Supplementary Material (ESI) for Chemical Communications

Controlled Enzymatic Synthesis of Natural-Linkage, Defined-Length Polyubiquitin Chains Using Lysines with Removable Protecting Groups.

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Electronic Supplemental Information

Materials and Methods

Construction of expression plasmid pTXB1-SynUb (K48TAG)

The coding sequence for Human Ub was codon-optimized for expression in *E. coli*. A full-length Ub gene was synthesized by overlap PCR using homologous oligonucleotides (Integrated DNA Technologies, Inc., Coralville, IA). Oligos were designed using an online tool DNAWorks (www.helixweb.nih.gov). Assembly PCR was performed in a 50 μL solution containing 0.2 mM dNTPs, 0.5 U of Phusion DNA polymerase (NEB), 1X Phusion HF reaction buffer (New England Biolabs, Inc., Ipswich, MA) and 2 μM of each UB2 through UB9 oligos. This mixture was thermal cycled as follows: initial denaturation at 98 ºC for 30 s, 25 cycles of 98 ºC for 10 s, 58 ºC for 30 s and 72 ºC for 20 s and final extension at 72 ºC for 10 min. The synthetic Ub gene was then amplified in the following conditions: 0.2 mM dNTPs, 0.5 U of Phusion DNA polymerase (NEB), 1X Phusion HF reaction buffer (NEB), 1 μL of assembly PCR product and 2 μM of each #1 and #10 oligo in 50 μL solution, under the same cycling conditions. The PCR product was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and digested with *Nde* I/*BamH* I. The digestion product was gel-purified and ligated into the *Nde* I/*BamH* I site of pET3a vector (EMD Chemicals Inc., San Diego, CA). The gene was confirmed by sequencing and then PCR amplified from pET3a vector using primers pTXB1Fwd and pTXB1Rev. The PCR product was digested with *Nde* I/*Sap* I, gel-purified and ligated into the *Nde*I/*Sap*I site of the pTXB1 vector (NEB) to generate pTXB1-SynUb plasmid. pTXB1-SynUb(K48TAG) plasmid was generated by site-directed mutagenesis PCR using template plasmid pTXB1-SynUb and primers K48TAGFwd and K48TAGRev.

Construction of tRNA/tRNA synthetase plasmid pSUP-PylT-PylS (Y384F)

A synthetic amber suppressor tRNA based on the *Methanosarcina mazei* pyrrolysine tRNA (PylT) was generated by assembly PCR using oligos PylT#1-6. This resulted in the tRNA downstream of the *E. coli* proK promoter.[1] The fragment was PCR amplified with PylTproKFwd and PylTproKRev. The PCR product was digested with *ApaL* I/*Xho* I, gel-purified and ligated into the same sites in the pSup vector.[1] The recombinant plasmid pSup-PylT was confirmed by sequencing. The gene encoding *Methanosarcina mazei* pyrrolysyl-tRNA-synthetase (PylS) was PCR amplified from genomic DNA (ATCC# BAA-159D-5) using primers PylRSFwd and PylRSRev. The PCR product was digested with *Nde*I/*Nsi*I, gel-purified and ligated into the same

sites in pSup-PylT, and the resultant plasmid pSUP-PylT-PylS was sequenced-confirmed. The point mutation Y384F that is known to enhance activity with Lys(Boc)[2] was introduced into PylS gene by site-directed mutagenesis PCR using pSup-PylT-PylS as the template and primers PylRSY384F Fwd and PylRSY384FRev.

Expression and purification of UbK48Lys(Boc)

