A Cryptic K48 Ubiquitin Chain Binding Site on UCH37 is Required for its Role in Proteasomal Degradation

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SUMMARY

Degradation by the 26S proteasome is an intricately regulated process fine-tuned by the precise nature of ubiquitin modifications attached to a protein substrate. By debranching ubiquitin chains composed of K48 linkages, the proteasome-associated ubiquitin C-terminal hydrolase UCHL5/UCH37 serves as a positive regulator of protein degradation. How UCH37 achieves specificity for K48 chains is unclear. Here, we use a combination of hydrogen-deuterium mass spectrometry, chemical crosslinking, small-angle X-ray scattering, NMR, molecular docking, and targeted mutagenesis to uncover a cryptic K48 ubiquitin chain specific binding site on the opposite face of UCH37 relative to the canonical S1 ubiquitin-binding site. Biochemical assays demonstrate the K48 chain-specific binding site is required for chain debranching and proteasome-mediated degradation of proteins modified with branched chains. Using quantitative proteomics, translation shutoff experiments, and linkage-specific affinity tools, we then identify specific proteins whose degradation depends on the debranching activity of UCH37. Our findings suggest that UCH37 and potentially other DUBs could use more than one S1 site to perform different biochemical functions.

INTRODUCTION

Protein ubiquitination is a dynamic posttranslational modification that has profound effects on cellular function (Oh et al., 2018). Once tagged with ubiquitin (Ub), a protein target can be forced to change cellular location, assemble into a multi-protein complex, or suffer the fate of degradation. These diverse functions are orchestrated by a wide array of different ubiquitin modifications. In addition to mono- and multi-mono-ubiquitination, Ub can be assembled into polymeric chains composed of different linkages (M1, K6, K11, K27, K29, K33, K48, and K63), lengths, and architectures (unbranched and branched) (Komander and Rape, 2012). Central to the regulation of Ub-dependent signaling events is the ability to remove Ub modifications. These actions are executed by a family of proteolytic enzymes called deubiquitinases (DUBs).

There are nearly 100 human DUBs that fall into seven distinct subfamilies: USPs, OTUs, UCHs, Josephin, MINDY, ZUP1, and JAMM/MPN+ (Clague et al., 2019; Leznicki and Kulathu, 2017; Mevissen and Komander, 2017). Members of the OTU, MINDY, JAMM, and ZUP1 families generally target specific linkages within Ub chains, leaving behind the Ub moiety directly attached to a substrate (Abdul Rehman et al., 2016, 2021; Hermanns et al., 2018; Hewings et al., 2018; Kwasna et al., 2018; Mevissen et al., 2013). USPs, on the other hand, tend to display selectivity for the protein directly anchored to the proximal Ub and are rather promiscuous toward different chain types (Faesen et al., 2011; Ritorto et al., 2014). In some cases, there are DUBs capable of targeting a specific chain type and removing Ub directly from a variety of different substrates (Gersch et al., 2017). These fundamentally distinct activities are thought to require the same S1 Ub-binding site, which steers the Ub C-terminal scissile bond into the active site for cleavage. A second binding site—a S1' site—enables substrate discrimination. With chain type-specific DUBs, for example, the S1' site positions the proximal Ub of a chain to allow only one linkage to gain access to the catalytic cleft (Mevissen et al., 2013, 2016). Since UCH family member UCH37 exhibits linkage specificity towards K48 chains, we thought the same rules would apply to UCH37.

Through association with the 26S proteasome and INO80 chromatin remodeling complexes, UCH37 (also called UCHL5) plays a fundamental role in many cellular processes (Hamazaki et al., 2006; Jørgensen et al., 2006; Qiu et al., 2006; Yao et al., 2006, 2008). Although its function in the context of the INO80 complex is poorly understood, we (Deol et al., 2020) and others (Song et al., 2021) recently reported that the proteasome-bound form specifically binds K48 chains and catalyzes the removal of K48 branchpoints to promote degradation. Protein substrates that depend on the Ub chain debranching activity of UCH37 for degradation, however, remain unknown. Complicating the identification of substrates is the fact that UCH37 has other known biochemical activities. As a member of the UCH family, UCH37 removes small adducts from the C-terminus of Ub and more recent studies have shown that the DUB can act as a peptidase by cleaving Ub from the N-terminus of proteins (Bett et al., 2015; Davies et al., 2021). Understanding how specificity is achieved for binding K48 chains and cleaving branchpoints is necessary for dissecting the impact of different biochemical functions on protein turnover.

Here, we report on the unexpected finding that the canonical S1 site is dispensable for K48 chain binding. Using a combination of hydrogen-deuterium exchange (HDX) mass spectrometry, NMR, chemical crosslinking, small-angle X-ray scattering (SAXS), and molecular docking, we find a cryptic K48 Ub chain binding site located on the opposite face of UCH37 relative to the S1 site. K48 linkage specificity is conferred by two regions: one composed of a helix-loop-helix motif and another containing L181. Targeted mutagenesis reveals that the K48-specific sites are necessary for chain binding and removing K48 branchpoints, but not for N-terminal cleavage. By reconstituting proteasome complexes with mutants of UCH37 either defective in Ub binding at the S1 site (frontside) or K48 chain binding (backside), we find that only the latter is required for regulating the degradation of substrates modified with branched chains. When the front- and backside mutants are expressed in cells lacking UCH37, the frontside mutant can rescue wild-type (WT) activity but the backside mutant cannot. With complete mutational control over the activity of UCH37, we are then able to identify branched chain-modified proteins whose abundance and turnover depend on K48 chain

binding. Our results suggest that not all DUBs use a single S1 site to perform all the necessary biochemical functions.

RESULTS

K48 Linkage Selectivity is Embedded in the Catalytic Domain of UCH37

Previously, we found that UCH37 exclusively interacts with K48 chains regardless of overall architecture but only cleaves the K48 linkage if it is present at a branchpoint (Deol et al., 2020). UCH37 is composed of a catalytic domain (CD) and a C-terminal helical domain termed the ULD. Whether both domains are required for K48 specificity is unclear. Thus, to address this issue, we first performed a series of pulldown experiments with a panel of homotypic Ub chains. Both inactive full-length UCH37 (UCH37^{C88A}) and the inactive form of the catalytic domain (CD^{C88A}) exclusively pulldown K48 chains (Figures 1A-B, S1A-C). K48 linkage specificity is also observed with the CD by isothermal titration calorimetry (ITC) and binding affinity improves with increasing chain length (Figures 1C-D, S1D-E).

Such linkage-specific avidity could arise from two or more CDs making simultaneous contacts with K48 chains or from multiple Ub-binding sites located within a single CD. Although the molar ratio obtained from the ITC data support the latter scenario, we wanted to confirm the stoichiometry of chain binding using size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS). SEC-MALS shows that indeed the CD forms a 1:1 complex with a K48-linked Ub trimer (tri-Ub) (Figures 1E-F). These results indicate that a single CD is necessary and sufficient for K48 specificity.

