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A cascading activity-based probe sequentially targets E1–E2–E3 ubiquitin enzymes

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Abstract

Post-translational modifications of proteins with ubiquitin (Ub) and ubiquitin-like (Ubl) modifiers, orchestrated by a cascade of specialized E1, E2 and E3 enzymes, control a staggering breadth of cellular processes. To monitor catalysis along these complex reaction pathways, we developed a cascading activity-based probe, UbDha. Akin to the native Ub, upon ATP-dependent activation by the E1, UbDha can travel downstream to the E2 (and subsequently E3) enzymes through sequential trans-thioesterifications. Unlike the native Ub, at each step along the cascade UbDha has the option to react irreversibly with active site cysteine residues of target enzymes, thus enabling their detection. We show that our cascading probe ‘hops’ and ‘traps’ catalytically active ubiquitin-modifying enzymes (but not their substrates) by a mechanism diversifiable to UbLs. Our founder methodology, amenable to structural studies, proteome-wide profiling and monitoring of

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M.P.C.M. and F.E. designed the study. M.P.C.M., K.W. and I.B. carried out all labeling experiments. I.B. and K.W. designed and executed in-cell labeling experiments with assistance from R.M. and I.B. collected and analyzed confocal microscopy data. Mass spectrometry and relevant data analysis were performed by J.C. and A.C.O.V. on samples prepared by K.W. and I.B. J.N.P. and D.K. performed structural and competition studies and analyzed NMR and X-ray data. K.P.W. and B.A.S. generated the panel of purified HECT and NEDD8 pathway enzymes and helped with data analysis. J.B. and M.G. provided UBA6. M.P.C.M., F.E. and H.O. managed the study. M.P.C.M. and I.B. wrote the manuscript with input from other authors.

Competing financial interests

H.O. and F.E. declare competing financial interests as co-founder and shareholder of UbiQ Bio BV. D.K. and H.O. are part of the DUB Alliance that includes Cancer Research Technology and FORMA Therapeutics.

Accession codes

The structure of the thioether-linked UBE2D3-UbDha adduct has been deposited to the Protein Data Bank (PDB) under PDB accession code 5IFR.

enzymatic activities in living cells, presents novel and versatile tools to interrogate the Ub/Ubl cascades.

Introduction

Post-translational modifications of cellular targets with ubiquitin (Ub) or ubiquitin-like (Ubl) modules are potent regulators of protein function and thus govern a wide range of biological processes¹. While the general biochemical logistics of Ub/Ubl activation, conjugation and ligation, orchestrated sequentially by the E1, E2, and E3 enzymes, are highly conserved among eukaryotes, the number and flavour of individual players in each organism's relevant enzymatic repertoire can differ widely¹. Humans are known to harbour 2 E1, ~30 E2s and ~600 E3s in the Ub conjugation cascade, and while some are highly specific for certain targets, others appear relatively promiscuous. The sheer complexity of such enzymatic networks, further inundated by ~80 specific proteases responsible for removal of these modifications¹, enables highly specialized and sensitive modes of regulation, necessary to accommodate dynamic cellular events. On the flip side, deregulation of these pathways is a common feature in cancer, neurodegenerative, and inflammatory diseases. Similarly, some pathogens have evolved to perturb or exploit the host's Ub/Ubl conjugation cascades to their advantage². Despite their importance, development of comprehensive tools to assess the enzymology of these processes has been a long-standing roadblock in the field.

An important class of reagents used to study enzymatic activity, structure and substrate specificity within the Ub/Ubl modification system are activity-based probes (ABPs)^{3,4}. In the last decade, we and others have developed various ABPs for deubiquitylating enzymes (DUBs) and Ubl specific proteases⁵⁻⁷. Among the advantages of such probes is their ability to report on DUB activities in cellular extracts and even intact cells, thus facilitating the study of these enzymes in their biological context⁸. Development of analogous tools for the ligation machinery has proven challenging. Unlike DUBs, which contain highly reactive cysteine (Cys) nucleophiles, ligases possess less nucleophilic active site Cys residues, rendering them more difficult to trap with electrophiles. Recently, Ubl-AMP probes were reported to selectively label cognate activating enzymes⁹⁻¹². However, because ligation requires transfer of Ub/Ubl between enzymes, the challenge of monitoring reactions *throughout* the cascade remained. To tackle this, we developed a mechanism-based ligase probe that undergoes sequential trans-thioesterification reactions as it cascades from the E1 to the E2 and subsequently the E3 stage. In addition, at each step of the cascade our probe has the option to irreversibly trap the active site Cys residues of the enzymes in question. This methodology, implemented and characterized using the Ub-specific conjugation machinery, is further extended to the NEDD8 enzyme family, demonstrating general applicability of our probe design to Ubls.

The unique properties of our cascading probe enable direct monitoring of sequential E1, E2 and (HECT) E3 activities in a wide variety of experimental settings. Given the ATP-dependence of its reactivity against ligases, combined with its lack of transfer to substrates, our probe is well suited for proteome-wide profiling of relevant enzymatic cascades. Furthermore, upon introduction into living cells, our probe monitors enzymatic activities of

interest and reports on changes in response to chemical or genetic inhibition. From the structural perspective, our stable mechanism-based trapping of catalytic Cys residues circumvents potential disadvantages incurred by traditional methods of stable E2-Ub conjugate preparation requiring active site mutagenesis^{13–17}. Collectively, these novel features of our ABP tool present previously inaccessible avenues for targeting and monitoring enzymatic activities along the Ub/Ubl conjugation cascades, with implications for drug discovery and cell as well as structural biology of these pathways.

Results

Design and synthesis of the cascading ABP

To initiate the Ub/Ubl modification cascade, the E1 activating enzyme adenylates the C-terminus of Ub at the expense of ATP, which results in a high energy E1~Ub thioester formed upon an intramolecular reaction of the intermediate adenylate with the E1 active site Cys nucleophile. Next, a trans-thioesterification reaction transfers the activated Ub to a conserved E2 Cys, thus forming an E2~Ub thioester intermediate, which subsequently enables transfer of Ub onto a substrate with the help of an E3¹⁸. It is known that a Ub-G76A mutant can also be processed by the E1–E2–E3 cascade, albeit with reduced efficacy^{19,20}. We reasoned that replacing the C-terminal alanine by a latent and electrophilic dehydroalanine (Dha) moiety, would retain recognition by the E1–E2–E3 enzymes, allowing the UbGly76Dha (UbDha) to traverse the cascade (Fig. 1). Analogous to ubiquitin activation, UbDha is activated by the E1 enzyme through the formation of an adenylate intermediate, which strongly increases the electrophilic character of the Michael acceptor (Dha moiety). The activated methylene group of the Dha moiety is now poised to *either* covalently trap the enzyme in an E1-UbDha thioether adduct (Fig. 1, pathway a) *or* follow the native ligation route resulting in an E1~UbDha thioester (pathway b). In the second scenario, UbDha is available for transfer to an E2 enzyme, during which an analogous two options of covalent thioether adduct with the probe (pathway a) or a native trans-thioesterification reaction (pathway b) arise. Lastly, similar options apply to the subsequent transfer of UbDha from the E2 to an active site cysteine-dependent E3 enzyme (i.e. from the HECT or RBR families of E3 ligases²¹).

