Acyl azide modification of ubiquitin C-terminus enables DUBs capture

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Supplementary Information

1 General

1.1 Reagents

Yeast extract, Tryptone, HEPES and MES were purchased from Sigma-Aldrich. HCl, NaOH, NaCl and Tris were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai). Sodium nitrite ($NaNO_2$) was purchased from Beijing Chemical Works Co., Ltd (Beijing). Tris (2-carboxyethyl) phosphine hydrochloride (TCEP HCl) and Sodium 2-mercaptoethanesulfonate (Mesna) were purchased from Adamas-beta, Titan Scientific Co., Ltd (Shanghai). Acetonitrile (HPLC grade) was purchased from J. T. Baker (Phillipsburg, NJ, USA). Ni-NTA resin was purchased from GE Healthcare. Chemically competent BL21 (DE3) cells were purchased from TransGen Biotech ltd (Beijing).

1.2 HPLC, Mass spectrometry and FPLC

Analysis by RP-HPLC (SHIMADZU, Prominence LC 20AT) was performed with an analytical column (Welch XB-C18, 4.6 × 250 mm, 5 μm, 120 Å, flow rate 1.0 mL/min). Analysis was monitored at 214 nm and 254 nm wavelengths. Solvents were sonicated for 30 minutes before use. Analysis condition: a linear gradient of 20%-70% acetonitrile (with 0.08% v/v TFA) in water (with 0.1% v/v TFA) over 30 min.

Tandem MS samples in Fig. 1c is the product of in gel trypsin digestion according to routine procedures. In particular, it's worth noting that iodoacetamide modified Cys would have the same MS value as the thioester bond on Cys residue, therefore we conducted the alkylation of Cys side chain using iodoacetic acid instead of iodoacetamide. Iodoacetic acid modified Cys has about 1 Da MS value more than thioester bond modification, therefore we can distinguish them without using an isotope-labeled iodoacetamide reagent. Mass spectrometry was performed on an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific). Protein samples in 0.1% formic acid were loaded onto a self-packed PicoTip column (New Objective, Woburn, MA) (360 μm od, 75 μm id, 15 μm tip), packed with 7–10 cm of reverse phase C18 material (ODS-A C18 5-μm beads from YMC America, Allentown, PA), rinsed for 5 min with 0.1% formic acid, and then eluted with a linear gradient from 2% to 35% B for 150 min (A = 0.1% formic acid, B = 0.1% formic acid in acetonitrile, flow rate ~200 nL/min) into the mass spectrometer.

FPLC was run on an AKTA (GE Healthcare Life Science) using a column of Superdex^{TM} 75 Increase 10/300 GL. Aqueous buffer (20 mM Tris, 150 mM NaCl, pH=7.4) was filtered with 0.22 μm filter paper, sonicated for 30 min and precooled for 10 min before use. The purification process was monitored at 280 nm.

1.3 Circular dichroism (CD) analysis

Protein samples were diluted in H_2O to a final concentration of ~ 0.1 mg/mL. Samples were measured on a Pistar p-180 spectrometer from 190 nm to 280 nm at room temperature in a quartz cell with 0.2 cm path length. Each protein sample was measured for three times.

1.4 SDS-PAGE and Immunoblotting

For SDS-PAGE, samples were loaded onto 4-20% SDS-PAGE gels with MOPS buffer and electrophoresed for 8 min at 90 V followed with 60 min at 120 V. All pictures of protein gels were taken on Chemi DocTM XRS+system (Bio-Rad).

For immunoblotting, protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF, Bio-Rad, 300 mA 1.5 h). Then the membranes were incubated with 5% (m/v) of skim milk powder dissolved in TBS buffer (20 mM Tris-HCl, 137 mM NaCl, pH=7.6) for 2 h at room temperature. After three times washing with TBST buffer (20 mM Tri-HCl, 137 mM NaCl, 0.1% Tween-20, pH=7.6) for 10

min at room temperature. The membranes were followed incubated with primary antibodies for 2 h at room temperature. Then washed with TBST buffer for four times again and incubated with secondary antibodies for another 2 h at room temperature. Finally, the membranes were washed with TBST buffer for four times and chemiluminescence solution (ECL) was used for protein detection.