HDX-MS Reveals Slower Exchange on the Backside of UCH37 upon Binding K48 Chains

To identify the K48 Ub chain specific binding sites in UCH37, we turned to hydrogen-deuterium exchange (HDX) mass spectrometry (MS). We decided to use UCH37^{C88A} bound to the DEUBAD (DEUBiquitinase ADaptor) domain of its partner protein RPN13 (referred to as the UCH37•RPN13^{DEUBAD} complex). Like the CD, the

UCH37•RPN13^{DEUBAD} complex binds K48 chains with 1:1 stoichiometry (Figures S1F-H). More importantly, the addition of RPN13^{DEUBAD} enables the investigation of a wide range of interactions from mono-Ub to branched trimers, which is necessary for uncovering cryptic Ub binding sites.

After obtaining initial data showing that high peptide coverage for UCH37 (97%) and RPN13^{DEUBAD} (98%) can be achieved at a resolution of 7-14 amino acids, we moved on to a proof-of-concept experiment with mono-Ub. Specifically, we compared the deuterium uptake between mono-Ub bound UCH37^{C88A}•RPN13^{DEUBAD} and free UCH37^{C88A}•RPN13^{DEUBAD}. A heat map corresponding to the differential deuterium uptake plots of individual peptides of UCH37^{C88A} shows a region near the C-terminus of the catalytic domain (residues 205-218) that undergoes slower exchange in the presence of Ub (Figure 2A). There is also slow exchange in the adjoining helical ULD domain (residues 257-270). The same regions are protected when Ub is covalently attached to the active site Cys (Figure 2B). Mapping these data on the extant structure (Sahtoe et al., 2015; VanderLinden et al., 2015) shows the protected regions coincide with the S1 site (Figures 2E-F).

We then shifted our attention to K48 chains. UCH37^{C88A}•RPN13^{DEUBAD} was mixed with either K48 di-Ub, tri-Ub, or K6/K48 tri-Ub and subjected to HDX-MS analysis (Figures S2A-C). Differential heat maps comparing the chain-bound to mono-Ub-bound form were calculated to identify sites undergoing slower exchange outside of S1 (Figures 2C, S2D-F). From these data it is apparent that slow exchange in residues comprising helices $\alpha 5$ and $\alpha 6$ is exclusive to K48 chains (Figures 2C, S2D-F). The $\alpha 5$ –6 motif is located on the backside of UCH37 relative to the S1 site and is not predicted to interact with Ub (Figure 2G).

We envisioned two possible scenarios that could account for protection of the $\alpha 5$ –6 motif. The first is that the distal K48-linked Ub subunit binds the S1 site while the proximal interacts with a portion of the CL. This would force the CL along with the N-terminus to move closer to the $\alpha 5$ –6 motif, decreasing its mobility and thus reducing the

exchange rate. The second is that lower deuterium uptake could simply reflect the presence of a cryptic Ub-binding site.

If the $\alpha 5-6$ motif serves as a Ub-binding site, then K48 chain binding should occur to the same extent regardless of whether the S1 site is occupied because the $\alpha 5-6$ motif is on a distinct face. However, if S1 together with the CL form a K48 specific binding motif, then covalent attachment of Ub to the active site Cys should ameliorate binding. Surprisingly, we find that mono-Ub still binds Ub~UCH37•RPN13^{DEUBAD}, albeit with two-fold lower affinity compared to the apo complex, and the binding of K48 di-Ub is not altered at all (Figures S2I-L). These results suggest the backside is necessary while the frontside is dispensable for binding K48 chains. HDX data confirm this supposition. Weak but reproducible protection of the $\alpha 5-6$ motif is observed upon addition of mono-Ub to Ub~UCH37•RPN13^{DEUBAD} and the degree of protection becomes much more pronounced when either K48 di-Ub or K6/K48 tri-Ub is added (Figures 2D, S2G-H). These data indicate the S1 site is dispensable for binding K48 chains and suggest that specificity is conferred through interactions mediated by the $\alpha 5-6$ motif on the backside of UCH37.

The reciprocal HDX data on the DEUBAD domain is also informative. Titrating in K48 di-Ub or K6/K48 tri-Ub to Ub~UCH37^{C88A}•RPN13^{DEUBAD} results in slower exchange in two partially overlapping peptides—338–348 and 344–356—corresponding to helices 5 and 6 of the DEUBAD domain (Figures 2J-K). The exchange rate for the same set of residues is not perturbed by mono-Ub (Figure 2I), indicating that slower deuterium uptake is unique to K48 chains. As the existing structure of Ub~UCH37•RPN13^{DEUBAD} shows, $\alpha 5-6$ of the DEUBAD interacts with the CL to form a contiguous surface facing the backside of the enzyme (Figures 2L-M). Thus, the DEUBAD along with the CL could provide one Ub-binding site while the $\alpha 5-6$ motif of UCH37 provides another. Alternatively, the DEUBAD could restrict the motion of the CL to afford a conformation conducive to chain binding.

In addition to the putative backside interactions, it is striking that the HDX data reveal few differences in the regions of UCH37 that are perturbed by unbranched and branched K48 chains. Although UCH37 binds K48 chains independent of chain architecture, branched chains are cleaved with higher efficiency than homotypic K48 chains (Deol et al., 2020; Shorkey et al., 2021; Song et al., 2021). Thus, we expected to find distinct regions that become more protected in the presence of branched chains. Instead, we only observe differences in the degree of deuterium uptake, as the exchange rates within the same regions of protection are faster with K6/K48 tri-Ub relative to K48 di- and tri-Ub (Figure S2M). These results suggest the interaction between UCH37•RPN13^{DEUBAD} and branched chains is more dynamic than that with unbranched K48 chains.

Backside Binding is Confirmed by Chemical Crosslinking

If the backside affords a K48-specific binding surface, we hypothesized that individual Ub subunits would crosslink distinct sites. To test this, we installed a chemical crosslinker on individual subunits of K48 di-Ub (Figures 3A-B) and K6/K48 tri-Ub (Figure 3C-E). We decided to place a photolabile, diazirine moiety (Lin et al., 2021) at position-46, as this is close to the I44 hydrophobic patch, which forms a hotspot for many interactions (Husnjak and Dikic, 2012). For comparison, mono-Ub was also modified with the diazirine crosslinker at the same position (Figure S3A). As expected, irradiation results in the formation of crosslinked products with each Ub variant (Figure S3B).

After band extraction and subsequent digestion, crosslinked sites on UCH37 were identified by MS (Figure S3C). The relative abundance of each site was estimated from the area under the curve (AUC) of the extracted ion chromatogram. Mapping these data onto the structure of UCH37 reveals that α 10 of the ULD is the primary crosslinking site when the diazirine is at position-46 of mono-Ub (Figure S3D). This result is entirely consistent with the Ub-bound structure, which shows A46 of Ub pointing directly toward α 10. Peptides corresponding to α 6, the C-terminal end of the CL, and 211–217 loop are also trapped by the diazirine-modified mono-Ub, but to a much lesser extent compared

to the α 10 peptide. These results confirm that mono-Ub also binds the backside α 5–6 motif in addition to the canonical S1 site.

