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Synthetic tools to study ubiquitin biology

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Chapter 2

Synthesis of isopeptide-linked diubiquitin chains

Adapted from:

Oualid, F.E., **D.S. Hameed**, D.E. Atmioui, H. Hilkmann, and H. Ovaa, *Synthesis of atypical diubiquitin chains.* Methods Mol Biol, 2012. 832: p. 597-609.

El Oualid, F., R. Merkx, R. Ekkebus, **D.S. Hameed**, J.J. Smit, A. de Jong, H. Hilkmann, T.K. Sixma, and H. Ovaa, *Chemical synthesis of ubiquitin, ubiquitin-based probes, and diubiquitin*. Angew Chem Int Ed Engl, 2010. 49(52): p. 10149-53.

Summary

Ubiquitination is an important post-translational modification that determines the fate of a target protein. Proteins can be modified with a single ubiquitin (Ub) or polyUb chains involving one of seven internal lysines or the N-terminal methionine of Ub. The concerted action of three enzymes (i.e., E1, E2 and E3) specify the target protein and also determine the type of ubiquitination involved. Access to all but K48 and K63 chains have been hampered due to the lack of specific E2 enzymes. We present native chemical ligation as an alternative to overcome the tedious biological enzyme ligation. In our approach, we used a thiolysine handle to mimic the property of E2 enzymes and facilitate diubiquitin (DiUb) synthesis. This way, we generated all seven iso-peptide linked diUb molecules.

Introduction

Diubiquitin synthesis using enzymes

Ubiquitination is the process of covalent modification of a target protein by another small protein called ubiquitin (Ub). It involves the concerted action of a specific E1 activating enzyme, an E2 ligase and an E3 conjugating enzyme leading to the formation of different types of ubiquitin conjugates. Targeted proteins can be modified with a single Ub or a polyubiquitin (polyUb) chain where Ub is self-conjugated onto itself via one of its seven internal lysine residues or the N-terminal methionine. Although functions of K48 and K63 linked Ub chains have been studied extensively, the lack of enzyme combinations has hampered access to other lysine-linked chains called the atypical chains.[1-3]

E1 enzymes activate Ub by forming a stable Ub-E1 thioester at the expense of an ATP molecule. In the presence of E2 enzymes, the Ub-E1 thioester undergoes trans-thioesterification to form Ub-E2 thioester. Subsequently, depending on the types of the E3 enzyme, Ub can be directly or indirectly transferred to a substrate protein to form the isopeptide link between the lysine of the target protein and the C-terminus of Ub (Figure 1). PolyUb chains are also formed by these enzyme combinations and lead to specific Ub signaling pathway.[4, 5] Also, mixed linkage polyUb chains have also been identified in cells.[6] The entire process of ubiquitination is reversed by enzymes called deubiquitinases (DUBs) that break down Ub conjugates releasing Ub monomers.[7]

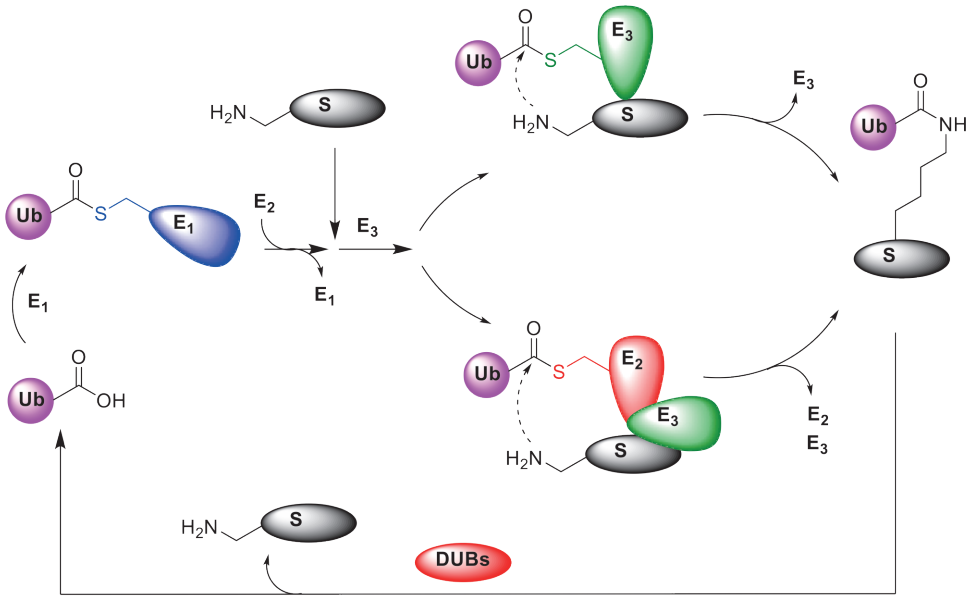

Figure 1: Schematic representation of Ubiquitination and deubiquitination. *Free Ub is activated by UbE1, which is then transferred via E2 and E3 onto a target protein, constituting the process of ubiquitination. On the other hand, Ub monomers are released from conjugates using deubiquitinases (DUBs).*

Enzymatic synthesis of Ub chains has been reported for all but K27 linked chains.[8] In general, K48 and K63 chains are easily generated using specific E2/E3 enzymes.[9] However, for the synthesis of atypical chains like K6, K11, K29 and K33, the enzymes are generally mutated to increase their fidelity to form only one type of chain.[10-15] However, the specificity of these enzymes is limited and there is still a minuscule formation of chains of other linkage types. Normally, these contaminating linkages are eliminated by the use of DUBs that are specific for the unwanted chains.[13, 15] Though enzymatic synthesis yield native isopeptide linked polyUb chains, the use of mutated enzymes and DUBs makes the whole process expensive and less yielding. Secondly, although it is possible to separate polyUb chains of different lengths, it is, however, difficult to restrict the chain-length using enzymatic synthesis approach. Hence, alternative ways to generate ubiquitin chains of specific length is needed. For many *in-vitro* assays to ascertain the linkage specificity of certain DUBs, a simple diubiquitin would be sufficient.[16] This diubiquitin has the basic components of a polyUb chain, containing both the distal and proximal ubiquitin and the different isopeptide linkages between them.

Native Chemical ligation as an alternative to enzymatic synthesis

Native chemical ligation was a technique introduced for the total or a semi-synthesis of a polypeptide.[17] Generally, this technique employs the use of an activated thioester-containing peptide and an N-terminal cysteine-containing peptide. When both peptides are added at neutral or slightly basic pH, the thioester-peptide would undergo trans-thioesterification to form a thioester with the cysteine of the second peptide. This then undergoes intramolecular rearrangement through the nucleophilic attack of the N-terminal

amino group in cysteine residue, thereby forming an irreversible and a stable peptide bond at this site. The kinetics of this reaction is dependent on the type of thioester linkage and the pH of the buffer.

In addition to cysteine which forms a peptide bond in a typical NCL, a novel unnatural amino acid called thiolysine is used in places where an isopeptide bond is needed.[18-20] In this case, a thiol handle is attached at either the δ or γ position of the lysine residue. During NCL, the thioester peptide undergoes trans-thioesterification and with thiolysine. This is followed by an S-N acyl transfer resulting in the intramolecular rearrangement to form the isopeptide bond at the ε amino group of the lysine residue. The thiol handle in thiolysine is then removed by radical-based desulfurization techniques. Thiolysines have been used in the synthesis of ubiquitinated peptides where the position of thiolysine determines the site of isopeptide linkage.[21] We have extended this technique to the synthesis of all seven isopeptide-linked diubiquitins (diUbs).

Chemical synthesis of ubiquitin mutants to facilitate diubiquitin synthesis

Ub has been synthesized using Fmoc-solid-phase peptide chemistry (Fmoc-SPPS) in both linear and modular fashions. Linear synthesis offers the advantage of yielding the desired product directly and in parallel. In particular, the use of dipeptides at a specific location during the linear synthesis of Ub has been instrumental in improving the yield and preventing the formation of aggregated intermediates during synthesis (Figure 2). [22]

One of the major advantages of chemical synthesis of Ub is the ability to specifically incorporate any amino acid at virtually any position. Among the unnatural amino acids, thiolysine has been useful in carrying our native chemical ligations where an isopeptide bond could be made specifically at a certain position.[18-20, 23, 24] For this purpose, Fmoc thiolysines were synthesized and incorporated into the Ub sequence by SPPS. This way, thiolysines was incorporated into positions 6, 11, 27, 29, 33, 48 and 63 thereby making synthetic mutant Ubs.

