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Supporting Information

# Release of Enzymatically Active Deubiquitinating Enzymes upon Reversible Capture by Disulfide Ubiquitin Reagents

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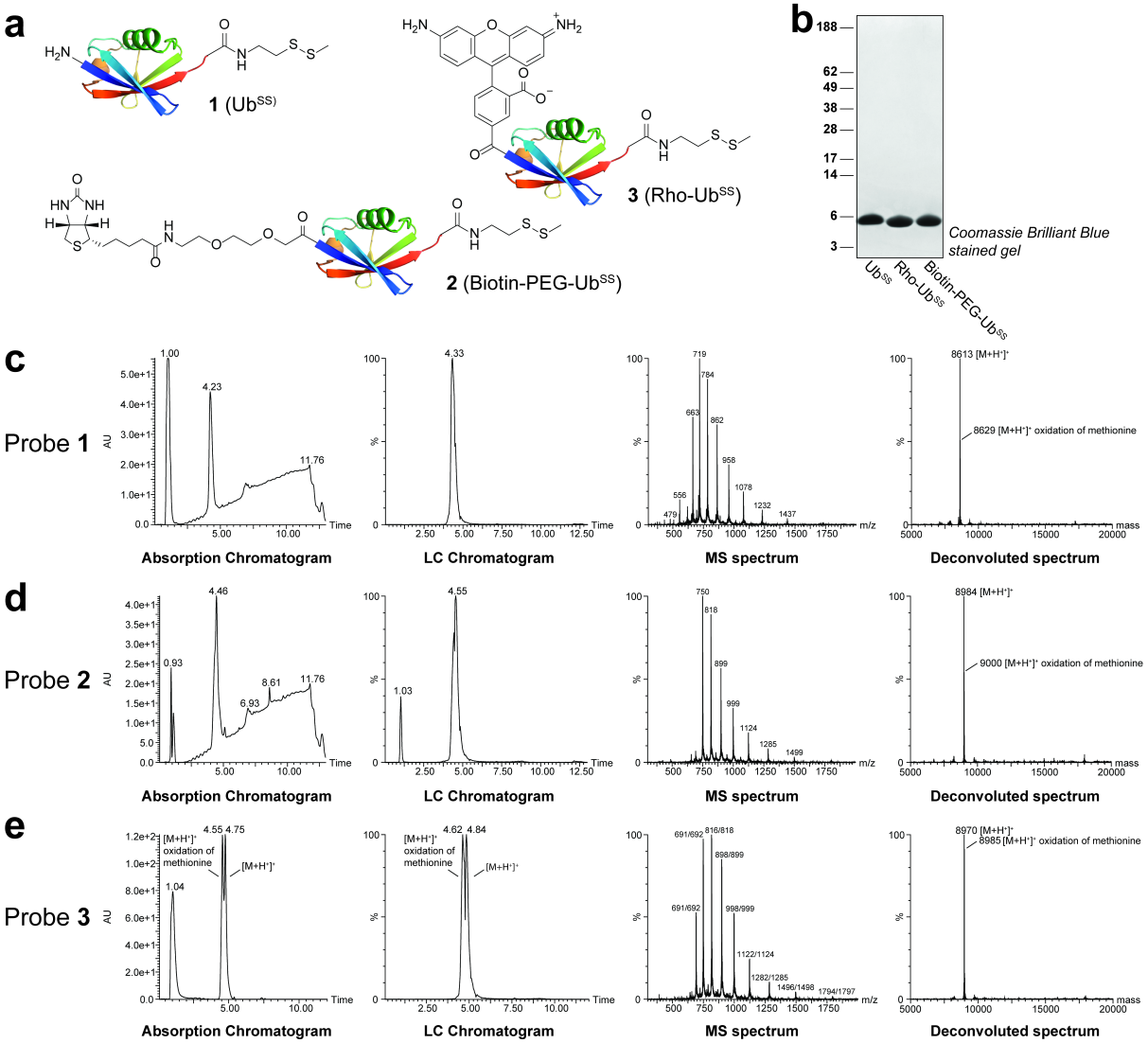
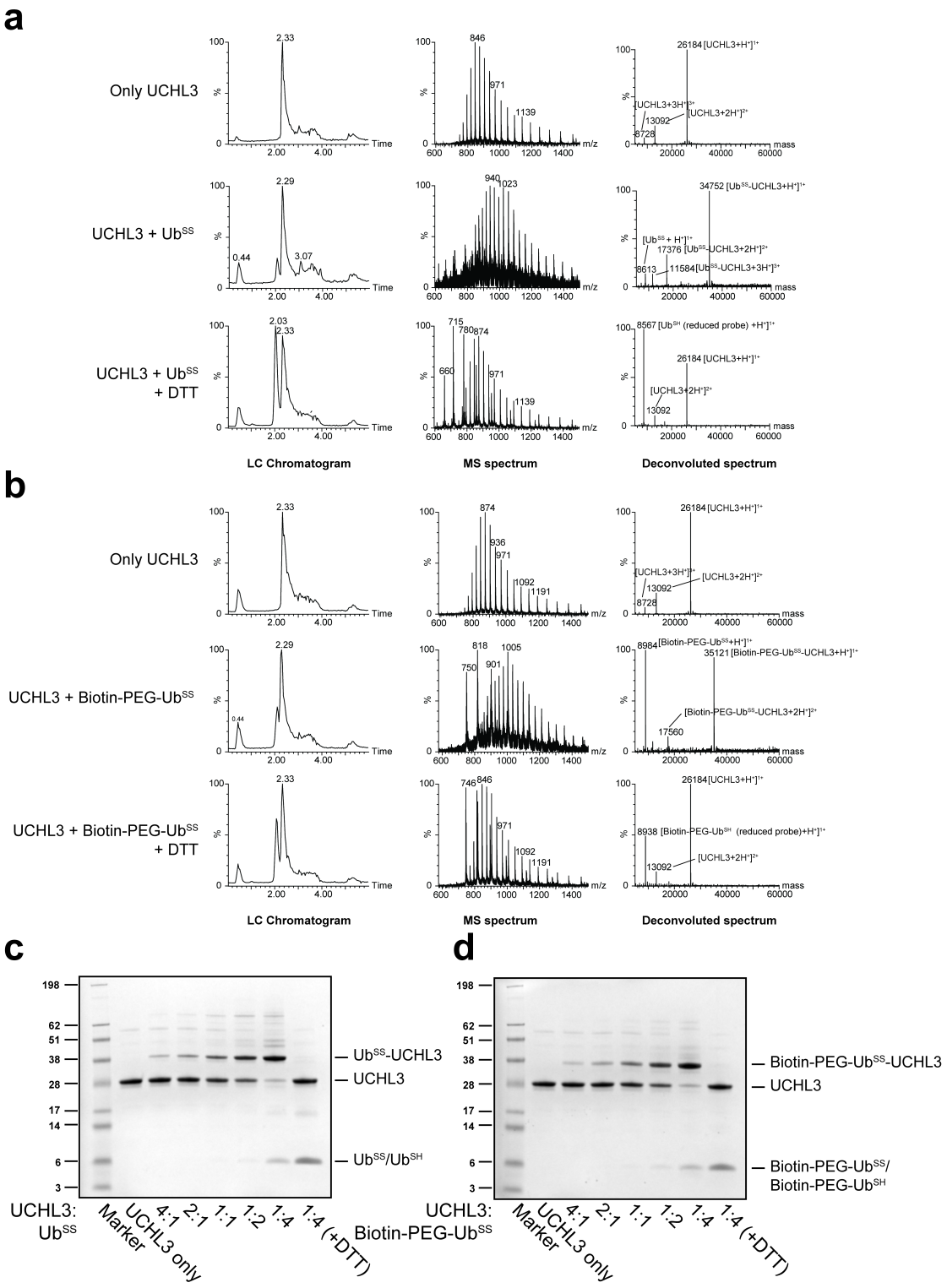
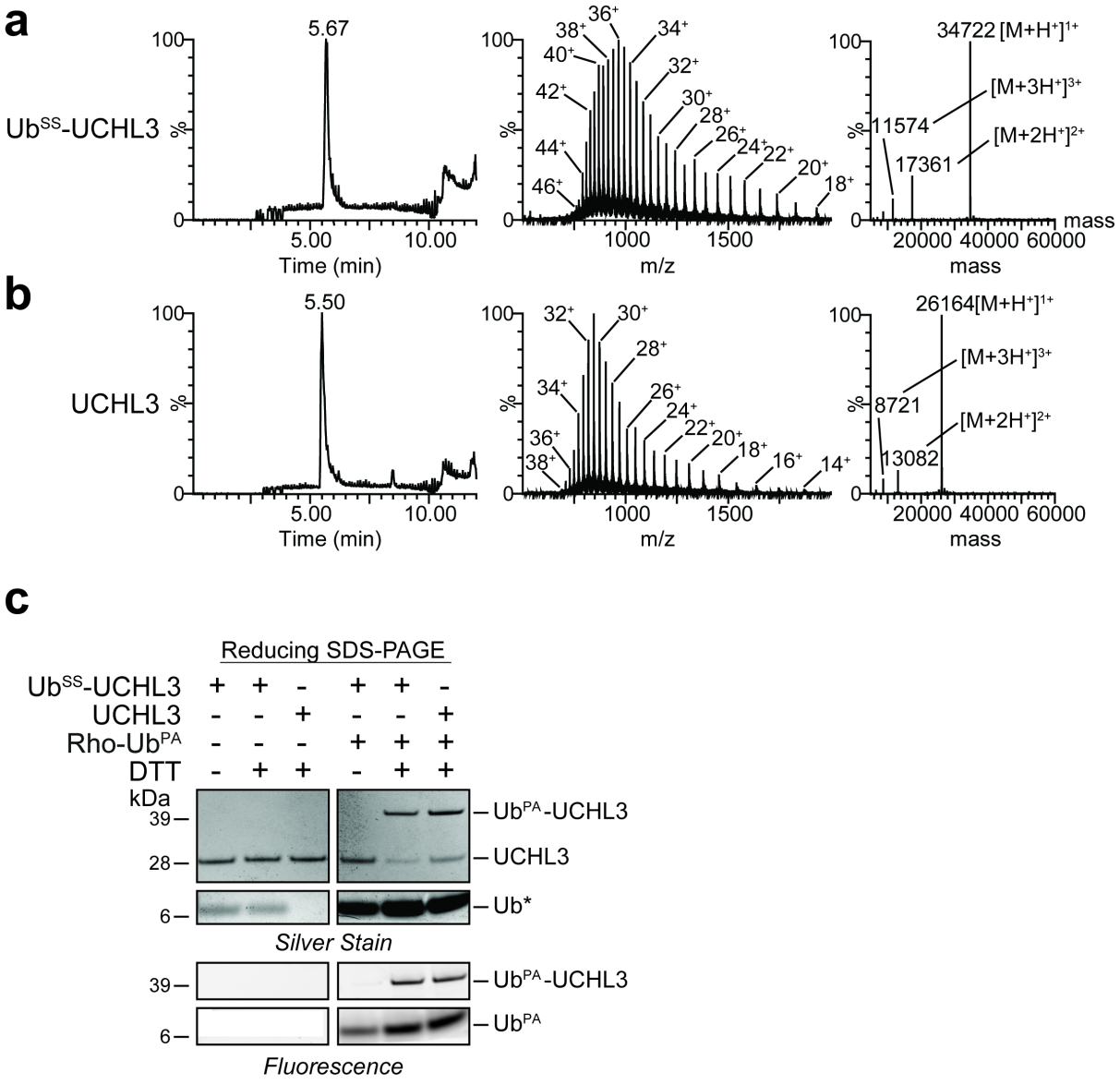
## Supporting Results