Plasmids pSup-PylT-PylS and pTXB1-SynUb(K48TAG) were double transformed into *E. coli* BL21(DE3) chemically competent cells. Expression was induced in condensed culture as described.[3] Briefly, a freshly transformed colony was grown in 2 liter 2×YT media supplemented with 100 mg/mL ampicillin and 35 mg/mL chloramphenicol at 37 ºC to OD_{600} of 0.8. The *E. coli* cells were pelleted, resuspended in 100 mL 2×YT media containing 100 mg/mL ampicillin, 35 mg/mL chloramphenicol, 1 mM IPTG and 2 mM Lys(Boc) and grown at 30 ºC for 16 hours. *E. coli* cells from the expression culture were harvested by centrifuge, washed three times with lysis buffer (20 mM HEPES, 500 mM NaCl, 1 mM PMSF, pH 8.5) and resuspended in 60 mL of lysis buffer. The resuspended cells were frozen and thawed three times, sonicated and then centrifuged to remove cell debris. Supernatant was applied to a column containing 20 mL of chitin-binding beads (NEB) which was pre-equilibrated with 100 mL lysis buffer. The beads with bound chitin-Ub fusion were washed with 400 mL wash buffer (20 mM HEPES, 500 mM NaCl, pH 8.5). On-column cleavage was induced by flushing the column with 60 mL wash buffer containing 50 mM DTT and column was kept at 37 ºC for 24 hours. Cleaved UbK48Lys(Boc) was eluted from the column using 60 mL of wash buffer. Proteins were further purified using size-exclusion chromatography to a final yield of 10 mg/L.

Expression and purification of $^{15}N\ Ub_{1-74}$ and $^{15}N\ Ub_{1-74}$K48Lys(Boc)

^{15}N-labeled Ub_{1-74} *and* Ub_{1-74}K48Lys(Boc) protein was expressed in auto-inducing minimal media. pET3a-Ub_{1-74} was transformed into BL21(DE3) pJY2 cells. pTXB1-SynUb_{1-74}(K48TAG) and pSup-PylT-PylS (Y384F) were double transformed into BL21 (DE3) cells. A freshly transformed single colony was inoculated into 4 mL 2×YT media supplemented with 100 mg/mL ampicillin and 35 mg/mL chloramphenicol to grow a stock culture. This stock culture was inoculated into a 2 liter culture of auto-inducing minimal media containing 10 mM Na_2SO_4, 50 mM KH_2PO_4, 50 mM Na_2HPO_4, 1 mM $MgSO_4$, 18 mM $^{15}NH_4Cl$ (Cambridge Isotopes), 0.04% ferric citrate, 0.5% glycerol, 0.05% glucose, 0.2% lactose and 2 mM Lys(Boc). This 2 liter culture was grown at 30 ºC for 24 hours. Purification was performed as above.

Enzymatic assembly of K48-linked di-Ub

Di-Ub was assembled enzymatically with equimolar amounts of UbK48Lys(Boc) and ^{15}N-labelled Ub_{1-74} using methods previously described.[4-6] Small-scale reactions (as presented in Figure 1) used 150 nM E1 and 1-2 μM E2-25K. Large-scale reactions used 10 mg of each Ub monomer, 150 nM E1 and 10-20 μM E2-25K and incubated overnight at 37 ºC. A post-reaction SDS-PAGE gel showed little or no reactant. Product was purified using both cation and size exclusion chromatography to > 60% yield, which is comparable to the yield achieved in our lab using the method of Pickart.[7]

De-protection of UbK48Lys(Boc)

The Boc protecting group of UbK48Lys(Boc) was removed by adding trifluoroacetic acid (TFA) to a final concentration of 2% and incubating at 37 ºC for 4 hours. The protein sample was then re-equilibrated to buffer containing 20 mM sodium phosphate, pH 6.8 using a Millipore 3K NMWL concentrator. No appreciable amount of protein was lost from TFA treatment of UbK48Lys(Boc) monomer or UbK48Lys(Boc) in di-Ub.

ESI-MS was performed on samples of ^{15}N-Ub$_{1-74}$, ^{15}N-Ub$_{1-74}$K48Lys(Boc) monomer and K48-linked di-Ub containing UbK48Lys(Boc) and ^{15}N-Ub$_{1-74}$ before and after TFA treatment (Figures S3-S5). MagTran[8] was used for deconvolution of ESI-MS spectra. Observed molecular weight of ^{15}N-Ub$_{1-74}$K48Lys(Boc) was 8649 Da (expected: 8649 Da). Observed molecular weight of di-Ub assembled from UbK48Lys(Boc) and ^{15}N-Ub$_{1-74}$ was 17200 Da (expected: 17200). After TFA treatment, a loss of 99 Da, which corresponds to the molecular weight of a single Boc protecting group, was seen in both monomer and di-Ub samples. No evidence of a Boc-containing peak was detected in either the monomer or di-Ub samples after TFA treatment. This is confirmed by NMR spectroscopy of monomeric ^{15}N-Ub$_{1-74}$K48Lys(Boc) before and after TFA treatment (see main text).