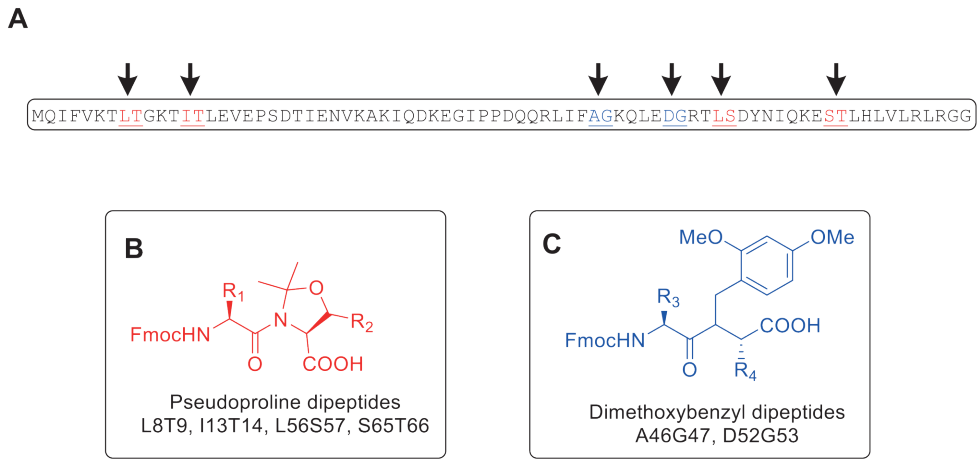

***Figure 2: Fmoc-SPPS strategy to make Ubiquitin**. **A:** Sequence of Ub with positions of dipeptides indicated by black arrow head. **B:** Pseudoproline dipeptides were used in four different positions as indicated. **C:** Dimethoxybenzyl dipeptides were used in two positions in the sequence.*

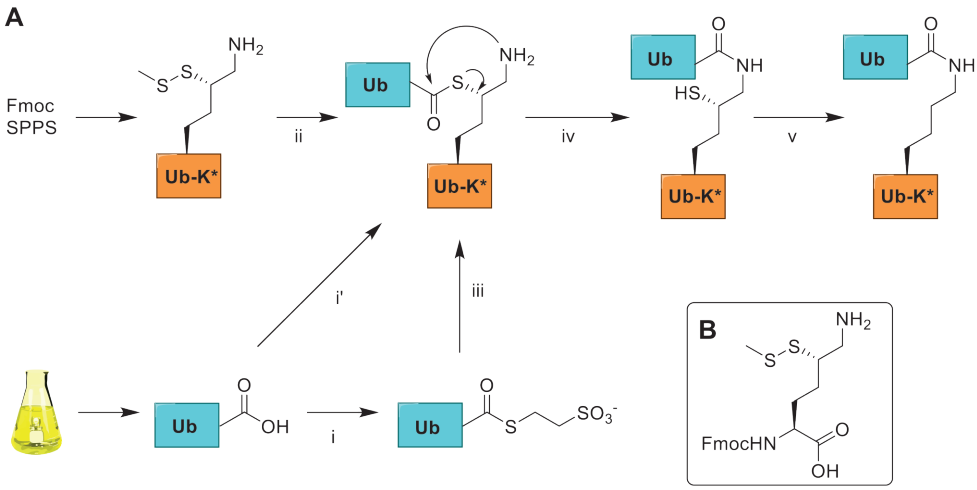

***Figure 3: Schematic representation of diubiquitin synthesis**. **A:** The distal Ub is obtained from bacterial expression system, while the proximal Ub is obtained from Fmoc-SPPS. i) UbE1, MESNa, ATP, $MgCl_2$, Sodium phosphate buffer pH 8.0 and then pH 6.5 after reaction is completed ; i') UbE1, MESNa, MPAA, ATP, $MgCl_2$, Sodium phosphate buffer pH 8.0; ii) TCEP, 6M Gnd.HCl pH 8.0; iii) MPAA, Gnd.HCl pH 8.0; iv) N→C acyl transfer; v) V-50, TCEP, Gnd.HCl pH 7.0; **B:** Structure of Fmoc δ-thiolysine used in the synthesis.*

Diubiquitin ligation reaction was carried out according to a native chemical ligation scheme described in figure 3. On one hand, Ub was synthesized or expressed as a full-length protein and activated as a thioester using the UbE1 enzyme and MESNa. This thioester can be generated *in-situ* during NCL by using UbE1 or isolated to yield Ub-MESNa thioester which is stable under acidic conditions. On the other hand, mutant Ub containing thiolysines at specific lysine positions was synthesized exclusively by SPPS. A native chemical ligation between the two-ubiquitin species formed a diubiquitin molecule with an isopeptide bond at the desired lysine position. A subsequent desulfurization step resulted in the removal of the thiol handle in lysine, leading to the formation of native isopeptide linked diubiquitin molecules.[25, 26]

Potential applications in assay of DUBs

Generally, diubiquitins can be used in characterizing the DUB preference for a particular linkage, especially in the context of polyubiquitin chains attached to a target protein.[27-29] DUBs normally fall under two categories – cysteine protease and metalloprotease class.[28, 30] The Cysteine protease class of DUBs is sub-divided into four families based on the conserved domains. These DUBs can either be specific for a substrate protein or non-specific where only the distal Ub is sufficient to invoke the DUB activity.[28] Also, linkage specificity is also attributed to DUBs. However, the linkage preference of the DUBs has been characterized only to a certain extent, and so it is important to know which DUB cleaves which kind of ubiquitin chain. This can also be used for establishing the functional role of DUBs in cells and thereby facilitating the development of therapeutic agents that can target a particular DUB based on its linkage specificity and functions.[31-33]

Results and Discussion

Expression of Ub and UbE1

Ub was expressed as a fully functional protein using a bacterial expression system. In our case, we expressed Ub in BL21 Rosetta cells. Harvesting Ub without any purification handle was possible due to its thermal stability. The expressed cells were simply heated up to 90 °C and then centrifuged to isolate Ub with more than 80% purity. Further purifications using FPLC followed by HPLC yielded lyophilized Ub (Figure S1).

Ub activating enzyme, E1 was also expressed in a bacterial expression system. Hexa-His-tagged UbE1 protein was expressed at 18 °C using IPTG induction and then the cells were lysed and spun down. Then, UbE1 in the supernatant was isolated by metal affinity chromatography using TALON beads followed by the removal of imidazole by buffer exchange using Amicon spin columns (Figure S2). Finally, the enzyme was concentrated and used in native chemical ligation experiments.

Solid Phase synthesis of Ub thiolysine

We have developed a high-yielding linear Fmoc-SPPS of Ub that allows the incorporation of desired tags and mutations as well as both the C- and N-terminal modifications. As linear syntheses yield desired products directly and in parallel, a significant advantage over modular approaches, we revisited the linear chemical synthesis of Ub. We decided to incorporate pseudoproline building blocks and dimethoxybenzyl (DMB) dipeptides (Figure 1A), which prevented the formation of folded and/or aggregated intermediates on-resin, resulting in a high yielding linear synthesis of Ub and Ub mutants.

We used Fmoc δ-thiolsyine that is protected in the free thiol as a methyl disulfide. This way, Fmoc thiolysine was incorporated in positions 6, 11, 27, 29, 33, 48 and 63 during the linear synthesis of Ub. After synthesis, these mutant Ub molecules are purified by FPLC and HPLC to yield the desired thiolysine-Ub molecule for native chemical ligation.

Native Chemical ligations and desulfurization

The native chemical ligation (NCL) reactions were carried out in both native buffer conditions and in denaturing buffer conditions. For reactions in the native buffer, UbE1 was added directly to the ligation mix containing Ub, ATP, $MgSO_4$ and the thiolysine-containing Ub. However, in such reactions, the thiolysine-Ub can also form thioester with E1 and result in the formation of polyUb chains. To avoid this, we used a mutant thiolysine-Ub that contained valine instead of glycine at position 76. This way, only the Ub w.t. that doesn't have the thiolysine handle, would form the activated Ub-thioester and react specifically with the thiolysine containing Ub. Initial experiments with E1 using only Ub and Ub thiolysine (G76V) also formed diubiquitins. However, positions 27 and 29 were difficult to make, owing to the inaccessibility to the enzyme-Ub intermediate (Figure 4A).

For NCL under denaturing conditions, it is necessary to make a stable Ub-thioester molecule. For that purpose, the addition of E1 along with Ub, ATP, MgCl2 and MESNa formed the Ub-MESNa thioester. After the reaction was complete, the Ub-MESNa thioester was kept stable under acidic conditions and purified as a lyophilized powder. This resulted in the formation of all seven isopeptide linked diubiquitin chains with a yield of around 15% (Figure 4B-I, S3).

