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High-affinity free ubiquitin sensors for quantifying ubiquitin homeostasis and deubiquitination

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tIVR

MGSSHHHHHHSSGLVPRGSHMKQEVQAWDGEVRQVSKHAFSLKQLDNPARIPPSG
WKCSKCDMRENLWNLTDGSILCGRRYFDGSGGNNHAVEHYRETGYPLAVKLGITP
DGADVYSYDEDDMVLDPSLAEHLSHFGIDMLKMQKGSCAAEEAELDLKAAIQESLRE
AGGGSDLLCKKGCGYYGNPAWQGFCSKWCWREEYHKARQK

tISR

MGSSHHHHHHSSGLVPRGSHMKQEVQAWDGEVRQVSKHAFSLKQLDNPARIPPSG
WKCSKCDMRENLWNLTDGSILCGRRYWDGSGGNNHAVEHYRETGYPLAVKLGITP
PDGADVWSYDEDDMVLDPSLAEHLSHFGIDMLKMQKGSCAAEEAEEQIAYAMQMSL
REAGGGSDLLCKKGCGYYGNPAWQGFCSKWCWREEAHKAAQK

tUI

MPSSHHHHHHSSGLVPRGSHMGSTVRFQQQLEQLSAMGFLNREANLQALIATCGDIN
AAIERLLGSGSEVQRQVSKHAFSLKQLDNPARIPPSGWKCSKCDMRENLWNLTDGSIL
CGRRYFDGSGGNNHAVEHYRETGYPLAVKLGITITPDGADVYSYDEDDMVLDPSLAEH
LSHFGIDMLKMQK

