Synthetic tools to study ubiquitin biology

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Chapter 6

A Ub-derived cyclic peptide inhibits UCHL5 associated with the 26S proteasome

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Summary

UCHL5 (UCH37 in humans) is one of the proteasome-associated deubiquitinase (DUB) enzymes that facilitate trimming of poly-ubiquitin (PolyUb) chains, an important regulatory mechanism in the proteasomal degradation pathway. Polyubiquitinated proteins that are deemed for degradation may escape from the proteasome when polyUb chains are cropped from the target protein before they are being translocated into the core particle of the 26S proteasome. Although UCHL5 is known to deubiquitinate substrates, its influence on 26S proteasome activity is poorly understood. Inhibition of UCHL5 is known to induce apoptosis in specific types of cancer. So far, there are no selective inhibitors identified for UCHL5. Here, we describe a peptide derived from Ub, that can bind and inhibit UCHL5. From the reported structure of UCHL5 bound to Ub-PRG probe, a β-sheet containing peptide derived from Ub was identified, synthesized, cyclized and validated against recombinant UCHL5. We report that this stable peptide derived from Ub can impart binding and inhibition of UCHL5 that is associated with the 26S proteasome.

Introduction

Ubiquitination is an important post-translational modification involved in various cellular metabolic processes. [1, 2] These processes are tightly regulated by a set of ubiquitinating enzymes involving the Ub-activating E1 enzymes, the E2 conjugating enzymes and an E3-ligases. [3] Substrates can be modified either with a single Ub or multiple Ub moieties or polyUb chains connected via different internal lysine residues or the N-terminal methionine residue of Ub. [4] The substrates can be deubiquitinated by enzymes called deubiquitinases (DUBs). [5]

One of the most important post-translational Ub modifications of substrates is the K48-linked polyubiquitin modification. [6] This is a key signal for protein degradation by the proteasome. [7] The 26S proteasomes in mammals contain many different subunits that work in conjunction with each other to facilitate effective protein degradation. [8] The degraded proteins are then converted to short peptides and amino acids that are recycled while a small portion of polypeptides is presented to the immune system by MHC Class I molecules. [9]

Proteasome activity is tightly regulated to prevent problems associated with unwarranted protein degradation. [10] Among the regulatory proteins associated with the proteasome are the deubiquitinases (DUBs). [11] So far, two DUBs have been identified that are tightly associated with the 26S proteasome complex: UCHL5 (UCH37 in humans) and USP14 (Ubp6 in humans), while Rpn11 (POH1 in humans) is an integral part of the 19S proteasome lid. [11, 12] UCHL5 and USP14 are cysteine-proteases, while Rpn11 is a JAMM/MPN+ metalloprotease. [13]

Although the structure of the 26S proteasome has been studied for years, the exact mechanism of action of the proteasomal DUBs is still under debate. [8] Evidence has pointed out that Rpn11, which is located at the base of the lid complex in close proximity to the 20S core complex, removes the polyUb chain *en-bloc* from substrate proteins while the substrate protein is concomitantly forced into the core complex for degradation. [14, 15] It has been proposed that USP14 functions as a chain-trimming DUB that removes Ub monomers from polyUb chains before the substrate is degraded by the proteasome. [16] However, much less is known about the mechanism of action of UCHL5 mainly due to the lack of good inhibitors.

Recently, the structure of UCHL5 in complex with the regulatory proteins Rpn13 and INO80G respectively has been reported. [17, 18] Based on the structural data, a model has been proposed that explains how the activity of UCHL5 is regulated by these two proteins.

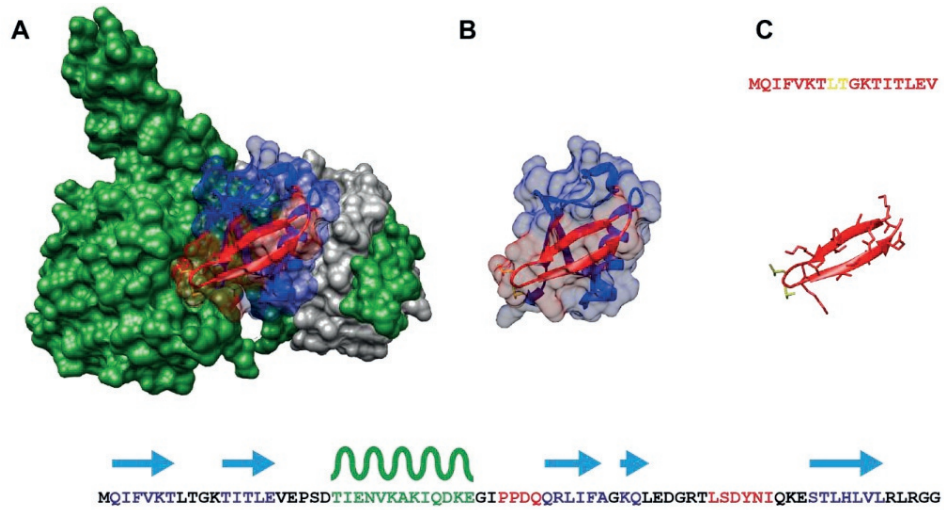

Figure 1*: Structure of Ub binding to UCHL5/Rpn13.* ***A:*** *X-ray structure of UCHL5 (in green) bound to Ub-PRG (in blue) and Rpn13 (grey) (PDB: 4UEL). Location of the binding pocket for Lue8 and Thr9 of Ub is represented in orange and the first* β*-sheet of Ub is shown in red.* ***B:*** *Isolated structure of Ub showing the orientation of the first β-sheet (in red). The sequence of Ub showing different secondary structures is shown below.* ***C:*** *Structure of the β-sheet peptide of Ub encompassing residues 1 to 17 in which the important residues, namely Leu8 and Thr9, are highlighted in yellow. D: Sequence of full-length Ub showing residues that comprises the β-sheet (blue arrows) and the α-helix (green curves). Residues that constitute the connecting loops are highlighted in red.*