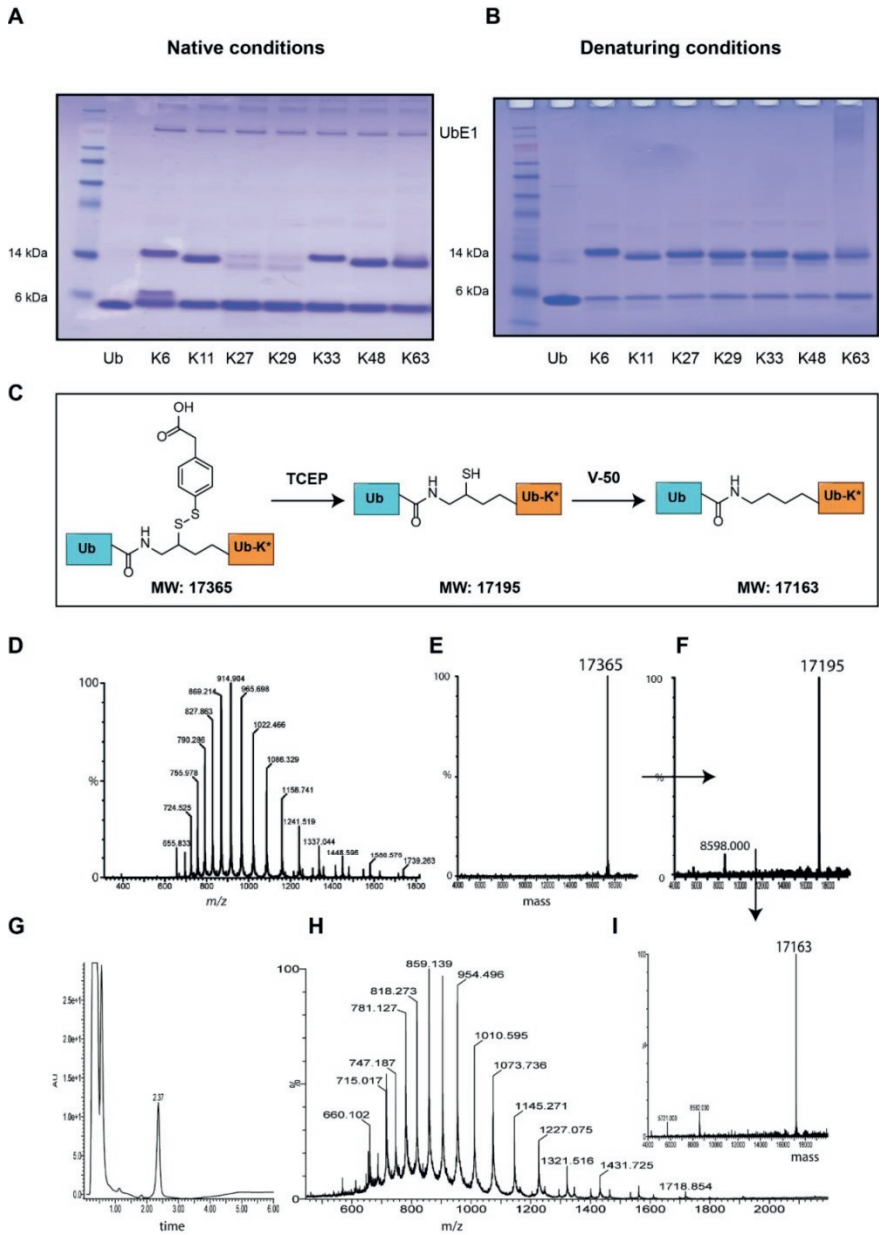

***Figure 4**: **A:** diUb synthesis under native buffer conditions using UbE1 enzyme; **B:** diUb synthesis under denaturing conditions using purified Ub-MESNa thioester; **C:** Schematic representation of desulphurization of diubiquitin using V-50; **D**: Mass spectra of K33 diUb showing the protein envelope; **E:** deconvoluted spectra showing MPAA adduct of K33 diUb; **F:** deconvoluted spectra of K33 diUb after reduction of MPAA using TCEP; **G:** UV chromatogram of desulfurized K33 diUb; **H:** Mass spectra of K33 diUb after desulfurization; **I:** deconvoluted mass spectrum of desulfurized K33 diUb showing removal of thiol (-32 Da).*

Purifications and validation

Normally, ubiquitins are purified using reverse-phase HPLC. Due to their efficient refolding capacity, the proteins can be lyophilized and subsequently refolded from DMSO into water or buffer of choice. However, separating diubiquitins from mono ubiquitin was difficult owing to similar hydrophobicity of both the molecules. However, the use of a 300 Å pore sized C18 columns in HPLC provided sufficient resolution to isolate diubiquitins from mono ubiquitin, as observed by SDS PAGE and validated using LCT-MS (Figures S4-S10).

Synthetic diUbs are validated for their biological function by assessing their recognition by DUBs. As mentioned earlier, DUBs can cleave ubiquitin chains based on the type of linkage.[28, 30] This requires recognition elements on the surface of DUBs which were identified using diUb probes. [34] To characterize the specificity of DUBs, diUb reagents are used. For example, an exclusive analysis of the linkage specificity of the USP family of DUBs has been done using our diUb reagents.[35] Moreover, our diUb reagents were also used to identify linkage specificity of an ovarian tumor (OTU) domain DUB called TRABID.[36] Very recently, our reagents were used to identify the specificity of USP7 and their role in cellular stress.[37]

Conclusions

We were able to synthesize diUbs from Ub precursors which were partly made synthetically. Ub expression was optimized to a yield higher quantity of protein and their purification was made easy due to the thermal stability of Ub to higher temperatures. UbE1 was also expressed by the auto-induction procedure yielding a higher quantity of active enzymes. These were used to successfully make Ub-thioester which can either be purified or used *in-situ*. Synthetic Ub containing thiolysine at specific sites were made using SPPS with high yield and efficiency due to the use of dipeptides in positions that are deemed difficult.

The synthesis of Ub thiolysine was instrumental in making all isopeptide linked diUbs without the need for E2 and E3 enzymes. This also restricts the synthesis to diUb and not the higher-order Ub chains which are one of the drawbacks when using the enzymatic route. This way, we synthesized all seven isopeptide linked diUb molecules.

Finally, we used these synthetic diUbs to establish the functional properties of enzymes from the USP family of DUBs. We observed that these DUBs preferred linkages that are easily accessible while there was some hydrolysis observed for the difficult linkages. These linkages were found in lysine positions that are hidden in the alpha-helix structure of the proximal Ub.

Experimental section

General

All aqueous solutions are prepared with ultrapure water (milliQ, prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25°C) and analytical grade reagents. Unless indicated otherwise, reagents are prepared and stored at room temperature. Unless indicated otherwise, chemicals are obtained commercially of the highest available grade. Fmoc-δ-thiolysine-OH (**D**, Fig. 1) was prepared as reported.[38] Solid Phase Peptide Synthesis was performed on a Syro II MultiSyntech Automated Peptide synthesizer. LC-MS measurements were made on a Waters 2795 Separation Module (Alliance HT) and Waters 2996 Photodiode Array Detector (190-750 nm) using the following conditions: Flowrate was at 0.8 mL/min for 6 min, using 2 mobile phases: A= 1% CH_3CN, 0.1% formic acid in water and B= 1% water and 0.1% formic acid in CH_3CN. Preparative cation-chromatography was used to purify Ub and Ub thiolysine at 4°C with an ÄKTA Unichromat 1500- "PRO" system

(15x185 mm column packed with Workbeads 40S™) using 2 mobile phases: 50 mM NaOAc, pH 4.5, and 1M NaCl in 50 mM NaOAc, pH 4.5. Flowrate is 5 mL/min. Purification of Ub and thiolysine-Ubs were performed on preparative reverse-phase HPLC purifications on a Shimadzu Prominence system using 2 mobile phases: A= 0.05% TFA in water and B= 0.05% TFA in CH_3CN. The column temperature was set at 40°C, flowrate was 7.5 mL/min and the UV-detection was performed at 230 and 254 nm. Quality analysis of the mass of synthesized Ub was done on the analytical column: Phenomenex Kinetex C18, (2.1x50 mm, 2.6 μM), the column at T= 40°C. For this, the following gradient was used: 0→0.5 min: 5% B; 0.5→4 min: 5 to 95%B gradient; 4→5.5 min: 95% B. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1 (deconvolution with Maxent 1 function).

Solid Phase Peptide Synthesis (SPPS) Ubiquitin polypeptide

SPPS reagents and standard fluorenylmethyloxycarbonyl (Fmoc) protected amino acid building blocks like benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate [PyBOP], diisopropylethylamine [DiPEA], 1-hydroxybenzotriazole (HOBt), acetic anhydride (Ac_2O), piperidine, trifluoroacetic acid [TFA] were obtained from Biosolve, Sigma-Aldrich and Novabiochem. Fmoc protected pseudoproline and dimethoxybenzyl (DMB) protected dipeptides like Fmoc-L-Ser(*t*Bu)-L-Thr($\Psi^{Me,Me}$pro)-OH, Fmoc-L-Leu-L-Ser($\Psi^{Me,Me}$pro)-OH, Fmoc-L-Ile-L-Thr($\Psi^{Me,Me}$pro)-OH; Fmoc-L-Leu-L-Thr($\Psi^{Me,Me}$pro)-OH, Fmoc-L-Asp(O*t*Bu)-(Dmb)Gly-OH and Fmoc-L-Ala-(Dmb)Gly-OH were bought from Novabiochem. Wang type resin pre-loaded with 0.2 mmol/g Fmoc-Gly-OH were obtained from Applied Biosystems. Peptide synthesis grade organic solvents that include *N*-methylpyrrolidinone [NMP]) were bought from Biosolve (*see* **Note 1**). Work-up solvents like phenol, triisopropylsilane [iPr_3SiH], diethyl ether [Et_2O], *n*-pentane were obtained from Aldrich. Two sets of cocktail mixtures were used for the synthesis. Cocktail A (TFA cleavage mix): TFA, H_2O, Phenol, iPr_3SiH (90.5/5/2.5/2 v/v/v/v). Cocktail B (Freeze dry mix): H_2O, CH_3CN, HOAc (65/25/10 v/v/v)

All Fmoc-protected amino acid building blocks were dried overnight under high vacuum (*see* **Note 8**). We used Fmoc-Gly-Wang resin (25 μmol) for Fmoc SPPS in NMP using PyBOP (4 equiv) and DiPEA (8 equiv). The following settings are applied:

For the first 30 cycles: a) single couplings for 45 min using 4 equiv of standard Fmoc amino acids; b) The Fmoc-δ-thiolysine-OH building block (Figure 3B) is coupled using 3 equiv; c) Fmoc removal with 20% piperidine in NMP for 2x 2 min and 1x 5 min;

After the first 30 cycles: a) extend the coupling time to 60 min; b) Fmoc deprotection with 20% piperidine in NMP for 4x 3 min; c) Thr12, Glu18 and Pro37 are attached with double couplings and elongated reaction times (90 min);

From cycle 40 to the final cycle, capping is performed with a mixture of Ac_2O/DiPEA/HOBt in NMP at 500, 125 and 15 mM respectively (2x 2 min and 1x 5 min). This solution is prepared fresh on the ice every 2 days. After the completion of all cycles in SPPS, the resins were washed with diethylether and dried under high vacuum. Following this, the resin was transferred to a 50 mL falcon tube. Cocktail A was degassed for 5 min with nitrogen and 5 mL of this was added to the resin. The resin was mixed with the TFA cleavage mix by rotation for 3 h at RT. After incubating with the TFA cleavage mix, the resin was filtered using syringe filter units and the mix was collected in a falcon tube containing a mixture of dry-ice cold Et_2O, *n*-pentane (3:1 v/v) (40 mL). The resulting pellet was precipitated by slow centrifugation (10 min at 2,000 rpm). The Et_2O, *n*-pentane mixture was removed by decantation, and the pellet was taken up in fresh Et_2O, *n*-pentane (3:1 v/v) (40 mL) at room temperature and centrifuged. This step was repeated twice. The pellet was

dissolved in 5 mL cocktail B and lyophilized. In general, a 25 μmol scale SPPS afforded ±120 mg of crude material.

