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

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Ubiquitin (Ub) conjugation is an essential post-translational modification that affects nearly all proteins in eukaryotes. The functions and mechanisms of ubiquitination are areas of extensive study, and yet the dynamics and regulation of even free (that is, unconjugated) Ub are poorly understood. A major impediment has been the lack of simple and robust techniques to quantify Ub levels in cells and to monitor Ub release from conjugates. Here, we describe avidity-based fluorescent sensors that address this need. The sensors bind specifically to free Ub, have dissociation constant \( K_d \) values down to 60 pM and, together with a newly developed workflow, allow us to distinguish and quantify the pools of free, protein-conjugated and thioesterified forms of Ub from cell lysates. Alternatively, free Ub in fixed cells can be visualized microscopically by staining with a sensor. Real-time assays using the sensors afford unprecedented flexibility and precision to measure deubiquitination of virtually any (poly)Ub conjugate.

In ubiquitination, free Ub (here referring to unconjugated monoUb or a polyUb chain with a free C terminus) is activated by formation of a C-terminal thioester first with E1 Ub-activating enzyme and then an E2 Ub-conjugating enzyme before it is transferred to substrates, usually to form an isopeptide bond with a protein lysine ε-amine. Thus, cells contain three classes of Ub: free, thioester-activated and (iso)peptide conjugated. Because ubiquitination contributes to the regulation of nearly every cellular process, Ub availability must be tightly controlled. Contributions to Ub homeostasis include Ub expression as peptidase or protein fusions and as polyubiquitin, processing to generate monoUb and recycling from Ub–protein conjugates by deubiquitinating enzymes (DUBs). Perturbations of these processes can deplete cellular free Ub and cause defects in cell development or neuronal functions and inhibit cell proliferation. Conversely, transgenic mice overexpressing Ub by just two- or three-fold exhibit neurological abnormalities.

Although the need to regulate free Ub is well established, studying intracellular Ub pools has been difficult. One approach has been ectopic expression of GFP-tagged Ub, but such experiments can be complicated by perturbations to the regulation of endogenous Ub and non-physiological behavior of the tagged Ub. For example, C-terminal tags would prevent Ub activation and conjugation, and N-terminal tags would block assembly of all M1-linked polyUb conjugates. Typically, to quantify endogenous free, conjugated or total Ub, anti-Ub antibodies in conjunction with ELISA or western blots are used. However, there are major drawbacks. Due to the aforementioned instrumentation and affinity-isolation steps makes its implementation challenging for most laboratories. Finally, none of the aforementioned approaches allow real-time quantitation of free Ub as it changes, for example, in deubiquitination reactions.

With the dual goals of establishing a simple, reliable method to quantify cellular Ub pools as well as a versatile real-time DUB assay, we embarked on development of sensors for free Ub. Our strategy was to fuse Ub binding domains (UBDs) of known structures that bind to non-overlapping Ub surfaces, and to exploit avidity effects to achieve high affinity and selectivity. To convert the binding proteins into sensors, we attached fluorescent dyes whose intensities changed in response to Ub binding. We then used the sensors to measure free, activated and conjugated intracellular Ub, to quantify deubiquitination of unlabeled conjugates in real-time and to identify endogenous free Ub by fluorescence microscopy of fixed cells.

Results

Design and characterization of the sensors. Ub binding proteins were assembled from multiple UBDs linked in tandem. Interdomain peptide linkers were kept short to promote avidity while minimizing the entropic cost of binding. Most UBDs bind through interactions with one of three different Ub surfaces: the hydrophobic patch surrounding residue 44, the C-terminal tail and the surface around D58 (Fig. 1a, upper panel). Individually, UBDs bind Ub with only modest affinities (\( K_d = 10^{-5} \) to \( 10^{-3} \) M), but by linking two or three weak-binding UBDs that target distinct surfaces, we predicted that high affinity could be achieved. An early version of an avidity-based binder that we call tIVR employed IsoT\(^{\text{IsoT}}\), Vps27\(^{\text{Vps27}}\) and Rabex5\(^{\text{Rabex5}}\) domains fused with flexible linkers (Fig. 1a, lower left panel and Supplementary Fig. 1). The IsoT\(^{\text{IsoT}}\) domain (\( K_d = 3 \) μM), which binds primarily to Ub C-terminal residues, conferred selectivity for free Ub, whereas the Vps27\(^{\text{Vps27}}\) domain (\( K_d = 117 \) μM) and Rabex5\(^{\text{Rabex5}}\) (\( K_d = 12 \) μM) domains worked synergistically with IsoT\(^{\text{IsoT}}\) to increase overall affinity and specificity.

