Electronic Supplementary Material (ESI) for Chemical Science.

Supporting information

Development of a DUB-selective fluorogenic substrate

Lorina Gjonaj,[a] Aysegul Sapmaz,[a] Dennis Flierman,[a§] George M. C. Janssen,[b] Peter A. van Veelen[b] and Huib Ovaa*[a]

[a] Oncode Institute and Department of Cell and Chemical Biology, Leiden University Medical Center, Einthovenweg 20, 2333 ZC, Leiden, The Netherlands.

[b.] Centre for Proteomics and Metabolomics, Leiden University Medical Center, Albinusdreef 2, 2333 ZA, Leiden, The Netherlands.

[§.] Current address: Pepscan, Zuidersluisweg 2, 8243 RC, Lelystad, The Netherlands.

Experimental section

General information:

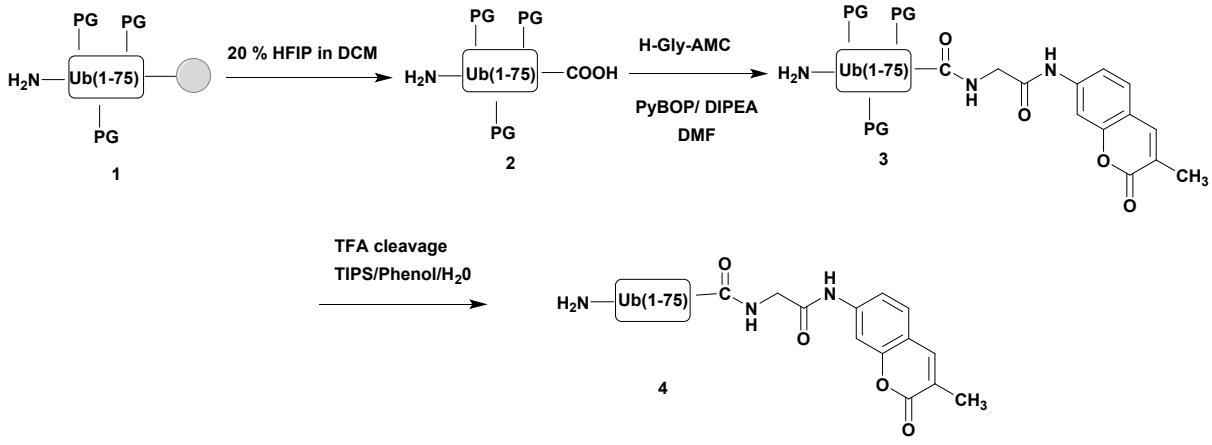
All solvents were HPLC grade or reagent grade. Chemicals (Aldrich, Fluka, Novabiochem) were used without further purification. LC-MS analysis were done with a system containing a Waters 2795 separation module (Alliance HT), Waters 2996 Photodiode Array Detector (190-750 nm), Phenomenex Kinetex XBridge-C18 (2.1 x 50 mm) reversed phase column and a Micromass LCT-TOF mass spectrometer. Samples were run at a flow rate of 0.80 mL/min (Kinetex C18) using a gradient of two mobile phases: A) aqueous formic acid (0.1 %), and B) formic acid in acetonitrile (CH_3CN) (0.1 %). Data processing was performed with Waters MassLynx 4.1 software (deconvolution with MaxEnt1 function). Preparative HPLC was performed with a Waters Auto-purifier System connected to a 3100-mass detector. The purification method was performed using Waters XBridge Prep C18 (19 x 150 mm, 5 µm OBD) column, run at a flow rate of 30 mL/min for 23 min using a gradient elution procedure [mobile phases: A) aqueous TFA (0.05 %) and B) TFA in CH_3CN (0.05 %)]. Gradient used: 18 % B for 2.5 min; 18 % B to 48 % B in 15 mins; washed at 95 % B for 2 mins; re-equilibrated back to 18 % B.

Softwares used during this research: FoldX: http://foldxsuite.crg.eu/, UCSF Chimera https://www.cgl..edu/chimera/, and Swiss-Model https://swissmodel.expasy.org/

Synthetic protocols:

Rh-ubiquitin ABP mutants were synthesized in parallel in a Fmoc-based solid phase peptide synthesis (SPPS) by following reported procedures[1].

Biotin-PEG-Ub-PA (Biotin-Ub-PA) was synthesized according to a previously reported procedure [2] : To the protected ubiquitin (1-75) (2 µmol) on trityl resin, 200 µL of a mixture in NMP consisting of PyBOP (5.2 mg, 5.0 eq), 2-[2-(Fmoc-amino)ethoxy]ethoxy}acetic acid (3.85 mg, 5.0 eq) and DIPEA (3.5 µL 10.0 eq) were added and allowed to react for 2h. This procedure was repeated twice. After overnight coupling, the resins were washed with 2 x NMP. The Fmoc protecting group was removed using 200 µL of 20 % piperidine/NMP and the resin was washed with NMP. This procedure was repeated three times, and the resin was washed thoroughly with NMP. Biotin was coupled to the PEG-modified Ub by adding a pre-activated mix containing PyBOP (5.2 mg, 5.0 eq), biotin (2.44 mg, 5.0 eq) and DIPEA (3.5 µL 10.0 eq) in 200 uL of NMP. After overnight coupling, the resins were washed twice with NMP and then four times with DCM. The modification of the C-terminus of Bio-PEG-Ub with propargylamine followed by global deprotection was done according to a previously reported procedure[1].



Scheme 1. Schematic overview of the synthesis of AMC reagents (PG=Protecting groups)

We followed a procedure modified from a previous publication for the synthesis of Ub-AMC and M20-AMC[3] (Scheme 1): Fully protected Ub (**1**) (2.0 µmol) was cleaved from the resin by adding a solution of 200 µL of 20 % HFIP in DCM. After 30 min, the flow-through was collected. The procedure was repeated twice. The flow-through was dried under N_2 flow and then freeze-dried using a mixture of CH_3CN/H_2O (1:1) overnight. The crude protected Ub (**2**) with a free C-terminus was dissolved in 100 µL DMF. To this mixture, we added 100 µL of a freshly prepared solution of PyBOP (5.2 mg, 5.0 eq), H-Gly-AMC (1.16 mg, 5.0 eq) and DIPEA (3.5 µL, 10.0 eq) and allowed to react overnight. Afterwards we added a mixture of CH_3CN/H_2O (1:1) to the reaction mix, prior to freeze-drying. To the crude material (**3**) was added 400 µL of freshly prepared cleaving mixture (TFA/TiPS/Phenol/H_2O; 92.5/2.5/2.5/2.5; v/v/v/v). After 3 hours, to the reaction mixture was added 1.6 mL of a dry-ice cold mixture of Et_2O/Pentane (3/1). The mixture was centrifuged at 1000 rpm for 7 min. The pellet was washed twice with 1.6 mL Et_2O, dried using N_2, and freeze-dried overnight. The desired AMC reagents (**4**) were purified using preparative HPLC.

Generating Ub-USP models

The new models of USP15 and USP16) (not taking in consideration the unstructured regions), generated via Swiss-Model, were aligned with USP7-Ub complex (PDB ID: 1NBF) using UCSF Chimera. The new pdbs (USP15-Ub and USP16-Ub) were adjusted using repairpdb, a function of FoldX (see below).

Computational predictions with FoldX

First the USP-Ub structures needed to be adjusted using the repairpdb function in order to obtain the optimal structure with the lowest energy. Mutational analysis protocol was performed next (each amino acids in the ubiquitin sequence was mutated towards 20 genetically encoded amino acids) and the newly generated binding energies were subtracted from the wild-type reference energies. From these data, we selected the residues which projected a positive effect by increasing the binding of Ub towards USP16, compared to binding of Ub with other evaluated USPs (USP7 and USP15).

Cell culture

HAP1 cells, USP7KO HAP1 (obtained from Horizon Genomics HAP1_USP7_00485-02) and UPS16KO HAP1 (obtained from Horizon Genomics HAP1_USP16_ HZGHC003566c010) were cultured in IMDM (Gibco) supplemented with 10% FCS and 1% Pen/Strep (FCS, Greiner). HeLa cells were cultured in DMEM (Gibco) supplemented with 10% FCS. Cells were cultured at 37°C and 5 % CO_2.

Labelling experiments

HAP1 cells were lysed in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 0.5 % Triton X-100, 2 mM DTT. HAP1 lysate (10 µL) was incubated for 30 min with 0.5 µM of the Ub probes. Samples were prepared in a sample buffer containing β-mercaptoethanol, boiled and separated by 4-12 % SDS-PAGE gel electrophoresis (Invitrogen, NP0321) in MOPS buffer (Invitrogen, NP0001,). Typhoon FLA 9500 (GE Healthcare LifeSciences) was used for fluorescence scanning using filters with an excitation wavelength of 473 nm and an emission wavelength of 532 nm.

Transfection protocol

$1x10^6$ HeLa cells were seeded in 10 cm dish (664160, Cellstar) for each sample. The following day we transfected the cells using Effectene Transfection Reagent (Qiagen, 301427). First, to 600 µL EC buffer we added the DNA (1.5 µg in MilliQ water) followed by 24 µL Enhancer. The mixture was incubated for 5 min. 30 µL Effectene was added to the DNA-Enhancer mixture. After mixing and 15 min incubation, we added the mixture dropwise to the cells. The next day, after removing the medium and washing with 1X PBS buffer, 400 µL lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5 % Triton X-100) was added to the cells. After scrapping the cells with lysis buffer,

the cells were further lysed by sonication for 10 min (alternation of 30 sec on and off) using Bioruptor Pico Sonication System and centrifuged for 30 min at 4 °C at maximum speed.

The plasmids were obtained fromAddgene: pCI-neo Flag HAUSP (16655); Flag-HA-USP5 (22590); Flag-HA-USP15 (22570) and Flag-HA-USP16 (22595). USP7 and USP15[4] were subcloned into Flag-C1 vector. Forward and reverse site-directed mutagenesis primers that contain the desired mutations were designed. Site directed mutagenesis PCR and DpnI digestion were performed as describe previously[5]. The mutated DNA was transformed into DH5α using LB medium. All of the obtained plasmids were verified by sequencing.

Western-blotting

Samples were transferred to a nitrocellulose membrane from Trans-Blot Turbo Mini Nitrocellulose Transfer Packs (Bio-Rad, 1704158) using a 7 min program for different molecular weight proteins (Trans-Blot Turbo Transfer System from Bio-Rad). 5 % BSA in Tris buffer pH 7.5 (50 mM Tris, 150 mM NaCl) was used for the blocking of the membranes. The membrane was incubated with a primary antibody solution in 1 % BSA in 0.1 % Tris buffer -Tween 20 (TBST) for 1 h, followed by three-times washing for 5 min in 0.1 % TBST. Afterwards, the membrane was incubated with the secondary antibody diluted in 1 % BSA in 0.1 % TBST for 30 min, then washed three times for 5 min in 0.1 % TBST. β-Actin antibody (Sigma-Aldrich, A5441) was used as a loading control in a 1:10000 dilution and USP16 antibody (Bethyl Laboratories, A301-614A) was used at a dilution of 1:1000. As secondary antibodies, we used from IRDye 680LT goat anti-mouse IgG (H+L) (926-68020, Li-COR) and IRDye 800LT goat anti-rabbit IgG (H+L) (926-32211, Li-COR). NeutrAvidin Protein, DyLight 680 (from Thermo Scientific, 22848, dilution 1:20000) was used to determine the levels of biotin. The signal of the secondary antibodies was determined using direct imaging by the Odyssey Classic imager (Li-Cor). VPA00705, A301-614A and sc-390683 are the USP16 antibodies used in this work.