UbDha was synthesized starting from Ub(1–75) (Supplementary results, Supplementary Fig. 1), using our previously reported linear Fmoc-based solid phase synthesis (SPPS) of Ub²², where coupling H-Cys(Bn)-OMe to the C-terminal carboxyl group of protected Ub(1–75) afforded Ub(1–75)-Cys(Bn)-OMe. This was subsequently transformed into UbDha-OMe by oxidative elimination with *O*-mesitylenesulfonylhydroxyl-amine (MSH)²³. Finally, the methyl ester was hydrolysed, to generate the UbDha probe. We also used the recently reported 2,5-dibromohexanediamide reagent to convert a Cys into a Dha moiety (Supplementary Fig. 1; Method B)^{23,24}. Importantly, in contrast to MSH, 2,5-dibromohexanediamide reacts with a C-terminal Cys residue and thus allows the use of recombinant Ubl G76C mutants to prepare probes.

Covalent bond formation with conjugating enzymes

To evaluate the ability of our probe to travel the cascade, we began by subjecting the Ub-activating UBE1 enzyme to UbDha *in vitro*. SDS-PAGE analysis of the reaction revealed formation of an UBE1-UbDha adduct (Fig. 2a), consistent with a thioether linkage due to its stability under reducing conditions. ATP-dependence of the reaction indicated that the ligation proceeds through the adenylate intermediate (Fig. 1). Similar observations were made for UBA6 (Fig. 2a, right panel), the second Ub E1 enzyme, which also activates the Ub1 modifier FAT10²⁵. To test whether the E1-UbDha thioester can transfer UbDha to the E2 stage, we added UBE2L3 to the reaction in Fig. 2a. Whereas SDS-PAGE analysis under non-reducing conditions facilitated labeling with both native Ub and UbDha (Fig. 2b), under reducing conditions only the UbDha probe was able to form a stable adduct with the E2 enzyme. As expected, labeling was not observed in the absence of ATP (Supplementary Fig. 2). Interestingly, the double UbDha loaded UBE1 intermediate²⁶ (Supplementary Figs. 2 and 3) observed in the absence of an E2, was sensitive to co-incubation with UBE2L3 (Supplementary Fig. 4), while adding UBE2L3 subsequent to UBE1 labeling with UbDha had no effect in this context. This may indicate a transfer of Ub from the adenylation active site to a nearby Cys in the adenylation domain of UBE1, which simply does not occur when UbDha is quickly transferred to the next step in the cascade, here transfer to E2²⁷. In addition to UBE2L3, UbDha showed labeling of 26 other ubiquitin E2s (Supplementary Fig. 5), but remained unreactive against Ub1 E2s (UBE2F, UBE2I, UBE2L6 and UBE2M). UbDha was also unable to label the Ub E2 UBE2Z downstream of UBE1 due to the enzyme's selectivity for the alternative E1, UBA6²⁵. Noncanonical catalytically inactive E2s UBE2V1 and UBE2V2²⁸ with scaffolding function also failed to react with the probe (Supplementary Fig. 5). Collectively, these results demonstrate broad utility of the UbDha probe in monitoring mechanism-based transfer of activated Ub from the E1 to a wide range of cognate E2s.

Under non-reducing conditions, (Fig. 2b, Supplementary Fig. 6) a ternary complex of E1~UbDha-E2 was revealed. Here, the acceptor E2 enzyme reacts directly with the Michael acceptor on the probe-donating E1-thioester adduct. This third pathway of probe action (Supplementary Fig. 6, right panels) was further confirmed by the stable oxyester linked E1-O~UbDha adduct (Supplementary Fig. 6).

Next, we investigated whether UbDha could be further delivered to an E3, bearing an active site Cys. The family of E3 ligases is subdivided into three major classes according to their mechanism of action²¹. In humans, the known thioester-forming E3s, which harbor catalytic Cys residues loaded with Ub by a mechanism analogous to the E1 and E2 enzymes, fall into the HECT (homologous to E6-AP terminus, 28 family members in humans) and RBR (RING-between-RING, 13 members in humans) classes. In contrast, the RING E3 ligases act as scaffolds between the E2~Ub thioester and the substrate protein, but do not themselves form thioesters²¹. We therefore examined a well-characterized HECT E3 ligase NEDD4L¹⁴. As expected, a Cy5-UbDha-NEDD4L thioether adduct was observed downstream of UBE1 and UBE2L3 (Fig. 2c). Similarly, a panel of 9 other HECT E3s also reacted with the probe (Supplementary Fig. 7). Because NEDD4L initially showed reactivity without ATP (Supplementary Fig. 7), we repeated this experiment with an active site Cys-to-

Ala mutant and a mutant in which the four non-catalytic Cys residues were mutated to Ala (Supplementary Fig. 8). Labeling was clearly visible under standard assay conditions, whereas omitting either ATP, UBE1, and UBE2D2 or ATP alone resulted in virtually no labeling. Interestingly, the catalytic Cys-to-Ala mutant showed reduced but not completely abolished labeling compared to the wild-type NEDD4L. A similar observation was made for the non-catalytic 4× Cys-to-Ala mutant, suggesting that NEDD4L has at least two cysteines competent to receive an activated UbDha. Not all HECT E3s have a Cys adjacent to a noncovalent Ub-binding site. For instance, the HECT domain of Smurf2 (54% identical to NEDD4L) lacks the candidate Cys in the noncovalent Ub binding site and showed no alternative labeling of any of its six non-catalytic Cys residues with our probe (Supplementary Fig. 9).

For a native Ub, the next step in the cascade following reactivity with an E3 would result in ligation to a target substrate. To test whether our probe behaves similarly, we chose WBP2²⁹ a known substrate for the UbDha-reactive E3 HECT ligases (NEDD4L, Rsp5, WWP1 and WWP2). While incubation with Ub showed multiple turnover ubiquitination on WBP2, no ubiquitination was observed using UbDha even with prolonged reaction times (Fig. 2d and Supplementary Fig. 10). This feature makes UbDha particularly advantageous for enzymatic profiling in cellular systems, where irrespective of the presence of substrates, our activated warheads will remain on the active enzymes themselves.

Generalizing the cascading ABP methodology to Ubls

To show that our probe design is applicable beyond the Ub cascade, we synthesized the NEDD8 G76C mutant by linear Fmoc based solid phase synthesis (SPPS)³⁰ and easily transformed the Cys into Dha by overnight incubation with 2,5-dibromohexanediamide. Following incubation of NEDD8Dha with UBA3/NAE1, SDS-PAGE analysis revealed formation of the expected UBA3-NEDD8 thioether adduct and a double NEDD8-loaded UBA3 adduct³¹ (Supplementary Fig. 11 and Supplementary Fig. 12). Co-incubation with UBE2M resulted in the formation of a NEDD8-UBE2M thioether adduct (Supplementary Fig. 11). As with the Ub E1 UBE1, formation of double NEDD8Dha-linked UBA3 was suppressed when UBE2M was present during the E1 labeling. Similarly, treatment with 2-mercaptoethanol had no effect on adduct formation, and labeling with NEDD8Dha failed in the absence of ATP (Supplementary Fig. 11). These experiments demonstrate that our founder cascading ABP design can be extended to other Ubl modification cascades.