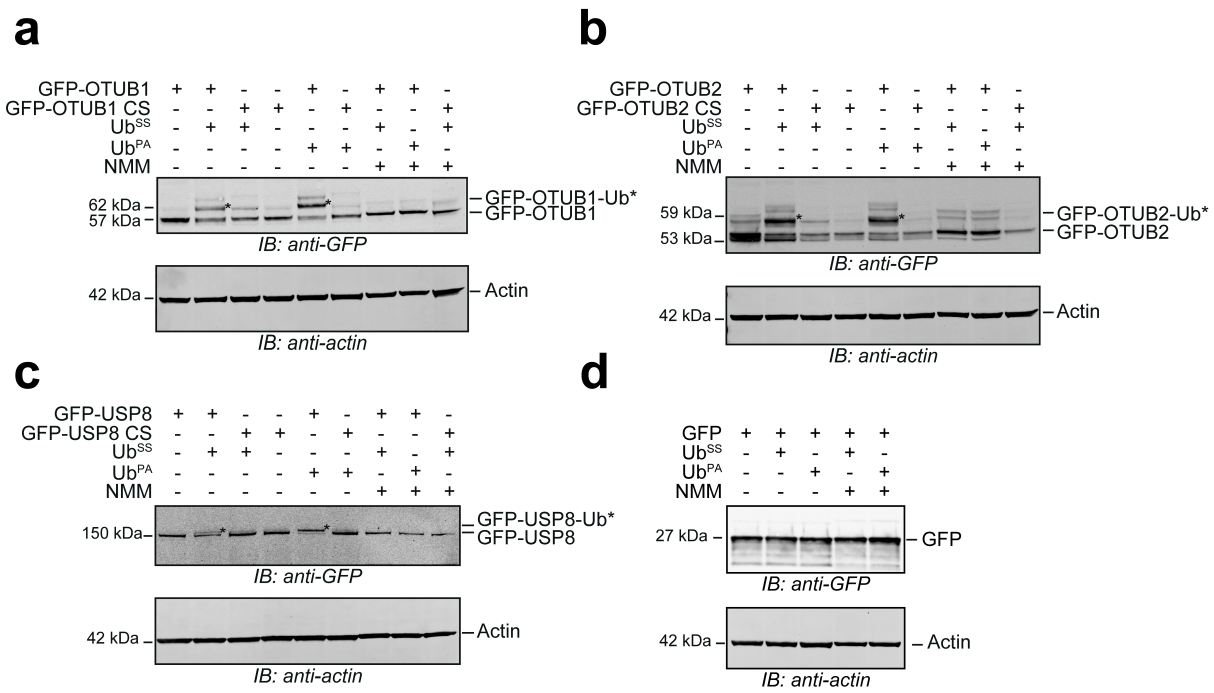

**Supplementary Figure 1:** a) Structures of probes **1** ($Ub^{SS}$), **2** (Biotin-PEG-$Ub^{SS}$) and **3** (Rho-$Ub^{SS}$). The ribbon style protein represents $Ub^{1-75}$. b) SDS-PAGE analysis of purified probes **1** ($Ub^{SS}$), **2** (Biotin-PEG-$Ub^{SS}$) and **3** (Rho-$Ub^{SS}$). c) LC-MS analysis of probe **1**; calculated $[M+H]^+$ 8613 Da, found $[M+H]^+$ 8613 Da. d) LC-MS analysis of probe **2**; calculated $[M+H]^+$ 8984 Da, found $[M+H]^+$ 8984 Da. e) LC-MS analysis of probe **3**; calculated $[M+H]^+$ 8969 Da, found $[M+H]^+$ 8970 Da.



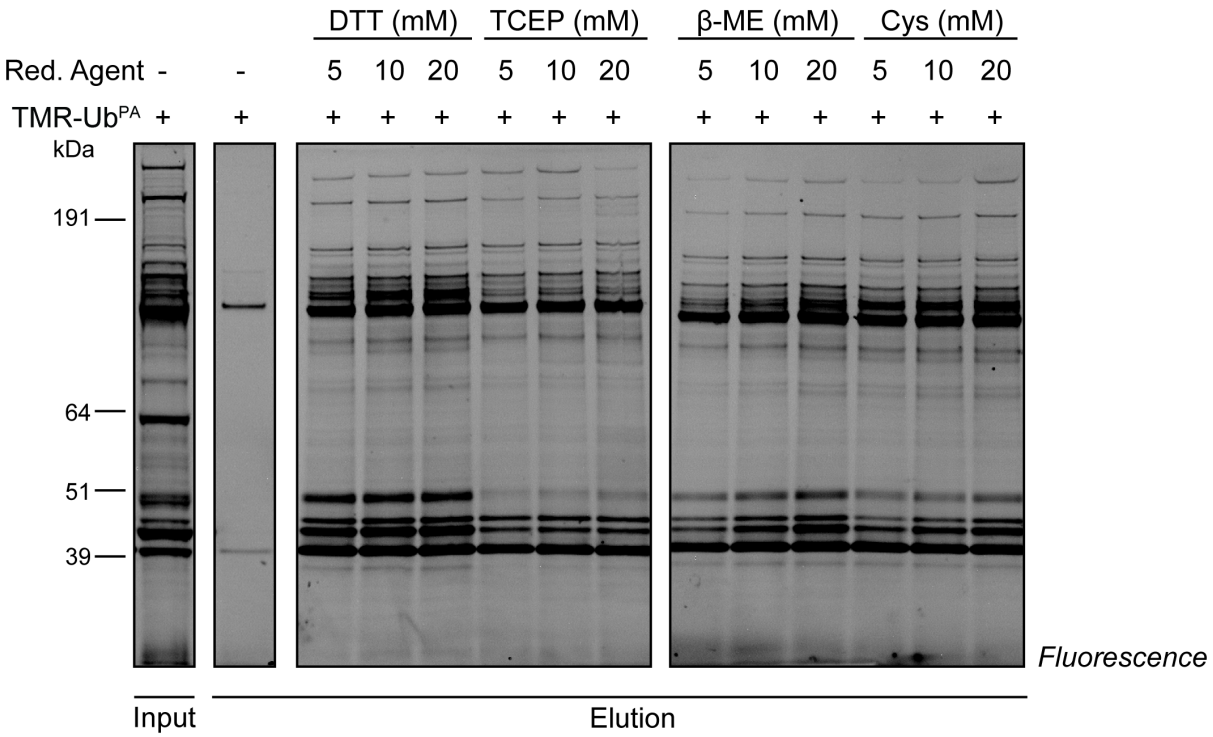

**Supplementary Figure 2:** a) Binding of $Ub^{SS}$ to UCHL3 monitored by LC-MS (molar ratio of UCHL3:$Ub^{SS}$ = 4:1). UCHL3 only is shown as reference. In addition, the incubation mixture was incubated with 10 mM dithiothreitol (DTT). b) Binding of Biotin-PEG-$Ub^{SS}$ to UCHL3 monitored by LC-MS (molar ratio of UCHL3:Biotin-PEG-$Ub^{SS}$ = 4:1). UCHL3 only is shown as reference. In addition, the incubation mixture was incubated with 10 mM DTT. c) Binding of $Ub^{SS}$ to UCHL3 using various molar ratios of UCHL3:$Ub^{SS}$ and additional incubation of DTT. Proteins were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue Staining. d) Binding of Biotin-PEG-$Ub^{SS}$ to UCHL3 using various molar ratios of UCHL3:Biotin-PEG-$Ub^{SS}$ and additional incubation of DTT. Proteins were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue Staining.