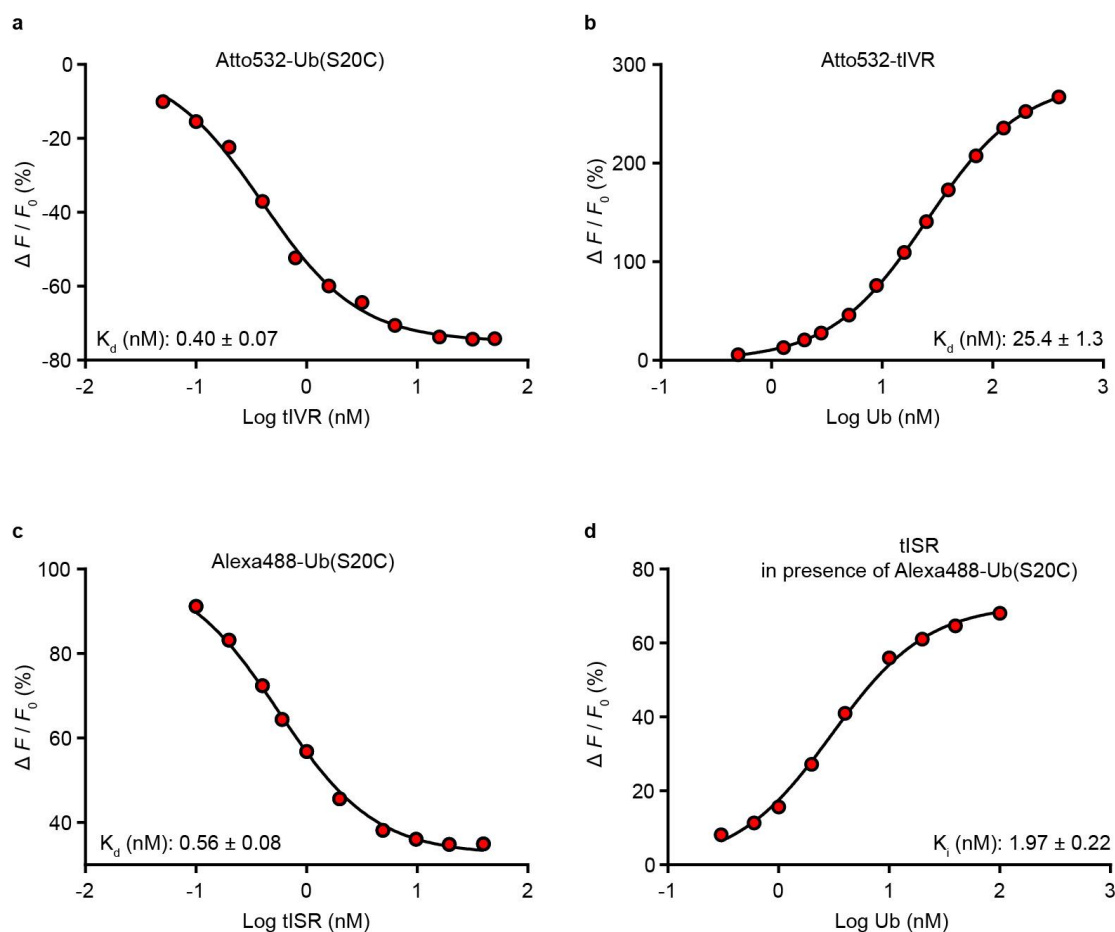
tUI-HA

MPSSHHHHHHSSGLVPRGSHMGSTVRFQQQLEQLSAMGFLNREANLQALIATCGDIN
AAIERLLGSGSEVQRQVSKHAFSLKQLDNPARIPPSGWKCSKCDMRENLWNLTDGSIL
CGRRYFDGSGGNNHAVEHYRETGYPLAVKLGITITPDGADVYSYDEDDMVLDPSLAEH
LSHFGIDMLKMQKCGSGSGYPYDVPDYAS

Supplementary Figure 1

Primary sequences of the free Ub sensors

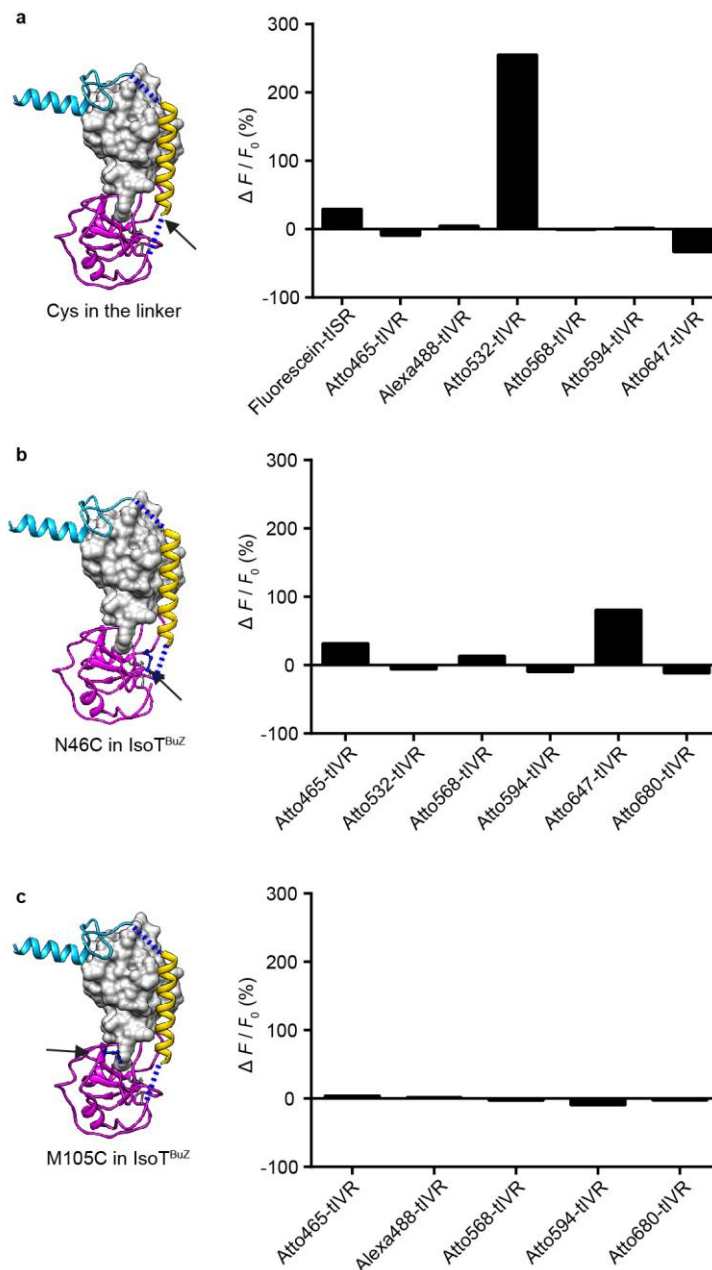
The bold, underlined residues indicate mutated residues, and the cysteines in red indicate fluorophore conjugation sites. The residues highlighted in cyan are linkers introduced to connect the domains, and the yellow and green highlighted sequences show peptides added to provide 6-His and HA-epitope tags, respectively.