Structure determination of a thioether-linked E2-Ub adduct

To evaluate the structural integrity of our thioether-linked adducts we performed both solution-based and crystallographic studies. Solution properties of the oxyester-linked UBE2N-O~Ub conjugate (in which the active site Cys has been mutated to Ser) have been thoroughly characterized by NMR spectroscopy and small angle X-ray scattering³², allowing us to validate the analogous thioether linkage as a suitable mimic. Analysis of chemical shift perturbations in the ¹H, ¹⁵N HSQC spectrum of UBE2N (Supplementary Fig. 13) arising from conjugation with Ub revealed several regions affected in both the thioether- and oxyester-linked samples (Fig. 3a; compare red to black) localized primarily to Loop 3, Helix 2, Loop 8, and the penultimate C-terminal helix (Fig. 3b, red surfaces). These

perturbed regions can be attributed to the “closed” conformation of the UBE2N-UbDha thioether adduct, which is predominant in solution³² and indicates the overall behavior of the thioether-linked adduct to be similar to the oxyester linkage. Certain resonances in the UBE2N-UbDha HSQC spectrum displayed markedly different characteristics from the oxyester-linked sample (Fig. 3a), mapped to the region directly surrounding the active site (Fig. 3b, blue surfaces). Given the exquisite sensitivity of amide resonances to their local chemical environment, such differences between the two linkage types are most likely due to their differing chemical properties, and not to a larger structural change.

While solution studies indicate normal inter-domain behavior within the thioether-linked adduct, they lack the atomic resolution of the linkage and the surrounding active site residues. To remedy this we crystallized the UBE2D3-UbDha adduct under conditions published for the oxyester-linked UBE2D3-O~Ub conjugate³³. The 2.2Å UBE2D3-UbDha thioether structure was strikingly similar to the published oxyester structure (PDB 3UGB), with Ca RMSD values of 0.23 and 0.35Å for UBE2D3 and Ub, respectively (Fig. 3c, Supplementary Table 1). The only significant deviation between the two structures was found in the Ub C-terminus near the linkage itself, manifested in an RMSD of 1.13Å for Ub residues 73–76. The thioether linkage was readily revealed in the corresponding electron density (Fig. 3d), although detailed features of the omit map did suffer from high B factors in the flexible Ub C-terminus (average B-factor of 105.7 for Ub residues 75–76 compared to 49.4 for all protein). Nearby residues within the UBE2D3 active site were found to adopt nearly identical conformations, with the only exception being Arg90, which was missing from the electron density (Fig. 3e). An overlay of the oxyester and thioether structures suggests that the additional carboxylate group of the thioether linkage could displace the Arg side chain from the E2 active site cleft, although to our knowledge there is currently no known role for this residue in E2 catalysis.

Preparation of stable E2-Ub conjugates has in the past relied on the DCA method¹³, oxyester^{14–16}, and isopeptide¹⁷ bonds, all necessitating mutations to the enzyme’s active site. Furthermore, oxyester-linked E2~O-Ub/ E2~O-Ubl conjugates suffer from susceptibility to hydrolysis, particularly in the presence of an active E3 ligase^{14–16}, thereby limiting their use in structural applications and preventing any potential utility in cell-based studies. In contrast, UBE2D3-UbDha and UBE2N-UbDha thioether-linked adducts remained inert in the presence of activating factors, such as the E3 ligases TRAF6 (RING-type) or NEDD4L (HECT-type), or an accessory E2-variant UBE2V2 with or without TRAF6, respectively (Figs. 3f and 3g). As a catalytically inert mimic of native thioester-linked conjugates, the thioether-linked adduct behaved as a competitive inhibitor of the ligation machinery in single-turnover assays monitoring diUb formation by UBE2N, UBE2V2, and the E3 ligase cIAP (Supplementary Fig. 14). Combined with solution and crystallographic data, these functional assays support the utility of thioether-linked E2-UbDha adducts as stable mimics in both structural and functional studies.

UbDha probe as a proteomics tool

Having validated activity and structural integrity of the UbDha probe, we turned to enzymatic cascade profiling in biological samples. Incubation of cell extracts with Cy5-

UbDha revealed robust labeling of both UBE1 (Fig. 4a) and UBA6 activating enzymes, reliably abrogated by apyrase treatment (Supplementary Fig. 15). Appearance of additional ATP-dependent bands was also detected on the same timescale, (Supplementary Fig. 15), supporting the in vitro data that the probe is passed downstream. To identify these proteins, we utilized the biotin-labeled probe variant for affinity-based proteomic profiling³ of human cervical cancer (HeLa, Fig. 4b) and melanoma (MelJuSo, Supplementary Fig. 16) cell extracts. We used the ATP-dependent reactivity of our probe to our advantage and performed affinity-based proteomic profiling in the presence of ATP, as compared to apyrase-mediated ATP-depletion. Mass spectrometric analysis of proteins associated with the probe in an ATP-dependent manner, retrieved both Ub E1 enzymes and numerous downstream E2 enzymes. Specifically, roughly half of known human E2 enzymes³⁴ charged by UBE1 (as well as UBE2Z, charged specifically by UBA6) were identified with high confidence in both cell lines. Among the most enriched proteins were UBE2L3, UBE2S and UBE2K, all of which can readily accept ubiquitin from UBE1 and UBA6²⁵. Interestingly, three different members of the E2D subfamily were recovered: UBE2D2, UBE2D3 and UBE2D4, in fact the largely uncharacterized UBE2D4 was the top hit in HeLa cells (Fig. 4b). By contrast, UBE2D4 was not recovered in MelJuSo cells, exemplifying how the UbDha probe can facilitate unbiased proteome-wide comparisons of enzymatic reactivities. In addition to canonical E2s, we also detected atypical E2/E3 hybrid enzymes (UBE2O and BIRC6) as well as HECT E3s. While the E3 ligases UBE3A and HECTD1 were found in both cell lines, TRIP12 was observed only in MelJuSo cells. UBE3A prefers to accept Ub from UBE2L3³⁵, which was recovered in high abundance from both cell lines, indicating isolation of a full E1–E2–E3 cascade. Enzymes of interest, immuno-precipitated directly from cells, can be subsequently investigated using the UbDha probe in the presence of supplemented reaction components of choice. This is demonstrated by the ability of GFP-UBE2J1 but not GFP-UBE2Z to accept activated Cy5-UbDha from UBE1 (Fig. 4c). As expected, mutation of active site Cys 91 of GFP-UBE2J1 to Ala (C91A) abrogated labeling, demonstrating suitability of UbDha for mutational studies.

We also identified four DUBs (OTUB1, OTUD7B, UCHL3 and USP15) in pull-downs with the ligase probe (Fig. 4b and Supplemental Fig. 16). Because DUBs harbor highly reactive active site Cys residues, we assessed potential cross reactivity by incubation of the Cy5-UbDha probe with cell lysates ectopically expressing various GFP- or FLAG-tagged DUBs (Supplemental Fig. 17a) alongside the recently reported DUB-specific ABP, Cy5-UbPA³⁰. While Cy5-UbPA readily modified all the active DUBs tested here, only incubation with excessive amounts of UbDha resulted in often marginal DUB labeling. Moreover, labeling of even highly reactive DUBs with Cy5-UbDha, such as OTUB1 and OTUB2 could be readily abolished by pretreatment with UbPA (Supplementary Fig. 17b). Of note, the DUBs recovered with UbDha in our proteomic experiment (particularly OTUB1) can interact with E2 enzymes³⁶. Since DUB-mediated catalysis proceeds independently of ATP, recovery of these DUBs in the ATP-dependent setting can be a result of co-isolation with their active partner ligases.

