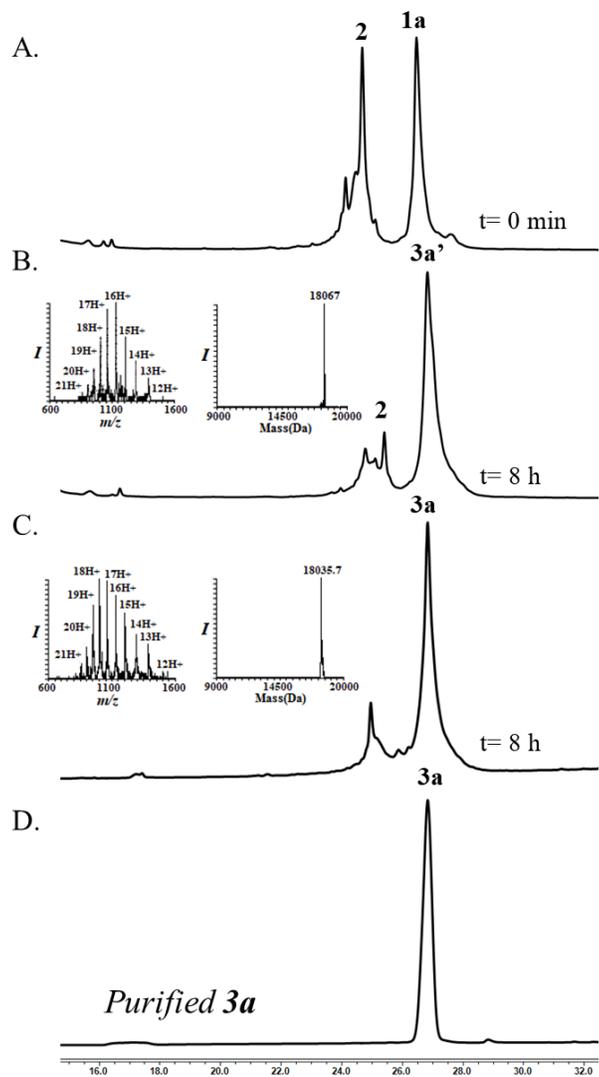


Figure S4: Synthesis of TAMRA-Cys(Acm)-Lys48-linked diUb **3a**. A) Analytical HPLC of ligation between **1a** and **2** at 0 min, B) after 8 h, and C) after one pot desulfurization overnight to form **3a** with the observed mass 18035 ± 0.7 Da, calcd 18036 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

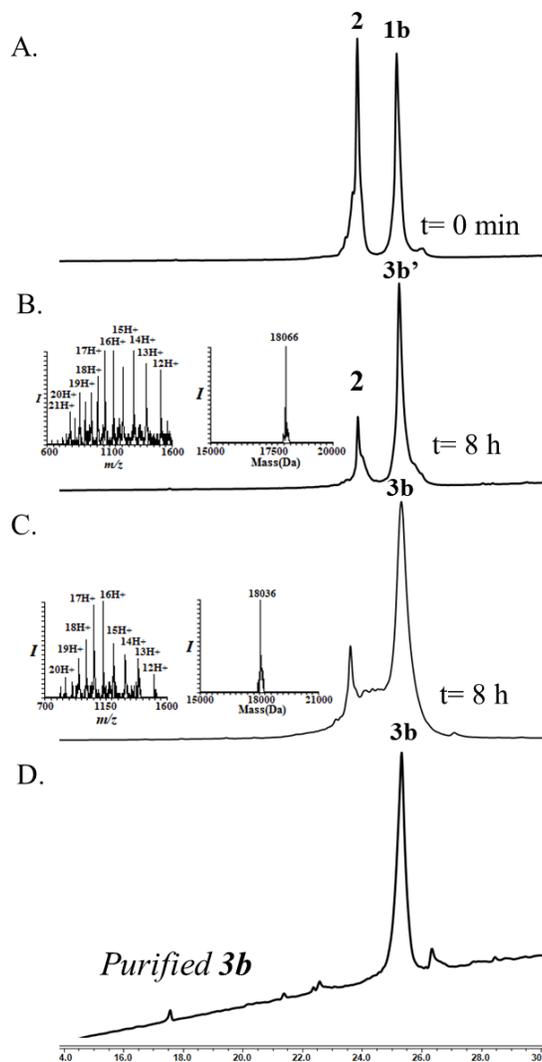


Figure S5: Synthesis of TAMRA-Cys(Acm)-Lys63-linked diUb **3b**. A) Analytical HPLC of ligation between **1b** and **2** at 0 min, B) after 8 h, and C) after one pot desulfurization overnight to form **3b** with the observed mass 18036 ± 0 Da, calcd 18036 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

12. Synthesis of diUbs with free Cys via ACM removal (4a & 4b)



Scheme S4: Synthetic scheme for 4a & 4b.

Cys(Acm) containing diUb **3a** or **3b** (5.8 mg, 0.322 μ mol, 1.0 eq) was dissolved in 222 μ L Gn·HCl buffer. 50 μ L Gn·HCl buffer containing MgCl₂ (100 eq) and 50 μ L Gn·HCl buffer containing PdCl₂ (20 eq) were incubated for 10 min at 37 °C.⁴ After, the PdCl₂ and MgCl₂ containing buffers were added to the diUb solution and incubated for 2 hours at 42 °C. The reaction was quenched by the addition of approximately 12 mg DTT and incubated for another 20 min. The formed precipitate was centrifuged, the supernatant was taken and kept for purification. The formed pellet was washed two times with 200 μ L Gn·HCl buffer. The supernatant was combined with the washing solutions to result in the cysteine deprotected diUb (**4a/4b**).

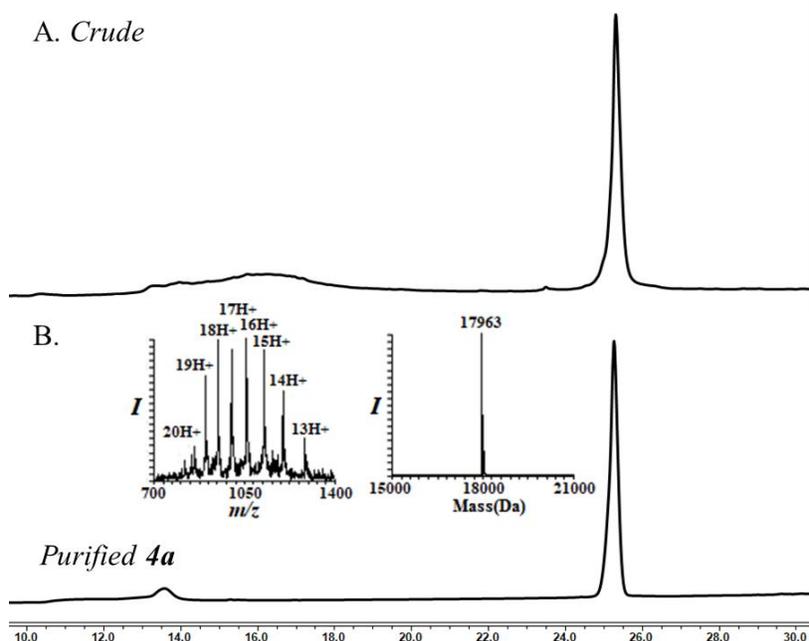


Figure S6: Synthesis of TAMRA-Cys-Lys48-linked diUb (**4a**): (A) Analytical HPLC of crude **4a**. (B) Analytical HPLC and mass analysis of purified **4a** with the observed mass 17963 ± 1 Da, calcd 17963 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

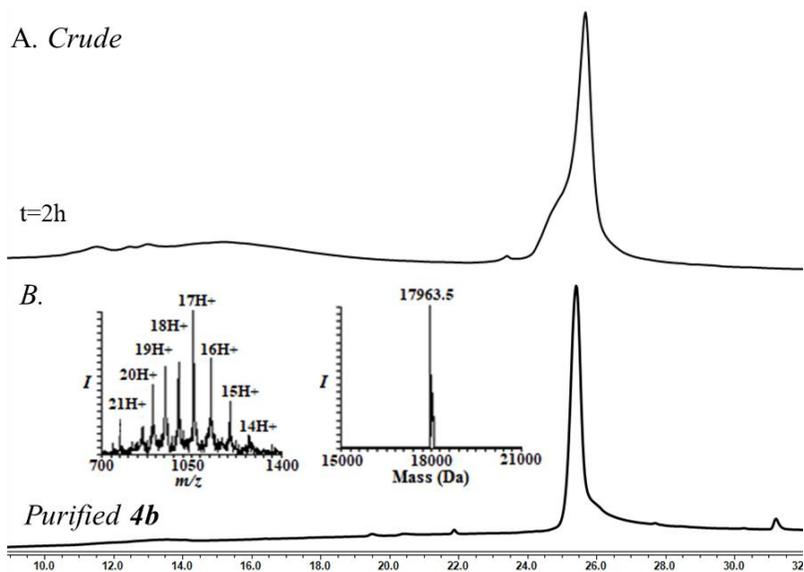
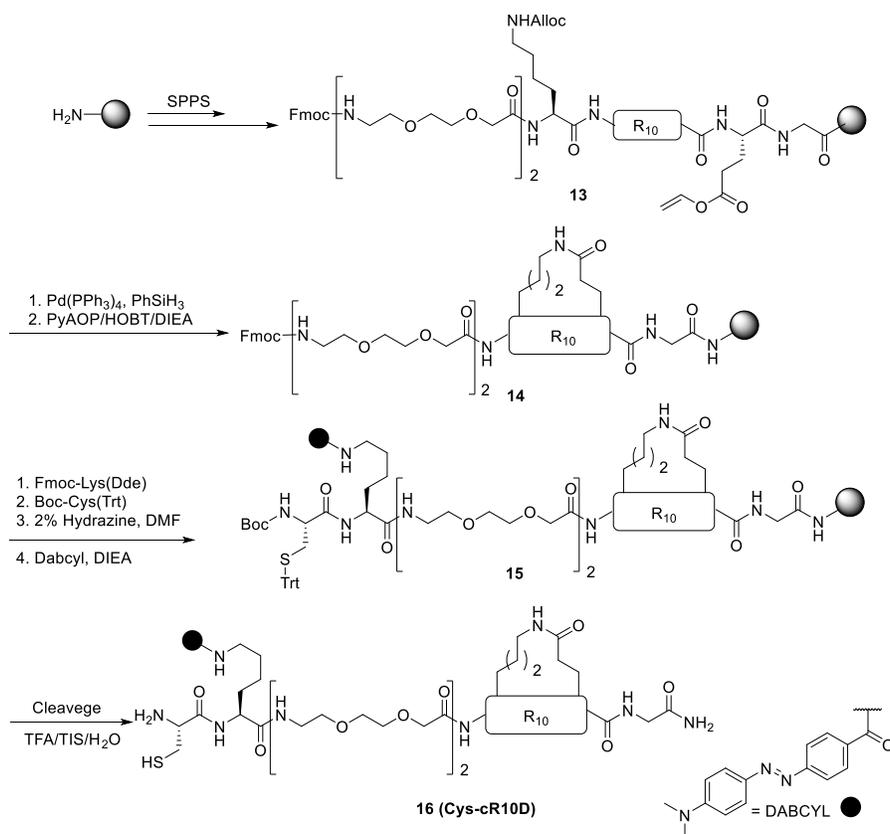