Pull-down experiment

$1x10^6$ HAP1 WT or USP16 KO HAP1 cells were seeded in 10 cm dish for each condition in triplicate. After 48 h, the cells were lysed in lysis buffer containing 50 mM Tris pH=7.5, 150 mM NaCl, and 0.5 % Triton X-100. HAP1 WT lysate was incubated with either Biotin-Ub-PA or no probe and USP16 KO HAP1 lysate was incubated with Biotin-Ub-PA for 30 min at 30°C, slowly shaking. Before the addition of the beads (Thermo Fischer Scientific, 29204,washed multiple times in lysis buffer), NMM 50 mM final concentration (N-methylmaleimide dissolved in DMSO, a cysteine protease inhibitor) was added to the samples to inhibit protease activity. 50 µL of the 50 % aqueous slurry was added to the lysates and they were incubated at 4°C for 2.5 h. The beads were collected with centrifugation at 1000 rpm at 4°C followed by thorough washing with lysis buffer. Then, the beads were washed in stringent conditions (twice with 0.1% SDS, twice with 0.1% SDS and 500 mM NaCl, twice with 8 M Urea). In the end, they were washed multiple times with lysis buffer. 10 % of the final beads were boiled at 100°C in loading buffer and then analysed by gel electrophoresis, followed by western blotting. The rest of the sample was analysed by gel electrophoresis and stained using Silver Staining (Thermo Fischer, 24612). The desired gel section was further analysed by LC-MS/MS.

Pull-down experiment with Rh-M20-PA

The experiments were performed with Rh-M20-PA probe as described above in triplicate for each condition. HAP1 WT lysates were incubated with either Biotin-Ub-PA probe or no probe (Control). Pull-down experiment were performed as indicated above. After the pull-down, 10 % of the final beads were boiled at 100°C in loading buffer and then analysed by gel electrophoresis, followed by western blotting. The rest of the sample was

analysed by gel electrophoresis and stained using Silver Staining (Thermo Fischer, 24612). The desired gel section was further analysed by LC-MS/MS.

Identification of proteins using LC-MS/MS

For MS analysis, gel slices were subjected to reduction with dithiothreitol, alkylation with iodoacetamide and in-gel trypsin digestion using a Proteineer DP digestion robot (Bruker). Tryptic peptides were extracted from the gel slices, lyophilized, dissolved in 95/3/0.1 v/v/v water/acetonitrile/formic acid and subsequently analysed by on-line C18 nano-HPLC MS/MS with a system consisting of an Easy nLC 1000 gradient HPLC system (Thermo, Bremen, Germany), and a LUMOS mass spectrometer (Thermo). Samples were injected onto a homemade precolumn (15 mm x 100 µm; Reprosil-Pur C18-AQ 3 µm, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (30 cm × 50 µm; Reprosil-Pur C18-AQ 3 µm). The gradient was run from 10% to 40% solvent B (20/80/0.1 water/acetonitrile/formic acid (FA) v/v) in 30 min. The nano-HPLC column was drawn to a tip of ∼5 µm and acted as the electrospray needle of the MS source. The LUMOS mass spectrometer was operated in data-dependent MS/MS mode for a cycle time of 3 seconds, with a HCD collision energy at 32 V and recording of the MS2 spectrum in the Orbitrap. In the master scan (MS1) the resolution was 120,000, the scan range 400-1500, at an AGC target of 400,000 @maximum fill time of 50 ms. Dynamic exclusion after n=1 with exclusion duration of 10 s. Charge states 2-5 were included. For MS2 precursors were isolated with the quadrupole with an isolation width of 1.2 Da. First mass was set to 110 Da. The MS2 scan resolution was 30,000 with an AGC target of 50,000 @maximum fill time of 60 ms.

In a post-analysis process, raw data were first converted to peak lists using Proteome Discoverer version 2.2 (Thermo Electron) and then submitted to the UniProt Homo sapiens database (67911 entries), using Mascot version 2.2.04 (www.matrixscience.com) for protein identification. Mascot searches were with 10 ppm and 0.02 Da deviation for precursor and fragment mass, respectively, and trypsin as enzyme. Up to two missed cleavages were allowed, and methionine oxidation was set as a variable modification; carbamidomethyl on Cys was set as a fixed modification. Protein was finally sorted and compared using Scaffold software version 4.8.4 (www. proteomesoftware.com).

Fluorescent activity assay

The purified DUBs or lysate were diluted in 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM cysteine, 0.5 mg/mL BGG (γ-Globulins from bovine blood from Sigma), 1 mg/ml CHAPS (Sigma). Activity-assays were done in 384-well plates (low volume, flat bottom, non-binding surface, black polystyrene, Corning). Each experiment was performed in duplicates. To 10 µL enzyme/lysate solution in a 384-well plate was added 10 µL of AMC substrate, spun down for 1 min at 1000 g. Afterwards, the hydrolysis of the substrate was measured every 30 sec using a Clariostar plate reader (BMG Labtechnologies) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. All experiments were performed simultaneously. Recombinant USP7FL and USP16FL were expressed and purified by Robbert Kim and Patrick Celie, respectively. Recombinant USP5 was obtained from Ubiquigent (64-0002-050) and USP15 from Enzo Life Sciences (BML-UW9845-0100)

Final enzyme concentration for the activity assay with purified DUBs: USP7 (1.25 nM), USP5 (2.55 nM), USP15 (5.0 nM) and USP16 (1.05 nM). Final concentration of the substrate (Ub-AMC or M20-AMC) was 200 nM. Lysates incubated with Ub-AMC were diluted 1/1000 prior to the measurement in the previously described buffer. Lysates incubated with M20-AMC were used without further dilution.

Kinetic measurements

In order to perform the kinetic measurement, Echo acoustic liquid handling technology (Labcyte) was used for an accurate dispensing of the AMC substrates in DMSO (Ub-AMC and M20-AMC). The substrate was diluted in 10 µL buffer (see above). After shaking for 30 sec, 10 µL of USP16, USP7 and USP15 (1 nM, 5nM and 0.53 nM

final concentrations of USP7, USP15 and USP16 were 1nM, 5nM and 0.53 nM, respectively) was added. Before starting the measurement, the plate was spun down for 1 min at 1000 g. The final concentration of the substrate was between 0.078-18.52 µM. The initial rates of the substrate hydrolysis were determined (based on the first 10 min of the measurements using GraphPad Prism 8.0.1) at different substrate concentration [S]. These data were fitted using nonlinear regression fit for enzyme kinetics. Michaelis-Menten equation $V = (V_{max} \times [S])/([S] + K_M)$ was used to determine V_{max}, K_M, and k_{cat}

V_{max} = maximal rate at saturating substrate concentrations

K_M = Michaelis constant

$k_{cat} = V_{max}/[E]_O$ $[E]_O$ is the enzyme concentration

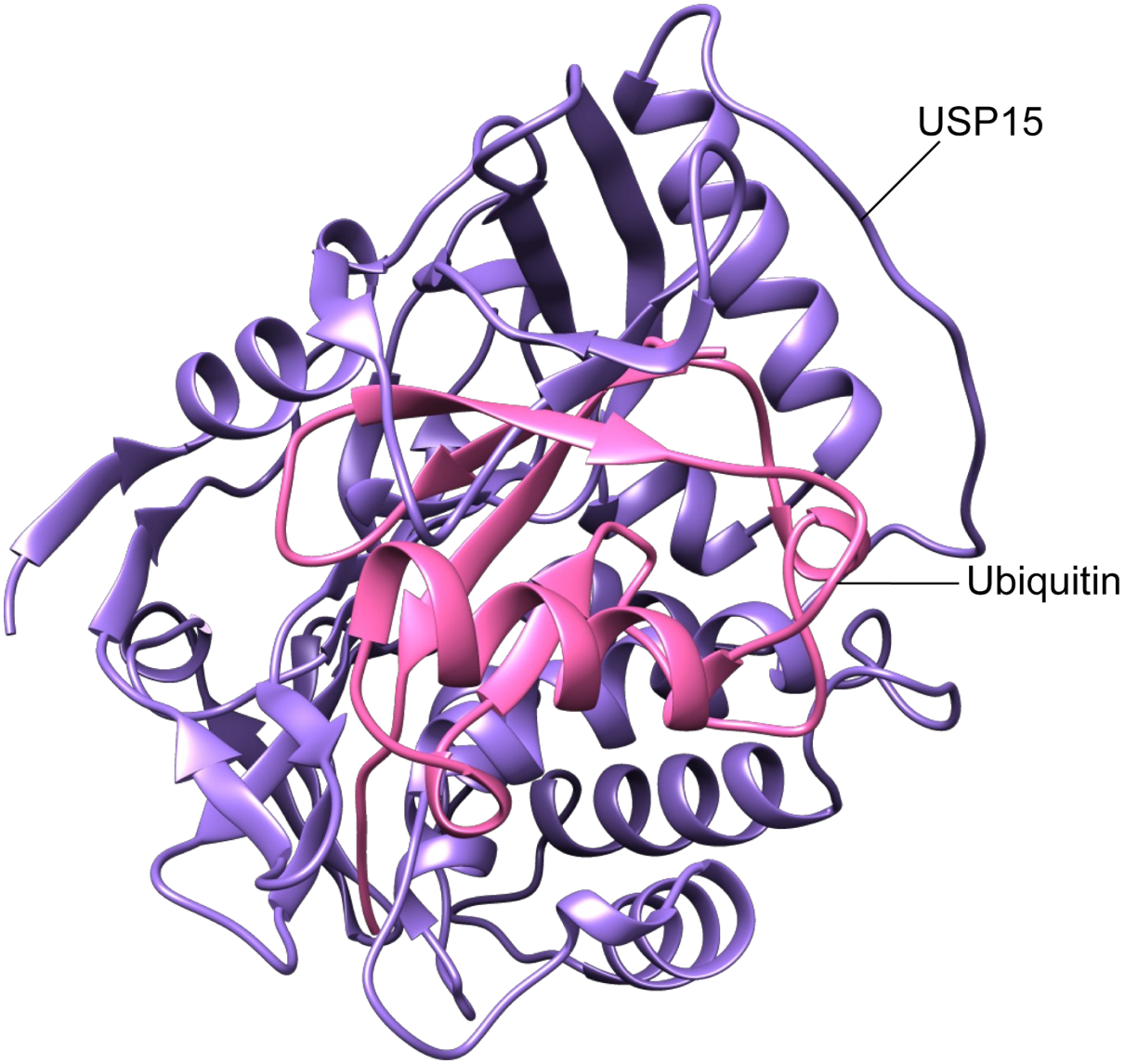


Fig. S1 Model of USP15 (purple) in complex with ubiquitin (pink).