In accord with the HDX-MS data, K48 chains shift the preferred crosslinking sites to the backside of UCH37. The proximal subunit of K48 di-Ub (Ub^{prox}) almost exclusively crosslinks a peptide corresponding to α 6 (Figure 3A). The distal subunit (Ub^{dist}), which generally displays weaker crosslinking compared to the proximal Ub, traps several sites (Figure 3B). The most prevalent is near the C-terminal end of the CL. Other less abundant sites include α 6, and a peptide fragment comprising residues 183-206. Crosslinked peptides corresponding to RPN13^{DEUBAD} were not detected. Our crosslinking data together with HDX-MS therefore uncover two distinct regions on the backside of UCH37 that are involved in the interaction with unbranched K48 chains: the α 5–6 motif and a more diffuse region composed of the C-terminal end of the CL along with the 176–190 and 211-217 loops. These data also suggest there is preferred orientation for the two subunits of K48 di-Ub, with Ub^{prox} binding α 5–6 and Ub^{dist} interacting with a series of flexible loops near the C-terminus of the CL.

We then wanted to know whether the K48-linked portion of a branched chain adopts a similar orientation. Like the Ub^{prox} from K48 di-Ub, we found that the diazirine at position-46 of the Ub^{prox} from K6/K48 tri-Ub shows a strong preference for crosslinking the α 6 region of UCH37 (Figure 3C), but there is significantly more crosslinking at the C-terminus of CL compared to the unbranched chain. The crosslinking pattern for the K48-linked Ub^{dist} also resembles the Ub^{dist} of the dimer (Figure 3D); however, there are subtle differences in the degree to which peptides from 183–206 and 211–217 are crosslinked (Figure S3C). Unbranched and branched chains might therefore engage UCH37 in a similar manner, but the K48-linked portion of a branchpoint might not be confined to an orientation in which the proximal subunit is localized to the α 5–6 motif. As a result, the K48-linked subunits of branched chain could be more prone to swapping positions.

The non-K48-linked subunit at a branchpoint could further enable subunits to swap positions. The crosslinking data shows that the 211–217 loop is one of the most prevalent sites trapped by the K6-linked Ub^{dist} of K6/K48 tri-Ub (Figure 3E). For position-46 of the K6-linked Ub^{dist} to be projected toward residues 211–217, Ub^{prox} would have to be positioned near the C-terminus of the CL thereby placing the K48-linked Ub^{dist} at the α 5–6 motif. If K48 cleavage occurs with a branched chain in this configuration, the α 5–6 motif would ostensibly be the S1 site dedicated to isopeptidase activity. Regardless of the precise orientation of the two K48-linked subunits, our data unexpectedly place the scissile bond on the opposite face relative to the canonical S1 site, suggesting cleavage occurs from the backside.

Structural Models of K48 di-Ub Bound to UCH37•RPN13^{DEUBAD}

Building on the results from HDX-MS and crosslinking, we sought to generate structural models of K48 chains bound to UCH37•RPN13^{DEUBAD}. Molecular dynamics (MD) simulations were performed to sample the conformational space of UCH37•RPN13^{DEUBAD} (Figure S4A). A total of three conformations were selected based on fitting to experimental SAXS data (Figures S4B-C). Each conformer then served as a starting point for docking K48 di-Ub using the program HADDOCK (Honorato et al., 2021; van Zundert et al., 2016). A complete list of docking restraints can be found in Table S1. The top-scoring solutions were further evaluated based on their agreement with the SAXS profile of K48 di-Ub:UCH37^{C88A}•RPN13^{DEUBAD} (Figures S4D-H). Excellent fits were obtained for three different poses (Figures 4A-C, S4H). In the first two, one subunit of K48 di-Ub forms an interface with the α 5–6 motif and the other subunit is positioned near the C-terminal end of the CL (Figure 4A-B). The K48 dimer adopts an extended conformation in each of the docking models. In the third pose, one of the Ub subunits can be seen interacting with the CL and the DEUBAD domain. Overall, the bulk of the structures correspond to poses 1 and 2, while pose 3 represents a minor fraction.

Taking all the best-fitting poses into account (59 in total), we wanted to obtain more insight into the specific sites involved in the interaction with K48 chains. We therefore

measured the frequency with which residues of UCH37 and Ub come into close contact (Figures S4E-F). Residues deemed significant are two standard deviations from the mean. Based on this analysis, it is evident that the conserved aromatic residues F117 and F121 form the nexus of the $\alpha 5-6$ motif (Figure S4E). This aromatic-rich region interacts with the I44 patch of Ub regardless of the orientation of K48 di-Ub (Figure S4F). When Ub^{prox} is docked at $\alpha 5-6$ (pose 1), I44 and F45 form contacts with both F117 and F121 of UCH37 (Figure 4D). Swapping Ub^{prox} with Ub^{dist} (pose 2), forces L8, I44, and V70 of Ub to interact with F117 and F121 (Figure 4E). The preference for Ub^{prox} binding to $\alpha 5-6$ could, in part, stem from polar contacts, as there are three residues in $\alpha 5-6$ (E116, S120, and D122) engaged with Ub^{prox} and only two when Ub^{dist} is bound (Figure 4F).

L181 of UCH37 appears to play an integral role in the second Ub binding site. In one orientation, L181 contacts L8 of Ub^{dist}, and in another it is near I44 of Ub^{prox} (Figures 4G-H). Additional contacts are established with other residues located in different loop regions, e.g., E160 and W58 (Figure 4G), or with a backbone amide of Ub^{prox} (Figure 4H). These models suggest that binding to both backside sites can only be accomplished with the K48 linkage, as other linkages do not place the two I44 patches in the correct orientation for engagement.

An Aromatic-Rich Motif in $\alpha 5-6$ is Required for Backside Binding

Targeted mutagenesis confirms the importance of the $\alpha 5-6$ motif and L181 in K48-specific binding. Replacing two of the key residues of the $\alpha 5-6$ motif, F117 and F121, with alanine (F117A and F121A) reduces the binding affinity toward K48 di-Ub to mono-Ub levels (Figures S5A-B) while retaining the same Ub-AMC hydrolytic activity as wild-type (WT) UCH37 (Figure S5C). The only other Ala substitution with a similar effect as F117A and F121A is L181A (Figures S5C-E). According to conservation analysis, F117 and L181 are invariably present in UCH37 homologs (Figure S5F).

HDX-MS and NMR data further underscore the importance of F117 in backside binding. HDX-MS analyses of UCH37^{C88A/F117A}•RPN13^{DEUBAD} (the F117A complex) shows that

the region encompassing residues 115-125 is no longer protected from exchange when either mono-Ub or K48 di-Ub are added (Figure 5A). Moreover, in contrast to UCH37^{C88A}•RPN13^{DEUBAD} (the C88A complex; Figures 5B-C), the F117A complex induces few perturbations in the Ile-region of the ¹H¹³C-methyl TROSY spectra of ILV-labeled K48-linked Ub^{prox} and Ub^{dist} (Figures 5D-E), indicating that the dimer largely remains in the unbound state. Binding to the frontside, however, is retained by the F117A complex, as evidenced by the nearly indistinguishable bound-state peaks for ¹H¹³C-methyl, ILV-labeled mono-Ub with the C88A and F117A complexes (Figure 5F). There are also minor peaks in the spectra of Ub^{prox} and Ub^{dist} with the F117A complex resembling the resonances for the bound state of mono-Ub (Figures 5D-E), suggesting that when the backside is inaccessible, each subunit can interact with the frontside to some extent.

