Ub-ProT reveals global length and composition of protein ubiquitylation in cells

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Protein ubiquitylation regulates diverse cellular processes via distinct ubiquitin chains that differ by linkage type and length. However, a comprehensive method for measuring these properties has not been developed. Here we describe a method for assessing the length of substrate-attached polyubiquitin chains, “ubiquitin chain protection from trypsinization (Ub-ProT).” Using Ub-ProT, we found that most ubiquitylated substrates in yeast-soluble lysate are attached to chains of up to seven ubiquitin molecules. Inactivation of the ubiquitin-selective chaperone Cdc48 caused a dramatic increase in chain lengths on substrate proteins, suggesting that Cdc48 complex terminates chain elongation by substrate extraction. In mammalian cells, we found that ligand-activated epidermal growth factor receptor (EGFR) is rapidly modified with K63-linked tetra- to hexa-ubiquitin chains following EGF treatment in human cells. Thus, the Ub-ProT method can contribute to our understanding of mechanisms regulating physiological ubiquitin chain lengths and composition.
Protein ubiquitylation is a dynamic multifaceted post-translational modification (PTM) responsible for regulating a diverse array of cellular processes, including protein degradation, protein trafficking, signal transduction, and the DNA damage response. Ubiquitylation is catalyzed by the concerted action of ubiquitin (Ub)-activating (E1), Ub-conjugating (E2), and Ub-ligating (E3) enzymes. Deubiquitylating enzymes (DUB) antagonize ubiquitylation by removing Ub modifications from their substrates. Ub can be covalently conjugated to substrates in several ways: as a single Ub conjugated to a single (monoubiquitylation) or multiple sites (multiple mono-ubiquitylation), or as polymeric chains (polyubiquitylation). Different Ub chains are formed through isopeptide linkages using seven internal lysine (K) residues, as well as its N-terminal methionine (M1). Effector proteins harboring Ub-binding domains (UBDs) function as readers/decoders by discriminating specific Ub linkages. In addition to homotypic Ub chains in which multiple linkages form mixed or branched chains. Furthermore, Ub undergoes phosphorylation and acetylation at specific S/T and K residues. Accumulating evidence indicates that linkage type, length, and chemical modification work in concert to affect the topology and dynamics of Ub chains and direct substrates to distinct biological pathways. In particular, linkage type is a critical determinant of chain function. For example, it is widely accepted that K48-linked chains function as targeting signals for proteasomal destruction, whereas K63-linked chains are generally involved in signal transduction, DNA repair, and trafficking of membrane proteins; other linkage types also have distinct cellular functions. In contrast, despite its fundamental importance, our knowledge regarding the functional relevance of Ub chain length remains limited. Earlier in vitro studies suggested that the proteasome recognizes K48-linked tetraubiquitin as the minimal targeting signal, and that binding strength increases markedly as chain length increases up to octaubiquitin. However, more recent studies showed that monoubiquitylation and multiple short Ub chains also constitute efficient proteasomal targeting signals. In endocytosis and endosomal targeting, the relative importance of monoubiquitylation and K63-linked polyubiquitylation of receptor proteins remains unclear.

To understand the biological significance of various Ub chain structures, it is essential to determine the linkage types, modifications, and lengths of endogenous, substrate-linked chains. Recent advances in mass spectrometry (MS) and antibody-engineering technologies allow us to determine and quantitate Ub linkages and PTMs in complex biological samples. In contrast, the lengths of substrate-attached Ub chains have only been determined by analyzing their gel mobility. However, since most endogenous substrates have multiple ubiquitylation sites, and attached chains might have heterogeneous lengths, more comprehensive and accurate techniques are required. Here we describe a novel biochemical method for determining Ub chain length, "Ub chain protection from trypsinization (Ub-ProT)." By combining this method with quantitative MS analysis, we identified the length and composition of Ub chains in yeast, and of ligand-activated epidermal growth factor receptor (EGFR) in mammalian cells.