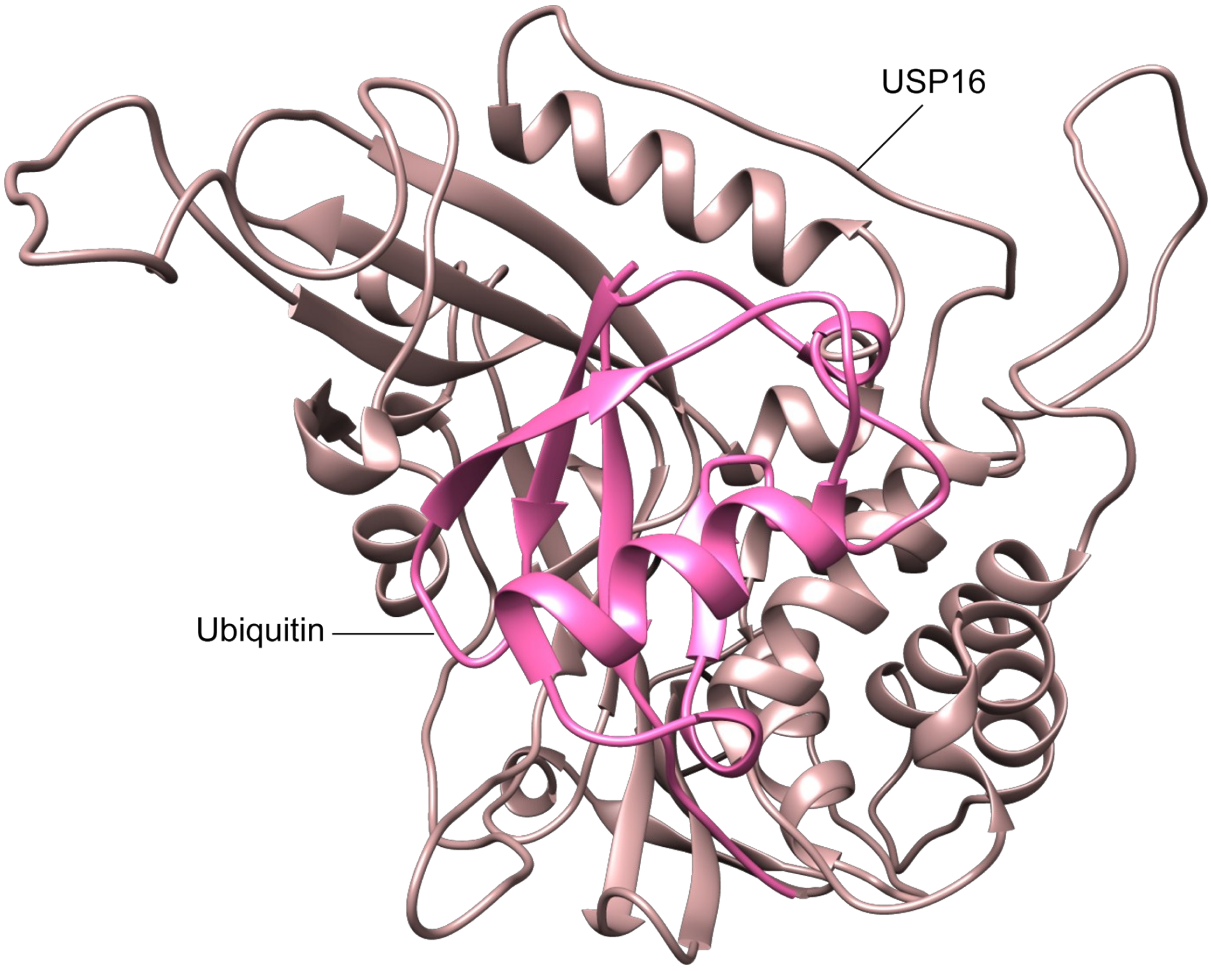


Fig. S2 Model of USP16 (light brown) in complex with ubiquitin (pink).

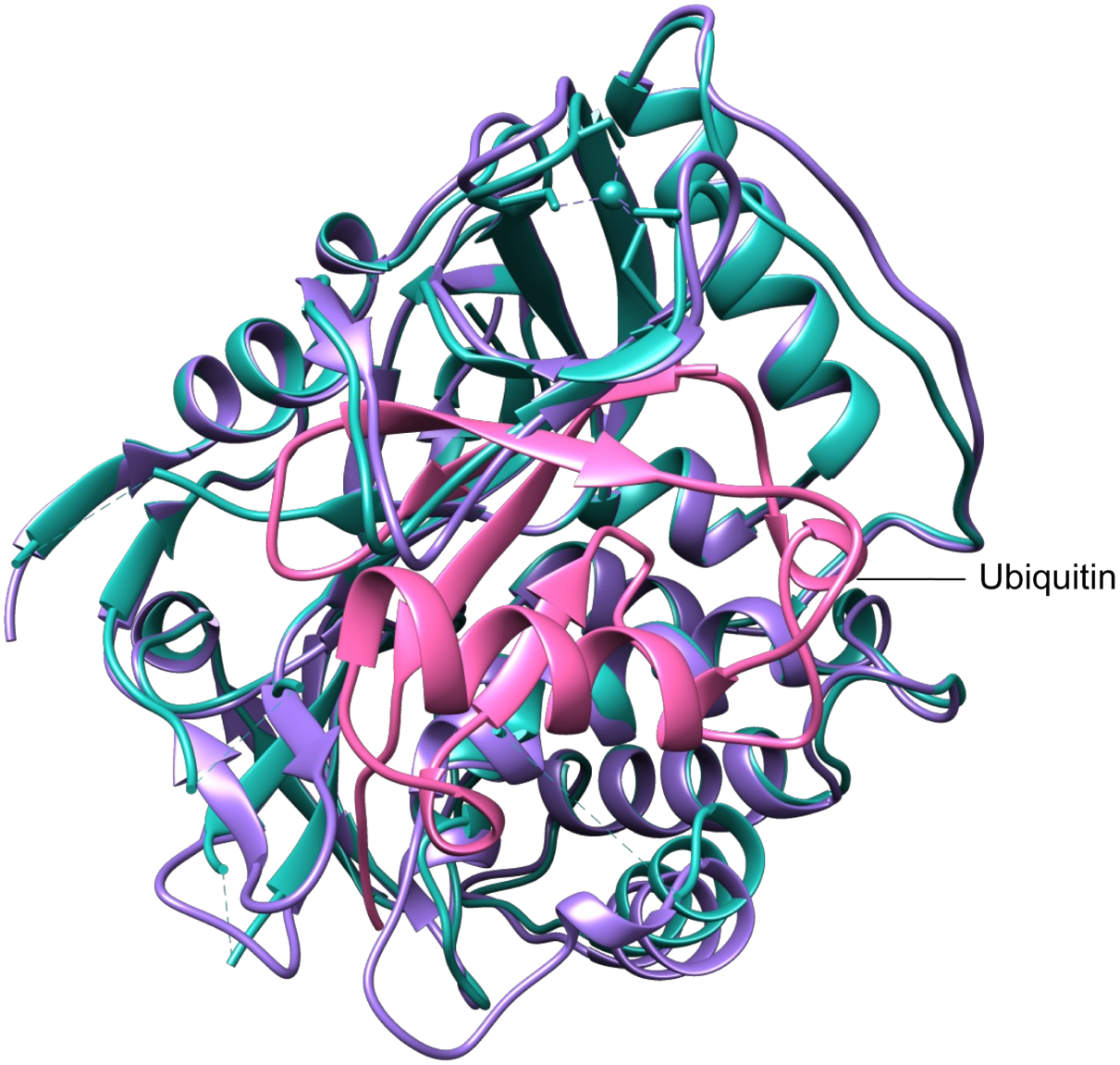


Fig. S3 Superimposition of USP15 model (purple) with the reported crystal structure (PDB ID: 6GHA, green)[6].

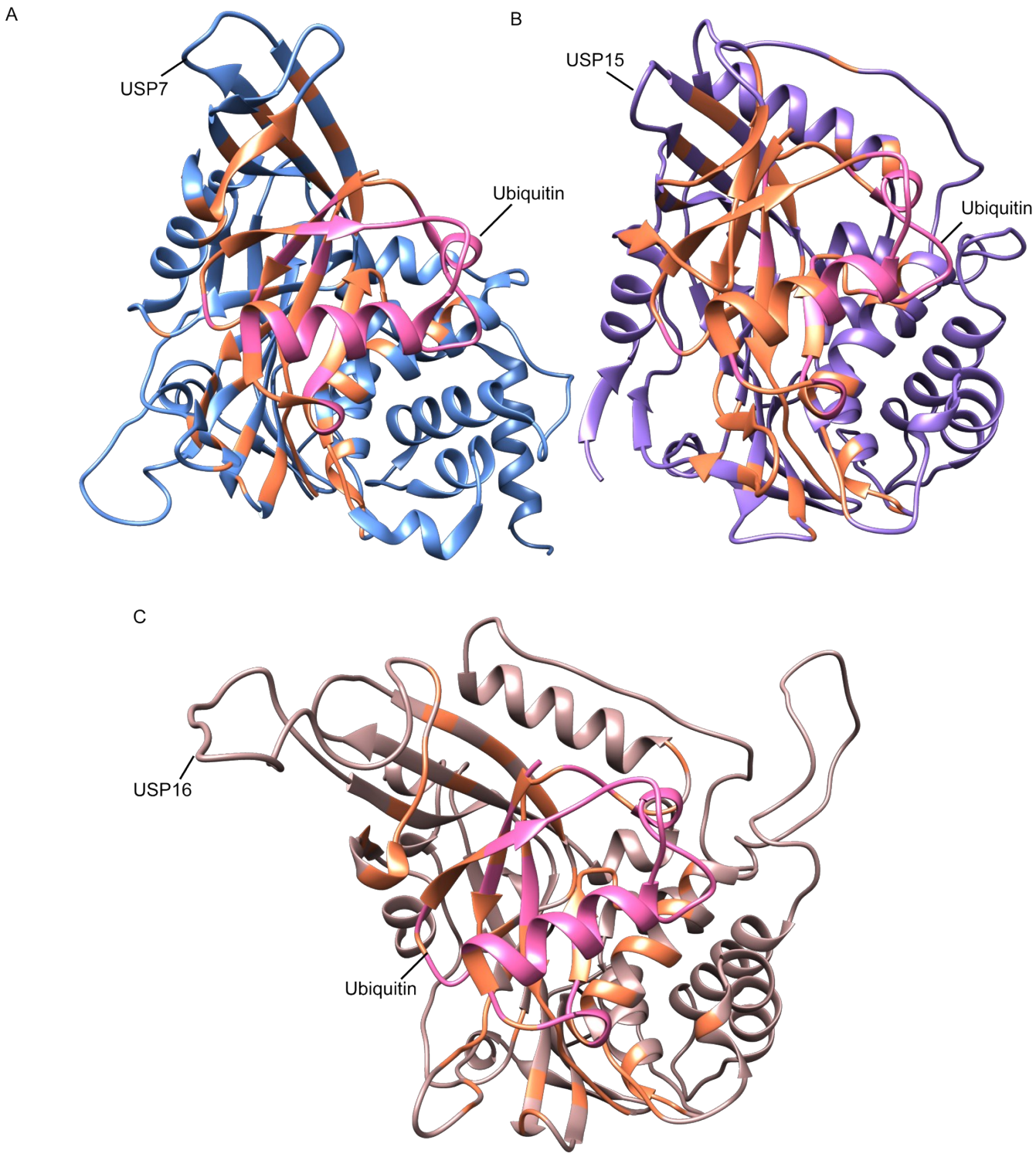


Fig. S4 Interacting residues on ubiquitin and the three USPs (all interactions coloured in orange).