Supplementary Figure 2

Sensor binding assays

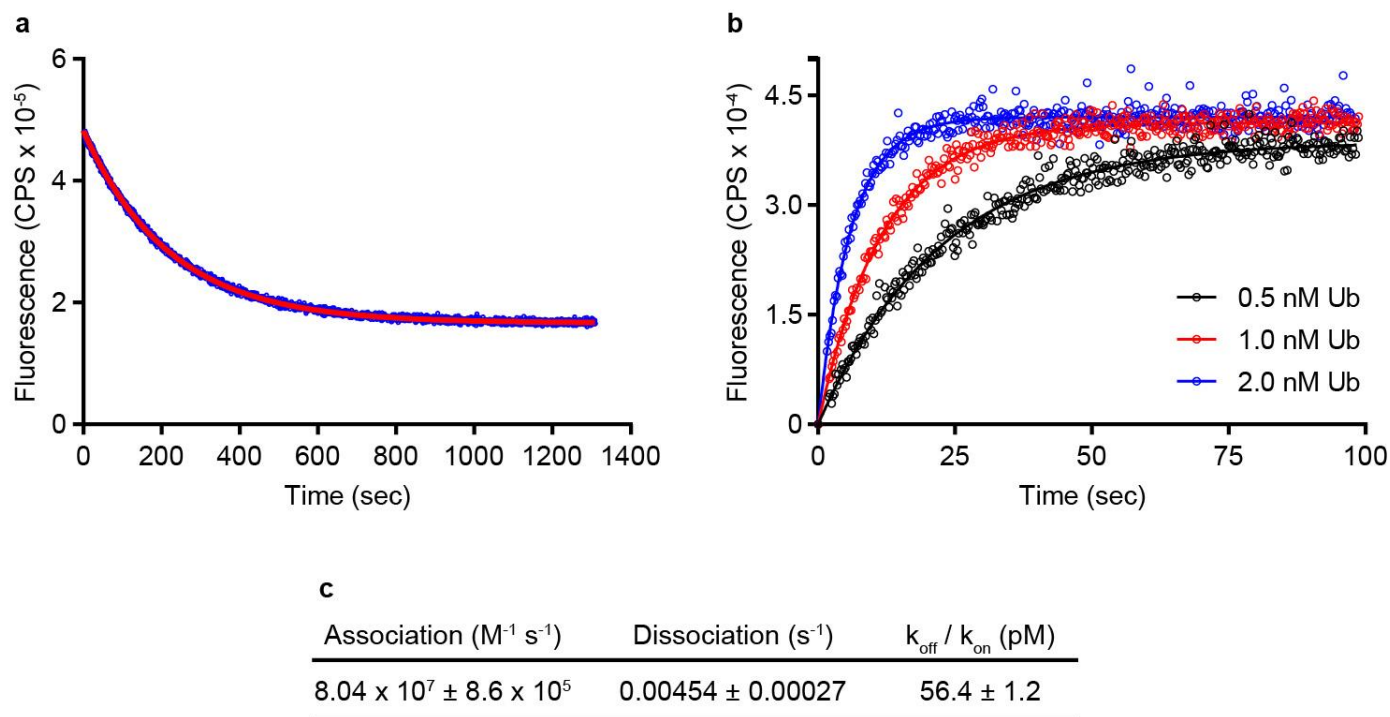
a, Atto532-Ub(S20C) titrated with tIVR. **b**, Atto532-tIVR titrated with Ub. **c**, Alexa488-Ub(S20C) titrated to tISR. **d**, tISR competition binding assay performed by titration of Ub in presence of Alexa488-Ub(S20C). In each titration, the fluorescence intensity change from ligand binding was measured. Error bars in **a**, which are mostly hidden by the point symbols, show standard deviations of the assay repeated two times independently; titrations in **b** - **d** were performed once. K_d values $\pm$ s.d. shown in each panel were determined from non-linear fits to the data.



