Supplementary Information

Non-hydrolyzable Ubiquitin-Isopeptide Isosteres as Deubiquitinating Enzyme Probes

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Materials and Methods.

LC-MS analysis was performed on a system equipped with a Waters 2795 seperation Module (Alliance HT), Waters 2996 Photodiode Array Detector (190-750 nm), Waters Alltima C18 (2.1x100 mm), Waters Symmetry300^{TM} C4 3.5 µM (2.1x100 mm) reversed phase column and LCT^{TM} Orthogonal Acceleration Time of Flight Mass Spectrometer. Samples were run at 0.40 ml/min using 2 mobile phases: A= 0.1% aq. formic acid and B= 0.1% formic acid in CH_3CN. Data processing was performed using Waters MassLynx 4.1 mass spectrometry software.

Preparative HPLC.

Preparative HPLC was performed on a Shimadzu LC-20AD/T equipped with a C4 Vydac column (Grace Davison Discovery Sciences^{TM}). Mobile phases: A= 0.05% aq. TFA and B= 0.05% TFA in CH_3CN. Column T= 20°C. Flow rate= 10.0 mL/min.

Solid phase peptide synthesis.

Peptide synthesis reagents were purchased from Novabiochem. Peptides were synthesized on a 25 or 50 µmol scale using a Syro II MultiSyntech automated peptide synthesizer and standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry. Starting with pre-loaded Fmoc amino acid Wang resin (0.2 mmol/g, Applied Biosystems), each successive amino acid was coupled in 4 molar excess for 45 min. with PyBOP and DiPEA. Deprotection of the Fmoc group was achieved with 20% piperidine in NMP (3×1.2 mL, 2×2 and 1×5 min). Peptides were cleaved with TFA/iPr_3SiH/H_2O (95/2.5/2.5), precipitated in cold n-hexane/diethyl ether and purified by RP-HPLC (C18). All peptides were analyzed by LC-MS.

Determining protein concentration.

To measure the total protein concentration, we used the "Pierce® BCA Protein Assay Kit" obtained from Thermo Scientific (Catalogue number: 23225). This assay allows the colorimetric detection and quantization of total protein using a bicinchoninic acid based reagent. The determination was performed according to the instructions of the manufacturer provided with the kit, with one modification. In place of BSA as a reference we used ubiquitin (obtained from Boston Biochem). Lyophilized Ubiquitin (2mg) was dissolved in deionized water (1 mL) to provide a stock solution from which dilutions were obtained. The colorimetric detection was performed using an Perkin Elmer Wallac Victor2 1420-014 spectrophotometer at 562 nm wave length.

Synthesis Ub_{74}-C4 aldehyde diethyl acetal (A).

A reaction mixture of 3 ml containing 20 mg ubiquitin (Boston Biochem), 25% aq. 4-aminobutyraldehyde diethyl acetal (Fluka) solution and 0.5 mg TPCK-treated trypsin (Worthington Biochemical Corporation, New Jersey) was adjusted to a pH of 7.5 with 2M HCl and shaken at 37ºC for 4 hrs. The reaction was quenched with 10 mg/ml trypsin inhibitor (from soybean, Merck) to a final concentration of 0.5 mg/ml. The reaction mixture was dialyzed against 50 mM NaOAc buffer (pH 4.5) and purified over a Resource S column (Pharmacia) using a gradient of 0 − 1 M NaCl in 50 mM NaOAc (pH 4.5). Eluted fractions containing product (as judged by LC-MS analysis) were pooled and concentrated using a Centriprep column (Amicon Ultra, Ultracel-3K). Using the BCA protein assay described above, the amount of protein in the concentrate was determined to be 6mg in the total volume (30% yield).