The deubiquitination efficiency of UCHL5 increases when it is associated with the DEUBAD domain of Rpn13. It was shown that Rpn13 augments deubiquitination of UCHL5 by providing additional recognition of ubiquitin, thereby bringing the C-terminus of Ub close to the active site of UCHL5. On the other hand, in association with full-length INO80G located inside the nucleus, the deubiquitination activity of UCHL5 is completely abolished. This is caused by the binding of INO80G to UCHL5 which in turn interferes with the binding of Ub to UCHL5. [17, 18]

In the X-ray crystal structure of Ub-UCHL5/Rpn13 (PDB: 4UEL), it can be seen that the covalent Ub-based probe called Ub-PRG [19] binds to UCHL5 in complex with Rpn13 through a hydrophobic patch consisting of residues Leucine 8, Threonine 9, and Isoleucine 44 of Ub in addition to the C-terminal tail of Ub (Figure 1A). [17] Specific residues in the N-terminus of Ub are in direct contact to a hydrophobic pocket in UCHL5 consisting of residues Phe218 and Leu38. The residues of Ub involved in this interaction include Lue8 and Thr9, Phe5 and Ile13 that are present in the first β-sheet of Ub. Therefore, we postulated that this particular β-sheet of Ub comprising of residues 1 to 17, could be used as a discrete binding motif for UCHL5/Rpn13 thereby functioning as an inhibitor of UCHL5.

Chemical synthesis of Ub has been reported by us and others using different strategies yielding multi-milligram quantities of synthetic ubiquitin. [20] We used the same strategy to synthesize the Ub-peptide consisting of residues 1 to 17 and refolded them to form the β-sheet. To trap the peptide in a more stable β-sheet conformation, we also cyclized the peptide directly after synthesis by SPPS. Both the linear version and cyclic version of this peptide

were checked for the β-sheet folding using circular dichroism (CD) measurements. The peptides were then tested against UCHL5/Rpn13 using enzyme-activity assays in a plate reader format, that revealed inhibition of UCHL5. Finally, we tested this peptide on the proteasome with functionally associated UCHL5 and USP14 where we used a Ub-based covalent probe (Ub-PRG) as a competitor to this peptide and observed that this peptide is an inhibitor of UCHL5 and not of USP14. We also confirmed that these peptides did not interfere with the activity of Rpn11, the metalloprotease DUB found in the lid of the 26S proteasome. This shows that both the linear and the cyclic versions of the peptide inhibits UCHL5 selectively within the context of the 26S proteasome activity.

Synthesis of linear and cyclic Ub-peptides

The synthesis of peptides by Fmoc-based solid phase peptide synthesis was carried out according to a previously reported procedure. [20] The cyclic and the terminally-protected linear version of ubiquitin peptides (1-17) were synthesized on TentaGel-TrtR resin and Rink-amide resin respectively. The Ub (1-17) peptide that was to be cyclized was synthesized on a Trt-resin with a free N-terminal methionine residue. The fully protected peptide was cleaved from the resin using 20% HFIP in DCM to afford the peptide with a free carboxy terminal group. After co-evaporating HFIP with DCM under reduced pressure, the protected Ub (1-17) peptide was obtained as a colourless oil. Cyclization of this peptide was carried out by adding 1.2 eq PyBOP and 1.2 eq DiPEA to the peptide dissolved in DMF at a concentration of 0.5 mg/ml. Completion of the reaction was followed by LC/MS analysis (Figure S1). After evaporating DMF under vacuum, TFA cleavage mix containing TFA: H_2O: TiPS: Phenol (92.5/2.5/2.5/2.5 v/v/v/v) was used to fully deprotect the final peptide. The peptide was then precipitated with dry-ice cooled ether:pentane (3:1) mix, followed by centrifugation and lyophilized to yield crude peptide that was then purified by reversed-phase HPLC. In the case of linear protected Ub (1-17) peptide containing an N-terminal acetyl group and a C-terminal amide group, the peptide was synthesized on a Rink amide resin with the N-terminal methionine residue protected with an acetyl group. Upon TFA cleavage using the TFA cleavage mix, the peptide was precipitated and lyophilized and purified using reversed phase HPLC.