LC-MS, cation-exchange FPLC, and HPLC

Liquid chromatography-mass spectrometry (LC-MS)-grade solvents like Formic acid and ULC/MS-grade water was obtained from Biosolve. Fast protein liquid chromatography (FPLC)-eluents like 50 mM sodium acetate, pH 4.5, and 1M NaCl, 50 mM sodium acetate, pH 4.5 were prepared according to the standard procedure to make buffers and salt solutions. High-performance liquid chromatography (HPLC-S)-grade solvents like acetonitrile [CH_3CN] were bought from Biosolve.

The crude lyophilized thiolysine Ub mutant (white powder, ±120 mg) was dissolved in DMSO (1 - 4 mL) and then added dropwise to 50 mM NaOAc, pH 4.5 (45 mL) (*see* **Note 10**). This solution was loaded onto an FPLC column (15x185 mm) packed with Workbeads 40S™. The Ub mutant was purified using a gradient of 0 to 1 M NaCl over 5 column volumes in 50 mM NaOAc, pH 4.5. The fractions containing the desired product was analysed by LC-MS (±0.2 M NaCl). The NaCl and NaOAc in the pure fractions can be removed by RP-HPLC or by exchanging it for milliQ water by spin-filtration over 3 kDa cutoff membrane filters (Amicon Ultra-15 Centrifugal Filter Units). For RP-HPLC, the crude lyophilized thiolysine Ub mutant (white powder, ±25 mg) was dissolved in DMSO (1 mL) and 2-3 drops of TFA was added and vortexed mixture for 5 min. Then, Ub mutant was purified by RP-HPLC (Atlantis C18 column, 10x150 mm, 5 μM) using a protocol that starts with a gradient of 3 min from 5% to 25% CH_3CN followed by a gradient of 11 min from 25% to 43% CH_3CN (in water containing 0.05% TFA). The Ub-(δ-thiolysine) mutant elutes from 15 to 16 min. Pure fractions were analysed in LC/MS and pooled together. These were lyophilized, and the resulting powder was lyophilized again after dissolving in 10 mL cocktail B. The overall yields of the Ub-(δ-thiolsyine) mutants varied between 15-20%. For MS analysis of synthesized Ub and diUbs, we used Kinetex C18 LC-MS column (2.1x50 mm, 2.6 μm) from Phenomenex. For HPLC purification of diUbs, we used Atlantis® dC_{18} OBD™ preparative RP-HPLC column (19x250 mm, 10 μM) and Atlantis® Prep T3, C18 column (10x150 mm, 5 μM) from Waters. For cation exchange FPLC purification of Ub, we used Work Beads™ 40 S from Bio-Works.

Ubiquitin-E1 expression and purification

Ubiquitin (Ub) and Ub activating enzyme (UbE1) were expressed in a bacterial expression system and purified. UbE1 was expressed from Rosetta BL21 cells by growing the cells in a 5 mL starter culture at 37 °C for 4 hours until the cells were grown till saturation. After this, they were transferred to a 2 L LB medium at a dilution rate of 100 x and grown until the OD reached 0.6. Then, 1 mM IPTG was added to this medium and the temperature was reduced to 18 °C for the cells to express UbE1 overnight. The following day, the cells were spun down at 2500xG for 5 min and the supernatant was removed. UbE1 cell pellets was homogenized in lysis buffer containing 20 mM Tris-HCl, 5 mM β-mercaptoethanol, 250 mM NaCl, 1 mM PhenylMethaneSulphonyl Fluoride (PMSF) and 1 Protease-inhibitor tablets (EDTA-free) for 50 mL lysis buffer at 4°C. The cell lysate was then subjected to lysis by the French press applying a final pressure between 5000-10,000 psi at 4 °C. The cells were lysed twice in the French press to ensure complete extraction of protein. Finally, the cell lysate was centrifuged at 22,000 rpm for 30 minutes at 4°C in an ultracentrifuge. The supernatant was then collected in a separate tube and imidazole was added to a final concentration of 25 mM. Pre-washed TALON beads were then added to the tube in a ratio of 1:20 (v:v) and left at 4 °C for 15 minutes in a rotor to ensure homogenization of the lysate. The solution was then transferred to a column packed with a glass filter and pressure was applied using a syringe

set-up. The filtrate flow-through was collected and stored separately. The column was then washed twice with 5 bed volumes of wash buffer. Elution buffer containing 20 mM Tris-HCl, 5 mM β-Mercaptoethanol, 250 mM NaCl and 250 mM Imidazole was added up to four times the bed volume to the column and homogenized by repeated pipetting. It was left at 4 ºC for 10 minutes. Pressure was applied to collect the filtrate containing the UbE1 enzyme. A portion of this filtrate was used for SDS-PAGE analysis to confirm the presence of UbE1.

The rest of the sample was loaded into a centrifugal filter with a molecular cut-off of 25 KDa. It was spun for 60 minutes at 4000 rpm at 4 ºC in a centrifuge to concentrate the enzyme. Dilution buffer (20 mM Tris-HCl pH 8.0, 5 mM β-Mercaptoethanol, 250 mM NaCl) was then added to the tube until the final volume was about 10 ml and centrifuged again for 60 minutes at 4000 rpm at 4 ºC. The supernatant was finally diluted with dilution buffer to a final volume of 10 ml and aliquoted into 250 µl per vial, immediately frozen in liquid nitrogen and stored at -80ºC.

Ubiquitin expression and purification

UbE1 was expressed by transforming Ub w.t. pET 15 plasmid (*Amp*+) into Rosetta cells and growing the cells in 5 mL starter culture in LB medium at 37 °C for 4 hours until the cells were grown till saturation. In the meanwhile, auto induction medium was prepared using the following components:

ZY medium (1L)

- 10 g Tryptone
- 5 g yeast extract
- 5 g NaCl
- MQ water was added to make up the final volume upto 1 L
- The medium was autoclaved at 121ºC for 15 minutes

NPS buffer (20×)

Component	mol/liter
dd H_2O	-
$(NH_4)_2SO_4$	0.5 M
KH_2PO_4	1 M
Na_2HPO_4	1 M

***Table 1: NPS buffer in 20x concentration**. The solution was prepared by adding the components in the sequence mentioned and stirred until they are completely dissolved. The pH of 20-fold dilution in water was around 6.75. The solution was autoclaved at 121ºC for 15 minutes.*

5052 (50×):

Component	concentration
Glycerol 100% solution	0.5 %
Glucose	0.05 %
α-lactose	0.2 %

***Table 2: 5052 addendum in 50x concentration**. The solution was prepared by adding the components in the sequence mentioned and stirred until they are completely dissolved.If necessary, the solution was snap heated in microwave (90ºC for about 30 seconds in intervals) until dissolved. The solution was autoclaved at 121ºC for 15 minutes.*

1 M $MgSO_4$

- 9.86 g $MgSO_4 \cdot 7H_2O$
- MQ water was added up to 40 ml
- Sterilization was performed using membrane filter (0.22 µm)

40% glucose (w/v)

- Glucose was added to the beaker filled with water and constantly stirred with a magnetic stirrer
- If necessary, the solution was snap heated in microwave (90ºC for about 30 seconds in intervals) until dissolved

ZYP-0.8G

Component	Concentration
ZY	-
1 M $MgSO_4$	1 mM
40% glucose	0.8%
20× NPS	1×
chloramphenicol (34 mg/mL)	34 µg/mL
ampicillin (50 mg/mL)	50 µg/mL

Table 3: ZYP 0.8G medium used for cell growth*. The 1M $MgSO_4$ was added before adding the 20x NPS to prevent precipitation of the medium. The saturated culture in ZYP 0.8G medium was slightly acidic around pH 6.0. The solution was autoclaved at 121ºC for 15 minutes.*

ZYP-5052 rich medium for auto-induction

Component	Concentration
ZY	-
1 M $MgSO_4$	1 mM
50x 5052	1×
20x NPS	1×
chloramphenicol (34 mg/mL)	34 µg/mL
ampicillin (50 mg/mL)	50 µg/mL

Table 4: ZYP 5052 medium used for autoinduction*. The 1M $MgSO_4$ was added before adding the 20x NPS to prevent precipitation of the medium. The solution was autoclaved at 121ºC for 15 minutes.*

After the starter culture reached saturation, 100 µl from this cell culture is inoculated in a 250 ml **ZYP-5052** medium supplemented with ampicillin in 2 L Erlenmeyer flask. They were left in the shaker at 300 rpm or more at 37ºC overnight. The next day (usually after 20-24 hours) when the OD_{600} was around 10, the cells were pelleted at 4000 rpm for 20 minutes. The supernatant was removed, and the cell pellet was resuspended in MQ containing protease inhibitor tablet. After homogenization, the cells were heated in a heating block at 90 °C for 30 minutes. After this, the cells were cooled down and DNAse I supplemented with 10 mM MgSO4 was added. After incubating for 15 min at 4 °C, the cells were heated again at 90 C for another 30 minutes and the lysate was then spun down at 20000 rpm for 30 minutes at 4 ºC. The supernatant was then used for purification using cation exchange chromatography followed by RP-HPLC using conditions mentioned in general methods. Finally, Ub was obtained as a lyophilized powder with a yield of 1 G per liter of cell culture.

