Electronic Supplementary Material (ESI) for RSC Chemical Biology.
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Supplementary Information for

"In vivo modulation of ubiquitin chains by N-methylated non-proteinogenic cyclic peptides"

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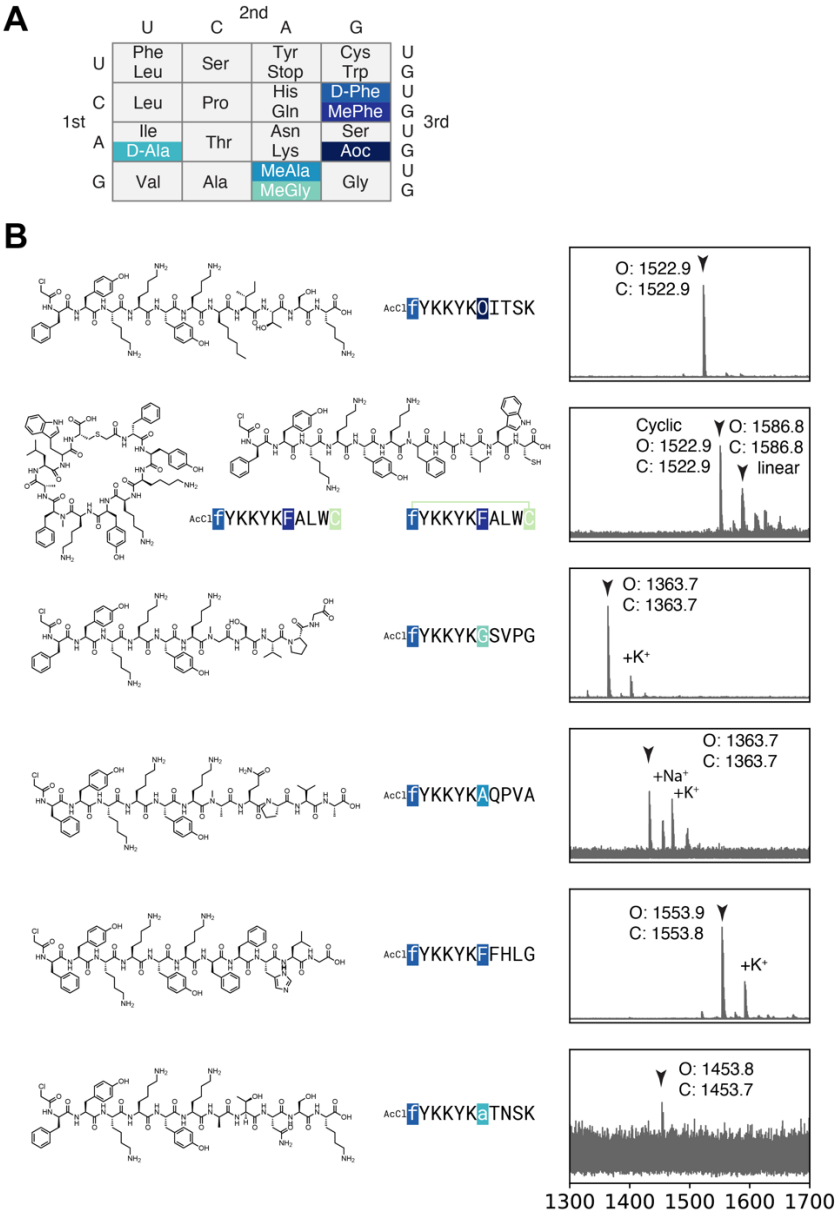

Figure S1. Reprogramming the genetic code. A. Genetic code used in cyclic peptide library construction, where the amino acids Met, Glu, Asp and Arg were replaced with the non-canonical amino acids described in the main text. Codons shown are those corresponding to the 'NNK' degenerate DNA codons used in the randomized regions of the cyclic peptides. In addition, the initiation 'AUG' codon was reprogrammed to AcCl-D-Phe. **B**. Test of translation fidelity. Six DNA sequences covering all the canonical and non-canonical amino acids from the reprogrammed genetic code were translated *in vitro* and the peptide products detected by MALDI-TOF MS. The expected peptide mass was observed in each case, with this being the only or the major product observed.

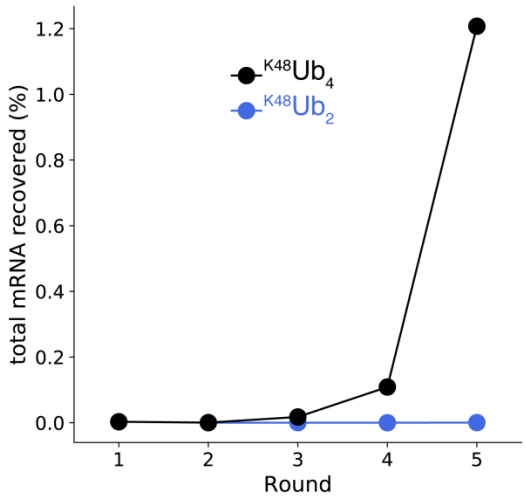

Figure S2. Cyclic peptide library increased in affinity after each round of selection. The percentage of mRNA:cDNA hybrids recovered after binding to the $^{K48}Ub_4$ target, measured by quantitative PCR, increased after each round. Suggests an increase in average affinity of the cyclic peptide library as it becomes enriched in higher affinity sequences.

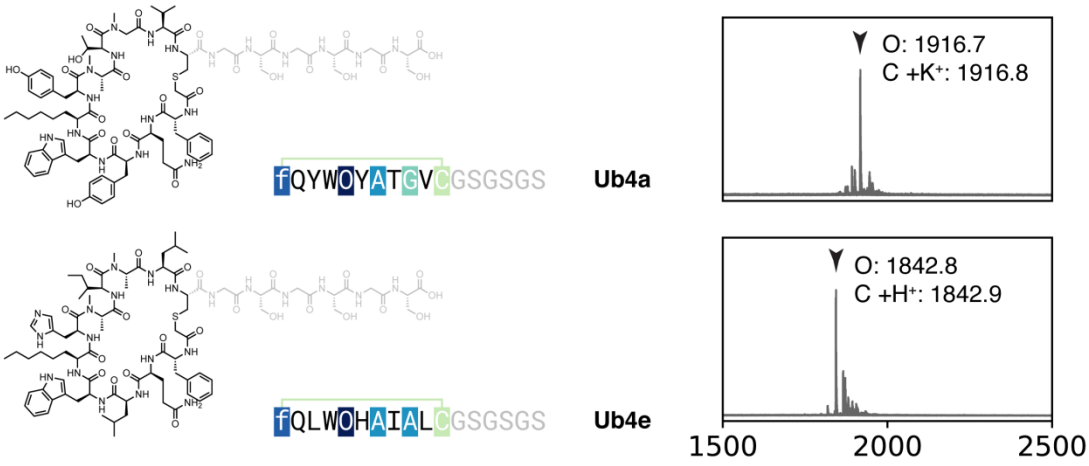

Figure S3. Confirmation that the selected DNA sequences are correctly translated by the reprogrammed genetic code. The DNA sequences for **Ub4a** and **Ub4e** were added to *in vitro* translation reactions with the reprogrammed genetic code described in the main text. The expected cyclic peptide products were the major products detected by MALDI-TOF MS.

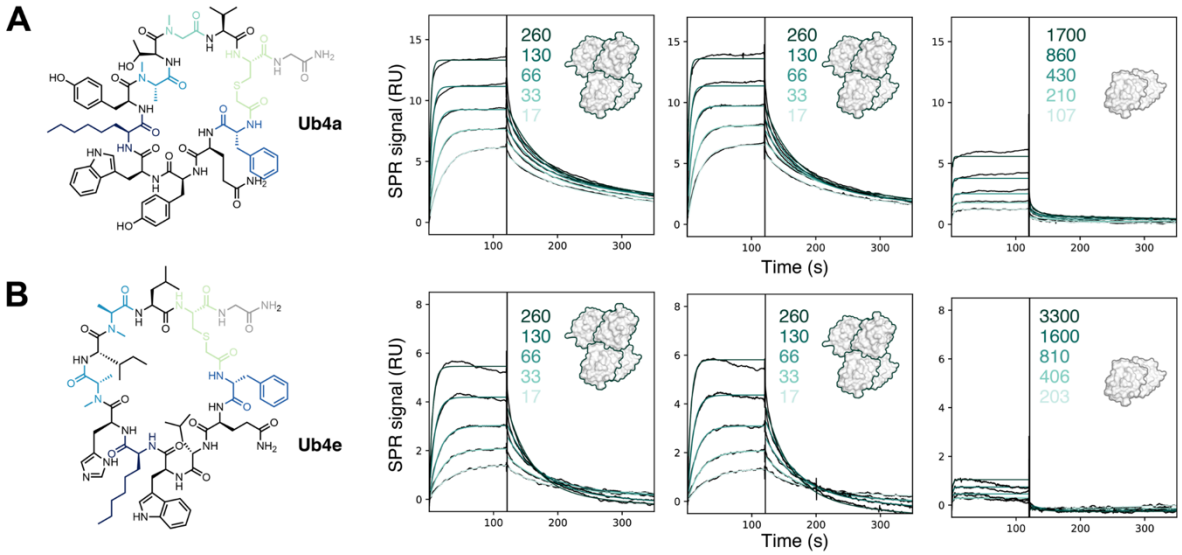

Figure S4. Cyclic peptides bind tightly to the desired target. A. SPR traces for **Ub4a** binding $^{K48}Ub_4$ (two repeat titrations) and $^{K48}Ub_2$. Ub chains were immobilized on the streptavidin chip, and cyclic peptide was flowed over at the (nM) concentrations indicated. **B**. SPR traces for **Ub4e** binding $^{K48}Ub_4$ and $^{K48}Ub_2$.