Supplementary Figure 3

Screening for an optimal fluorescent dye and conjugation site on the tIVR sensor

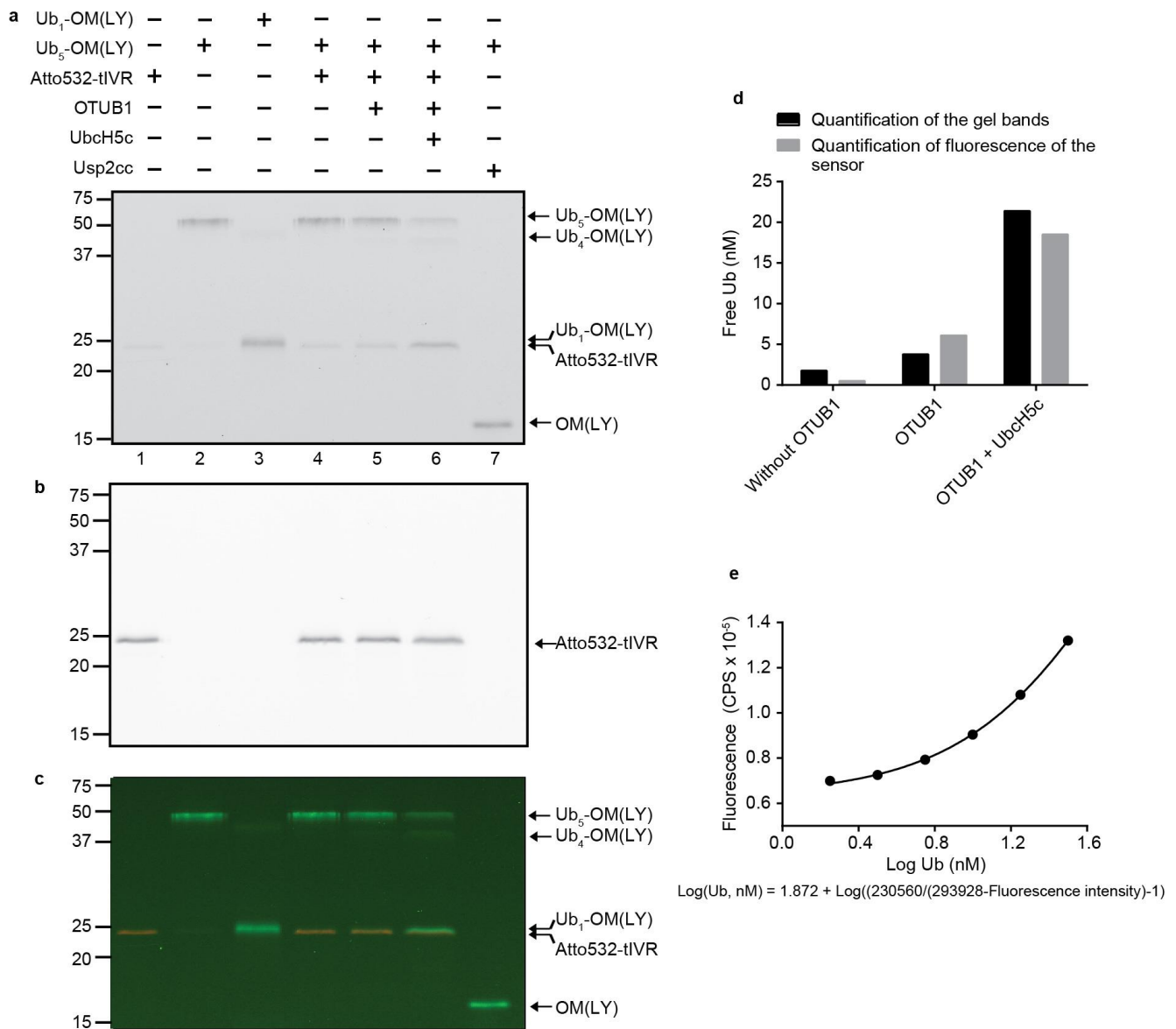
a, Cys residue in the linker connecting IsoT^{Buz} and Vps27^{UIM}; **b**, N46C; and **c**, M105C in IsoT^{Buz} were selected to conjugate various fluorescent dyes. In each panel, the arrow shows the conjugation site on the tIVR model and the histograms show performance with different fluorophores installed at that site. To determine which site and fluorescent dye gave the largest fluorescence change upon Ub binding, fluorescence intensities of the labeled sensors (2 nM) were measured with and without saturating Ub. F_0 is fluorescence intensity of the labeled sensors without Ub, and ΔF is the intensity change upon addition of 200 μ M Ub.