NMR experiments

NMR samples of Ub$_{1-74}$ and di-Ub (150 μM – 1 mM) were prepared in the appropriate buffers containing 8% $^{2}H_2O$ and 0.02% (w/v) NaN_3. For pH 4.5 and 6.8, the buffer composition was 50 mM ammonium acetate and 20 mM sodium phosphate, respectively. All NMR studies were performed on a Bruker DRX-600 spectrometer equipped with a TXI cryoprobe at 23ºC. {^{15}N-^{1}H} HSQC and TROSY-HSQC spectra were acquired with spectral widths of 6010 Hz and 2100 Hz for the ^{1}H and ^{15}N dimensions, respectively. A total of 160 t_1 increments were collected with 2048 complex points in each. Assignments for Ub$_{1-74}$ and di-Ub were determined by comparison with previously published spectra of human Ub and K48-linked Ub2.[6] Spectra were processed using NMRPipe[9] and Sparky.[10] Chemical shift perturbations were determined using identical buffer and temperature conditions for both monomeric Ub and the proximal ubiquitin in K48-linked di-Ub.

Oligonucleotides

Restriction sites are listed in italics, mutations underlined.

Ub1 (*Nde*I)	GGAATTC*CATATG*CAGATTTTTGTGAAAACCCTGACCG
Ub2	ACTTCCAGGGTAATGGTTTTGCCGGTCAGGGTTTTCACAAA
Ub3	AAAACCATTACCCTGGAAGTGGAACCGAGCGATACCATTGA
Ub4	CCTGAATTTTCGCTTTCACATTTTCAATGGTATCGCTCGGT
Ub5	ATGTGAAAGCGAAAATTCAGGATAAAGAAGGCATTCCGCCG
Ub6	CCGCAAAAATCAGACGCTGCTGATCCGGCGGAATGCCTTCT
Ub7	AGCGTCTGATTTTTGCGGGCAAACAGCTGGAAGATGGTCGT
Ub8	CTGAATATTGTAATCGCTCAGGGTACGACCATCTTCCAGCT
Ub9	CCTGAGCGATTACAATATTCAGAAAGAAAGCACCCTGCATC
Ub10 (*BamH*I)	CGC*GGATCC*TTAGCCGCCACGCAGACGCAGCACCAGATGCAGGGTGCTTTCTT
pTXB1Fwd (*Nde*I)	GGAATTC*CATATG*CAGATTTTTGTGA
pTXB1Rev (*Sap*I)	GGTGGTT*GCTCTTC*CGCAGCCGCCACGCAGACGCA
K48TAGFwd	CGTCTGATTTTTGCGGGC<u>TAG</u>CAGCTGGAAGAT
K48TAGRev	GCCCGCAAAAATCAGACGCTGCTGATCCGG
PylTproKFwd(*ApaL*I)	AACCAA*GTGCAG*TGATCAAAAGCGGCCGCAAAACTAGTGGCAGCG
PylTproKRev(*Xho*I)	AACAA*CTCGAG*TTTGTCGACCAAAAAAGCCT
PylRSFwd(*Nde*I)	AAAGCGGCCGCAAA*CATATG*GATAAAAAACCACTAAACACTCT
PylRSRev(*Nsi*I)	ACACA*ATGCAT*TTACAGGTTGGTAGAAATCCCGTTATAGTA
PylRSY384FFwd	GCGATTCCTGCATGGTCT<u>T</u>TGGGGATACCCT
PylRSY384FRev	AGACCATGCAGGAATCGCCTACGATCTTGA