Figure S7: Synthesis of TAMRA-Cys-Lys63-linked diUb (**4b**): (A) Analytical HPLC of crude **4a**. (B) Analytical HPLC and mass analysis of purified **4b** with the observed mass 17963.5 ± 1 Da, calcd 17963 Da (average isotopes). Detection of HPLC chromatogram at 214 nm.

13. Synthesis of Cell-Penetrating peptides (CPPs)

13.a. Synthesis of Cys-cR10D

Cys-cR10D sequence: CK(DABCYL)(Linker)₂KRrRrRrRrRrEG



Scheme S5. Synthesis of **cR10D**. Capital letters correspond to L-amino acids and small letters – D-amino acids.

For synthesizing the **Cys-cR10D**³ peptide **16** (sequence shown in **Scheme S5**) all amino acids (until the linker) were manually double coupled on pre-swollen Rink amide resin (0.1 mmol) using the standard procedure and the linkers [2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic were manually coupled using Fmoc linker (2 eq.), HATU (2 eq.) and DIEA (4 eq.) for 2 h to give **13** (as reported previously).³ Then, Alloc and Oallyl protecting groups were simultaneously removed with the mentioned conditions, and the cyclization was performed on the solid support using PyAOP (5

eq.), HOBt (5 eq.), and DIEA (10 eq.) in DMF (8 ml for 0.1 mmol) for 90 min to afford **14**. After cyclization, the sequence was completed and was followed by Dde protecting group removal by three cycles of 2% hydrazine in DMF for 30 min each cycle to give **15**. Upon deprotection, DABCYL was coupled using the acid (4 eq.), HATU (4 eq.), and DIEA (8 eq.) for 2 h. The peptide was then cleaved as mentioned above and purified by semi-preparative HPLC using a C18 column with a gradient of 0-60% buffer B over 30 min to afford the corresponding **cR10D** peptide **16** in ~25 % yield.

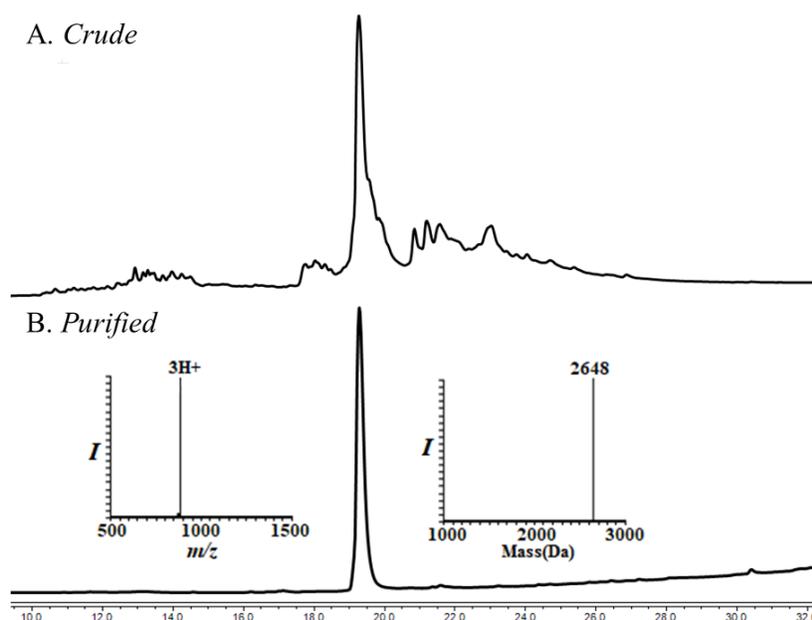
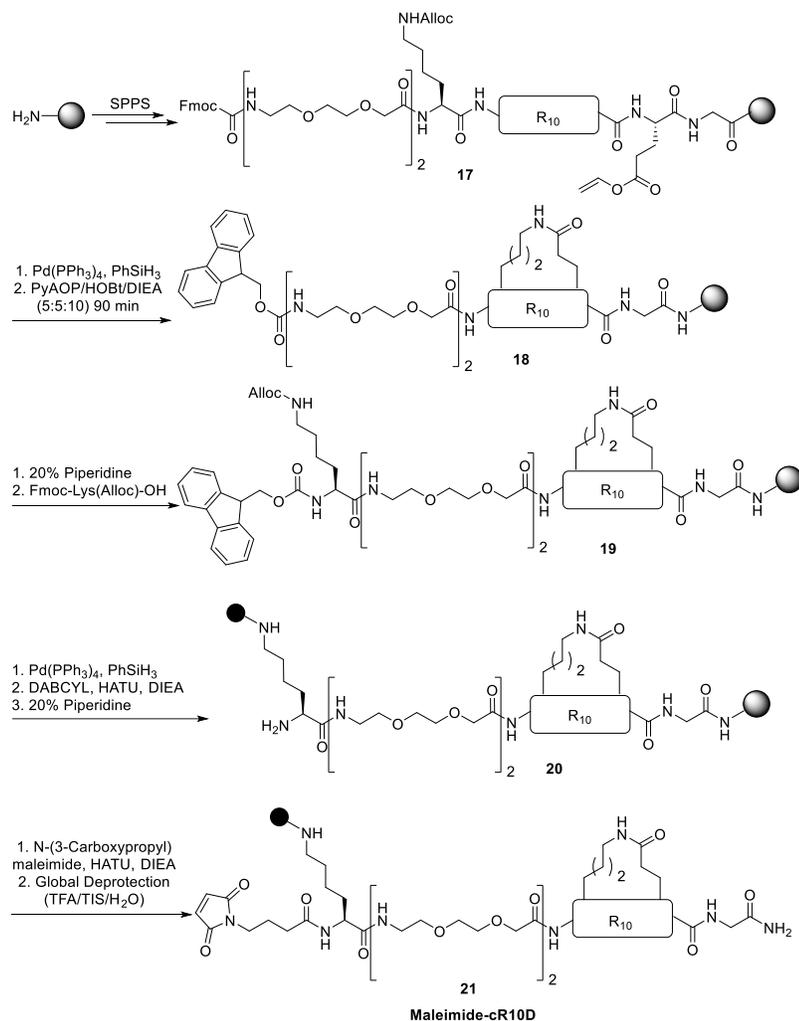


Figure S8: Synthesis of **Cys-cR10D** peptide: (A) Analytical HPLC of crude **Cys-cR10D**. (B) Analytical HPLC and mass analysis of purified **Cys-cR10D** with the observed mass 2648 ± 0.4 Da, calcd 2648 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

13.b. Synthesis of Mal-cR10D

Mal-cR10D sequence: Propionic acid maleimide-K(DABCYL)(Linker)₂KRrRrRrRrRrRrEG



Scheme S6. Synthesis of **maleimide-cR10D**. Capital letters correspond to L-amino acids and small letters – D-amino acids.

For synthesizing the maleimide-cR10D peptide **21** (sequence shown in **Scheme S6**) all amino acids (until the linker) were manually double coupled on pre-swollen Rink amide resin (0.1 mmol) using the standard procedure and the linkers [2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid (abbreviated as linker) were manually coupled using Fmoc linker (2 eq.), HATU (2 eq.) and DIEA (4 eq.) for 2 h to give **17**. Then, Alloc and Oallyl protecting groups were simultaneously removed with the

mentioned conditions, and the cyclization was performed on the solid support using PyAOP (5 eq.), HOBT (5 eq.), and DIEA (10 eq.) in DMF (8 ml for 0.1 mmol) for 90 min to afford **18**. After cyclization and Fmoc deprotection, Fmoc-Lys(Alloc)-OH was coupled followed by the coupling of DABCYL using this acid (4 eq.), HATU (4 eq.), and DIEA (8 eq.) for 2 h. Upon Fmoc deprotection, N-(3-Carboxypropyl) maleimide was coupled using the acid (2 eq.), HATU (2 eq.), and DIEA (4 eq.). The peptide was then cleaved as mentioned above and purified by semi-preparative HPLC using a C18 column with a gradient of 0-60% buffer B over 30 min to afford the corresponding maleimide-cR10D peptide **21** in ~18 % isolated yield.