Supplementary Figure 4

Kinetics of Atto532-tUI binding to Ub

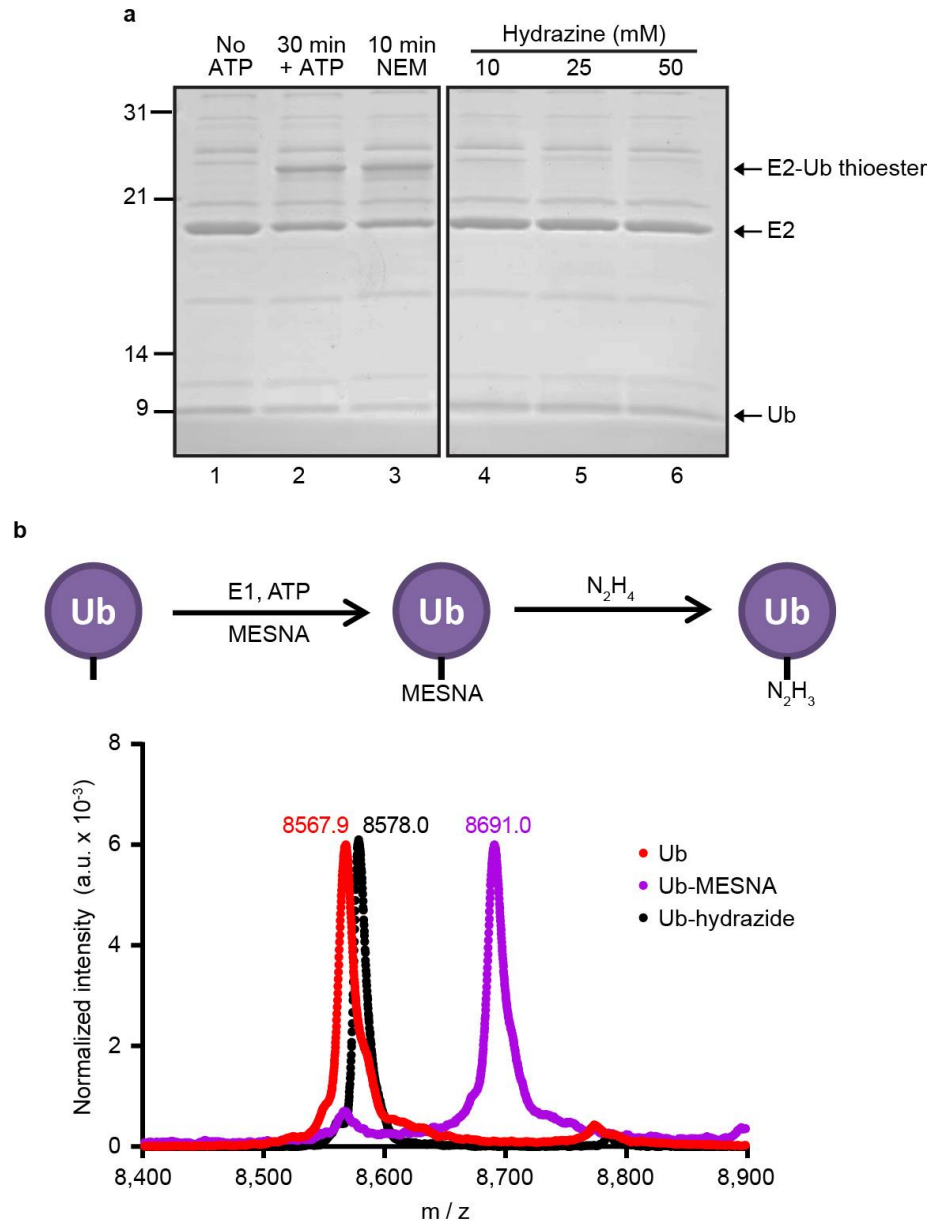
a, To determine the dissociation rate of the Atto532-tUI•Ub complex, Atto532-tUI (1.0 nM) was pre-incubated at 25 °C with 1.0 nM Ub, and then excess unlabeled tUI (1 μ M) was rapidly added and the fluorescence was measured every 0.25 s using a stopped-flow fluorimeter. Three independent reactions were performed, and all the data were fit with a single-exponential decay model to determine k_{off} . The experimental data (blue) are shown superimposed with the fitted curve (red). **b**, The change in fluorescence intensity of 50 pM Atto532-tUI immediately after addition of 0.5, 1.0, or 2.0 nM Ub was monitored at 25 °C. The fluorescence was measured every 0.25 s. From each curve, an observed rate (k_{obs}) was determined from the best-fitting exponential curve, and from these k_{on} was determined from the equation $k_{obs} = k_{on} \times [Ub] + k_{off}$. Each reaction to generate the association data was performed once. **c**, The experimentally determined association and dissociation rates $\pm$ s.d. for the Atto532-tUI•Ub complex are shown together with the $K_d \pm$ s.d. calculated from their ratio $\pm$ s.d.