PylT#1	AAAGCGGCCGCAAAACTAGTGGCAGCGGCTAACTAAGCGGCCTGCTGACTTTCTCGCCGATCAAAAGGC
PylT#2	CAGATACGCCCTCGTCAATCCCTTAATAGCAAAATGCCTTTTGATCGGCGAGAAAGTC
PylT#3	AGGGATTGACGAGGGCGTATCTGCGCAGTAAGATGCGCCCCGCATTGGGAACCTGA
PylT#4	CCCGGCTGAACGGATTTAGAGTCCATTCGATCTACATGATCAGGTTCCCAATGCGGGGC
PylT#5	GACTCTAAATCCGTTCAGCCGGGTTAGATTCCCGGGGTTTCCGCCAAATTCGAAAAGCCTG
PylT#6	TTTGTCGACCAAAAAAGCCTGCTCGTTGAGCAGGCTTTTCGAATTTGGCGGAAAC

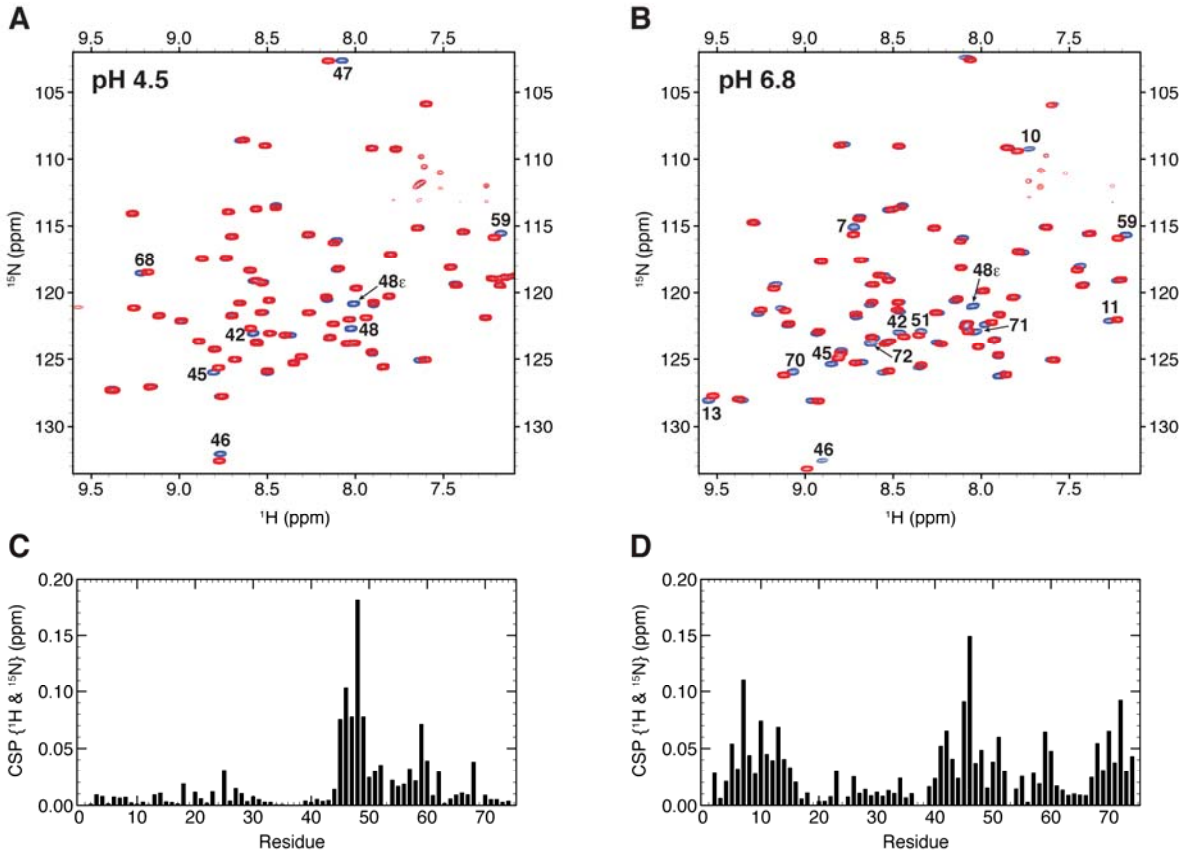

Figure S1. {^{15}N-^{1}H} TROSY-HSQC spectra of the proximal Ub_{1-74} (blue) in TFA-treated K48-linked di-Ub and of the corresponding monomeric Ub (red) at pH 4.5 (A) and pH 6.8 (B). (C,D) The spectral differences between di-Ub and mono-Ub, quantified as amide chemical shift perturbations (CSPs), are plotted as a function of the residue number for pH 4.5 (C) and pH 6.8 (D). The CSPs were calculated as $CSP = [(\Delta\delta_H)^2 + (\Delta\delta_N/5)^2]^{1/2}$, where $\Delta\delta_H$ and $\Delta\delta_N$ are chemical shift differences for ^{1}H and ^{15}N, respectively. Residues with significant CSPs are indicated on the spectra above. The amide crosspeak arising from the isopeptide linkage to the side chain of Lys-48 is labeled as 48ε.