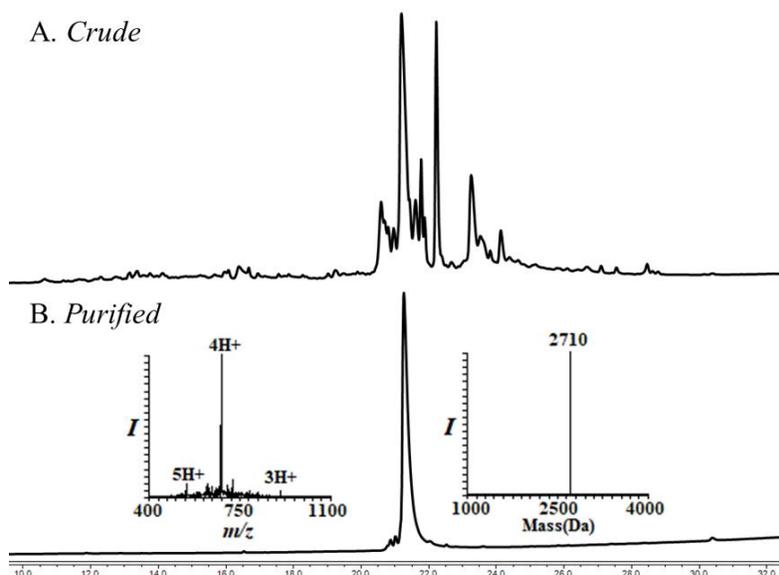
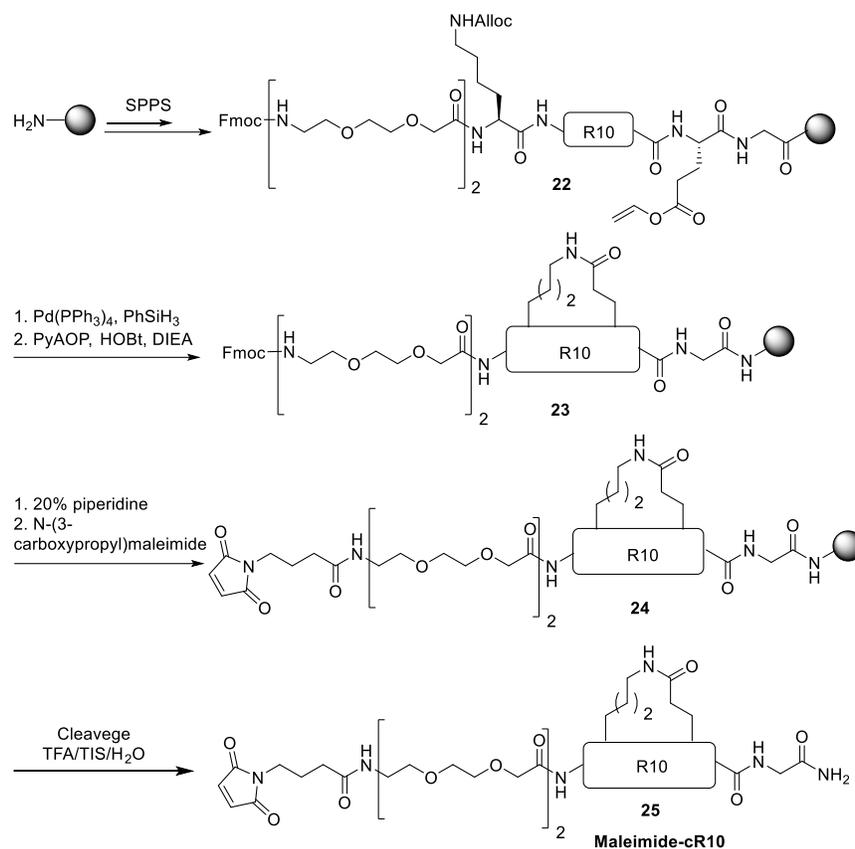


Figure S9: Synthesis of **maleimide-cR10D** peptide: (A) Analytical HPLC of crude **maleimide - cR10D**. (B) Analytical HPLC and mass analysis of purified **maleimide-cR10D** with the observed mass 2710 ± 0.0 Da, calcd 2710 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

13.c. Synthesis of Mal-cR10

Mal-cR10 sequence: Propionic acid maleimide-(Linker)₂KRrRrRrRrRrEG



Scheme S7. Synthesis of **maleimide-cR10**. Capital letters correspond to L-amino acids and small letters – D-amino acids.

The synthesis of **maleimide-cR10**, **25** (scheme S7) was carried out according to section 13. b. where after cyclization followed by Fmoc group removal, N-(3-carboxypropyl) maleimide was coupled using the acid (2 eq.), HATU (2 eq.), and DIEA (4 eq.) to get **24**. The peptide was then cleaved as mentioned above and purified by preparative HPLC using a C18 column with a gradient of 0-60% buffer B over 30 min to afford the corresponding **maleimide-cR10** peptide in ~27% isolated yield.

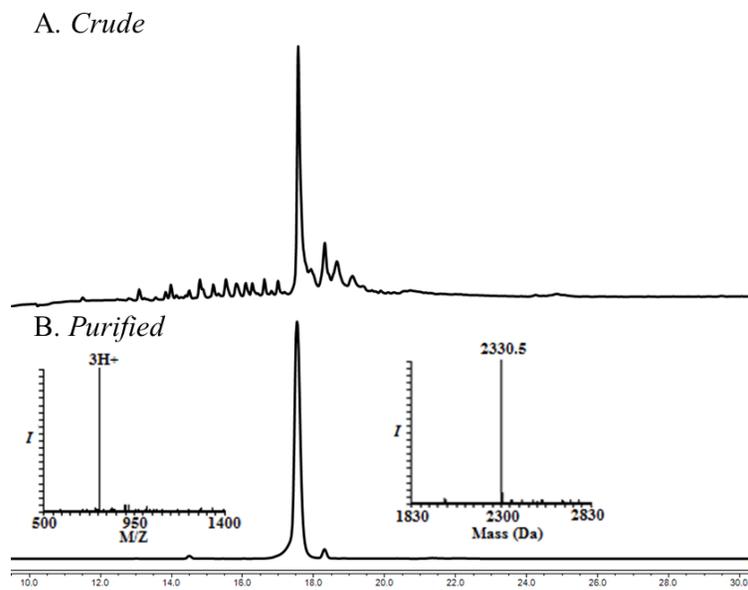
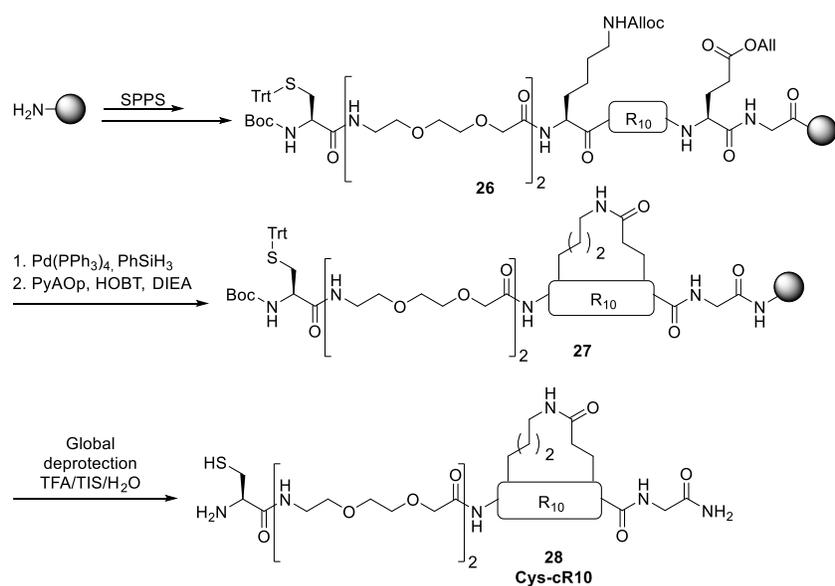


Figure S10: Synthesis of **maleimide-cR10** peptide: (A) Analytical HPLC of crude **maleimide-cR10**. (B) Analytical HPLC and mass analysis of purified **maleimide-cR10** with the observed mass 2330.5 ± 0.5 Da, calcd 2330 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

13.d. Synthesis of Cys-cR10

Cys-cR10 sequence: Cys-(Linker)₂KRrRrRrRrRrEG



Scheme S8. Synthesis of **Cys-cR10**. Capital letters correspond to L-amino acids and small letters – D-amino acids.

The synthesis of **cR10**³ peptide **28** (**Scheme S8**) was carried out according to the section 13.a. to get **26** followed by cyclization to get **27**. The peptide was then cleaved as mentioned above and purified by preparative HPLC using C18 column with a gradient of 0-60% buffer B over 30 min to afford the corresponding **Cys-cR10** peptide in ~17 % isolated yield.

