Supplementary information

De novo macrocyclic peptides that specifically modulate Lys48-linked ubiquitin chains

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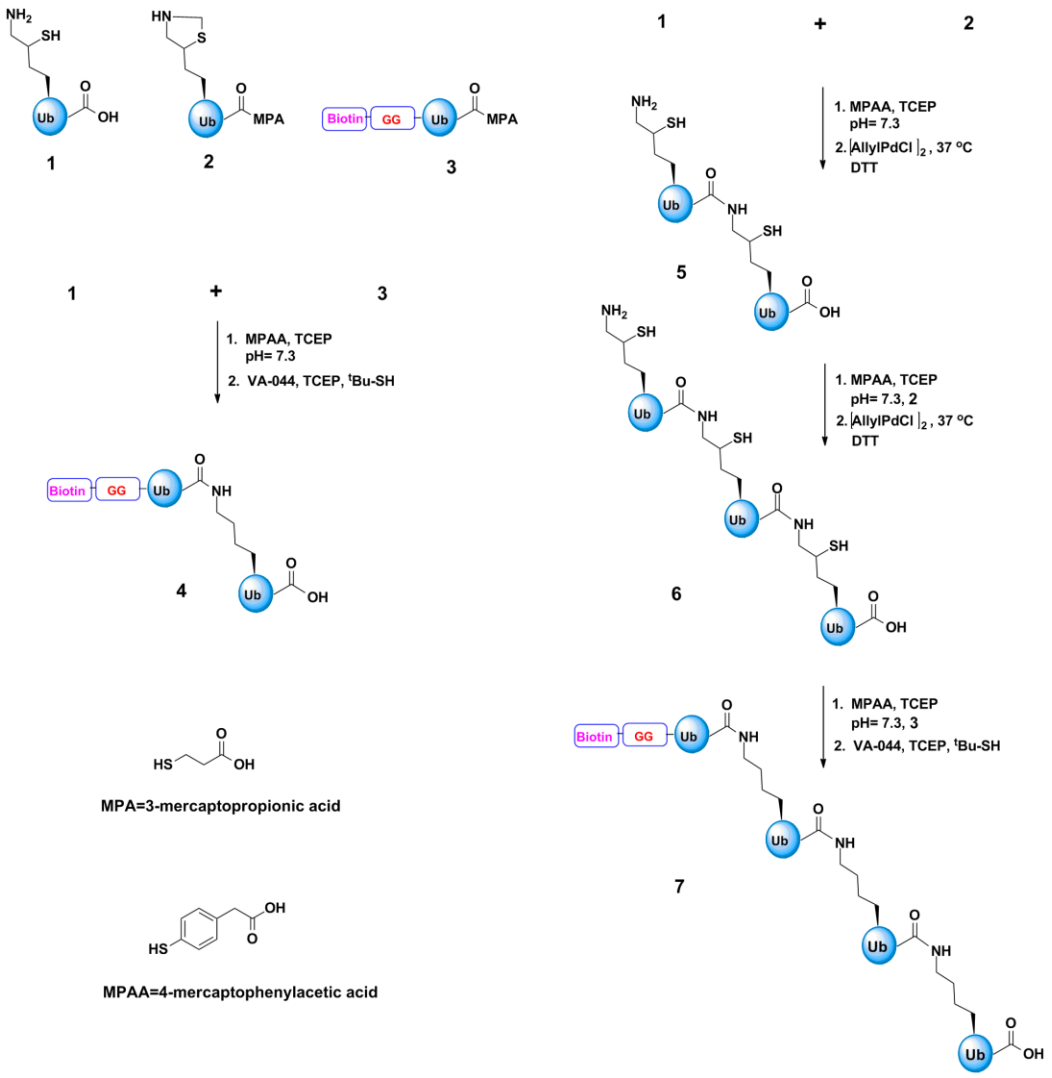
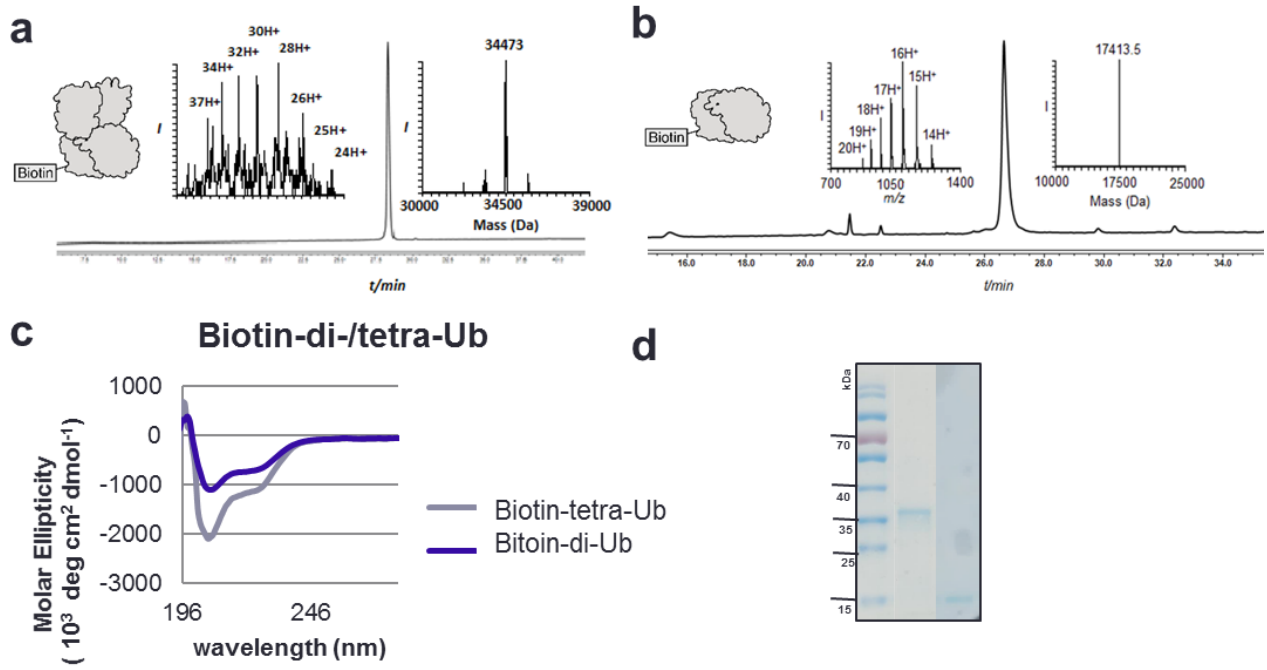
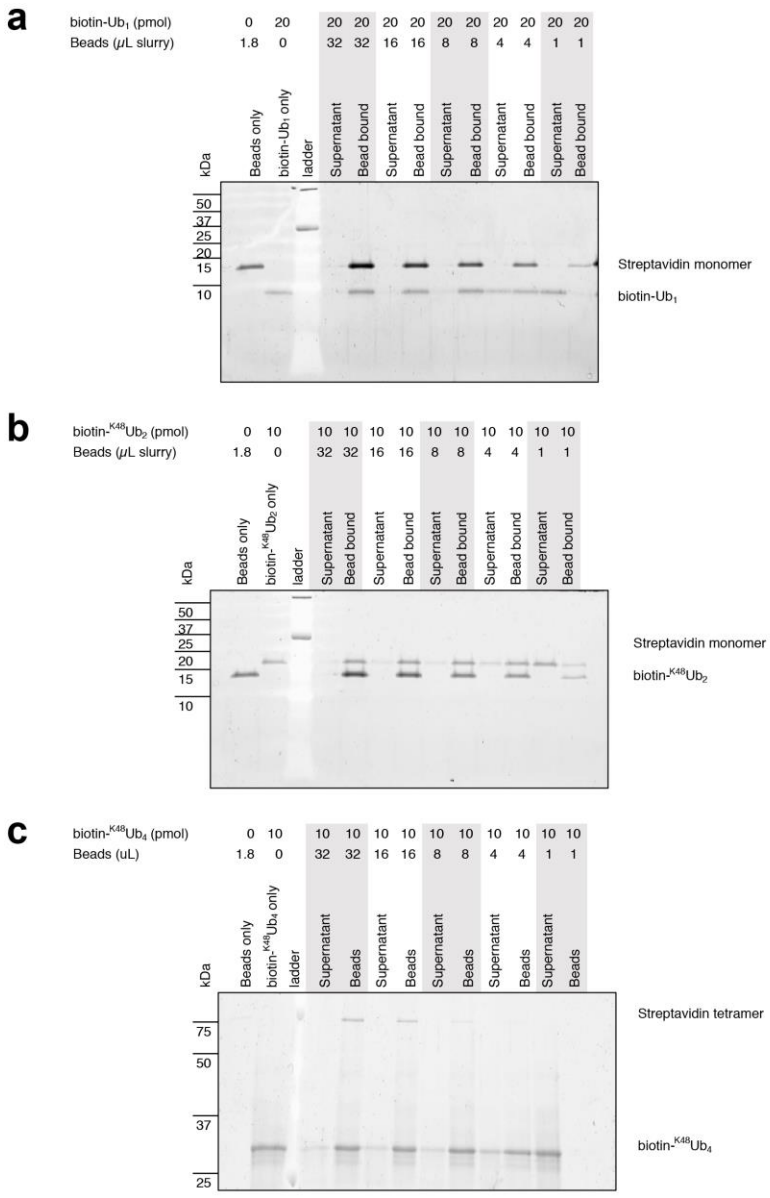


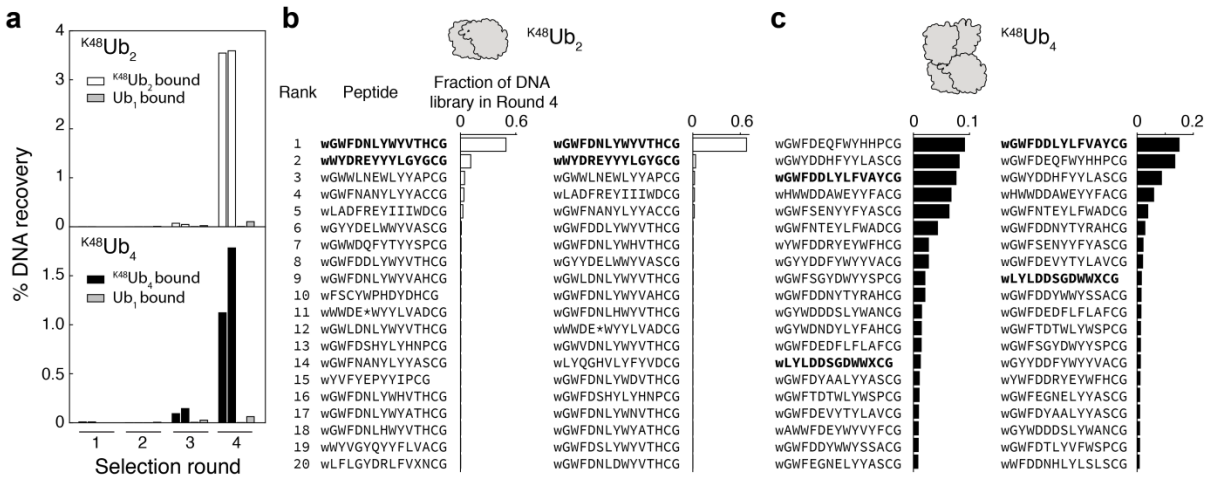
Supplementary Figure 1. **Our strategy for the synthesis of biotin Ub chain**. The synthesis was carried out according to Ref[1] with the mentioned modifications. Briefly, for the synthesis of the Ub chains we prepared Ub building blocks **1**, **2** and **3** (biotin-Ub-MPA) directly on solid support as described previously. The synthesis of the biotin-$^{K48}Ub_2$ chain was achieved by ligating **1** and **3** building blocks. The synthesis of biotin-$^{K48}Ub_4$ chain was carried out by using a sequential approach, first by ligating building blocks **1** and **2** to get **5** which was treated with allylpalladium chloride dimer $[AllylPdCl]_2$ to unmask the thiazolidine protection. This enabled us to ligate building block **2** in order to get $^{K48}Ub_3$, **6**. The ligation between **6** ($^{K48}Ub_3$) and **3** (biotin-Ub-MPA) gave the biotin-$^{K48}Ub_4$, which was subjected to the radical mediated desulfurization and HPLC (employing C4 column and gradient 0-60% B) and FPLC purification steps to give the desired native $^{K48}Ub_4$ chain with high purity.