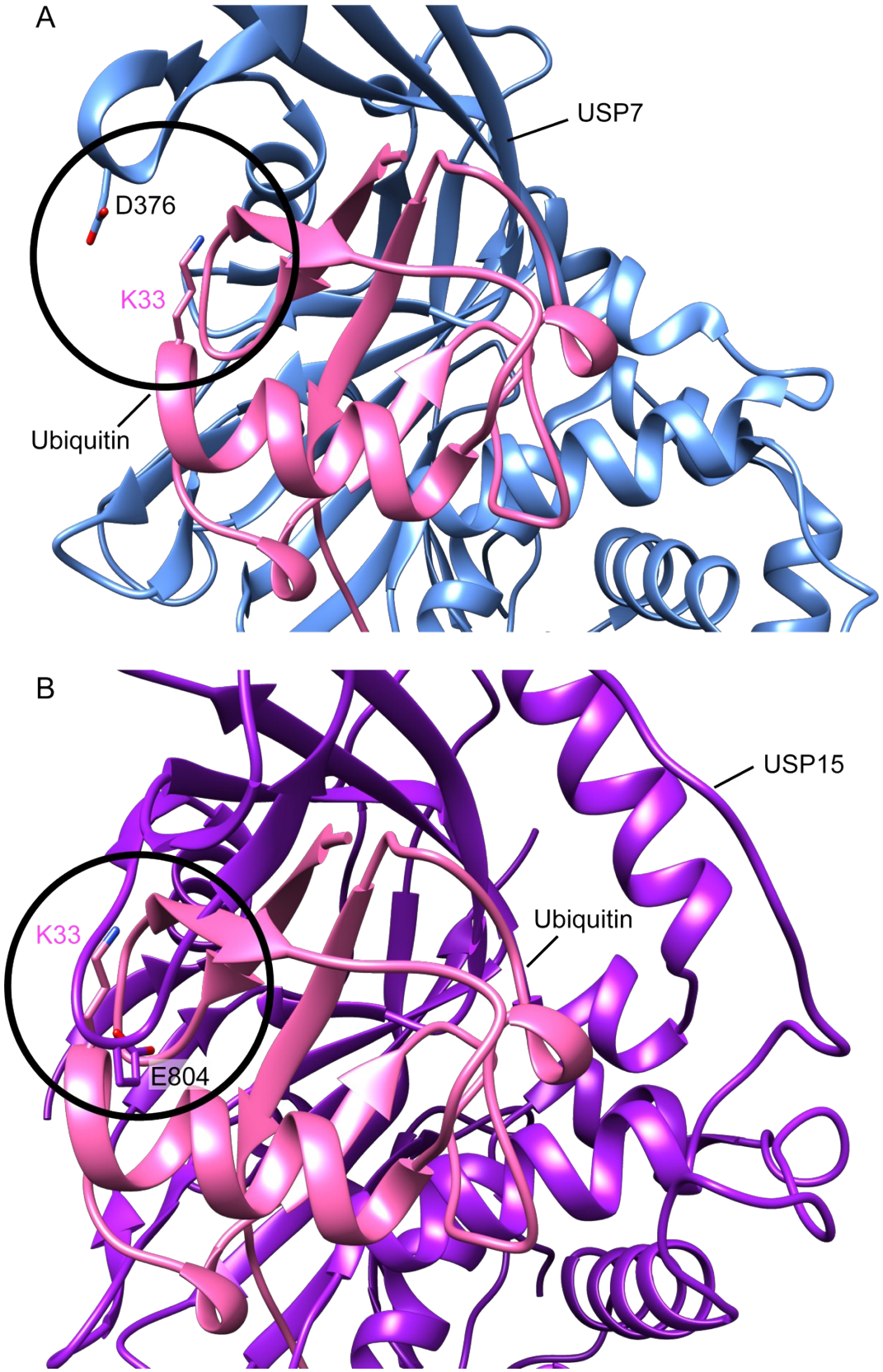


Fig. S5 Interaction of K33 of Ub (highlighted) with A) D376 (USP7 structure) and B) E804 (USP15 structure).

Table S1. List of the ubiquitin mutants

ID	Sequence	Mutation
M1	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDKLGIPPDQQRLIFAGKQLEDGRTLSZYNIZKESTLHAVARLRG-PA	T7D,L8Y,I13R,E34L,D58K-bio,Q62K-bio,L69A,L71A
M2	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDKLGIPPDQQRLIFAGKQLEDGRTLSZYNIRKESTLHAVARLRG-PA	T7D,L8Y,I13R,E34L,D58K-bio,Q62R,L69A,L71A
M3	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDKLGIPPDQQRLIFAGKQLEDGRTLSZYNIDKESTLHAVARLRG-PA	T7D,L8Y,I13R,E34L,D58K-bio,Q62D,L69A,L71A
M4	Rh-MQIFVKDYTGKWYTLEVEPSDTIENVKAKIQDKIGNPPDNQRLIFAGKQLEDGRTLSZYNIQKESTLHIVGRLRG-PA	T7D,L8Y,T12W,I13Y,E34I,I36N,Q40N,D58K-bio,L69I,L71G
M5	Rh-MQIFVKDYTGKWYTLEVEPSDTIENVKAKIQDKIGNPPDNQRLIFAGKQLEDGRTLSZYNIQKESTLHAVARLRG-PA	T7D,L8Y,T12W,I13Y,E34I,I36N,Q40N,D58K-bio,L69A,L71A
M6	Rh-MQIFVKSYTGKWRTLEVEPSDTIENVKAKIQDKIGNPPDNQRLIFAGKQLEDGRTLSZYNIQKESTLHAVARLRG-PA	S7D,L8Y,T12W,I13R,E34I,I36N,Q40N,D58K-bio,L69A,L71A
M7	Rh-MQIFVKDYTGKWRTLEVEPSDTIENVKAKIQDKIGNPPDQQRLIFAGKQLEDGRTLSZYNIQKESTLHIVGRLRG-PA	T7D,L8Y,T12W,I13Y,E34I,I36N,D58K-bio,L69I,L71G (unsuccessful)
M8	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIZKESTLHAVARLMG-PA	T7D,L8Y,I13R,E34L,Q40N,D58K-bio,Q62K-bio,L69A,L71A,R74M
M9	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIZKESTLHAVARLZG-PA	T7D,L8Y,I13R,E34L,Q40N,D58K-bio,Q62K-bio,L69A,L71A,R74K-bio
M10	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIZKESTLHAVARLWG-PA	T7D,L8Y,I13R,E34L,Q40N,D58K-bio,Q62K-bio,L69A,L71A,R74W
M11	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIDKESTLHAVARLMG-PA	T7D,L8Y,I13R,E34L,Q40N,D58K-bio,Q62D,L69A,L71A,R74M
M12	Rh-MQIFVKSYTGKWRTLEVEPSDTIENVKAKIQDKIGNPPDNQRLIFAGKQLEDGRTLSZYNIQKESTLHAVARLZG-PA	S7D,L8Y,T12W,I13R,E34I,I36N,Q40N,D58K-bio,L69A,L71A,R74K-bio
M13	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIZKESTLYIVGRLMG-PA	T7D,L8Y,I13R,E34L,Q40N,D58K-bio,Q62K-bio,H68Y,L69I,L71G,R74M
M14	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIQKHATLHAVARLRG-PA	T7D,L8Y,I13R,E34L,Q40N,D58K-bio,E64H,S65A,L69A,L71A,
M15	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIQKHATLHAVARLZG-PA	T7D,L8Y,I13R,E34L,Q40N,D58K-bio,E64H,S65A,L69A,L71A, R74K-bio
M16	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIQKFSTLHAVARLZG-PA	T7D,L8Y,I13R,E34L,Q40N,D58K-bio,E64F,L69A,L71A, R74K-bio
M17	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIZKESTLHAVARLMG-PA	T7D,L8Y,I13R,E16K-bio,E34L,Q40N,D58K-bio,Q62K-bio,L69A,L71A,R74M
M18	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIQKESTLHAVARLRG-PA	T7D,L8Y,I13R,E16K-bio,E34L,Q40N,D58K-bio,L69A,L71A
M19	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDELGIPPDNQRLIFAGKQLEDGRTLSZYNIQKESTLHAVARLRG-PA	T7D,L8Y,I13R,E16K-bio,K33E,E34L,Q40N,D58K-bio,L69A,L71A
M20	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDEEGIPPDNQRLIFAGKQLEDGRTLSZYNIQKFSTLHAVARLWG-PA	T7D,L8Y,I13R,E16K-bio,K33E,Q40N,D58K-bio,E64F, L69A,L71A,R74W
M21	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDELGIPPDNQRLIFAGKQLEDGRTLSZYNIQKESTLHAVARLMG-PA	T7D,L8Y,I13R,E16K-bio,K33E,E34L,Q40N,D58K-bio,L69A,L71A,R74M
M22	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDELGIPPDQQRLIFAGKQLEDGRTLSZYNIDKESTLHAVARLRG-PA	T7D,L8Y,I13R,E16K-bio,K33E,E34L,D58K-bio,Q62D,L69A,L71A
M23	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDELGIPPDQQRLIFAGKQLEDGRTLSZYNIDKESTLHAVARLZG-PA	T7D,L8Y,I13R,E16K-bio,K33E,E34L,D58K-bio,Q62D,L69A,L71A,R74K-bio
M24	Rh-MQIFVKSYTGKWRTLZVEPSDTIENVKAKIQDKIGNPPDNQRLIFAGKQLEDGRTLSZYNIDKESTLHAVARLZG-PA	T7S,L8Y,T12W,I13R, E16K-bio,K33E, I36N,Q40N,D58K-bio,L69A,L71A,R74K-bio
M25	Rh-MQIFVKDYTGKWRTLZVEPSDTIENVKAKIQDKIGNPPDQQRLIFAGKQLEDGRTLSZYNIQKESTLHIVGRLMG-PA	T7D,L8Y,T12W,I13Y,E16K-bio,E34I,I36N,D58K-bio,L69I,L71G, R74M
M26	Rh-MQIFVKDYTGKWRTLZVEPSDTIENVKAKIQDEEGNPPDQQRLIFAGKQLEDGRTLSZYNIQKESTLHIVGRLMG-PA	T7D,L8Y,T12W,I13Y,K33E,E16K-bio,I36N,D58K-bio,L69I,L71G, R74M
M27	Rh-MQIFVKDYTGKWRTLZVEPSDTIENVKAKIQDEIGNPPDQQRLIFAGKQLEDGRTLSZYNIQKESTLHIVGRLMG-PA	T7D,L8Y,T12W,I13Y,K33E,E16K-bio,E34I,I36N,D58K-bio,L69I,L71G, R74M
M28	Rh-MQIFVKDYTGKWRTLZVEPSDTIENVKAKIQDEIGNPPDQQRLIFAGKQLEDGRTLSZYNIZKESTLHIVGRLMG-PA	T7D,L8Y,T12W,I13Y,K33E,E16K-bio,E34I,I36N,D58K-bio,Q-K-bio, L69I,L71G, R74M
M29	Rh-MQIFVKDYTGKWYTLZVEPSDTIENVKAKIQDKIGNPPDNQRLIFAGKQLEDGRTLSZYNIQKESTLHAVARLZG-PA	T7D,L8Y,T12W,I13Y,E16K-bio,E34I,I36N,Q40N,D58K-bio,L69A,L71A,R74K-bio
M30	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDKLGIPPDQQRLIFAGKQLEDGRTLSZYNIDKESTLHAVARLMG-PA	T7D,L8Y,I13R,E16K-bio,E34L,D58K-bio,Q62D,L69A,L71A,R74M
M31	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDELGIPPDQQRLIFAGKQLEDGRTLSZYNIQKESTLHAVARLMG-PA	T7D,L8Y,I13R,E16K-bio,K33E,E34L,D58K-bio,Q62D,L69A,L71A,R74M
M32	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDEEGIPPDQQRLIFAGKQLEDGRTLSZYNIDKESTLHAVARLMG-PA	T7D,L8Y,I13R,E16K-bio,D58K-bio,K33E,L69A,L71A,R74M
M33	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDELGIPPDQQRLIFAGKQLEDGZTLSZYNIQKESTLHAVARLMG-PA	T7D,L8Y,I13R,E16K-bio,K33E,E34L,R54K-bio,D58K-bio,L69A,L71A,R74M
M34	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDELGIPPDQQRLIFAGKQLEDGZTLSZYNIQKESTLHAVARLWG-PA	T7D,L8Y,I13R,E16K-bio,K33E,E34L,R54K-bio,D58K-bio,L69A,L71A,R74W
M35	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIQKHATLHAVARLRG-PA	T7D,L8Y,I13R,E16K-bio,E34L,Q40N,D58K-bio,E64H,S65A,L69A,L71A,
M36	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIQRHATLHAVARLZG-PA	T7D,L8Y,I13R,E34L,Q40N,D58K-bio,K63R,E64H,S65A,L69A,L71A, R74K-bio
M37	Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDELGIPPDNQRLIFAGKQLEDGRTLSZYNIQRHATLHAVARLMG-PA	T7D,L8Y,I13R,K33E,E34L,Q40N,D58K-bio,K63R,E64H,S65A,L69A,L71A, R74M
M38	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDELGIPPDQQRLIFAGKQLEDGZTLSZYWIQKESTLHAVARLMG-PA	T7D,L8Y,I13R,E16K-bio,K33E,E34L,R54K-bio,D58K-bio,N60W,L69A,L71A,R74M
M39	Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDELGIPPDQQRLIFAGKQLEDGZTLSZY$IQKESTLHAVARLWG-PA	T7D,L8Y,I13R,E16K-bio,K33E,E34L,R54K-bio,D58K-bio,N60E-bio,L69A,L71A,R74W
M40	Rh-MQIFVKDYWGKTRTLZVEPSDTIENVKAKIQDKLGIPPDNQRLIFAGKQLEDGRTLSZYNIDKESTLHAVARLMG-PA	T7D,L8Y,T9W,I13R,E16K-bio,E34L,Q40N,D58K-bio,Q62D,L69A,L71A,R74M