Anti-biotin (AB53494) and anti-OTUB1 (AB175200) was purchased from Abcam. Anti-β-actin antibody was purchased from Cell Signaling Technology (4970). Goat anti-Rabbit IgG (HRP) secondary antibody (D111018) and goat anti-mouse IgG (HRP) secondary antibody (D110087) were purchased from Sangon Biotech (Shanghai) Co., Ltd (Shanghai).

2.Construction of protein expression plasmids

The following genes were codon optimized and synthesized by Genscript (Nanjing).

Ub76C: The Ub76C mutant was cloned into pET-22b vector.

Amino Acid sequence:

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLR
LRGC

avi-Ub76C: The avi-Ub76C mutant was cloned into pET-22b vector.

Amino Acid sequence:

GSGLNDIFEAQKIEWHEMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRT
LSDYNIQKESTLHLVLRLRGC

UCHL1: UCHL1 was cloned into pET-28a vector with *Nde*I and *Xho*I as restriction site.

Amino Acid sequence:

MQLKPMEINPEMLNKVLSRLGVAGQWRFVDVLGLEEESLGSVPAPACALLLLFPLTAQHENFRKKQIEEL
KGQEVSPKVYFMKQTIGNSCGTIGLIHAVANNQDKLGFEDGSVLKQFLSETEKMSPEDRAKCFEKNEAIQ
AAHDAVAQEGQCRVDDKVNFHFILFNNVDGHLYELDGRMPFPVNHGASSEDTLLKDAAKVCREFTERE
QGEVRFSAVALCKAA

OTUB1: OTUB1 was cloned into pET-28a vector with *Nde*I and *Xho*I as restriction site.

Amino Acid sequence:

MAAEEPQQQKQEPLGSDSEGVNCLAYDEAIMAQQDRIQQEIAVQNPLVSERLELSVLYKEYAEDDNIYQ
QKIKDLHKKYSYIRKTRPDGNCFYRAFGFSHLEALLDDSKELQRFKAVSAKSKEDLVSQGFTEFTIEDFHN
TFMDLIEQVEKQTSVADLLASFNDQSTSDYLVVYLRLLTSGYLQRESKFFEHFIEGGRTVKEFCQQEVEPM
CKESDHIHIIALAQALSVSIQVEYMDRGEGGTTNPHIFPEGSEPKVYLLYRPGHYDILYK

USP7 catalytic domain: USP7catalytic domain (208-560) was cloned into pET-28a vector with *Nde*I and *Xho*I as restriction site.

Amino Acid sequence:

KKHTGYVGLKNQGATCYMNSLLQTLFFTNQLRKAVYMMPTEGDDSSKSVPLALQRVFYELQHSDKPVG
TKKLTKSFGWETLDSFMQHDVQELCRVLLDNVENKMKGTCVEGTIPKLFRGKMVSYIQCKEVDYRSDR
REDYYDIQLSIKGKKNIFESFVDYVAVEQLDGDNKYDAGEHGLQEAEKGVKFLTLPPVLHLQLMRFMYD
PQTDQNIKINDRFEFPEQLPLDEFLQKTDPKDPANYILHAVLVHSGDNHGGHYVVYLNPKGDGKWCKFD
DDVVSRCTKEEAIEHNYGGHDDDLSVRHCTNAYMLVYIRESKLSEVLQAVTDHDIPQQLVERLQEEKRIE