One of the challenges in assessing the contribution of individual Ub residues to the interaction with UCH37 using NMR is that front- and backside binding are conflated. This issue is certainly germane to Ub^{prox} considering the spectrum of this subunit in the presence of the C88A complex is nearly identical that of mono-Ub bound to the same complex. The spectra collected for Ub^{dist}, however, tell a different story. A marked upfield shift in the ¹H dimension is observed for Ub^{dist} I44 in the presence of the C88A complex (Figure 5C). The same peak is completely absent from the spectrum of Ub^{dist} bound to the F117A complex (Figure 5E). These data suggest the shift observed for Ub^{dist} I44 is a signature of backside binding and that I44 of the distal subunit plays an important role in the backside interaction.

F117A and F121A Impair Chain Debranching and Proteasomal Degradation

Our data thus far demonstrate the importance of the α5–6 motif in binding, but do not address its role in the catalytic activity of UCH37. To this end, we monitored the cleavage of a branched trimer substrate (K6/K48 tri-Ub) over time by following the formation of di- and mono-Ub using SDS-PAGE. The steady-state parameters reveal that F117A and F121A each reduce the debranching activity by ~20-fold; L181A has a similar effect (Figure 6A). F117A and F121A also compromise the cleavage of high-

molecular weight (HMW) Ub chains bearing different branchpoints (Figure 6B). These results indicate that impaired binding to K48 chains translates to a marked decrease in debranching activity.

We then asked whether a defective S1 site affects chain debranching. By engaging I36 of Ub, I216 plays an integral role in the S1 site. Replacing I216 with Glu (I216E) diminishes the hydrolytic activity toward Ub-AMC by ~11-fold, consistent with previous reports (Figure S5C) (Sahtoe et al., 2015). Interestingly, I216E only reduces the binding affinity for mono-Ub by ~2-fold relative to the inactive C88A complex and has no effect on binding K48 di-Ub (Figure S6A). Chain debranching is also unaffected by the I216E substitution (Figures 6A-B), suggesting either the canonical S1 site is not involved in the isopeptidase activity or a Ub moiety is situated differently than what is predicted by the extant mono-Ub-bound structures.

To test whether the backside is necessary for the role of UCH37 on the proteasome, 26S proteasomes deficient of WT UCH37 and RPN13 (Δ UCH37/ Δ RPN13 Ptsms) were reconstituted with different RPN13-bound forms of UCH37: F117A, F121A, or I216E (Figure S6B-J). In the presence of K6/K48 and K48/K63 HMW Ub chains, WT- and I216E-replenished (rWT and rI216E) Ptsms generate smaller Ub conjugates, but F117A- and F121A-replenished (rF117A and rF121A) Ptsms are essentially inactive (Figure 6C). Degradation is also affected by F117A and F121A. Using K11/K48-polyUb-UBE2S-UBD and K48/K63-polyUb-titin-I27^{V15P} as substrates, a decrease in polyubiquitinated species and a concomitant increase in Ptsm-derived peptides is observed with rWT and rI216E Ptsms but not with rF117A and rF121A Ptsms (Figure 6D).

The in vitro results suggest that degradation of branched chain-modified proteins depends on the K48-specific binding of UCH37, raising the question of whether degradation in cells also depends on the same backside motif. To test this, UCH37^{I216E} and UCH37^{F117A} were ectopically expressed from a doxycycline-inducible promoter in UCH37^{KO} cells stably expressing the Ptsm activity reporter GFP^u (Figure

6E). Translation shutoff experiments were then performed to measure the turnover of GFP^u. For comparison, the same experiments were conducted with WT HEK293 cells and UCH37^{KO} cells as previously reported. In accord with the biochemical assays, UCH37^{I216E} restores GFP^u turnover in the null background, whereas the expression of UCH37^{F117A} is unable to rescue the WT phenotype (Figure 6F). In an orthogonal assay, we examined the effect of UCH37^{I216E} and UCH37^{F117A} on the turnover of newly synthesized proteins (NSPs). Azidohomoalanine (AHA) is incorporated into NSPs in lieu of methionine during a short pulse to allow for visualization (Figure 6G) (Dieterich et al., 2006; Howden et al., 2013; McShane et al., 2016). Degradation is then monitored after replenishing the growth medium with excess methionine. In agreement with the GFP^u data, we found that AHA-labeled NSPs dissipate over time when UCH37^{KO} cells are complemented with UCH37^{I216E} but not with UCH37^{F117A} (Figure 6G).

Uncovering the Targets of UCH37-Dependent Chain Editing

Having complete mutational control over UCH37 activity in a null background allows us to identify proteins whose turnover is specifically affected by the loss of K48 chain-specific binding and debranching. To this end, we started by measuring total protein levels in unperturbed WT and UCH37^{KO} cells; however, the differences between the two cell lines turned out to be minimal (Figures S7A-B). We then decided to treat cells with H₂O₂, as UCH37 has previously been implicated in the cellular response to oxidative stress (Harris et al., 2019).

In the presence of H₂O₂, two separate TMT-based proteomic experiments were performed: one comparing the proteomes of UCH37^{F117A}- and UCH37^{WT}-expressing UCH37^{KO} cells, and another comparing the proteomes of UCH37^{I216E}- and UCH37^{WT}-expressing UCH37^{KO} cells (Figure 7A). This setup allowed us to analyze duplicate or triplicate samples of each H₂O₂-treated cell line in a single experiment and eliminate strain background effects. High-resolution MS quantified reproducibly ~2500 proteins from the 10-plex analysis using an identification cutoff of at least 2 unique peptides. Protein abundances between replicates show higher correlations (Pearson's correlation coefficient ≥0.91) than those observed between different cell lines (Figure S7C).

Statistical analysis reveals UCH37^{F117A} and UCH37^{I216E} induce significant, but distinct, changes in protein abundance relative to WT ($\log_2$ ratio < -0.5 or > 0.5, corrected $\log_{10}$ p-value > 1.3) (Figures 7B-C).

Gene Ontology (GO) enrichment analysis further illuminates the differences between UCH37^{F117A} and UCH37^{I216E}. Proteins that are uniquely upregulated by UCH37^{F117A} participate in mitochondrial membrane organization, mitochondrial transport, oxidative phosphorylation, and nucleotide metabolism (Figure 7D). These data suggest that the backside, and thus K48 chain binding and debranching, is important for regulating the levels of proteins related to mitochondrial function and nucleotide metabolism during oxidative stress. By contrast, the frontside appears to regulate the abundance of proteins involved in chromosome organization, DNA metabolism, and DNA repair, as evidenced by an accumulation upon expression of UCH37^{I216E}. The nuclear localized INO80 complex is instrumental in many of the same biological processes specific to the UCH37^{I216E} mutant (Poli et al., 2017). Thus, it is plausible that the canonical S1 site is critical to the function of UCH37 in INO80.