Supplementary Figure 5

Analysis of the DUB reaction products by SDS-PAGE

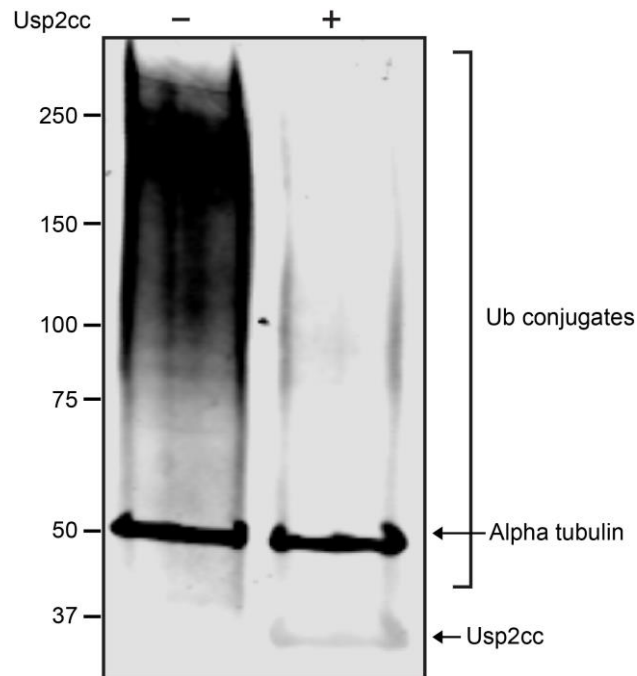
Samples from the end-points of the real-time DUB reactions in Fig. 2 were separated by SDS-PAGE together with the following control samples: Atto532-tIVR in lane 1, Ub₅-OM(LY) in lane 2, Ub₁-OM(LY) in lane 3, and the Usp2cc-digested product in lane 7. Fluorescent gel bands were detected with a Typhoon laser scanner using excitation at either **(a)** 488 nm, **(b)** 532 nm, or **(c)** both to visualize Lucifer Yellow-labeled ovomucoid protein (OM(LY)) or Atto532-labeled tIVR. **d**, The bar graph compares the Ub released in the DUB reactions in Fig. 2 quantified by either the protein band fluorescence (panel **a**) or the sensor fluorescence (Fig. 2). **e**, Atto532-tIVR (2 nM) was titrated with Ub (2 - 32 nM) and the data were fit with a 1:1 binding model as described in Methods. The fitted equation was used to convert fluorescence intensities from the real-time DUB reaction curves (Fig. 2, left panel) to free Ub concentrations (Fig. 2, right panel).



Supplementary Figure 6

Generation of Ub-hydrazide from Ub thioesters

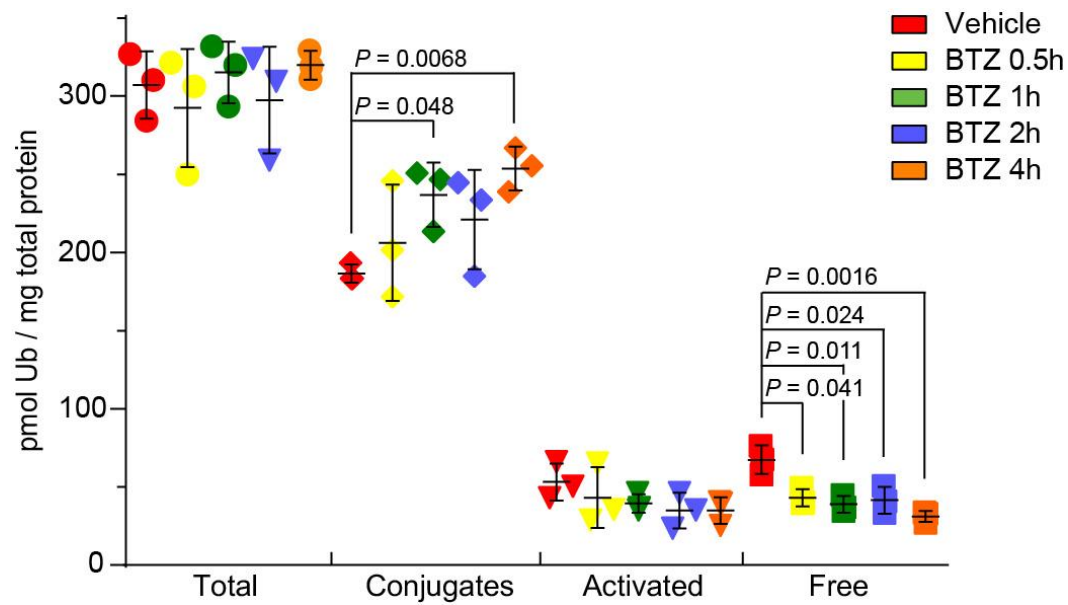
a, SDS-PAGE was used to monitor E2-Ub thioester formation and subsequent hydrazinolysis. Ub was incubated with E1 and E2 (UbchH5c) enzymes without (lane 1) or with (lane 2) ATP to form Ub~UbchH5c thioester. After treatment with NEM (lane 3) to inactivate enzymatic activities, hydrazine was added at the concentrations indicated; lanes 4 – 6 show that hydrazinolysis appeared to be complete (i.e., E2-Ub thioester was gone) under all three conditions. **b**, Upper scheme shows the reaction to generate Ub-hydrazide (see Methods) used in Fig 1b-d titrations. Lower panel shows overlaid MALDI-TOF mass spectra of Ub and products (i.e., Ub-MESNA and Ub-hydrazide) from each step in the synthesis. The results in **a** and **b** are representative of 3 independent experiments.