Activity based protein profiling in cells

To address the efficacy of UbDha in monitoring the Ub-conjugating cascade in the cellular context, we next introduced Cy5-UbDha into HeLa cells by electroporation. In-gel fluorescence analysis followed by immunoblotting revealed speedy engagement of both human Ub activating enzymes (Fig. 5a) on a time-scale comparable to (if not faster than) that observed in lysate labeling experiments (Fig. 4a). Furthermore, treatment of cells with the UBE1 inhibitor PYR-41³⁷ prior to introducing the probe noticeably reduced detectable UBE1 activity (Fig. 5b), indicating that the probe can be used to monitor enzymatic inhibition in living cells.

Cells harboring Cy5-UbDha were found to exhibit normal morphology, with the probe being evenly distributed throughout the nuclear and cytoplasmic space, as expected when small molecules such as ubiquitin move unrestricted across the nuclear membrane³⁸ (Fig. 5c, top panels). Of note, in cells undergoing late stages of cell division, accumulation of Cy5-UbDha was consistently observed at the cytokinetic bridge (Supplementary Fig. 18), consistent with the site of BIRC6 activity at this time in the cell cycle³⁹. Having detected BIRC6 in high abundance in our proteome-wide active ligase analysis (Fig. 4b), these observations suggest that our probe could be used to study spatial and/or temporal aspects of relevant enzymatic cascades. For instance, introduction of Cy5-UbDha into HeLa cells ectopically expressing predominantly nuclear GFP-UBE1 resulted in the corresponding nuclear accumulation of the probe (Figs. 5c, and 5d) relative to untransfected cells. In-gel fluorescent analysis and immunoblotting of corresponding lysates confirmed formation of a GFP-UBE1-UbDha adduct (Fig. 5e).

To investigate whether the probe, while inside the cell, can be passed downstream of the E1, we introduced Cy5-UbDha into cells expressing UBE2J1 or its catalytically inactive C91A or C91S mutants. Catalysis-dependent modification of the E2 with the probe was indeed observed (Figs. 6a and 6b, left panel) and found to be sensitive to inhibition of the upstream UBE1 (Fig. 6b, right panel). UBE2J1, which we isolated with UbDha from MeJuSo cell lysates (Supplementary Fig 16), localizes to the endoplasmic reticulum (ER), where it functions in ER-associated degradation (ERAD)⁴⁰. In cells, Cy5-UbDha was found to readily colocalize with wild type, but not catalytically dead UBE2J1 (Figs. 6c and 6d). Importantly, such colocalization was sensitive to inhibition of the upstream E1 (Figs. 6c and 6d), indicating that imaging of intact cells harboring fluorescently labeled UbDha can report on relevant enzymatic cascades. Collectively, these experiments illustrate a wide range of utilities for UbDha in the study of ubiquitin ligase activities in cells. To the best of our knowledge, UbDha is the first probe that allows cascade-dependent profiling of ubiquitin conjugating enzymes in a physiologically relevant setting.

Discussion

Given the critical roles of E1, E2 and E3 enzymes in a wide range of biological processes and their resulting emergence as drug targets^{41,42}, there is a critical need for suitable assay reagents to study their function. The pyramidal structure of the Ub/Ubl conjugation systems, their complex cross-reactivities, and the reactive nature of the E2~Ub and E3~Ub thioester intermediates present practical challenges in dissecting interactions between Ub-loaded

partner enzymes. To monitor these enzymatic cascades, we present a unique probe designed to hop from one active site to the next, leaving a detectable covalent mark at every step of the way.

Relatively inert on its own, our cascading probe (UbDha) requires ATP-dependent activation by the E1 enzyme, which increases the electrophilic character of the Dha moiety, making it suitable to follow the cascade of trans-thioesterification reactions downstream. The key conceptual advantage of Dha-based methodology lies in its unprecedented ability to choose at any point along the cascade between a native-like thioester and irreversible thioether bond formation. Indeed, we show that UbDha readily labels active site Cys residues of E1, E2 and HECT E3 enzymes. Importantly, UbDha does not get transferred to substrates. This feature endows our cascade probe with advantageous capabilities over the native Ub, particularly in complex biological settings, where enzymes are present together with their substrates. Under such circumstances, UbDha enables a direct measure of enzyme activity, rather than merely detecting consequences thereof.

Because entry of Ub(/Ubl)Dha into its cognate enzymatic cascade requires ATP, much of the background binding to the probe can be easily discriminated by eliminating ATP with apyrase. This simple feature makes UbDha suitable for activity-based profiling of Ub/Ubl cascades not only *in vitro*, but also in complex biological circumstances, as demonstrated by the proteome-wide analysis of Ub conjugation machineries isolated from two different cancer cell lines. The straightforward nature of the experimental setup is expected to be readily adaptable to comparative profiling of E1, E2 (and to some degree E3 enzymes) as a function of various biological perturbations (i.e. stimulation or starvation, infection, etc.). The same reagent can subsequently be used to study the effects of mutations in enzymes isolated directly from organisms of interest, as well as test for relevant factors upstream or downstream in the cascade. Standard biochemical techniques presently relied upon for such studies typically involve laborious expression and purification protocols. Furthermore, no observable reactivity in such preparations may be attributable to misfolding or lack of necessary modifications acquired in the carrier organism. Our methodology bypasses these difficulties by offering a relatively quick and easy way to assess reactivity of enzymes isolated directly from cells using simple immuno-precipitation. Then, taking cellular enzymology one step further, UbDha can be introduced into living cells to directly monitor enzymatic activities occurring in their natural context. In this way, the versatility of the UbDha cascade probe may prove invaluable in dissecting how aberrant activities of E1–E2–E3 cascades contribute to pathogenesis^{45,46}, as well as for diagnosis and monitoring efficacy of UPS targeting therapy. Furthermore, by generating a NEDD8-based counterpart of the UbDha probe capable of labeling the NEDD8 conjugating machinery, we show our method to be diversifiable towards ubiquitin-like proteins. As such, the technology described here may be used to interrogate presently less well-defined ligation machineries of various UbIs.

In addition to cell-based applications, Ub/UblDha may prove useful *in vitro*, particularly for structure determination. The thioether adducts described here bypass the need for the often relied upon active site mutagenesis^{13–17} thus avoiding potential disturbances to catalytic properties of enzymes in question. Stability of our thioether adducts under reducing conditions, in the presence of an activating E3 ligase and in functional assays allowed us to

perform NMR and X-ray crystallography studies. High degree of similarity to the published oxyester-linked structure supports their utility as stable mimics in both structural and functional studies. We foresee that UbDha may be used to expedite generation of crystal structures of E1, E2 or E3 enzymes and their complexes²⁷. In addition, we hypothesize that the stability of our E2-UbDha adducts immobilized on affinity beads could enable proteomic profiling of cognate RING E3 enzymes, which cannot themselves be directly trapped in a mechanism-dependent manner⁴³.