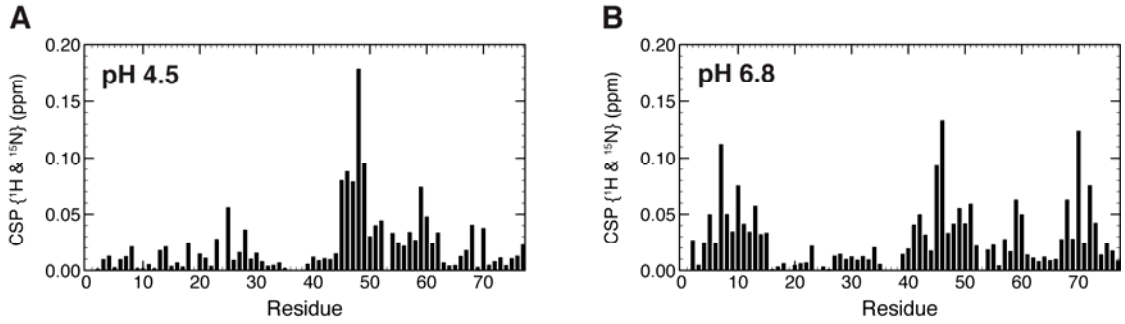

Figure S2. Shown are the chemical shift perturbations at pH 4.5 (A) and pH 6.8 (B) for the proximal ubiquitin in K48-linked di-Ub constructed from UbK48R and UbD77 ubiquitins. The corresponding monomer is UbD77. The overall CSP patterns at both pHs are very similar to K48-linked di-Ub constructed from UbK48Lys(Boc) and Ub_{1-74} (Figure S1).