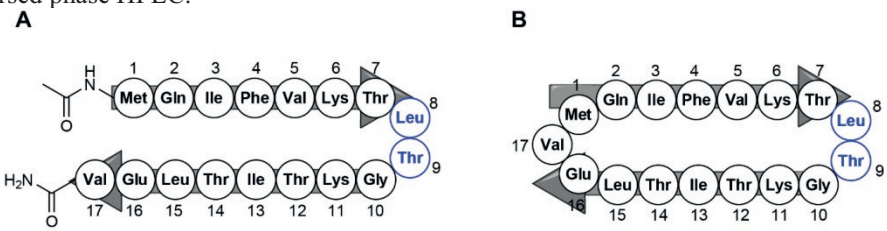

Figure 2*: Illustration of the linear end-protected peptide (**A**) and the cyclic peptide (**B**) based on Ub sequence from residues 1 to 17. The* β*-strand is represented by the arrow. Leu8 and Thr9 residues are the key residues of interaction with UCHL5 in this sequence (highlighted in blue.).*

Results

The structure of UCHL5 in complex with Ubiquitin and Rpn13 had been reported. [17, 18] This structure shows the binding pocket for residues 1 to 17 of Ub. Therefore, a peptide containing residues 1 to 17 of Ub was synthesized using Fmoc-SPPS. Since the structural stability of this β-sheet was not known, we synthesized both terminally-protected

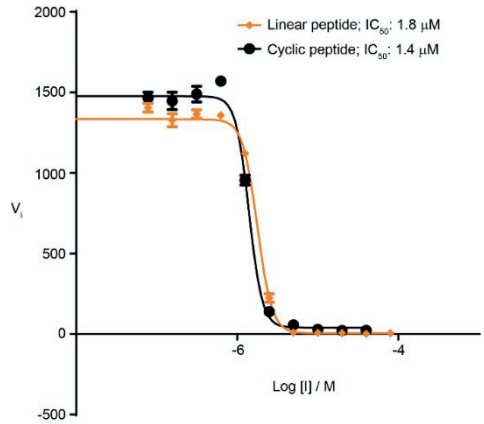

Figure 3: IC_{50} curves of Ub β-sheet peptide*. Both linear and cyclic version of the peptides were tested for inhibition of UCHL5 activity in a standard plate-reader assay using Ub-Rho as a substrate. Both the peptides inhibited the enzyme in a similar fashion.*

linear peptide and a cyclized peptide (Figure 2). In order to check for their folding, peptides were analysed using circular dichroism, which revealed a β-sheet conformation in both the cases (Figure S2).

First, we tested whether our peptides were able to inhibit UCHL5 activity using a standard Ub-rhodamine substrate in a plate-reader assay. As expected, both the linear and cyclic peptides were able to inhibit the activity of UCHL5. The linear peptide inhibited UCHL5 with an IC_{50} of 1.78 µM while the cyclic version inhibited at an IC_{50} of 1.4 µM (Figure 3).

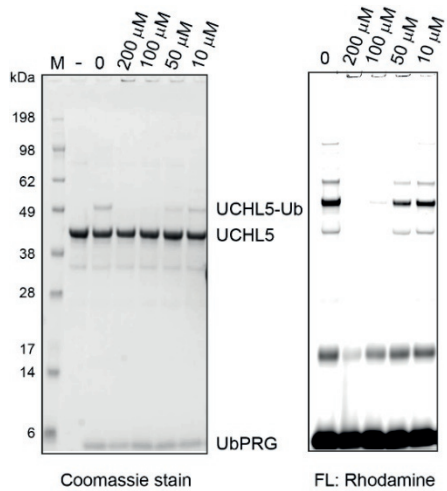

Figure 4: SDS-PAGE analysis of UCHL5 labelling using Rho-Ub-PRG in the presence of cyclic Ub β-sheet peptide*. **Left**: Coomassie stain showing inhibition of UCHL5 labelling by cyclic peptide at concentrations higher than 50 µM. **Right**: Fluorescence scan of the same gel showing disappearance of Rho-UbPRG-UCHL5 signal at higher concentrations of Ub peptide.*

For comparison, a known 19S DUB inhibitor called b-AP15, that has been reported to inhibit both UCHL5 and USP14, has an IC_{50} value of 16 μM. [21] This suggests that these peptide inhibitors are 10-fold more potent in inhibiting UCHL5 alone. Next, we tested whether our peptide can compete with Ub-PRG labelling of UCHL5. Ub-PRG acts by binding covalently to the active site cysteine with the alkyne group at its C-terminus. We incubated UCHL5 with different concentrations of the cyclic peptide Ub (1-17) for 30 minutes at RT. Rho-Ub-PRG was then added to this sample and incubated for 5 minutes. The samples were run on SDS-PAGE and labelling of UCHL5 was measured using a fluorescence scanner. The results show that the peptide was able to completely inhibit Ub-PRG labelling of UCHL5 at a concentration of 100 μM. (Figure 4).

Since our cyclic peptide was able to prevent binding of Ub-probe with UCHL5, we verified whether it is specific for UCHL5. UCHL5 is mainly associated with proteasome activity, along with USP14, another cysteine protease DUB associated with the proteasome. We verified whether our cyclic peptide can inhibit UCHL5 selectively over USP14 in the 26S proteasome. Isolation and purification of the proteasome in complex with UCHL5 and USP14 were carried out as reported previously. [22] (Figure 5A).

We incubated 5 μM of the cyclic peptide with 0.5 μg purified 26S proteasome for 30 minutes at 37 °C. Then, Rho-Ub-PRG was added to this sample for 15 minutes at 37 °C and analyzed on a denaturing SDS-PAGE gel. Visualization under a fluorescence scanner showed that the cyclic peptide was able to inhibit labelling of UCHL5 by Rho-Ub-PRG but not of USP14 (Figure 5B). On the other hand, 5 μM of IU1, a USP14 specific inhibitor was unable to inhibit UCHL5 (Figure 5C). This shows that our peptide inhibitor can inhibit UCHL5 in a specific manner in the context of the 26S proteasome.