AQKRKERQE

3. Protein expression and purification

3.1 Expression and purification of Ub76C and avi-UbG76C

The plasmids containing UbG76C and avi-UbG76C gene were transformed into E. coli BL21 (DE3) cells respectively. The E. coli was grown in LB containing 0.1mg/mL ampicillin at 37 ℃ to an OD600 of 0.8. IPTG with a final concentration of 0.4 mM was added to LB medium and then shook it for 4 hours at 37 ℃. Cell precipitation was harvested at 4000 rpm at 8 ℃ for 10 minutes, then cells were resuspended in lysis buffer (20 mM Tris, 150 mM NaCl, pH=7.4) and lysed by ultra-sonication in ice bath. Perchloric acid was then added to lysis buffer to 0.7% (V/V) with vigorous stirring. The suspension was centrifuged at 11000 rpm at 8 ℃ for 30 min, and then the supernatant was filtrated with 0.22 μm filter paper followed by dialyzed into dialysis buffer (50 mM Tris-HCl, pH=7.4) to 15 mg/mL for hydrazinolysis reaction.

3.2 Expression and purification of DUBs

DUBs (UCHL1, OTUB1, USP7 catalytic domain) plasmids were transformed into E. coli BL21 (DE3) cells. The E. coli was grown in LB containing 0.1mg/mL Kanamycin at 37 ℃ to an OD600 of 0.8. IPTG with a final concentration of 0.4 mM was added to LB medium and then shook it for 12 hours at 16 ℃. Cell precipitation was harvested at 4000 rpm at 8 ℃ for 10 minutes, then cells were resuspended in lysis buffer (20 mM Tris, 150 mM NaCl, pH=7.4) and lysed by ultra-sonication in ice bath. The suspension was centrifuged at 8 °C for 30 minutes at 11000 rpm, and the supernatant was obtained. All DUBs were fused to N terminal His6 tag and purified with Ni-NTA column. Mix the supernatant with Ni-NTA column at 4 °C for 1 h. The column was washed with washing buffer (20 mM Tris-HCl, 150 mM NaCl, 40 mM imidazole, pH=7.4) and then eluted with elution buffer (20 mM Tris-HCl, 150 mM NaCl, 250 mM imidazole, pH=7.4). After the centrifugation the DUBs were further purified using FPLC with SuperdexTM 75 Increase 10/300 GL in aqueous buffer (20 mM Tris, 150 mM NaCl, pH=7.4). Purified DUBs were stored at -80 °C until use.

4. Preparation of protein-hydrazides

4.1 Preparation of Ub_{1-75}-$NHNH_2$

Ub_{1-75}-$NHNH_2$ was prepared through reported hydrazinolysis strategy. The purified UbG76C mutant was concentrated to 15 mg/mL. Then the hydrazinolysis reagent (Mesna: 100 mg/mL, NH_2NH_2-HCl: 50 mg/mL, TCEP: 5 mg/mL) was added. The hydrazinolysis reaction (final pH=7.0) was carried out at 50 °C under N_2 protection for 36 h. The raw material could be completely transformed into Ub_{1-75}-$NHNH_2$. Subsequently, Ub_{1-75}-$NHNH_2$ was dialyzed into 20mM MES, 100mM NaCl, pH=6.0 buffer for refolding. About 30 mg of Ub_{1-75}-$NHNH_2$ can be obtained from per liter expression.

4.2 Preparation of (biotin)avi-Ub_{1-75}-$NHNH_2$

The purified avi-UbG76C mutant was concentrated to 10 mg/mL. First, the biotin tag was installed by BirA enzyme. A final concentration of 5 mM magnesium chloride, 2 mM ATP, 0.45 mM D-Biotin, and 1 μM BirA enzyme were added. (biotin) avi-UbG76C was obtained by 1 h reaction at 37 ℃ and monitored by HPLC and ESI MS (Fig. S2). Then N-S migrates hydrazine to prepare (biotin) avi-Ub_{1-75}-$NHNH_2$. The (biotin)avi-Ub_{1-75}-$NHNH_2$ was dialyzed into 20 mM MES, 100 mM NaCl, pH=6.0 buffer for refolding. About 24 mg of (biotin)avi-Ub_{1-75}-$NHNH_2$ can be obtained from per liter expression.