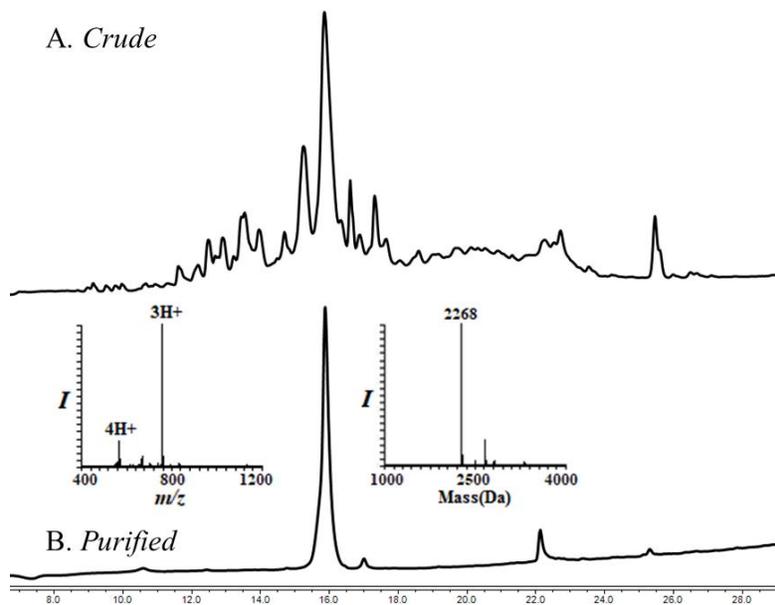
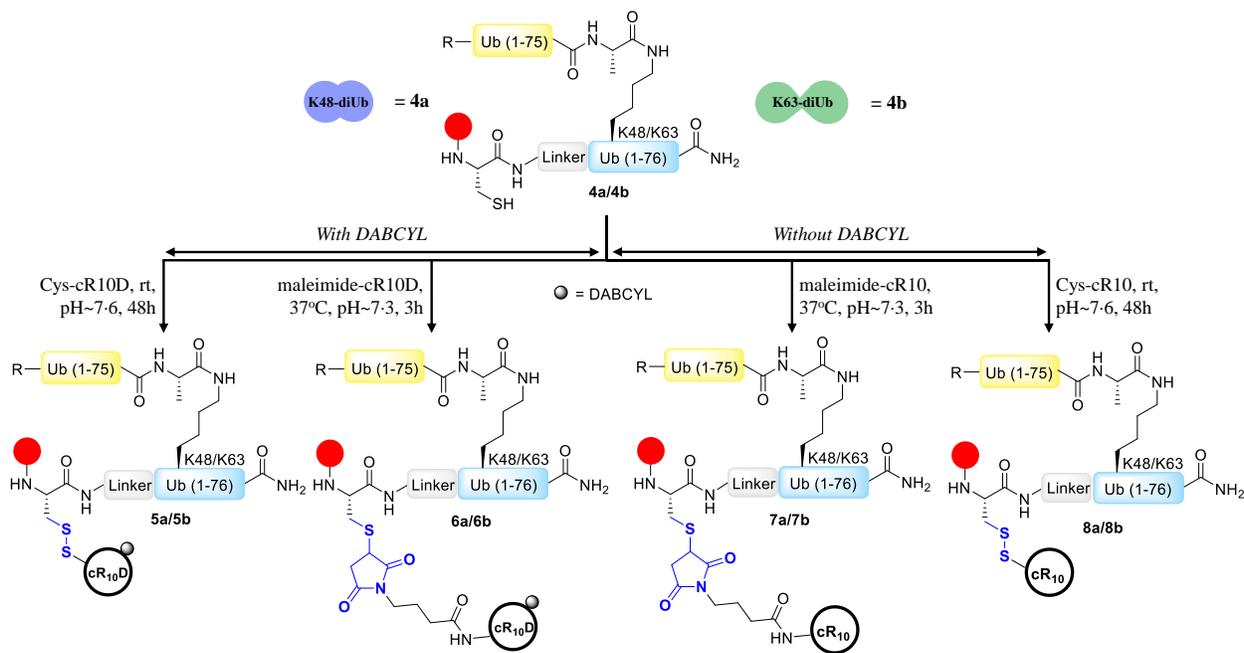
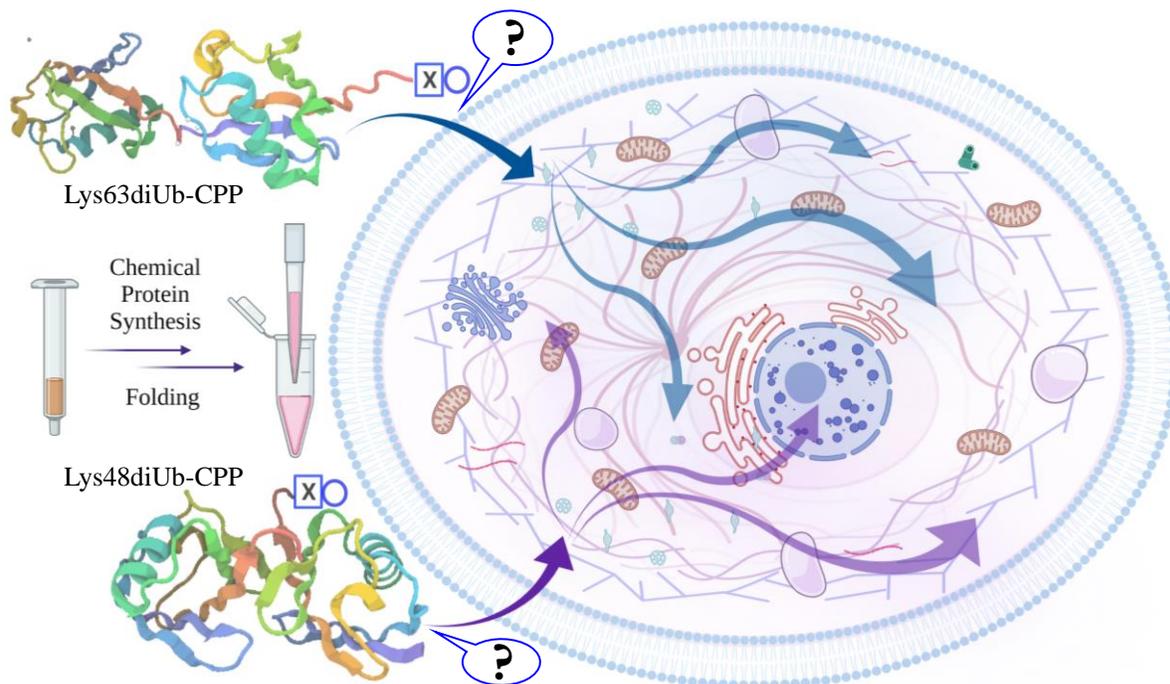


Figure S11: Synthesis of **Cys-cR10** peptide: (A) Analytical HPLC of crude **Cys-cR10**. (B) Analytical HPLC and mass analysis of purified **Cys-cR10** with the observed mass 2268 ± 0.6 Da, calcd 2269 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

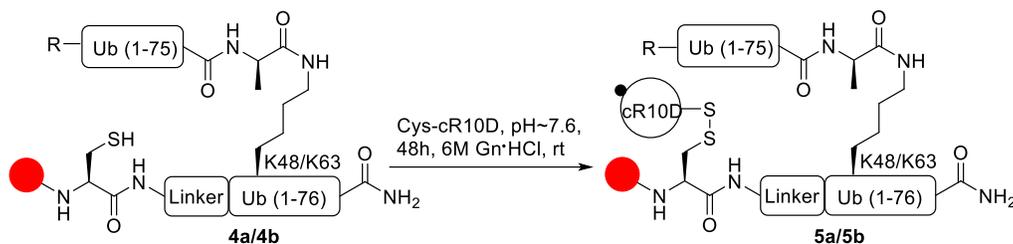
14. Synthetic scheme of all the diUb-CPP conjugates (5a-8a; 5b-8b)



15. Schematic Representation of the general strategy for the live-cell delivery of the fully synthesized diUb proteins conjugated through a linker to CPP (“X” denotes a linker and “o” for cyclic CPP). Created with BioRender.com.



16. Conjugation of Cys-cR10D to diUbs to generate cleavable diUb conjugates **5a** & **5b** for cell delivery



Scheme S9. Synthetic scheme for cleavable diUb-SS-cR10D conjugates **5a** & **5b**.

To conjugate the **Cys-cR10D** to diUb **4a** and **4b**, the diUb was dissolved in 6M Gn·HCl (pH=7.6) in a final concentration of 1mM. The diUb containing solution was added to CPP (lyophilized powder, 15 eq.) and the mixture was kept at room temperature for 48 h. Next, the reaction mixture was purified by preparative HPLC using a C4 column with a gradient of 0-60% buffer B over 30 min to afford the corresponding **diUb-SS-cR10D** conjugates **5a** and **5b** in ~22 % isolated yield. Interestingly, activation of the Cys with 2, 2'-dithionitropyridine (DTNP) in Cys-cR10D to efficiently prepare our desired conjugates as per the previous report did not proceed well (data not shown).

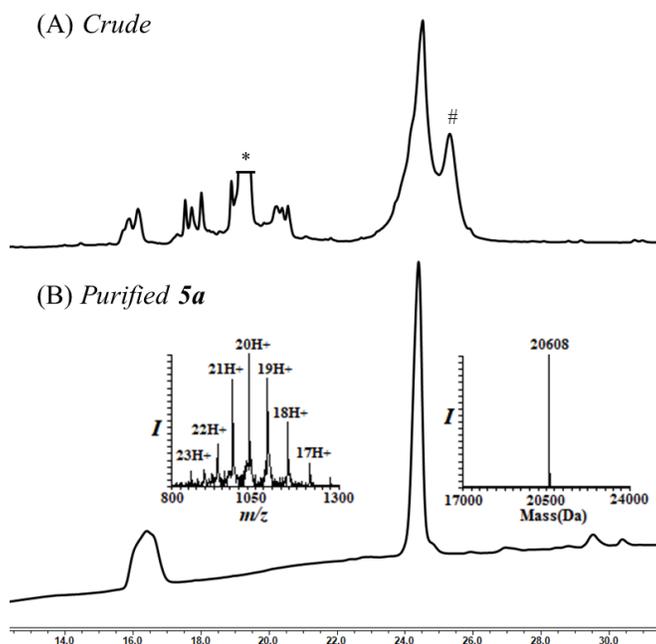


Figure S12: Synthesis of **Lys48-linked diUb-SS-cR10D**: (A) Analytical HPLC of crude **5a**. (B) Analytical HPLC and mass analysis of purified **5a** with the observed mass 20608 ± 2 Da, calcd 20608 Da (average isotopes). Detection of HPLC chromatogram at 214nm. * is CPP dimer; # unidentified mass.