**Supplementary Figure 3:** Analytical LC-MS profile of a) $Ub^{SS}$-UCHL3 complex and b) untreated UCHL3, purified by anion exchange chromatography. c) Activity-based protein profiling assay to determine the activity of DTT-treated and untreated purified $Ub^{SS}$-UCHL3 complex and purified UCHL3 using the fluorescent DUB probe Rho-Ub-PA.[1] Proteins were resolved by reducing SDS-PAGE and proteins were visualized using Silver Stain and in-gel fluorescence scanning. The $Ub^{SS}$-UCHL3 complex cannot be visualized using reducing SDS-PAGE as a result of complete reduction of the complex during sample preparation just before electrophoresis. Ub* indicates reduced disulfide probe $Ub^{SH}$ (left panel) or Rho-Ub-PA and $Ub^{SH}$ (right panel).




**Supplementary Figure 4:** GFP-fusion DUBs OTUB1 (a), OTUB2 (b) and USP8 (c), or GFP alone (d), were overexpressed in HEK293T cells, and lysates were incubated with probe $Ub^{SS}$ or Ub-PA[1]. Overexpression of catalytic inactive mutants (CS) and preincubation with N-methyl maleimide (NMM) was used as negative control. Proteins were separated by SDS-PAGE before immunoblotting followed by profiling with the indicated antibodies. DUBs labeled with $Ub^{SS}$ or Ub-PA are marked with an asterisk. In Figures a-c GFP-*"DUB"*-Ub* indicates GFP-*"DUB"* bound to either $Ub^{SS}$ (lane 2) or Ub-PA probe (lane 5). IB: Immunoblot. Anti-actin immunoblotting was used as loading control.




**Supplementary Figure 5:** HeLa cell extract (lane 1) was incubated with the disulfide probe Biotin-PEG-Ub$^{SS}$ and labeled DUBs were captured on neutravidin resin. The captured DUBs were released from the resin using the indicated concentrations of the reducing agents dithiothreitol (DTT) (lanes 3-5), tris-(2-carboxyethyl)phosphine (TCEP) (lanes 6-8), 2-mercaptoethanol (β-ME) (lanes 9-11) and cysteine (Cys) (lanes 12-14). Active DUBs in the input cell extract and elutions were labeled with the DUB activity probe TMR-Ub-PA[1]. As negative control, no reducing agent was added to the elution buffer. Proteins were resolved by SDS-PAGE and fluorescently labeled DUBs were visualized using fluorescence scanning of the SDS-PAGE gel.

## Experimental Section

### General

All chemicals that were used in this project were acquired from Biosolve or Sigma–Aldrich, unless otherwise indicated, and at the highest commercially available grade. Peptide building blocks were purchased from Novabiochem (EMD Millipore) and glycine-functionalized trityl resin (TentaGel R TRT-Gly Fmoc) from Rapp Polymere (Tübingen, Germany). All chemicals and solvents were used as received. Ubiquitin carboxyl-terminal esterase L3 (UCHL3) was recombinantly expressed and purified by Remco Merkx from The Netherlands Cancer Institute, according to published procedures.[2] OTUB2 was expressed and purified by the protein facility of The Netherlands Cancer Institute. $USP7^{1-560}$ was kindly provided by Robbert Kim from the Netherlands Cancer Institute. Labeled and unlabeled Ub-PA probes were synthesized by Remco Merkx and Gerbrand van der Heden-van Noort from the Leiden University Medical Center according to published procedures.[1] GFP-fusion DUBs were kindly provided by Ilana Berlin from the Leiden University Medical Center.

Preparative High-Performance Liquid Chromatography (HPLC) was performed on a AutoPurification HPLC/MS System (Waters) equipped with a 2767 Sample Manager, 2545 Binary Gradient Module, two 515 HPLC pumps, System Fluidics Organizer, a 2998 Photodiode Array Detector (210 to 650 nm), a 3100 mass detector, and an XBridge BEH C18 OBD Prep. Column (130Å, 5 µm, 30 mm x 150 mm) by using two mobile phases: A (water) and B (acetonitrile) with a flow rate of 74.8 mL $min.^{-1}$; 1,5% trifluoroacetic acid (TFA) in acetonitrile was pumped through the system with a flow rate of 2.48 mL $min.^{-1}$. Run time 21 min.; column temp. 20 °C; gradient: 0–2.5 min. 10% B; 2.5–17.5 min. →40% B; 17.5–19.5 min. →95% B; 19.5–20.9 min. 95% B; 20.9–21 min. →5% B. 1.5 %. After column separation, 0.02% of the sample was split and diluted in water (10%) and formic acid (0.1%) in acetonitrile with a flow rate of 1 mL $min.^{-1}$ and sent to the mass detector.