**Results**

**Establishing a method for determining Ub chains.** Because Ub can form polymeric chains, a given composition of ubiquitylation can form numerous structures, e.g., a substrate protein bearing four Ubs can form five distinct topologies even if linkage types and branching are not considered (Fig. 1a). Thus, the gel mobilities of ubiquitylated proteins do not accurately reflect individual chain Ub lengths. Analysis of Ub chains cleaved from substrate proteins at the proximal Ub moiety would be the optimal way to determine chain length. DUBs could be used for this purpose, but unfortunately, the known DUBs do not discriminate linkage positions. Although the proteasomal DUB Rpn11 can remove Ub chains by cleaving the proximal Ub, the reaction is coupled to substrate unfolding by ATPase subunits. Therefore, we designed an alternative approach using trypsin and a Ub chain protector. A previous study showed that Ub is specifically cleaved at Arg74 by trypsin digestion under native conditions. This cleavage occurs for all different Ub linkage types (Fig. 1b, i). However, if substrate-attached chains are masked by a Ub chain protector, intact polyubiquitin chains should remain after trypsinization, allowing the substrate-bound poly-Ub chains to be easily analyzed using a gel-based assay. We named this approach Ub-ProT (Fig. 1b, ii). As a Ub chain protector, we used the tandem Ub-binding entity (TUBE), a high-affinity probe for Ub chains. TUBE is an artificial protein, consisting of four repeats of the Ub-associated (UBA) domain of human RAD23A or UbQLN1. Ub TUBE binds K48- and K63-linked tetraubiquitins with a K_{d} of 9 and 0.7 nM, respectively. To prevent trypsin digestion, we constructed trypsin-resistant (TR-)TUBE consisting of a biotin tag, a hexahistidine tag, and six tandem repeats of the UbQLN1 UBA domain in which the Arg residues were replaced by Ala (Supplementary Fig. 1). We confirmed that TR-TUBE could efficiently pull down all eight types of di-Ubs (Supplementary Fig. 2a). Then, we tested whether free K48-, K63-, K48-, and K11-linked Ub chains, K48/K63 branched chains, and di-Ubs consisting of all eight different linkage types could be protected from trypsin digestion by TR-TUBE (Fig. 1c, d; Supplementary Figs. 2–5). This condition was sufficient to digest all unprotected Ub chains to at least monomers. TR-TUBE protected all chain types from digestion (Fig. 1c, d; Supplementary Fig. 2c). Greater than 90% of input K48-, K63-, and M1-linked di-Ubs were protected from trypsinization, and 40–60% of K6-, K27-, K29-, and K33-linked chains were protected (Supplementary Figs. 2c and d; Supplementary Data 1). TR-TUBE could also protect K48/K63 branched chains from digestions, although the protection efficiency was lower than for homogeneous chains (Fig. 1d; Supplementary Fig. 5). Since our TR-TUBE construct consists of six Ub-binding domains, one concern about our method was that it might only protect Ub chains up to hexamers. However, as seen in Fig. 1c, TR-TUBE could efficiently protect much longer chains, suggesting that multiple molecules of TR-TUBE can bind to a single chain to restrict trypsin accessibility. To further examine whether the number of Ub-binding domains in TR-TUBE may artificially influence the length of protected Ub chains, we constructed TR-TUBEs with four and eight Ub-binding domains, and compared the lengths of protected Ub chains (Supplementary Fig. 4). All three different TR-TUBE constructs protected M1-linked chains equally, and protected chains were indistinguishable from untrypsinized control chains.

We next applied the Ub-ProT method to tetraubiquitin-fused Sic1 (4×Ub-T7-Sic1) as a model substrate with a defined Ub chain length (Fig. 2a). The tetraubiquitin of 4×Ub-T7-Sic1 was digested to Ub monomers by trypsinization, but almost completely protected in the presence of TR-TUBE. In contrast, the same construct containing the I44A mutation in each Ub moiety (4×UbI44A-T7-Sic1) was completely digested, validating our method. Several Ub enzymes are efficiently self-ubiquitylated in vitro: Cdc34 is self-ubiquitylated with K48-linked chains, Rsp5 is self-ubiquitylated with K63-linked chains, and MBP-tagged Parkin is multiply monoubiquitylated. In each case,
ubiquitylated proteins were detected as a smear at high molecular weight, which disappeared following trypsinization (Fig. 2b). In the presence of TR-TUBE and trypsin, the protein substrate was digested leaving typical Ub ladders. Comparison with free Ub chains (used as a length marker) revealed that Cdc34 and Rsp5 were modified with K48- and K63-linked chains, respectively, of up to ~10-mers (Fig. 2b, left and middle). In contrast, self-ubiquitylated Parkin was modified with monoubiquitin and, to a lesser extent, short Ub chains (Fig. 2b, right). Because TR-TUBE captured almost all ubiquitylated Parkin, we concluded that TR-TUBE can bind not only Ub chains but also multiply monoubiquitylated substrates.

Measurement of chain length of yeast ubiquitylated proteins. To characterize global Ub chain lengths in an entire organism, we next investigated the mean length of substrate-attached Ub chains in yeast lysates. In this experiment, we used a drug-sensitive pdr5 mutant, which increases sensitivity to the proteasome inhibitor MG132. This allowed us to compare Ub chain lengths in control lysates and in lysates from yeast in which proteasomal activity was inhibited by MG13223. Soluble lysates were prepared from exponentially growing cells cultured with or without MG132, and soluble lysates and in lysates from yeast in which proteasomal activity was inhibited by MG13223. Soluble lysates were prepared from exponentially growing cells cultured with or without MG132, and

ubiquitin chains were used as markers (Ub marker). TR-TUBE: – – + –– +

Blot: anti-Ub

Ub-ProT assay of ubiquitylated proteins. Self-ubiquitylated GST-Cdc34 (Ub)n-Cdc34, left), self-ubiquitylated GST-Rsp5 (Ub)n-Rsp5, middle), and self-ubiquitylated MBP-Parkin (Ub)n-Parkin, right) were subjected to Ub-ProT. Free ubiquitin was probed with anti-ubiquitin antibody. Positions of ubiquitin chains with different lengths are numbered. Asterisks denote branched ubiquitin chains (Supplementary Fig. 5)