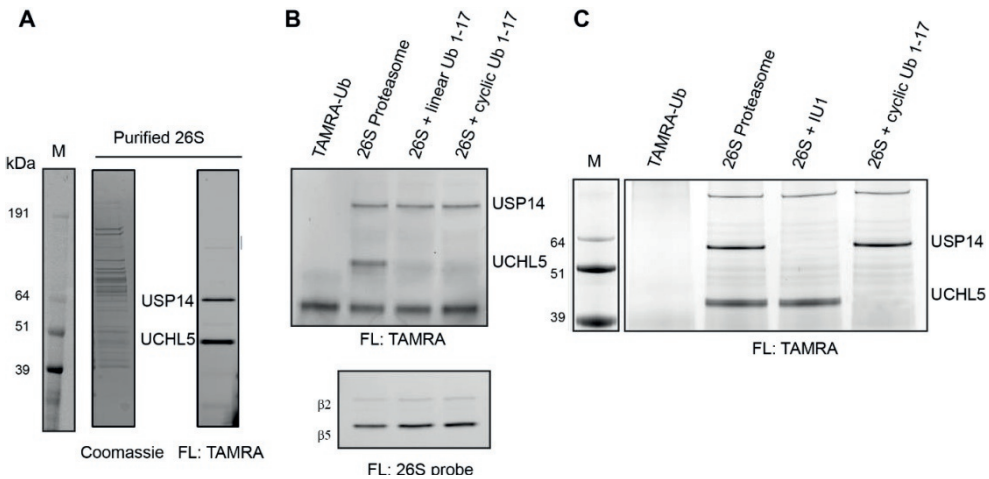

Figure 5: Inhibition of UCHL5 associated with the 26S proteasome. A: *The purified 26S proteasome (0.5 μg) is shown in Coomassie stain on the left. TAMRA-Ub-PRG labelling of the two cysteine-protease DUBs UCHL5 and USP14 is shown in the fluorescence scan on the right.* ***B:*** *Both the linear and cyclic Ub β-sheet peptide inhibits UCHL5 labelling by TAMRA-Ub-PRG but showing no change in USP14 labelling; the peptides did not affect the 26S protease activity as shown below.* ***C:*** *Selective inhibition of USP14 using IU1 and UCHL5 using cyclic Ub β-sheet peptide.*

In order to check whether our peptide can interfere with the activity of Rpn11, we checked its inhibition in a standard Ub-FP assay. Rpn11/Rpn8 heterodimer was expressed and purified using bacterial expression as described. [15] Using a Ub-FP substrate containing Ub linked by an isopeptide bond to a TAMRA-labelled Ub peptide, comprising residues 41to 54 of Ub, was used in a fluorescence polarization assay. [23] We observed that the cyclic peptide was unable to inhibit Rpn11/Rpn8 activity, suggesting that the peptide is specific to UCHL5 among other DUBs in the proteasome complex (Figure S3).

Discussions

The Ub interaction with UCHL5/Rpn13 has been studied using X-ray crystallography. [17, 18] These studies revealed the mode of binding of Ub to the surface of UCHL5. We used this information to design a linear and a cyclic beta-sheet peptide based on residues 1-17 of Ub. This provided competitive binding with UCHL5, as revealed with competitive labelling using a Ub-PRG probe. These peptides inhibited UCHL5/Rpn13 with an IC_{50} in the range of 1.4 μM to 1.8 μM, which is similar to the IC_{50} of the known UCHL5 inhibitor, b-AP15. [24] In addition, using Rho-Ub-PRG labelling of UCHL5, we show how the peptide competes with Ub binding by inhibiting labelling of UCHL5 with a Rho-Ub-PRG probe.

All of the known proteasomal DUBs have been implicated in the regulation of proteasomal degradation, upstream to its protease activity. Two of the DUBs namely UCHL5 and USP14 are strongly associated with chain trimming activity, which prevents degradation of polyUb-substrate. On the other hand, the metalloDUB Rpn11, positioned between the lid and the core complex is involved in the removal of Ub chains from the substrate when it is translocated into the core complex. This prevents the spontaneous degradation of Ub. Although specific inhibitors are known for USP14 and Rpn11, the lack of inhibitors for UCHL5 has hampered studies on substrate processing. The specificity of our peptide to UCHL5 in the context of the proteasome will help to study the role of each of the three DUBs in the activity of the 26S proteasome.

Conclusion

Studies on proteasome inhibition are indispensable for both fundamental and therapeutic research. It is essential that this process is studied in detail for further understanding of proteasomal function and the development of better pharmacological agents that can be used to treat diseases including cancer. So far, bortezomib has been used in the treatment of cancer including multiple myeloma and mantle cell lymphoma. It functions by blocking proteolytic activities in the core particle. However, several enzymes act upstream of the proteasome that tightly regulates its function. Such enzymes can also be targeted to enhance or inhibit proteasome activity, potentially leading to alternative therapy for specific types of cancers. [25, 26]

Among such enzymes are the deubiquitinases that function as regulators of Ub-dependent proteasomal degradation. UCHL5 and USP14 are two cysteine-protease class of DUBs that essentially trim polyUb chains before the substrate is being processed by the proteasome core particle. On the other hand, Rpn11 which is a metalloprotease DUB, is part of the proteasome and facilitates the removal of polyUb en-bloc from the substrate before it is degraded by the core particle.