For the synthesis of Ub-MESNa thioester, a buffer containing 0.2 M magnesium(II) chloride ($MgCl_2$), 0.5 M adenosine triphosphate (ATP), 2.0 M 2-mercaptoethane sulfonate sodium salt (MESNa), 50 mM sodium phosphate buffer, pH 8.0 were prepared according to standard procedure. To synthesize Ub-MESNa, 10 mg of ubiquitin was dissolved in 0.5 mL DMSO and then into 10.2 mL 50 mM sodium phosphate buffer, pH 8.0. To this was added 0.59 mL $MgCl_2$ (0.2 M; final conc. 10 mM), 0.59 mL MESNa (2.0 M; final conc. 100 mM) and finally 0.23 mL ATP (0.5 M; final conc. 10 mM). The pH was adjusted to pH 8.0 using 1N NaOH. Then 75 µL of Ub activating enzyme E1 (39 µM; final conc. 0.25 µM) was added and incubated at 37 °C for 3 hours (final Ub conc is now 100 µM) (*see* **Note 11**). The resulting Ub-MESNa thioester was purified by RP-HPLC (Atlantis C18 column, 10x150 mm, 5 µM) using an 18 min gradient from 10% to 60% CH_3CN in water containing 0.05% TFA. The conversion was almost 100 % and the final yield was approximately 85 % pure.

Native Chemical Ligation

Native chemical ligation (NCL) in the native buffer as performed in 0.2 M sodium phosphate buffer, containing 50 mM NaCl at pH 8. For this, 200 ηM of UbE1 was added to 100 µM of Ub w.t. and 100 µM of Ub(G76V) thiolysine (of each linkage type) in the presence of 6 mM ATP, 6 mM MgSO4 and 50 mM MESNa at 37 °C overnight.

In the case of NCL under denaturing conditions, Cocktail C buffer containing 6 M guanidine-HCl (Gdn·HCl) in 0.2 M sodium phosphate buffer at pH 7.0 (*see* **Note 3**) containing 50 mM tris(2-carboxyethyl)phosphine-HCl, pH 7.0 (TCEP) (Piercenet, *see* **Note 5**) and 100 mM 4-mercaptophenylacetic acid at pH 8.0 (MPAA) (AlfaAesar, *see* **Note 4**) was prepared. For the diUb ligation, 1 mL cocktail C was added to 10 mg of UbMESNa powder. To this solution was added 10 mg of Ub thiolysine mutant (of each linkage type) and incubated >6 h at 37°C. If LC-MS analysis shows any unreacted Ub thiolysine mutant, a fresh portion of UbMESNa (5 mg) powder was added, followed by an additional incubation for >6 h at 37°C. The resulting diubiquitin conjugate was purified by RP-HPLC (Waters Atlantis® dC_{18} OBD™ preparative RP-HPLC column (19x250 mm, 10 µm) using a protocol that starts with a gradient of 5½ min from 20% to 32% CH_3CN followed by a gradient of 15 min from 32% to 42% CH_3CN (in water containing 0.05% TFA) (*see* **Note 12**). The ligation product elutes from 12-15 min.

For the subsequent desulfurization step, cocktail D containing 6 M Gdn-HCl in 0.2 M sodium phosphate buffer pH 6.5 (*see* **Note 6**), 50 mM 2,2'-azobis(2-amidinopropane) dihydrochloride (**V-50**) (Wacko Chemicals USA), 50 mM Glutathione (GSH) (Aldrich) and 250 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (ChemImpex) at pH 6.5 was prepared. It is to be noted that the diUb synthesized under native conditions was not desulphurized because the yield was generally lower than that used in denaturing conditions.

To execute radical desulfurization, Cocktail D was first degassed by argon bubbling for 10 min. Then, 10 mL cocktail D was added to 10 mg of the diUb conjugate. After this, 2,2'-Azobis(2-amidinopropane) dihydrochloride (542 mg, final conc= 0.2 M) was added to the mixture. The solution was vortexed for 1 min and incubate for >6 h at 60°C (*see* **Note 13**). After confirming complete desulfurization by LC-MS analysis, the native linked diubiquitin conjugate was purified by RP-HPLC Waters Atlantis® dC_{18} OBD™ preparative RP-HPLC column (19x250 mm, 10 µm) using a protocol that starts with a gradient of 7 min from 5% to 25% CH_3CN followed by a gradient of 20 min from 25% to 45% CH_3CN (in water containing 0.05% TFA). The product elutes from 16-19 min. The overall yields of the Ub-Ub chains vary between 40-70%.

The diUb conjugates were analysed using pre-cast polyacrylamide gels NuPAGE® Novex® 12% Bis-Tris Gels (Invitrogen) run in MES buffer (20x) NuPAGE®: diluted to 1x

using Demi water. The protein marker used was SeeBlue® Plus 2 Prestained Standard 1x (Invitrogen). The sample buffer used contained NuPAGE® LDS Sample Buffer 4x (Invitrogen) and 10% of 14.3 M 2-Mercaptoethanol (Aldrich).

After ligation, the diUb conjugates were analysed by SDS-PAGE gel electrophoresis. For this, 1 mg of diUb conjugate was dissolved in 100 µL DMSO and diluted into 900 µL milliQ water. 5 µL of this sample was added to 10 µL of 3x loading buffer (prepared by diluting 4× NuPAGE® SDS Sample Buffer (900 µL) with 90 µL 2-mercaptoethanol and 210 µL milliQ water). The samples were heated for 10 min at 70°C. Then, 10 µL of each sample was loaded onto a Pre-cast polyacrylamide gels NuPAGE® Novex® 12% Bis-Tris Gel. As a protein marker, 1x SeeBlue ® Plus 2 Prestained Standard and 10 µL of a Ub standard, respectively. The samples were run the gel containing 1x MES buffer at 190V (≈50 min). Finally, the samples were stained with Coomassie Brilliant Blue and destained with H_2O, EtOH, AcOH (50/40/10 v/v/v).

Notes

1. Only use peptide grade NMP. A potential problem with NMP (and DMF) is the presence of amines, which can give rise to partial Fmoc cleavage. To ensure that the NMP is of good quality, we recommend incubating Fmoc-Phe-OMe (5 mg), overnight in 1 mL of NMP and test for Fmoc cleavage by LC-MS analysis.
2. 50 mM Sodium phosphate pH 8.0 is prepared as follows: 284.1 mL of 0.2 M Na_2HPO_4 (Fluka, prepared by dissolving 17.8 g Na_2HPO_4 in 500 mL water) and 15.9 mL of 0.2 M NaH_2PO_4 (Fluka, prepared by dissolving 13.8 g in 500 mL water) were mixed and diluted with 300 mL water. This 0.1 M Sodium phosphate buffer pH 8.0 can then be diluted twice for 50 mM Sodium phosphate buffer pH 8.0.
3. 6 M Gdn·HCl in 0.2 M sodium phosphate buffer pH 7.0 is prepared by dissolving 229.2 g Gdn-HCl in up to 400 mL 0.2 M sodium phosphate buffer pH 7.0. The final pH is adjusted using to 7.0 with 10N NaOH.
4. MPAA does not dissolve readily in water until the pH is adjusted to 8.0. The MPAA stock at pH 8 is prepared as follows: 1.68 g of 4-mercaptophenylacetic acid is added to 7.5 mL water and the pH is adjusted to 8.0 using 10N NaOH. The final volume is around 10.8 mL, corresponding to a 4-mercaptophenylacetic acid concentration of 925 mM.
5. This can also be prepared by dissolving TCEP-HCl (ChemImpex) in milliQ water and adjusting the pH to 7.0 with 10N NaOH followed by 1N NaOH. The solubility of TCEP-HCl in water is >310 g/L (1.08 M); best is to start with a 1.0 M solution since this allows for dilution during the adjustment of the pH.
6. 6 M Gdn.HCl in 0.2 M sodium phosphate pH 6.5 is prepared as follows: 22.9 g of Gdn.HCl is dissolved in 40 mL 0.2 M sodium phosphate buffer pH 8.0 (see ***Note 2***). The pH is adjusted to 6.5 with 1N NaOH.
7. 50 mM NaOAc pH 4.5 is prepared as follows: A 5M NaOAc (Sigma Aldrich) stock is prepared by dissolving 205 g NaOAc in 143 mL acetic acid mixed with 800 mL water and the total volume is adjusted to 1 L with water. The final pH was 4.5. Next, 5 mL of this 5M stock was diluted with 495 mL of water.
8. Besides removing moisture, this also ensures that no traces of acetic acid (or other acids) are present, which can be introduced in trace amounts during the preparation and/or purification of these building blocks.
9. Safety precaution: it is important to cover the rotor baskets with a lid since the diethylether/n-pentane solution is highly volatile.

10. If the material does not dissolve well, the DMSO can be warmed carefully and vortexed. Adding a few drops of TFA is also beneficial, but here it is important to monitor its effect on the pH of the NaOAc buffer used for the cation chromatography.
11. In general, we found the reaction to be complete within 6 h. Best is, to follow the reaction by LC-MS analysis, and once completion is verified, to perform the RP-HPLC directly. Thioesters are labile under basic conditions, but they do show enhanced stability under these conditions when thiols (such as MESNa) are present. If storage is required, we recommend acidifying the reaction (for example by adding 100 mM NaOAc, pH 4.5).
12. It is important to remove any MPAA for the following radical-initiated desulfurization step as MPAA is known to act as a radical scavenger. After preparative HPLC purification of the ligation mixture, the anticipated δ-thiolysine linked diUb conjugates are isolated (partially) as MPAA disulfides. Incubation with TCEP (during the subsequent desulfurization step) gives clean formation of the reduced δ-thiolysine linked diUb conjugates. The amount of MPAA released during this *in situ* reduction has no inhibitory effect on the radical-initiated desulfurization.
13. The **V-50** does not dissolve completely upon addition; this is achieved upon incubation at 60°C. Since N_2-gas is released during the reaction, we recommend to slightly puncture any lid of a plastic reaction vessel.