Based on the proof-of-concept studies described herein, we anticipate our cascading probe reagents to greatly facilitate future discoveries on Ub/Ubl conjugation.

Online Methods

Chemical synthesis

Detailed procedures for the synthesis of Dha-based ABPs can be found in Supplementary Note 1.

Protein expression and purification

UBE1—The UBE1 enzyme (N-terminal His-tagged) was expressed from a pET3a vector in autoinduction⁴⁴ media at 37°C using *E. coli* BL21 (DE3). After 2–3 hours the temperature was lowered to 18°C and the bacteria were allowed to grow an additional 12h. His-tag mediated purification was performed using TALON beads (Clontech) in Buffer A containing 50 mM TRIS (pH 8.0), 150 mM NaCl and 0.25 mM TCEP, washing with 5 mM imidazole (pH 8.0) and eluting with 500 mM imidazole (pH 8.0). Further purification was performed by anion exchange using a Resource Q column (GE Healthcare) using a gradient to 1M NaCl in Buffer A, which was followed by a Superdex 200 column (GE Healthcare).

UBE1 C632S mutant—The active site cysteine in UBE1 (C632) was mutated to a serine using the protocol provided with the Quik Change Site-Directed Mutagenesis Kit (Invitrogen). The mutant was expressed and purified as described for UBE1.

6His-UBA6—A bacmid containing the verified human 6His-UBA6 cDNA sequence was purified from *E. coli* strain DH10Bac using the Quiagen plasmid mini kit (Quiagen) and transfected into SF21 insect cells using the TransIT 2020 transfection reagent (Mirus) leading to the production of baculovirus during three amplification steps according to standard protocols. SF21 cells were infected with the 6His-UBA6 encoding baculovirus and the 6His-UBA6 protein was purified by nickel affinity chromatography using HisTrap matrix (GE Healthcare). The function of the 6His-UBA6 protein was verified by in vitro activation assays of recombinant human ubiquitin and the recombinant human ubiquitin-like modifier FAT10.

UBE2N, UBE2V2, and UBE2D3—Full length constructs of UBE2N, UBE2V2, and UBE2D3, and NEDD4 residues 521–900, and cIAP residues 363–614 were expressed from pGEX6P-1 constructs in Rosetta 2 (DE3) pLacI cells (Novagen). TRAF6 residues 50–211 were expressed similarly from the pOPIN-K vector. ¹⁵N-labeled UBE2N was expressed in M9 minimal medium supplemented with ¹⁵N-NH₄Cl. Transformed cells were cultured at

37°C to an O.D. of 0.6–0.8 and induced with 0.2mM IPTG at 18°C for 16 hours. Cells were lysed by sonication in a buffer containing 25mM Tris (pH 8.5), 200mM NaCl, and 4mM DTT. The clarified lysate was bound to glutathione resin, washed with additional lysis buffer, and the resin was incubated with GST-tagged 3C protease overnight at 4°C for cleavage of the GST fusion tag, with the exception of NEDD4 and cIAP which were eluted to retain the GST tag. Following cleavage, the released protein was washed through the resin, concentrated, and further purified by size exclusion chromatography on a Superdex 75 column (GE Healthcare) equilibrated in either 20mM Tris (pH 7.4), 100mM NaCl, 1mM DTT for reactions, or 25mM sodium phosphate (pH 7.0), 150mM NaCl for NMR spectroscopy. Mouse His-UBE1 was expressed from the pet-24 vector as above and purified by conventional Ni-NTA methods followed by anion exchange on a Resource Q column and size exclusion chromatography on a Superdex 200 column (GE Healthcare). Pure fractions of all proteins were concentrated and flash frozen for storage at –80°C.

NAE1/UBA3, HECT E3s and WBP2—Complex of GST-tagged NAE1 and UBA3 were co-expressed in *E. coli* RIL strain at 16°C for 16 hours. GST-tagged HECT E3s were expressed in *E. coli* (RIL) at 18°C overnight. GST-proteins were eluted by 10 mM glutathione followed by proteolysis (thrombin for GST-NAE1, TEV protease for all GST-HECT) at 4°C overnight and further purified by ionic exchange and size exclusion chromatography on a Superdex 200 (GE Healthcare) column.

His-MBP-tagged WBP2 was expressed in *E. coli* (RIL) at 20°C overnight and eluted from Ni-NTA column. TEV protease was applied to removed His-MBP tag. Full-length WBP2 was further purified by ionic exchange using fast flow Q column and size exclusion chromatography on a Superdex 200 column.

Pure proteins were concentrated, aliquoted, flash frozen by liquid nitrogen and stored at –80 °C.

E1–E2–E3 labeling assay conditions—Detailed assay conditions can be found in Supplementary Note 2.

Structure Determination

Preparation of thioether-linked ¹⁵N-UBE2N-UbDha and UBE2D3-UbDha—25 μM UBE1, 250 μM E2 (¹⁵N-UBE2N or UBE2D3), 1 mM UbDha, 5 mM ATP, and 10 mM MgCl₂ were incubated at room temperature for 16 hours. Any remaining thioester linkages were reduced by the addition of 10 mM DTT at the end of the reaction. The ¹⁵N-UBE2N-UbDha adduct was purified to ~95% homogeneity by size exclusion chromatography on a Superdex 75 column (GE Healthcare) equilibrated in 25 mM sodium phosphate (pH 7.0), 150 mM NaCl. The UBE2D3-UbDha adduct was subsequently purified to >98% homogeneity by repeated cation exchange chromatography on a 6 mL Resource S column (GE Healthcare) with a 50–500 mM NaCl gradient in 50 mM Tris (pH 8.5) buffer, followed by a final size exclusion chromatography step on a Superdex 75 column (GE Healthcare) equilibrated in 25 mM HEPES, 50mM NaCl pH 7.

NMR spectroscopy ^{15}N -UBE2N-UbDha— ^1H , ^{15}N BEST-TROSY experiments⁴⁵ on 200 μM ^{15}N -UBE2N or ^{15}N -UBE2N-UbDha were acquired with optimized pulse sequences on a Bruker Avance2+ 700MHz spectrometer equipped with a cryogenic triple resonance TCI probe. Data sets were processed using Topspin (Bruker) and visualized with NMRViewJ (One Moon Scientific). Chemical shift perturbations were calculated using the equation $\delta_j = [(\delta_j^{15}\text{N}/5)^2 + (\delta_j^1\text{H})^2]^{1/2}$. Perturbation values greater than 0.05 ppm were mapped onto the UBE2N crystal structure (PDB 1J7D), such that the analysis is directly comparable to previous work on the oxyester-linked UBE2N-O~Ub conjugate³².