To measure the affinity between tIVR and free Ub, we first titrated Atto532-Ub(S20C) with tIVR and determined a 0.4 nM \( K_d \) for the 1:1 complex (Supplementary Fig. 2a). Then, taking advantage of this high affinity and the 3.5-fold lower fluorescence of Atto532-Ub(S20C) in the complex, we determined tIVR affinities for Ub...
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Kamino acids are identical; nonetheless, tIVR has a 3,000-fold Ub in its sequence and tertiary structure, and its C-terminal four

that the dye promotes the interaction (ΔG, Affinities (K averages from duplicate samples. The points shown are

measured by direct titrations with the Ub or UbL derivatives indicated and fit with a 1:1 binding model as described in Methods. The points shown are averages from duplicate samples. Affinities (K or K) of the three sensors determined for the indicated Ub and UbL ligands. Effects of Ub–Ub linkage type were assessed from competition binding assays with 0.8 nM Atto532-Ub(S20C) and 6.0 nM tIVR titrated with 7–4,000 nM of the indicated polyUb ligands. Errors listed are standard deviations from the fits. Because in d the binding curve with Ub-hydrazide might reflect trace contamination by free Ub, a K value is not shown.

and other ligands by competition (Fig. 1b). We found that free Ub binds tIVR with a K of 19 nM (measured as an inhibition constant K, see Fig. 1b); the higher affinity of Atto532-Ub(S20C) indicates that the dye promotes the interaction (ΔG ≈ 0.2 kcal mol⁻¹). The competition assays additionally showed that tIVR has high selectivity against the Ub-like (UbL) protein Ned8 and Ub derivatives that lack a free C-terminal carboxylate (Fig. 1b). Among the many different UbL proteins in eukaryotes, Ned8 is most similar to Ub in its sequence and tertiary structure, and its C-terminal four amino acids are identical; nonetheless, tIVR has a 3,000-fold preference for Ub. A similarly large discrimination was observed against Ub-GB1, a mimic of a Ub–protein conjugate in which the Ub C terminus is extended by the Protein G B1 domain, and even addition of the small adduct hydrazine to the Ub C terminus decreased tIVR affinity 150-fold (Fig. 1b).

To provide a direct readout for Ub binding, we explored site-specific labeling with fluorescent dyes (Supplementary Fig. 3). tIVR(C130) modified with Atto532-maleimide showed a threefold fluorescence increase on Ub binding (Supplementary Fig. 2b). By replacing Vps27 with the S5a domain and introducing two amino acid substitutions in the IsoT domain (Fig. 1a and Supplementary Fig. 1), we developed a second-generation sensor, tISR, with nearly ten times the affinity of tIVR for free Ub (Supplementary Fig. 2c,d). Atto532-tISR fluorescence increased
six-fold on binding Ub and its \( K_d \) was 24.3 nM (Fig. 1c and Supplementary Fig. 2b).

A third-generation sensor was developed with the goal of even greater affinity. In tUI, the UIM-RUZ domains of tIVR/tISR were replaced by ubiquilin-1 associated UBA (UQ1\textsuperscript{184}), which binds to the Ub hydrophobic patch (\( K_d = 22 \mu M \))\textsuperscript{12,22}. Only two amino acids (versus five with tIVR or tISR) were needed to connect the UQ1UBA C terminus with the N terminus of IsoFluor, which we expected might reduce the entropic cost of complex formation. We conjugated Atto532 to UQ\textsuperscript{184} G573C in tUI (Fig. 1a, lower right panel); titration with Ub revealed a three-fold fluorescence increase and a remarkably low \( K_d \) of 66 ± 16 pM (Fig. 1d,e). We confirmed this by measuring the association and dissociation rate constants for the Atto532-tUI-Ub complex, from which we calculated a key component of the hydrophobic patch. Because interactions with Buz and UBA domains are likely to be disrupted in the minor conformer, the Ub versus Ub(pS65) \( K_d \) difference could in part reflect this equilibrium where only a fraction of Ub(pS65) is in the binding-competent conformation.