Glu(biotinyl-PEG)=$ (E-bio); Lys(biotin)=Z (K-bio)

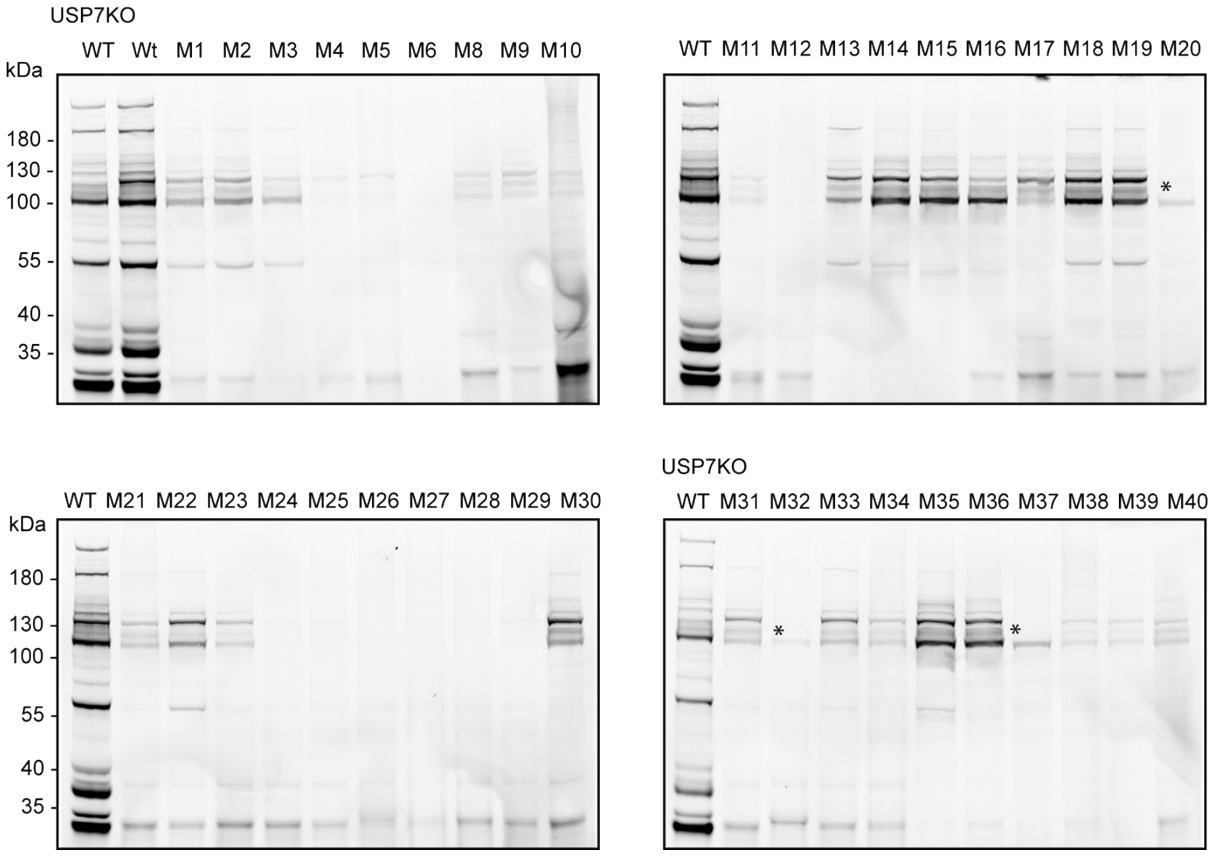


Fig. S6 DUB activity-based probe assay analysed using in-gel fluorescence scanning. *= suspected labelled USP16.

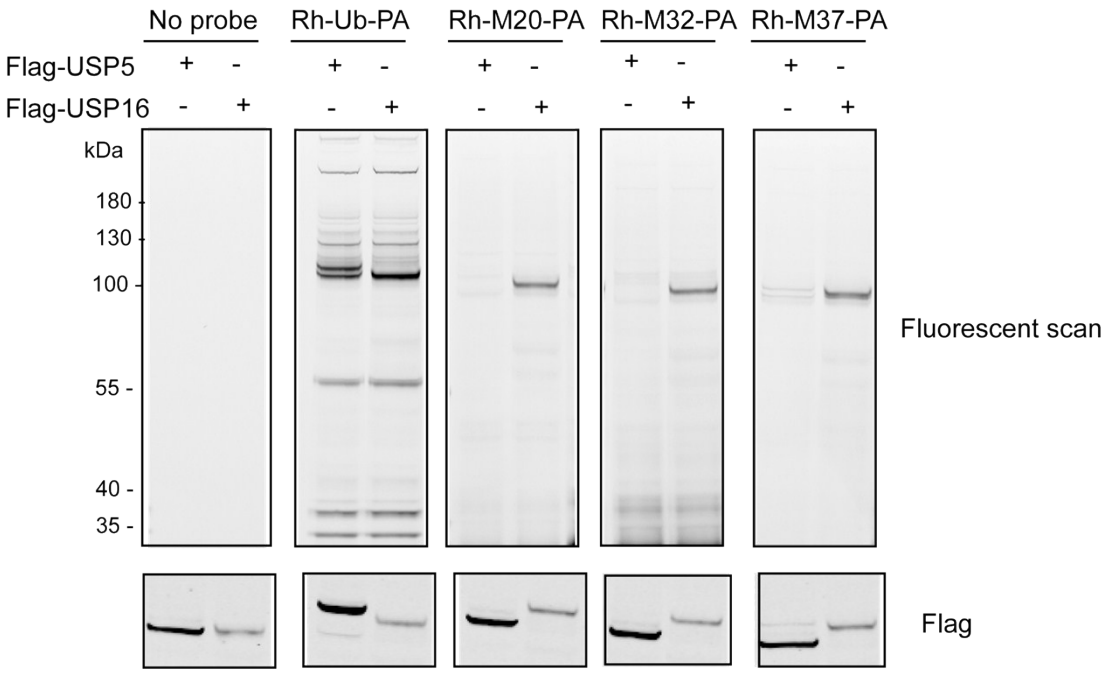


Fig. S7 Labelling of the lysate from HeLa cells overexpressing Flag-USP5 and Flag-USP16 with the best mutants and controls analysed using in-gel fluorescence scanning. Rh-M37-PA showed slight reactivity with overexpressed Flag-USP5.