To validate these findings, we wanted to examine the effects of UCH37^{I216E} and UCH37^{F117A} on the turnover of specific proteins exhibiting higher abundance upon expression of the latter but not the former. We chose to focus on POLR2D and MFN2. POLR2D (also referred to as Rpb4) regulates various stages of the mRNA life cycle through its interactions with RNA Pol II (Farago et al., 2003; Goler-Baron et al., 2008; Harel-Sharvit et al., 2010). Mitofusin-2 (MFN2) is a conserved dynamin-like GTPase localized to the outer membrane of mitochondria, and along with MFN1, regulates mitochondrial dynamics (Zorzano et al., 2010). We first compared the levels of POLR2D and MFN2 in WT and UCH37^{KO} cells by immunoblotting and found that both proteins are more abundant in the absence of UCH37 (Figures 7E). We then assessed relative turnover rates. In WT cells, POLR2D and MFN2 are rapidly depleted after shutting off translation; however, in UCH37^{KO} cells, both proteins remain relatively stable, indicating that turnover is impaired without UCH37 (Figure 7E). Degradation of POLR2D and MFN2 can only be rescued in UCH37^{KO} cells expressing UCH37^{I216E} (Figures 7F &

S7D), suggesting the turnover of these proteins is regulated by the K48 chain-specific binding and debranching activity of UCH37.

Lastly, we sought to check whether heterotypic chains bearing K48 linkages accumulate on the putative substrates in the absence of UCH37. MFN2 has previously been shown to be modified with K6-linked chains during mitochondrial stress (Gersch et al., 2017). Thus, we used a K6-specific affimer (Michel et al., 2017) to isolate polyubiquitinated proteins from WT and UCH37^{KO} cells treated with the proteasome inhibitor MG132 (Figure 7G). Western blot analysis of the K6-linked conjugates shows higher levels of polyubiquitinated MFN2 (polyUb-MFN2) and K48 linkages in UCH37^{KO} cells relative to WT (Figure 7H). Treatment with H₂O₂ increases the overall amounts. Addition of the K48 linkage-specific DUB OTUB1 results in the cleavage of Ub conjugates attached to MFN2, indicating the K6 chains also contain K48 linkages, consistent with previous reports (Michel et al., 2017). Moreover, the ability of UCH37•RPN13 to hydrolyze polyUb-MFN2 argues that the K6/K48 chains have branchpoints. These data therefore point to specific substrate proteins whose degradation is potentiated by the ability of UCH37 to debranch K48 Ub chains.

DISCUSSION

Here, we describe how UCH37—a DUB with fundamental roles in chromatin biology and protein degradation—achieves specificity for K48 Ub chains. The surprising finding is that the canonical S1 site, which is thought to be the principal Ub-binding site, is dispensable for K48-linkage-specific binding. Specificity is instead accomplished by avid binding to the opposite face of the enzyme relative to the S1 site. Mutational analysis shows the backside is not only required for K48 chain binding but also chain debranching and the ability of UCH37 to promote proteasomal degradation. Defects in the S1 site and backside have distinct effects on the turnover of a subset of proteins, enabling the identification of branched chain-modified substrates whose degradation depends on UCH37. These results raise an intriguing question of whether some DUBs use more than one S1 site to perform different biochemical activities (Figure 7I).

A Model for Chain Debranching

Considering the mechanisms by which some USP DUBs target both Ub chains and ubiquitinated substrates, the most logical model for the different activities of UCH37 would invoke a single S1 site. USP30, for example, is thought to use the same S1 site to cleave K6-linked Ub chains and remove Ub from numerous mitochondrial outer membrane proteins (Gersch et al., 2017). Chain type specificity and substrate promiscuity are both possible due to an accessible active site geometry and the presence of a second Ub-binding site, termed the S1' site. A similar scenario can be envisioned for UCH37. When the substrate is a C-terminal adduct or an N-terminally ubiquitinated protein, the Ub moiety interacts with the canonical S1 site and the scissile peptide bond enters the active site because the flexible crossover loop (CL) moves out of the way. By contrast, when the substrate is a branched Ub chain, the distal moiety binds the S1 site and the proximal Ub interacts with a portion of the CL only if a K48 linkage is present between the two subunits.

Our data, however, suggest that in addition to the canonical S1 site there is another S1 site located on the backside dedicated to binding and catalyzing K48 chain debranching. Whether the $\alpha 5-6$ motif or the region containing the CL and L181 serve as the S1 site depends on the orientation of the K48 chain most conducive to catalysis. Unbranched K48 chains, which are generally poor substrates, appear to preferentially bind with the proximal Ub at the $\alpha 5-6$ motif and the distal occupying the CL and L181 region. In this orientation, molecular docking analysis suggests the isopeptide bond would be sterically shielded from the catalytic Cys. An arrangement that might be more catalytically productive is one in which the two subunits swap positions. With the proximal Ub near the CL and L181, the K48 distal Ub can still interact with the $\alpha 5-6$ motif, but the scissile bond could be in a better position for cleavage.

How the third Ub that branches off from the proximal subunit facilitates isopeptidase activity remains an open question. One possibility is that it provides enough steric bulk to shift the equilibrium in favor of the proximal subunit binding near the CL and L181, which would allow the isopeptide bond to move closer to the catalytic Cys. However,

this model does not explain why Ub must be at all three positions of a branchpoint, as we have previously shown (Deol et al., 2020), nor does it explain the enhanced selectivity and debranching activity conferred by RPN13. Another scenario, more consistent with the data, is one in which the non-K48 distal Ub forms weak interactions with the frontside of the enzyme, e.g., the 211–217 loop. The precise orientation is unknown but considering the I216E substitution does not affect the rate of debranching, the nature of the interaction between the non-K48 distal Ub and the frontside is unlikely to resemble mono-Ub binding the canonical S1 site. With the non-K48 distal Ub on the frontside, the proximal Ub would be forced to reside near the CL and L181 and the K48 distal Ub would be bound to the $\alpha 5$ –6 motif. Cleavage of the branched chain would mean the $\alpha 5$ –6 motif acts as the backside S1 site. Thus, UCH37 could potentially use distinct S1 sites for different biochemical functions. Future studies will focus on elucidating the precise mechanism by which UCH37 together with RPN13 discern between unbranched and branched chains.

Substrates Requiring the Debranching Activity of UCH37 for Degradation

The different biochemical activities of UCH37 present challenges in pinpointing which proteins depend on the K48 chain debranching activity for degradation. This issue can be addressed with mutants that specifically disrupt K48 chain binding and debranching without altering hydrolytic activity, i.e., F117A and F121A. By comparing the effects of UCH37^{F117A} and UCH37^{I216E}, which causes defects in the canonical S1 site, on total protein abundance we found that the mitochondrial fusion protein MFN2 and the RNA polymerase II subunit POLR2D depend on the K48 chain binding and debranching activity of UCH37 for degradation. Previous studies have shown that MFN2 is polyubiquitinated and degraded by the proteasome in response to cellular stress. The E3 ligase HUWE1 modifies MFN2 with Ub chains bearing a mixture of K6, K11, and K48 linkages (Leboucher et al., 2012; Michel et al., 2017; Senyilmaz et al., 2015). Our results are not only consistent with these findings, but also provide direct evidence that the chains attached to MFN2 contain branchpoints that are removed by UCH37 to facilitate degradation.