**Real-time deubiquitination assays with label-free substrates.** Although desirable, quantitative DUB activity assays rarely employ physiological substrates and have been hampered by the lack of good methods to quantify products. Moreover, real-time monitoring of activity—the preferred approach to high-precision kinetics studies—has been virtually impossible with physiological DUB substrates. For these reasons, artificial substrates such as Ub-(7-amido-4-methylcoumarin) or conjugates (for example, diUb) modified with fluorophores for fluorescence resonance energy transfer (FRET)-based assays are used\textsuperscript{13,14}. Our free Ub sensors make possible less restrictive DUB assays that can employ virtually any Ub conjugate as a substrate. As an example, we used Atto532-tIVR to monitor DUB-catalyzed release of free Ub (or anchored polyUb) in a continuous fluorometric assay. As a substrate, we used K48-linked Ub, conjugated to ovomucoid first domain (OM); the ovomucoid first domain moiety also was modified with Lucifer Yellow dye (LY) for detection after SDS–PAGE\textsuperscript{30,31}. For the DUB, we used human OTUB1, which selectively cleaves K48 Ub–Ub linkages\textsuperscript{33} and is activated allosterically by certain E2 enzymes\textsuperscript{34}. Without OTUB1, Ub4 linking-competent conformation.

**Ub pool quantitation in cell lysates.** We next developed methods using the sensors to quantify cellular Ub pools. Our goal was to generate a workflow that would not depend on expensive, time-intensive steps requiring chromatography or MS. The general approach, in which sensor (for example, Atto532-tUI) fluorescence is measured with and without addition of cell lysate or other sample, promised to be simple and direct. The main challenge was to develop conditions to prevent appearance of free Ub due to disassembly of conjugates by endogenous DUBs or from spontaneous hydrolysis of Ub thioesters.

Our strategy was to lyse cells and quickly inactive endogenous DUBs and other proteases, and then treat each sample in three ways.
to differentially convert Ub pools to the free-Ub form for measurement with the sensor (Fig. 3a). To measure endogenous free Ub without interference from chemically labile Ub thioesters, samples were treated with hydrazine to rapidly convert all Ub thioesters into Ub C-terminal hydrazide; Ub-hydrazide is stable and, relative to free Ub, gives a negligible response with the sensor (Fig. 1b–e and Supplementary Fig. 6). Thus, sensor fluorescence of hydrazine-treated samples will measure endogenous free Ub. With a second portion of the sample, β-mercaptoethanol was used to release Ub from thioesters; measurement with the sensor then will report the sum of the endogenous free and thioesterified Ub pools35. A third portion was incubated with Usp2cc, a truncated DUB that can deubiquitinate virtually all forms of conjugated Ub36; when used in combination with a thiol reducing agent, all forms of Ub are converted to free Ub (Supplementary Fig. 7) and the sensor readout will report the total Ub. By deducting the sum of the thioester-activated and free Ub from the total Ub, we can quantify the conjugated-Ub pool.

We first determined the amounts of free, activated and conjugated Ub in HeLa cells (Fig. 3); the results are in good agreement with other reports15,37,38. The sensor assays then were used with HeLa cells treated with inhibitors of the E1 Ub-activating enzyme or proteasome (Fig. 3b,c). As expected8, the E1 inhibitor C1 dramatically increased free Ub with a concomitant loss of activated Ub and most Ub–protein conjugates (Fig. 3b). Conversely, proteasome inhibition by bortezomib (BTZ) promoted accumulation of Ub conjugates that reached a maximum at 1 h and then persisted through a 4h treatment (Fig. 3c and Supplementary Fig. 8). The conjugate increase was accompanied by a modest depletion of activated Ub and a two-fold decrease in free Ub, presumably due to impaired proteasome-dependent recycling of Ub from conjugates. Different from this result, proteasome-inhibited mouse embryonic fibroblast (MEF) cells exhibited little change in free Ub levels, even though conjugated Ub increased 50%; instead total Ub increased, likely due to increased expression of Ub genes (Fig. 3c)40.