Crystallization, data collection and structure determination UBE2D3-UbDha—

The thioether-linked UBE2D3-UbDha adduct was crystallized following conditions published for the oxyester-linked form³³. Purified conjugate was concentrated to 0.9 mM (23.5 mg/mL) and screened against conditions surrounding the published 200 mM tripotassium citrate, 20% PEG 3350 condition in 200nL sitting drops at a 1:1 protein:reservoir ratio. The resulting crystals were cryoprotected in LV CryoOil (MiTeGen) and vitrified prior to data collection at Diamond Light Source beamline I04-1. Data was collected at 100K with a wavelength of 0.92Å. Diffraction images were processed using XDS⁴⁶ and scaled using AIMLESS⁴⁷. Isolated UBE2D3 and Ub molecules from the oxyester UBE2D3-O~Ub structure (PDB 3UGB) were used as search models for molecular replacement using PHASER⁴⁸. Iterative model building and refinement were performed using COOT⁴⁹ and PHENIX⁵⁰, respectively. The thioether linkage could be built unambiguously into the electron density. The final model contained 96.9% in favored, 2.7% in allowed, and 0.4% in outlier Ramachandran space. Data collection and refinement statistics can be found in Table 1. Structural figures were generated using PyMOL (www.pymol.org).

***In vitro* ubiquitination assays**

Stability—The stability of thioether-linked UBE2D3-UbDha adduct was assessed by incubating 5 μM purified thioether conjugate at 37°C in combination with 2 μM TRAF6 or 2 μM GST-NEDD4. The stability of the UBE2N-UbDha adduct was assessed similarly, but using the accessory E2 variant UBE2V2 with or without TRAF6. Samples were collected at 0, 1, 3, and 24-hour timepoints for visualization on Coomassie-stained SDS-PAGE gels. Control reactions containing 0.5 μM UBE1, 5 μM E2, 20 μM Ub, and the relevant activating factors demonstrate the Ub chain formation activity under normal *in vitro* conditions.

Competitive inhibition—Single-turnover ubiquitination assays were established for UBE2N with an initial activation stage containing 10 μM UBE2N, 0.5 μM UBE1, 20 μM Ub, 2.5mM ATP, and 5mM MgCl₂ to generate native thioester linked UBE2N~Ub after 30min at 37°C. This reaction was then quenched by addition of apyrase to prevent any further formation of UBE2N~Ub. Pre-activated UBE2N~Ub was then diluted twofold into a reaction containing 5 μM UBE2V2 alone or in addition to 1 μM GST-cIAP, and samples were collected at 0, 15, and 30-minute timepoints for visualization of diUb product formation on Coomassie-stained SDS-PAGE gels. To test for competitive inhibition using the thioether-linked UBE2N-UbDha adduct, increasing concentrations (1.25 μM , 2.5 μM , 5 μM , 10 μM , and 20 μM) of purified conjugate were added into reactions containing UBE2N~Ub, UBE2V2,

and GST-cIAP as specified above, and 30-minute timepoints were collected to compare levels of diUb product formation with the control experiment.

Mammalian cell lines—HEK293T and HeLa cell lines used in this study originated from ATCC and were cultured under standard conditions in DMEM (Gibco) supplemented with 10% FCS (Sigma-Aldrich) at 37°C with 5% CO₂. The previously characterized human melanoma cell line MelJuSo⁵¹ was cultured in IMDM (Gibco) supplemented with FCS. All cell lines were routinely tested for mycoplasma contamination with consistently negative outcome.

Labeling of overexpressed DUBs in cell lysates—For overexpression of epitope-tagged DUBs in HEK293T cells, previously described wild type and catalytic point mutants of USP8, OTUB1 and OTUB2 were used³⁰. Additionally, human USP15 and OTUD1 were subcloned from pDEST-cDNA constructs (Addgene) into 2×FLAG-C1 vector (Clontech) at HindIII/XbaI and XhoI/EcoRI respectively, and mutagenesis of catalytic residues C269S in USP15 and C320S in OTUD1 was performed using standard protocols (Stratagene)). DNA was delivered into HEK cells using polyethylenimine (PEI, Polysciences, Inc.) according to manufacturer's instructions. 24h following transfection, cells were harvested by scraping in lysis buffer (150 mM NaCl, 50 mM Tris HCl pH 7.5, 0.5% Triton-X100, Protease Inhibitor tablet (Roche)) and lysate was clarified by centrifugation. Reactions were incubated in the presence of indicated components with either Cy5-Ub-PA (1.0 µg/reaction), stationary for 30–45 min at RT or Cy5-Ub-Dha (1.5 µg/reaction) with gentle agitation for 70 min at 37°C in the presence of 10 mM ATP and 10 mM MgCl₂. 10 mM NMM was added where indicated. Pre-incubation with unlabeled Ub-PA (1.5 µg/reaction) was performed as for Cy5-Ub-PA, followed by Cy5-Ub-Dha chase as above. Reactions were stopped by the addition of sample loading buffer supplemented with 100 mM DTT, followed by boiling for 10 min. Samples were resolved using standard SDS-PAGE, and probe reactivity was assessed by Cy5 fluorescence scanning (λ_{ex} = 625 nm; λ_{em} = 680 nm), followed by gel transfer onto Nitrocellulose membranes and immunoblotting using either rabbit anti-GFP serum⁵², mouse anti-Flag (1:1000 dilution; Sigma Aldrich, Sigma F3165) or mouse anti- β -actin (1:10000 dilution; Sigma A544), as indicated. Fluorescent secondary antibodies anti-mouse-800 (1:10000 dilution; LiCOR, 926–3210) and anti-rabbit-800 (1:10000 dilution; LiCOR, 926–3211) were used for visualization of labeled proteins on LICOR Odyssey system v3.0.

Cell lysate labeling—Cell lysates were prepared by resuspending cell pellets in 3 pellet volumes of HR buffer (50mM TRIS, pH 7.4, 5 mM MgCl₂, 250 mM sucrose, 1 mM DTT) and lysed by sonication. After clarification by centrifugation (20,000 rpm, 4°C, 20 min), total protein concentration was determined by Nanodrop. For the labeling experiments, 100 µg of lysate was incubated with 0.5µg Cy5-Ub-Dha, 10 mM ATP, 10 mM MgCl₂, in Labeling Buffer (50 mM HEPES, 100 mM NaCl, pH 7.5) at 37°C for 1h or as indicated. Additional 1 mM ATP and MgCl₂ were added to the reaction every 20 minutes to replenish consumed ATP. In case of the negative control, lysates were treated with 2 Units of Apyrase (Sigma Aldrich) prior to addition of the Cy5-UbDha probe (ATP and MgCl₂ were omitted). The reaction was terminated by the addition of 3× SDS-PAGE Loading Buffer (Invitrogen) containing beta-mercaptoethanol. Samples were resolved by SDS-PAGE and visualized by

fluorescence scanning ($\lambda_{\text{ex}} = 625 \text{ nm}$; $\lambda_{\text{em}} = 680 \text{ nm}$). For visualizing the reactivity of endogenous UBE1 and UBA6 western blotting was performed as previously described and membranes were probed with rabbit UBE1 (1:1000 dilution; Abcam, ab 181225) or rabbit UBA6 antibody (1:1000 dilution; Abcam, ab 177514).