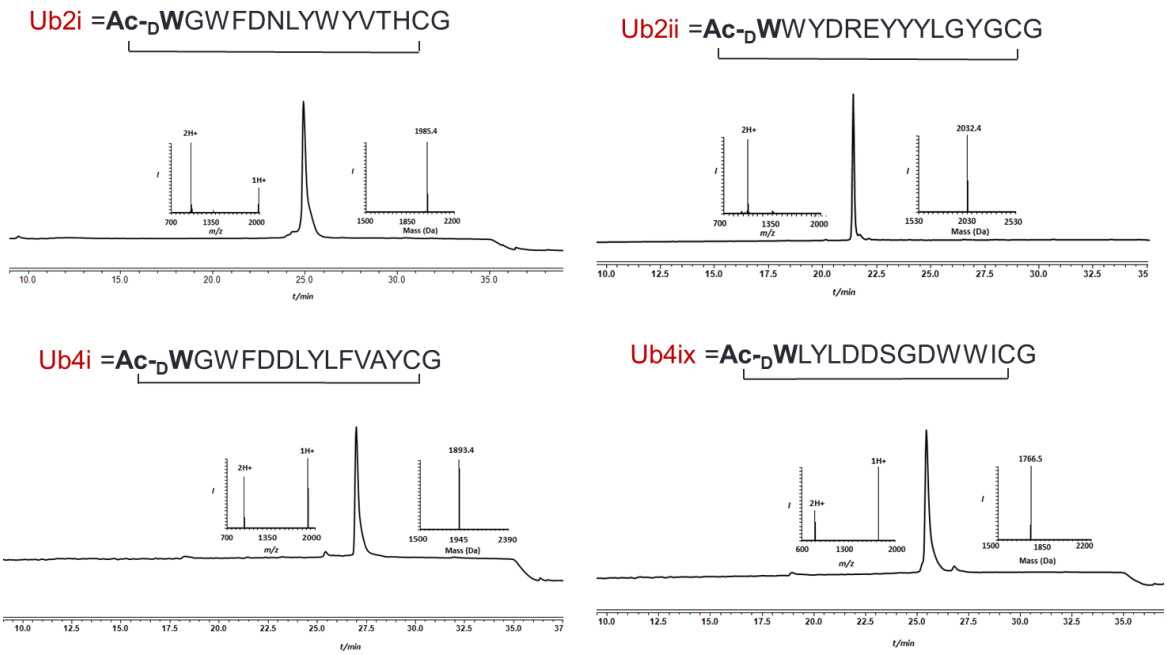
Supplementary Figure 2. Characterization of biotin-$^{K48}Ub_{2,4}$ chains. a) Analytical HPLC and ESI-MS of the purified biotin-$^{K48}Ub_4$ with the observed mass 34473 ± 1.3 Da (calculated 34473.3). **b)** Analytical HPLC and ESI-MS of the biotin-$^{K48}Ub_2$ with the observed mass 17,413.5 ± 0.8 Da. (calculated 17,413), **c)** Circular Dichroism (CD) analyses of the native biotin-$^{K48}Ub_{2,4}$ revealed correct folding compared to the secondary structure of Ub and **d)** SDS-PAGE gel of the purified biotin-$^{K48}Ub_{2,4}$ chains.



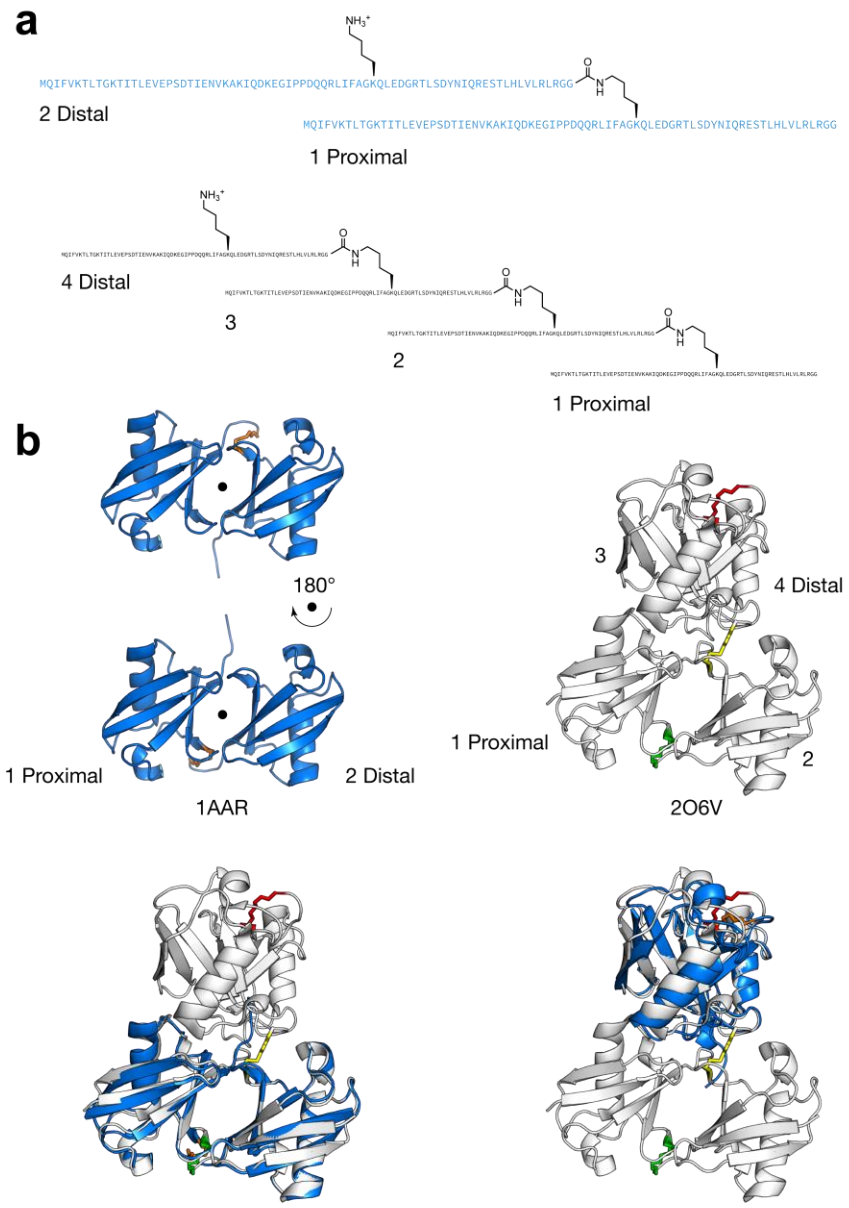
Supplementary Figure 3. Biotin Ub chains bind to streptavidin magnetic beads. a) Biotin-Ub_1 can be recovered bound to streptavidin magnetic beads, with increasing amount of biotin-Ub_1 removed from the supernatant with greater amounts of beads. **b**) Same for biotin-$^{K48}Ub_2$ and biotin-$^{K48}Ub_4$ (**c**).



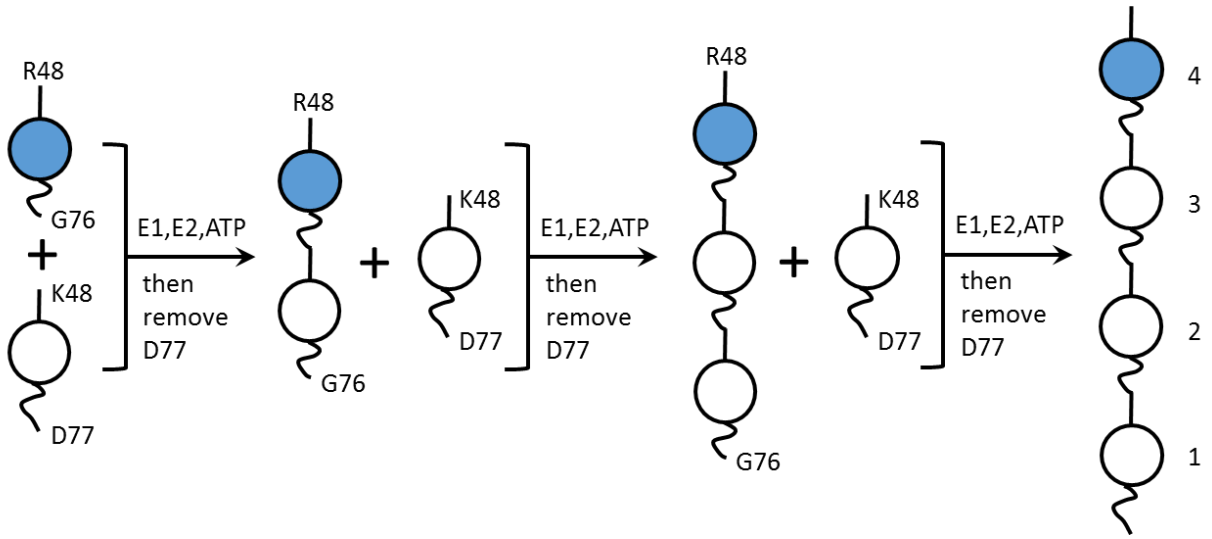
Supplementary Figure 4. Total DNA recovered increased and specific peptide sequences are enriched during RaPID selections. a) Yield of DNA recovered after each round of selection against $^{K48}Ub_2$ and $^{K48}Ub_4$ targets. An increased in recovered DNA with each round is an indication that the libraries were becoming enriched in tight binding cyclic peptides. **b**) For the two repeats of the $^{K48}Ub_2$ selection, sequencing of the DNA library after round 4 shows enrichment for the same two peptides (bold), corresponding to **Ub2i** and **Ub2ii**. * = stop codon, X = start 'ATG' codon, only the top 20 peptides are shown. **c**) For the two repeats of the $^{K48}Ub_2$ selection, similar peptides appeared in the top peptides. **Ub4i** and **Ub4ix** shown in bold. In the synthesized peptide **Ub4ix**, X was replaced with isoleucine.