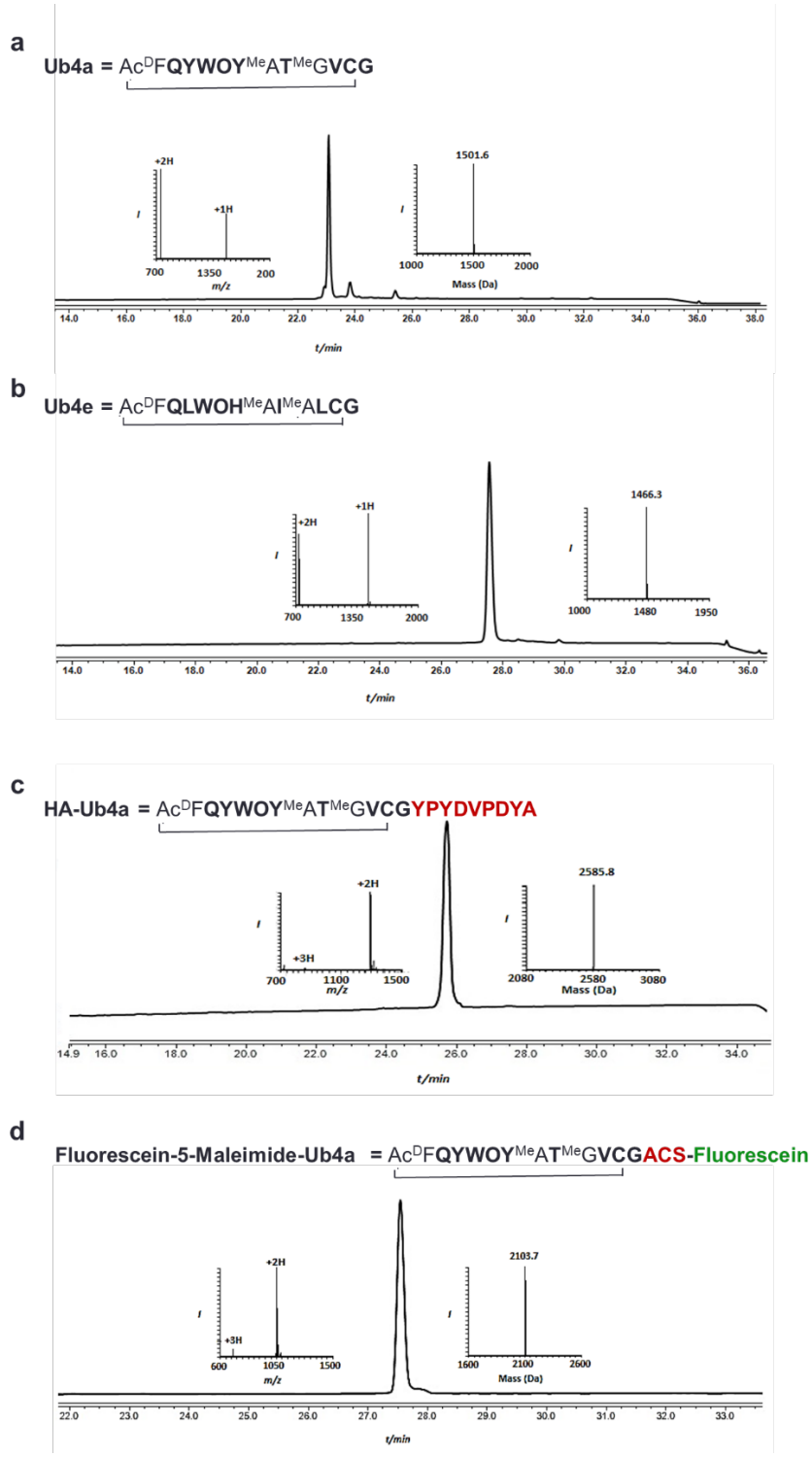

Figure S5. Analytical HPLC and ESI-MS analysis of the purified cyclic peptides. A. **Ub4a**, **B**. **Ub4e**, **C**. **Ub4a**-HA and **D**. **Ub4a**-fluorescein with the observed mass 1501.6 ± 0.1 Da, 1466.3 ± 0.1 Da, 2585.8 ± 0.3 Da and 2103.7 ± 0.2 Da respectively (calculated 1501.9 Da, 1466.3 Da, 2585.8 Da and 2103.7 Da respectively).

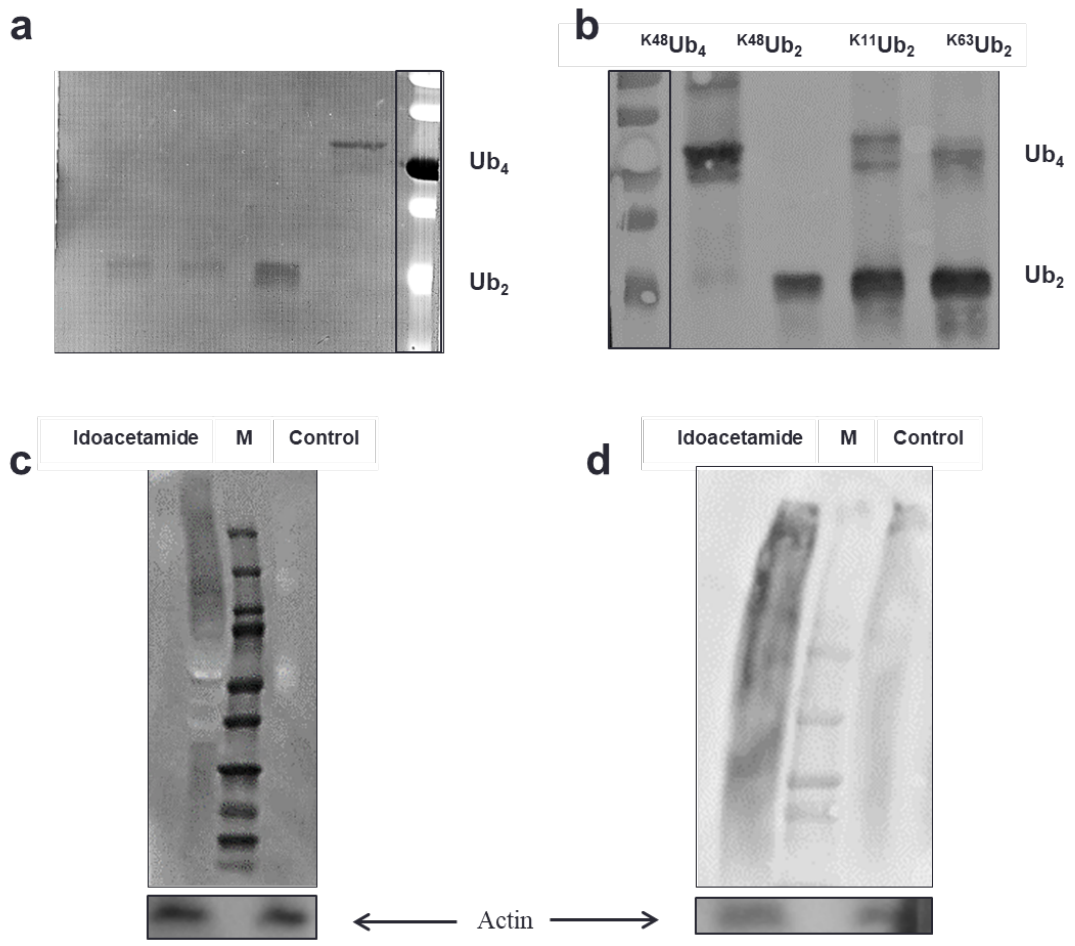

Figure S6. **Detection of blotted Ubiquitin polymers using labelled cyclic peptides. A**. Fluorescein labelled cyclic peptide, **Ub4a**-fluorescein could be used to detect Ub chains transferred onto a nitrocelluose membrane following a standard Western blot protocol. Staining was strongest for K48-linked chains. **B**. Control Western blot of the same Ub-chain samples using anti-Ub antibody. **C**. Cyclic peptide could also be used to detect Ub-conjugates from cells. Cells were lysed in the presence of the general DUB-inhbitor Iodoacetamide, transferred to nitrocellulose and Ub-conjgates detected by HA-tagged **Ub4a**, visualized using an anti-HA antibody. **D**. Control Western blot where the same samples were detected using anti-Ub antibody.