Quantitation of endogenous free Ub in fixed cells. Atto532-tUI, with its high affinity and specificity, could be an ideal tool to localize endogenous, intracellular free Ub. Initially, fixed cells were stained directly with Atto532-tUI, but high background fluorescence, possibly from non-specific binding by the fluorophore, reduced sensitivity (data not shown). Therefore, we used instead hemagglutinin-tagged tUI (HA-tUI) followed by detection with anti-HA antibody. We confirmed the specificity of HA-tUI in control experiments where fixed cells were incubated with HA-tUI together with excess free Ub. The fluorescence observed in these competition experiments was negligible, suggesting that the staining with HA-tUI is specific for cellular Ub (Supplementary Fig. 9). A diffuse intracellular distribution of free Ub was expected based on its small size (8.6 kDa) and negligible self-association41. Staining with HA-tUI was observed throughout the cytoplasm and nuclei of HeLa, U2OS, MEF and retinal pigment epithelium (RPE)1 cells (Fig. 4a). Compared to K48-linked chains and (poly)ubiquitylated proteins, free Ub staining appears evenly distributed throughout the cell (Supplementary Fig. 10). GFP-Ub

![Fig. 3](https://example.com/fig3.png)

**Fig. 3 | Effects of cellular stresses on Ub pools.** a, Scheme used for the in-solution Ub pool measurements. b,c, Quantitation of Ub pools in lysates of indicated cell lines after treatment with vehicle (DMSO) or E1 inhibitor, C1, at 10 μM (b) or proteasome inhibitor, BTZ, at 1μM for 1h (c). Statistical analyses by t-test (b) and one-way analysis of variance with Bonferroni’s adjustment (c); error bars represent ± s.d. (n = 3).
Fig. 4 | Free Ub staining in fixed and permeabilized HeLa, U2OS, MEF and RPE1 cells. a, Maximum projection images of free Ub staining with HA-tUI in HeLa, U2OS, MEF and RPE1 cells after 1 h incubation with 1 μM proteasome inhibitor, BTZ or 10 μM E1 inhibitor, C1. Scale bars, 20 μm. b, Mean fluorescence for HeLa, U2OS, MEF and RPE1 cells after 1 h incubation with 1 μM proteasome inhibitor or 10 μM E1 inhibitor. AU, arbitrary units. Cells analyzed per condition: HeLa, control n = 131, BTZ n = 125, C1 n = 116; U2OS, control n = 161, BTZ n = 170, C1 n = 175; MEF, control n = 80, BTZ n = 79, C1 n = 69; RPE1, control n = 133, BTZ n = 87, C1 n = 49. Bars show mean ± s.d. Statistical analyses used two-tailed unpaired Student’s t-test with Welch’s correction where appropriate. c, Relative free Ub from staining (mean fluorescence ± s.d.) of untreated cells or after proteasome or E1 inhibition. d, Representative interphase (left panel) and mitotic (right panel) RPE1 cells stained with HA-tUI (red) and DAPI (blue). Intensity measurements from three-dimensional reconstructions employed Imaris software. Total cell fluorescence (arbitrary units; mean ± s.d.) for interphase and mitotic RPE1 cells were 23.7 ± 2.5 (n = 5) and 38.4 ± 3.5 (n = 3), respectively, whereas fluorescence per unit volume was 134.1 ± 8.6 for interphase cells and 463.2 ± 70.6 for mitotic cells. Scale bars, 5 μm.
mutated to prevent its conjugation to other proteins was similarly diffuse when expressed in mammalian cells.

Staining with HA-tUI offers an alternative to solution-based assays to monitor changes in free Ub during growth or in response to different stresses. After proteasome inhibition with BTZ for 1 h, HeLa, U2OS, MEF and RPE1 cells showed 1.3–1.5-fold less staining with HA-tUI relative to control cells. As expected, E1 inhibition increased the free Ub (two-fold in MEF and RPE cells; three-fold in HeLa and U2OS cells) (Fig. 4b,c). Although proteasome inhibition decreased free Ub in all four cell lines, the intracellular distributions of free Ub appeared unaffected. For RPE1 cells, ratios of cytoplasmic to nuclear staining were not changed by incubation with BTZ (control cells, 0.85 ± 0.07, n = 5; BTZ, 0.89 ± 0.08, n = 4), whereas increased staining was observed after E1 inhibition and cell-to-cell variability was greater overall (Fig. 4a–c). The changes in staining of HeLa cells are consistent with the in-solution assays, although MEF cell staining indicated ~25% less free Ub after proteasome inhibition. HeLa, U2OS, MEF and RPE1 cells showed 1.3–1.5-fold less staining with HA-tUI than cells in interphase (Fig. 4d). The increase in free Ub may be due, at least in part, to large-scale deubiquitination of H2A histones in mitotic cells.