5. General cell culture procedure

HeLa cells (CCL-2™) were purchased from ATCC® and grown in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin in a 37 °C incubator at 5% CO_2. To detach cells from culture flasks, the media was aspirated and the flask was washed with cold PBS before cells treated with 0.25% Trypsin and returned to 37 °C incubator for 1 min. Trypsin was quenched by adding the supplemented media. The cell suspension was collected and the cells were pelleted (5 min at 145xg). Media was then aspired and the cell pellet was resuspended in fresh media.

6. Labeling of DUBs with Ub probes

6.1 Labeling of DUBs with Ub probes in buffers

1mM $Ub_{1-75}NHNH_2$ and (biotin)avi-$Ub_{1-75}NHNH_2$ were oxidized with $NaNO_2$ respectively at pH=3.0 in ice water bath for 20 min respectively, then the reaction pH was adjusted to 6.0. The in situ generated Ub acyl azide probe was diluted to 16μM in aqueous buffer (20 mM Tris, 150 mM NaCl, pH=7.4), and 8 μM purified DUB was added to incubate for another 1 h on ice. Then the reaction was quenched with 4 × loading buffer without DTT and analyzed by SDS-PAGE.

For iodacetamide treatment, 1 mM iodacetamide was pre-incubated with UCHL1 for 30 minutes, then the UCHL1 was diluted to 8 μM in aqueous buffer and reacted with Ub_{1-75}-N_3 for another 30 min. The reaction was quenched with 4 × loading buffer without DTT and analyzed by SDS-PAGE.

For DTT treatment, 16 μM Ub_{1-75}-N_3 was incubated with 8 μM UCHL1 for 30 min on ice, then 5 mM DTT was added and incubated for another 10 min. The reaction was quenched with 4 × loading buffer without DTT and analyzed by SDS-PAGE.

6.2 Labeling of DUBs with Ub probes in cell lysates

Hela cells were lysed with NP-40 lysate (P0013F, Beyotime) supplemented with 1 mM PMSF and incubated on ice for approximately 30 min. Subsequently, the cell debris was removed by centrifugation at 11000 rpm at 4 ℃ for 10 min. Total protein concentration was determined using Bradford assay (Sangon Biotech). Finally Hela cell lysates were diluted to 3mg/mL and stored at -80 ℃ until use. Ub_{75}-N_3 and (biotin)avi-Ub_{75}-N_3 were obtained as described above. Then the probe was diluted to 16 μM in 120 μg cell lysates and incubated at 4 ℃ for 1 h. Immunoblot analysis was performed with anti-OTUB1 antibodies.

7. Pulldown and Mass Spectrometry Analysis

Hela cells were lysated with NP-40 buffer and diluted to 3mg/mL. 1 mM (biotin)avi-Ub1-75-NHNH2 and 1 eq. NaNO2 were reacted in 20 mM MES, 100 mM NaCl, pH=3.0 buffer in ice water bath for 20 min to generated probe 2 in situ, subsequently the pH was adjusted to 6.0. Then the in situ generated Ub acyl azide probe was diluted to 16μM in Hela cell lysates and incubated for another 1 h on ice. Streptavidin agarose beads (Promega) were incubated for 3h at 4 ℃. Next, Magnetic beads were treated with 1 × binding buffer (25 mM Tris, 150 mM NaCl, 0.5% V/V NP-40, pH=7.6), 2 × 0.5% SDS in PBS, 2 × 1 M NaCl in PBS, 2 × Tris-buffered saline and 10 × 50 mM NH_4HCO_3 at pH=8.0 in sequence. The enriched proteins were eluted with 10mM DTT in 2 × loading buffer. Then the samples were resolved by SDS-PAGE and analyzed by LFQ mass spectrometry. Each pull-down experiment was performed in three replicates.