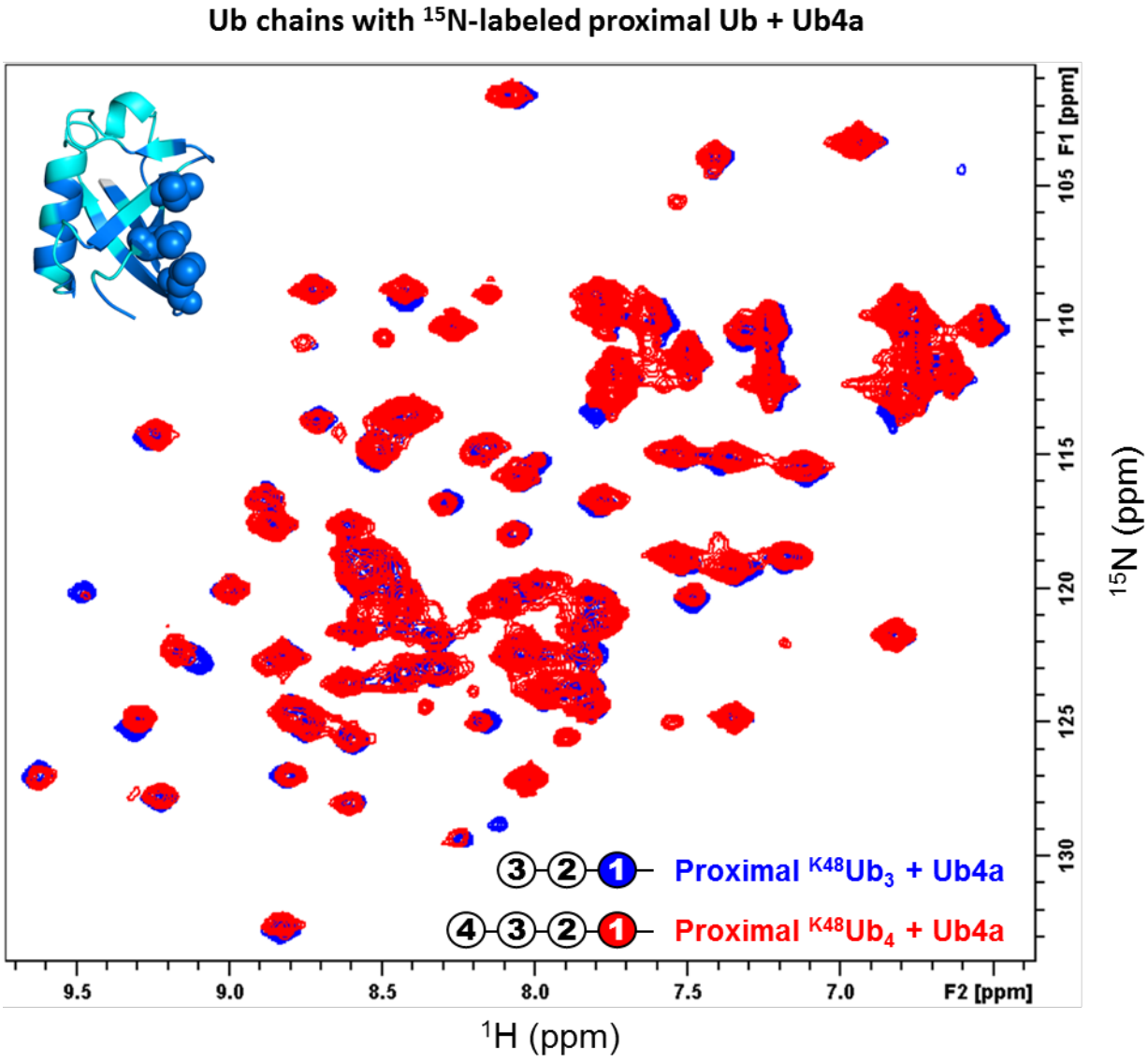

Figure S7. Overlay of ^{1}H-^{15}N NMR spectra of the proximal Ub in ^{K48}Ub$_3$ (blue) and in ^{K48}Ub$_4$ (red) at the endpoint of titration with **Ub4a** (peptide:polyUb molar ratio = 0.75:1 and 1:1, respectively). Note strong similarity of the two spectra. Shown in the upper right corner is the structure of Ub with residues exhibiting strong CSPs mapped (colored marine); the side chains of the hydrophobic patch residues L8, I44, V70 are shown as spheres.

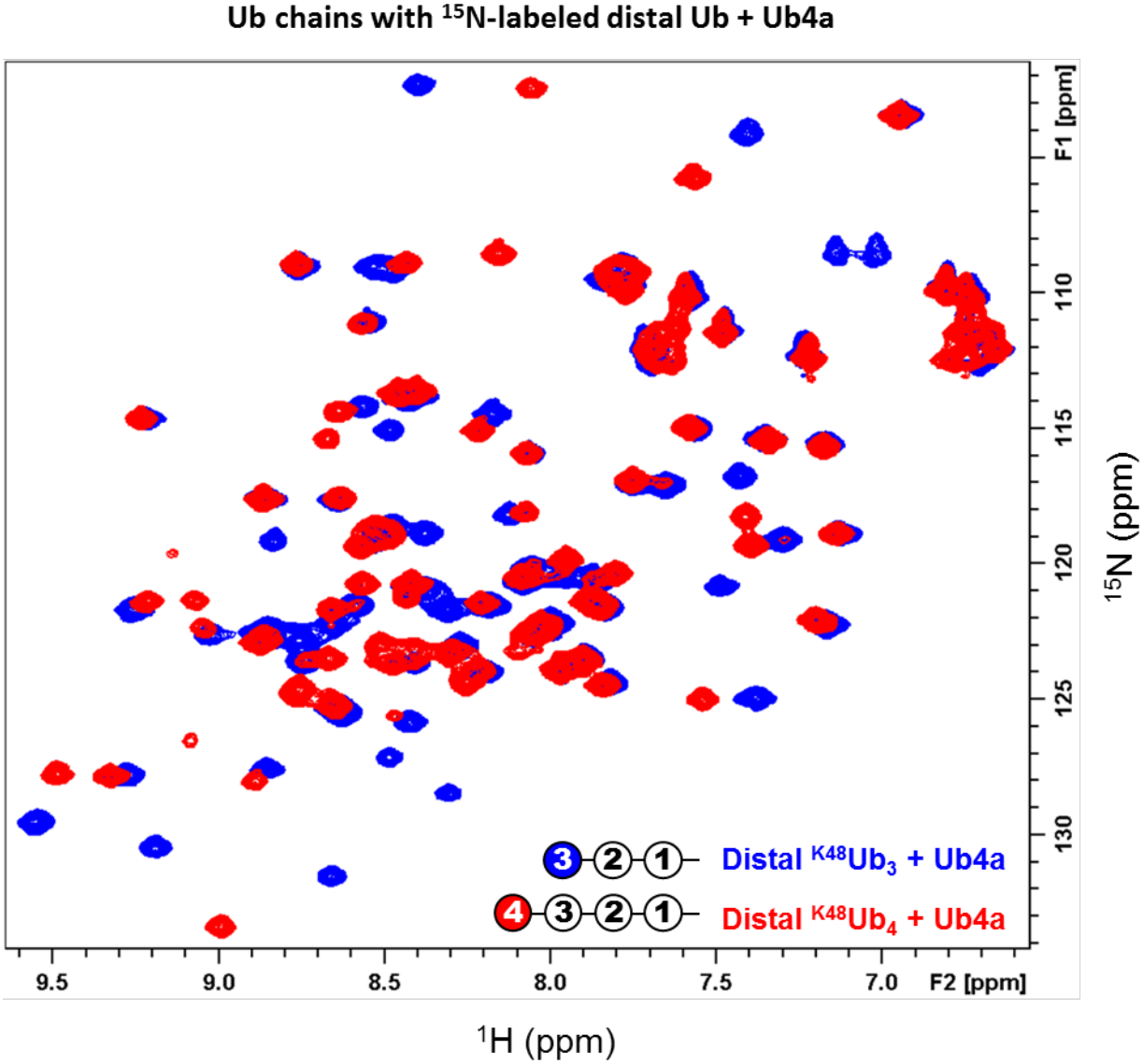

Figure S8. Overlay of ^{1}H-^{15}N NMR spectra of the distal Ub in $^{K48}Ub_3$ (blue) and in $^{K48}Ub_4$ (red) at the endpoint of titration with **Ub4a** (peptide:polyUb molar ratio = 1:1 and 2:1, respectively). Note substantial differences between the two spectra.

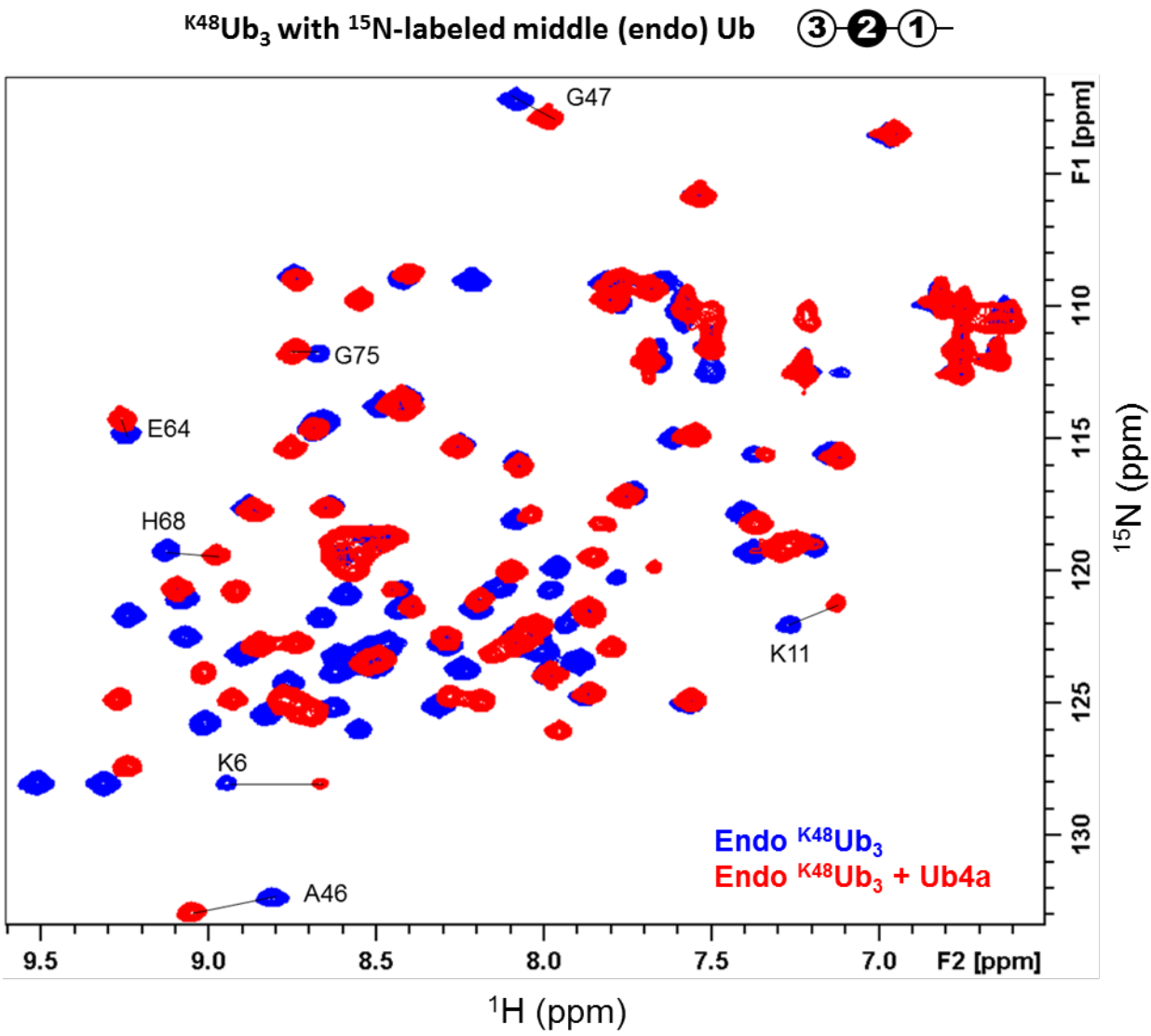

Figure S9. Overlay of ^{1}H-^{15}N NMR spectra of the middle (endo) Ub in ^{K48}Ub$_3$ free in solution (blue) and upon addition of **Ub4a** (red) (peptide:polyUb molar ratio = 2:1). Signals of select residues are indicated. These spectra were recorded at 25ºC.