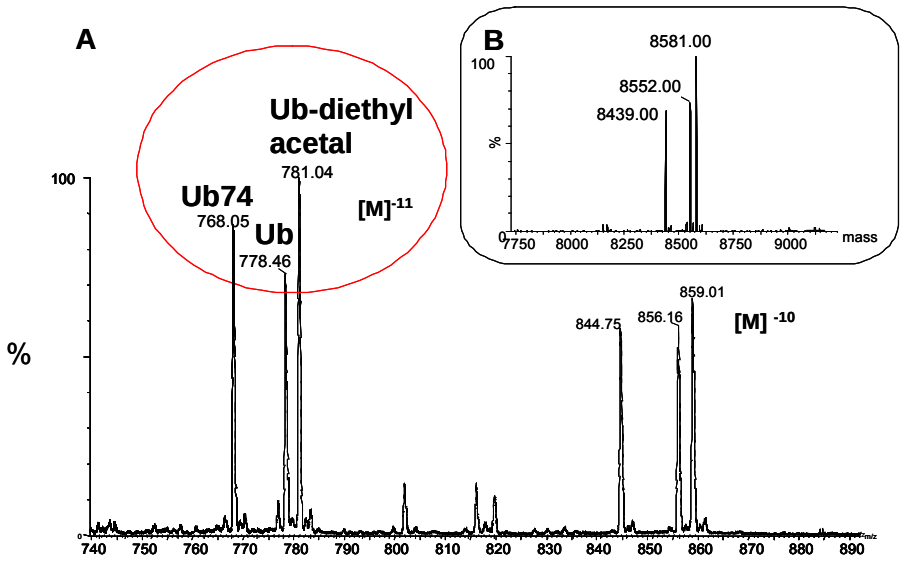

Figure 1. (**A**) Mass spectrum zoomed in at the $(M+12H)^{+12}$ species and (**B**) deconvoluted mass spectrum of normal isotopic abundance corresponding to the reverse trypsinolysis product mixture after 4 hrs.

General protocol for oxime conjugate formation.

1 mL of Ub74-C4 aldehyde diethyl acetal (1 mg/ml) is incubated with 1.5 equiv of the appropriate aminoxy functionalyzed peptide dissolved in 50 µL deionized water. Aqueous HCL was added from a 4 M stock to a final concentration of 0.5 M HCl and the reaction mixture was incubated for 30 minutes at 37ºC. This resulted in *in situ* acetal deprotection and ensuing oxim formation. Full consumption of acetal and aldehyde was observed by LC-MS analysis in all cases. Excess peptide was removed by purification with preparative reversed phase HPLC. Following purification, the

product yield was determined to be in the range of 80-85% of the input Ub74-C4 aldehyde diethyl acetal in all cases as determined with the BCA protein assay kit.

Biotinylation of Ubiquitin.

Ubiquitin (1 mg, Boston Biochem) was mixed with a buffer containing 25 mM Hepes, pH 7.6 and an equivalent amount of EZ-Link-Sulfo-NHS-LC-Biotin (Pierce). The mixture was incubated at r.t. for 2 hours, after which the product was purified by gel filtration using Hepes buffer (25 mM, pH 7.6). Mass spectrometry analysis of the ubiquitin fraction from the column confirmed 50% as mono-biotinylated Ub while the rest remained as free Ub. This fraction was free from traces of unreacted biotin.

DUB expression and purification.

A codon-optimized Usp7 CD (residues 208 to 560) was cloned into the pGEX-6P-1 vector backbone using the BamHI and NotI restriction sites, respectively. Expression was perfomed using BL21(DE3) T1 resistant *E.coli* cells (Sigma). Induction was achieved by autoinduction (reference autoinduction medium) at 16°C using a 16 hour induction time. After centrifugation, cell pellets were resuspended in 50 mM Hepes (pH 7.5), 300 mM NaCl, 1 mM DTT and supplemented with Complete Protease Inhibitor-EDTA free tablets (Roche). Lysis was achieved using a high-pressure homogenizer (Emulsiflex, Avestin). After centrifugation for 30 minutes at 20.000 rpm using a 25.50 rotor (Avanti, Beckman), the supernatant was applied to GST beads (Amersham GE). After extensive washing with 50 mM Hepes pH 7.5, and elution with 50 mM reduced glutathione in Hepes pH 7.5 (Sigma), the eluate was purified by gel filtration on a S75 1660 column with a coupled GST FF column using an Akta system (Amersham GE). This typically yielded 10 mg of pure protein per liter culture. Full length USP7 (residues 1-1102) was a gift from Boston Biochem, Boston, MA, USA.