Ub-ProT protects substrate-attached ubiquitin chains in vitro. a Ub-ProT assay of tetraubiquitin-fused Sic1. M1-linked tetraubiquitin-fused T7-Sic1, cleaved tetraubiquitin, and ubiquitin monomer are indicated. A ubiquitin mutant (I44A in each ubiquitin moiety, resulting in a defect in TR-TUBE binding) was used as a control. Unanchored M1 chains were used as ubiquitin markers.

b Ub-ProT assay of ubiquitylated proteins. Self-ubiquitylated GST-Cdc34 (Ub)n-Cdc34, left), self-ubiquitylated GST-Rsp5 (Ub)n-Rsp5, middle), and self-ubiquitylated MBP-Parkin (Ub)n-Parkin, right) were subjected to Ub-ProT. Free ubiquitin was used as markers

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Fig. 1 Establishment of a method for determining ubiquitin chain length. a Different combinations of ubiquitylation with four ubiquitin molecules. Ubiquitins are depicted as red circles. This schematic does not take into account different linkage types or branching chains, which greatly increase the number of combinations. b Conceptual schematic of the “ubiquitin chain protection from trypsinization (Ub-ProT)” method. (i) Trypsinization under non-denaturing condition results in near-complete digestion of substrate proteins and partial digestion of ubiquitin chains, (ii) whereas a protector such as TR-TUBE protects ubiquitin chains from digestion. c Ub-ProT assay of free ubiquitin chains. K48-linked (left), K63-linked (center left), M1-linked (center right), and K11-linked (right) chains were pulled down with TR-TUBE (PD) and subjected to trypsinization. Ubiquitin was probed with anti-ubiquitin antibody. Positions of ubiquitin chains with different lengths are numbered. Monomeric Ub is more resistant to trypsinization compared to Ub chains, and sometimes remains after trypsinization of input samples (lane 2 in the middle two gels). d Ub-ProT assay of K48/K63 branched chains. K48/K63 branched chains were captured by TR-TUBE, subjected to trypsinization, and detected using anti-ubiquitin antibody. Positions of ubiquitin chains with different lengths are numbered. Asterisks denote branched ubiquitin chains (Supplementary Fig. 5)
captured endogenous ubiquitylated proteins efficiently (Fig. 3a, lanes 1 and 5). MG132 treatment increased the intensity of various ubiquitylated bands in both lysates and TR-TUBE pull-downs (lanes 2 and 6), indicating that ubiquitylated substrates accumulated upon proteasomal inhibition. We also compared the compositions of Ub linkages between lysate and pulled-down samples by quantitative MS analysis and Ub-absolute quantification (AQUA)/parallel reaction monitoring (PRM)\(^\text{24}\). In lysates, K48 and K63 linkages were predominant, and the abundance of other linkages was as follows: K29 > K11 > K6 > M1 \(\cong\) K27 \(\cong\) K33 (Fig. 3b; Supplementary Data 2). Linkage compositions were similar between lysates and pulled-down samples, confirming that TR-TUBE does not generate significant linkage bias during pull-downs. Upon trypsinization of unprotected lysates, signals from Ub conjugates completely disappeared (Fig. 3a, lanes 3 and 4), while trypsinization of TR-TUBE-captured proteins generated a
Ub-ProT analysis of UPS-related mutant cells. Soluble lysate from wild-type (WT), ubp6Δ, rad23Δ dsk2Δ (r23Δ d2Δ), or CDC48 mutant (cdc48-3) cells were subjected to Ub-ProT analysis. Gel regions subjected to MS quantitation are indicated by red letters (a–l). Free ubiquitin chains were used as markers. Absolute amounts of ubiquitin linkages in lysate from MG132-treated and cdc48-3 cells. The gel region above 49 kDa, where ubiquitylated proteins were detected, was subjected to Ub-AQUA/MS5 analysis (mean ± s.e.m.; n = 5 biological replicates; Supplementary Data 5). Length distributions of total ubiquitin and five major linkages in MG132-treated wild-type or cdc48-3 cells. Gel fractions in a were analyzed by quantitative mass spectrometry (mean ± s.e.m.; n = 3 biological replicates; Supplementary Data 6).