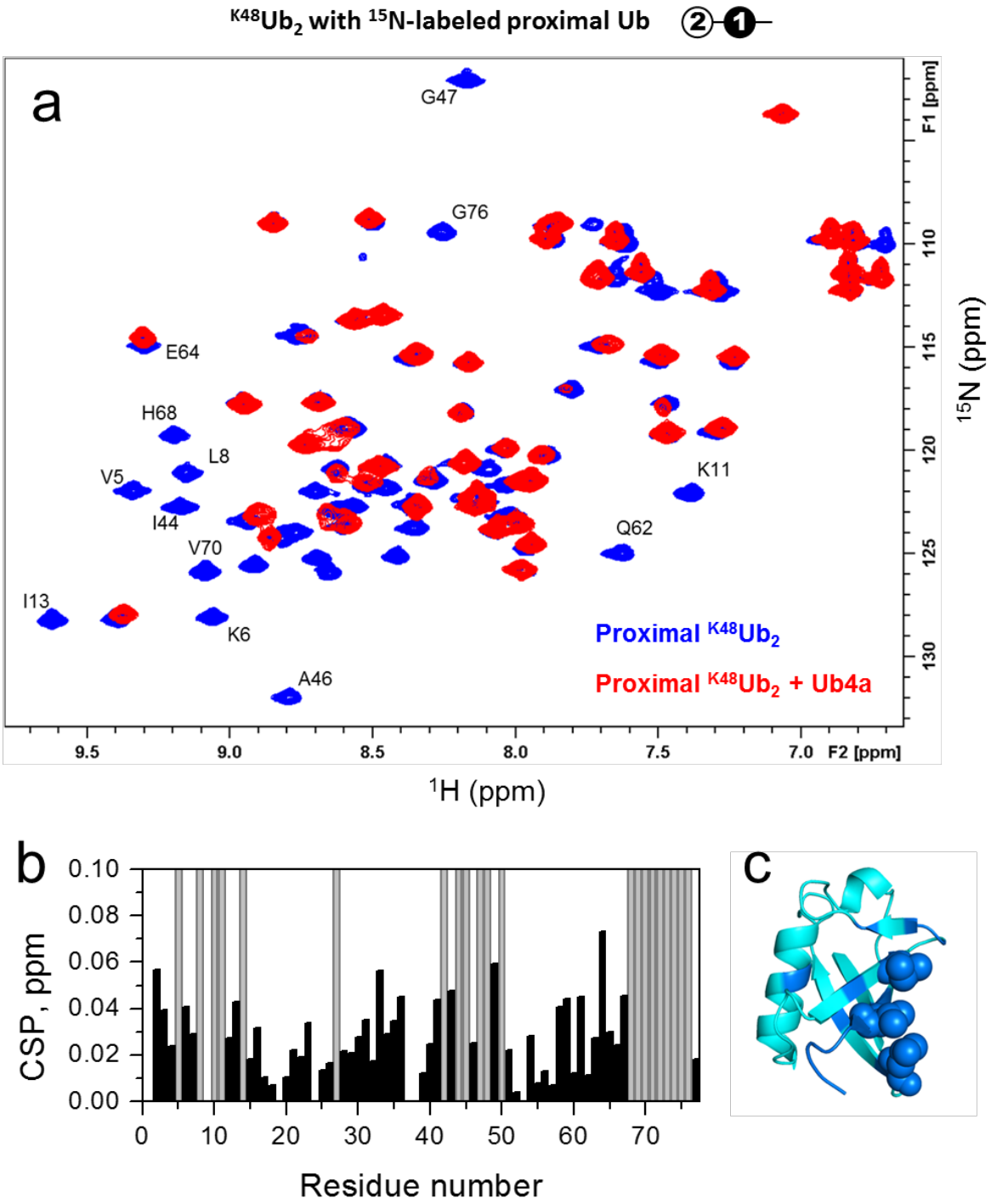

Figure S10. Ub4a binding to the proximal Ub of $^{K48}Ub_2$. **A**. Overlay of ^{1}H-^{15}N NMR spectra of the proximal Ub of $^{K48}Ub_2$ free in solution (blue) and upon addition of **Ub4a** (red) (peptide:polyUb molar ratio = 1.5:1). **B**. Residue-specific CSP values (black bars). Grey bars indicate residues that exhibited signal disappearance during the titration. C. Map of the perturbed residues (signals disappeared) on the structure of Ub. The side chains of the hydrophobic patch residues L8, I44, V70 are shown as spheres.

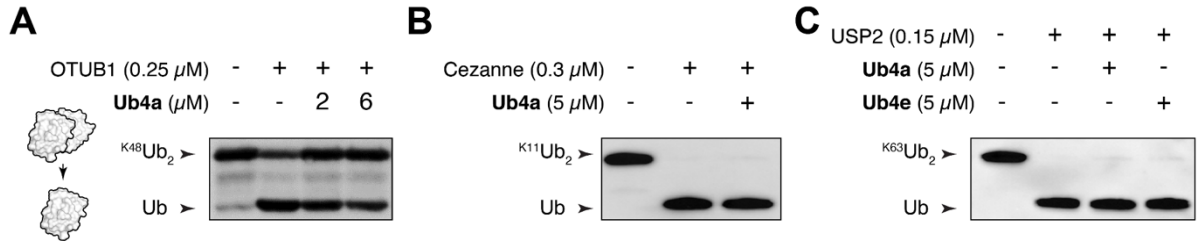

Figure S11. Cyclic peptides exhibit selectivity against K48-linked chains. **A**. Even though **Ub4a** binds longer Ub chains with significantly greater affinity, at high (μM) concentrations **Ub4a** is also able to bind/protect Ub dimer $^{K48}Ub_2$ (2 μM) from cleavage by the DUB OTUB1. This is consistent with the "high" μM K_D between this cyclic peptide and $^{K48}Ub_2$ observed in the SPR analysis. **B**. **Ub4a** offers no protection to K11-linked Ub dimer from cleavage by the DUB Cezanne, consistent with poor affinity of these cyclic peptides for Ub-chains with this linkage. **C**. Neither **Ub4a** or **Ub4e** protect K63-linked Ub dimer against cleavage by the DUB USP2, consistent with poor affinity of these cyclic peptides for Ub-chains with this linkage. We assume that, at these high (μM) peptide concentrations, some degree of binding/protection of alternatively-linked dimers would be observed, if these cyclic peptides also bound longer alternatively-linked Ub chains.

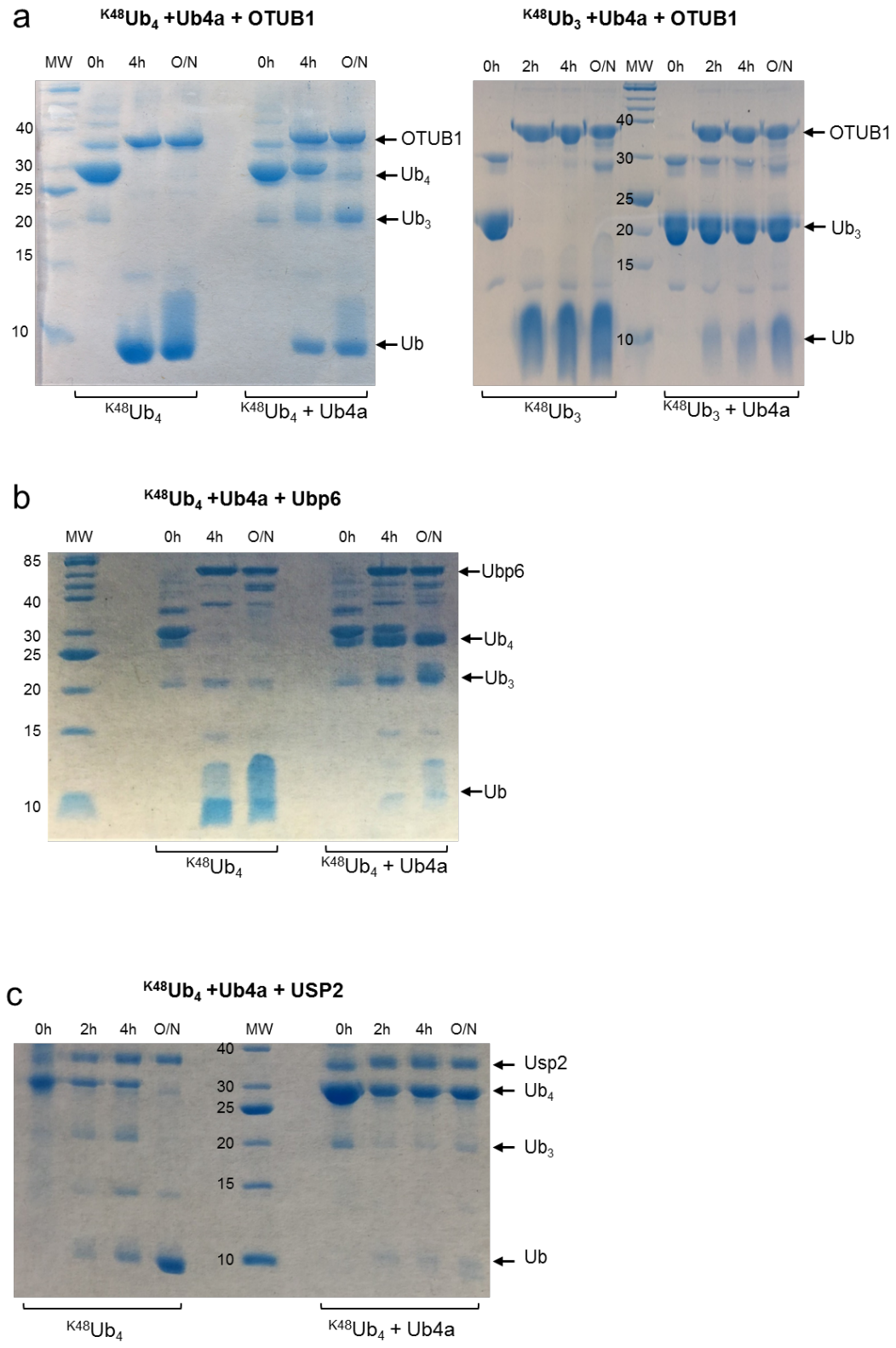

Figure S12. DUBs. **Cyclic peptide Ub4a protects K48-linked Ub-chains from disassembly by deubiquitinases. A**.. Time course of disassembly of $^{K48}Ub_4$ (left) and $^{K48}Ub_3$ (right) by K48-linkage specific OTUB1 in the absence and presence of **Ub4a**. **B**. Time course of disassembly of $^{K48}Ub_4$ by the proteasome-associated DUB Ubp6 in the absence and

presence of **Ub4a**. **C**. Time course of disassembly of $^{K48}Ub_4$ by linkage-nonspecific DUB USP2 in the absence and presence of **Ub4a**. In all these assays the Ub_4 or Ub_3 concentration was 10 μM, the DUB was at 2 μM (5.4 μM for USP2), the peptide was added in a 2:1 molar ratio to each chain. MW indicates molecular markers, the corresponding molecular weight values are shown in kDa. O/N = overnight (~16 h).