Inhibition of UCHL5 and USP14 is known to increase the activity of the proteasome. [21] Although specific inhibitors of USP14 exist, UCHL5-specific inhibition has been pursued for a long time. [11] We now report the design and synthesis of a small Ub-derived peptide that can inhibit UCHL5 associated with the proteasome. Our design is based on a known Ub-binding site and the resulting peptide was synthesized and validated against a UCHL5/Rpn13

heterodimeric complex. Peptide-based inhibitors that can retain their secondary structure in a cellular environment and that simultaneously resist exopeptidase cleavage potentially can be used for therapeutic purposes in treating diseases such as cancer.

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Supplementary information:

General methods:

All commercial materials (Aldrich, Fluka, Novabiochem) were used without further purification. All solvents were reagent grade or HPLC grade. LC/MS analysis was performed with a system containing a Waters 2795 separation module (Alliance HT), Waters 2996 Photodiode Array Detector (190–750 nm), Phenomenex Kinetex XB-C18 (2.1 x 50 mm) reversed phase column and a MicromassLCT-TOF mass spectrometer. Samples were run at 0.80 mL min (Kinetex C18) with the use of a gradient of two mobile phases: A) aq. formic acid (0.1 %), and B) formic acid in CH_3CN (0.1 %). Data processing was performed using Waters MassLynx 4.1 software (deconvolution with Maxent1 function). Preparative HPLC was performed on a Waters XBridge™ Prep C18 Column (30 x 150 mm, 5µm OBD™) at a flow rate of 37.5 mL/min using aq. 0.05% TFA (Solvent A) and acetonitrile containing 0.05% TFA (Solvent B) as eluents. The gradient used for purification of peptides was from 25% B to 95% B over 18 minutes. All samples containing pure peptide were pooled together and lyophilized.

Fmoc-SPPS of peptides:

SPPS was performed on a Syro II MultiSyntech Automated Peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry at 25 µmol scales. All amino acids were used in excess and coupling mix containing PyBOP and DIPEA were used accordingly. Linear Ub (1-17) peptide was synthesized on H-Rink amide Chemmatrix® resin (Sigma-Aldrich), and the N-terminus of this peptide was capped with acetyl group using acetic anhydride. After synthesis, the peptide was completely deprotected using TFA cleavage mix (TFA: H_2O: TiPS: Phenol (92.5/2.5/2.5/2.5 v/v/v/v)). The cyclic peptide of Ub (1-17) was synthesized on TentaGel Trt R resin with a free N-terminus. Immediately after synthesis, the cyclic peptide was cleaved from the resin using HFIP in DCM (20% v/v) so that the side chains remain protected while the N- and C-terminus are freed. Cyclization was done using 1.2 equiv. PyBOP, 1.4 equiv. DIPEA in DMF at a peptide concentration of 0.5 mg/mL, overnight at Room Temperature. After cyclization, the peptide was completely deprotected using the same TFA cleavage mix used for linear peptide. Following deprotection, the peptide was precipitated in the dry-ice cold ether:pentane (v:v 1:1), dried and lyophilized. The lyophilized peptides were then dissolved in aqueous DMSO (5%) and purified using preparative RP-HPLC.

Circular Dichroism (CD) measurements:

For Circular Dichroism (CD) measurements, a JASCO CD J1000 machine was used (UMC, Utrecht, the Netherlands). Samples were diluted in 20 mM TrisHCl, 20 mM NaCl pH 7.4, to a final concentration of 4 µM. Measurements were performed at 25 °C using wavelengths ranging from 260 nM to 185 nM in a span of 100 mdeg. The scanning speed was 20 nM/min and measurements from 10 experiments were accumulated. Based on the observed values of CD measurements and concentration used in measurements, CD plots were drawn.

IC_{50} assay of linear and cyclic peptides:

The DUB mediated hydrolysis of Ub-rho yields free rhodamine and Ub. The rhodamine signal is measured in a fluorescence intensity assay using a buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl, 5 mM DTT, 0.05% Tween-20 and 0.1 mg/ml BGG at pH 8 and at room temperature [1]. The enzyme and the substrates were added to a 384-well Corning™ low volume flat bottom plates. Fluorescence intensity was monitored at an excitation wavelength of 487 nm and an emission wavelength of 535 nm using BMG CLARIOstar® plate reader. The enzyme stock was diluted into the assay buffer at a final concentration of 1 nM. The Ub-rhodamine substrate was prepared synthetically according to the previously reported procedure [2]. The substrate dissolved in DMSO was diluted out in the water and later in the assay buffer to a final concentration of 100 nM. For dissolving the peptides, samples were prepared in DMSO as a 5 mM stock solution. Then they were dissolved in MQ water and then into one of the fluorogenic assay buffers. Serial dilution of the peptides was prepared in the assay buffer. After 30 minutes of incubation, the substrate (Ub-Rho or UbFP assay reagent) was added. They are then immediately measured in a plate-reader over a period of up to 90 minutes at RT.

Covalent Ub-probe labelling of UCHL5:

Recombinant UCHL5/Rpn13 was taken at a concentration of 1 μM in a labelling buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl and 5 mM DTT. To this, cyclic peptide Ub (1-17) at different concentrations ranging from 200 μM to 10 μM was added and incubated for 30 minutes at RT. After this, 2 μM of Rho-Ub-PRG probe was added and incubated for 5 minutes at RT. The samples were run on an SDS-PAGE agarose gel using MES buffer. The gel was scanned for fluorescence of Rhodamine in a Typhoon FLA 9500 (GE Healthcare Lifesciences) using filters set at 473 nm (excitation wavelength) and 532 nm (emission wavelength). Coomassie staining was carried out using InstantBlue™ Protein Stain (Sigma-Aldrich).