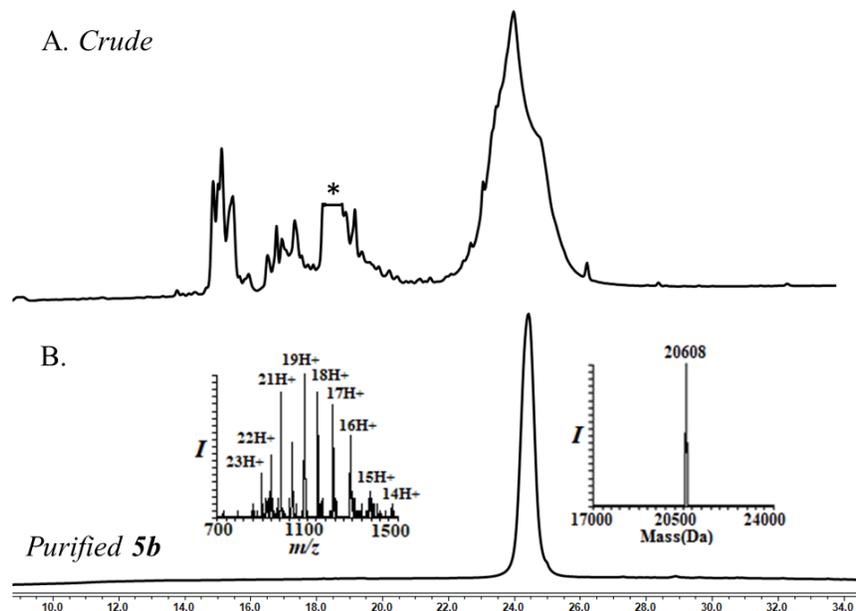
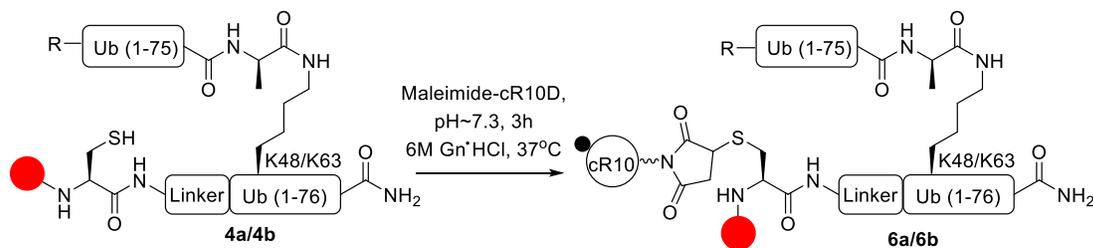


Figure S13: Synthesis of **Lys63-linked diUb-SS-cR10D**: (A) Analytical HPLC of crude **5b**. (B) Analytical HPLC and mass analysis of purified **5b** with the observed mass 20608 ± 0.4 Da, calcd 20608 Da (average isotopes). Detection of HPLC chromatogram at 214nm. * is CPP dimer.

17. Conjugation of maleimide-cR10D to diUbs to generate stable diUb conjugates **6a** and **6b** for cell delivery



Scheme S10: Synthetic scheme for the stable diUb-maleimide-cR10D conjugates **6a** & **6b**.

To conjugate, the Maleimide-cR10D to diUbs, both of the CPP and diUb were dissolved in 6M Gn·HCl (pH=7.3) in a final concentration of 2mM. The CPP-containing solution was added to the diUb solution, and the mixture was kept at 37 °C for 3 h. Next, the reaction mixture was purified by preparative HPLC using a C4 column with a gradient of 0-60% buffer B over 30 min to afford the corresponding **6a** or **6b** conjugates in a ~30 % isolated yield.

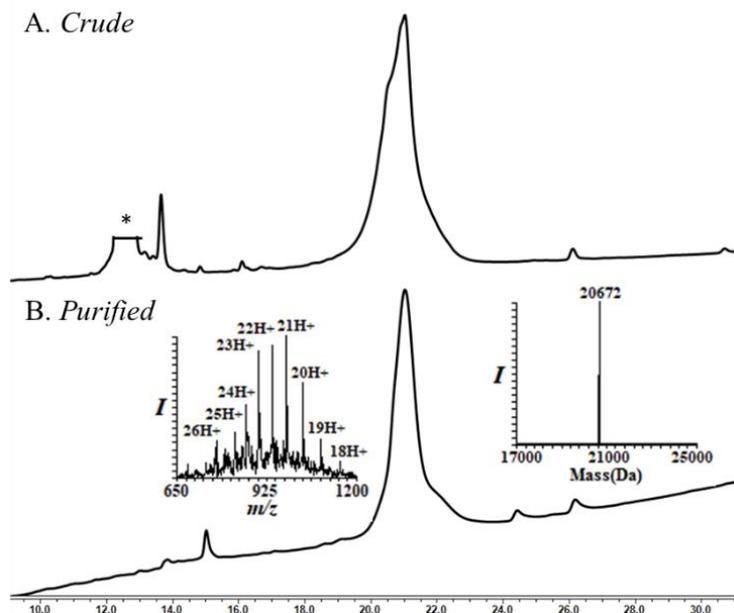


Figure S14: Synthesis of **Lys48-linked diUb-maleimide-cR10D**: (A) Analytical HPLC of crude **6a**. (B) Analytical HPLC and mass analysis of purified **6a** with the observed mass 20672 ± 0.1 Da, calcd 20672 Da (average isotopes). Detection of HPLC chromatogram at 214nm. * is excess CPP.

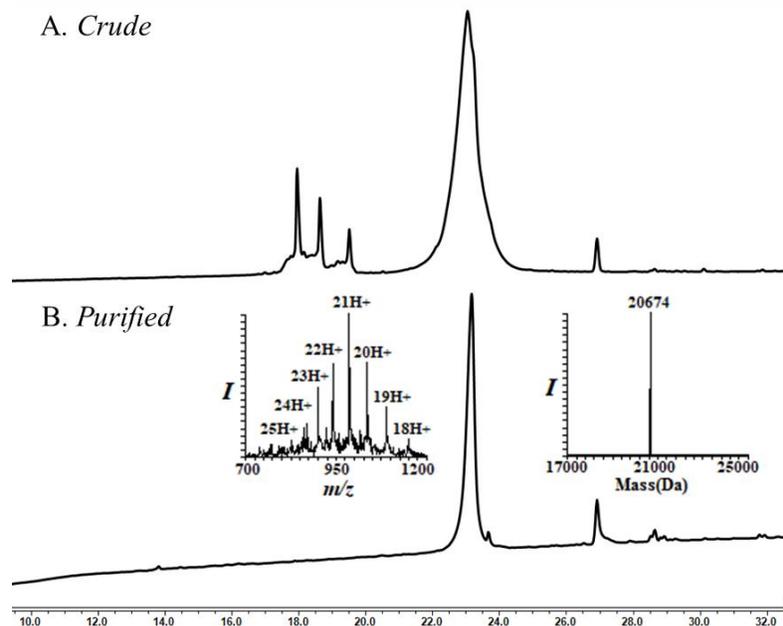
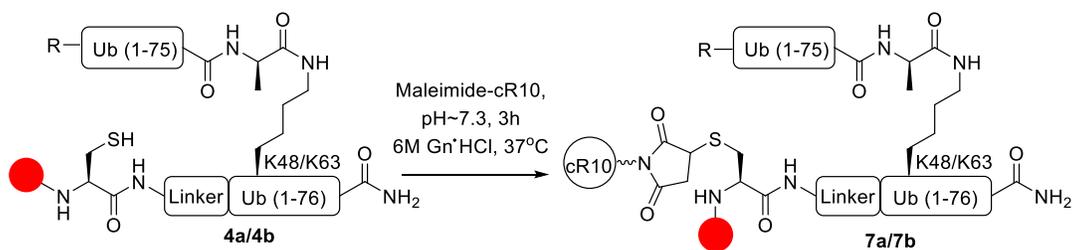


Figure S15: Synthesis of **Lys63-linked diUb-maleimide-cR10D**: (A) Analytical HPLC of crude **6b**. (B) Analytical HPLC and mass analysis of purified **6b** with the observed mass 20674 ± 2 Da, calcd 20672 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

18. Conjugation of maleimide-cR10 to diUbs to form the stable diUb conjugates **7a** & **7b** for cell delivery



Scheme S11: Synthetic scheme for the stable diUb-maleimide-cR10 conjugates **7a** & **7b**.

The synthesis was carried out according to the procedure described in section 18. the reaction mixture was purified by preparative HPLC using a C4 column with a gradient of 0-60% buffer B over 30 min to afford the corresponding **7a** or **7b** conjugates in ~35 % isolated yield.

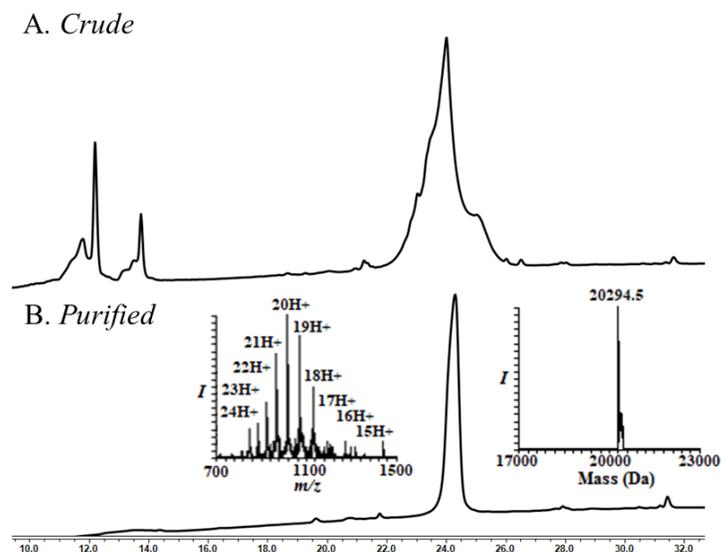


Figure S16: Synthesis of **Lys48-linked diUb-maleimide-cR10**: (A) Analytical HPLC of crude **7a**. (B) Analytical HPLC and mass analysis of purified **7a** with the observed mass 20294 ± 1 Da, calcd 20294 Da (average isotopes). Detection of HPLC chromatogram at 214nm. * is excess CPP.