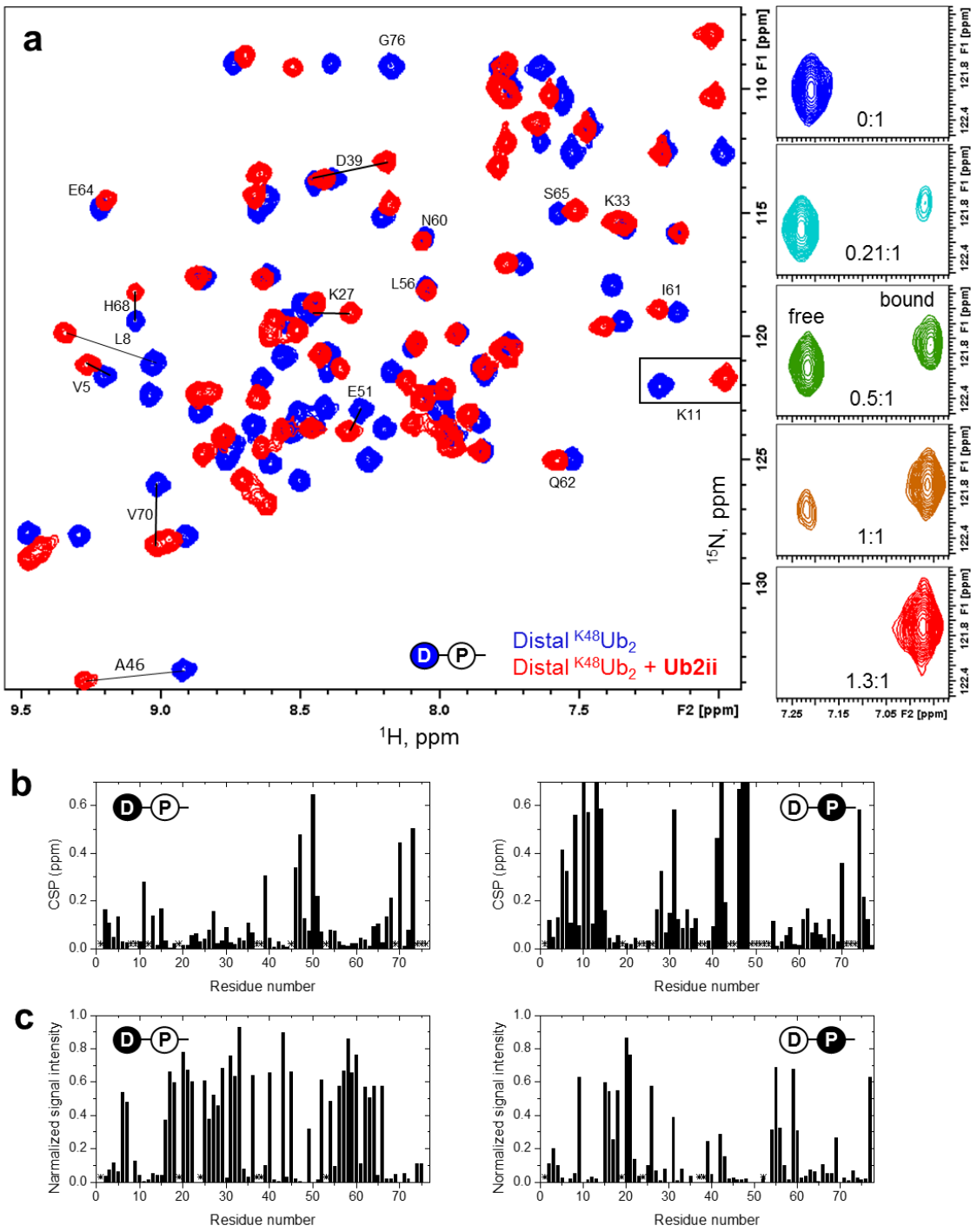
Supplementary Figure 5. Synthesis and characterization of the cyclic peptides. a) Analytical HPLC and ESI-MS analysis of the purified cyclic peptides Ub2i, Ub2ii, Ub4i and Ub4ix with the observed mass 1985.4 ± 0.1 Da, 2032.4 ± 0.1 Da, 1893.4 ± 0.1 and 1766.5 ± 0.2 Da, respectively (calculated 1985.4 Da, 2032.4 Da, 1893.4 and 1766.5 Da, respectively).



Supplementary Figure 6. Structure and symmetry of K48-linked Ub chains. a) Topology of $^{K48}Ub_2$ and $^{K48}Ub_4$, with the isopeptide bonds shown **b**) Structure of $^{K48}Ub_2$ (blue), PDB 1AAR showing the rotational symmetry. Structure of $^{K48}Ub_2$ (white), PDB 2O6V. $^{K48}Ub_2$ can be superimposed twice on $^{K48}Ub_4$, or four times if the isopeptide linkage is not taken into account.

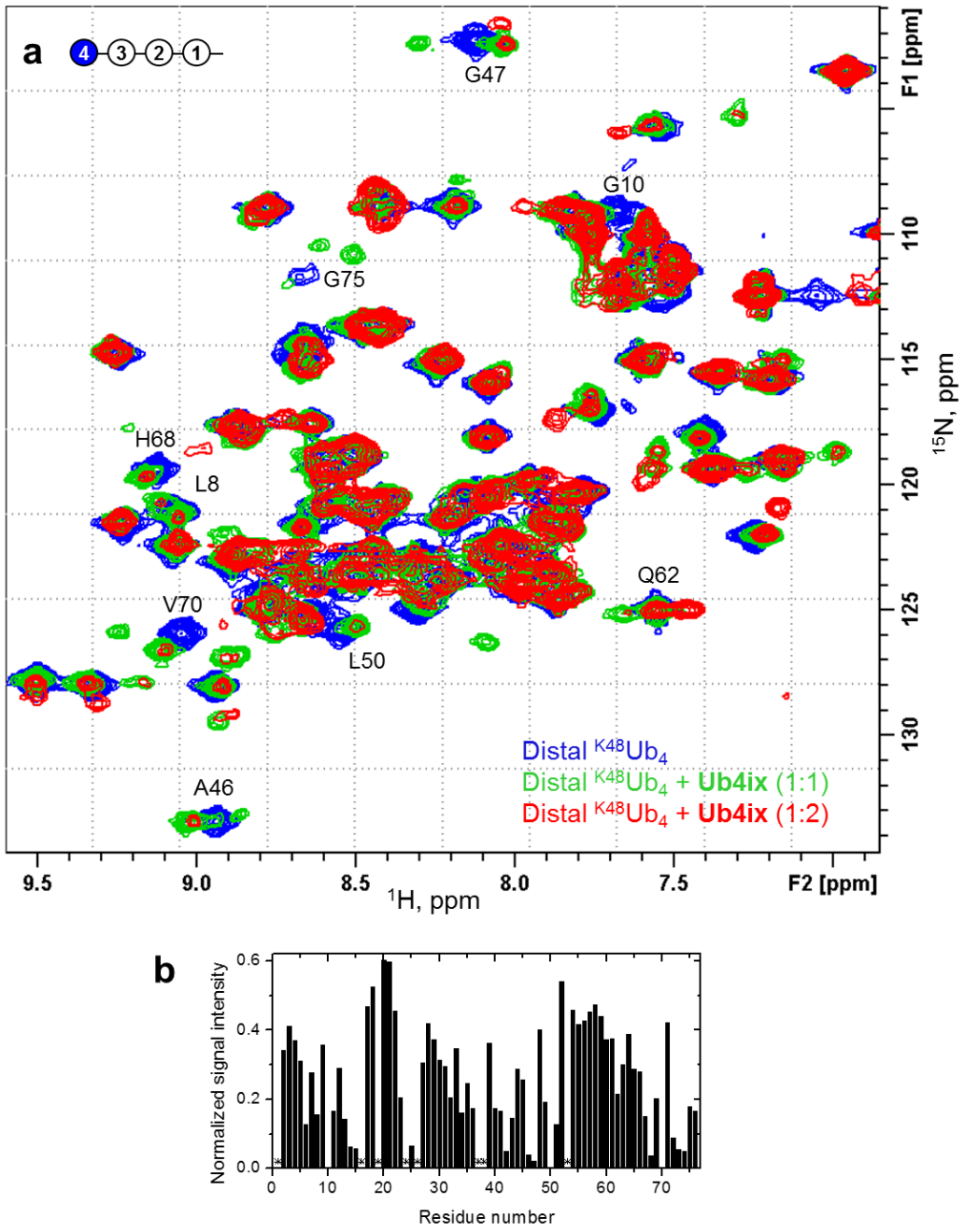


Supplementary Figure 7. Our strategy of controlled enzymatic assembly of selectively isotope-labeled K48-linked di-, tri-, and tetra-Ub chains. Shown is assembly of Ub chains having the most distal Ub unit (colored) isotope enriched for NMR studies. Chains with other Ub units isotope-labeled were made in a similar manner. Also indicated is the numbering of Ub units in tetra-Ub used in the text.-



Supplementary Figure 8. Residue-specific perturbations in the distal Ub unit of $^{K48}Ub_2$ upon Ub2ii binding. a) Overlay of the ^{1}H-^{15}N correlation spectra of $^{K48}Ub_2$ (distal Ub) free (blue) and in the presence of 1.3 molar equivalent of **Ub2ii** (red). Signal shifts for select residues are indicated. To illustrate the slow-exchange regime of binding, insets on the right show the

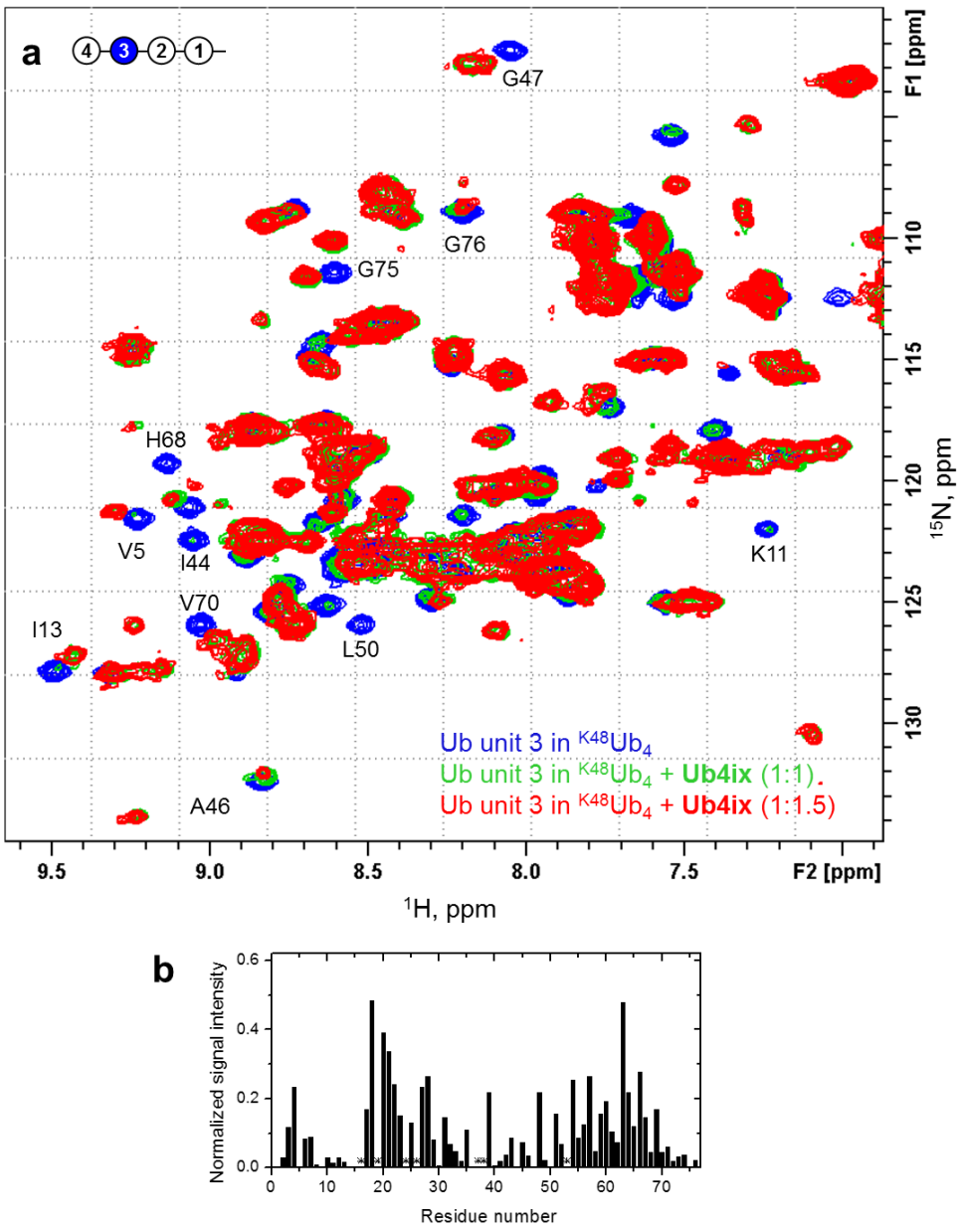
behavior of the signal of K11 (boxed) during the titration with the peptide, at the indicated peptide:Ub_2 molar ratios. In agreement with the very slow (~1 per minute) off-rates observed by SPR, we were unable to detect exchange cross-peaks in zz-exchange NMR spectra even with exchange times up to 2 s. **b)** Residue-specific chemical shift perturbations at the endpoint of titration with **Ub2ii** in the distal (left) and proximal (right) Ub units in $^{K48}Ub_2$. **c)** Intensities of the unbound signals of the distal (left) and proximal (right) Ub units in $^{K48}Ub_2$ at the endpoint of titration with **Ub2ii**. The signal intensity for each residue was normalized to the corresponding intensity measured in the absence of peptide. The spectral perturbations in $^{K48}Ub_2$ were accompanied by a concomitant decrease in the transverse relaxation time (T_2) of amide protons, from ~25 ms to ~16 ms, consistent with an increase in the overall size of $^{K48}Ub_2$ upon complexation with the peptide[3]. The drawings indicate which Ub unit in Ub_2 is analyzed. Asterisks indicate residues that could not be detected or could not be reliably quantified due to signal overlap.