Ub chain ladder (Fig. 3a, lanes 7 and 8). In addition, different Ub chains with three or more Ubs exhibited different gel mobilities (Fig. 3c; Supplementary Fig. 6). Comparison with free Ub chains revealed that the lengths of the substrate-attached Ub chains were mainly in the monomer to heptamer range (Fig. 3c, left and middle). Although MG132 treatment increased the amounts of chains of each length, maximal lengths were unchanged.

To investigate the relationship between linkage types and chain lengths, we cut gel lanes into 12 pieces to separate monomers and chains of each length, maximal lengths were unchanged.

Fig. 4 Cdc48/p97 regulates ubiquitin chain length.Ub-ProT analysis of UPS-related mutant cells. Soluble lysate from wild-type (WT), ubp6Δ, rad23Δ dsk2Δ (r23Δ d2Δ), or CDC48 mutant (cdc48-3) cells were subjected to Ub-ProT analysis. Gel regions subjected to MS quantitation are indicated by red letters (a–l). Free ubiquitin chains were used as markers. Absolute amounts of ubiquitin linkages in lysate from MG132-treated and cdc48-3 cells. The gel region above 49 kDa, where ubiquitylated proteins were detected, was subjected to Ub-AQUA/MS5 analysis (mean ± s.e.m.; n = 5 biological replicates; Supplementary Data 5). Length distributions of total ubiquitin and five major linkages in MG132-treated wild-type or cdc48-3 cells. Gel fractions in a were analyzed by quantitative mass spectrometry (mean ± s.e.m.; n = 3 biological replicates; Supplementary Data 6).

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substrates were reduced in ubp6Δ and rad23Δ dsk2Δ cells (Fig. 4a left; ubp6Δ and r23Δ d2Δ). This result is consistent with those of a previous study demonstrating that Rad23 and Dsk2 protect Ub chains from DUB29. In contrast, in cdc48-3 mutant cells, we observed significant accumulation of ubiquitylated substrates, with elevated levels of five Ub linkage types (K6, K11, K29, K48, and K63) (Fig. 4a left and 4b; Supplementary Data 5). Strikingly, a subsequent Ub-ProT assay revealed that Ub chains were significantly elongated in the cdc48-3 mutant, but unaffected in ubp6Δ and rad23Δ dsk2Δ cells (Fig. 4a, middle and right). Linkage quantification suggested that the elongated chains in cdc48Δ cells contained all five major linkages (Fig. 4c, Supplementary Data 6). Because chain elongation was not observed in proteasome-inhibited cells, this effect was not simply due to the accumulation of ubiquitylated proteins. Instead, since Cdc48/p97 is involved in inhibited cells, this effect was not simply due to the accumulation of Ub chains linked through K11, K48, and K63 (Fig. 4a left and 4b; Supplementary Data 5). Strikingly, a subsequent Ub-ProT assay revealed that Ub chains were significantly elongated in the cdc48-3 mutant, but unaffected in ubp6Δ and rad23Δ dsk2Δ cells (Fig. 4a, middle and right). Linkage quantification suggested that the elongated chains in cdc48Δ cells contained all five major linkages (Fig. 4c, Supplementary Data 6). Because chain elongation was not observed in proteasome-inhibited cells, this effect was not simply due to the accumulation of ubiquitylated proteins. Instead, since Cdc48/p97 is involved in segregation/ remodeling of protein complexes, and major ubiquitylating enzymes build Ub chains on their substrates through processive elongation30, 31. Cdc48-dependent segregation/remodeling likely inhibits processive Ub chain elongation in substrate complexes. Substrate-attached Ub chains could be remodelled on Cdc48 complexes by multiple cofactors, such as Ub-binding cofactors (Ufd1, Npl4, Ufd3, and Shp1), the DUB Otu1, and E4 Ub elongation factor Ufd232, 33. We investigated the role of these cofactors in regulation of substrate-attached Ub chain lengths (Fig. 5), and found that Ub chains were significantly elongated in a npl4-1 mutant, which is defective for extraction of ubiquitylated membrane proteins24 (Fig. 5a). Ub chains were also slightly elongated in shp1Δ cells, but unaffected in otu1Δ, ufd2Δ, and ufd3Δ cells (Fig. 5b). Previously, we reported that Ub-binding of Npl4 was completely abolished by the npl4-1 mutation (G323S) in vitro24. Thus, we conclude that the recognition and segregation of ubiquitylated substrate by Cdc48 and Npl4 is important in regulating Ub chain lengths in cells.