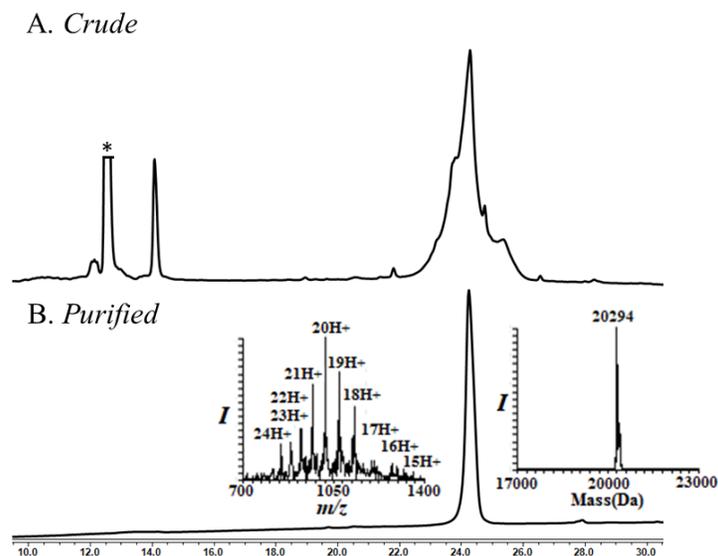
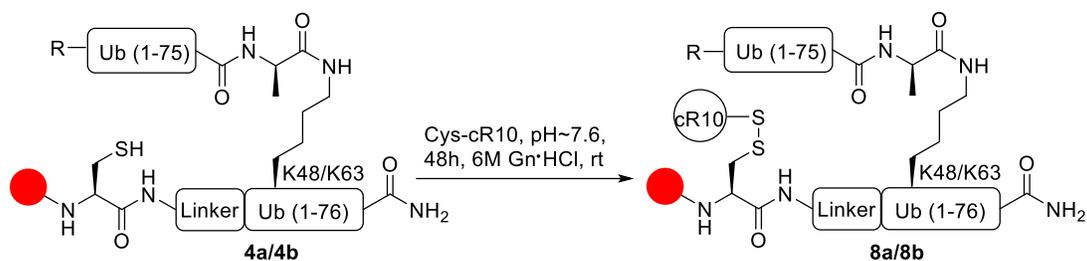


Figure S17: Synthesis of **Lys63-linked diUb-maleimide-cR10**: (A) Analytical HPLC of crude **7b**. (B) Analytical HPLC and mass analysis of purified **7b** with the observed mass 20294.5 ± 0.5 Da, calcd 20294 Da (average isotopes). Detection of HPLC chromatogram at 214nm.

19. Conjugation of Cys-cR10 to diUbs to generate cleavable diUb conjugates **8a** & **8b** for cell delivery



Scheme S12: Synthetic scheme for cleavable diUb-SS-cR10 conjugates **8a** & **8b**.

The synthesis was carried out according to the procedure described in section 17. the reaction mixture was purified by preparative HPLC using a C4 column with a gradient of 0-60% buffer B over 30 min to afford the corresponding **8a** or **8b** conjugates in ~17 % isolated yield.

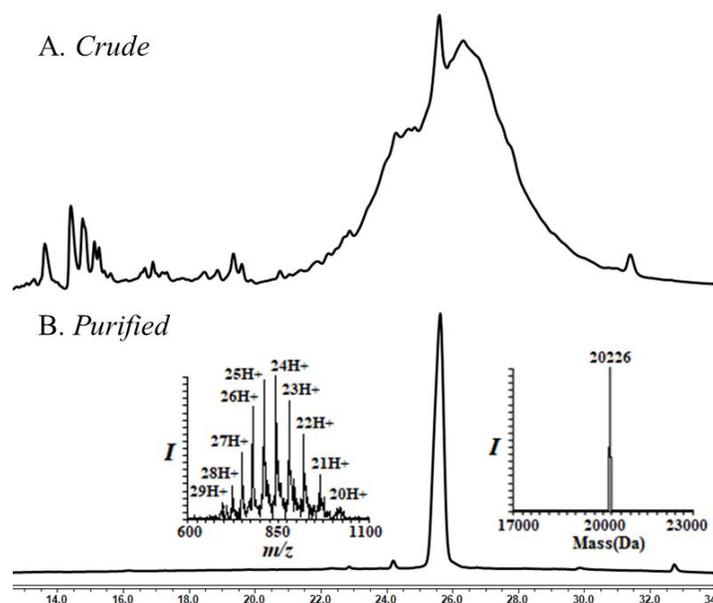


Figure S18: Synthesis of **Lys48-linked diUb-SS-cR10**: (A) Analytical HPLC of crude **8a**. (B) Analytical HPLC and mass analysis of purified **8a** with the observed mass 20227 ± 1 Da, calcd 20226Da (average isotopes). Detection of HPLC chromatogram at 214nm.

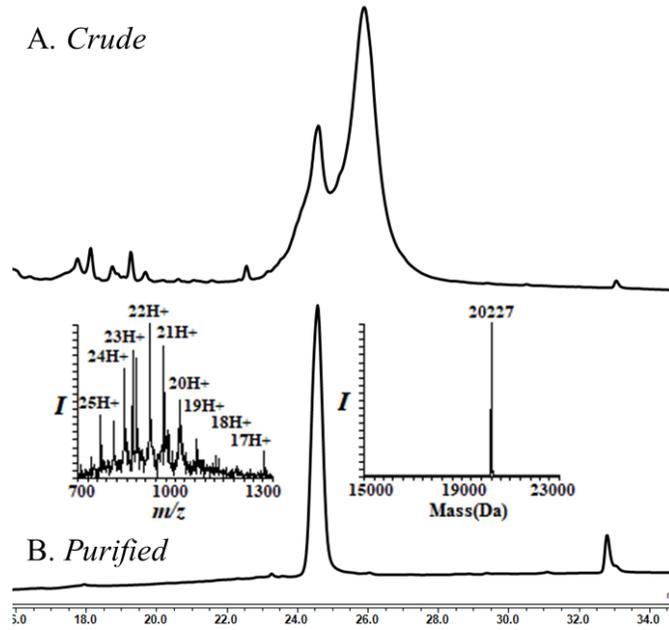


Figure S19: Synthesis of **Lys63-linked diUb-SS-cR10**: (A) Analytical HPLC of crude **8b**. (B) Analytical HPLC and mass analysis of purified **8b** with the observed mass 20226 ± 0.0 Da, calcd 20226Da (average isotopes). Detection of HPLC chromatogram at 214nm.

20. Quantification for the delivery of 5a and 5b in live U2OS cells

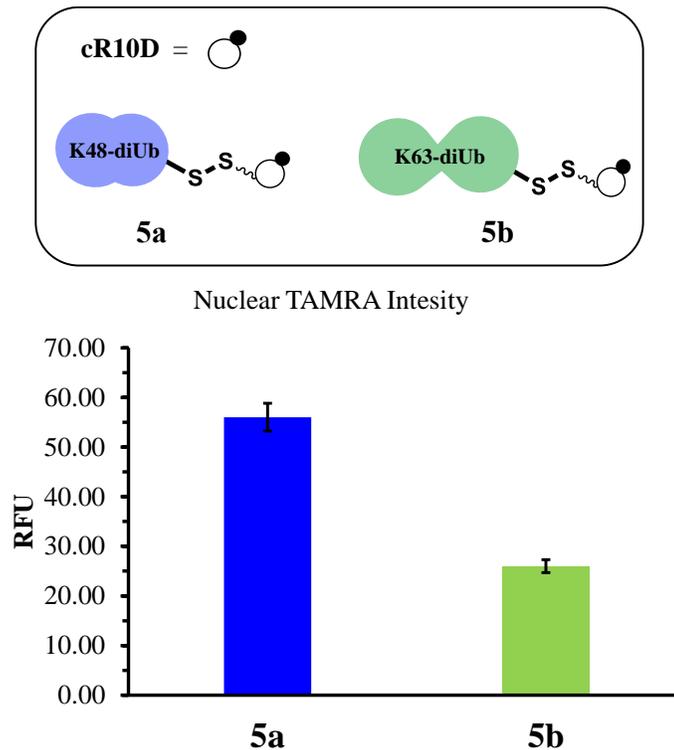


Figure S20: Schematic structures of the conjugates **5a** and **5b**. Cellular uptake quantification of **5a** and **5b**. The result is the average of the three independent sets of experiments, each in biological duplicates. Error bars are the standard deviation of averages.