Supplementary Figure 7

Processing cell lysates with Usp2cc releases most conjugated Ub

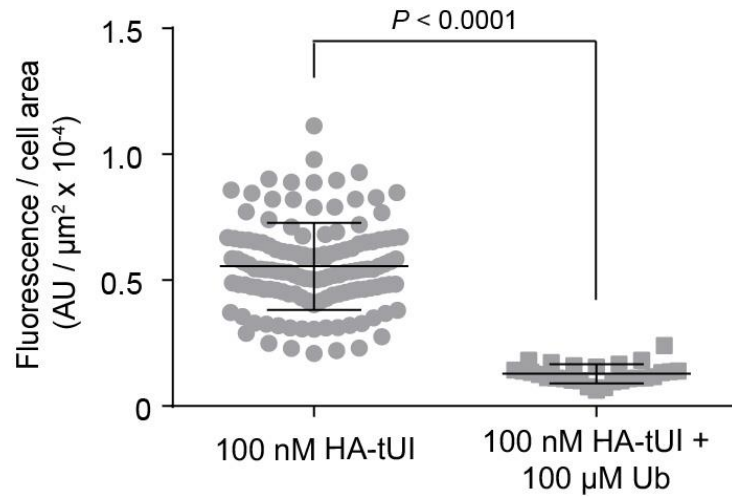
SDS-PAGE and immunoblotting with anti-Ub antibody (P4G7) shows the levels of conjugated Ub in HeLa cell lysates prepared as described in the Methods for in-solution measurements of Ub pools. The lysate was generated and incubated without or with Usp2cc for 1 h at 37 °C as described in Methods. This analysis was performed once.



Supplementary Figure 8

Ub pools in proteasome-inhibited HeLa cells do not change significantly after 1 h

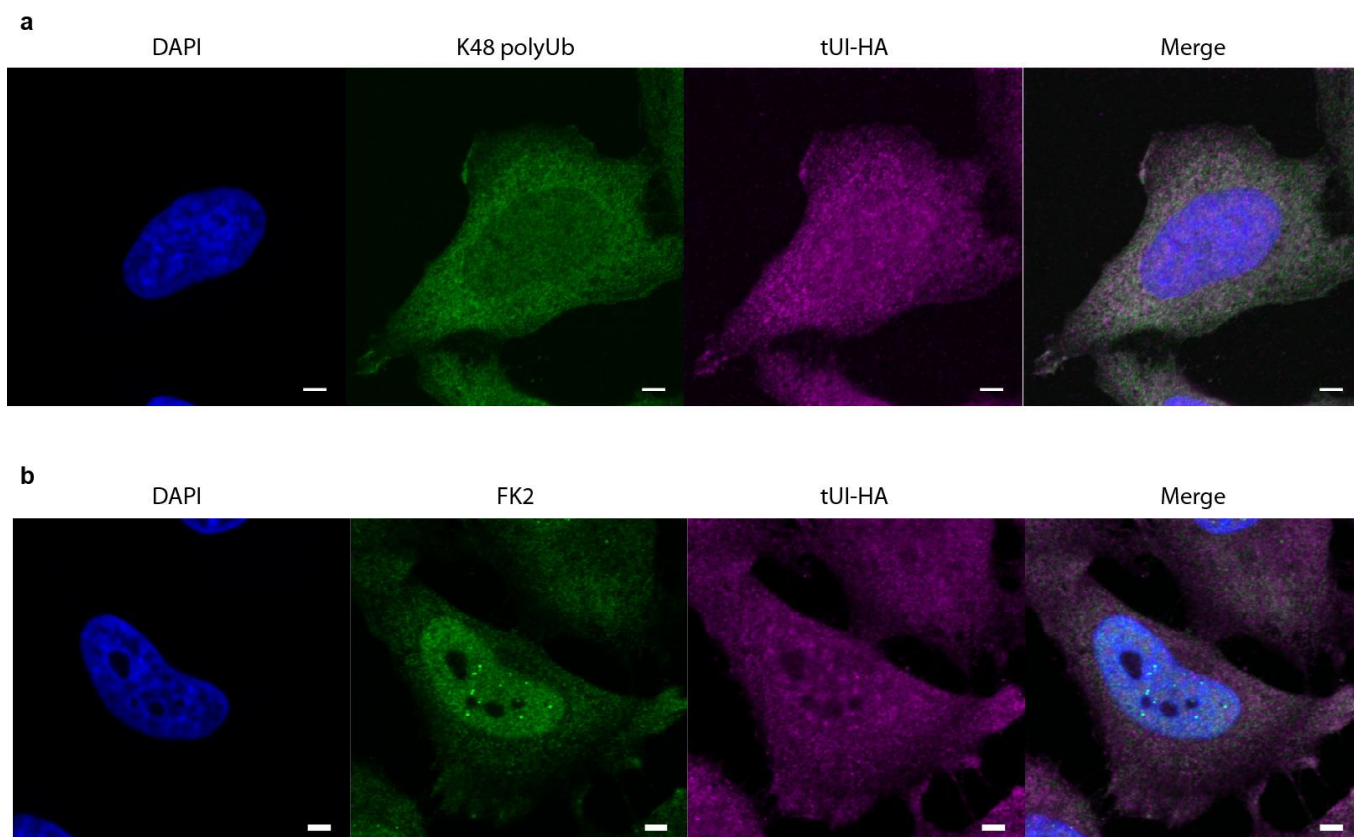
In-solution quantification of Ub pools in lysates after treatment with vehicle (DMSO) or the proteasome inhibitor, BTZ (1 μ M). Statistical analysis of the sample mean was by one-way ANOVA with Bonferroni's adjustment; error bars represent $\pm$ s.d. ($n = 3$).