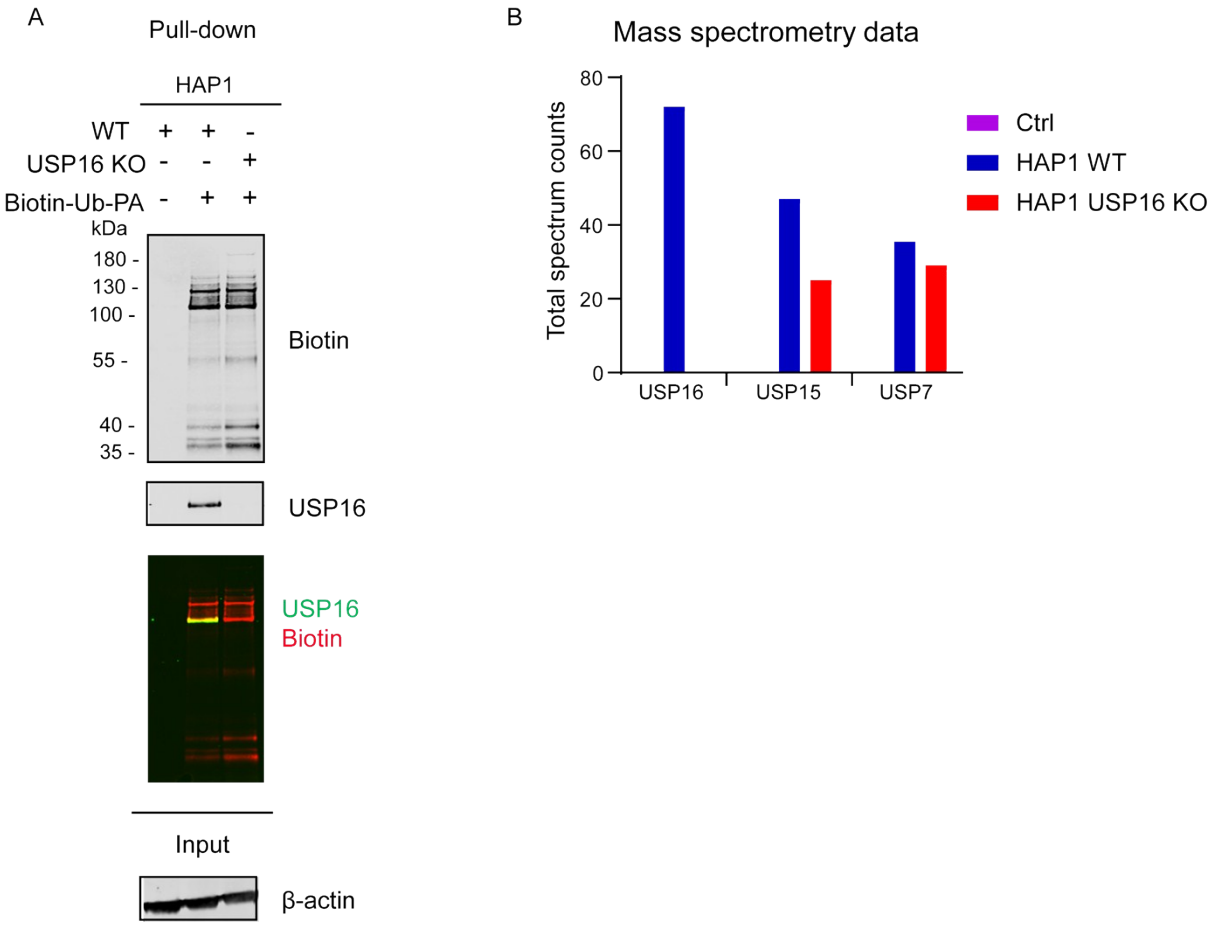


Fig. S8 Pull-down experiment with Biotin-Ub-PA and identification of the presence of USP16 using mass spectrometry. A) DUB activity-based probe assay of HAP1 and USP16 KO HAP1 lysate with Biotin-Ub-PA. Labelled DUBs were analysed using western blotting. B) Representation of the total spectrum counts for USP7, USP15 and USP16. β-actin is used as a loading control. A301-614A is the USP16 antibody used in this experiment. Control= HAP1 WT lysate incubated only with neutravidin beads. See also Supplementary data 2.

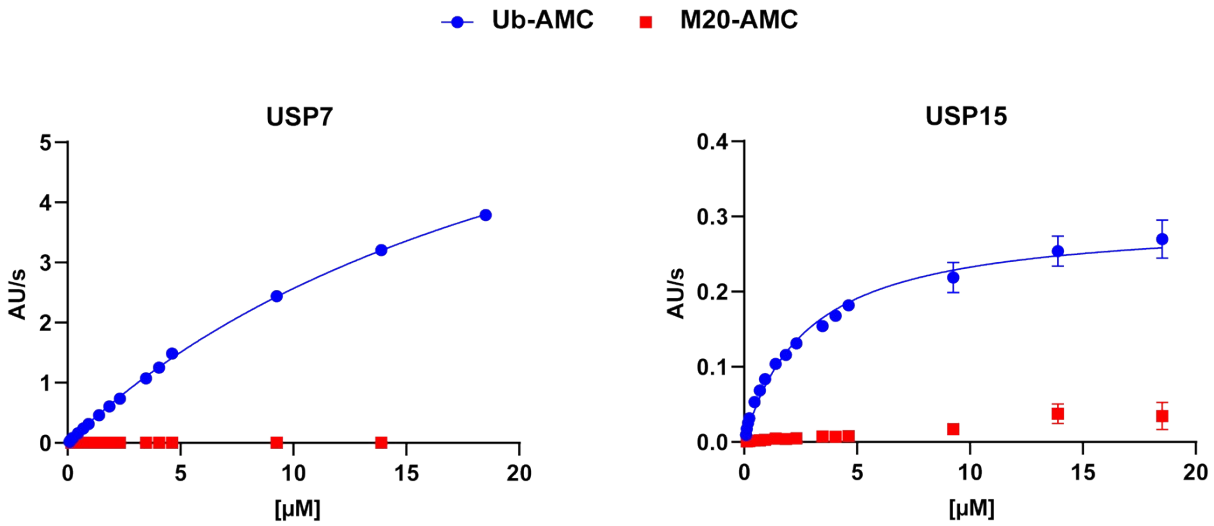
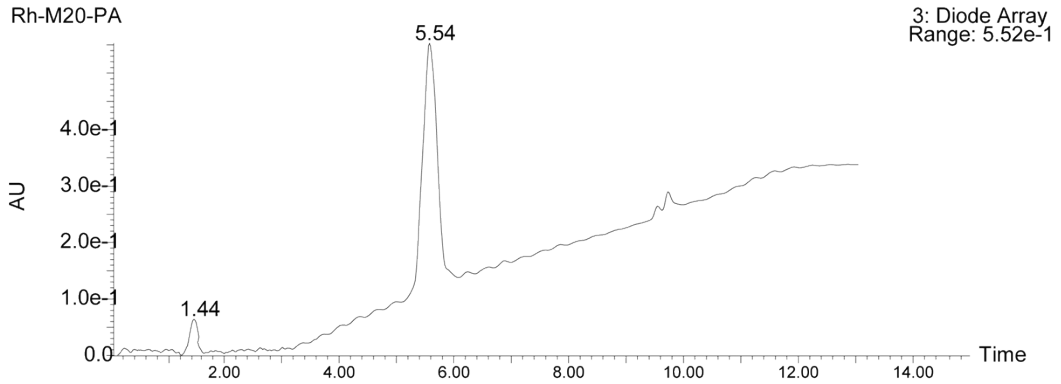


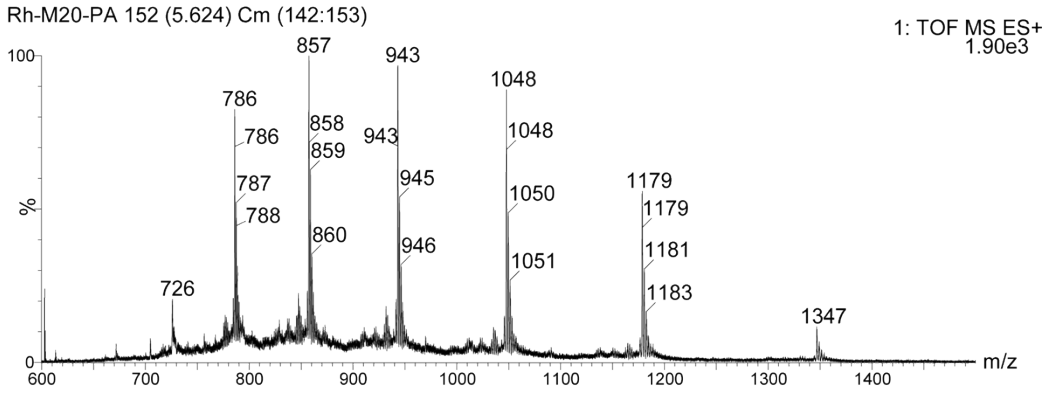
Fig. S9 Kinetic measurements of Ub-AMC and M20-AMC in presence of USP7 (left panel) and USP15 (right panel) using Michaelis-Menten fit achieved by plotting the initial rates (V_0) at different AMC reagent concentrations Final concentrations of USP7 and USP15 is 1 nM and 5 nM, respectively . The measurements were performed in four independent replicates. In the case of Ub-AMC, km of USP7 (23.95 µM) and USP15 (2.78 µM) while no k_M could be determined for M20-AMC

Analytical data: For the main reagents used in this work are shown the analytical data using LC-MS: A) UV chromatogram trace (λ =190-600 nm); B) mass spectrum of the main peak; C) deconvoluted mass of the spectra using MaxEnt 1.

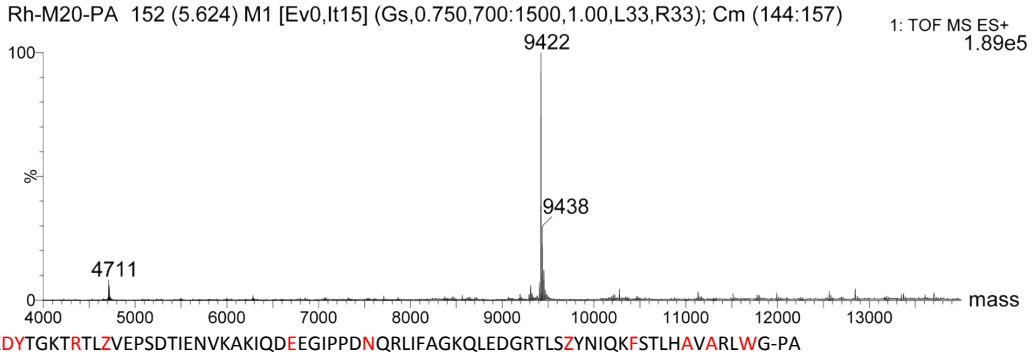
A



B



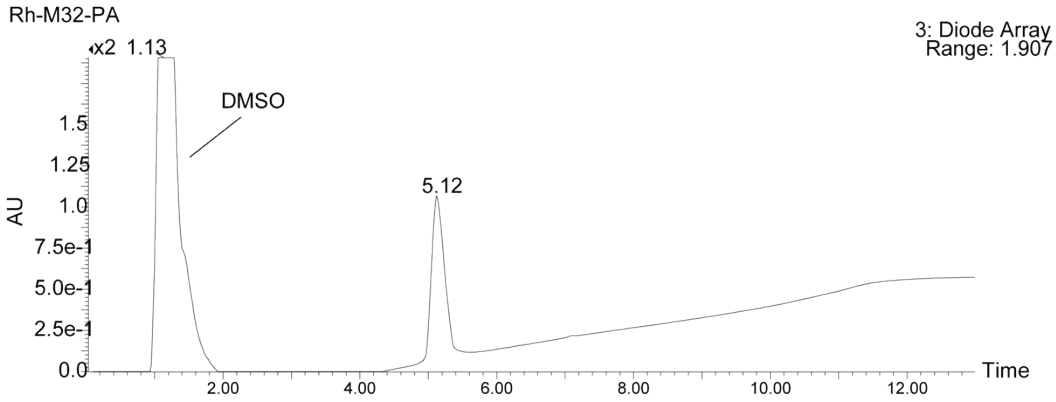
C



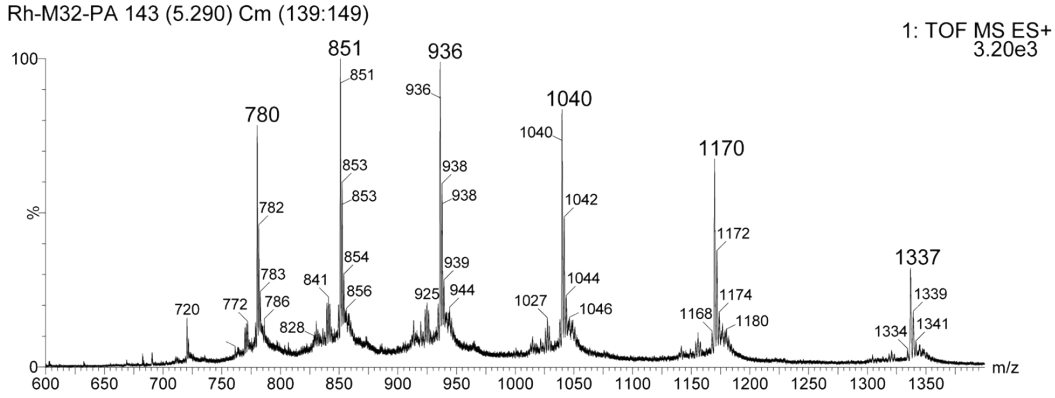
Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDEEGIPPDNQRLIFAGKQLEDGRTLSZYNIQKFSTLHAVARLWG-PA

Fig S10. Analytical data for Rh-M20-PA. ESI-Mass [M+H] Calculated: 9424 / Found: 9422. Mass of 9438 corresponds to the oxidized methionine.