26S Proteasome purification:

The 26S proteasome was purified from mammalian cells using a previously reported procedure [3]. HTBH-Rpn11-HEK293T cells were treated with 5 mM of the indicated compounds for 16 hours after which cells were washed and collected by scraping in lysis buffer (100 mM NaCl, 50 mM sodium phosphate, 10% glycerol, 5 mM ATP, 1 mM DTT, 5 mM $MgCl_2$, 13 protease inhibitor (Roche), 13 phosphatase inhibitor (Roche), and 0.5% NP-40 (pH 7.5)). After a centrifugation step (21,100 g for 15 minutes) HTBH-tagged Rpn11-containing 26S proteasomes were isolated from the lysate by overnight incubation at 4°C with streptavidin beads. After 3 washes with wash buffer (50 mM Tris-HCl (pH 7.5)), 10% glycerol, 1 mM ATP), 26S proteasomes were cleaved from the beads by treatment with 1% TEV enzyme (protein expressed and purified in-house by the NKI protein facility) for 1 hour at 30°C in wash buffer. Protein concentration was determined using NanoDrop. For proteomic analysis, proteasome isolation was performed four separate times for each condition. HEK293T cells stably expressing HTBH-tagged Rpn11 were kindly provided by Lan Huang (University of California, Irvine).

Supplementary figures:

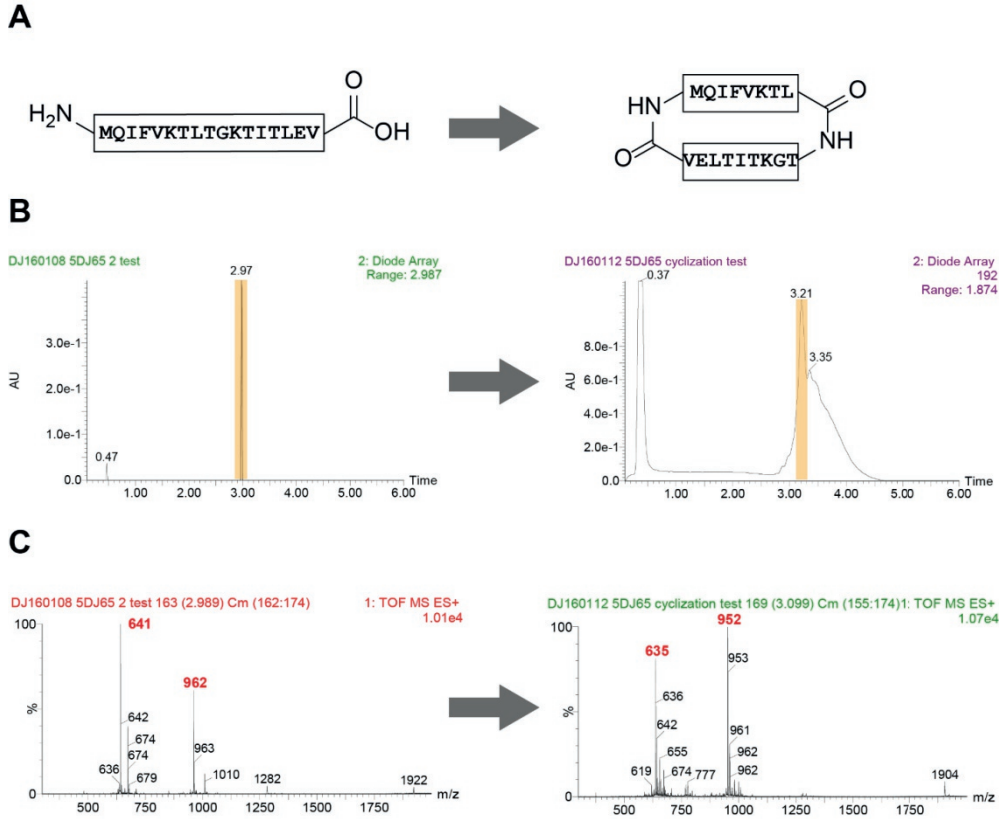

Figure S1: Cyclization of Ub (1-17) peptide. ***A***: *Schematic illustration of cyclization procedure where the N- and the C-terminus of the peptide is conjugated together using peptide coupling procedure.* ***B***: *UV chromatogram from the LC/MS measurement of the peptide before (left) and after (right) cyclization procedure.* ***C***: *Mass spectrometric signals of Ub (1-17) peptide before (left) and after(right) the completion of cyclization.*

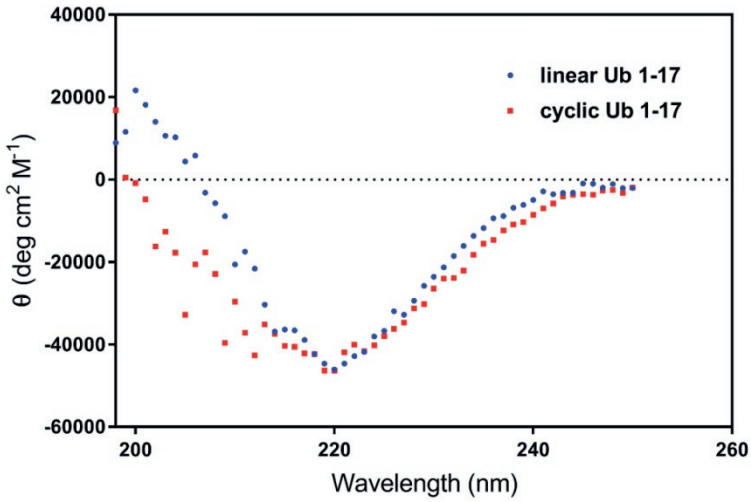

Figure S2: Circular Dichroism spectrum of both the linear and the cyclic Ub (1-17) *peptide. The pattern is similar to what is generally observed for a β-sheet peptide.*