The *E.coli* host Rosetta2(DE3) was used for the large scale protein expression of His-tagged USP4, USP21 and USP25. 5 mL of an overnight pre-culture was used to inoculate 500 mL autoinduction medium in 3 L baffled flasks and grown at 37°C until an OD_{600} of 2-3 units was reached. The temperature was then lowered to 21°C for overnight induction. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol and 1 mM PMSF. The cells were broken by subjecting the cell suspension to a 10 second sonification pulse with a pause of 30 seconds after each pulse for a total of 5 minutes using the Misonics sonicator S-4000 at 80% maximum setting. The lysate was

centrifuged at 20k for 30 minutes at 4°C to remove cellular debris and unbroken cells. The resulting supernatant was incubated with washed Talon metal affinity resin (Clontech, Inc., Palo Alto, CA) for 20 minutes at 4°C and the beads were then washed with lysis buffer. Protein was eluted with lysis buffer containing 400 mM imidazole. This step was followed by cation exchange for USP25 and by anion exchange step for USP4 and USP21. USP25 was diluted 10-15 times with 50 mM BisTris pH 6.5 followed by cation exchange chromatograpy purification using an Akta FPLC system (GE Healthcare). The diluted sample was applied to a Poros S column equilibrated with buffer A (20 mM BisTris pH 6.5, 10 mM NaCl and 5 mM β-mercaptoethanol). The bound protein was eluted with buffer A containing 1 M NaCl using a 60% gradient in 20 column volumes. USP4 and USP21 were applied to a PorosQ column equilibrated with buffer B (20mM Hepes pH7.5, 100mM NaCl and 5mM β-mercaptoethanol). The bound protein was eluted with buffer B containing 1M NaCl using a 60% gradient in 20 column volumes.

After ion exchange gelfiltration was applied. Peak fractions were pooled and concentrated by ultrafiltration using an Amicon Ultra centrifugal unit (Millipore) and then applied to a Superdex 200 (GE Healthcare) gelfiltration column equilibrated with 25 mM Tris-HCl (pH 8.0), 150 mM NaCl and 5 mM β-mercaptoethanol. Peak fractions from the gelfiltration column were pooled and concentrated in an Amicon Ultra unit to 10 mg/ mL. The concentrated protein was flash frozen in liquid nitrogen and stored at −80°C.

USP2a CD (residues 259-605) was a gift from Life Sensors, Malvern, PA, USA.

DUB binding assay.

Binding of DUBs to Ub isospeptide isosteres was assessed with a Biacore T100 apparatus. Biotinylated Ub and Ub isopeptide isosteres were immobilized separately (to 50 RU) onto streptavidin-coated Biacore sensor chips (SA chips) by resuspending them in 10 mM Hepes,100 mM NaCl, 2 mM β-mercaptoethanol, pH 7.5. A streptavidin-coated sensor surface without ligand was used as a control surface in order to subtract unspecific binding. Binding experiments were performed at a flow rate of 30 µL/min. The DUBs were applied in concentrations ranging from 100 nM to 25 µM at room temperature. After each binding event, surfaces were regenerated by a short stripping pulse of 50 mM NaOH. Evaluation of the binding data was performed by steady-state analysis plotting saturated binding vs the respective analyte concentration. K_d values

were calculated using Prism (GraphPad Software, Inc., San Diego, CA, USA) employing non-linear regression analysis.

DUB activity assays.