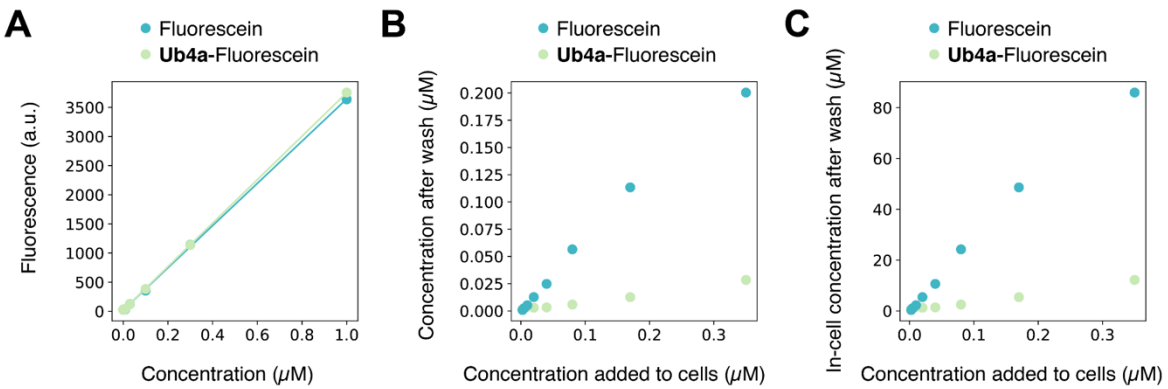

Figure S13. Fluorescently labeled cyclic peptides accumulate within cells. A. Fluorescence reading are linear with fluorescein and **Ub4a**-fluorescein concentration, allowing a calibration curve to be made. **B**. After incubating cells with fluorescein or **Ub4a**-fluorescein, the amount inside cells could be detected by washing, lysis and fluorescence measurement. This showed significant uptake of **Ub4a**-fluorescein realtive to fluorescein alone. **C**. Adjustment for celluar volume shows that **Ub4a**-fluorescein was concentrated within cells.

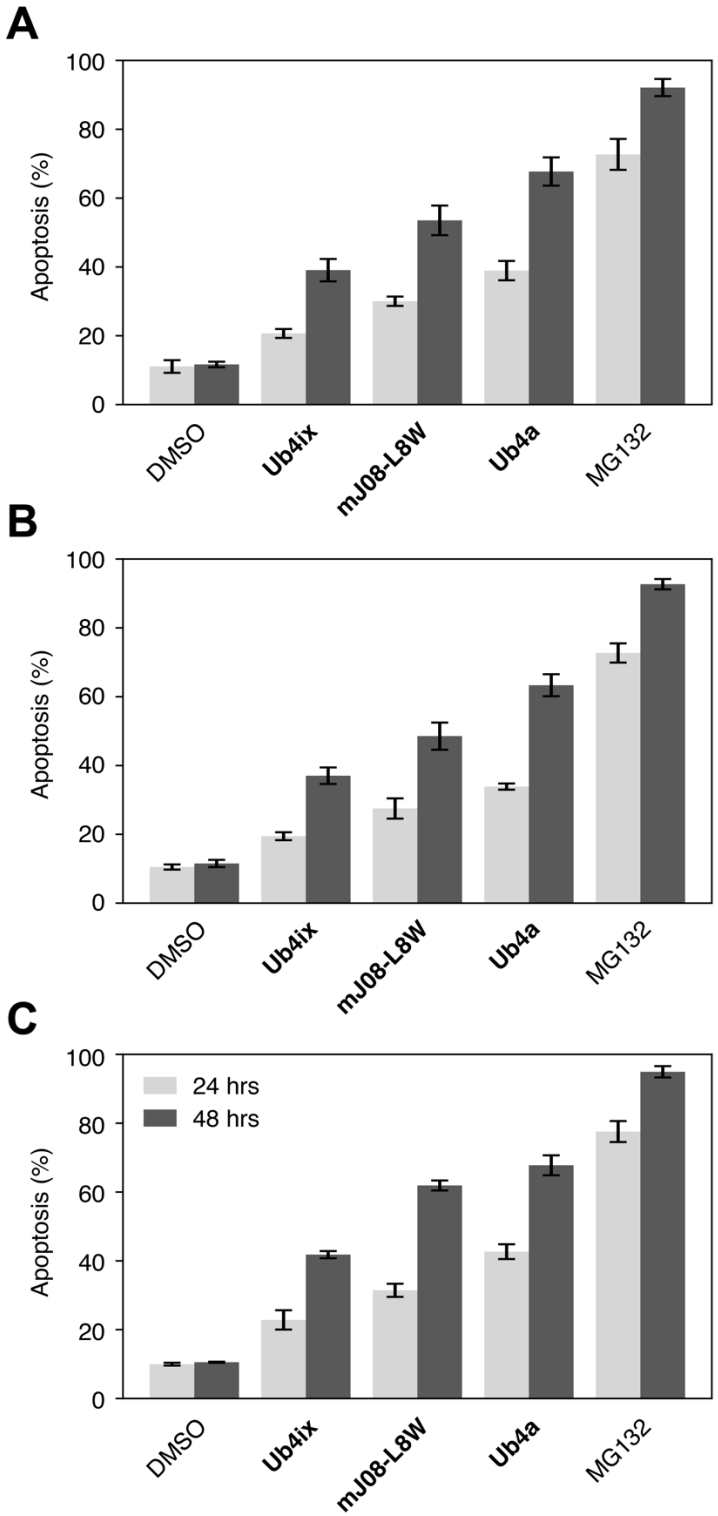

Figure S14. **Ub4a cyclic peptide induced apoptosis in several cancer cell lines**. **A**. Cyclic peptides **Ub4ix**, **mJ08-L8W** and **Ub4a** induced apoptosis in SH-SY5Y neuroblastoma cells (bone marrow), similar to the direct proteasome inhibitor MG132. **B**. Similarly, the cyclic peptides induced apoptosis in MDA-MB-231 cells (epithelial human breast cancer). **C**. The cyclic peptides also induced apoptosis in HeLa cells (cevical cancer).

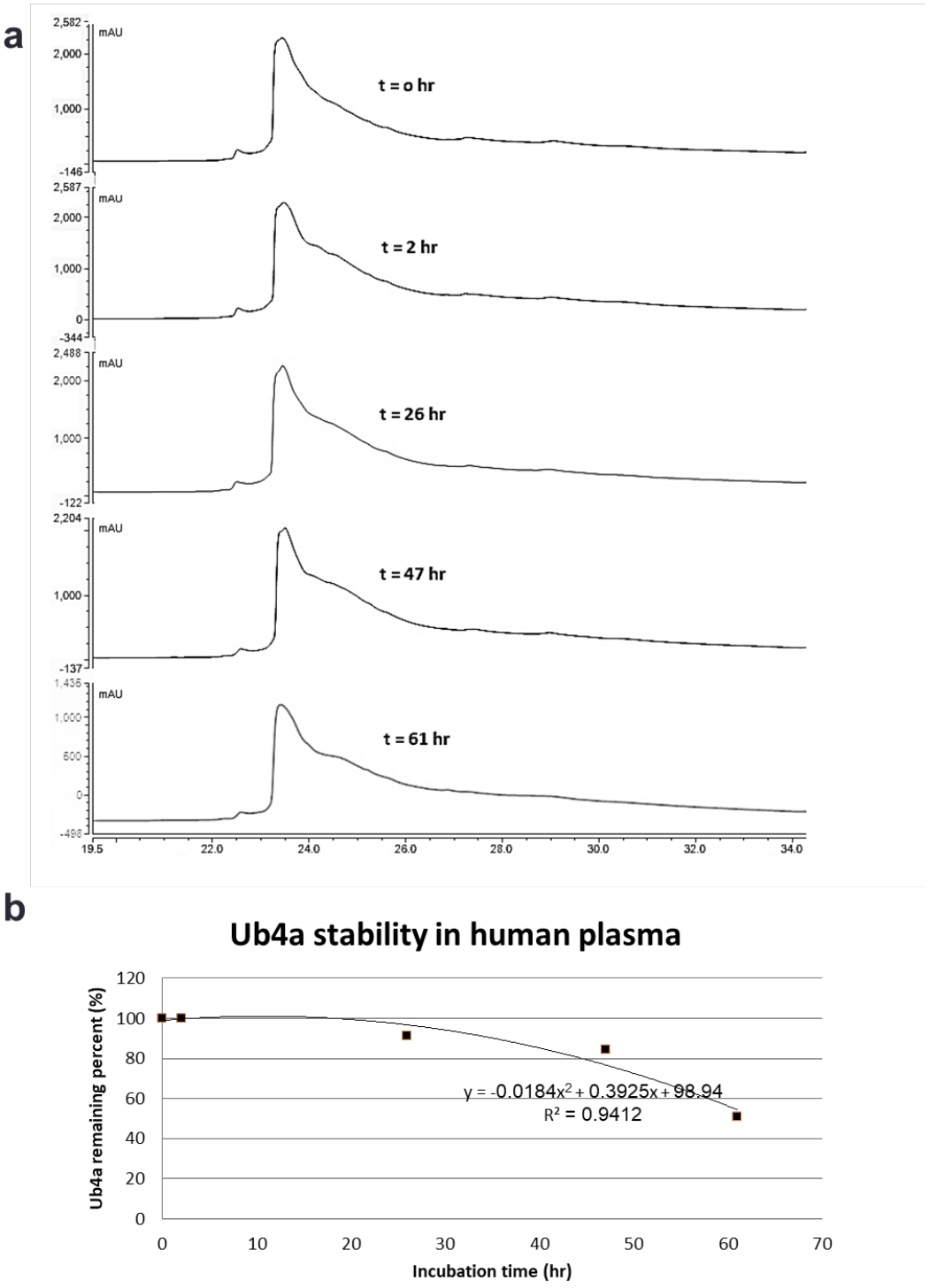

Figure S15. Ub4a is stable for multiple days in human plasma. A. **Ub4a** peptide was dissolved in phosphate-buffered saline buffer (PBS) (2 mg/ml) and incubated in human plasma (Sigma-Aldrich) at 37°C and investigated for its stability by HPLC at different time intervals such as t = 0 h, 2 h, 26 h, 47 h and 61 h. As the time advanced, the intensity of peptide was decreased and indicate that the peptide **Ub4a** may slowly degraded. **B**. **Ub4a** remaining percent %, showing a reduction to ~50% after 61 hrs.