Discussion

Individually, UBDs have only modest affinity for Ub and are typically found together with other binding domains (and sometimes additional UBDs) to promote binding to specific types of (poly)Ub–protein conjugates. By fusing multiple UBDs, we have engineered new proteins with specificity and high affinity for monomeric free Ub that have been shown to be uniquely suited for in vitro sensing of free Ub. Our basic strategy was to use the Buz domain to directly bind to Ub with an unconjugated C terminus, and to increase affinity with one or two additional UBDs that interact with Ub on non-overlapping surfaces. Critical to this design strategy was maximizing avidity. This was achieved by having multiple UBDs bind simultaneously while minimizing the entropy lost on complex formation through careful selection of peptides linking the UBDs. Avid binding can boost affinity by combining the contributions of individual binding domains in a complex assembled from a multivalent ligand and a corresponding multivalent binder. The Gibbs free energy for binding overall (ΔGtotal) can be approximated as the sum of the individual binding domain interaction free energies (ΔGBD1, ΔGBD2 and so on) plus the unfavorable free energy due to reduction in entropy (predominantly, losses in translational and rotational entropy) from having all the binding domains linked together (ΔGS): ΔGtotal = ΔGBD1 + ΔGBD2 + ... + ΔGBDn + ΔGS (1)

To achieve ‘perfect’ avid binding and maximize affinity, ΔGS should be close to zero. For Ub binding by a tandem-UBD (tUBD) protein such as tIVR:

ΔGUbd = ΔGUBD1 + ΔGUBD2 + ΔGUBDn + ΔGS (2)

ΔGUbd can be calculated for each UBD based on the Kd values reported for Ub binding by the individual IsoTDep, Vps27TM and Rabex5Dep domains (that is, −7.53, −5.25 and −6.62 kcal mol−1, respectively); similarly, ΔGUBD = −10.52 kcal mol−1 can be calculated from the tIVR–Ub Kd of 19.3 nM (Fig. 1e). Thus, although avidity promotes tight binding by tIVR, it is at the cost of ΔGS, which is −10.52 (−19.40) = 8.88 kcal mol−1. This substantial penalty reduced tIVR affinity by >10-fold from a theoretical Kd of 5.9 × 10−15 M.

Remarkably, tUIs affinity for Ub (Kd = 194 pM, see Supplementary Fig. 11) is only three times weaker than what would be predicted for perfect avid binding by the combination of the UQ1UBA (Kd = 22 μM)2,25 and Buz (Kd = 3 μM)16 domains. Contributing to this highly efficient avidity effect is that UI1 has only one linker peptide (versus two in tIVR), and it is very short (Supplementary Fig. 1). Thus, a likely route to increase affinity for tIVR or tISR is further optimization of the interdomain linkers. On the other hand, a large increase in tUIs affinity would have to come from tighter-binding versions of one or both UBDs, as the linker already is nearly optimal.

There are several ways in which our free Ub sensors could be improved. Affinity might be increased, as noted above, with either modified linkers or alternative UBDs. Our designs have utilized only a few of more than 20 types of UBD44,45; moreover, other Ub binding proteins such as catalytically inactive DUBs might be used as components of tUBD-type fusion constructs46. Particularly intriguing is the prospect of tailoring recognition by incorporation of a Ub that would select either for or against specific modifications on the Ub. For example, although tIVR binds equally well to free polyUb chains of different Ub–Ub linkage types (Fig. 11), modified Ruz or UIM domains could be developed in which steric clash prevents binding to Ub conjugated at particular lysine(s). Similarly, Ub (or linker) modifications might add selectivity for phosphoUb derivatives. Finally, alternative fluorophores and attachment sites can be explored to improve sensor sensitivity and dynamic range.

The free Ub binders and the fluorescent sensors developed from them provide new tools to capture, deplete, quantify or visualize free Ub in vitro and in cells. Multiple DUBs have been implicated in human disease, but DUB inhibitor screens generally have been limited to non-physiological substrates. The real-time sensor-based assays will make possible high-throughput screens with virtually any Ub conjugate as the substrate. Other examples include neurological disorders where Ub homeostasis is disrupted and accompanied by Ub-containing aggregates or a general depletion of free Ub. Staining with tUI also revealed cell cycle-dependent differences in HA-tUI than cells in interphase (Fig. 4d). The increase in free Ub may be due, at least in part, to large-scale deubiquitination of H2A histones in mitotic cells.