Whole proteomic analysis was performed using LFQ MS. The processing procedures for protein samples have been basically described. Briefly, gel bands of protein samples were digested, followed by disulfide reduction with 25 mM DTT and alkylation with 55 mM iodoacetamide. Sequencing-grade modified trypsin was used for in-gel digestion at 37 °C overnight in 50 mM NH_4HCO_3 buffer. The peptide was extracted twice with 1% TFA in a

1:1 acetonitrile /H_2O (v/v) solution for 30 min and then centrifuged to reduce volume. For LC-MS/MS analysis, peptides were separated by gradient elution using an Easy-NLC 1000 system at a flow rate of 0.300 μL/min for 120 min, directly connected to a Thermo Orbitrap Fusion mass spectrometer. Proteins were identified from their MS spectra for peak detection and quantification using MaxQuant (version 1.5.5.1), supported by the Andromeda search engine and searched in the UniProt human database.

8. Supplemental Figures and Tables

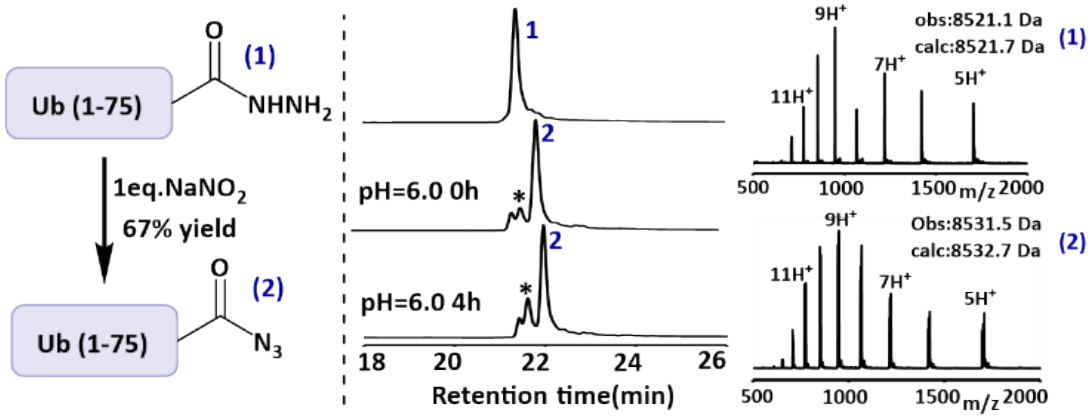

Fig. S1 Analytical RP-HPLC (214 nm) and ESI-MS (Electro Spray Ionization-Mass Spectroscopy) trace for the stability of $Ub_{1\text{-}75}$-N_3. The asterisk indicates the hydrolysis product from acyl azide to carboxylic acid.

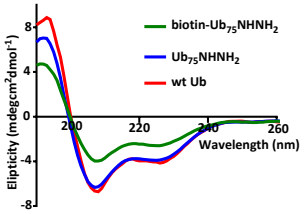

Fig. S2 CD spectra of wildtype Ub in pH=7.4, $Ub_{1\text{-}75}NHNH_2$ in pH=6.0 and (biotin)avi-$Ub_{1\text{-}75}NHNH_2$ in pH=6.0.

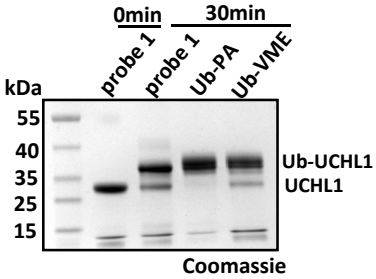

Fig. S3. SDS-PAGE analysis of labelling of UCHL1 by $Ub_{1\text{-}75}$-N_3 (probe 1) with Ub propargylamide (Ub-PA) and Ub vinyl methyl esters (Ub-VME). 8 μM UCHL1 was incubated with 16 μM probe 1, Ub-PA and Ub-VME respectively for 30min. Then the reaction was quenched with 4 × loading buffer without DTT and analyzed by SDS-PAGE.