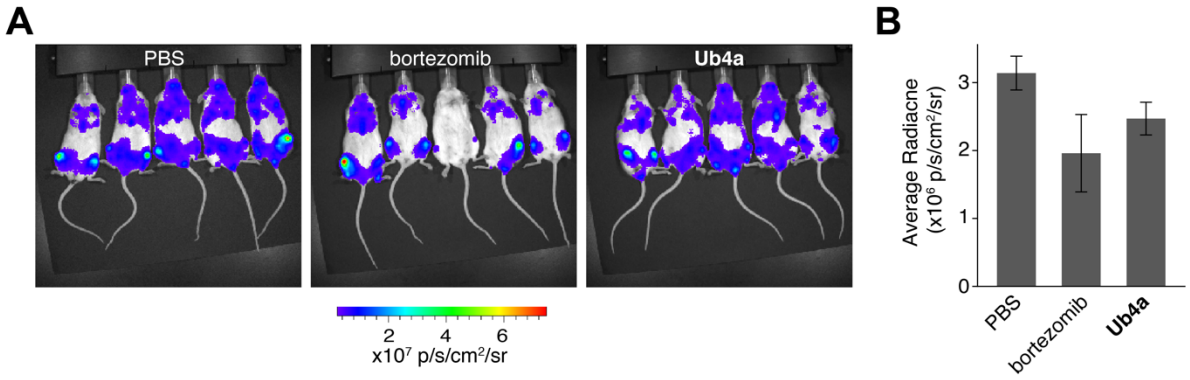

Figure S16. Inhibition of tumor growth after 21 days of treatment with bortezomib or Ub4a. A. Imaging of luciferase expressing human CAG myeloma cells in mice shows that treatment with either the approved anti-cancer drug bortezomib or the cyclic peptide Ub4a, inhibit the growth of tumors *in vivo*. **B**. Quantification of luminescence from human tumor cells in mice shows that 21 days of treatment with Ub4a causes reduction in tumor growth similar to treatment with bortezomib.

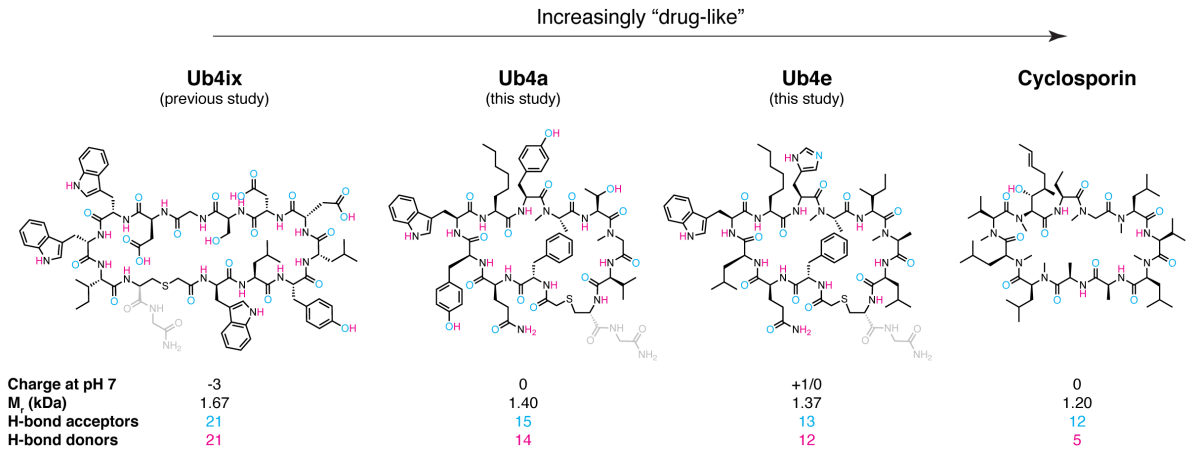

Figure S17. Physicochemical properties of cyclic peptides approach those of cyclosporin. Cyclic peptides **Ub4a** and **Ub4e** have properties (charge, mass, and H-bonding atoms) approaching that of the cyclic peptide drug cyclosporin, and are a significant improvement over the first-generation Ub-binding cyclic peptide **Ub4ix**. Linker used in mRNA display shown in grey.

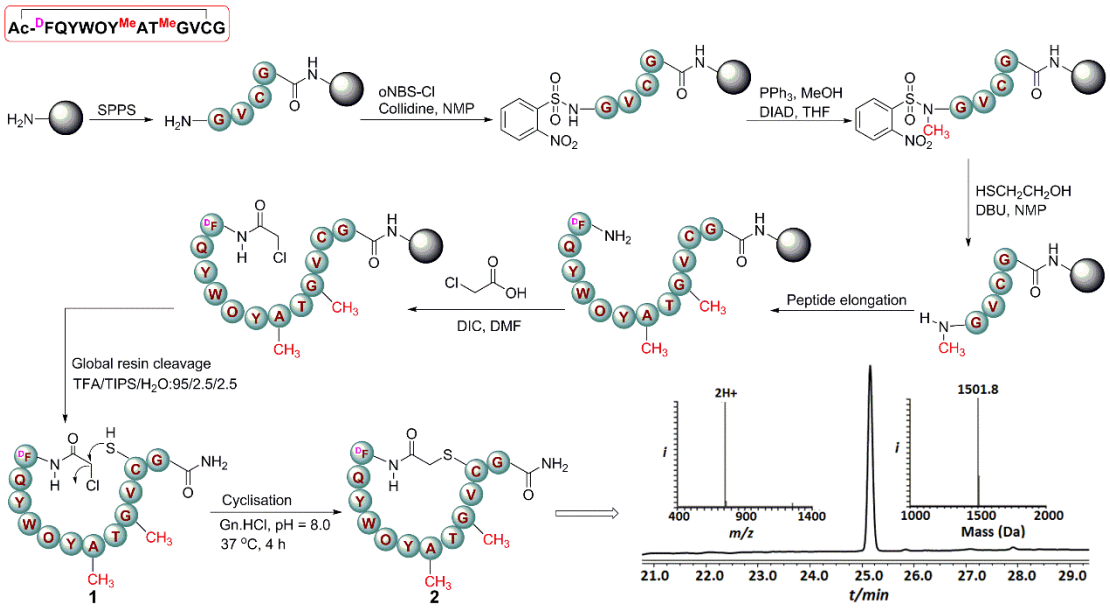

Figure S18. Schematic representation for the synthesis of **Ub4a** wild type. HPLC-MS analysis of **Ub4a** (WT) with the observed mass 1501.8 ± 0.1 Da (calcd 1501.9 Da, average isotopes). The amino acid "O" used in the sequence was Fmoc-L-2-aminooctanoic acid.

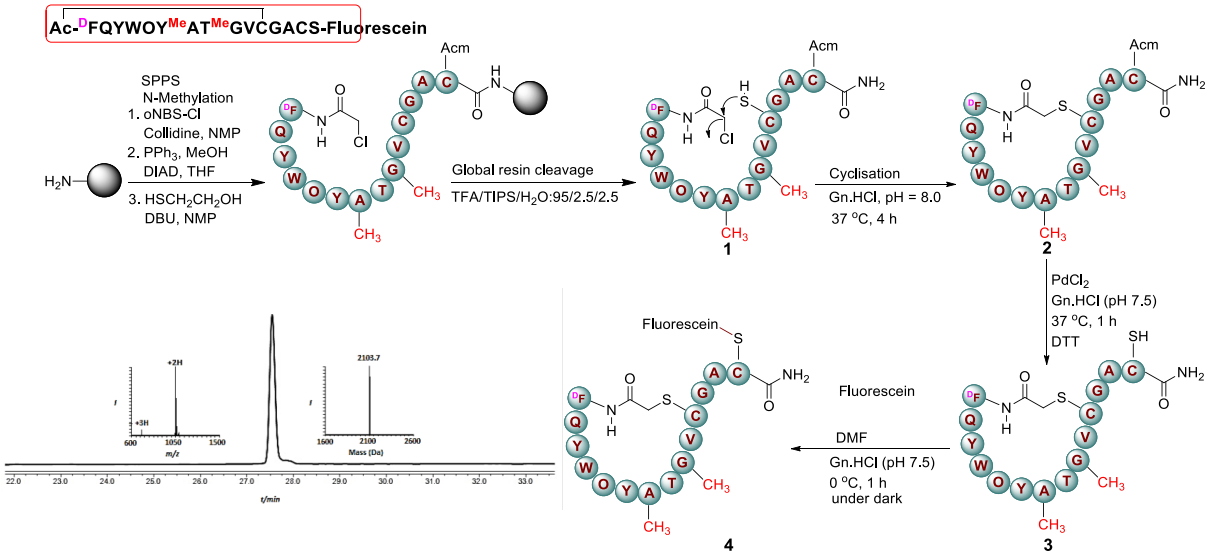

Figure S19. Schematic representation for the synthesis of **Ub4a** with fluorescein. HPLC-MS analysis of labelled **Ub4a** with the observed mass 2103.7 ± 0.2 Da (calculated 2103.7 Da).

Table S1. SPR kinetic fits. Average value from two titrations shown in main text Fig. 1d. Largest standard deviation from either titration shown as the error.

Target	Cyclic peptide	$k_{association}$ ($M^{-1}s^{-1}$)	$k_{dissociation}$ (s^{-1})	K_D (nM)
$^{K48}Ub_4$	**Ub4a**	2.1 (± 0.6) x10^6	0.018 (± 0.002)	9 (± 3)
$^{K48}Ub_4$	**Ub4e**	1.2 (± 0.4) x10^6	0.021 (± 0.003)	18 (± 7)

Table S2. SPR amplitude fits. Average fit K_D value from two titrations shown in main text Fig. 1d (with individual values in parentheses), or individual fit value.