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Author contributions
Y.S.C. and R.E.C. conceived and designed the ubiquitin sensor reagents. Y.S.C. produced the sensors and characterized them in vitro. S.A.B., T.Y. and R.E.C. characterized them in cell-based studies, which were done by S.A.B., L.F.P. and F.S. All authors contributed to writing or commenting on the manuscript.

Competing interests
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**Materials and protein preparation.** tVR, dsR, UI, Ub-G81 and UbC85c were cloned into pET28a and transformed into BL21-CodonPlus (DE3) Escherichia coli cells for protein expression. Expression was induced by the addition of 0.4 mM IPTG to cells grown at 37 °C to an optical density (OD600nm) of 0.6–0.8, and then growth was continued at 25 °C for 8 h. The cells were gathered by centrifugation at 3,200g, resuspended in ice-cold buffer A (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 10 mM imidazole and 10 mM β-mercaptoethanol) and lysed by sonication; the lysates were clarified by centrifugation for 30 min at 4 °C at 20,199g. A His trap HP column (GE Healthcare, no. 17-5248-02) was used to purify the proteins from the lysates. Samples were applied to the column equilibrated with buffer A, and, after washing with 20 column volumes, bound proteins were eluted with a linear gradient to 500 mM imidazole in buffer B. The proteins were further purified by gel filtration through a Superdex 75 column (GE Healthcare, no. 29-1480-21) eluted with pH 7.4 PBS and 1 mM DTT or 1 mM TCEP. Purity was confirmed by SDS–PAGE. Ub14, Neddf (ref. 10), Uspp2cc and Ub–OMLY11 were prepared as described. OTU1B was a gift from C. Wolberger (Johns Hopkins University). K6, K11, K27, K29, K33, K48 and K63-linked Ub2 chains were measured in the binding assays. All binding assays were done in PBS buffer, pH 7.4, 100 mM NaCl for 2 h at 25 °C. Excess dyes were quenched by incubation with 250 mM ATP, 250 mM NaCl and 10 mM DTT) to reduce the urea to less than 2 M; to this, Uspp2cc was added at a 1:10 (Uspp2cc:total protein) ratio and incubated at 37 °C for 1 h. To another fraction of the extract, 100 mM CHES, pH 9, containing 150 mM β-mercaptoethanol was added and incubated at 37 °C for 1 h. The third fraction was incubated at 37 °C for 1 h with freshly made 200 mM hydrazine-HCl, pH 8.5. These samples were then diluted using PBS and 0.2 mM m-ovalbumin to ensure that Ub was within the linear range of the assay (for example, with Atto532-UI, from 2–60 μM). Dilution was also performed to reduce the concentrations of free Ub, β-mercaptoethanol (<20 mM), hydrazine (<20 mM), which can otherwise interfere with binding by the sensor.

**Fluorophore labeling.** Sensor proteins were labeled at cysteine with fluorophore-maleimide dyes from ATTO-TEC (Atto dyes, see Supplementary Fig. 3), Molecular Probes (Alexa Fluor 488) or Anaspec (fluorescein). Fluorophore-maleimide dyes (1.5–5-fold molar excess) were incubated with 50 μM sensor in 50 mM HEPEs, pH 7.4, 100 mM NaCl for 2h at 25 °C. Excess dyes were quenched by incubation with 100 mM β-mercaptoethanol for 10 min at 25 °C. To remove excess dyes, the reaction product was bound to Ni-NTA resin (Thermo Fisher) equilibrated with buffer A, the resin was washed five times with the Histrap binding buffer, and sensor proteins were eluted with Histrap elution buffer. Labeling was confirmed by SDS–PAGE and then scanning the gel for fluorescence using a Typhoon FLA 9500 (GE Healthcare Life Sciences). Degree of labeling (DOL) and concentrations of the labeled proteins were calculated by the equations below.

\[
\text{DOL} = \frac{A_{\lambda} \times \varepsilon_{\lambda}}{(A_{\lambda} - A_{\varepsilon}) \times \varepsilon_{\varepsilon}} \times \frac{C_{\text{prot}}}{r_{\text{prot}}}
\]

\[
\text{Protein concentration (M)} = \frac{A_{\lambda} - A_{\varepsilon}}{A_{\lambda} \times \varepsilon_{\lambda}} \times \frac{C_{\text{prot}}}{r_{\text{prot}}}
\]

In the equations, \(A_{\lambda}\) represents the absorbance at the dye absorption maximum, \(A_{\varepsilon}\) is absorbance at 280 nm of the labeled protein, \(r_{\text{prot}}\) is the extinction coefficient at 280 nm of the protein, \(r_{\varepsilon}\) is the extinction coefficient at 280 nm of the dye alone, \(\varepsilon_{\lambda}\) is the extinction coefficient at the absorption maximum of the dye and \(C_{\text{prot}}\) is the correction factor at 280 nm.