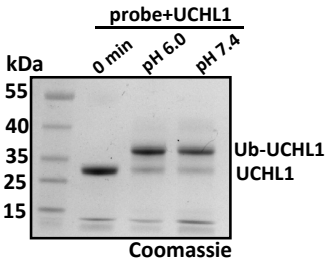

Fig. S4 SDS-PAGE analysis of labelling of UCHL1 by Ub_{1-75}-N_3 in different pH. 1 mM $Ub_{1-75}NHNH_2$ and 1 eq. $NaNO_2$ were reacted in 20 mM MES, 100 mM NaCl, pH=3.0 buffer in ice water bath for 20 min, subsequently the pH was adjusted to 6.0 and 7.4 respectively, and UCHL1 was added to incubate for another 0.5 h. Then the reaction was quenched with 4 × loading buffer without DTT and analyzed by SDS-PAGE.

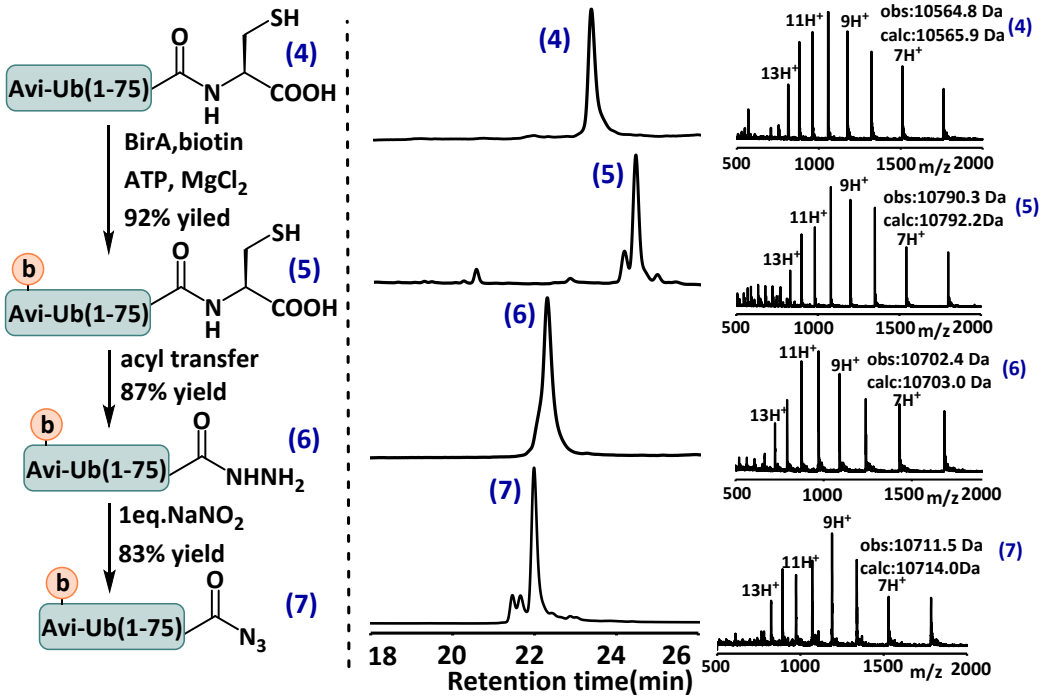

Fig. S5 Analytical RP-HPLC (214 nm) and ESI-MS trace for the preparation of (biotin)avi-Ub_{1-75}-N_3.

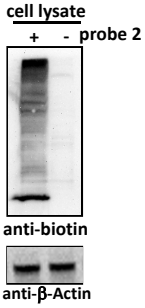

Fig.S6 Hela cell lysate Immunoblotting using probe 2 was performed with an anti-biotin antibody.