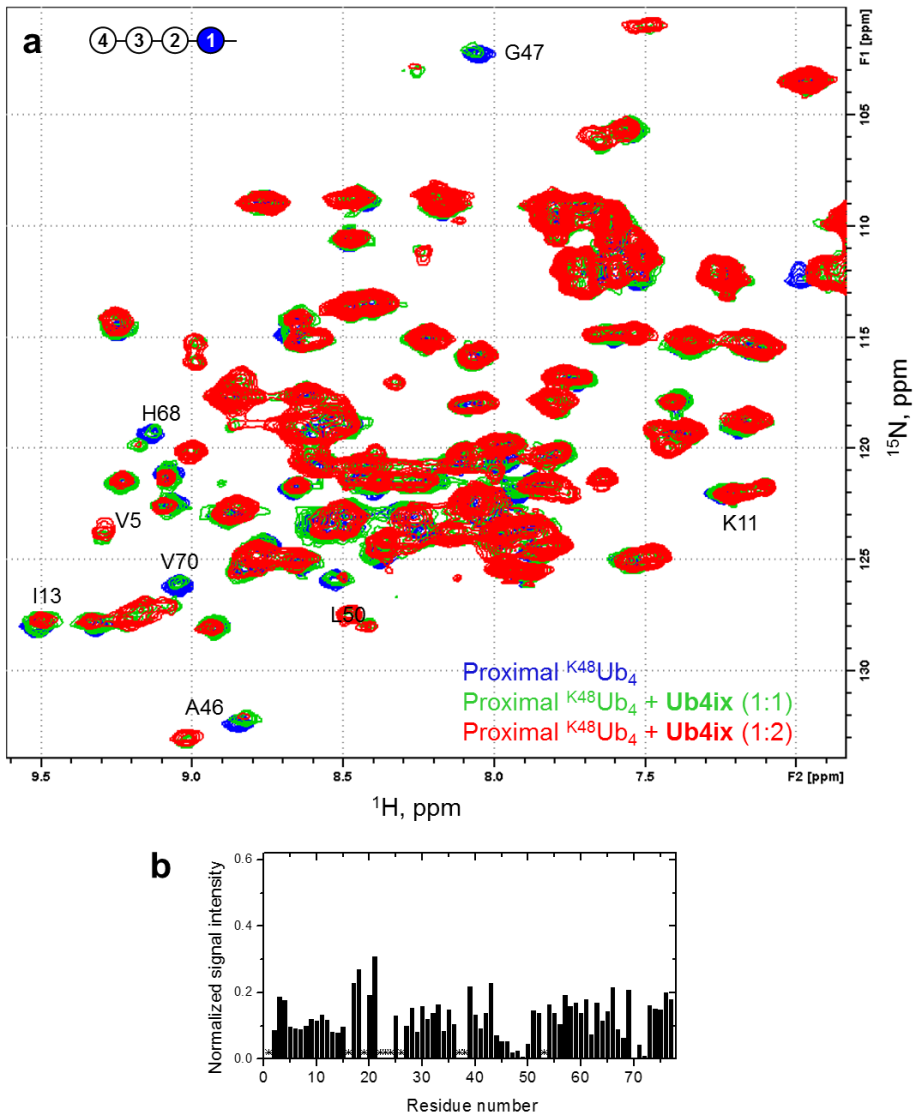
Supplementary Figure 9. Residue-specific perturbations in the distal Ub unit of $^{K48}Ub_4$ upon Ub4ix binding. a) Overlay of the ^{1}H-^{15}N correlation spectra of $^{K48}Ub_4$ (distal Ub) free (blue) and in the presence of 1 (green) and 2 (red) molar equivalents of **Ub4ix** (red). Select perturbed residues are indicated. Note several alternate (bound) peaks, for example, for residues G47, Q62, and G75. **b)** Intensities of the unbound signals at the endpoint of titration with **Ub4ix**. The signal intensity for each residue was normalized to the corresponding intensity measured in

the absence of peptide. Asterisks indicate residues that could not be detected or could not be reliably quantified due to signal overlap.

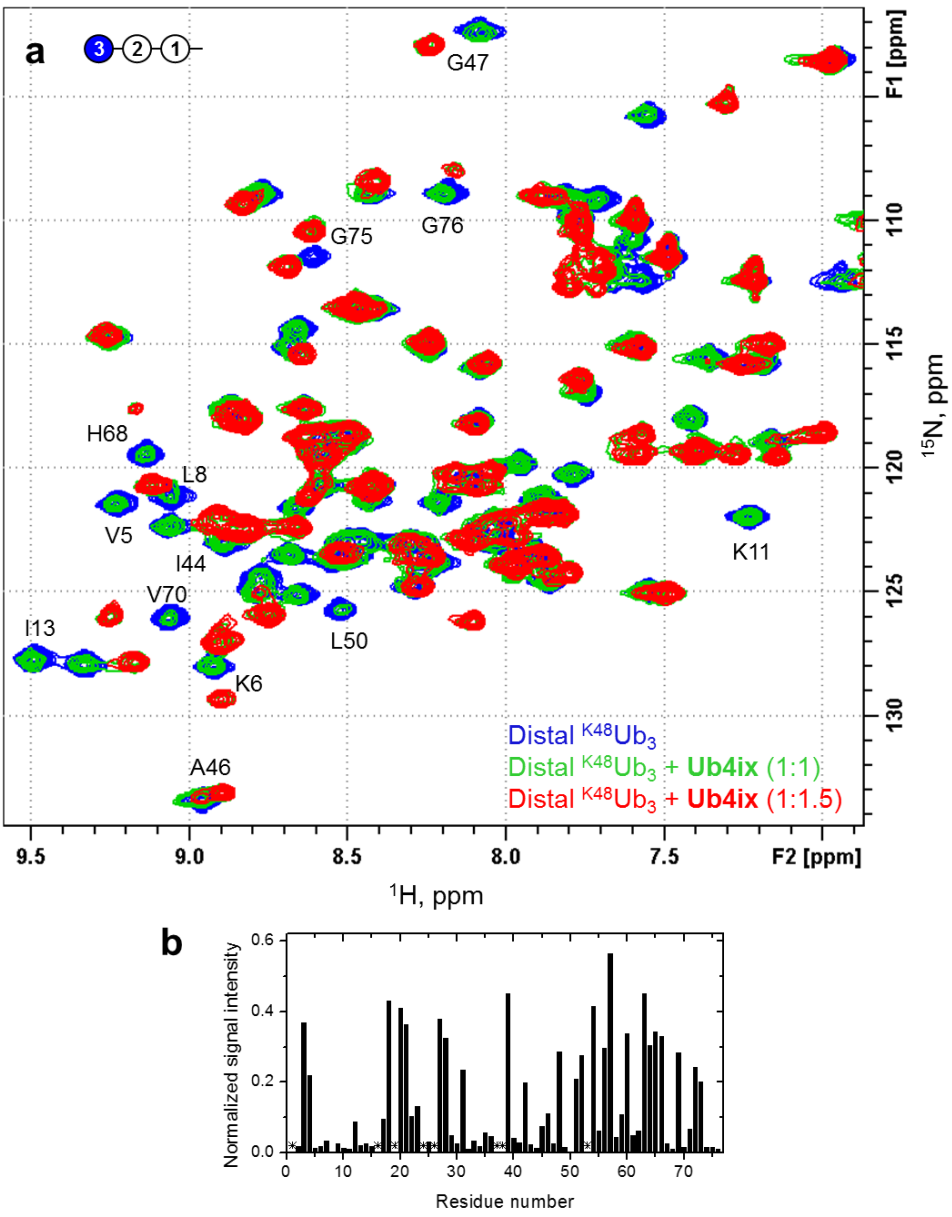
In contrast to Ub unit 3 (see Fig S14), the unbound signals of the distal Ub did not completely disappear even at 2 molar equivalents of the peptide, suggesting occurrence of a second binding event. This is also corroborated by the continuing decrease in ^{1}H T_2 from 13 ms (free Ub_4) to 10 ms to 8 ms (at 1 and 2 molar equivalents of the peptide, respectively). Moreover, for some residues in the distal Ub we observed more than one alternate signal upon titration (e.g. G47, G75 in panel **a**), indicative of more than one bound conformation of the peptide.



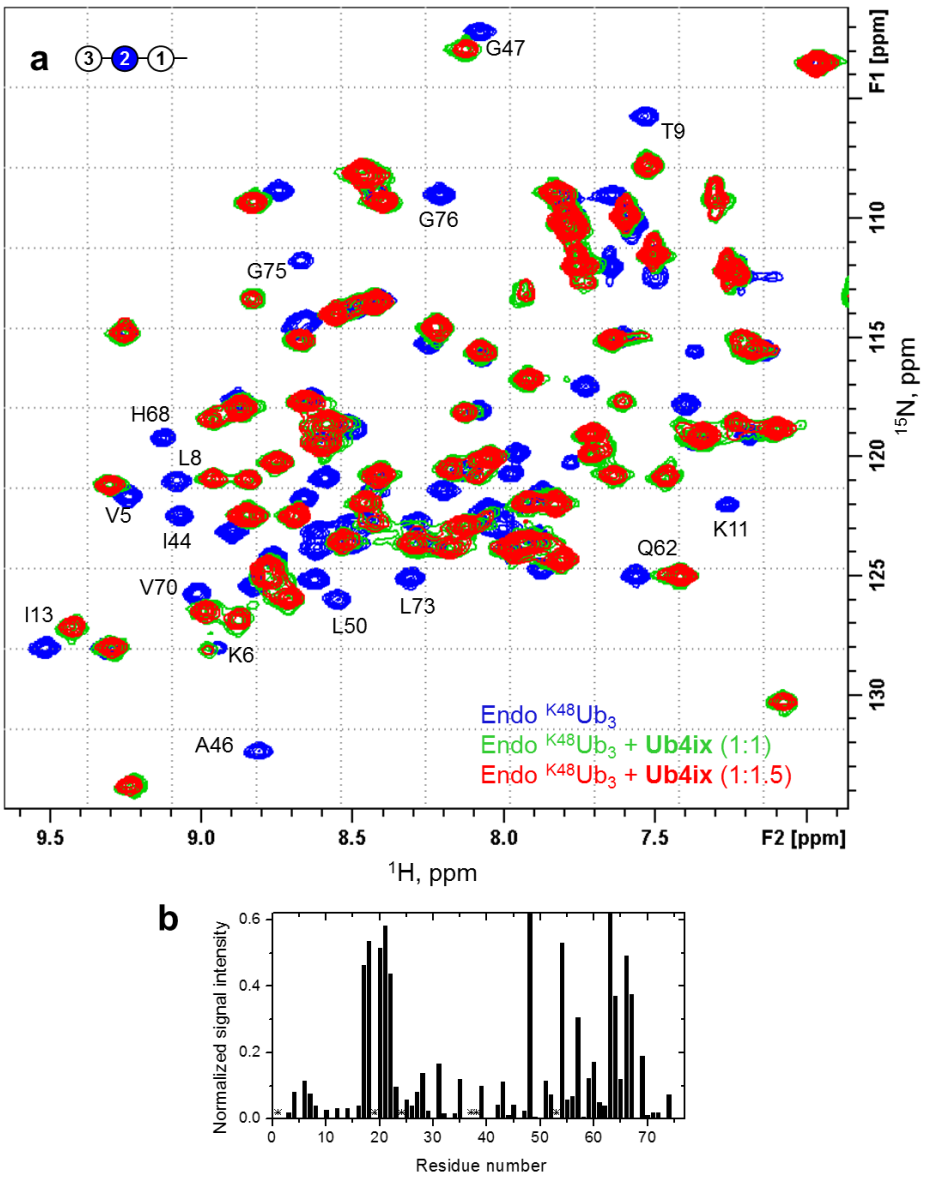
Supplementary Figure 10. Residue-specific perturbations in Ub unit 3 of $^{K48}Ub_4$ upon Ub4ix binding. a) Overlay of the ^{1}H-^{15}N correlation spectra of $^{K48}Ub_4$ (unit 3 ^{15}N-enriched) free (blue) and in the presence of 1 (green) and 1.5 (red) molar equivalents of **Ub4ix** (red). Select perturbed residues are indicated. **b)** Intensities of the unbound signals at the endpoint of titration with **Ub4ix**. The signal intensity for each residue was normalized to the corresponding intensity measured in the absence of peptide. Asterisks indicate residues that could not be detected or could not be reliably quantified due to signal overlap.