Mass Spectrometry (MS) analysis was performed on a LC-MS system equipped with a Alliance 2795 Separation Module (Waters), 2996 Photodiode Array Detector (190–750 nm), and LCT Orthogonal Acceleration Time of Flight Mass Spectrometer. UCHL3 and probe-UCHL3 complex samples were run over a XBridge BEH C18 Column (130Å, 3.5 µm, 2.1 mm x 30 mm), with flow rate 0.8 mL $min.^{-1}$, runtime 6 min., column temperature 40 °C and two mobile phases: A (acetonitrile (1%) and formic acid (0.1%) in water) and B (water (1%) and formic acid (0.1%) in acetonitrile); gradient: 0–0.2 min. 5% B; 0.2–3.2 min. →95% B; 3.2–4.2 min. 95% B; 4.2-4.4 min. →5% B; 4.4-6.2 min. 5% B. Analytical LC-MS analysis for probes **1** ($Ub^{SS}$), **2** (Biotin-PEG-$Ub^{SS}$) and **3** (Rho-$Ub^{SS}$) and purified UCHL3 and $Ub^{SS}$-UCHL3 complex was performed using a XBridge BEH C18 Column (130Å, 3.5 µm, 2.1 mm x 100 mm), with flow rate 0.4 mL $min.^{-1}$, runtime 13 min., column temperature 40 °C, and two mobile phases: A (acetonitrile (1%) and formic acid (0.1%) in water) and B (water (1%) and formic acid (0.1%) in acetonitrile); gradient: 0–0.4 min. 5% B; 0.4–8 min. →95% B; 8–9.9 min. 95% B; 9.9-10.10 min. →5% B; 10.10-13 min. 5% B.
Data processing was performed with MassLynx Mass Spectrometry Software 4.1 (deconvolution with Maxent1 function; Waters).

Anion exchange chromatography for UCHL3 and $Ub^{SS}$-UCHL3 purification was performed by the NKI Protein Facility on an ÄKTA Micro chromatography system from GE Healthcare using a MiniQ PC 3.2/3 column from GE Healthcare. The protein was eluted using a sodium chloride (NaCl) gradient in Tris (tris(hydroxymethyl)aminomethane) buffer (50 mM, pH 7,5).

Gel electrophoresis (SDS-PAGE) was performed on NuPAGE Novex Bis-Tris Mini-gels (Invitrogen) and run in MOPS or MES buffer at 170 V depending on molecular weight of the protein.

In-gel fluorescence scans were obtained by either using a ProXPRESS 2D Proteomic imaging system (Perkin–Elmer) with filter settings ($\lambda_{ex}/\lambda_{em}$) 550/590 nm (TMR) or by using a Typhoon FLA 9500 (GE

Healthcare) using a 473 nm laser (Rho) or 532 nm laser (TMR) and emission filters of 530 nm (Rho) or 575 nm (TMR).

Immunoblots stained with infrared fluorescent secondary antibodies were visualized by using an Odyssey Infrared Imaging System (LI-COR,Lincoln, NE).

Cleavage of Ub-Rho110 by UCHL3 was monitored using a Clariostar spectrophotometer using filter settings ($\lambda_{ex}/\lambda_{em}$) of 487/535 nm.

**Chemical synthesis of active-site directed Ub-based probes**

Probes **1** ($Ub^{SS}$), **2** (Biotin-PEG-$Ub^{SS}$) and **3** (Rho-$Ub^{SS}$): The amino acid sequence of ubiquitin$^{1-75}$ ($Ub^{1-75}$) was chemically synthesized on trityl resin by following Fmoc solid-phase peptide synthesis using a described procedure[3] with minor adjustments. Double couplings were used for all amino acids and reacted for 25 min. The N-terminal Fmoc protecting group was removed from Fmoc-Ub(1-75) for all three probes by washing the resin with dichloromethane (DCM) (2x) and *N*-Methyl-2-pyrrolidone (NMP) (1x), followed by cleavage with 20% piperidine/NMP (5 x 5 min.). After Fmoc deprotection the resin was washed with NMP and DCM.

For probe **1** ($Ub^{SS}$), the N-terminus was Boc-protected. The resin was suspended in NMP and a solution of di-*tert*-butyldicarbonate (4 equiv.) in NMP and *N*,*N*-Diisopropylethylamine (DIPEA) (4 equiv.) were added. The reaction mixture was shaken for 1 h. and the resin was washed with NMP/DCM (5x). The Boc-protection was repeated one time.