Why chain debranching is required for the efficient removal of MFN2, or any other protein, by the proteasome is less clear. There could be several reasons. For example, the removal of branchpoints could assist in the transfer of a polyubiquitinated protein from an effector protein to the proteasome. The proteasomal shuttle protein HHR23A contains two Ub-binding UBA domains, one with a preference for K48 linkages and the other displaying no linkage selectivity (Sims et al., 2009; Varadan et al., 2005). Coordination between the two UBA domains could enable high affinity binding to a K48 branched chain (Haakonsen and Rape, 2019). This would prioritize branched chain-modified substrates for degradation, but debranching would have to occur to allow the proteasome to takeover. A similar scenario can be envisioned for the transfer of substrates between the AAA+ ATPase p97/VCP and the proteasome, as p97 is required for the degradation of MFN2 (Tanaka et al., 2010) and been shown to act as an effector of branched chains (Yau et al., 2017). Another reason for chain debranching is that it might allow for the Ub chain to stabilize a conformation of the proteasome that is conducive to efficient degradation.

CONCLUSION

In summary, our data uncover an entirely unique region of UCH37 required for K48 chain specificity, chain debranching, and promoting the proteasomal degradation of branched chain-modified proteins. What is particularly exciting is the prospect that a DUB can use different S1 sites to target Ub chains and ubiquitinated substrates, suggesting inhibitors could be designed to block one activity but not the other. For instance, a small molecule that specifically blocks Ub chain binding and debranching could serve as a powerful tool to probe the role of UCH37-catalyzed debranching in many biological paradigms.

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AUTHOR CONTRIBUTIONS

J.D., S.B., Y.L., K.K.D., S.J.E., J.F., M.T., and E.R.S. designed the experiments. E.R.S. supervised the experiments. J.D., S.B., Y.L., K.K.D., and M.T. conducted the experiments. J.D., S.B., Y.L., and E.R.S. wrote the manuscript.

FIGURE 1. The Catalytic Domain of UCH37 Confers K48 Linkage-Specificity.

(A) SDS-PAGE analysis of 6xHis-UCH37^{C88A}-mediated pulldowns with M1-, K6-, K11-, K29-, K33-, K48-, and K63-linked Ub chains. Each chain (50 pmol) was mixed with 6xHis-UCH37^{C88A} (5 nmol) immobilized on Ni-NTA resin.

(B) SDS-PAGE analysis of 6xHis-UCH37(CD)^{C88A} with M1-, K6-, K11-, K29-, K33-, K48-, and K63-linked Ub chains. Each chain (50 pmol) was mixed with 6xHis-UCH37(CD)^{C88A} (5 nmol) immobilized on Ni-NTA resin.

(C-D) ITC analysis of UCH37(CD)^{C88A} binding to K48 tri-Ub (C), and K63 tri-Ub (D).

(E) SEC-MALS analysis of the interaction between UCH37(CD)^{C88A} and K48 tri-Ub.

(F) Theoretical and calculated molar mass of complexes detected by SEC-MALS.

See also Figure S1.

FIGURE 2. HDX-MS Uncovers a Cryptic K48 Chain-Specific Binding Site.

(A) Differential deuterium uptake plot comparing mono-Ub bound

UCH37^{C88A}•RPN13^{DEUBAD} to free UCH37^{C88A}•RPN13^{DEUBAD}.

(B) Differential deuterium uptake plot comparing UCH37•RPN13^{DEUBAD} covalently linked

to Ub propargylamine at the active site Cys (Ub~UCH37•RPN13^{DEUBAD}) to free

UCH37^{C88A}•RPN13^{DEUBAD}.

- (C) Differential deuterium uptake plot comparing K48 di-Ub-bound UCH37^{C88A}•RPN13^{DEUBAD} to mono-Ub-bound UCH37^{C88A}•RPN13^{DEUBAD}.
- (D) Differential deuterium uptake plot comparing K48 di-Ub-bound Ub~UCH37•RPN13^{DEUBAD} to free Ub~UCH37•RPN13^{DEUBAD}.
- (E-G) Structure of UCH37 (PDB: 4UEM) showing regions with statistically significant differences in exchange upon non-covalent binding to mono-Ub (E), covalent attachment of Ub to the active site (F), and non-covalent binding to K48 di-Ub (G). Data correspond to 2 h of deuterium labeling. CL: crossover loop.
- (H) Structure of UCH37 (PDB: 4UEM) showing statistically significant differences in exchange upon non-covalent binding of K48 di-Ub to Ub~UCH37•RPN13^{DEUBAD}. Data correspond to 2 h of deuterium labeling. CL: crossover loop.
- (I-K) Differential deuterium uptake plot comparing the effects of mono-Ub (I), K48 di-Ub (J) and K6/K48 tri-Ub (K) binding to Ub~UCH37•RPN13^{DEUBAD} on the exchange of residues in RPN13^{DEUBAD}.
- (L-M) Heat map showing regions of RPN13^{DEUBAD} with statistically significant differences in deuterium exchange upon non-covalent binding to K48 di-Ub (L), and non-covalent binding to K6/K48 tri-Ub (M). Data correspond to 2 h of deuterium labeling.
- See also Figure S2.

FIGURE 3. Chemical Crosslinking Confirms the Presence of a K48 Chain-Specific Binding Site on the Backside of UCH37.

(A-E) A photolabile diazirine-based crosslinker was appended to individual Ub subunits of K48 chains. Crosslinked peptides were mapped onto the structure of UCH37 (PDB: 4UEL) according to their relative abundance based on the area under the curve (AUC) of the extracted ion chromatogram.

(A) Map of the crosslinking data for K48 di-Ub binding to UCH37^{C88A}•RPN13^{DEUBAD} with the proximal Ub subunit labeled with the diazirine colored by % peptide crosslinked.

(B) Map of the crosslinking data for K48 di-Ub binding to UCH37^{C88A}•RPN13^{DEUBAD} with the distal Ub subunit labeled with the diazirine colored by % peptide crosslinked.

(C) Map of the crosslinking data for K6/K48 tri-Ub binding to UCH37^{C88A}•RPN13^{DEUBAD} with the proximal Ub subunit labeled with the diazirine colored by % peptide crosslinked.

(D) Map of the crosslinking data for K6/K48 tri-Ub binding to UCH37^{C88A}•RPN13^{DEUBAD} with the K48-linked distal Ub subunit labeled with the diazirine colored by % peptide crosslinked.

(E) Map of the crosslinking data for K6/K48 tri-Ub binding to UCH37^{C88A}•RPN13^{DEUBAD} with the K6-linked distal Ub subunit labeled with the diazirine colored by % peptide crosslinked. See also Figure S3.

FIGURE 4. Structural Models of the K48 di-Ub:UCH37•RPN13^{DEUBAD} Complex.