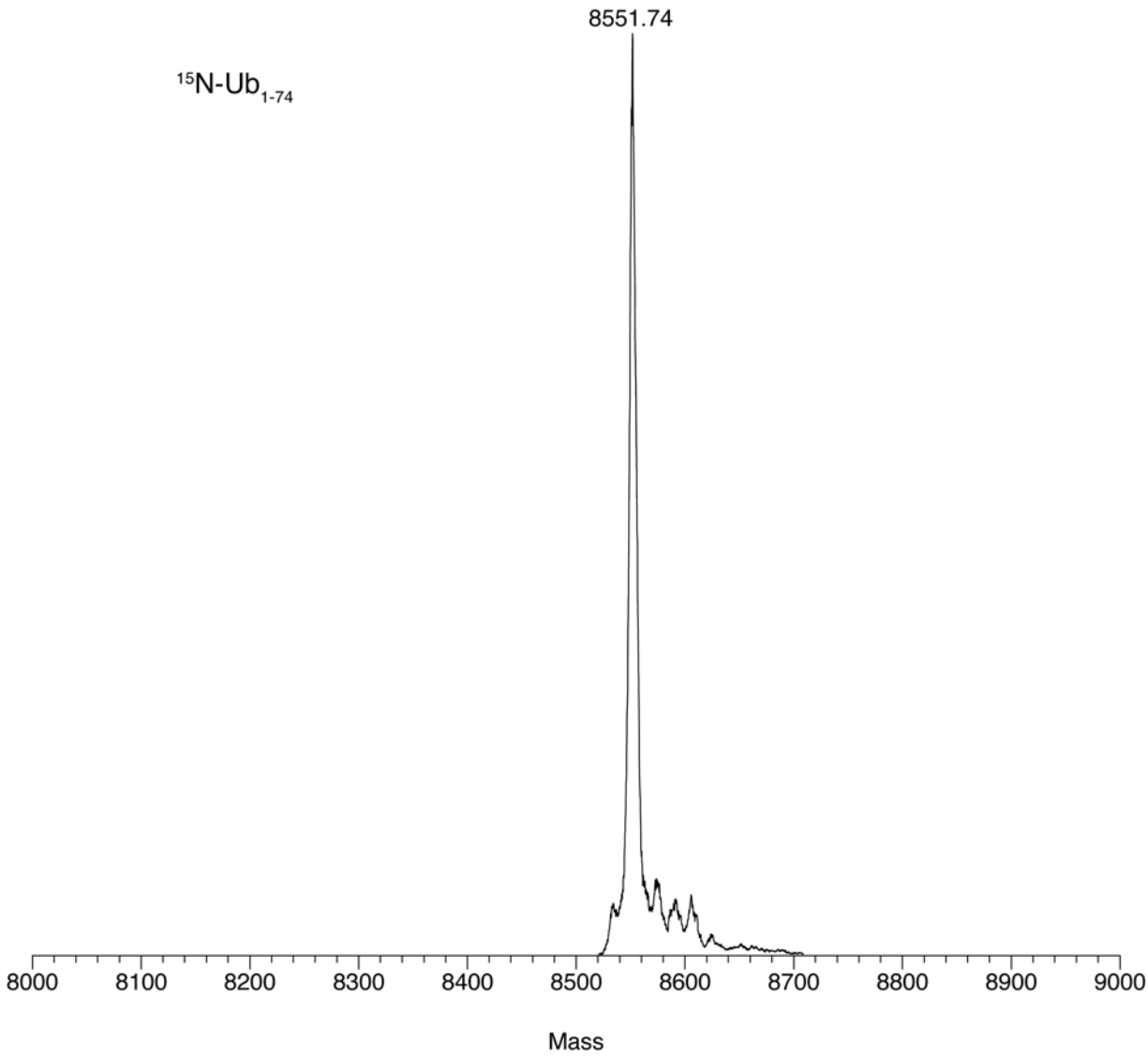

Figure S3. ESI-MS deconvolution of ^{15}N-Ub$_{1-74}$. Observed molecular weight of 8552 Da (expected 8552 Da assuming 99% ^{15}N incorporation).