Supplementary information

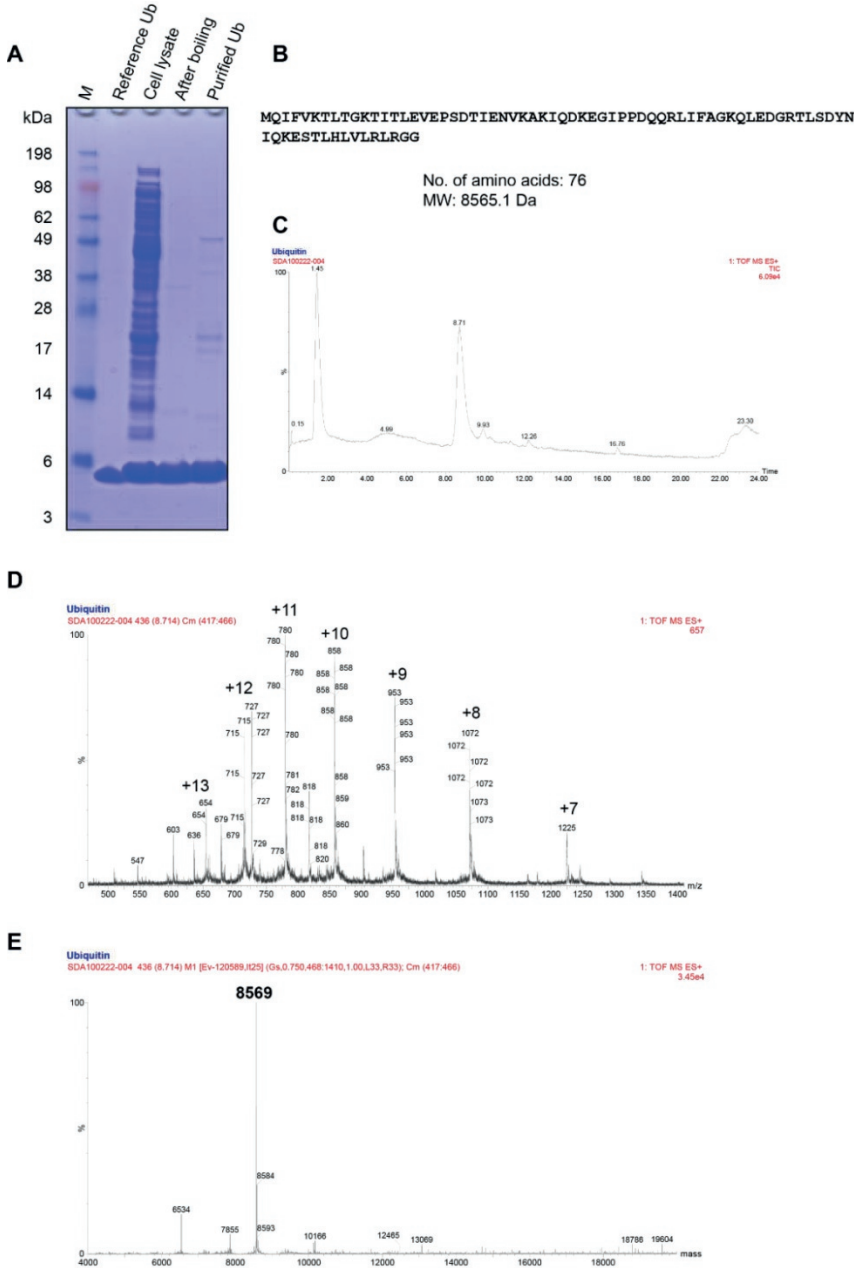

Figure S1: Expression and purification of Ub. ***A:*** *SDS-PAGE analysis of Ub expression in Rosetta BL21 cells and purification by boiling the lysate followed by reversed phase HPLC.* ***B:*** *Sequence of Ub.* ***C:*** *Combined UV chromatogram of Ub purified from bacterial cell lysate.* ***D:*** *Combined Mass spectra of Ub from C.* ***E:*** *Deconvoluted mass of Ub from mass spectra (Expected MW: 8564.8 Da, Observed: 8569 Da).*

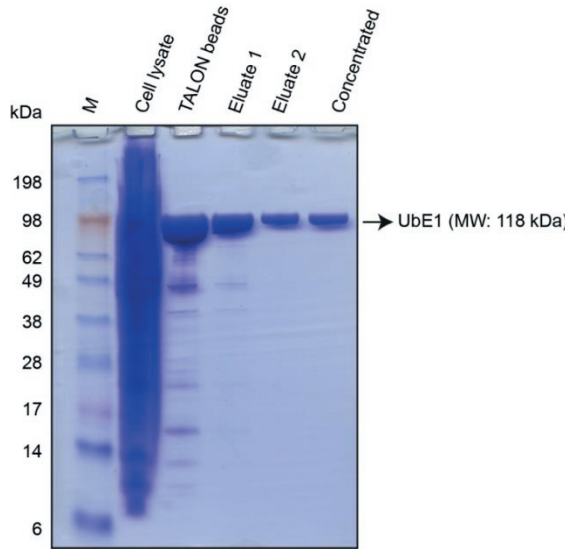

Figure S2: *Expression and purification of human UbE1 enzyme analyzed by SDS-PAGE.*

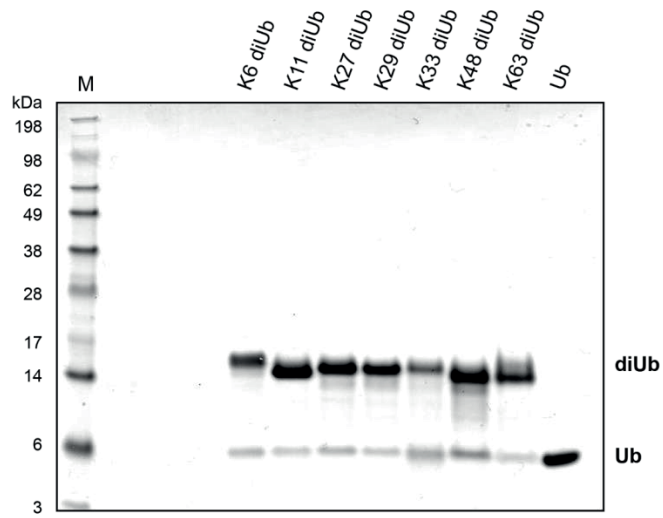

Figure S3: *Isopeptide linked diUbs generated by Native chemical ligation between Ub thioester and Ub K-δ-thiolysine, performed under denaturing conditions. All seven diUbs were prepared, desulfurized and purified using reversed phase HPLC.*

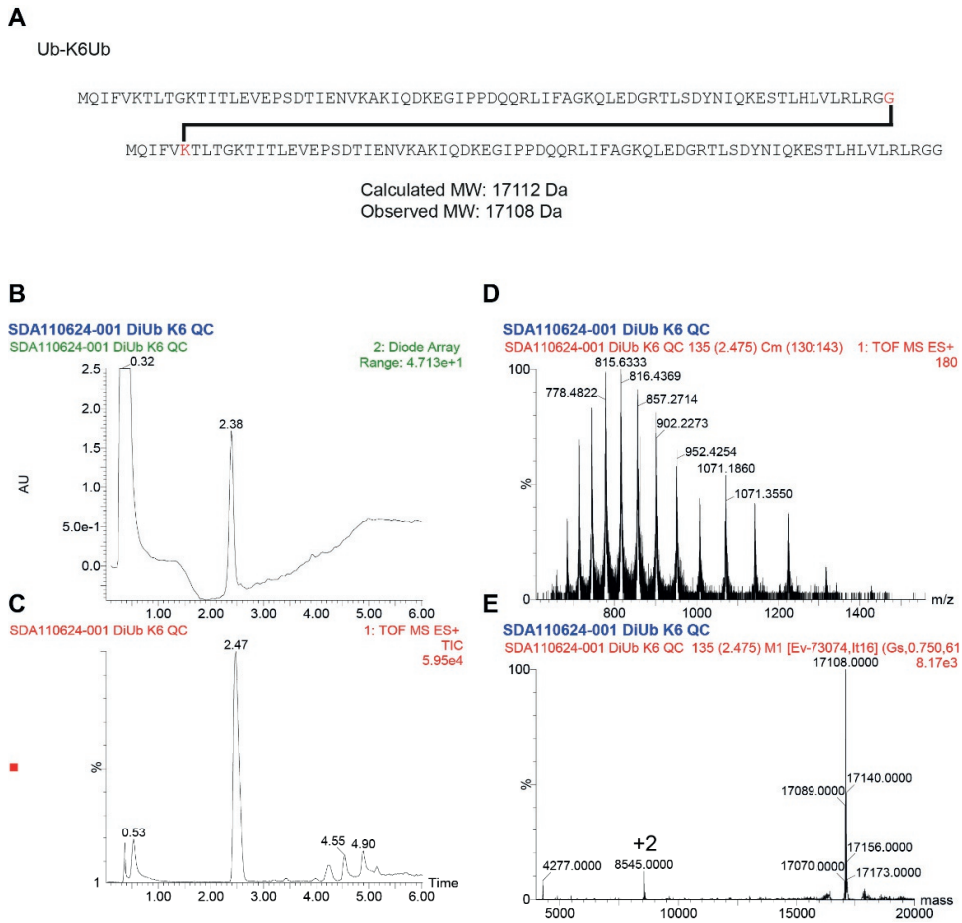

***Figure S4: LC-MS analysis of K6diUb sample prepared under denaturing conditions**. **A:** Sequence of K6diUb showing the position of isopeptide bond. **B:** UV chromatogram (λ – 280 nm). **C:** Mass spectrum. **D:** Combined mass spectrum of peak at 2.47 min. **E:** Deconvoluted mass of mass spectra.*