21. CLSM characterization of fluorescently labelled **6a and **6b** conjugates in live U2OS cells and quantification**

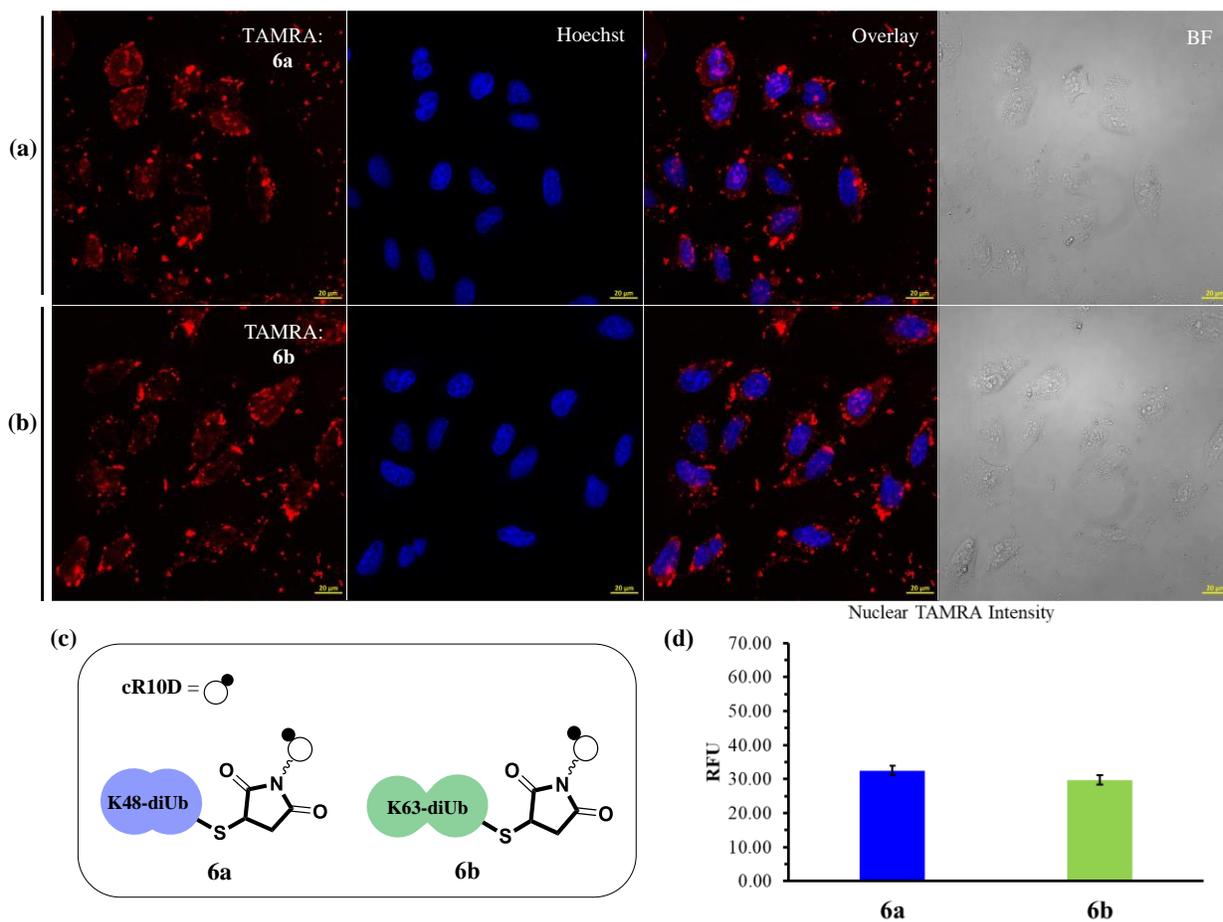


Figure S21: (a) TAMRA fluorescence from **6a** (red), with the corresponding nuclear stain (blue), and bright-field image. The overlay shows both the Hoechst and TAMRA (**6a**) channel together (b) TAMRA fluorescence from **6b** (red), with corresponding nuclear stain (blue) and bright-field image. The overlay shows both the Hoechst and TAMRA (**6b**) channel together (scale bar 20 μm). (c) Schematic structures of conjugates **6a** and **6b**. (d) Cellular uptake quantification of **6a** and **6b**. The result is the average of the three independent sets of experiments, each in biological duplicates. Error bars are the standard deviation of averages.

22. Quantification for the delivery of 7a and 7b in live U2OS cells

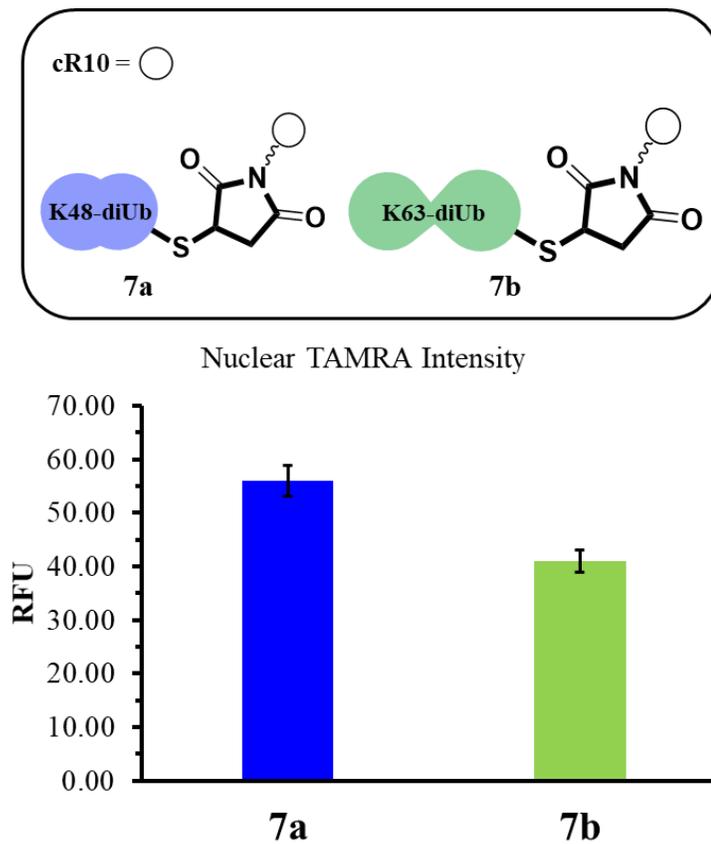


Figure S22: Schematic structures of the conjugates **7a** and **7b**. Cellular uptake quantification of 5a and 5b. The result is the average of the three independent sets of experiments, each in biological duplicates. Error bars are the standard deviation of averages.

23. Representative images for the delivery of 7a & 7b in 6 μ M and 10 μ M concentrations in live U2OS cells

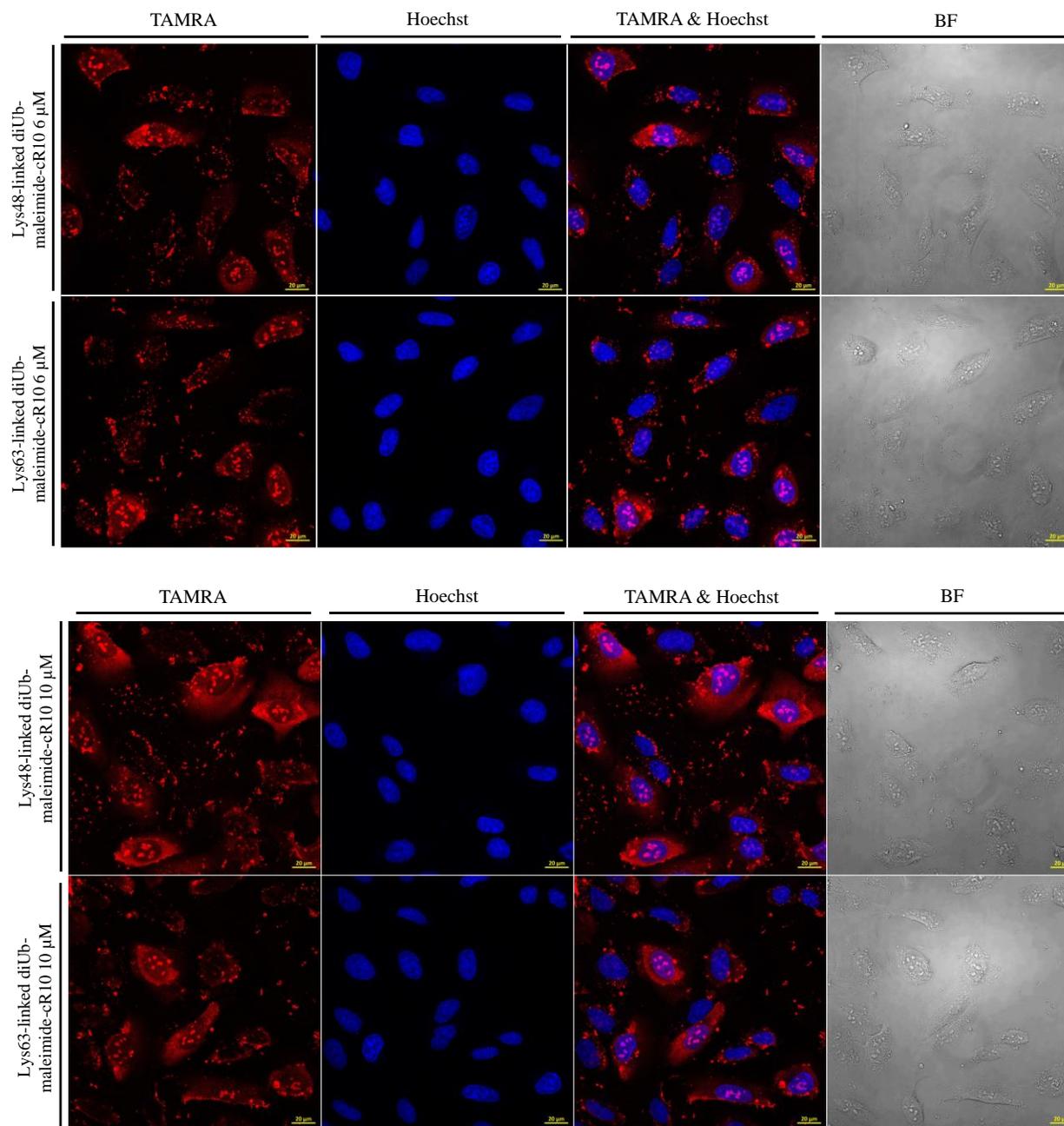


Figure S23. Live-cell delivery at 37 $^{\circ}$ C at varying Concentrations. Scale bars for each of the confocal images 20 μ m.

24. Cellular uptake of 7a can be achieved at reduced temperature

Cells were incubated for 1 hour at 4 °C. Then the cells were placed on ice and the protein complex in solution (also kept in ice previously for 1 hour in a PBS buffer) was added to the cells and incubated for an extra 40 min at 4 °C. The cells were washed three times with PBS and imaged immediately at the confocal microscope.⁵

In both the cases, uptake has been observed. In 5 μ M concentration, the uptake is low but at 6 μ M, the uptake is more pronounced. The diUb has been found in most cells freely distributed in the cytosol and labeling distinctively the nucleolus. This experiment rules out the requirement of any energy dependent cellular uptake pathway, including all endocytosis pathways.