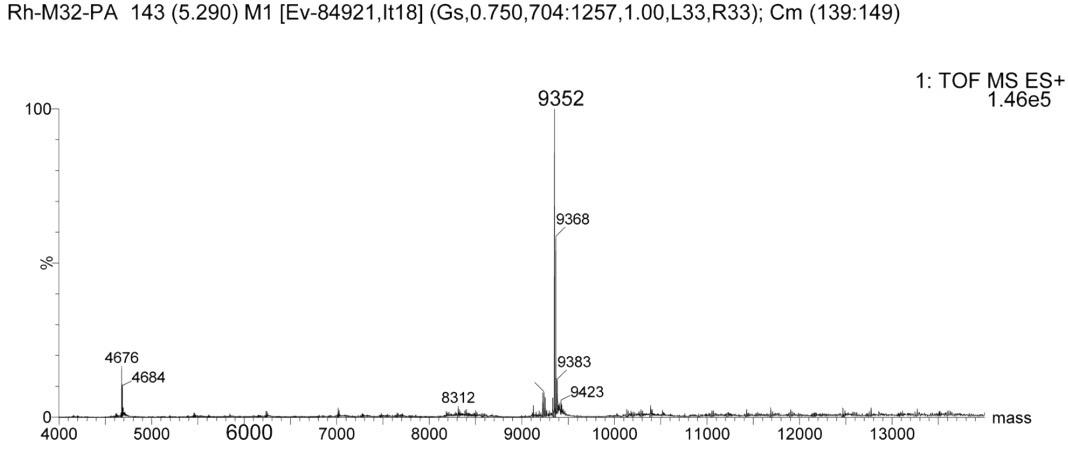
A



B



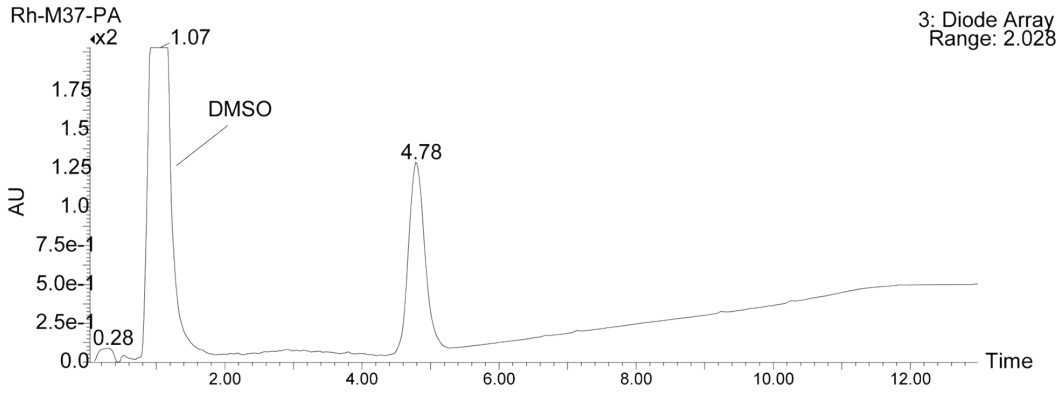
C



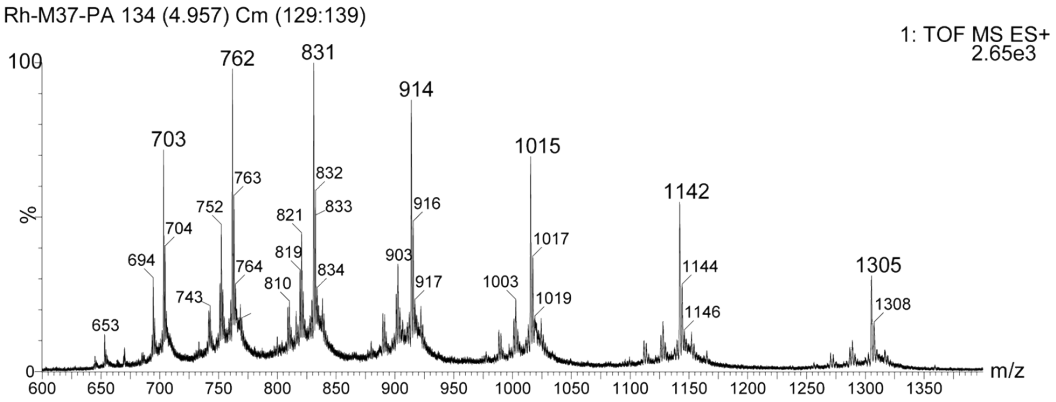
Rh-MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDEEGIPPDQQRLIFAGKQLEDGRTLSZYNIDKESTLHAVARLMG-PA

Fig S11. Analytical data for Rh-M32-PA. ESI-Mass [M+H] Calculated: 9351 / Found: 9352. Mass of 9368 corresponds to the oxidized methionine.

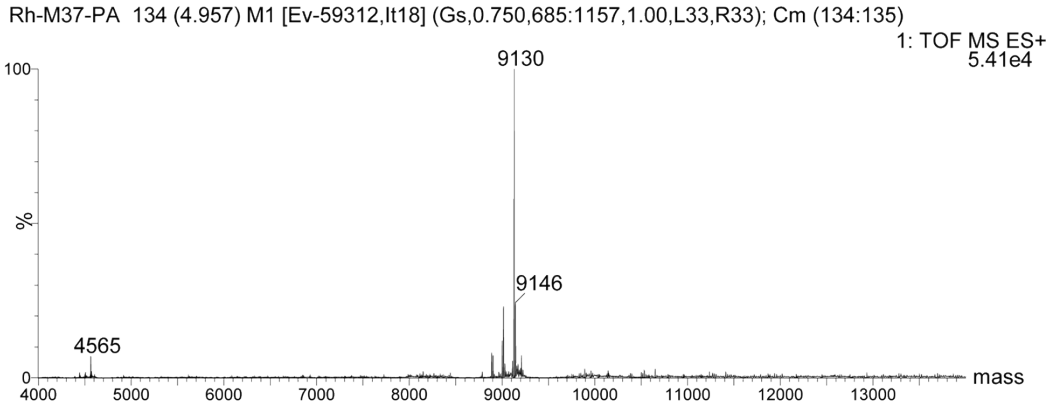
A



B



C

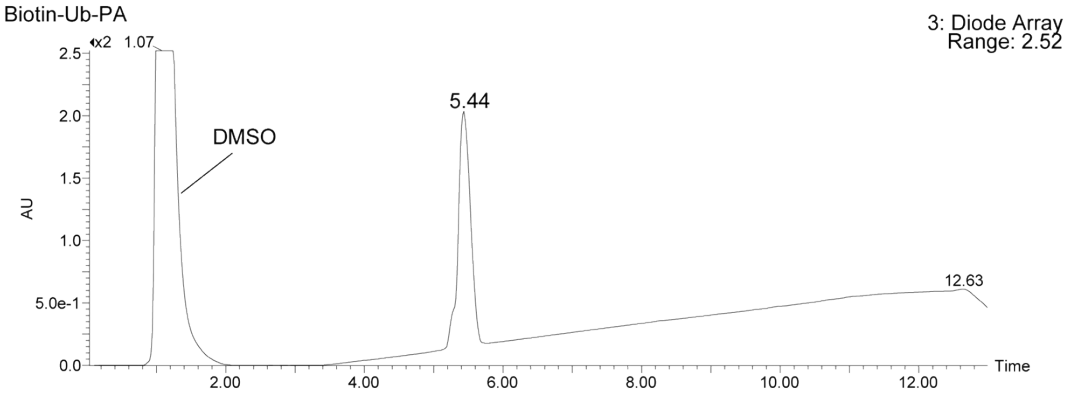


Rh-MQIFVKDYTGKTRTLEVEPSDTIENVKAKIQDELGIPPDNQRLIFAGKQLEDGRTLSZYNIQRHATLHAVARLMG-PA

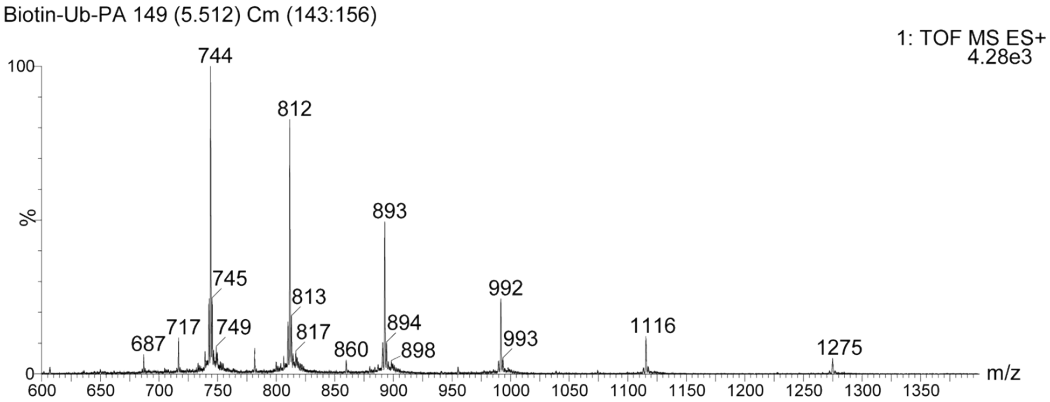
Fig S12. Analytical data for Rh-M37-PA. ESI-Mass [M+H] Calculated: 9129 / Found: 9130 Mass of 9146 corresponds to the oxidized methionine.