For probe **2** (Biotin-PEG-$Ub^{SS}$), a PEG spacer (8-Fmoc-amino)-3,6-dioxaoctanoic acid, AK Scientific, Inc., Union city, CA), was coupled to the N-terminus using PyBOP (4 equiv.), DIPEA (4 equiv.), (8-Fmoc-amino)-3,6-dioxaoctanoic acid (4 equiv.) in NMP for 25 min. at ambient temperature. The Fmoc protection group was removed by piperidine in NMP (3 x 10 min.) at ambient temperature. Subsequently, biotin was coupled using HBTU (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, 4 equiv.), HOBt (1-hydroxybenzotriazole, 4 equiv.), DIPEA (8 equiv.), and carboxy-functionalized biotin (4 equiv.) (Sigma-Aldrich) in NMP and reacted for 2.5 h.

For probe **3** (Rho-$Ub^{SS}$), ***N,N′***-Boc-protected 5-carboxyrhodamine 110 was coupled to the N-terminus using PyBOP (4 equiv.), DIPEA (4 equiv.), ***N,N′***-Boc-protected 5-carboxyrhodamine 110 (4 equiv.) in NMP and reacted for 16 h. at ambient temperature.

The protected sequences (~20 µmol or ~40 µmol) were cleaved from the resin with hexafluoroisopropanol (HFIP)/DCM (1:4 (v/v)) 2 x 20 min.) and the filtrates were collected. The reaction mixture was evaporated and the residues were co-concentrated with dichloroethane (3x) to obtain the protected sequences as clear oils. After cleavage the protected sequences were dissolved in 4 mL DCM and 2-(methyldisulfanyl)ethan-1-amine (5 equiv.), PyBOP (5 equiv.) and triethylamine (10 equiv.) were added. 2-(methyldisulfanyl)ethan-1-amine was synthesized according to published procedures.[4] The resulting reaction mixtures were stirred overnight at ambient temperature. The reaction mixtures were evaporated to dryness and a mixture of trifluoroacetic acid/water/triisopropylsilane/phenol (90:5:2.5:2.5 (v:v:v:v) ) (10 mL) was added. After stirring for 3.5 h. at ambient temperature, the reaction mixture was precipitated by slow addition of the reaction mixture to ice-cold ether/pentane (3:1). The suspension was centrifuged and the pellet was washed with ice-cold ether (2x). The resulting pellet was dissolved in water/acetonitrile/acetic acid (65:25:10 (v:v:v)), frozen, and lyophilized. The resulting probes were purified using preparative HPLC, and lyophilized. SDS-PAGE and LC-MS analyses of the probes are shown in Figure S2. The resulting powders were dissolved in dimethylsulfoxide (DMSO) and stored at -20 °C.

**Cell culture and preparation of cell extracts**

HeLa, HEK293T and EL4 cell lines used in this study originated from ATCC and were grown in Gibco EMDM medium or Gibco RPMI 1640 medium (Life Technologies) medium (for EL4 cells) supplemented with FCS (10 %) at 37 °C in a 5% $CO_2$ atmosphere.

Cells were lysed by sonication in lysis buffer (Tris (50 mM), sucrose (250 mM), magnesiumchloride (5 mM) supplemented with CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate, 0.5%) and nonidet P-40 (0.1%), clarified by spinning (16 000 g,10 min., 4 °C) and removal of the pellet unless stated otherwise.

**Binding of disulfide probes to recombinant DUBs**

UCHL3 (0.3 nmol, 12 µM) depleted from reducing agents was incubated with probe **1** ($Ub^{SS}$) or probe **2** (Biotin-PEG-$Ub^{SS}$) (0, 3, 6, 12, 24, 48 µM) in Tris buffer (50 mM, 100 mM NaCl, pH 7.5) for 45 min. at ambient temperature. One extra sample of 12 µM UCHL3 and 48 µM of probe **1** ($Ub^{SS}$) and **2** (Biotin-PEG-$Ub^{SS}$) was prepared and incubated with 10 mM of DTT (10 min.). Before LC-MS analysis, 2 µL aliquots were taken to which we added non-reducing sample buffer (Invitrogen) (no boiling) for non-reducing SDS-PAGE-analysis.

OTUB2, UCHL3 and $USP7^{1-560}$ (1 µM) were incubated with probe **3** (Rho-$Ub^{SS}$, 5 µM) in Tris buffer (50 mM, 100 mM NaCl, pH 7,5) for 30 min. at ambient temperature and either treated with 10 mM of DTT (10 min., ambient temperature) or left untreated. Non-reducing sample buffer was added (Invitrogen) and labeling was monitored by non-reducing SDS-PAGE, followed by in-gel fluorescence scanning. As control, the corresponding unlabeled DUBs were taken along.