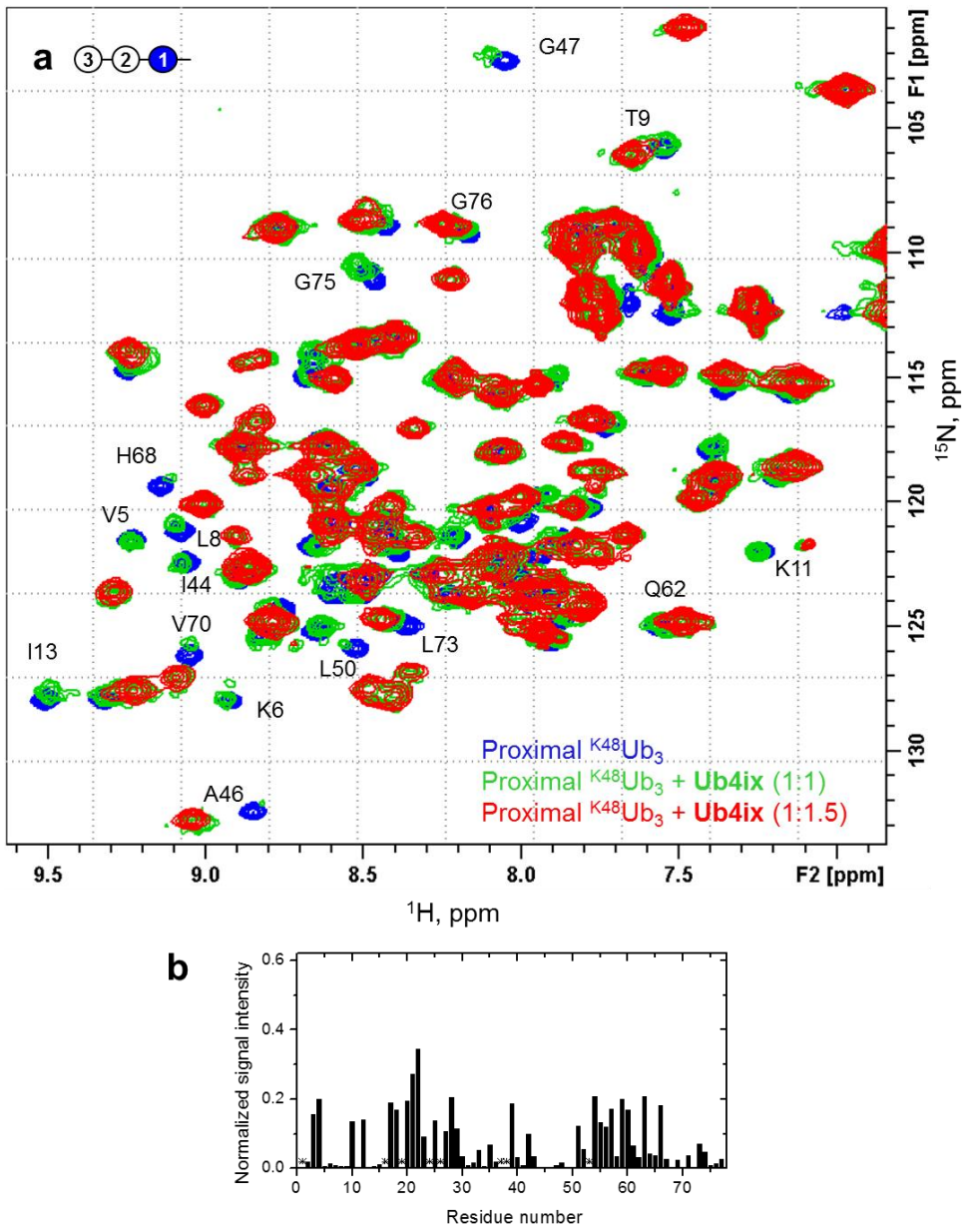
Supplementary Figure 11. Residue-specific perturbations in the proximal Ub of $^{K48}Ub_4$ upon Ub4ix binding. a) Overlay of the ^{1}H-^{15}N correlation spectra of $^{K48}Ub_4$ (proximal) free (blue) and in the presence of 1 (green) and 2 (red) molar equivalents of **Ub4ix**. Select perturbed residues are indicated. **b)** Intensities of the unbound signals at the endpoint of titration with **Ub4ix**. The signal intensity for each residue was normalized to the corresponding intensity measured in the absence of peptide. Asterisks indicate residues that could not be detected or could not be reliably quantified due to signal overlap. The average amide ^{1}H T_2 decreased from ~13 ms (free Ub_4) to ~10 ms at 1:1 to 8 ms at 1:2 Ub_4:peptide molar ratio, indicative of gradual increase in the overall size of Ub_4 upon **Ub4ix** binding and suggesting a 1:2 stoichiometry of binding.



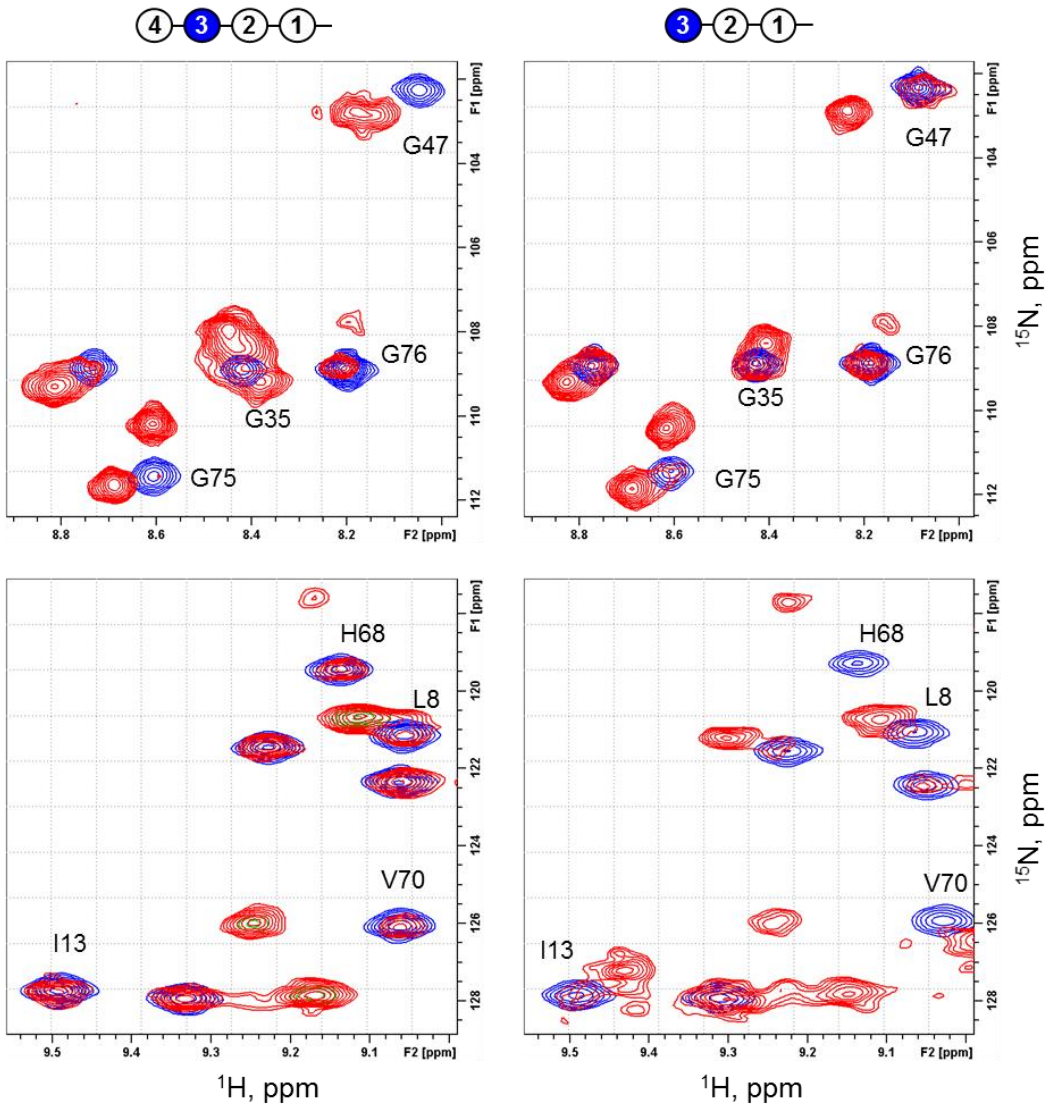
Supplementary Figure 12. Residue-specific perturbations in the distal Ub of $^{K48}Ub_3$ upon Ub4ix binding. a) Overlay of the ^{1}H-^{15}N correlation spectra of $^{K48}Ub_3$ (distal) free (blue) and in the presence of 1 (green) and 1.5 (red) molar equivalents of **Ub4ix**. Select perturbed residues are indicated. **b)** Intensities of the unbound signals at the endpoint of titration with **Ub4ix**. The signal intensity for each residue was normalized to the corresponding intensity measured in the absence of peptide. Asterisks indicate residues that could not be detected or could not be reliably quantified due to signal overlap.