Chain topology in ubiquitylated EGFR. Ubiquitylation of ligand-activated EGFR promotes clathrin-independent endocytosis and endosomal targeting of the receptor for lysosomal degradation11. EGFR is modified by multiple mono- and polyubiquitin chains linked through K11, K48, and K63 linkages35, but the topology and functional relevance of these modifications are still under debate30–32. To explore this issue, we applied Ub-ProT to EGFR ubiquitylation. For this purpose, we generated HeLa cells stably expressing EGFR-EFEGF-3xFLAG from the AAVS1 locus (Fig. 6a). Upon EGFR treatment (100 ng ml−1), EGFR-EFEGF-3xFLAG was rapidly ubiquitylated, and was subsequently degraded after 60 min (Fig. 6b). Microscopic analysis confirmed that the tagged EGFR initially localized to the plasma membrane, but moved to endosomes after 15 or 30 min of EGF stimulation, and then translocated to lysosomes after 1 h (Supplementary Fig. 8). Ub-AQUA/PRM analysis revealed that ubiquitylated EGFR at the 5-min time point contained the following Ub chain composition: K63 linkage (~50% of total Ub), K48 linkage (~40%), K11 linkage (~2%), and mono/endocap Ub (~4%) (Fig. 6c; Supplementary Data 7). These results are consistent with those from a previously published study35. We next subjected the ubiquitylated EGFR to Ub-ProT and observed a Ub ladder corresponding to K63-linked chains of four to six Ub molecules specifically in EGF-treated cells (Fig. 6d, lane 5). To determine whether these were true K63 chains, we used linkage-selective DUBs. Recently, Komander et al. developed a versatile method for analyzing the higher-order architecture of heterotypic chains by linkage-selective DUBs, termed "Ub chain restriction (UbiCRest)"39. Among the DUBs, we used K48-chain-selective OTUB1, K63-chain-selective AMSH, and non-selective USP2. As expected, the Ub ladder was completely digested by treatment with AMSH or USP2, but not with OTUB1 (Fig. 6d, lanes 6-8). Thus, ligand-activated EGFR is primarily modified with K63-linked tetra- to hexa-ubiquitins. When present, K48 and K11 linkages are formed at sites distal to the K63 chains.

To further investigate the relationship between EGFR trafficking and Ub chain length, we performed Ub-ProT at various times after EGF stimulation (Fig. 6e). Signals for monoubiquitylation, which may consist of multiple monoubiquitylations on the substrate, or of multiple monoubiquitylations that branch off of K63 chains, and Ub chains were elevated after 5 min of EGF treatment, and reached a maximum after 30 min, suggesting that EGFR is continuously ubiquitylated from the plasma membrane to the endosome. After 60 min of stimulation, the intensities of Ub bands decreased, consistent with deubiquitylation at multivesicular bodies, and lysosomal degradation. Thus, although certain additional bands were also detected, the major Ub modifications to EGFR were multiple monoubiquitylations and K63-linked polyubiquitylation during the trafficking process (Fig. 6f). Consistent with this result, recent studies suggested that the K63-linked chains function in endosomal sorting rather than internalization of EGFR36, 40. It remains unclear why the K63 chains had intermediate length, i.e., why they were tetra- to hexa-ubiquitins rather than di- or tri-ubiquitins. One possible explanation is that long chains may be advantageous for collaborative recognition by the highly organized ESCRT complexes (0, I, II, and III), which contain multiple UBDs31.

Discussion
In this study, we developed a novel method for determining the chain length of ubiquitylated substrates. By combining this method (Ub-ProT) with MS-based Ub quantitation (Ub-AQUA/
Ub-ProT regulates ubiquitylation of over a thousand proteins, explaining complex rather than limitations of ubiquitylating enzymes. Cdc48 that chain length is regulated by sequestration by the Cdc48 immunoblotting with indicated antibodies. Parental wild-type HeLa cells were also analyzed (ctrl). Degraded EGFR is indicated by an asterisk.

Accumulation of elongated Ub chains (Figs. 4 and 5), suggesting and protect long Ub chains. However, it is important to note the limitations to our method. While we demonstrate that Ub-ProT using TR-TUBE can bind and protect all Ub linkage types, there is up to a twofold variability in the amount of protection. While 90% of K11-, K48-, K63-, and M1-linked dimeric Ub is protected by Ub-ProT using TR-TUBE, K6-, K27-, K29-, and K33-linked Ubs are protected at 40–60% efficiency (Supplementary Fig. 2). Therefore, our method may slightly underestimate the occurrence of certain linkage types. In addition, while TR-TUBE does not bind free monomeric Ub, we see some monomeric Ub when we analyze chain length and composition type after Ub-ProT treatment of certain Ub-ProT using TR-TUBE, K6-, K27-, K29-, and K33-linked Ubs are protected at 40–60% efficiency (Supplementary Fig. 2).

Immunoprecipitated EGFR was treated with K63 linkage-specific OTUB1, or nonspecific USP2, followed by Ub-ProT. 

Time-dependent changes of ubiquitylated chain lengths on EGFR. After EGFR treatment, EGFR-EGFP-3×FLAG was immunoprecipitated with anti-FLAG antibody and subjected to Ub-ProT analysis of ubiquitylated EGFR combined with UbiCRest assay. Immunoprecipitated EGFR was treated with K63 linkage-specific AMSH, K48 linkage-specific OTUB1, or nonspecific USP2, followed by Ub-ProT. 