Target	Cyclic peptide	K_D (nM)
$^{K48}Ub_4$	**Ub4a**	24 (26; 23)
$^{K48}Ub_4$	**Ub4e**	87 (80; 94)
$^{K48}Ub_2$	**Ub4a**	>2300
$^{K48}Ub_2$	**Ub4e**	>18000

Table S3. Aminoacylation parameters

Amino acid	Activating ester	tRNA	Flexizyme	Time (hrs)
ClAc-D-Phe	CME	$tRNA^{fMet}_{CAU}$	eFx	2
D-Ala	DNB	$tRNA^{EnPro1E2\#3}_{CAU}$	dFx	2
D-Phe	CME	$tRNA^{EnPro1E2\#3}_{GCG}$	eFx	2
MePhe	CME	$tRNA^{EnGlu}_{CCG}$	eFx	6
MeAla	DNB	$tRNA^{EnGlu}_{GUC}$	dFx	2
MeGly	DNB	$tRNA^{EnGlu}_{CUC}$	dFx	2
Aoc	CME	$tRNA^{EnGlu}_{CCU}$	eFx	6

Supplementary Methods

Amino acids and flexizymes

Ester activated amino acids were prepared as described previously[1, 2]. Flexizymes prepared as described previously[3, 4].

Preparation of tRNA

tRNA were prepared as described previously[4, 5]. Briefly, an appropriate DNA template was transcribed by T7 RNA polymerase and the resulting RNA purified using 8% denaturing PAGE, dissolved in water and the concentration adjusted to 250 μM and stored at -80 °C.

$tRNA^{EnPro1E2\#3}_{CAU}$:

GGGTGATTGGCGCAGCCTGGTAGCGCACTTCGCTCATAACGAAGGGGTCAGGGG
TTCGAATCCCCTATCACCCGCCA

$tRNA^{EnPro1E2\#3}_{GCG}$:

GGGTGATTGGCGCAGCCTGGTAGCGCACTTCGCTGCGGACGAAGGGGTCAGGGG
TTCGAATCCCCTATCACCCGCCA

$tRNA^{EnGlu}_{CCG}$:

GTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTCCGGAGGCGGTAACAGGGGT
TCGAATCCCCTAGGGGACGCCA

$tRNA^{EnGlu}_{GUC}$:

GTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTGTCAAGGCGGTAACAGGGGTT
CGAATCCCCTAGGGGACGCCA

$tRNA^{EnGlu}_{CUC}$:

GTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTCTCAAGGCGGTAACAGGGGTT
CGAATCCCCTAGGGGACGCCA

$tRNA^{EnGlu}_{CCU}$:

GTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTCCTAAGGCGGTAACAGGGGTT
CGAATCCCCTAGGGGACGCCA

Aminoacylation of tRNA

Aminoacylation was carried out following protocols described previously[2, 6]. 25 μM tRNA, 25 μM flexizyme and 5 mM ester activated amino acid (in DMSO) were incubated in 50 mM HEPES-KOH pH 7.5 buffer (final concentration 20% DMSO), 600 mM $MgCl_2$ and incubated at 0 °C for the times described in **Table S3**. tRNA, flexizyme and buffer were first combined, heated to 95 °C for 2 mins, cooled to room temperature for 5 mins before addition of activated Mg_2Cl, incubated at room temperature for 5 mins before cooling on ice and addition of activated amino acid. The reaction was stopped by adding to 4 reaction volumes of 0.3 M NaOAc pH 5.2, then reactions involving different loaded tRNA were mixed and then precipitated together using 10 reaction volumes of EtOH. Pellets of aminoacylated tRNA were 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*[7]. 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). 16 of the 20 canonical amino acids were included at 500 μM i.e. excluding Met, Asp, Arg and Glu.

The translation reaction was supplemented with 5 μM EF-P, expression and purification of which described in[8], and loaded tRNAs: 40 μM ClAc-D-Phe-$tRNA^{fMet}_{CAU}$, 20 μM D-Ala-

$tRNA^{EnPro1E2\#3}_{CAU}$, 20 µM D-Phe-$tRNA^{EnPro1E2\#3}_{GCG}$, 40 µM MePhe-$tRNA^{EnGlu}_{CCG}$, 20 µM MeAla-$tRNA^{EnGlu}_{GUC}$, 20 µM MeGly-$tRNA^{EnGlu}_{CUC}$, 20 µM Aoc-$tRNA^{EnGlu}_{CCU}$.

Ub-BTN targets

$^{K48}Ub_4$-BTN and $^{K48}Ub_2$-BTN (biotin) prepared using solid phase peptide synthesis and native chemical ligation as described previously[4]. Binding to Streptavidin magnetic beads tested as described previously[4].

First round of selection to find $^{K48}Ub_4$ binding peptides

A DNA library was prepared with T7 promoter, ribosome binding site Shine Dalgarno, 'ATG' start codon, 6-12 'NNK' degenerate codons, 'TGCGGCAGCGGCAGCGGCAGCTAG' containing Cys for cyclization, $(GlySer)_3$ linker, and amber (RF1) stop codon, and puromycin attachment sequence. DNA library was transcribed to mRNA, and puromycin ligated to the 3' end, as described previously[4, 6]. Initial cyclic peptide library was formed by adding this puromycin ligated mRNA library (100 pmol) to a 100 µL scale reprogrammed *in vitro* translation system and incubating 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 $^{K48}Ub_4$-BTN. Bead amounts were chosen so that the final concentration of $^{K48}Ub_4$ 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 extracted and ethanol precipitated for use in subsequent rounds.

Subsequent rounds of selection, including negative selection to $^{K48}Ub_2$

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 $^{K48}Ub_2$-BTN, with sufficient beads for 200 nM in the suspension. After incubation at 4 °C 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 $^{K48}Ub_4$-BTN, incubated at 4 °C 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 °C and collecting the supernatant. As for the first round, DNA was quantified using qPCR, amplified using PCR, transcribed and ligated to puromycin.

Deep sequencing of recovered enriched DNA libraries

DNA recovered from rounds 2,3,4 of the 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.

Surface Plasmon Resonance (SPR) studies

The interaction between cyclic peptides **Ub4a**, **Ub4e** 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 **Fig. S4, and Table S1**. SPR amplitude fits using standard K_D binding equation $\frac{x*max}{KD+x}$, where max is the fit amplitude when all cyclic peptide is bound and x is the concentration of the cyclic peptide. Fit max from each cyclic peptide binding $^{K48}Ub_4$ was fixed when fitting titrations to $^{K48}Ub_2$.

NMR studies

NMR studies were performed on Bruker Avance III 600 MHz spectrometer equipped with TXI or TCI cryoprobe. The sample temperature was set to 23°C, unless indicated otherwise. Purified selectively ^{15}N-labelled $^{K48}Ub_4$, $^{K48}Ub_3$ and $^{K48}Ub_3$ samples (^{15}N-enriched in the proximal or distal Ub, as indicated in the main text) were prepared in 20 mM sodium phosphate buffer (pH 6.8) containing 5-10% D_2O and 0.02% (w/v) NaN_3. The **Ub4a** peptide was dissolved in 40% D_6-DMSO, 30% D_2O and 30% 20 mM sodium phosphate pH 6.8 buffer before use. The peptide was titrated in µL amounts into 200 µM $^{K48}Ub_4$, $^{K48}Ub_3$ or $^{K48}Ub_3$ up to 1.0-1.5 molar equivalents. As shown previously[4] 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. The spectra of free protein and at the titration endpoint were also recorded at 37°C in order to reduce line broadening (for signal assignment purposes) and to check the reproducibility of the spectral changes at a physiologically relevant temperature. Spectra were processed and analyzed using TopSpin (Bruker Inc.) and Sparky[9]. Assignment of peptide-bound NMR signals of Ub_3 and Ub_4 was complicated by signal broadening (due to slow tumbling of tri- and tetra-Ub) and overlap (as well as) by the slow-exchange regime which precluded tracing gradual shifts of the NMR signals upon titration. Generally, in the cases of slow or intermediate exchange, the residues affected by peptide binding were identified based on strong attenuation/disappearance of their unbound signals in the spectra. In addition, ^{15}N-edited TOCSY and NOESY spectra were recorded at the titration endpoint for assignment purposes, which allowed us to assign a significant portion of the peptide-bound NMR signals.

NMR signal shifts were quantified as chemical shift perturbations (CSP) 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.

Synthesis of Ub4a wild type peptide

Fmoc-SPPS was performed on Rink amide resin (0.26 mmol/g, 0.10 mmol scale) for the synthesis of the **Ub4a** cyclic peptide. Peptide synthesis was carried out in the presence of amino acid (4.0 equiv), HCTU (4.0 equiv) and DIEA (8.0 equiv) at room temperature. Initially, the pre-swollen resin was treated with 20% piperidine in DMF containing 0.10 mmol HOBt (5:10:5 min) for the Fmoc deprotection. Unless specified, all amino acids in the sequence were coupled on an automated peptide synthesizer. N-Methylation at specific sites were performed manually.

N-Methylation:

Step1: Wash the peptide with free NH_2 on resin sequentially with DMF and NMP. A solution of o-nitrobenzene sulfonyl chlroride, o-NBS, (89.0 mg, 4.0 equiv) in 2 mL of *N*-methylpyrrolidone (NMP) and sym. collidine (132.0 µl, 10.0 equiv) was loaded onto the resin and shaken for 20 min at room temperature. This step was repeated one more time and the resin was washed with NMP (3 X 5 mL). **Step2**: The resin was washed with dry tetrahydrofuran, THF, (3 X 5 mL). A solution of triphenylphsophine, PPh_3, (131.2 mg, 5.0 equiv) in 2.85 mL of dry THF and anhydrous methanol (~40 µL, 10.0 equiv) was loaded onto the resin and shaken for 2 min at room temperature. A solution of diisopropyl azodicarboxylate, DIAD, (~98.5 µL, 5.0 equiv) in 1 mL of dry THF was split into 5 portions. Each portion was loaded onto the resin at a time interval of 10 min and shaken at room temperature. Then the resin was washed with dry THF and the same step was repeated one more time.