**Binding assays.** All binding assays were done in PBS buffer, pH 7.4, supplemented with 0.05% Brij35 and either 0.2 mg/ml ovobumin or GB1 protein, and either 1 mM DTT or 1 mM TCEP. A Fluoromax-4 spectrofluorimeter (HORIBA Scientific) was used to measure fluorescence intensity in the binding assays. We fixed cells at <80% confluence with 4% paraformaldehyde in PBS for 30 min at 37 °C, permeabilized them with 0.1% Triton X-100 for 10 min at room temperature and blocked for 1 h with 5% BSA and 0.5% Tween-20 in PBS. Cells were stained for free Ub with 100 mM UIA-HA diluted in blocking solution for 30 min at room temperature. As a negative control, the sensor was pre-incubated for 5 min at room temperature with 100μM Ub in blocking solution before addition to the samples. Next, cells were incubated overnight at 4 °C with anti-HA antibody (Sigma-Aldrich clone HA-7 or Bethyl Laboratories A190-108A; 1:1,000 dilution), stained with Alexa Fluor 680-conjugated goat anti-mouse IgG (Thermo Scientific) and then washed with PBS and 100 nM tUI-HA diluted in blocking solution for 1 h. Imaging was done with a LI-COR Odyssey.

**Sample preparation for microscopy.** HeLa (ATCC), U2OS and MEF cells were cultured in DMEM/Ham’s F-12, 50/50 mix (Corning). The U2OS and MEF cells were used previously described11. Cells were incubated for 1 h with 1 μM BTZ (UBiquitin-Proteasome Biotechnologies), 10μM E1 inhibitor (Compound 1; provided by Takeda Oncology) or vehicle alone (0.1% DMSO). Each experiment was performed a minimum of two times. Western blotting. Cells were lysed and prepared as for the in-solution pool assays. Lysate samples were separated by SDS–PAGE followed by western blotting onto nitrocellulose (0.2 μm, Bio-Rad). After blocking in PBS with 0.05% Tween-20 and 5% non-fat dry milk, membranes were incubated with anti-Ub (clone P4G7, Santa Cruz Biotechnology, 1:1,000 dilution) and anti-alpha tubulin (clone D5C12, Abcam, 1:5000 dilution) mouse monoclonal antibodies overnight at 4 °C, washed with blocking buffer and then incubated with IRDye 680CW-conjugated goat anti-mouse (1:10,000, LI-COR Odyssey).
Fisher; 1:500 dilution) and mounted on slides using ProLong Diamond Antifade medium (Thermo Fisher). Some coverslips were also stained with anti-Ub (clone FK2, Enzo Life Sciences; 1:1,000 dilution) or anti-K48 Ub (clone Apu2, Millipore; 1:200 dilution) primary antibodies and, subsequently, with Alexa Fluor 568-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit (Thermo Fisher; 1:400 dilution) secondary antibodies. The HCS CellMask dye (Thermo Fisher) was added to the cells for 0.5 h as a marker of cell boundaries for high-content fluorescence intensity-based measurements.

**Microscopy and image analysis.** Cells were imaged using a Zeiss LSM 880 confocal microscope with C-Apochromat ×40/1.20 W or Plan-Apochromat ×63/1.40 Oil DIC M27 objectives. z-stack images were acquired with ZEN Black software (v.14.0.9.201) at 0.746 μm intervals. ImageJ 1.51 h (National Institute of Health, NIH) was used to perform maximum intensity projections of z-sections and to calculate cell mean fluorescence intensity values; cell contours were drawn using HCS CellMask dye as a reference and nuclei were identified with DAPI stain. Autofluorescence intensities recorded from unstained cells were subtracted from the tUI fluorescence. Three-dimensional reconstructions of RPE1 cells were obtained with Imaris software (v.9.1.1, Bitplane) from serial z-sections acquired at 0.242 μm increments. The Imaris surface creation tool was used to generate volume renderings and to quantify tUI fluorescence intensities in interphase and mitotic cells.