A possible model for ubiquitin chain architecture of ubiquitylated EGFR

**Methods**

**Plasmids**

*TUBE expression plasmids:* We modified a previously reported high-affinity probe for Ub chains, TUBE, which consists of four tandem repeats of the UBA domain of UBQLN1. To capture ubiquitylated proteins more efficiently and...
prevent trypsinization to allow MS analysis, we mutated all Arg residues in the UBA domain to Ala and fused four to eight repeats together44. Next, the gene encoding TUB-T7-Sic1 was inserted into a multiple cloning site (A vector) of pET15b-1×Ub, and pETIb5-4×Ub-His6, in which four Ub were tandemly fused without a linker, followed by T7-Sic1PT inserted using the In-Fusion cloning kit (Takara). The template plasmid, pETIb5-1×Ub, was kindly provided from Dr. K. Sakamoto (RIKEN institute, Japan). The resultant plasmids, pETIb5-4×UbI44A-T7-Sic1PY, and 4×Ub44A-T7-Sic1PY-His6, respectively.

DUB expression plasmids. To construct bacterial expression vectors for linkage-specific DUBs, cDNAs codon-optimized for bacterial expression of human AMSH and OTUB1 (Eurofins Genomics) were cloned into the pGEX6P1 expression vector (GE Healthcare). The bacterial expression vector for USP2, pET15b-USP2cc, was a gift from Dr. R. Baker43.

TALE nuclease and EGFR Plasmids. To generate HeLa cells stably expressing EGFR-EGFP-3×FLAG, we used the TALE nuclease (TALEN) method. AAVS1 site-specific TALE plasmids were constructed as described previously44 using TALE monomer template plasmids and TALE backbone plasmids (Addgene #32192). To construct donor vector pZDonor-AAVS1-EGFR-EGFP-3×FLAG, the sequences encoding the EFl promoter, human EGFR cDNA (NM_002258), EGFP, 3×FLAG, and SV40 polyA were tandemly inserted into pZDonor-AAVS1-Neo, in which the puromycin resistance gene of pZdonor-AAVS1-purmycin (Sigma-Aldrich) was replaced with the G418 resistant gene.

Expression and purification of DUBs. Recombinant AMSH, OTUB1, and USP2 were prepared as described previously39, 41. In brief, His6-USP2 was expressed in E. coli BL21 (DE3) harboring pETIc5-USP2cc and purified on TALON resin. GST-AMSH and GST-OTUB1 were expressed in E. coli BL21 (DE3) harboring pGEX6P1-AMSH or pGEX6P1-OTUB1 and purified on glutathione-Sepharose 4B beads. The glutathione S-transferase (GST) tag was removed by PreScission Protease (GE Healthcare). DUB activity was verified by cleaving free K48- or K63-linked chains.

Ub-ProT assay for in vitro substrates. Because the trypsin sensitivity of proteins vary with their structural properties, the amount of trypsin was titrated in each experimental setup. Di-Ub chains (500 ng) and TR-TUBE (5 μg) were incubated overnight at 37 °C in 20 μl of trypsin solution (50 mM ammonium bicarbonate (AMBC), 0.01% Rapigest SF, and 15 ng trypsin (Trypsin Gold; Promega)). For unanchored polyubiquitinated proteins, M1-, K11-, K48-, K63-linked, or K48/K63 branched Ub chains (50 ng) were incubated overnight at 37 °C in 20 μl of trypsin solution (50 mM AMBC, 0.01% Rapigest SF, and 2.5 ng trypsin). Ub4-sci1 protein (500 ng) and TR-TUBE (5 μg) were incubated overnight at 37 °C in 20 μl of trypsin solution (50 mM AMBC, 0.01% Rapigest SF, and 5 ng trypsin). For self-ubiquitylated E2 or E3s, conjugates (1 mg) were immobilized on beads were incubated at 37 °C with TR-TUBE (5 μg) in 20 μl trypsin solution (50 mM AMBC, 0.01% Rapigest SF, and 15–25 ng trypsin).

Ub-ProT assay for yeast lysates. For Ub-ProT assay of yeast extracts, 30 OD600 units of log-phase cells were harvested and lysed with glass beads in 300 μl of lysis buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 10 mM MgCl2, 10 mM MgCl2, 1 mM DTT, and 5 mM ATP. Self-ubiquitylated GST-Cdc43 was prepared by incubating 100 μg ml−1 GST-Cdc43 on glutathione-Sepharose 4B beads (GE Healthcare) for 15 h at 37 °C in the presence of 333 μm bovine Hsc70-1, 50 μg ml−1 bovine Immunoglobulin G (IgG) (NEB, Hsc70-1), 10 mM MgCl2, 0.1 mM DTT, and 2 mM ATP, as described previously20. Self-ubiquitylated GST-Rsp5 was carried out by incubating 50 μg ml−1 GST-WW-HECT1 (5 μg) on glutathione-Sepharose 4B beads for 15 h at 28 °C in the presence of 6 μg ml−1 human Hsc70-1, 50 μg ml−1 Ub4c, 50 μg ml−1 Ub in 50 mM sodium-HEPES (pH 7.5), 100 mM NaCl, 10% glycerol, 10 mM MgCl2, 1 mM DTT, and 5 mM ATP.