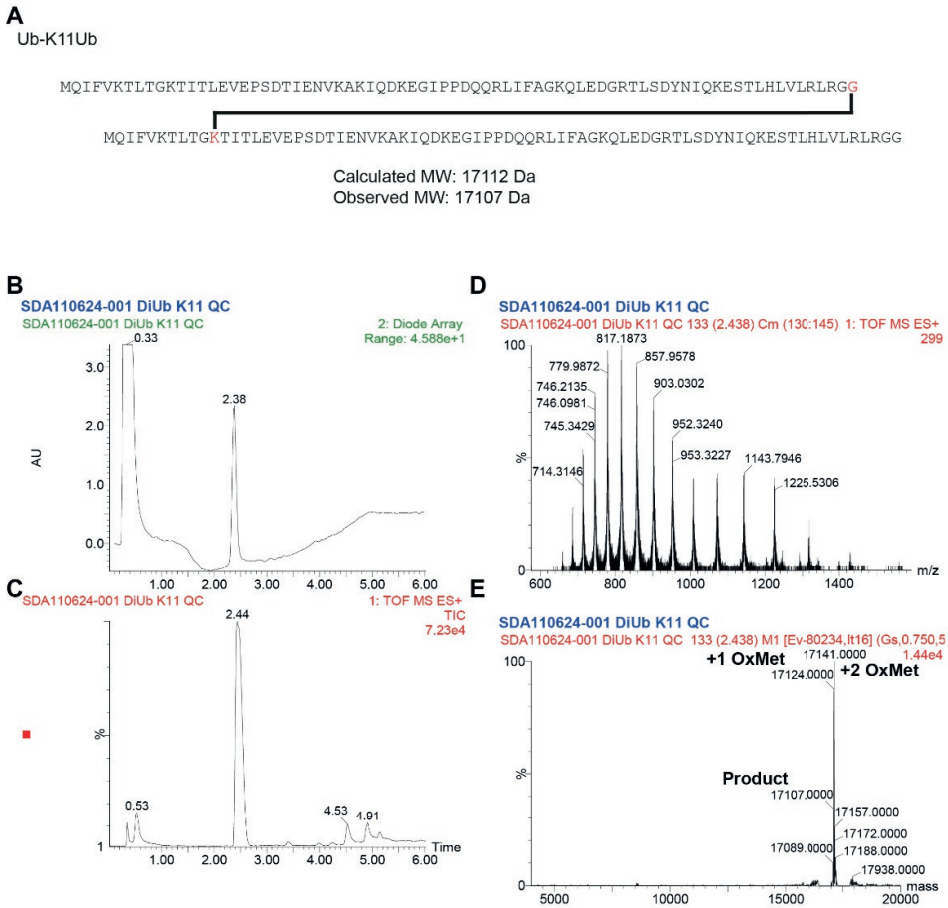

Figure S5: LC-MS analysis of K11diUb sample prepared under denaturing conditions. ***A:*** *Sequence of K11diUb showing the position of isopeptide bond.* ***B:*** *UV chromatogram (λ – 280 nm).* ***C:*** *Mass spectrum.* ***D:*** *Combined mass spectrum of peak at 2.44 min.* ***E:*** *Deconvoluted mass of mass spectra. The two additional masses correspond to oxidized state of the two methionine residues in the diUb molecule.*

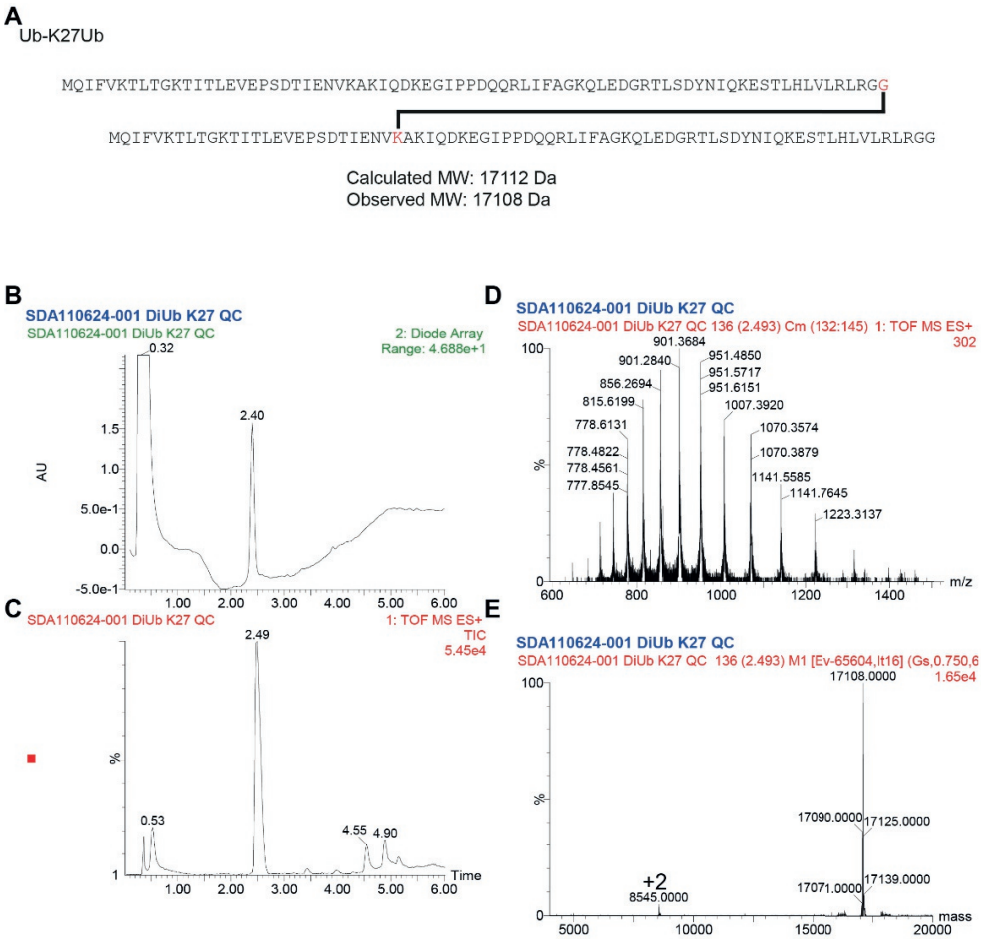

***Figure S6: LC-MS analysis of K27diUb sample prepared under denaturing conditions**. **A:** Sequence of K27diUb showing the position of isopeptide bond. **B:** UV chromatogram (λ – 280 nm). **C:** Mass spectrum. **D:** Combined mass spectrum of peak at 2.49 min. **E:** Deconvoluted mass of mass spectra.*

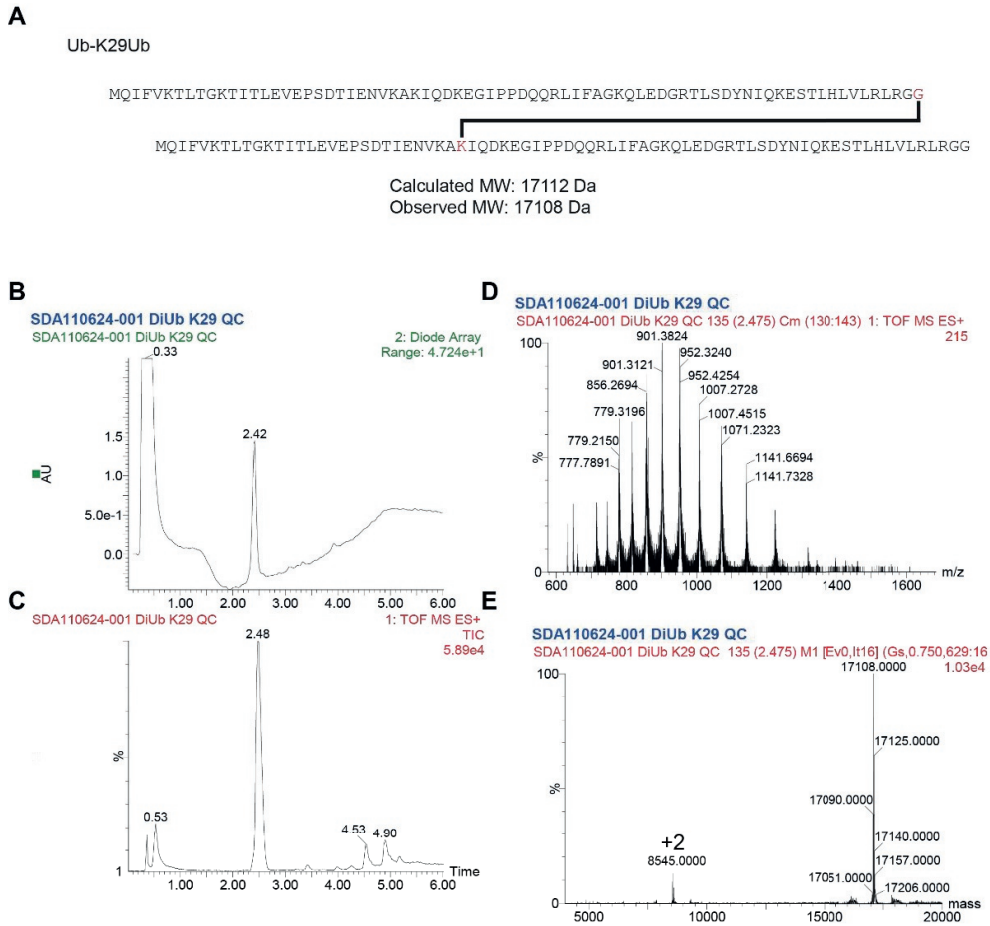

Figure S7: LC-MS analysis of K29diUb sample prepared under denaturing conditions. ***A:*** *Sequence of K29diUb showing the position of isopeptide bond.* ***B:*** *UV chromatogram (λ – 280 nm).* ***C:*** *Mass spectrum.* ***D:*** *Combined mass spectrum of peak at 2.48 min.* ***E:*** *Deconvoluted mass of mass spectra.*