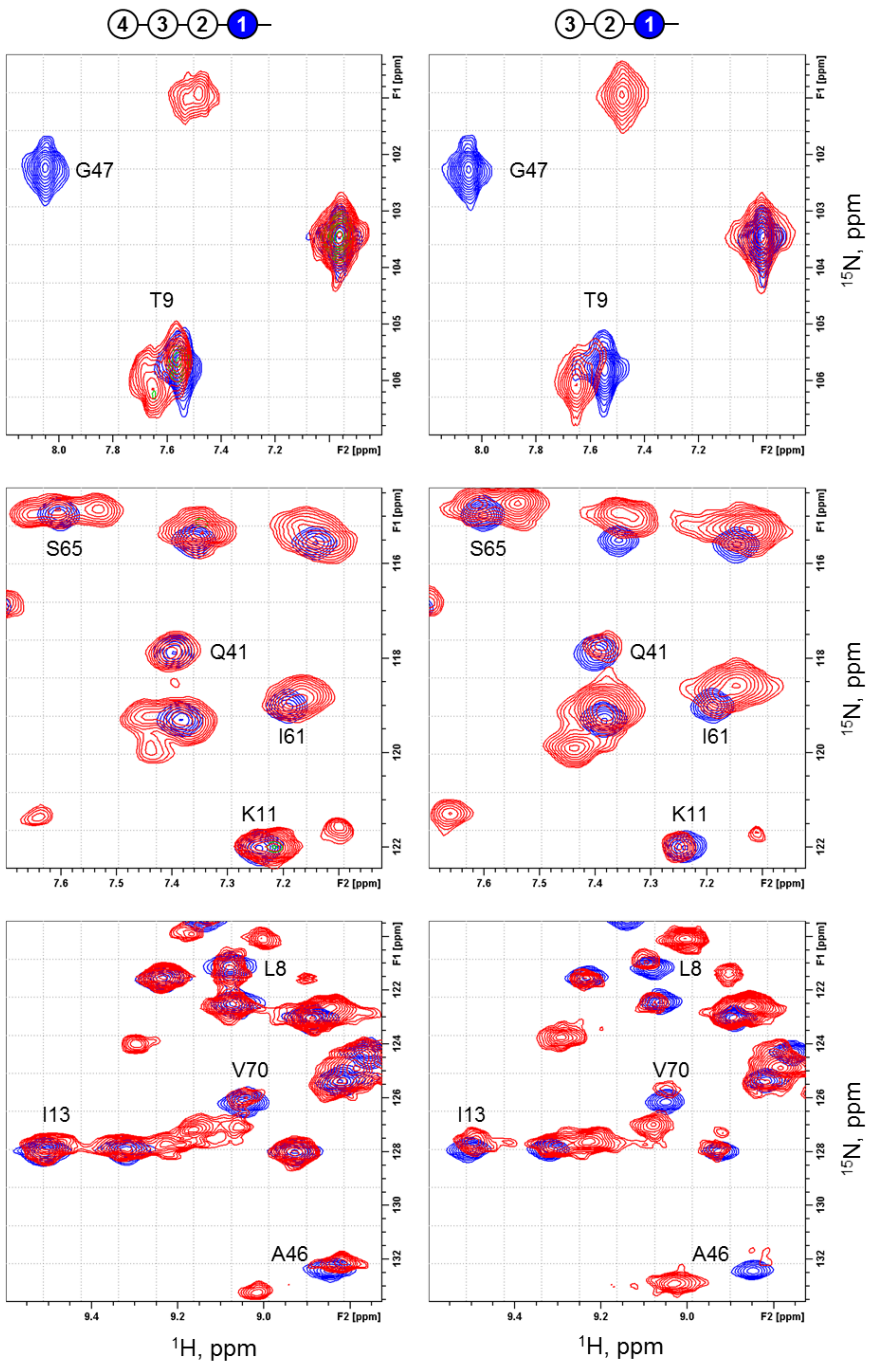
Supplementary Figure 13. Residue-specific perturbations in the endo Ub of $^{K48}Ub_3$ upon Ub4ix binding. **a)** Overlay of the ^{1}H-^{15}N correlation spectra of $^{K48}Ub_3$ (endo Ub ^{15}N enriched) free (blue) and in the presence of 1 (green) and 1.5 (red) molar equivalents of **Ub4ix**. Select perturbed residues are indicated. Note little change between the green and red spectra, indicating that most signals are in the bound state already at the 1 molar equivalent of the peptide. **b)** Intensities of the unbound signals at the endpoint of titration with **Ub4ix**. The signal intensity for each residue was normalized to the corresponding intensity measured in the absence of peptide. Asterisks indicate residues that could not be detected or could not be reliably quantified due to signal overlap.



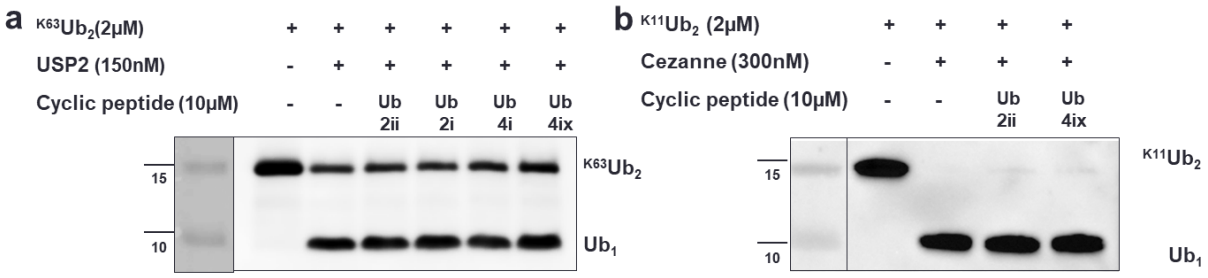
Supplementary Figure 14. Residue-specific perturbations in the proximal Ub of $^{K48}Ub_3$ upon Ub4ix binding. a) Overlay of the ^{1}H-^{15}N correlation spectra of $^{K48}Ub_3$ (proximal) free (blue) and in the presence of 1 (green) and 1.5 (red) molar equivalents of **Ub4ix**. Select perturbed residues are indicated. **b)** Intensities of the unbound signals at the endpoint of titration with **Ub4ix**. The signal intensity for each residue was normalized to the corresponding intensity measured in the absence of peptide. Asterisks indicate residues that could not be detected or could not be reliably quantified due to signal overlap.



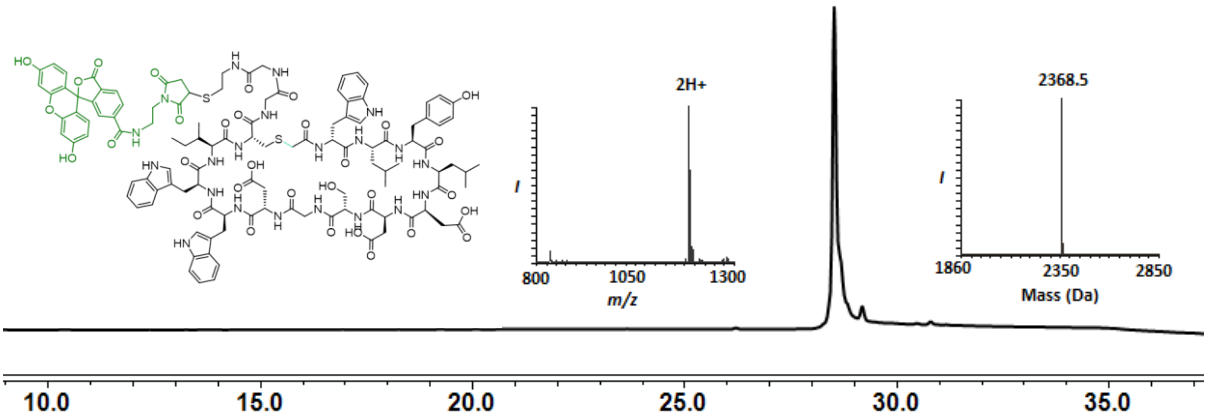
Supplementary Figure 15. Ub unit 3 in $^{K48}Ub_4$ and the distal Ub in $^{K48}Ub_3$ exhibit similar patterns of residue-specific NMR signal shifts upon Ub4ix binding. Shown is an overlay of regions of the ^{1}H-^{15}N correlation spectra of Ub unit 3 in $^{K48}Ub_4$ (left column) and the distal Ub in $^{K48}Ub_3$ (right column), free (blue) and in the presence of 1 molar equivalent (red) of **Ub4ix**. Unbound signals corresponding to select residues are indicated.



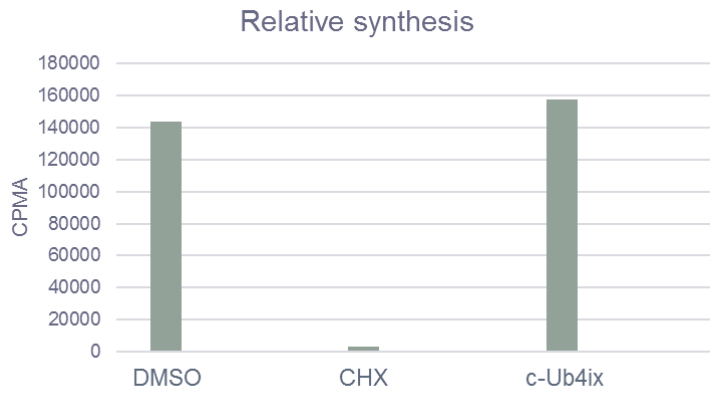
Supplementary Figure 16. Proximal Ub units in $^{K48}Ub_4$ and $^{K48}Ub_3$ exhibit similar patterns of residue-specific NMR signal shifts upon Ub4ix binding. Shown is an overlay of regions of the ^{1}H-^{15}N correlation spectra of the proximal Ub in $^{K48}Ub_4$ (left column) and in $^{K48}Ub_3$ (right column), free (blue) and in the presence of 1 molar equivalent (red) of **Ub4ix**. Unbound signals corresponding to select residues are indicated.



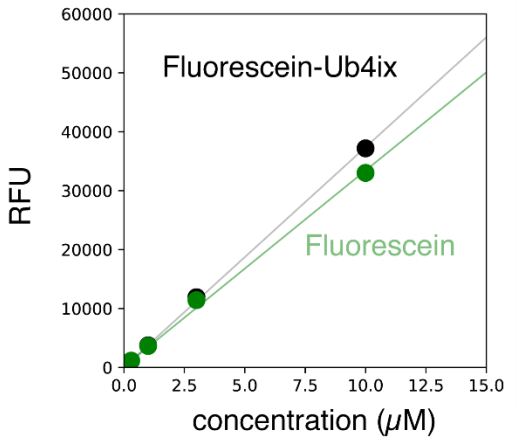
Supplementary Figure 17. **Cyclic peptides exhibit selectivity against K48-linked chains**. **a)** $^{K63}Ub_2$ was incubated for 1 hour with active USP2 enzyme and panel of cyclic peptides. **b)** $^{K11}Ub_2$ was incubated with active Cezanne enzyme and **Ub2ii** or **Ub4ix** cyclic peptides for 1 hour. The same amounts were loaded on 14% SDS-PAGE gel, electro-blotted to nitrocellulose membrane and probed with anti-Ub antibody. Bands were quantified with Image Quant LAS 4000 (GE Healthcare).



Supplementary Figure 18. Synthesis and characterization of fluorescein-5-maleimide-Ub4ix cyclic peptide. Analytical HPLC and ESI-MS analysis of the purifed fluorescein-5-maleimide-**Ub4ix** cyclic peptide with the observed mass 2368.5 ± 0.1 Da (calculated 2368.5 Da).



Supplementary Figure 19. Cyclic Ub4ix does not interfere with protein synthesis. HeLa cells were incubated with either DMSO, ribosomal inhibitor cycloheximide (CHX) (100µg/ µl), or **Ub4ix** (10 µM**)**. After 4 hours, media were replaced with media containing radioactive Met and Cys, and cells where pulsed for 4 hours. Cells were thoroughly washed, proteins were extracted from the cells, and the radioactive readings of each sample were measured using a scintillation counter, indicating the extent of incorporation of radiolabeled Met and Cys into newly synthesized proteins.



Supplementary Figure 20. Calibration of the absorption response (RFU) (ex= 480, em= 525 nm) from known concentrations of fluorescein-Ub4ix and fluorescein.

Supplementary Methods

Preparation of eFx

All oligonucleotides were purchased from Operon (Japan). The DNA template for the flexizyme eFx (DNA_eFx, below) was assembled by primer extension and PCR as described previously[5]. The DNA template was transcribed by T7 RNA polymerase and purified using 12% denaturing PAGE. The RNA product (RNA_eFx) was dissolved in water, adjusted to 250 μM and stored at -80 °C.

DNA_eFx =

GGCGTAATACGACTCACTATAGGATCGAAAGATTTCCGCGGCCCCGAAAGGGGATT
AGCGTTAGGT

RNA_eFx = GGAUCGAAAGAUUUCCGCGGCCCCGAAAGGGGAUUAGCGUUAGGU

Preparation of $tRNA^{fMet}_{CAU}$

The DNA template for $tRNA^{fMet}_{CAU}$ (DNA_tRNA, below) was assembled by primer extension and PCR as described previously[6]. The DNA template was transcribed by T7 RNA polymerase and purified using 8% denaturing PAGE, the RNA product (RNA_tRNA) was dissolved in water and the concentration adjusted to 250 μM and stored at -80 °C.