Activity-based Ub cascade profiling in intact cells

Electroporation of UbDha into living cells—HeLa cells cultured under standard growth conditions were transfected with GFP, GFP-UBE1⁵³, GFP-UBE2J1, GFP-UBE2J1 or mutants C91A or C91S, Flag-UBE2J1 or mutant Flag-C91A using Effectene (Qiagen), according to manufacturer's instructions. To facilitate the incorporation of the probe, the growth medium was refreshed 4–6 hours following transfection and again 1–2 hours prior to electroporation. For inhibition of UBE1, cells were treated with 50 μM PYR-41 for 30 min prior to introducing the probe. Following removal of the growth medium, cells were kept on ice for the duration of the protocol. Cells were washed twice with cold electroporation buffer (2mM HEPES, pH 7.4, 15 mM $\text{K}_2\text{HPO}_4/\text{KHPO}_4$, 250 mM Mannitol, 1mM MgCl_2). Next, 1.5 mL of a solution of the Cy5-UbDha, Cy5-Ub or Rho-UbDha probe in electroporation buffer (0.4 mg/mL) was added to each of the wells and electroporation was performed on ice using a Biorad GenePulser Xcell with CE and PE module Pulse Generator equipped with a Petri PulserTM electroporation applicator (BTX) using the following settings: square wave, voltage=75V, pulse length=3ms, pulse interval=1.5s, number of pulses=5, cuvette width=2mm. The electroporation applicator was turned 90 degrees and electroporation was repeated once. The probe solution was replaced by cold electroporation buffer and cells were allowed to recover on ice for 2 min. Following treatment, cells were washed twice with ice-cold PBS and allowed to recover under standard growth conditions as indicated (15–120 min).

For gel-based analysis, samples were lysed using reducing SDS-PAGE loading buffer followed by brief sonication, heated at 98°C for 10 min before being analyzed on SDS-PAGE gel, and subsequently visualized by fluorescence scanning ($\lambda_{\text{ex}} = 625 \text{ nm}$; $\lambda_{\text{em}} = 680 \text{ nm}$ (Cy5-UbDha) or $\lambda_{\text{ex}} = 473 \text{ nm}$; $\lambda_{\text{em}} = 530 \text{ nm}$ (Rho-UbDha)). Western blotting was performed as previously described and membranes were probed with either rabbit anti-GFP serum⁵² or mouse anti-Flag (1:1000 dilution; Sigma Aldrich, Sigma F3165).

Confocal microscopy—For microscopy experiments, the samples were fixed in 4% Formaldehyde (Merck) in PBS and mounted onto glass slides (Thermo Scientific) using Prolong Gold mounting medium with DAPI (Invitrogen). Z stacks of 5 images per cell were collected on a Leica SP5 confocal microscope equipped with HyD detectors, using a 63 $\times$ magnification lens in combination with 2.5–4 $\times$ digital zoom and represented as maximum z projections. Image processing and fluorescence intensity analysis was performed using ImageJ64 software, and colocalization was expressed in the form of Mander's overlap coefficients calculated using JACoP. Pixel plot analyses were generated using Leica LASAF software.

Statistics and reproducibility—Statistical data are presented as standard deviation (s.d.). Comparisons between the samples were made using a two-sided t-test (assessed

against the control), with the p values reported above the corresponding data in the graphs. Data analyzed was derived from at least two biologically independent experiments (n) as indicated in figure legends.

Proteomic activity profiling of Ub-conjugating enzymes

Sample preparation—For identification of enzymes reactive towards the Biotin-UbDha probe, 15 cm dishes of subconfluent HeLa or MelJuSo cells were cultured under standard conditions. Cells were washed with PBS, trypsinized and centrifuged at 1500 rpm. The pellet was washed once with PBS, resuspended in 3 pellet volumes HR lysis buffer (50 mM Tris pH 7.4, 5 mM MgCl₂, 250 mM sucrose, 1 mM DTT, and Protease Inhibitor Tablet (Roche)) and lysed by sonication. After sonication, the cell extracts were clarified by centrifugation (20,000 rpm, 4°C, 20 min) and the total protein concentration determined using the Nanodrop. Labeling was performed by adding 10 µg Biotin-UbDha, 10mM ATP, 10mM MgCl₂ to 10 mg cell lysates (total protein concentration) in Labeling Buffer (50mM HEPES, 100mM NaCl, 1mM DTT, pH 8.0) in a volume of 400µL for 1h at 37°C while gently shaking. To ensure that the ATP supply is constant, 1mM ATP and 1mM MgCl₂ were added to the reaction every 15 min. For the negative controls, ATP was depleted prior to the reaction using 2 Units of Apyrase (Sigma Aldrich) for 15 min at 37°C. In case of the negative controls, the labeling reaction was performed as previously described, but with the omission of ATP and MgCl₂. After reaction with the probe, the lysates were incubated with pre-equilibrated High Capacity Neutravidin Agarose (Thermo Fisher) for 3h at 4°C in a total volume of 700 µL, while rotating. Subsequently, the supernatant was removed and the resin was washed with 1 mL each of the following wash buffers⁵⁴: buffer 1 (2% SDS in dH₂O), buffer 2 (50 mM HEPES, pH 7.5, 1 mM EDTA, 500 mM NaCl, 1% Triton-X 100, 0.1% deoxycholate), buffer 3 (10 mM Tris, pH 8.0, 1 mM EDTA, 0.5% NP-40, 250 mM LiCl) and buffer 4 (50 mM Tris, pH 7.4, 50 mM NaCl). After washing, the samples were eluted in 40 µL 3× SDS PAGE Loading Buffer (Invitrogen) with additional 2-mercaptoethanol and boiled at 95°C for 5 min before loading onto a 10% gel (NuPAGE,Invitrogen). The proteins were run 1cm into the gel, and subsequently stained overnight with Coomassie Brilliant Blue before being destained in H₂O. After destaining, the band was cut into small pieces using a sterile scalpel and processed further for LC-MS/MS analysis.

LC-MS/MS analysis—In-gel digested peptides were cleaned and desalted by 1 ml 0.1% formic acid twice on OASIS HLB cartridge (Waters), before being eluted twice with 100 µL 80% acetonitrile in 0.1% formic acid. Desalted peptides were vacuum centrifuged at room temperature until complete dryness then dissolved in 0.1% formic acid, prior to online nanoflow liquid chromatography-tandem mass spectrometry. The analysis of in-solution digested peptides was performed using an EASY-nLC system (Proxeon) connected to a Q-Exactive (Thermo) using Higher-Collisional Dissociation (HCD) fragmentation. Separation of peptides was performed using 13 cm long analytical columns (ID 75 µm, Polymicro Avantes) packed in-house with 1.8 µm C18 beads (Reprospher-DE, Pur, Dr. Maisch), using a 30 minute gradient from 2% to 95% acetonitrile in 0.1% formic acid and a flow rate of 200 nL per minute. The mass spectrometer was operated in data-dependent acquisition mode using a top 10 method. Full-scan MS spectra were acquired with a target value of 3E6 and a resolution of 70,000, with a scan range from 400 to 1,400 m/z. HCD tandem MS/MS spectra

were acquired using a target value of 1E5, a resolution of 17,500, and a normalized collision energy of 25%. All charges lower than two and higher than six were rejected, and all unknown charges were rejected. The underfill ratio was set to 1.0%, and a dynamic exclusion of 60 seconds was used.