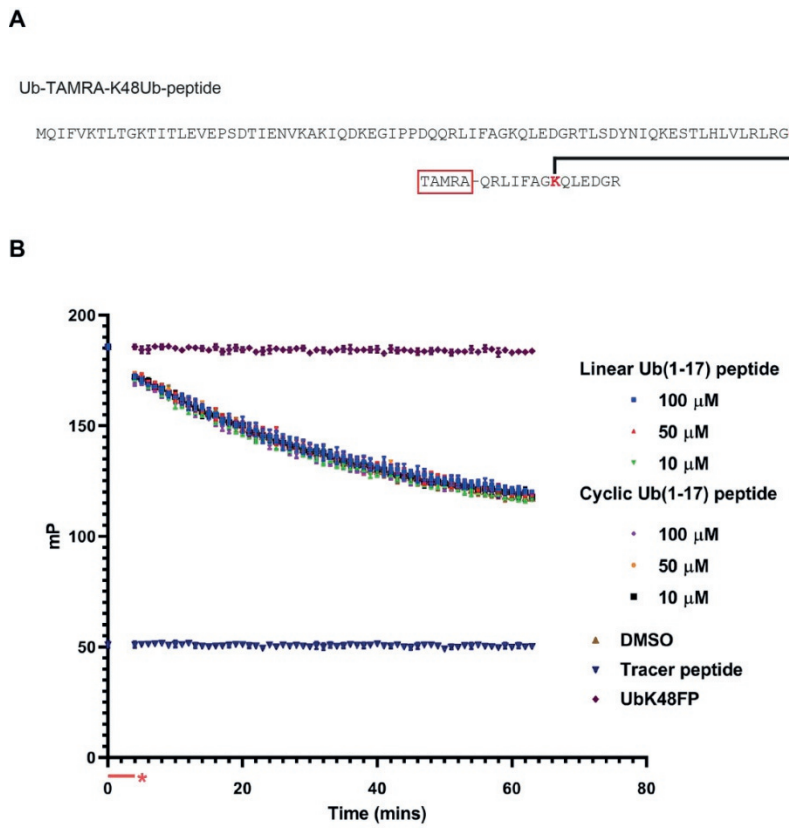

***Figure S3**: **A**: Sequence of U-TAMRA-K48Ub-peptide (UbK48FP) substrate used in Rpn11 enzyme activity assay. **B**: Fluorescence polarization data of Rpn11/Rpn8 activity in the presence of different concentrations of both linear and cyclic Ub (1-17) peptide. UbK48FP reagent was used as a substrate and TAMRA-K48(Ub) peptide was used as tracer peptide. The first four minutes were not measured due to the time taken from adding substrates to measuring the plate.*