Yeast strains and media. Saccharomyces cerevisiae strains used in this study are listed in Supplementary Table 1. All strains are isogenic to W303 or BY4741. Standard media and genetic techniques were used to manipulate yeast strains44. A deletion mutant of PDR5 was used to increase sensitivity to the proteasome inhibitor MG13225. Yeast cells were grown at 28 °C in SC medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, 2% glucose, 10 mM potassium phosphate (pH 7.5), 400 μg ml−1 adenine sulfate, 10 μg ml−1 uracil, and 20 μg ml−1 tryptophan).

Cell lines and culture. HeLa cells (laboratory stock of M. Komada) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin in a humidified 1% CO2 incubator with 5% CO2. To introduce EGFR-EGFP-3×FLAG to the AAVS1 locus tandemly fused without a linker, followed by T7-Sic1PY inserted using the In-Fusion cloning kit (Takara). The template plasmid, pETIb5-1×Ub, was kindly provided from Dr. K. Sakamoto (RIKEN institute, Japan). The resultant plasmids, pETIb5-4×UbI44A-T7-Sic1PY, and 4×Ub44A-T7-Sic1PY-His6, respectively.

Expression and purification of TR-TUBE. TR-TUBE was expressed in Escherichia coli Rosetta2 (DE3) for 15 h at 22 °C. Cells were lysed by passage through a precooled French pressure cell (Ohtake Works) in lysis buffer (50 mM sodium phosphate (pH 7.0), 300 mM NaCl, 10% glycerol, and 1 mM Tris-2-carboxyethyl phosphinehydrochloride), and the lysate was clarified by 30-min centrifugation at 29 300 × g. The supernatant was incubated with TALON resin (Clontech), and TR-TUBE was eluted with elution buffer (50 mM sodium-HEPES (pH 7.1), 100 mM NaCl, and 200 mM imidazole). Then, TR-TUBE was biotinylated with EZ-link Maleimide-PEG2-Biotin (Thermo Scientific), and further purified by gel filtration on Superdex 75 (GE Healthcare), pre-equilibrated with 50 mM sodium-HEPES (pH 7.1), 100 mM NaCl, and 10% glycerol. Prior to biotinylated TR-TUBE was divided into small aliquots and stored at −80 °C. Biotinylated 6×TR-TUBE was used throughout the study unless otherwise noted.

Preparation of Ub chains and ubiquitylated proteins. For Fig. 1c and Supplementary Fig. 2, the following commercial Ub reagents were obtained from Boston Biochem; K48-linked polyubiquitin chains (1–7), K63-linked polyubiquitin chains (1–7), and di-ubiquitin. K11-linked polyubiquitin was kindly provided from Dr. Y. Sugiyama (National Institute of Advanced Industrial Science and Technology). For other figures, we prepared unanchored M1-, K48-, and K63-linked polyubiquitin chains using pet-tU-BUBC, UE2B/E2-25K, and UE2B/NUBC1-UE2V1, as described previously45. For K48/K63 branched chains, 2 μg of bovine Ub (Sigma-Aldrich), 50 ng of human Hsc70-1, (Boston Biochem), and 400 ng of Usp2a was incubated in 20 μl of TR-TUBE (pH 7.5), 5 mM MgCl2, 2 mM ATP, and 0.5 mM dithiothreitol (DTT) (Sigma-Aldrich) for 3 h. Then, 400 ng of E2-25K was added and incubated at 37 °C for 3 h. Self-ubiquitylated GST-Cdc43 was prepared by incubating 100 μg ml−1 GST-Cdc43 on glutathione-Sepharose 4B beads (GE Healthcare) for 15 h at 37 °C in the presence of 333 μm bovine Hsc70-1, 50 μg ml−1 bovine Immunoglobulin G (IgG) (NEB, Hsc70-1), 10 mM MgCl2, 0.1 mM DTT, and 2 mM ATP, as described previously20. Self-ubiquitylated GST-Rsp5 was carried out by incubating 50 μg ml−1 GST-WW-HECT1 (5 μg) on glutathione-Sepharose 4B beads for 15 h at 28 °C in the presence of 6 μg ml−1 human Hsc70-1, 50 μg ml−1 Ub4c, 50 μg ml−1 Ub in 50 mM sodium-HEPES (pH 7.5), 100 mM NaCl, 10% glycerol, 10 mM MgCl2, 1 mM DTT, and 5 mM ATP. Self-ubiquitylated MBP-Parkin was prepared by incubating 20 μg ml−1 MBP-Parkin on Amylose resin (New England Biolabs) for 3 h at 32 °C in the presence of 1.6 μg ml−1 human Hsc70-1, 100 μg ml−1 Ub4c, 50 μg ml−1 Ub in 50 mM sodium-HEPES (pH 8.8), 200 mM NaCl, 2 mM DTT, and 4 mM ATP, as described previously22. After the reactions, the beads were washed with PBS plus 5% Tween 20 (PBS-T) and stored at 4 °C.
presence of 0.7 μg of AMSH, 2 μg of OTUB1, or 2 μg of USP2 in 200 μl of DUB buffer. For Ub-ProT assays, the beads were washed three times with TX-100 buffer and incubated for 1 h in 0.5% FBS/DMEM supplemented with 0.5% sodium azide (Na3) and 1 mM EDTA, and twice with 50 mM AMBc. Finally, the beads were washed twice with 100 μl of TT-GFP buffer, twice with detergent-free buffer (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 1 mM EDTA), and twice with 50 mM AMBc. Then, the beads were washed twice with TX-100 buffer, twice with detergent-free buffer (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 1 mM EDTA), and twice with 50 mM AMBc. Finally, the beads were washed twice with TT-GFP buffer, twice with detergent-free buffer, and twice with PBS. Cells were subsequently incubated with HRP-conjugated goat anti-mouse Ig (1:10,000; Abcam) used as a secondary antibody, was purchased from Jackson Immunoresearch Laboratories. Immunoblotting was performed using ECL Western Blotting Detection Reagent (GE Healthcare), and analyzed on an ImageQuant LAS4000 (GE Healthcare). Uncropped blots images are shown in Supplementary Fig. 9.