Data processing—MaxQuant version 1.5.1.2 was used to analyze all RAW data.^{55,56} The experiment was performed in biological triplicate, and measured as three technical replicates. MS/MS spectra were filtered and deisotoped and the 18 most abundant fragments for each 100 m/z were retained. MS/MS spectra were filtered for a mass tolerance of six ppm for precursor masses, and a mass tolerance of 20 ppm was used for fragment ions. Peptide and protein identification was performed through matching the identified MS/MS spectra versus a target-decoy version of the complete human Uniprot database (03 December 2015, 92040 proteins), in addition to a database of 245 commonly observed mass spectrometry contaminants. Up to two missed tryptic cleavages were allowed. Cysteine carbamidomethylation was set as a fixed peptide modification. Protein N-terminal acetylation and methionine oxidation were set as variable peptide modifications. Peptides were accepted with a minimum length of six amino acids, a maximum size of 6kDa, and a maximum charge of six. The processed data was filtered by posterior error probability (PEP) to achieve a protein false discovery rate (FDR) of below 1%, a peptide-spectrum match FDR of below 1%. Modified peptides were additionally filtered to have an Andromeda score of at least 40.

Label-Free Quantification (LFQ)—Label-Free Quantification was performed using MaxQuant LFQ. Quantification was performed over three biological replicates. The fast LFQ algorithm was used with default settings (min. ratio count of two, min. neighbors of three, average neighbors of six). Protein LFQ values were Log2 transformed for further processing. Proteins identified by site only, reverse sequence only, and potential contaminants were removed. Proteins identified by at least 4 MS/MS spectra, at least 2 razor +unique peptides and at least one unique peptide were retained. To ensure biological reproducibility, only proteins detected in at least two out of the three biological replicates in at least one group were retained. Subsequently, missing values were imputed using Perseus software version 1.5.1.6, with standard settings. LFQ values were averaged within all treatment conditions. Proteins were considered to have a specific interaction with the ubiquitin ligase probe when they were enriched at least two-fold compared to the negative control with a T-test p value of less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mechanism and synthesis of the activity based probe Ub-Dha

In situ activation of Ub-Dha with E1 and ATP results in a mechanism based ABP for E1, E2 and Cys dependent E3 enzymes. Pathway a describes the covalent trapping of the enzyme (E-UbDha = thioether-linked adduct), while pathway b depicts the native transthioesterification processing of the probe (E~UbDha = thioester intermediate of conjugate) by the cascade.

Figure 2. Covalent thioether bond formation of UbDha with conjugating enzymes

a) ATP-dependent labeling of the E1 enzymes UBE1 (left) and UBA6 (right); **b)** Reactivity of UBE2L3 towards Ub and UbDha under reducing and non-reducing conditions; **c)** Fluorescent scan showing NEDD4L HECT labeling with the Cy5-UbDha; **d)** Multiple turnover ubiquitination on substrate WBP2 doesn't occur with UbDha. Asterisks (*) indicate modified forms of UBE1, UBA6, UBE2L3 and NEDD4L. For uncut gels, see Supplementary Figure 19.

Figure 3. Structural studies of thioether-linked E2-Ub adducts

a) UBE2N chemical shift perturbations upon Ub activation for oxyester (black) and thioether (red) linkages. Resonances perturbed beyond facile re-assignment were plotted using maximum perturbation value. **b)** Changes (from a) mapped onto the UBE2N structure (PDB 1J7D) (similarities in red; differences in blue). Active site Cys is colored yellow. **c)** Superposition of thioether-linked UBE2D3-UbDha conjugate (gray) over the oxyester-linked form (cyan, PDB 3UGB). **d)** Simulated annealing omit map of electron density surrounding the thioether linkage. 2|Fo|-|Fc| electron density (blue) contoured at 1σ, |Fo|-|Fc| density

(green) contoured at 3σ . Inlay: diagram illustrating the thioether linkage. **e)** Overlay of E2 active site residues in thioether (gray) and oxyester (cyan) structures. Stability of the thioether-linked UBE2D3- and UBE2N-UbDHA adducts, incubated with **f)** RING E3 ligase TRAF6 or the HECT E3 ligase NEDD4, or **g)** accessory E2-variant UBE2V2, alone or in combination with the RING E3 ligase TRAF6.

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Figure 4. Proteome-wide activity profiling of Ub-conjugation machinery

a) Time-course of UBE1 labeling in HeLa cell extracts with Cy5-UbDha in the absence (–) or presence (+) of ATP scavenger apyrase. **b)** Proteomic profiling of the Ub activation, conjugation and ligation machineries in HeLa cells. Volcano plot of pairwise comparison of proteins bound to the Biotin-UbDha probe relative to apyrase treatment (negative $\log_{10}$ p-value, y-axis) as a function of fold enrichment (average $\log_2$, x-axis). Confidently identified proteins (average $\log_2$ ratio >1 , $p < 0.05$) are marked as follows: E1 (green), E2 (red), HECT E3 (blue), hybrid E2/E3 (purple) and DUBs (light blue); proteins unrelated to the Ub cycle

are marked in black, and those falling below the threshold are shown in gray. Several known cascade connections are highlighted with gray lines. **c)** Labeling of GFP-tagged enzymes isolated from HeLa cells (UBE2J1 or its catalytic mutant C91A versus GFP-UBE2Z) with Cy5-UbDha downstream of purified UBE1. Asterisks (*) indicate modified forms of E1 and E2 enzymes. For uncut gels, see Supplementary Figure 20.

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Figure 5. Activation of UbDha in vivo

a) In vivo labeling of endogenous E1 enzymes with Cy5-UbDha. Fluorescence scanning and immunoblotting of lysates from HeLa cell electroporated with the probe and harvested at indicated time intervals following electroporation with the probe. **b)** In vivo labeling of UBE1 with Cy5-UbDha following UBE1 inhibitor PYR-41 (50 μM) treatment. Fluorescence scan and quantification (% labeling in the absence of PYR-41; n=3, error bars correspond to SD, with significance (p) assessed using a two-sided t-test) are shown. **c)** Distribution of Cy5-UbDha (magenta) in cells ectopically expressing GFP-UBE1 (green) relative to

untransfected cells. Representative 3D confocal compilations of fixed cells treated as indicated are shown with DAPI (blue) overlays and nuclear insets; scale bars = 10 μm . **d)** Pixel traces of DAPI and Cy5-UbDha (marked with dotted lines in d) plotted as fluorescence over distance. **e)** Formation of the GFP-UBE1-UbDha adduct in cells. Asterisks (*) indicate modified forms of E1 enzymes. For uncut gels, see Supplementary Figure 21.

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Figure 6. Probing in vivo E1–E2 cascade with UbDha

a) In vivo UbDha adduct formation with Flag-UBE2J1 versus its catalytic mutant C91A by immunoblot against Flag. **b)** Reactivity of GFP-UBE2J1 versus its mutants C91A or C91S (left panel) or as a function of UBE1 inhibition (50 μ M PYR-41, right panel) with Cy5-UbDha or -Ub electroporated into HeLa cells. Asterisks (*) indicate modified forms of E1 and E2 enzymes. **c)** Representative 3D confocal compilations of fixed cells of fixed cells (from c) treated as indicated. Overlays of GFP (green) with Cy5-UbDha (magenta) along with corresponding pixel plots are shown; scale bars = 10 μ m. **d)** Colocalization (Mander's

overlap coefficient) of Cy5-UbDha with wild type (J1) or mutant (CS) GFP-UBE2J1 ($n = 2$, error bars correspond to SD, with significance (p) assessed using a two-sided t-test). For uncut gels, see Supplementary Figure 22.

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