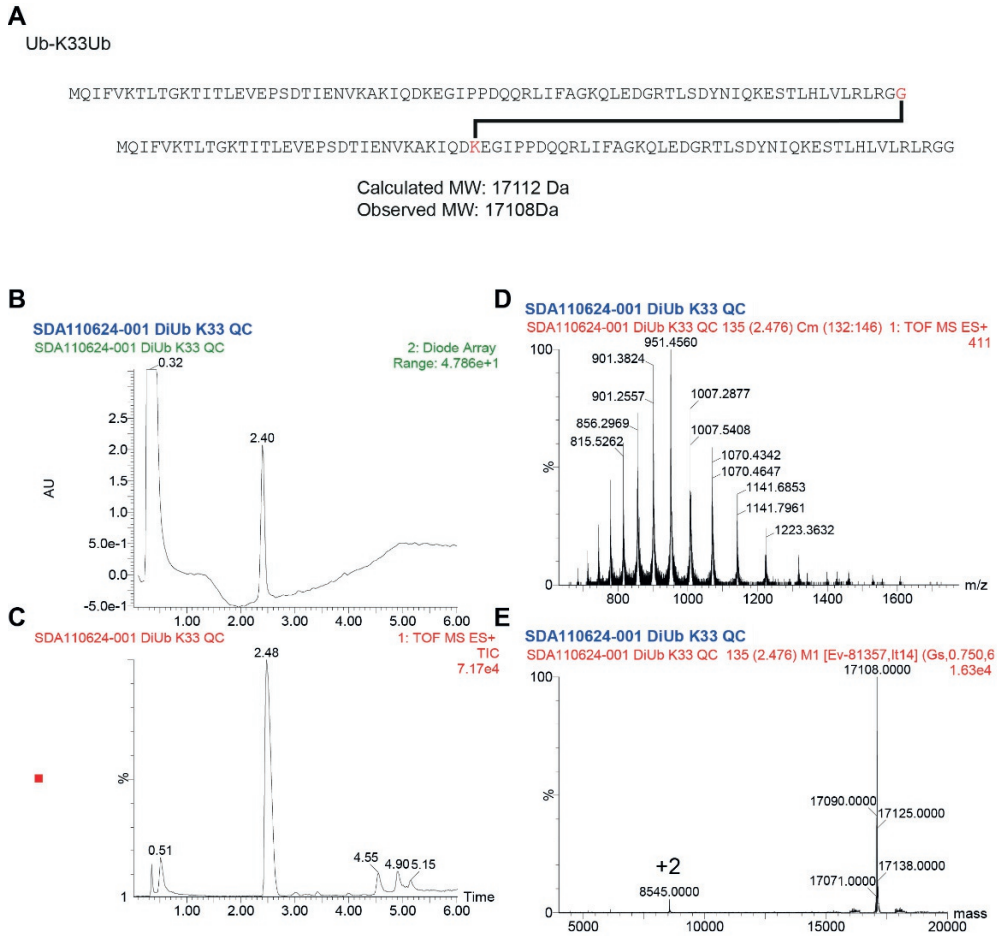

Figure S8: LC-MS analysis of K33diUb sample prepared under denaturing conditions. ***A:*** *Sequence of K33diUb showing the position of isopeptide bond.* ***B:*** *UV chromatogram (λ – 280 nm).* ***C:*** *Mass spectrum.* ***D:*** *Combined mass spectrum of peak at 2.48 min.* ***E:*** *Deconvoluted mass of mass spectra.*

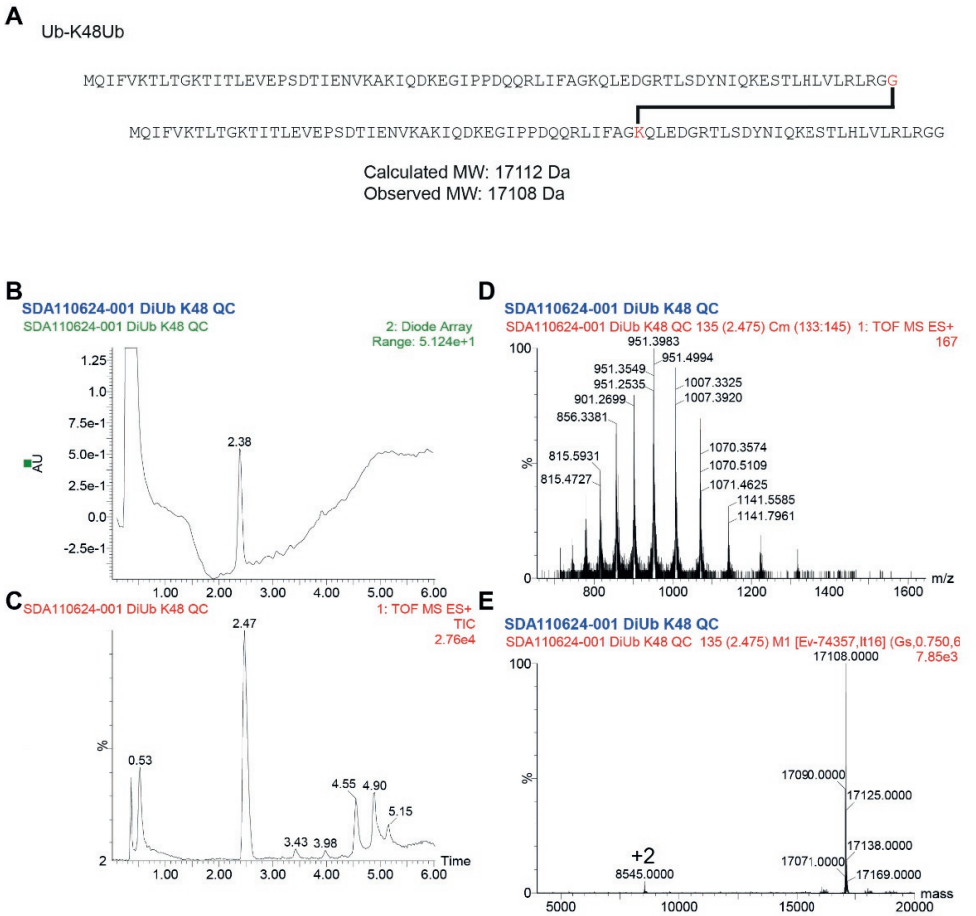

Figure S9: LC-MS analysis of K48diUb sample prepared under denaturing conditions. ***A:*** *Sequence of K48diUb showing the position of isopeptide bond.* ***B:*** *UV chromatogram (λ – 280 nm).* ***C:*** *Mass spectrum.* ***D:*** *Combined mass spectrum of peak at 2.47 min.* ***E:*** *Deconvoluted mass of mass spectra.*

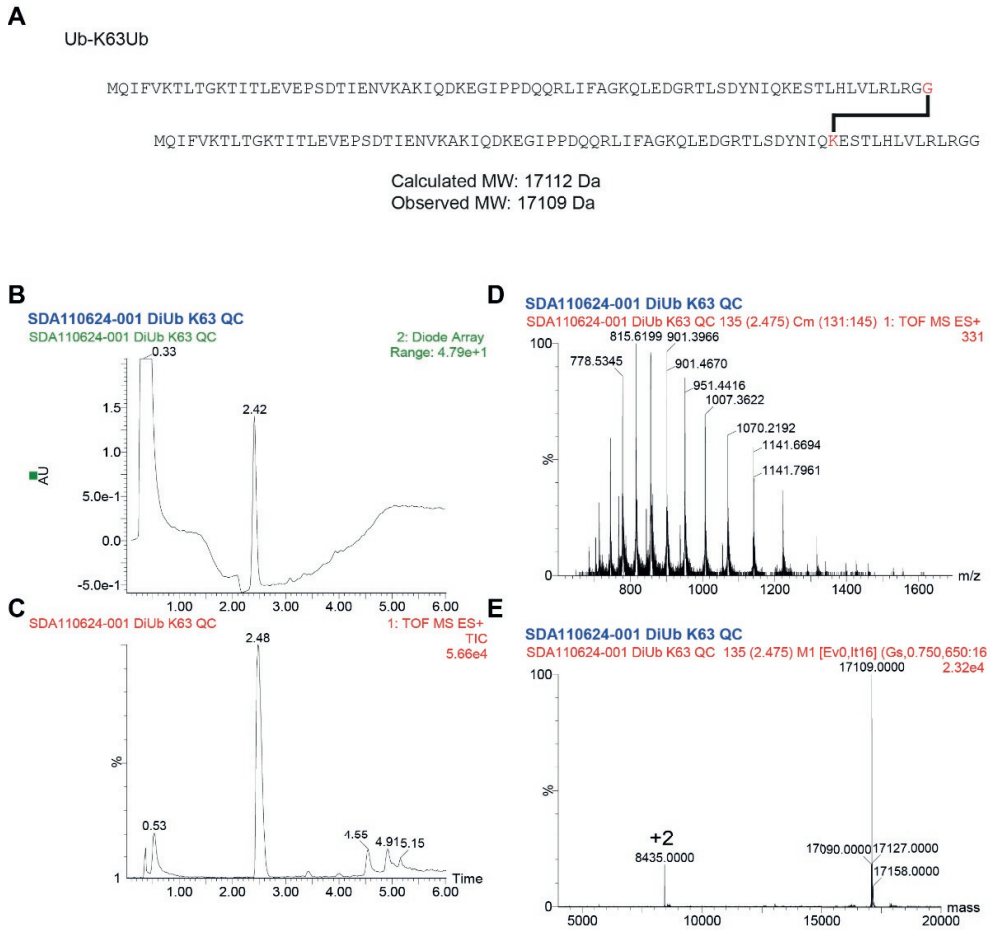

Figure S10: LC-MS analysis of K63diUb sample prepared under denaturing conditions. ***A:*** *Sequence of K63diUb showing the position of isopeptide bond.* ***B:*** *UV chromatogram (λ – 280 nm).* ***C:*** *Mass spectrum.* ***D:*** *Combined mass spectrum of peak at 2.48 min.* ***E:*** *Deconvoluted mass of mass spectra.*

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