**Ubiquitin-AQUA/PRM**

MS/MS-based absolute quantification (AQUA) of Ub peptides by PRM was performed as described previously. For the yeast lysates and TR-TUBE-pull-down samples shown in Figs. 3b and 4b, proteins (10 μg) were fractionated on NuPAGE gels with a full run (8 cm); gel lanes were cut into 12 fractions, starting at the position corresponding to the Ub monomer, using a grid cutter (2 mm long × 7 mm wide, Gel Company); the resultant slices were subjected to trypsinization. Digests were extracted on an X II Blot Module (Life Technologies). After verify transfer by Pon championships, we performed immunoblotting with the following antibodies: mouse monoclonal antibody against Ub (P4D1, horseradish peroxidase (HRP)-conjugated; used at 1:500 for immunoblotting; Santa Cruz Biotechnology); mouse monoclonal antibody against the FLAG-tag (M2, HRP-conjugated; used at 1:1000; Abcam); rabbit monoclonal antibody against K48-linked (EP8590-448; 1:1000; Abcam). HRP-conjugated goat anti-mouse Ig (1:10,000), used as a secondary antibody, was purchased from Jackson Immunoresearch Laboratories. Immunoblotting was performed using ECL Western Blotting Detection Reagent (GE Healthcare), and analyzed on an ImageQuant LAS4000 (GE Healthcare). Uncropped blots images are shown in Supplementary Fig. 9.

Fluorescence microscopy. To monitor EGFR traffic, HeLa cells stably expressing EGF-EGFP-3×FLAG were serum-starved in DMEM supplemented with 0.5% FBS for 24 h, followed by 2 h in 0% FBS/DMEM. Subsequently, cells were stimulated with 100 ng/ml of Texas Red-conjugated EGFP (Thermo Scientific) at 37 °C for the indicated times. Cells were fixed in 3% formaldehyde/PBS for 15 min on ice, permeabilized with 0.5% Triton X-100/PBS for 10 min, and blocked with 10% FBS/PBS. Cells were subsequently incubated with anti-EEA1 antibody (1 μg/ml, E46112, BD). In Transduction Laboratories) in blocking buffer for 1 h at room temperature, washed three times with PBS, and incubated with Alexa Fluor 647-conjugated anti-mouse IgG antibody (1:1000; Invitrogen) in blocking buffer for 1 h at room temperature. Nuclei were stained with 4’,6-diamidino-2-phenylindole. Fluorescence images were captured on a LSM710 laser-scanning confocal microscope (Carl Zeiss) equipped with a PLANN/APO ×63 oil objective and the ZEISS ZEN 2010 software.

**Data availability**

The authors declare that the RAW files of Ub-AQUA/PRM data have been deposited in the PeptideAtlas repository under data set ID PASS01132. All other data supporting the findings of this study are available within the manuscript and its Supplementary Files or are available from the corresponding author upon request.

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Author contributions

Y.S. designed the study; H.T., D.B., N.A., A.K., and Y.S. performed experiments; H.T., D.B., M.K., F.O., and K.T. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Additional information

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