2 nM HAUSP (Boston Biochem) or 10 nM USP16 were used to cleave 30 nM Ub-PLA2 (1) (a gift from Progenra, Malvern, USA) in a reaction with or without 150 nM monoUb/Ub-isopeptide isostere. Proteolytic cleavage of Ub results in the release of PLA2 which in turn cleaves its substrate 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (NBD-C6-HPC, Molecular Probes, Leiden, the Netherlands), 20 µM, liberating fluorescent NBD(2). Reactions were carried out in a volume of 100 µl in black-walled 96 well plates (Optiplate-96F, Perkin-Elmer) in 20 mM Tris-HCl, pH 8.0, 2 mM CaCl2 and 2 mM β-mercaptoethanol. The increase in fluorescence intensity over time was determined using a Wallac Victor2 (Perkin-Elmer) plate reader with excitation and emission filters of 475 nm and 555 nm,respectively, at 37 ºC.

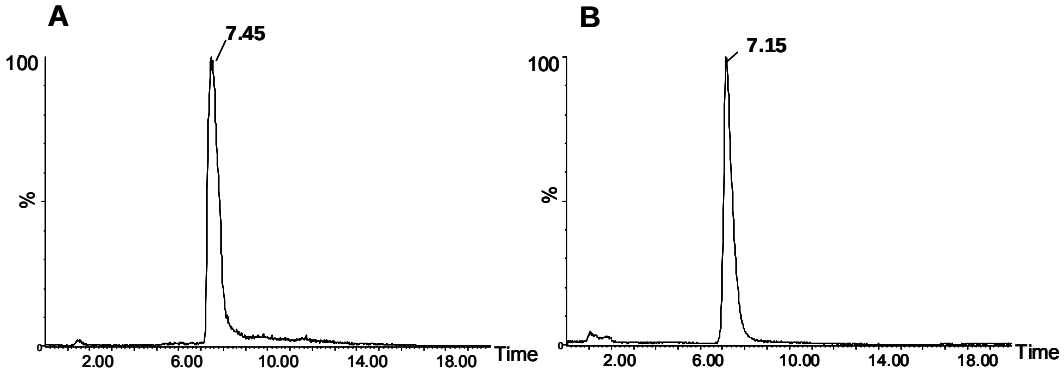

Figure 2. HPLC profile of (**A**) UbK48- and (**B**) UbK63-isopeptide isosteres. The 230 nm absorption profile of purified conjugates is shown, analyzed by reversed phase HPLC analysis using a C4 column. A single major product peak eluting around 7 min. corresponds to the desired product.

In order to test hydrolytic stability of Ub isopeptide isostere towards cleavage by a DUBs, conjugates were analyzed after cleavage reactions. The effect of 40 nM of USP2a CD on 15 µM Ub-K48 isopeptide isostere was compared with native 15 µM K48 linked di-Ub. USP2a CD is known to cleave K48 linked poly Ub chains (2). Following

(1) Nicholson, B.; Leach, C.A.; Goldenberg, S. J.; Francis, D. M.; Kodrasov, M. P.; Tian, X.; Shanks, J.; Sterner, D. E.; Bernal, A.; Mattern, M. R.;, Wilkinson, K. D.; Butt, T. R. *Protein Sci.* **2008**, *17*, 1035.

(2) Komander, D.; Reyes-Turcu, F.; Licchesi, J. D.; Odenwaelder, P.; Wilkinson, K. D.; Barford, D. *EMBO Rep.* **2009**, *10*, 466.

incubation in assay buffer for 2 hrs, the assay contents were analyzed on a 12% Bis-Tris gel (NuPage, Invitrogen), next to a molecular weight marker (SeaBlue plus2, Invitrogen). Results are shown in Figure 3, native K48 linked diUb was cleaved by USP2a CD to a significant extent whereas the Ub-K48 isopeptide isostere remained stable evidencing resistance to DUB-mediated hydrolysis.