**Activity assays using purified $Ub^{SS}$-UCHL3 disulfide complex**

46 µM UCHL3 was incubated with 116 µM $Ub^{SS}$ for 60 min. at 37°C (2% DMSO). As reference, UCHL3 was incubated with 2% DMSO. Both UCHL3 and $Ub^{SS}$-UCHL3 were purified by anion exchange chromatography and analytical LC-MS was performed. The complex was either left untreated or treated with 10 mM of DTT. DUB activity was monitored using a Ub-Rho110 cleavage assay[5] and a DUB ABP assay using Rho-Ub-PA.[1] The Ub-Rho110 assay was performed using 200 nM of Ub-Rho110 substrate and 0,06 nM of UCHL3, $Ub^{SS}$-UCHL3 complex or DTT-treated $Ub^{SS}$-UCHL3 complex in Tris-NaCl buffer (50 mM, pH 7.5). The Rho-Ub-PA assay was performed using 90 nM of UCHL3, $Ub^{SS}$-UCHL3 complex or DTT-treated $Ub^{SS}$-UCHL3 complex in Tris-NaCl buffer (50 mM, pH 7.5) and 4 µM of Rho-Ub-PA and incubated for 40 min. at 37°C. Samples were either treated with non-reducing sample buffer or reducing sample buffer containing β-mercaptoethanol and resolved by either reducing or non-reducing SDS-PAGE. Fluorescently labeled proteins were visualized by in-gel fluorescence scanning and subsequently the gels were Silver Stained.

**DUB inhibition in cell extract**

HeLa cells were lysed as described above and the cell extract (1 mg/mL) was incubated with 1 µM of $Ub^{SS}$ for 1 h. at ambient temperature in or without the presence of DTT. Subsequently, 1 µM of DUB activity-based probe TMR-Ub-PA was added and incubated for 15 min. at ambient temperature.

**Binding of $Ub^{SS}$ to GFP-fusion DUBs in cell extract**

For overexpression of GFP-fusion DUBs in HEK293T cells, the previously described constructs for wild-type and catalytically inactive OTUB1, OUTB2, and USP8 were used.[1] DNA was transfected into HEK293 cells using polyethylenimine (PEI, Polysciences, Inc.) according to the manufacturer's instructions. After 24 h. of expression, cells were harvested by scraping into ice cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.5% TritonX-100) supplemented with Complete Protease Inhibitor (Roche) followed by brief sonication on ice. Clarified lysates were incubated with $Ub^{SS}$ or Ub-PA[1] (25 or 50 µM, 30-45 min. at room temperature). As a negative control, lysates were preincubated with 10 mM *N*-methyl maleimide (NMM) before labeling with probe. The reaction was quenched by addition of SDS-PAGE loading buffer without reducing agents. Samples were resolved using SDS-PAGE, followed by transfer onto nitrocellulose membranes and immunoblotting with anti-GFP serum[6]

and β-actin antibody as indicated. Infrared fluorescent secondary antibodies from LiCOR were used for visualization of the labeled enzymes on the LiCOR Odyssey system 3.0.

**Pull down of active DUBs using probe Biotin-PEG-Ub$^{SS}$ and various reducing agents**

HeLa cells were lysed by sonication in lysis buffer (Tris (50 mM), sucrose (250 mM), $MgCl_2$ (5 mM), pH 7.5, supplemented with CHAPS (0.5%) and nonidet P-40 (0.1%)), clarified by spinning (16 000 g,10 min., 4 °C) and removal of the pellet and diluted to 5 mg/mL. 3.9 µM Biotin-PEG-Ub$^{SS}$ was incubated with 5 mg/mL lysate for 75 min. at 37°C (0.2% final DMSO concentration). 5 mg/mL lysate was incubated with 0.2% DMSO as negative control. Pre-equilibrated High Capacity Neutravidin Agarose resin (Thermo Fisher Scientific) was added to the incubation mixtures and rotated for 16 h. at 4°C. The resins were washed with Tris buffer (50 mM, 100 mM NaCl, pH 7.5) (6 x) and divided into small samples. The resins were treated with elution buffer (50 mM Tris, 100 mM NaCl, pH 7.5) containing various concentrations of the reducing agents DTT, TCEP, β-mercaptoethanol and cysteine or no reducing agent. The DUB ABP TMR-Ub-PA[1] was included in the elution buffers. The negative control resin was treated with Tris buffer without reducing agents to which TMR-Ub-PA was added. Reducing sample buffer was added to the elutions and active DUBs were visualized by resolving the proteins by reducing SDS-PAGE followed by in-gel fluorescence scanning.

## References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