Supplementary Figure 9

Competition by free Ub for cell staining by HA-tUI

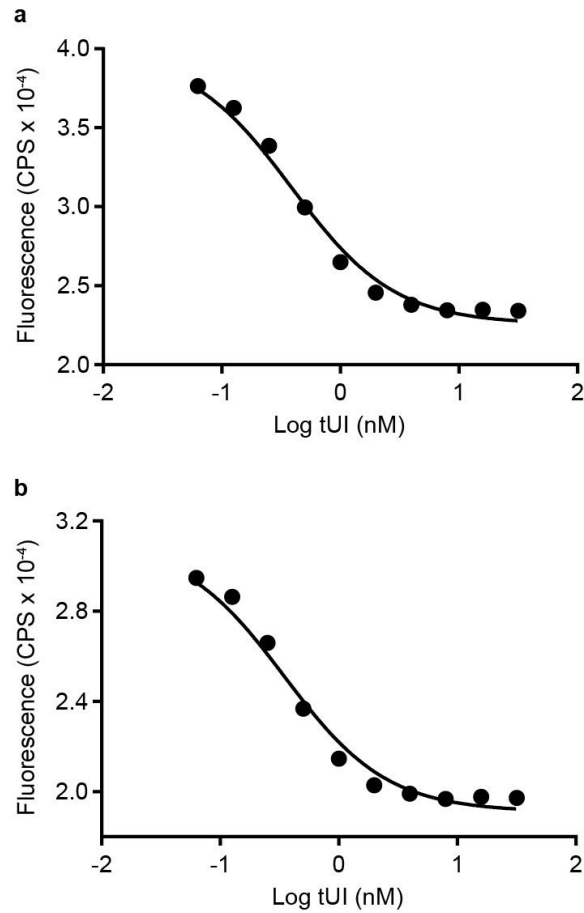
Quantitation of mean fluorescence in HeLa cells (maximum projection images) after incubation with HA-tUI with or without excess free Ub. For 100 nM HA-tUI, $n = 131$; for 100 nM HA-tUI + 100 μM Ub, $n = 28$. AU, arbitrary units; error bars indicate mean $\pm$ s.d. Statistical analysis used two-tailed unpaired Student's *t*-test with Welch's correction.



Supplementary Figure 10

Free Ub staining by HA-tUI shows a different subcellular distribution than K48-linked polyUb or total conjugated Ub

HeLa cells were fixed with 4% PFA and stained with DAPI (blue), anti-K48 polyUb antibody (green), and HA-tUI (magenta) in panel **a**, or DAPI (blue), anti-Ub FK2 antibody (green), and HA-tUI (magenta) in panel **b**. For each cell, the images are from a single z-stack plane that shows both nucleus and cytoplasm. The cells shown are representative of more than 10 cells analyzed on each of two coverslips.



Supplementary Figure 11

Competition binding assays to measure tUI affinity for Ub

Competition binding assays were performed with **a**, 70 pM Atto532-tUI or **b**, 50 pM Atto532-tUI in the presence of 80 pM Ub. tUI was titrated from 0.063 nM to 32 nM, from which a mean K_i of 194 pM was determined from fittings to the two independent data sets.