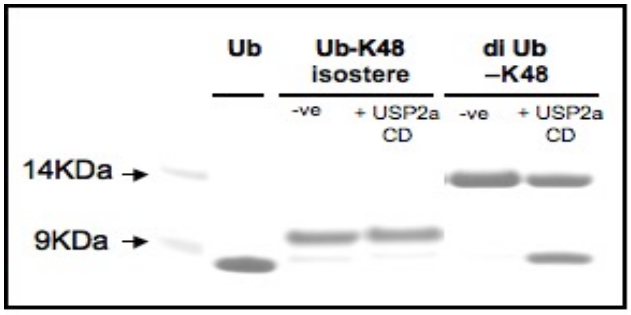

Figure 3. Ub-K48 isopeptide isostere resists hydrolysis by USP2a CD which can cleave K48 linked di-Ub.

We tested if the Ub isopeptide isosteres can specifically inhibit DUB action side by side with Ub as Ub is known to inhibit the activity of several DUBs (3). The effect on the activity of the DUBs USP7/HAUSP and USP16 were compared upon treatment with monoUb and the linkage specific isostere using the *in vitro* Ub DUB reporter assay. As can be seen in Figure 4, the non-hydrolyzable UbK48 isopeptide isostere inhibits USP7/HAUSP potently compared to the Ub control confirming the specific interaction of USP7/HAUSP with the peptide sequence flanking the UbK48 site. In contrast, incubation with USP16 gave the opposite effect: Ub itself can inhibit USP16.

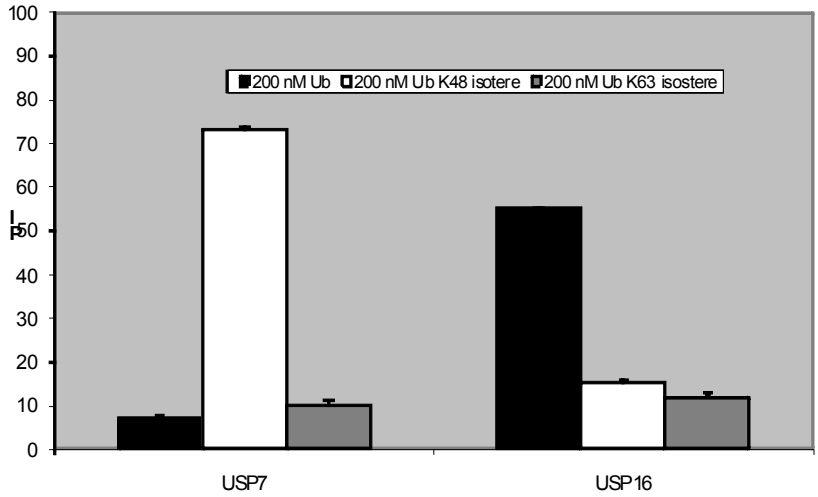

Figure 4. Specific inhibition of DUBs (USP7 and USP16) by Ub isopeptide isosteres.

(3) Yin, L.; Krantz, B.; Russell, N. S.; Deshpande, S.; Wilkinson, K. D. *Biochemistry* **2000**, *39*, 10001.