DNA_tRNA =

GGCGTAATACGACTCACTATAGGCGGGGTGGAGCAGCCTGGTAGCTCGTCGGGCTC

ATAACCCGAAGATCGTCGGTTCAAATCCGGCCCCCGCAACCA

RNA_tRNA =

GGCGGGGUGGAGCAGCCUGGUAGCUCGUCGGGCUCAUAACCCGAAGAUCGUCGGU

UCAAAUCCGGCCCCCGCAACCA

Aminoacylation of $tRNA^{fMet}_{CAU}$ with ClAc-D-Trp-CME

Initiator $tRNA^{fMet}_{CAU}$ was aminoacylated with the non-canonical amino acid N-ClAc D-Trp following protocols described previously[7, 8]. 25 µM $tRNA^{fMet}_{CAU}$, 25 µM eFx and 5 mM ClAc D-Trp-CME were incubated in 50 mM HEPES-KOH pH 8.4, 600 mM $MgCl_2$ in 20% DMSO for 2 hours at 0 °C. The reaction was stopped by adding 4 reaction volumes of 0.3 M NaOAc pH 5.2, and the product precipitated using 10 reaction volumes of EtOH. The ClAc-D-Trp-$tRNA^{fMet}_{CAU}$ pellet was washed twice with 0.1 M NaOAc pH 5.2 70% EtOH, once with 70% EtOH, dried and stored at -80 °C.

Reprogrammed *in vitro* translation system

A custom *in vitro* translation mixture was assembled, with each protein/RNA component purified separately from *E .coli*. Final concentrations: 1.2 µM ribosome, 0.1 µM T7 RNA polymerase, 4 µg/mL creatine kinase, 3 µg/mL myokinase, 0.1 µM pyrophosphatase, 0.1 µM nucleotide-diphosphatase kinase, 2.7 µM IF1, 0.4 µM IF2, 1.5 µM IF3, 30 µM EF-Tu, 30 µM

EF-Ts, 0.26 µM EF-G, 0.25 µM RF2, 0.17 µM RF3, 0.5 µM RRF, 0.6 µM MTF, 0.73 µM AlaRS, 0.03 µM ArgRS, 0.38 µM AsnRS, 0.02 µM CysRS, 0.06 µM GlnRS, 0.23 µM GluRS, 0.09 µM GlyRS, 0.02 µM HisRS, 0.4 µM IleRS, 0.04 µM LeuRS, 0.03 µM MetRS, 0.68 µM PheRS, 0.16 µM ProRS, 0.04 µM SerRS, 0.09 µM ThrRS, 0.03 µM TrpRS, 0.02 µM ValRS, 0.13 µM AspRS, 0.11 µM LysRS, 0.02 µM TyrRS. Additionally, 50 mM Hepes-KOH (pH 7.6), 100 mM potassium acetate, 2 mM GTP, 2 mM ATP, 1 mM CTP, 1 mM UTP, 20 mM creatine phosphate, 12 mM $Mg(OAc)_2$, 2 mM spermidine, 2 mM DTT, and 1.5 mg/mL *E. coli* total tRNA (Roche). 19 of the 20 canonical amino acids were included at 500 µM: methionine was not added nor the formyl donor, usually 10-formyl-5,6,7,8-tetrahydrofolic acid, required for initiation using formyl methionine. The translation reaction was supplemented with 50 µM ClAc-D-Trp-$tRNA^{fMet}_{CAU}$.

mRNA library preparation

The DNA template for mRNA with 6-12 randomized codons (DNA_NNK, below) were assembled by primer extension and PCR as described previously[8]. The DNA template was transcribed by T7 RNA polymerase and purified using 8% denaturing PAGE, the mRNA products were dissolved in water and stored at -80 °C. mRNA with NNK lengths of 6,7,8,9,10,11,12 were mixed in a ratio of 0.0004:0.01:0.1:1:1:1 to avoid oversampling the smaller NNK sequences, with the aim of having a greater number of unique sequences in the final mRNA library. The mRNA library was ligated with puromycin linker (5'-Phosphate - CTCCC GCCCC CCGTC C-(SPC18)5-CC- puromycin-3') by T4 RNA ligase. The ligated product was purified by phenol-chloroform extraction and ethanol precipitation.

DNA_NNK =

TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACAT

ATG$(NNK)_{6\text{-}12}$TGCGGCAGCGGCAGCGGCAGCTAGGACGGGGGGCGGAAA

N = equal C:G:A:T, K = equal G:T

Reconstitution of biotin-Ub_n

Lyophilized biotin-Ub_n, after SPPS and purification, were resuspended in 6 M Urea, followed by diluted x200 in 50 mM HEPES pH 7.3, 150 mM NaCl. Any precipitate was removed by centrifugation at 13,000 g for 30 mins. Final concentration of soluble protein was measured using Qubit Protein Assay Kit (ThermoFisher), identity confirmed using MALDI-TOF (Bruker) and flash frozen $N_{2(l)}$ before storage at -80°C.

Binding of biotin-Ub_n polymers to Streptavidin magnetic beads

Various volumes of Dynabeads M280 Streptavidin (ThermoFisher) 10 mg mL^{-1} bead slurry were washed with selection buffer (50 mM HEPES pH 7.3, 150 mM NaCl, 0.05% Tween20, 2 mM DTT) before removal of the buffer and addition of 10 µL of 1 µM biotinylated protein, and incubated for 15 mins at 4 °C. Free biotin was added to a concentration 25 µM and incubated for an additional 15 mins at 4 °C. Supernatant and washed beads were run on denaturing on Tricine-SDS-PAGE for biotin-Ub_1 and biotin-$^{K48}Ub_2$, 10% Tris-SDS-PAGE for biotin-$^{K48}Ub_4$ and stained using SYPRO Ruby Protein Gel Stain (ThermoFisher) (Supplementary figure x). Binding capacities were 3 pmol μL^{-1} slurry for biotin-Ub_1 and 2 pmol μL^{-1} slurry for biotin-Ub_2 and biotin-Ub_4.

First round of selection to find $Ub_{2,4}$ binding peptides

The two initial cyclic peptide libraries (one each for $^{K48}Ub_2$ and $^{K48}Ub_4$) was formed by adding puromycin ligated mRNA library (150 pmol) to a 150 µL scale reprogrammed *in vitro* translation system and incubated at 37 °C for 30 mins. This was cooled to 25 °C for 12 mins to promote mRNA-puromycin ligation to the translated peptides. To this solution 15 µL of 200 mM EDTA (pH 8.0) was added and incubated at 37 °C to dissociate the ribosome from the mRNA-puromycin-peptides products and promote cyclization of the peptides. 165 µL x2 blocking solution (100 mM HEPES pH 7.3, 300 mM NaCl, 0.1% Tween20, 0.2% acetylated BSA, 4 mM DTT) was added, and the resulting solution added to magnetic beads loaded with either biotin-Ub_2 or biotin-Ub_4. Bead amounts were chosen so that the final concentration of Ub_n in the suspension equaled 200 nM. Bead suspensions were incubated with rotation for 30 mins at 4 °C. Beads were quickly washed x3 with ice-cold selection buffer (50 mM HEPES pH 7.3, 150 mM NaCl, 0.05% Tween20, 2 mM DTT). The bead bound mRNA were then reverse transcribed using MLV-RTase H(-) (Promega) for 1 hour at 42 °C using a DNA primer matching the 3' end of the mRNA . The suspensions were diluted in PCR buffer, heated to 95°C and the DNA-containing supernatant collected. qPCR was used to quantify the amount of recovered DNA. This DNA was PCR amplified using taq polymerase and transcribed overnight using T7 polymerase to make mRNA, acidic phenol chloroform extracted and isopropanol precipitated, ligated to puromycin (see above), extracted and ethanol precipitated for use in subsequent rounds.

Subsequent rounds of selection, including negative selection to Ub_1

Ligated mRNA from a previous round (5 pmol) was added to a 5 µL scale reprogrammed *in vitro* translation system. This was incubated at 37 °C 30 mins, 25 °C for 12 mins, then 1 µL 100 mM EDTA (pH 8.0) added and 30 mins at 37°C. mRNA was then reverse transcribed with

MLV-RTase H(-) to form mRNA-DNA duplexes. x2 blocking solution was added, and this solution added to magnetic beads loaded with biotin-Ub_1, with sufficient beads for 200 nM or 2 µM Ub_1 in the suspension. After incubation at 4 ℃ for 30 mins with rotation, and supernatant was removed this incubation constitutes a "negative selection". Two further negative selections were carried out using fresh Ub_1 loaded beads. The supernatant was then added to beads loaded with either biotin-Ub_2 or biotin-Ub_4, incubated at 4 ℃ for 30 mins, and the supernatant discarded a "positive selection". The beads were then washed x3 with ice-cold selection buffer. PCR buffer added to the beads and DNA recovered by heating to 95 ℃ and collecting the supernatant. As for the first round, DNA was quantified using qPCR, amplified using PCR, transcribed and ligated to puromycin. An increase in % recovered DNA was observed with each round in all selections (Supplementary figure y a), indicating the increase in the average affinity of the peptide library for the target as the library becomes enriched in stronger target binders.

Deep sequencing of recovered enriched DNA libraries

DNA recovered from rounds 2,3,4 of the two selection were prepared for MiSeq sequencing (Illumina). Single read 150 bp was sufficient to cover the NNK region. DNA reads with average Phred33+ score > 25 in the NNK region were translated and unique peptide sequences were tallied using custom python scripts.

Synthesis and characterization of the cyclic peptides

The cyclic peptides were synthesized using Fmoc-solid phase peptide synthesis (Fmoc-SPPS). To the the N-terminal chloroacetyl chloride (ClAc-NHS) was coupled and then the peptide was cleaved from the resin using the mixture of TFA/H_2O/TIS (95:2.5:2.5) followed by precipitation and lyophilization. The peptides were dissolved in DMSO, followed by pH adjusted to >8 by

adding Triethylamine) and incubated at room temperature for 30 min for complete cyclization. DMSO was removed under reduced pressure using rotaevaporator and the peptide was purified by HPLC employing C4 column and gradient 0-60% B.

Synthesis and characterization of Fluorescein-5-Maleimide-Ub4ix cyclic peptide

Similar procedure to the synthesis of the cyclic peptides was employed for the labeled peptides, however, Fmoc-Cys(Acm) was coupled as the first amino acid. After cyclization of the linear peptide as described above, the Acm was removed using 10 Equiv. of $PdCl_2$ in 6 mM Gn.HCl was added[2] and the reaction mixture was incubated at 37 ^{o}C for 30 min. For the labelling, we added 25 Equiv of fluorescein-5-maleimide to cyclic peptide in DMF solution. The reaction was completed within 3 hours under dark conditions at room temperature. Finally, the peptide was purified by HPLC employing C4 column and gradient 0-60% B.

Surface Plasmon Resonance (SPR) studies

The interaction between cyclic peptides **U2i**, **U2ii**, **U4i**, **U4ix** and $^{K48}Ub_2$, $^{K48}Ub_4$, Ub_1, $^{K11}Ub_2$, $^{K63}Ub_2$ was determined using BIACORE T100 instrument (GE healthcare) equipped with a biotin CAPture kit Series S chip. The buffer in all experiments was 50 mM HEPES, pH 7.3, 150 mM NaCl, 0.05% Tween20, 2 mM DTT with 0.2% DMSO. Biotinylated Ub_1, $^{K48}Ub_2$ and $^{K48}Ub_4$ were loaded to approximately equal molar amounts on the SPR chip, and various concentrations of cyclic peptides were flowed over at 100 µL s^{-1} for 120 s, before initiation of dissociation by flowing buffer over at 100 µL s^{-1} for a further 700 s. Traces were fit to the simplest, two state model for binding. Association was fit to a single exponential assuming pseudo-first order conditions, whereas the dissociation trace was fit to single exponential plus a drift term. The results are shown in Table 1. Binding of **U2i**, **U2ii** and **U4i** to $^{K48}Ub_4$ had approximately double

the amplitude of the equivalent trances for $^{K48}Ub_2$ (Fig. 1d), suggesting a higher stoichiometry of binding; the respective K_d values are reported as apparent $K_{d,app}$. Due to the poor quality of the traces for **U4ix** binding $^{K48}Ub_2$, the amplitude of the association kinetic trace at different concentrations of peptide was fit to estimate the lower bound for K_d (Table 1). "No detectable binding" (main text) was declared when flowing cyclic peptide over an immobilized Ub chain did not produce significant SPR signal (significant being > 2 RUs), and did not show any dose dependent signal, for peptide concentrations of 0.12 μM, 0.25 μM, 0.5 μM, 1 μM, and 2 μM.