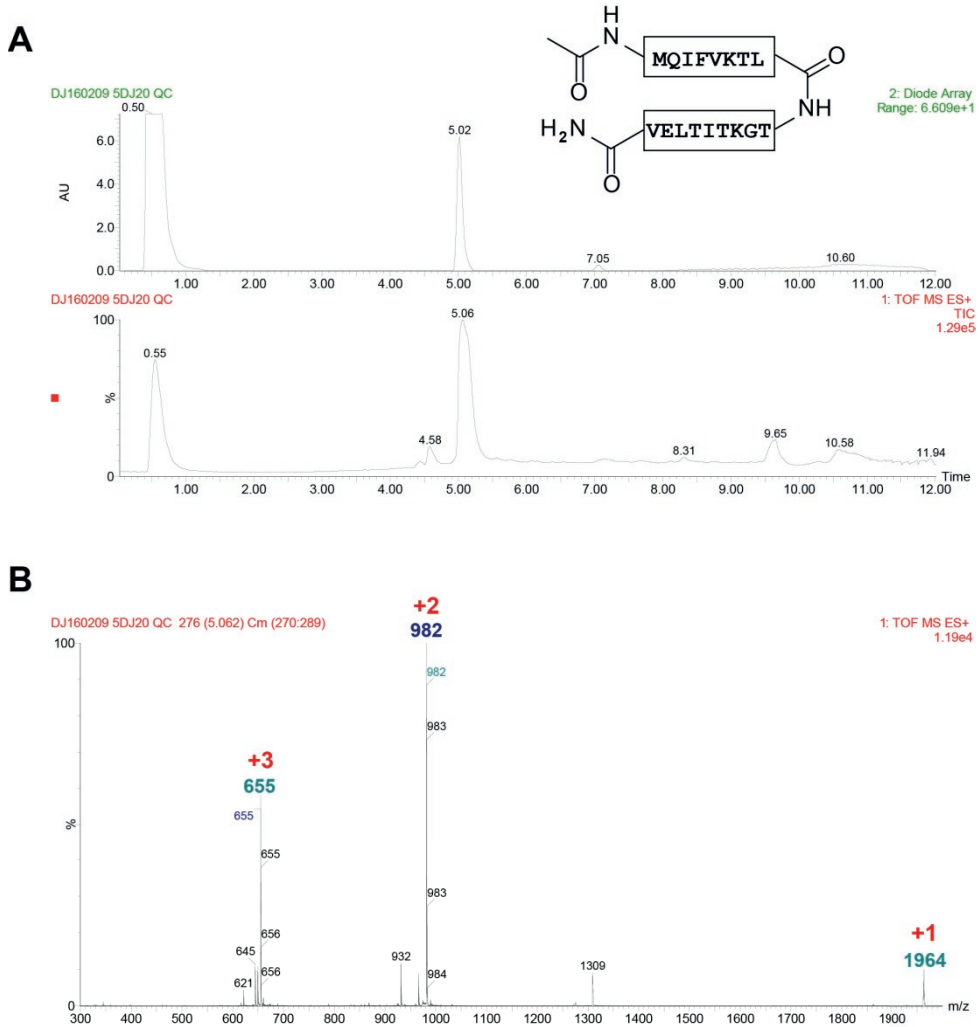

Figure S4: *LC-MS analysis of linear Ub (1-17) peptide.* ***A:*** *Top: UV chromatogram; Bottom: Combined Mass spectrum; Inset: illustration of linear peptide.* ***B:*** *Combined mass spectrum of UV peak at 5.02 min. Calculated mass: 1963.4 Da. Observed mass: 1964(M+1), 982 (M+2) and 655 (M+3).*

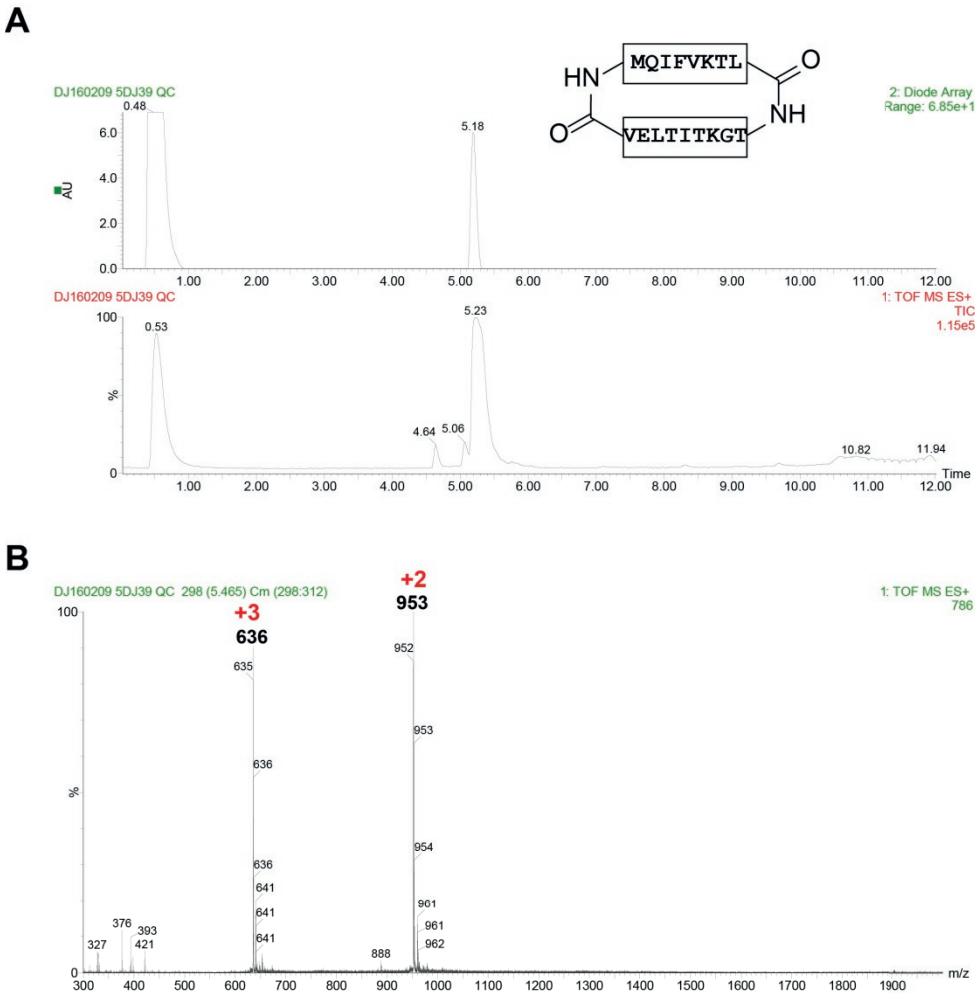

Figure S5: *LC-MS analysis of cyclic Ub (1-17) peptide.* ***A:*** *Top: UV chromatogram; Bottom: Combined Mass spectrum; Inset: illustration of cyclic peptide.* ***B:*** *Combined mass spectrum of UV peak at 5.18 min. Calculated mass: 1922.4 Da. Observed mass: 953 (M+2) and 636 (M+3).*

Supplementary references:

1. Sahtoe, D.D., et al., *Mechanism of UCH-L5 activation and inhibition by DEUBAD domains in RPN13 and INO80G.* Mol Cell, 2015. **57**(5): p. 887-900.
2. El Oualid, F., et al., *Chemical synthesis of ubiquitin, ubiquitin-based probes, and diubiquitin.* Angew Chem Int Ed Engl, 2010. **49**(52): p. 10149-53.
3. Wang, X., et al., *Mass spectrometric characterization of the affinity-purified human 26S proteasome complex.* Biochemistry, 2007. **46**(11): p. 3553-65.