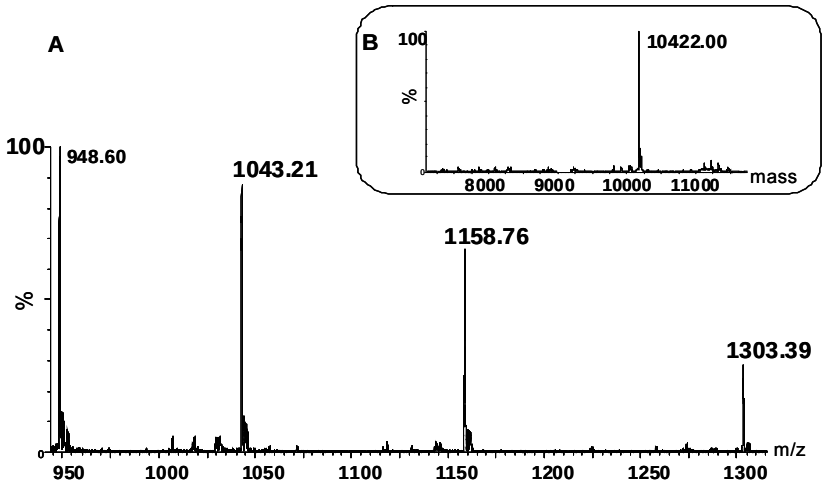

Figure 5. (**A**) Mass spectrum and (**B**) deconvoluted mass spectrum of normal isotopic abundance corresponding to a Ub-K561 FANCD2 isopeptide isostere.

Determination of Kds for DUBs towards Ub and Ub-isosteres

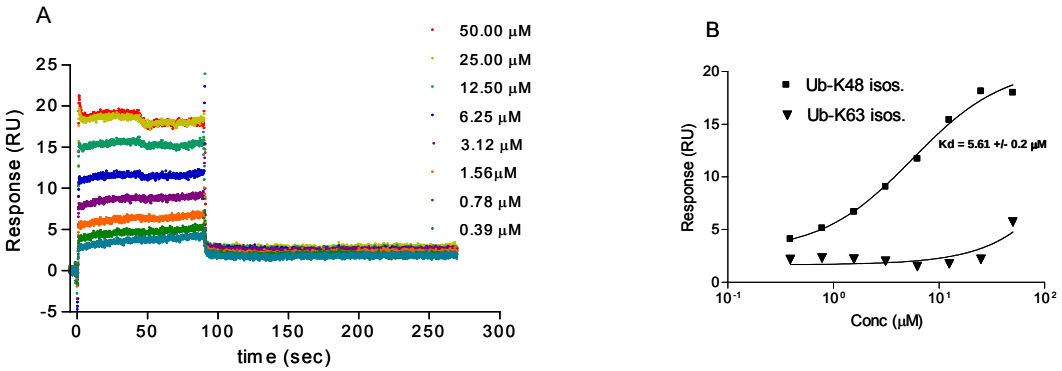

Figure 6. Binding of USP7 CD to Ub isopeptide isosteres. SPR response curves for the binding of USP7 CD (concentrations ranging from 0.39 to 50 µM, bottom curve to top curve respectively) to **(A)** biotinlylated Ub-K48 isopeptide isostere, no interaction was observed for the K63 isostere **(B)** The maximum RUs for every curve is plotted against the corresponding concentrations of USP7 CD used for binding measurements. Employing non-linear regression analysis on the plotted curves, the K_Ds for binding of USP7 CD to respective Ub isopeptide isosteres are calculated.