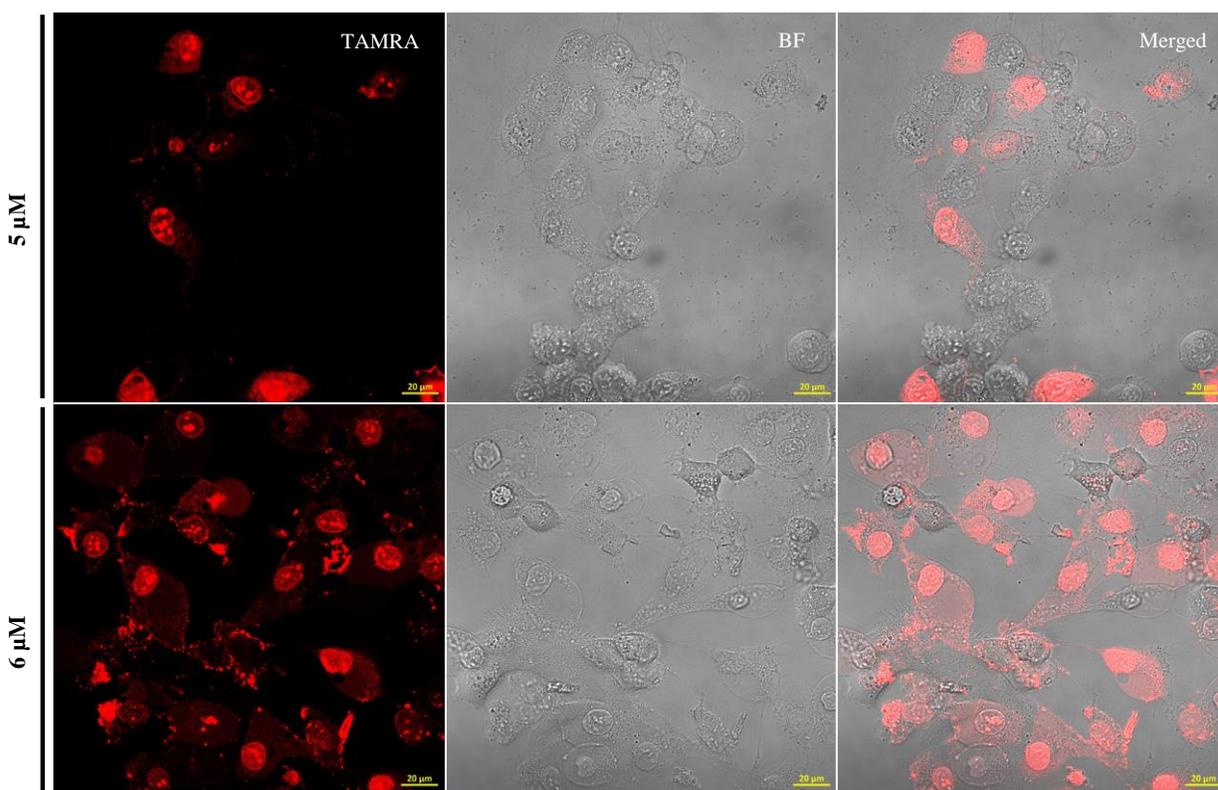


Figure S24. Uptake of 5 μ M and 6 μ M of 7a at 4 °C. Scale bar 20 μ m.

25. CLSM characterization of fluorescently labelled **8a** and **8b** conjugates in live U2OS cells and quantification

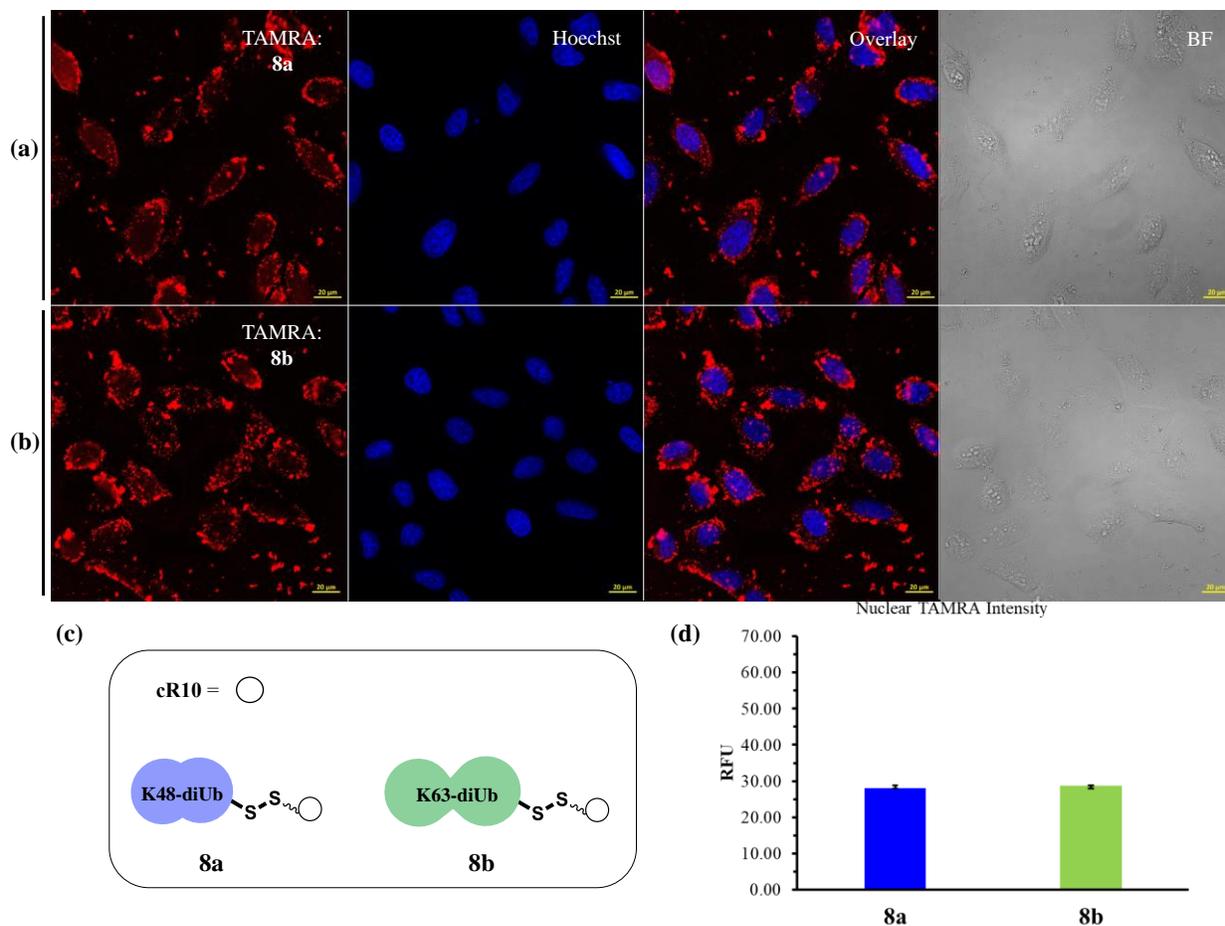


Figure S25. (a) TAMRA fluorescence from **8a** (red), with corresponding nuclear stain (blue), and bright-field image. The overlay shows both the Hoechst and TAMRA (**8a**) channel together (b) TAMRA fluorescence from **8b** (red), with corresponding nuclear stain (blue) and bright-field image. The overlay shows both the Hoechst and TAMRA (**8b**) channel together (scale bar 20 μm). (c) Schematic structures of the conjugates **8a** and **8b**. (d) Cellular uptake quantification of **8a** and **8b**. The result is the average of the three independent sets of experiments, each in biological duplicates. Error bars are the standard deviation of averages.

26. Visualization of the surface charge distribution of the diUbs

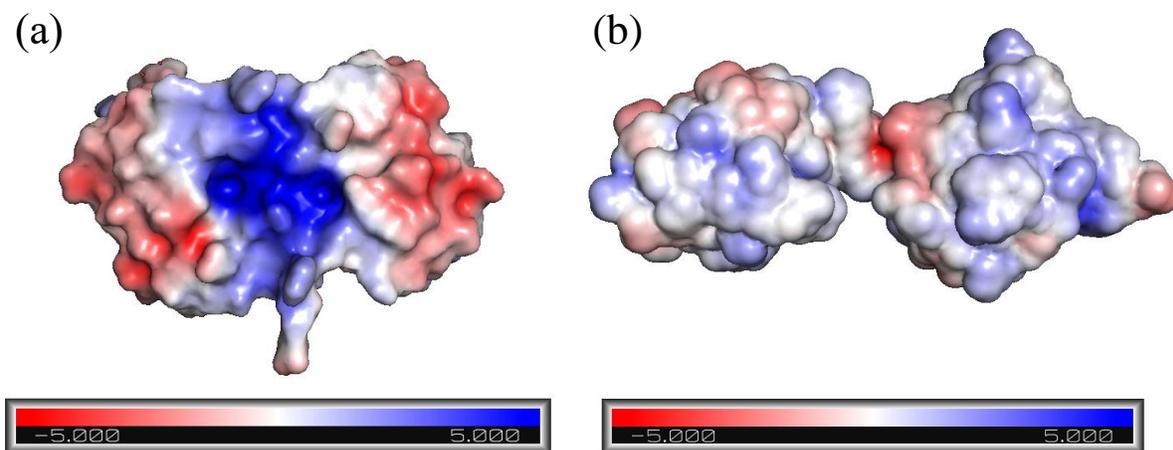


Figure S26. Electrostatic surface visualization of (a) Lys48-linked diUb (PDB: 1AAR) and (b) Lys63-linked diUb (PDB: 2JF5) by APBS plugin in PYMOL. The diUbs are represented by their electrostatic surface with equipotential contours from $-5kT/e$ to $+5kT/e$; the positive potential is painted blue and negative red.

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