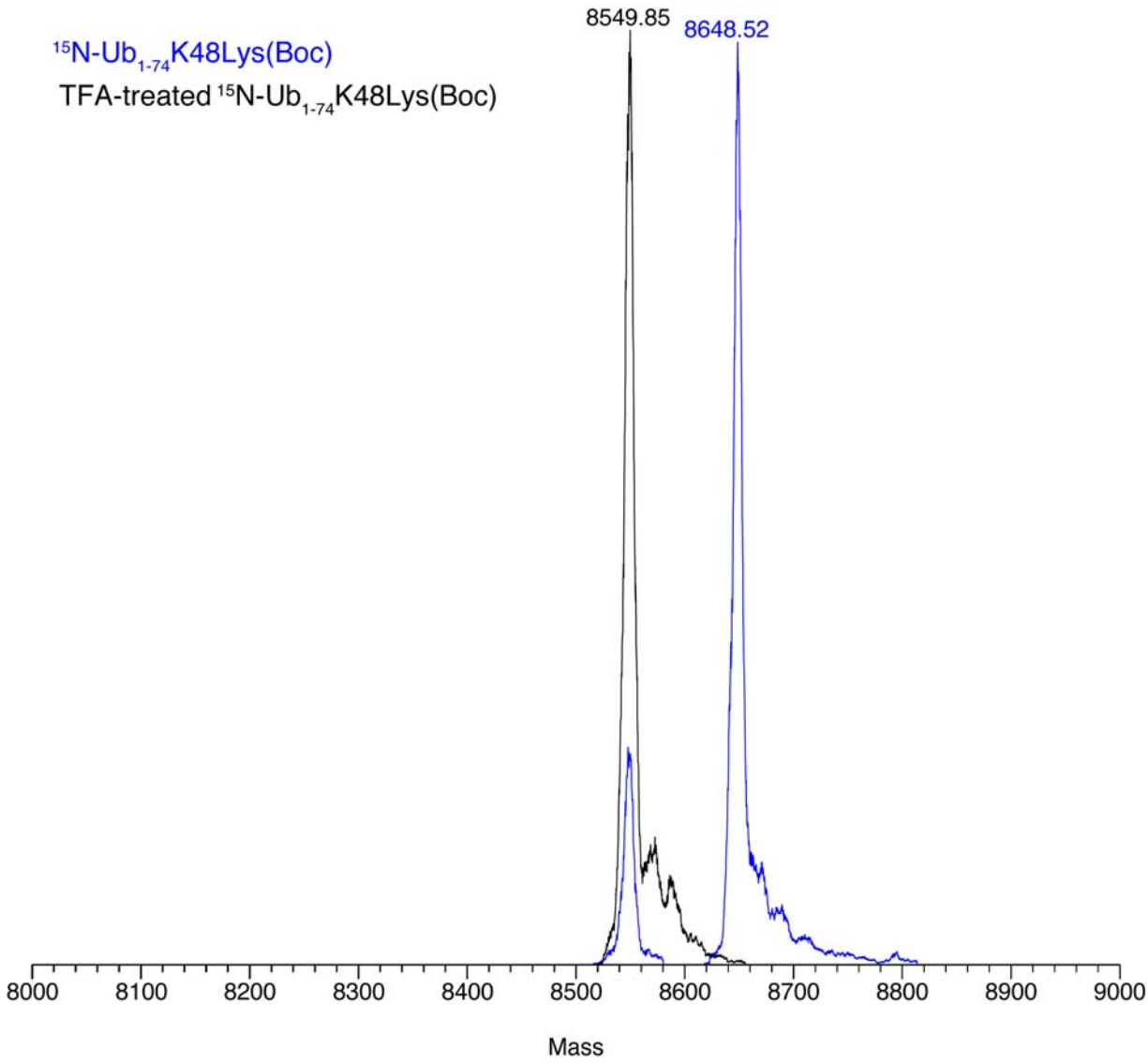

Figure S4. ESI-MS deconvolution of ^{15}N-Ub$_{1-74}$K48Lys(Boc) before (blue trace) and after TFA treatment (black trace). Prior to TFA treatment, the observed molecular weight was 8649 Da (expected: 8649 Da assuming 99% ^{15}N incorporation). Minor peak at 8550 Da is due to partial removal of the Boc group from electrospray ionization. After TFA treatment, the mass is decreased by 99 Da (molecular weight of one Boc protecting group) to 8550 Da. Mass of TFA-treated ^{15}N-Ub$_{1-74}$K48Lys(Boc) differs by 2 Da from that of ^{15}N-Ub$_{1-74}$ due to incorporation of ^{14}N-labeled Lys(Boc) amino acid at position 48.