Step3: Wash the resin with NMP (3 X 5 mL). A solution of 2-mercaptoethanol (70 µl, 10 equiv) in 2 mL of NMP and 1, 8-Diazabicyclo[5.4.0] undec-7-ene, DBU, (75.6 µl, 5.0 equiv) was loaded onto the resin and shaken for 15 min at room temperature. The resin was washed with NMP and the same step was repeated one more time followed by wash the resin with DMF (3 X 5 mL).

After N-Methylation, the next amino acid in the sequence was manually coupled using HATU (4.0 equiv) and DIEA (8.0 equiv) for 1 h (2x). To the N-terminal chloro acetic acid (141.8 mg, 15.0 equiv) was coupled using N, N'-diisopropylcarbodiimide, DIC, (~156.6 µl, 10 equiv) in 2 mL of DMF and then the peptide was cleaved from the resin using the mixture of TFA/H_2O/TIS (95:2.5:2.5) followed by subjected to precipitation and lyophilization. The crude peptide **1** (**Fig. S18**) was dissolved in 6M Gn.HCl followed by pH adjusted to 8.0 (NaOH) and incubated at 37 ºC for 4h for cyclization. The HPLC analysis was carried out on a C18 analytical column with a gradient of 0-60% B over 30 min. The crude peptide was purified using a C18 preparative column with the gradient flow of 0-60% B over 60 min give the peptide **2** in 52% yield (**Fig. S18**).

Synthesis and purification of Fluorescein-5-Maleimide-Ub4a cyclic peptide

The synthesis of linear peptide **1** (**Fig. S17**) with Cys (Acm) at C-terminus was carried out in similar way as described above. Cyclization was performed under the similar conditions as mentioned above. **Acm removal**: A solution of $PdCl_2$ was prepared by dissolving $PdCl_2$ (10.1 mg, 10.0 equiv) in 6 M Gn·HCl/200 mM phosphate buffer (pH 7.3, 100 µl) at 37 °C for 20 min to dissolve completely. The peptide **2** (10.0 mg, 5.72 mmol, 1.0 equiv) was dissolved in 6 M Gn·HCl/200 mM phosphate buffer (pH 7.3, 2858.5 µl, 2 mM) at room temperature. Then $PdCl_2$ solution was added to the peptide solution and the reaction mixture was incubated at 37 °C. The progress of the reaction was monitored by taking aliquot from the reaction mixture and quenched with DTT, followed by centriugatation and injection of the supernatant into HPLC using a C18 analytical column with a gradient of 0-60% B in 30 min. The reaction was found to complete in 1 h. After quenching with DTT, the crude peptide was purified using a C18 semi-preparative column with the gradient flow of 0-60% B over 60 min give the peptide **3** in 42% (4.0 mg) in yield (**Fig. S19**). **Labelling of Ub4a with of Fluorescein-5-Maleimide**: The peptide 3 (4.0 mg, 2.38 mmol, 1.0 equiv) was dissolved in 6 M Gn·HCl/200 mM phosphate buffer (pH 7.3, 2386 µl, 1 mM) and cooled to 0 ºC for 5 min. A solution of of **Fluorescein-5-Maleimide** (2.70 mg, 4.5 equiv) dissolved in 100 µl of DMF at 0 ºC under dark conditions was added to the peptide solution and the reaction was kept at 0 ºC under dark. Under dark conditions, the HPLC analysis was carried out on a C4 analytical column with a gradient of 0-60% B over 30 min and found the reaction was completed in 1 h. Under dark conditions, the crude peptide was purified using a C4 semi-preparative column with the gradient flow of 0-60% B over 30 min give the peptide **4** in 70% (3.23 mg) in yield (**Fig. S19**).

In vitro DUB assays

In vitro DUBs 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.

$^{K48}Ub_{2/4}$ (2 μM) were incubated for 1 hour with OTUB1 (0.25 μM) in the presence and absence of cyclic peptide **Ub4a** (2/ 6 μM). $^{K11}Ub_2$ (2 μM) was incubated with active Cezanne enzyme (0.3 μM) without or with 5 μM of **Ub4a** peptide and $^{K63}Ub_2$ (2 μM) was incubated for with USP2 (0.15 μM) enzyme, with or without **Ub4a** or **Ub4e** (5 μM) cyclic peptides for 1 hour. 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. Samples 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).

OTUB1, Ubp6, or USP2 used in the *in vitro* DUB assays for the disassembly reactions of $^{K48}Ub_4$ and $^{K48}Ub_3$ were initially prepared in 50 mM Tris (pH 8.0) buffer before being added to the Ub chains used. To start the hydrolysis process, substrate chains (18 μM) and individual DUB (3.6 μM or 9 μM for USP2) solutions were mixed at a 1:0.2 molar ratio (1:0.5 for USP2) and incubated at 37 °C for a total of ~16 hrs (overnight). Time point samples were taken at the indicated intervals, and the reactions were stopped by taking aliquots and adding 2X Laemmli sample buffer before being resolved by SDS–PAGE on 15% polyacrylamide gels. The gels were stained with Coomassie blue.

In vitro 26S Proteasome assay

In vitro 26S proteasome reaction were performed in 12.5 μL reaction mixture volume which contain: 5.3 μM of synthetic HA_α-globin-K48-linked tetra-Ub 150 nM[10], 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. Before and after incubation, products were separated 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).

Ub-conjugate assay

HeLa cells were incubated with cyclic peptides. Cells were lysed using RIPA buffer, resolved using SDS-PAGE, proteins were transferred to a nitrocellulose membrane, and the membrane was blotted using an antibody against Ub-conjugates (1:10000) and anti-actin antibody (1:10000).

Microscopy

Cyclic peptides were incubated for 2 hours at 37°C with Hela cells were seeded on glass bottomed (#1.5) 96-well dish, and were incubated in the presence of either DMSO or **Ub4a**-fluorescein **or Ub4e**-fluorescein 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.

Quantification of fluorescein and fluorescein-labelled peptide uptake into live cells

Cyclic peptides were incubated for 16 hr at 37°C with Hela cells (40,000 cell~230 μg protein) in 96 well plate (glass bottomed (#1.5)). Plates were washed (with PBS (three times)), cells lysed using RIPA (lysis buffer). A calibration curve was made using fluorescein or fluorescein-cyclic peptide to calculate the concentration after lysis. Using a cell volume of (50 μl) and a cell count (80,000) (480 μg protein) the intracellular concentration was estimated. Concentrations were calculated using fluorescence (ex= 480, em= 525 nm) and the calibration in **Fig. S8A**.

Western blots using Ub4a-Fluorescein

The same molar amount of different pure synthetic chains (2 μM) was run on 14% SDS-PAGE gel, electro-blotted to nitrocellulose membranes followed by blocking the membrane with 5% skim milk in TBST for 1 hour, washing with TBST followed by incubation of the membrane with **Ub4a**-fluorescein cyclic peptide (1:30,000) 1 hour. Acquisition of the Ub4a membrane-binding assay was performed using Typhoon FLA 9500 (GE Inc.), using fluorescence mode and the appropriate filters.

The experiment was repeated with the same concentration of Ub chains, but the membrane was blotted using an antibody against Ub-conjugates (1:10000) (primary antibody) followed by anti-rabbit secondary antibody conjugated to peroxidase (HRP) (1:10000) and anti-actin antibody. Bands were quantified with Image Quant LAS 4000 (GE Healthcare).

Detection of Ub conjugates in cell lysate using HA-tagged Ub4a cyclic peptide

Hela cells were incubated in the presence or absence of the non-specific DUB inhibitor iodoacetamide for 2 hours. Following incubation, the cells were lysed in RIPA buffer (50mM Tris-cl pH 8, 150mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1 SDS). 14% SDS-PAGE gel, electro-blotted to nitrocellulose membranes and probed with HA-**Ub4a** peptide (1:30,000) followed by anti-HA secondary antibody (1: 1000) and anti-actin antibody (1:10000). For comparison, another membrane was blotted using an antibody against Ub-conjugates and Actin. Bands were quantified with Image Quant LAS 4000 (GE Healthcare).

Apoptosis assays

Cyclic peptides, MG132, bortezomib, ixazomib or carfilzomib, at 100 nM, were incubated with the described cells for 24 and 48 hr, FACS was used to follow apoptosis, bars represent mean values. Induction of apoptosis in Hela cell lines by treatment with cyclic peptides was determined using annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Biosciences) according to the manufacturer`s protocol. Annexin V positive cells were counted as apoptotic, regardless of PI staining. 2 x 10^5 cells/well were treated with inhibitors for 24- and 48-hours in a dose dependent manner. DMSO and MG132 were used as negative and positive controls respectively. Samples were acquired on a Becton Dickinson LSR II flow cytometer (BD Biosciences) and analyzed using Flowjo software.

Measurement of Ub4a stability in human plasma.

Peptides were incubated in human plasma at 37°C and sampled at 0, 2, 26, 47, and 61 hours. HPLC and ESI-MS analysis were performed. **Ub4a** peptide was dissolved in phosphate-buffered saline buffer (PBS) (2 mg/ml) and incubated in human plasma (Sigma-Aldrich) at 37°C and investigated its stability by HPLC at different time intervals such as t = 0 h, 2 h, 26 h, 47 h and 61 h. At each time point, 10 µl of aliquot was drawn from the reaction mixture

and dilute with 10 µl of 50% Acetonitrile in water and injected into analytical HPCL using C18 analytical column and a gradient of 0-60% buffer B at 280 nm (U.V) over 30 min. As the time advanced, the intensity of peptide was decreased and indicate that the peptide **Ub4a** may slowly destabilizing as presented in the **Fig. S10**.

In vivo assay of tumor cell growth inhibition

Luciferase-labeled CAG human myeloma cells (5×10^6/mouse) were injected *i.v.* to NOD/SCID mice. After 7 days animals were sorted into three different groups (n=5) and treatment was initiated. Treatment groups included Bortezomib (0.5 mg/kg, twice a week, i.p), **Ub4a** (1 mg/kg, twice a week, i.p) and PBS served as control. Tumor growth was evaluated by IVIS bioluminescent imaging[11, 12]. Briefly, mice were injected intraperitoneally with D-luciferin substrate (150 mg/kg) and anesthetized with continuous exposure to isoflurane (EZAnesthesia, Palmer, PA). Light emitted from the bioluminescent cells was detected by the IVIS camera system, and tumor burden was quantified by measurement of total photon counts per second, using Living Image software (Xenogen).

Supplementary References

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