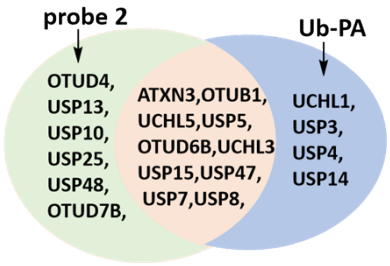

Fig. S7 Venn diagram showing the overlap of significantly enriched DUBs by probe 2 and previous reported Ub-PA probe

	control			probe2		
Gene name	**1**	**2**	**3**	**1**	**2**	**3**
ATXN3	**22.6005**	**22.9318**	**22.5806**	**27.0762**	**27.0626**	**26.5133**
OTUB1	**23.7319**	**22.4476**	**21.4131**	**31.1545**	**31.0145**	**31.0311**
OTUD3	**22.0334**	**22.7709**	**23.6154**	**21.8885**	**22.4986**	**22.8413**
OTUD4	**23.6127**	**21.7414**	**23.4148**	**27.9689**	**27.9615**	**27.8288**
OTUD6B	**23.6558**	**22.0669**	**22.0298**	**27.0716**	**26.8824**	**27.6126**
OTUD7B	**22.9793**	**22.5564**	**22.4769**	**26.3418**	**26.5287**	**26.0348**
UCHL1	**22.7233**	**23.5955**	**22.0881**	**23.9343**	**24.3191**	**23.0906**
UCHL3	**22.8933**	**26.4574**	**26.0797**	**27.4344**	**27.907**	**27.9498**
UCHL5	**23.0623**	**23.7188**	**23.1011**	**28.307**	**28.2967**	**28.2982**
USP10	**24.4176**	**22.2209**	**22.5912**	**25.6339**	**25.2754**	**25.8695**
USP13	**21.5889**	**22.2292**	**23.2566**	**28.2264**	**28.068**	**27.9011**
USP15	**23.0164**	**22.6255**	**23.051**	**26.2344**	**26.2299**	**25.6541**
USP16	**25.3456**	**24.7183**	**25.6224**	**26.2449**	**26.0794**	**26.3224**
USP19	**23.5532**	**23.5561**	**21.7266**	**22.8526**	**24.988**	**25.4274**
USP22	**23.6988**	**24.2799**	**22.8569**	**24.9121**	**23.4984**	**23.5823**
USP24	**24.1958**	**22.8162**	**21.4544**	**22.7037**	**24.3591**	**23.0317**
USP25	**23.1826**	**21.697**	**22.2039**	**25.8519**	**25.2473**	**25.5833**
USP3	**24.3813**	**22.5012**	**22.9543**	**22.5546**	**24.7885**	**22.8092**
USP36	**23.7087**	**22.4442**	**23.1628**	**22.5327**	**23.4122**	**23.0485**
USP47	**22.6398**	**22.3617**	**21.8831**	**24.547**	**25.2071**	**25.3229**
USP48	**24.8405**	**22.2694**	**21.9046**	**26.867**	**27.2078**	**26.8183**
USP5	**24.7655**	**23.3988**	**22.1467**	**29.7449**	**29.8271**	**29.7869**
USP7	**22.3309**	**24.0098**	**23.7354**	**27.3005**	**27.1823**	**27.7697**
USP8	**22.7659**	**21.242**	**22.8421**	**28.7216**	**28.9971**	**28.9034**
USP9X	**23.8034**	**22.0875**	**21.2141**	**22.5591**	**24.4754**	**22.0358**
YOD1	**23.5119**	**22.4362**	**22.0248**	**24.0613**	**21.6047**	**24.1483**

Table S1. Label-free quantification (LFQ) intensity scores for DUBs captured in HeLa cell lysate proteome-wide profiling using 16 μM probe 2 and streptavidin agarose beads were selected as control.