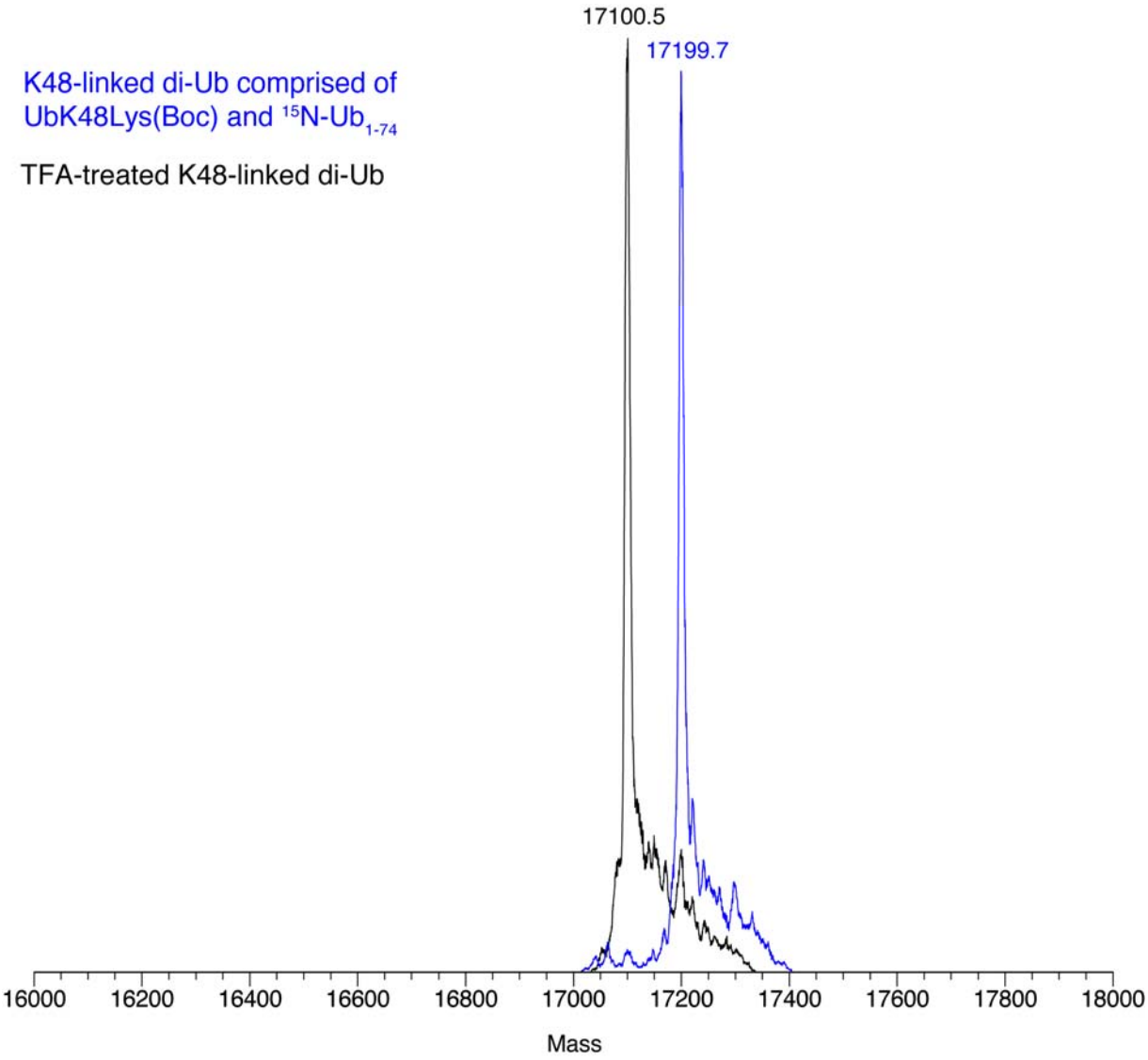

Figure S5. ESI-MS deconvolution of K48-linked di-Ub comprised of the UbK48Lys(Boc) monomer and ^{15}N-Ub$_{1-74}$ monomer before (blue trace) and after TFA treatment (black trace). Prior to TFA treatment, the observed molecular weight is 17200 Da (expected: 17200 Da). After TFA treatment of di-Ub, the Boc-containing peak is eliminated and a loss of 99 Da is detected. This loss is in accord with the molecular weight of one Boc-protecting group.

References

1. Y. Ryu and P. G. Schultz, *Nat Methods*, 2006, **3**, 263-265.
2. T. Yanagisawa, R. Ishii, R. Fukunaga, T. Kobayashi, K. Sakamoto and S. Yokoyama, *Chem Biol*, 2008, **15**, 1187-1197.
3. J. Liu, C. A. Castaneda, B. J. Wilkins, D. Fushman and T. A. Cropp, *Bioorg Med Chem Lett*, 2010, **20**, 5613-5616.
4. S. Raasi, R. Varadan, D. Fushman and C. M. Pickart, *Nat Struct Mol Biol*, 2005, **12**, 708-714.
5. R. Varadan, M. Assfalg, S. Raasi, C. Pickart and D. Fushman, *Mol Cell*, 2005, **18**, 687-698.
6. R. Varadan, O. Walker, C. Pickart and D. Fushman, *J Mol Biol*, 2002, **324**, 637-647.
7. C. M. Pickart and S. Raasi, *Methods Enzymol*, 2005, **399**, 21-36.
8. Z. Zhang and A. G. Marshall, *J Am Soc Mass Spectrom*, 1998, **9**, 225-233.
9. F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer and A. Bax, *J Biomol NMR*, 1995, **6**, 277-293.
10. T. D. Goddard and D. G. Kneller. SPARKY 3.