Biotin-PEG-MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG-PA

A



B



C

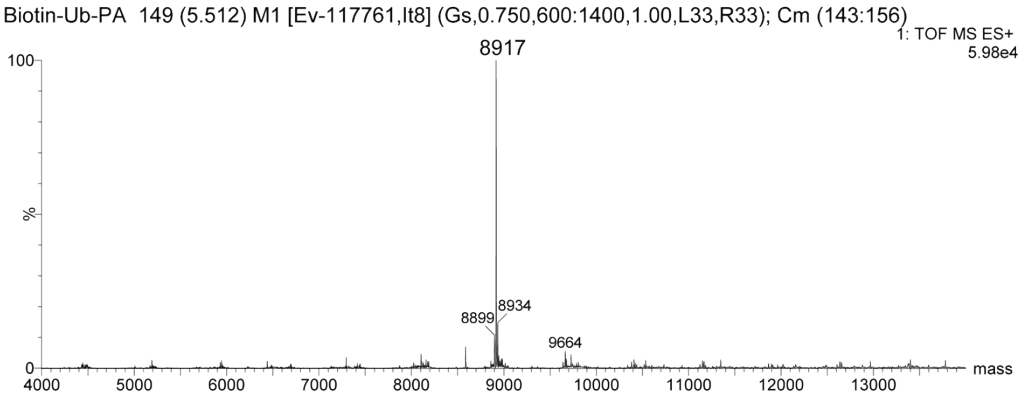
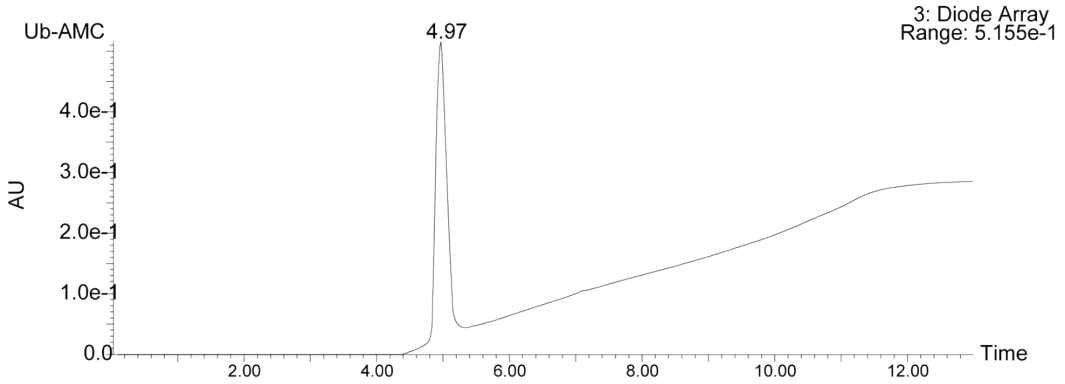


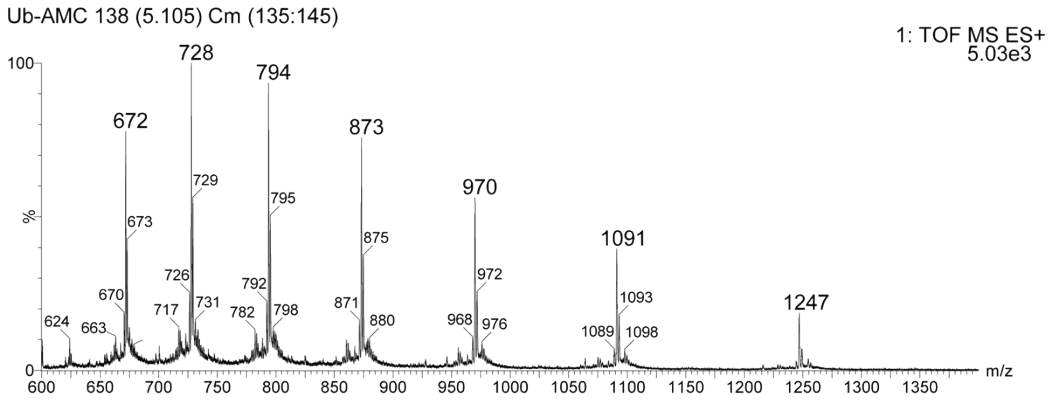
Fig S13. Analytical data for Biotin-Ub-PA. ESI-Mass [M+H] Calculated: 8917 / Found: 8917.

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG-AMC

A



B



C

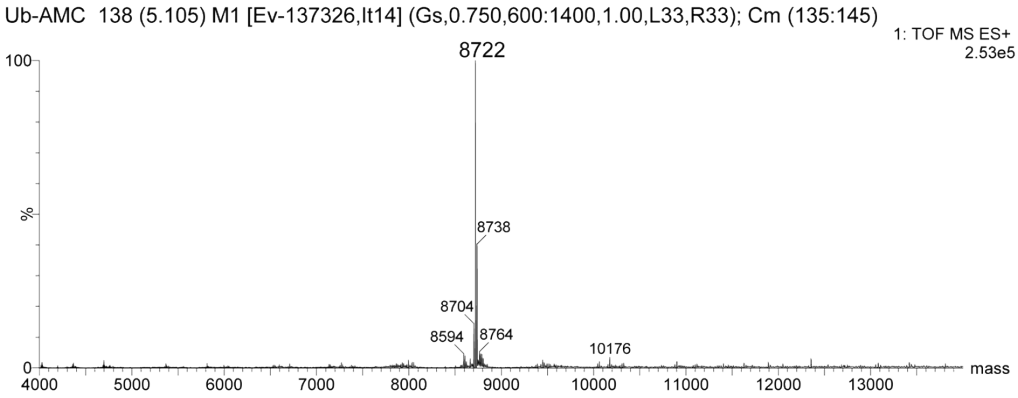


Fig S14. Analytical data for Ub-AMC. ESI-Mass [M+H] Calculated: 8720/ Found: 8722. Mass of 8738 corresponds to the oxidized methionine.

MQIFVKDYTGKTRTLZVEPSDTIENVKAKIQDEEGIPPDNQRLIFAGKQLEDGRTLSZYNIQKFSTLHAVARLWGG-AMC

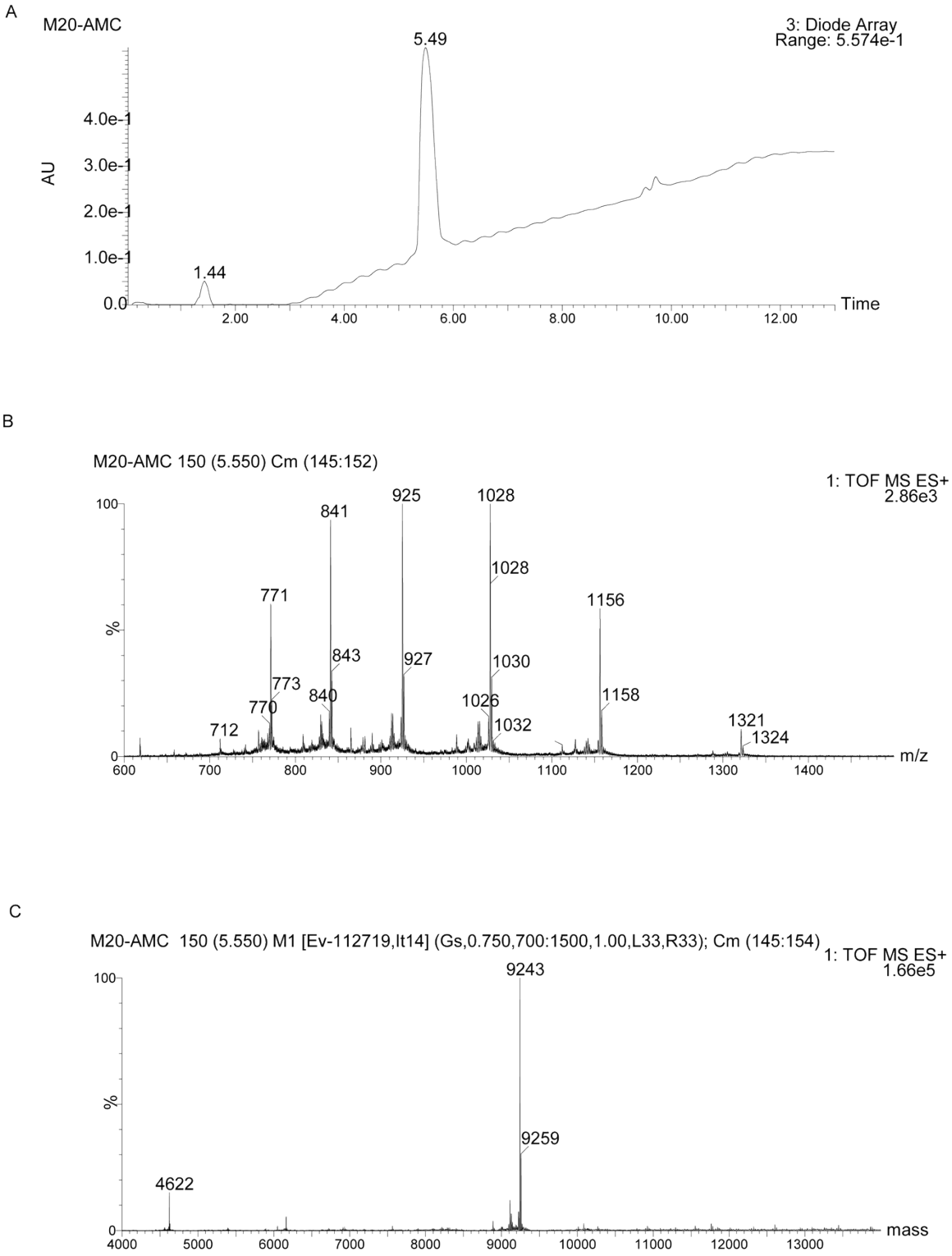


Fig S15. Analytical data for M20-AMC. ESI-Mass [M+H] Calculated: 9243/ Found: 9243. Mass of 9259 corresponds to the oxidized methionine.

References:

1. Gjonaj, L.; Sapmaz, A.; Gonzalez-Prieto, R.; Vertegaal, A. C. O.; Flierman, D.; Ovaa, H., USP7: combining tools towards selectivity. *Chem Commun (Camb)* **2019**.
2. de Jong, A.; Witting, K.; Kooij, R.; Flierman, D.; Ovaa, H., Release of Enzymatically Active Deubiquitinating Enzymes upon Reversible Capture by Disulfide Ubiquitin Reagents. *Angew Chem Int Ed Engl* **2017,** *56* (42), 12967-12970.
3. El Oualid, F.; Merkx, R.; Ekkebus, R.; Hameed, D. S.; Smit, J. J.; de Jong, A.; Hilkmann, H.; Sixma, T. K.; Ovaa, H., Chemical synthesis of ubiquitin, ubiquitin-based probes, and diubiquitin. *Angew Chem Int Ed Engl* **2010,** *49* (52), 10149-53.
4. Jongsma, M. L.; Berlin, I.; Wijdeven, R. H.; Janssen, L.; Janssen, G. M.; Garstka, M. A.; Janssen, H.; Mensink, M.; van Veelen, P. A.; Spaapen, R. M.; Neefjes, J., An ER-Associated Pathway Defines Endosomal Architecture for Controlled Cargo Transport. *Cell* **2016,** *166* (1), 152-66.
5. Sapmaz, A.; Berlin, I.; Bos, E.; Wijdeven, R. H.; Janssen, H.; Konietzny, R.; Akkermans, J. J.; Erson-Bensan, A. E.; Koning, R. I.; Kessler, B. M.; Neefjes, J.; Ovaa, H., USP32 regulates late endosomal transport and recycling through deubiquitylation of Rab7. *Nat Commun* **2019,** *10* (1), 1454.
6. Ward, S. J.; Gratton, H. E.; Indrayudha, P.; Michavila, C.; Mukhopadhyay, R.; Maurer, S. K.; Caulton, S. G.; Emsley, J.; Dreveny, I., The structure of the deubiquitinase USP15 reveals a misaligned catalytic triad and an open ubiquitin-binding channel. *J Biol Chem* **2018,** *293* (45), 17362-17374.