**Statistical analysis.** Statistical calculations were performed with GraphPad Prism software and are described in the relevant figure legends. P values less than 0.05 were considered significant.

**Material availability.** Plasmids for bacterial expression of tUI, tUI-HA, tIVR and tISR are available from Addgene.org (Addgene ID 122661, 122662, 122663 and 122664, respectively).

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The data that support the findings of this paper are available from the corresponding author on reasonable request.

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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  Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- Microscopy data were collected with Zeiss ZEN Black software (version 14.0.9.201); laser-scanned images on the Li-COR used Image Studio version 5.0.21, and on the Typhoon FLA 9500 used software version 1.0 build 1.0.0.185; spectrofluorometer data were acquired using HORIBA JobinYvon software FluorEssence version 25.1.1 and Origin 7.5878

Data analysis
- Image analyses used Zeiss ZEN Black software (Version 14.0.9.201), Image J (NIH; 1.51h), and Imaris software (version 9.1.1, Bitplane AG). Non-linear fitting to kinetic and binding data used Prism 6 (GraphPad Software)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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- A list of figures that have associated raw data
- A description of any restrictions on data availability

Data used for this study are available from the corresponding author on reasonable request.
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size  Numbers of cells scored for quantitative single-cell imaging were chosen for P < 0.05. For biochemical assays, sample size (typically, n = 3) was chosen to have s.d. < 10%

Data exclusions  As a pre-established criterion for fluorescence quantification from cell images, cells with poorly-defined cell boundaries (e.g., overlapping cells or cells near edges of the field) were excluded from analysis.

Replication  The procedures described in the study were used in two or more independent experiments and found to yield reproducible results.

Randomization  All assays used to characterize the sensors were in vitro, and randomization was not used; the validity of the experimental design was supported by the high quality of fits to the data and that distributions of residuals obtained from the fits were generally random.

Blinding  Investigators were not blinded regarding sample identities, but biochemical assay replicates generally were done at different times by different individuals.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a  Involved in the study       | n/a  Involved in the study |
| ☐ ☒ Antibodies                   | ☐ ChIP-seq |
| ☐ ☒ Eukaryotic cell lines        | ☐ Flow cytometry |
| ☐ ☒ Palaeontology                | ☐ MRI-based neuroimaging |
| ☒ Animals and other organisms    |         |
| ☒ Human research participants    |         |
| ☒ Clinical data                  |         |

Antibodies

Antibodies used  Monoclonal Anti-HA, Clone HA-7 (mouse IgG1 isotype); Sigma-Aldrich; Cat # H3663; Lot # 038M4810V
HA antibody, rabbit polyclonal; Bethyl Laboratories; Cat # A190-108A; Lot # A190-108A-2
Anti-Ubiquitin, Lys48-Specific, clone Apu2 (rabbit monoclonal); Millipore; Cat # 05-1307; Lot # 3018752
Anti-ubiquitin; clone FK2 (mouse); Enzo Life Sciences; Cat # BML-PW8810; Lot # 08281228
Anti-ubiquitin (clone P4G7), Santa Cruz Biotechnology; anti-alpha tubulin (clone DMIA), Abcam
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568; ThermoFisher; Cat # A10037; Lot # 1696197
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488; ThermoFisher; Cat # A11008; Lot # 1761258

Validation  Specificity of the anti-HA antibodies for immunofluorescence applications was validated by observing negligible staining of cells lacking HA-tagged protein. Specificity of the anti-(K48)-ubiquitin mAb (clone Apu2) is documented by the developer (Newton, Kim, et al. Cell, 134: 668-78 (2008)) and in several papers referenced at the vendor’s website. Specificities of the other antibodies used are documented by data available at the manufacturers' websites.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  HeLa, U2OS, and RPE-1 (tert) cell lines were from the ATCC. The immortalized MEF cell line was from Averil Ma (UCSF) and is described in Pasupala et al. (2018) J Biol Chem, doi: 10.1074/jbc.RA118.004677
| Authentication                  | The cell lines were not authenticated by us. |
|--------------------------------|---------------------------------------------|
| Mycoplasma contamination       | All cells used were tested for Mycoplasma and were negative for contamination. |
| Commonly misidentified lines   | No commonly misidentified cell lines were used. (See ICLAC register) |