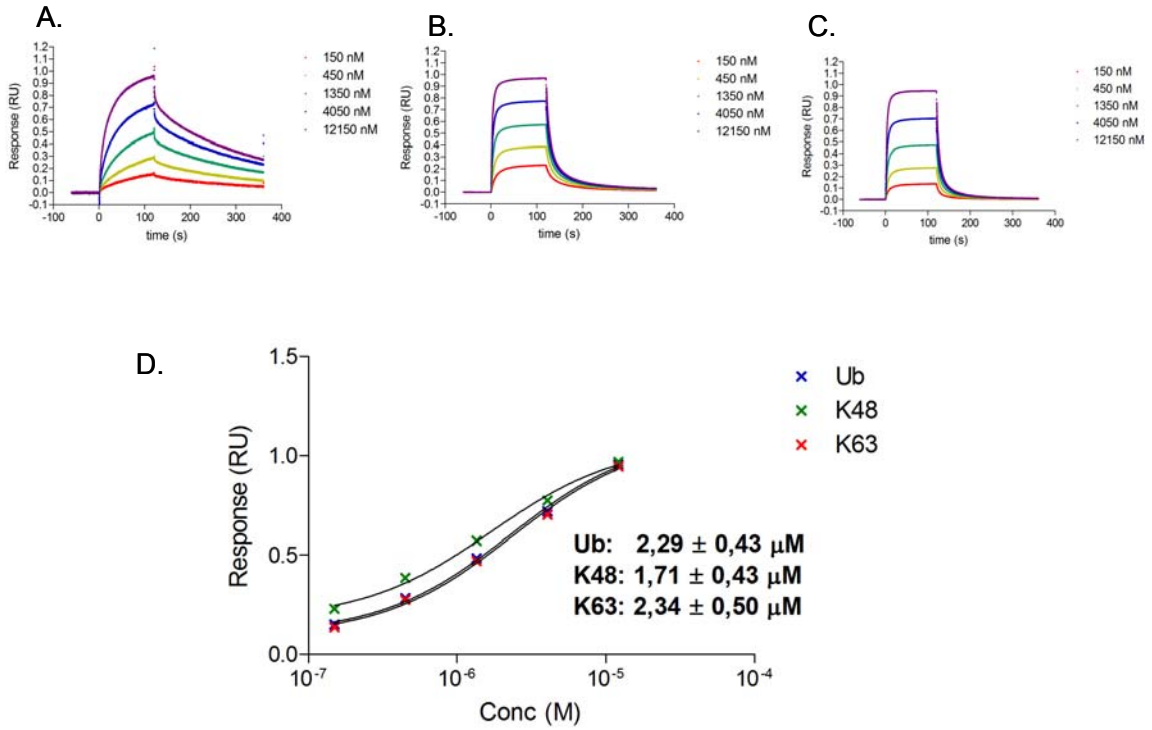

Figure 7. Binding of USP4 CD to Ub isopeptide isosteres. SPR response curves for the binding of USP4 CD (concentrations ranging from 0.15 to 12.15 µM, bottom curve to top curve respectively) to **(A)** biotinlated Ub, **(B)** biotinlylated Ub-K48 isopeptide isostere and **(C)** biotinylated Ub-K63 isopeptide isostere, all immobilized to a SA chip on separate lanes. (**D**) The maximum RUs for every curve in figure A, B and C are plotted against the corresponding concentrations of USP4 CD used for binding measurements. Employing non-linear regression analysis on the plotted curves in D, the K_Ds for binding of USP4 CD to Ub and Ub isopeptide isosteres are calculated.