(A-C) HADDOCK models show three poses corresponding to the interaction between K48 di-Ub and UCH37 along with their fit to experimental SAXS data of the K48 di-Ub:UCH37^{C88A}•RPN13^{DEUBAD} complex. The goodness of fit to the experimental intensity is represented by χ^2 values. In the first pose (A), the proximal Ub (Ub^{prox};

green) interacts with $\alpha 5-6$ of UCH37. In the second pose (B), the distal Ub (Ub^{dist}; orange) interacts with $\alpha 5-6$ of UCH37. The third pose (C) shows Ub^{dist} (orange) interacting with RPN13^{DEUBAD} while Ub^{prox} (green) is situated near the C-terminal end of the crossover loop (CL).

(D) Residues highlighting the interaction between the aromatic-rich region of UCH37 $\alpha 5-6$ and the I44 patch of Ub^{prox} in pose 1 (A).

(E) Residues highlighting the interaction between the aromatic-rich region of UCH37 $\alpha 5-6$ and the I44 patch of Ub^{dist} in pose 2 (B).

(F) Polar contacts between Ub^{prox} and UCH37 $\alpha 5-6$ in pose 1 (A).

(G) Contacts between Ub^{dist} and residues of UCH37 located outside the $\alpha 5-6$ motif in pose 1 (A).

(H) Contacts between Ub^{prox} and residues of UCH37 located outside the $\alpha 5-6$ motif in pose 2 (B).

See also Figure S4.

FIGURE 5. Backside Mutant Impairs K48 Chain Binding.

(A) Deuterium uptake plots showing how the F117A mutation affects hydrogen-

deuterium exchange in a peptide corresponding to residues 115-125 located in the $\alpha 5-6$ motif of UCH37. For comparison, the uptake plots corresponding to a region located outside the $\alpha 5-6$ motif (residues 200-218) are also shown. The data on the top represents the rate of exchange with UCH37^{C88A}•RPN13^{DEUBAD} and the data on the bottom corresponds to UCH37^{C88A/F117A}•RPN13^{DEUBAD}.

(B) ¹H/¹³C-methyl TROSY NMR spectra of the Ile region of the proximal subunit (Ub^{prox}) of K48 di-Ub free in solution (grey) and bound to UCH37^{C88A}•RPN13^{DEUBAD} (the C88A complex; green).

- (C) $^1\text{H}^{13}\text{C}$ -methyl TROSY NMR spectra of the Ile region of the distal subunit (Ub^{dist}) of K48 di-Ub free in solution (grey) and bound to the C88A complex (orange).
- (D) $^1\text{H}^{13}\text{C}$ -methyl TROSY NMR spectra of the Ile region of K48-linked Ub^{prox} free in solution (grey) and bound to $\text{UCH37}^{\text{C88A/F117A}} \bullet \text{RPN13}^{\text{DEUBAD}}$ (the F117A complex; green).
- (E) $^1\text{H}^{13}\text{C}$ -methyl TROSY NMR spectra of the Ile region of K48-linked Ub^{dist} free in solution (grey) and bound to the F117A complex (orange).
- (F) $^1\text{H}^{13}\text{C}$ -methyl TROSY NMR spectra of the Ile region of K48-linked Ub^{dist} free in solution (grey) and bound to the F117A complex (orange).

FIGURE 6. Backside Mutants Impair K48 Chain Debranching and UCH37-Dependent Proteasomal Degradation.

(A) Steady-state kinetic analyses of the cleavage of K6/K48 tri-Ub with different UCH37 variants (WT, F117A, F121A, and I216E) in complex with RPN13.

- (B) SDS-PAGE analysis of the cleavage of K6/K48 and K48/K63 high-molecular weight (HMW) Ub chains with different UCH37 variants (WT, F117A, F121A, and I216E) in complex with RPN13. Gels were imaged by SyproTM Ruby staining.
- (C) Western blot analysis of cleavage of K6/K48 HMW Ub chains by WT Ptsm, WT UCH37•RPN13-replenished Ptsm (rWT), UCH37^{F117A}•RPN13-replenished Ptsms (rF117A), UCH37^{F121A}•RPN13-replenished Ptsms (rF121A), and UCH37^{I216E}•RPN13-replenished Ptsms (rI216E). 0.2 mg of Ptsm complex were used in each reaction. Immunoblotting was performed with Alexa Fluor 647-conjugated streptavidin.
- (D) Fluorescent SDS-PAGE analyses of the degradation of Cy5-labeled, K48/K63-Ub_n-titin-I27^{V15P}-23-K-35 and K11/K48-polyUb-UBE2S-UBD using WT, rWT, rC88A, rI216E, rF117A, and rF121A Ptsm complexes. Reactions were performed under multi-turnover conditions and quenched after 1 h. Gels stained for total protein are shown for reference.
- (E) Western blot analysis of UCH37 levels in WT, UCH37^{KO} (KO), and KO HEK293 cells expressing UCH37 variants under a doxycycline-inducible promoter. Immunoblotting was performed with α-UCH37 and α-actin antibodies.
- (F) Schematic showing the different degradation profiles of the Ptsm activity reporter GFP^u in WT and UCH37^{KO} HEK293 cells expressing UCH37 variants under a doxycycline-inducible promoter.
- (G) Plots showing percent GFP^u remaining in the indicated cell lines after shutting off translation with emetine.
- (H) Scheme showing the labeling and visualization of newly synthesized proteins (NSPs) with azidohomoalanine (AHA) in UCH37^{KO} cells expressing either UCH37^{I216E} or UCH37^{F117A}.
- (I) Fluorescent SDS-PAGE analysis of the turnover of AHA-labeled NSPs in UCH37^{KO} cells expressing either UCH37^{I216E} or UCH37^{F117A}.
- (J) Quantitation of the results shown in (I).
- Error bars represent SEM of three independent experiments. *p < 0.1, ** p < 0.01, *** p < 0.001 and **** p < 0.0001
- See also Figure S6.

FIGURE 7. Identifying Proteins Dependent on the K48 Chain Binding and Debranching Activity of UCH37 for Degradation.

- (A) Scheme for tandem mass tagging (TMT)-based proteomics of UCH37^{KO} cells expressing either WT UCH37, UCH37^{I216E}, or UCH37^{F117A}.
- (B-C) Volcano plots comparing the proteomes of UCH37^{F117A}- and WT UCH37-expressing UCH37^{KO} cells (B), and UCH37^{I216E}- and WT UCH37-expressing UCH37^{KO} cells (C). In both cases, the cells are treated with H₂O₂ to induce oxidative stress.
- (D) Biological process analysis of proteins upregulated in UCH37^{F117A}- and UCH37^{I216E}-expressing UCH37^{KO} cells.
- (E) Western blot analysis of the turnover of POLR2D and MFN2 after shutting off translation with emetine in WT and UCH37^{KO} HEK293 cells.
- (F) Western blot analysis of the turnover of POLR2D and MFN2 after shutting off translation with emetine in UCH37^{KO} cells reconstituted with either UCH37^{F117A} or UCH37^{I216E}. Immunoblotting with α-POLR2D, α-MFN2, and α-actin antibodies.
- (G) Scheme showing the enrichment and analysis K6-linked Ub chains.
- (H) Western blot analysis of K6-affimer-enriched ubiquitinated species from WT and UCH37^{KO} HEK293 cells. The enriched conjugates were treated with OTUB1 (1 μM) or UCH37•RPN13 (2 μM) at rt for 1 h. Immunoblotting with α-MFN2 and α-K48-linkage specific antibodies.
- (I) Proposed model showing distinct biochemical activities on and front- and backside of UCH37.