Supplementary Table 1. Fit kinetic and equilibrium parameters for cyclic peptides binding to Ub chains.

Target	Peptide	k_{on} (M^{-1} s^{-1})	k_{on} (error)	k_{off} (s^{-1})	k_{off} (error)	K_d (nM)	K_d (error)
$^{K48}Ub_2$	Ub2i	5.93E+05	1.78E+05	0.024	0.002	40	12
$^{K48}Ub_4$	Ub2i	7.79E+05	1.91E+05	0.011	0.001	14	4
$^{K48}Ub_2$	Ub2ii	4.89E+05	1.09E+05	0.016	0.001	33	8
$^{K48}Ub_4$	Ub2ii	8.77E+05	2.33E+05	0.012	0.001	14	4
$^{K48}Ub_2$	Ub4i	2.62E+05	8.01E+04	0.024	0.001	91	28
$^{K48}Ub_4$	Ub4i	4.07E+05	1.10E+05	0.011	0.001	26	7
$^{K48}Ub_2$	Ub4ix					>1100	
$^{K48}Ub_4$	Ub4ix	3.52E+06	7.30E+05	0.021	0.002	6	1

Synthesis of selectively isotope-enriched Ub chains for NMR studies

Recombinant Ub monomers carrying chain-terminating mutations (either Ub^{K48R} or Ub^{K63R} mutations or C-terminal D77 extension, Ub^{D77}) were expressed and purified as detailed elsewhere[9]. Isotopic enrichment (by ^{15}N or $^{13}C/^{15}N$) of Ub^{WT} and the abovementioned Ub variants was achieved as detailed[10]. Following cell lysis, the proteins were purified by cation exchange and then size-exclusion chromatography. The purity, molecular mass, and proper folding (where applicable) of the purified proteins were confirmed by SDS PAGE, mass spectrometry (MS), and NMR.

Di-ubiquitins with isotope-enriched distal or proximal Ub were made using controlled Ub chain assembly method utilizing E1 and linkage-specific E2 enzymes, as detailed in[10 11 12] . K48-linked Ub dimers were obtained by conjugating Ub^{K48R} and Ub^{D77} using E2-25K as the E2 enzyme while K63-linked dimers were assembled from Ub^{K63R} and Ub^{D77} using a combination of Ubc13 and MMS2. The dimers were subsequently purified by cation exchange chromatography and confirmed by SDS PAGE, MS, and NMR.

The assembly of selectively isotope-enriched K48-linked Ub trimers and tetramers was achieved using the following steps, illustrated in Fig S10. First, the Ub^{K48R}--Ub^{D77} dimers with desired isotope-labeling scheme were made using E1 and E2-25K as detailed above. Then the chain-terminating D77 at the C-terminus of the proximal Ub was removed enzymatically using yeast Ub C-terminal hydrolase YUH1, thus making the dimer available for further conjugation. As the next step, this dimer was conjugated to Ub^{D77} to form a trimer. Subsequently, the C-terminal D77 of the trimer was removed to enable assembly of the K48-linked tetramer. At each reaction step,

the products were purified by cation exchange chromatography and confirmed by SDS PAGE, MS, and NMR (where applicable).

NMR studies

All NMR studies were performed on Bruker Avance III 600 MHz spectrometer equipped with TCI cryoprobe. The sample temperature was set to 23^{o}C for **Ub2ii** binding studies or 25^{o}C for **Ub4ix** studies, unless indicated otherwise. The protein (monomeric Ub or Ub chains) samples were prepared in 20 mM sodium phosphate buffer (pH 6.8) containing 5-10% D_2O and 0.02% (w/v) NaN_3. The **Ub2ii** peptide was dissolved in deuterated DMSO (up to 20 mM concentration) and titrated in μL amounts into 50 μM ^{15}N-Ub^{WT} or K63-linked Ub_2 (^{15}N-enriched in the distal domain) to two molar equivalents. **Ub2ii** was titrated into 100 μM solutions of proximally- or distally-^{15}N-enriched $^{K48}Ub_2$ to 1:1 or 1:1.3 molar ratio, respectively, as well as into 200 μM $^{K48}Ub_2$ with the ^{13}C/^{15}N-enriched proximal or distal Ub unit. **Ub4ix** titration into K48-linked Ub_2, Ub_3, and Ub_4 was performed in a similar manner, with the starting Ub chain concentration of 200 □M; the chain/peptide molar ratio at the endpoint varied from 1:1 (Ub_2) to 1:1.5 or 1:2 for Ub_3 and Ub_4. Several of these titrations were performed twice and at differing protein concentrations, to verify the reproducibility of the observed spectral changes. Separately, a similar amount of DMSO was added to ^{15}N-labeled Ub to verify that the presence of DMSO in the peptide-containing stock solution had negligible effect on Ub spectra.

1D ^{1}H spectra and 2D ^{15}N-^{1}H SOFAST-HMQC spectra were acquired at each titration point. To facilitate NMR signal assignment of K48-Ub_2 in the **Ub2ii** -bound state, triple-resonance HNCA, HN(CO)CA, HNCO, and HN(CA)CO experiments were performed using Ub_2 samples with

either distal or proximal Ub $^{13}C/^{15}N$-enriched. The triple-resonance experiments for the distal Ub were performed at 37^{o}C in order to reduce line broadening. Standard Bruker pulse sequences and pulse sequences generously provided by Ananya Majumdar (Johns Hopkins University) were used for these purposes. Spectra were processed and analyzed using TopSpin (Bruker Inc.) and CCPNMR[13]. Assignment of the peptide-bound NMR signals of Ub_4 was complicated by signal broadening (due to slow tumbling of tetra-Ub) and overlap and by the slow-exchange regime which precluded tracing gradual shifts in the NMR signals upon titration. The residues affected by peptide binding were identified based on strong attenuation/disappearance of their unbound signals combined with the absence of new, bound signals in the close proximity in the spectra.

NMR signal shifts were quantified as chemical shift perturbations using the following equation:

$$CSP = [(\delta_{HA} - \delta_{HB})^2 + ((\delta_{NA} - \delta_{NB})/5)^2]^{1/2},$$

where δ_H and δ_N are chemical shifts of ^1H and ^{15}N, respectively, for a given backbone N-H group, and A and B refer to the unbound and bound species, respectively.

To quantify the reduction in intensity of the unbound NMR signals of Ub chains upon peptide binding, intensities in the ^{15}N-^1H spectra recorded at each titration point were measured at the positions corresponding to peaks of the peptide-free species, and then normalized for each residue by dividing by the intensity of the corresponding signal in the spectra recorded before the addition of the peptide. This allowed us to identify and monitor residues involved in slow exchange wherein the unbound signal gradually decreases in intensity upon addition of the peptide (see Fig 2a and Fig S11a).

***In vitro* deubiquitination assay**

In vitro deubiquitination reactions were performed in a TRIS buffer (50 mM TRIS, 1 mM TCEP, pH 7.7), containing 2 µM $^{K48}Ub_{2/4}$, then the specific DUB (50 mM Tris, 0.5 mM EDTA, 1 mM TCEP and 0.5 mg/ml ovalbumin, pH 7.5) was added. The reaction mixtures were incubated at 37 °C and at the indicated time points the reactions were stopped by taking aliquots and mixed them with 3X sample buffer and boiling. The same amount were loaded on 14% SDS-PAGE gel, electro-blotted to nitrocellulose membrane and probed with anti-Ub antibody. Bands were quantified with Image Quant LAS 4000 (GE Healthcare).

***In vitro* proteasome degradation assay**

12.5 µL: 5.3 µM of synthetic HA_α-globin-K48-linked tetra-Ub^{14} 150 nM proteasome (Enzo) were incubated in presence of 2 mM ATP, 40 mM Tris, 2 mM DTT and 5 mM $MgCl_2$, at 37 °C for 50 min. The reactions were stopped by the addition of 3x sample buffer. The reactions were loaded on 10% SDS-PAGE gel, electro-blotted to nitrocellulose membrane and probed with rabbit anti-HA antibody. Quantification was carried out using the ECL camera software (Fuji).

Radioactive Pulse and Chase

For the assay of inhibitions of protein synthesis, cells were incubated with DMSO, ribosomal inhibitor cycloheximide (CHX), or **Ub4ix**. After 4 hours, media were replaced with medium containing ^{35}S-methionine and cysteine (20µCi) and cells where pulsed for 4 hours. Plates were extensively washed with ice-cold PBS and proteins were extracted from cells using TCA precipitation. The radioactive readings were measured using a scintillation counter.

For degradation-inhibition assays, cells were labeled with ^{35}S-methionine and cysteine (20µCi) for 16 hours, followed by a chase in cold medium for 4 hours, in the presence of DMSO, proteasome inhibitor MG132, or **Ub4ix**. Media were then collected, and amino acids were

extracted using TCA precipitation. Radioactive readings were measured using a scintillation counter.

Ub4ix cell intake

HeLa cells were seeded on glass bottomed (#1.5) 96-well dish, and were incubated in the presence of either DMSO or **Fluorescein-5-Maleimide-Ub4ix** for the indicated times. Medium was then aspired; fresh medium was added to wells and live cell imaging was carried out using Zeiss LSM-700 confocal microscope equipped with an environmental control module. We chose a relatively low magnification (20x, NA=0.85) in order to emphasize the broad penetrance of the peptide into cells, the images presented are of 1.8μm thick sections (obviously thinner than HeLa cells), and the focal plane was chosen based on the best focus of nuclei. The media were replaced (to media without the fluorescent peptide) and cells were washed prior to acquisition.

Screening of the cellular uptake of fluorescein-Ub4ix

HeLa cells (40,000 cell~230 μg protein) were seeded on glass bottomed (#1.5) 96-well dish, and were incubated in the presence of either DMSO, **Fluorescein** or **Fluorescein-5-Maleimide-Ub4ix** (different concentrations) for 16 hr. Medium was then aspired; PBS (three times), RIPA (lysis buffer) were added to wells (480 μg protein), the absorption (ex= 480, em= 525 nm) was screened using the calibration in Supplementary Fig. 20.

Cell viability assays and kinetic experiments

In order to assess the effect of the cyclic peptides on Hela cells toxicity was determined using the MTT assay. Equal amount of cells (8,000 cells/well) were plated in 96-well plates in triplicate. After 24 hours, attached cells were exposed to increasing concentrations of the cyclic peptides for another 24 hours. Thereafter, 10 μL of the 12 mM MTT (Vybrant® MTT Cell Proliferation

Assay Kit, ThermoFisher) was added per well according to the manufacturer`s protocol an incubated for 4 hours at 37 °C. This was followed by addition of 100 µL of the SDS-HCl solution and thorough mixing incubated for 4 hours at 37 °C. Optical density was recorded at 590 nm with a reference measurement at 630 nm. EC50 values were defined as the concentrations that correspond to a reduction of cell growth by 50% when compared to values of untreated control cells and depicted as means of relative activity ± standard deviation.

FACS Analysis

Induction of apoptosis in Hela cell lines by treatment with cyclic peptides were determined after 24 and 48 hours incubation in a dose dependent manner, using annexin V-FITC apoptosis detection kit (BD Biosciences) according to the manufacturer`s protocol. 2 x 10^5 cells/well were seeded in 6-well plates and treated with inhibitor for 24/48 hours in a dose dependent manner. The increase in fluorescence, which indicates the apoptosis level in the treated cells, were monitored using flow cytometry and compared to untreated cells containing DMSO as a control and MG132 as a positive control.

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detailed synthesis will be published elsewhere.