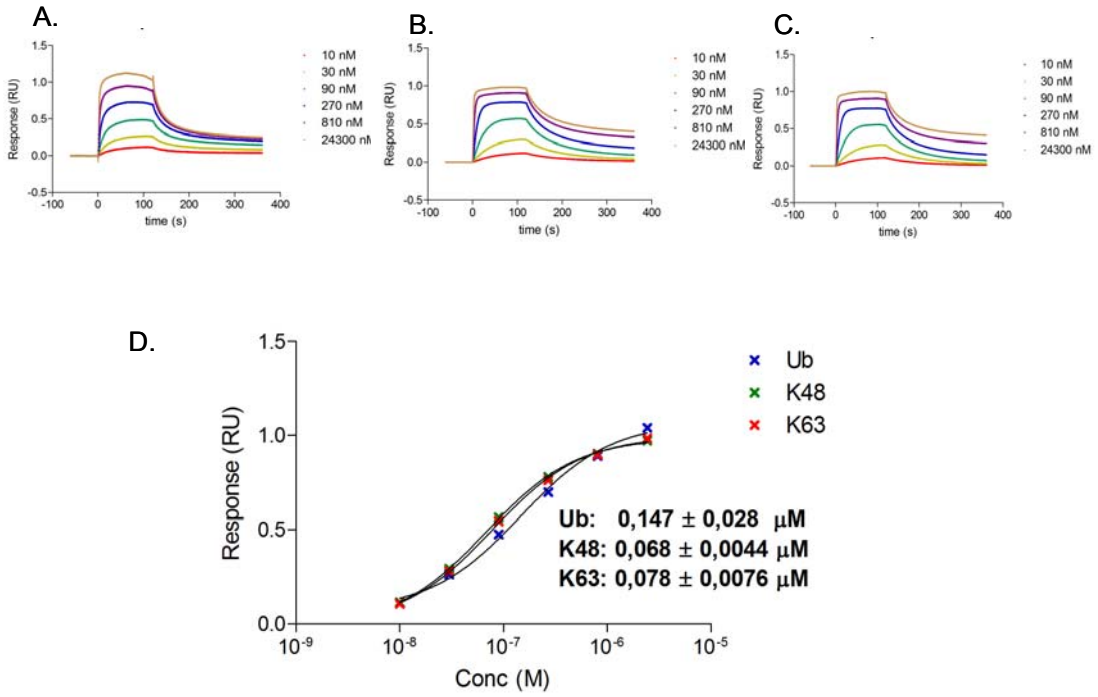

Figure 8. Binding of USP21 CD to Ub isopeptide isosteres. SPR response curves for the binding of USP4 CD (concentrations ranging from 0.01 to 24.3 µM, bottom curve to top curve respectively) to **(A)** biotinlated Ub, **(B)** biotinlylated Ub-K48 isopeptide isostere and **(C)** biotinylated Ub-K63 isopeptide isostere, all immobilized to a SA chip on separate lanes. (**D**) The maximum RUs for every curve in figure A, B and C are plotted against the corresponding concentrations of USP21 CD used for binding measurements. Employing non-linear regression analysis on the plotted curves in D, the Kds for binding of USP21 CD to Ub and Ub isopeptide isosteres are calculated.

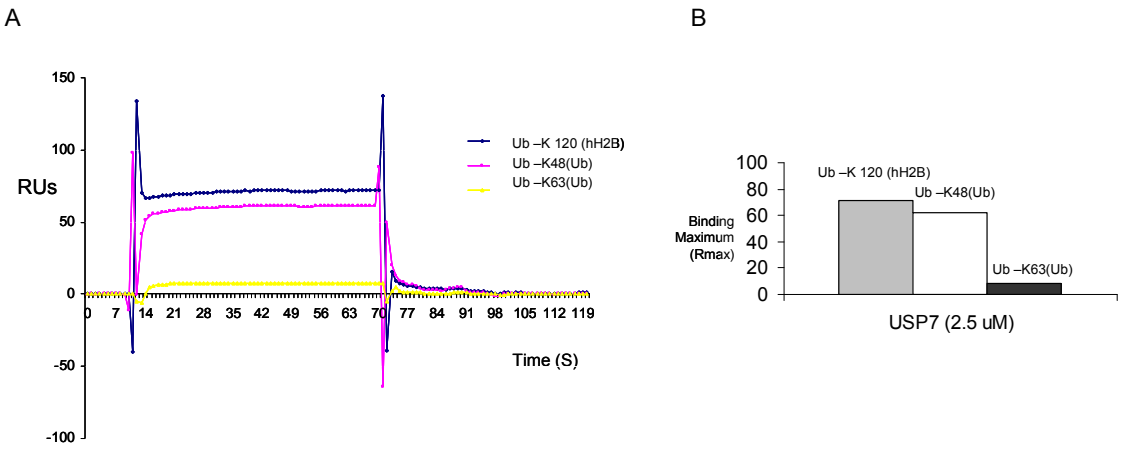

Figure 9. Binding of Ub isopeptide isosteres to USP7. **(A)** Three different Ub isosteres, UbH2B (K120), UbK48Ub and UbK63Ub, are immobilized on three separate lane on a biacore (CM5) chip surface. Binding of 2.5 μM to the three immobilites over a period of 60 s are measured in parallel by SPR in a Biacore T-100 apparatus. The maximum binding response units (Rmax) in each case is substracted from the Rmax in the control surface (lane 4). **(B)** The maximum binding of enzyme ligand is compared: USP7 binds well to both the UbH2B (K120) and the UbK48Ub, but not to the UbK63Ub isostere.