Error bars represent SEM of two independent experiments. *p < 0.1, ** p < 0.01, *** p < 0.001 and **** p < 0.0001.

See also Figure S7.

(F) SEC-MALS analysis of the interaction between UCH37^{C88A}•RPN13^{DEUBAD} and K48 tri-Ub.

(G) SEC-MALS analysis of the interaction between UCH37^{C88A}•RPN13^{DEUBAD} and K6/K48 tri-Ub.

(H) Theoretical and calculated molar mass of complexes detected by SEC-MALS.

FIGURE S2. HDX-MS Uncovers a Cryptic K48 Chain-Specific Binding Site.

(A) Differential deuterium uptake plot comparing K48 di-Ub-bound UCH37•RPN13^{DEUBAD} and free UCH37•RPN13^{DEUBAD}.

- (B) Differential deuterium uptake plot comparing K48 tri-Ub-bound UCH37•RPN13^{DEUBAD} and free UCH37•RPN13^{DEUBAD}.
- (C) Differential deuterium uptake plot comparing K6/K48 tri-Ub-bound UCH37•RPN13^{DEUBAD} and free UCH37•RPN13^{DEUBAD}.
- (D) Differential deuterium uptake plot comparing K48 di-Ub-bound UCH37•RPN13^{DEUBAD} and mono-Ub-bound UCH37•RPN13^{DEUBAD}.
- (E) Differential deuterium uptake plot comparing K48 tri-Ub-bound UCH37•RPN13^{DEUBAD} and mono-Ub-bound UCH37•RPN13^{DEUBAD}.
- (F) Differential deuterium uptake plot comparing K6/K48 tri-Ub-bound UCH37•RPN13^{DEUBAD} and mono-Ub-bound UCH37•RPN13^{DEUBAD}.
- (G) Differential deuterium uptake plot comparing mono-Ub-bound Ub~UCH37•RPN13^{DEUBAD} and Ub~UCH37•RPN13^{DEUBAD}.
- (H) Differential deuterium uptake plot comparing K6/K48 tri-Ub-bound Ub~UCH37•RPN13^{DEUBAD} and Ub~UCH37•RPN13^{DEUBAD}.
- (I) ITC analysis of mono-Ub binding to Ub~UCH37•RPN13^{DEUBAD}.
- (J) ITC analysis of K48 di-Ub binding to Ub~UCH37•RPN13^{DEUBAD}.
- (K) Competitive fluorescence polarization analysis of the interaction between mono-Ub and Ub~UCH37•RPN13^{DEUBAD}.
- (L) Competitive fluorescence polarization analysis of the interaction between K48 di-Ub and Ub~UCH37•RPN13^{DEUBAD}.
- (M) Deuterium uptake profiles comparing the effects of K6/K48 tri-Ub and K48 di-Ub on three different regions of UCH37.
- Data are representative of at least two replicates.

FIGURE S4. Structural Models of the K48 di-Ub:UCH37•RPN13^{DEUBAD} Complex.

(A) MD trajectories for eight independent simulations plotted as C α -RMSD of UCH37 over time.

(B) Overlay of UCH37•RPN13^{DEUBAD} structures that afford the best fit to experimental SAXS data. Flexible N- and C-termini of RPN13^{DEUBAD} are omitted.

(C) Individual structures of UCH37•RPN13^{DEUBAD} (Models 1-3) with the corresponding fits to the SAXS data. Flexible N- and C-termini of RPN13^{DEUBAD} are omitted.

(D) Theoretical and experimental SAXS data for the best-fitting multi-state model comprising Models 1-3. Pairwise C α -RMSD is shown between Models 1-3.

(E-H) SEC-SAXS analyses of UCH37^{C88A}•RPN13^{DEUBAD} (blue) and K48 di-Ub:UCH37^{C88A}•RPN13^{DEUBAD} (red) showing distance distribution (E-F), Guinier analyses (G), and Kratky plots (G).

(H) Frequency analysis of pairwise interactions between Ub^{prox} or Ub^{dist} of K48 di-Ub and F117/F121 of UCH37 shown in the form of a bubble plot.

(I) Contact frequencies shown for UCH37 residues that are interacting with either Ub^{prox} or Ub^{dist} of K48 di-Ub in poses 1 and 2. Contact frequencies for K48 di-Ub residues interacting with UCH37 in poses 1 and pose 2.

FIGURE S5. Backside Mutant Impairs K48 Chain Binding.

(A) ITC analyses of mono-Ub and K48 di-Ub binding to UCH37^{C88A/F117A}•RPN13.

(B) ITC analyses of mono-Ub and K48 di-Ub binding to UCH37^{C88A/F121A}•RPN13.

(C) Hydrolysis of Ub-AMC by the indicated UCH37 variants. Each variant is in complex with RPN13.

(D) Table of binding affinities for different UCH37 variants measured by ITC.

(E) ITC analyses of mono-Ub and K48 di-Ub binding to UCH37^{C88A/L181A}•RPN13.

(F) Surface representation of UCH37 colored by conservation.

Data are representative of at least two replicates.

FIGURE S6. Backside Mutants Impair K48 Chain Debranching and UCH37-Dependent Proteasomal Degradation.

(A) ITC analyses of mono-Ub and K48 di-Ub binding to UCH37^{C88A/I216E}-RPN13.

(B-H) Western blot analyses of the purification of different 26S Ptsm complexes with and without UCH37 variants.

(I) Hydrolysis of Ub-AMC by the indicated 26S Ptsm complexes.

(J) Hydrolysis of suc-LLVY-AMC by the indicated 26S Ptsm complexes.

Data are representative of at least two replicates.

	UCH37	DEUBAD
Active Residues	117,121,154	
Passive Residues	81,82,83,84,85,118,119,120,122,123,124,125,126,127,128,155,156,157,158,159,160,161,162,181,182,183,184	403,406,410,419,420,422
Flexible Residues		329-347,444-468
	Ub2K48-proximal subunit	Ub2K48-distal subunit
Active Residues	8,44,70	8,44
Passive Residues	36,42,43,45,46,49,50,57,58,59,60,68,71,72,73,74,75,76	42,43,45,46,47,48,49,50,57,58,59,60,68,70,71
Flexible Residues	48	72-76

Table S1. Active and Passive Restraints Used for HADDOCK Modeling.

MATERIALS & METHODS

****All materials written in the following format: REAGENT/ RESOURCE (Source, Identifier)***

Antibodies

Anti UCH37 (Abcam, Cat. # ab124931)
 Anti RPN11 (Abcam, Cat. # ab109130)
 Anti RPN13 (Cell Signaling Technology, Cat. # D9Z1U)
 Anti RPT2 (Abcam, Cat. # ab3317)
 Anti PSMB7 (R&D Systems, Cat. #MAB7590)
 Anti USP14 (Abcam, Cat. # ab56210)
 Anti Ub (P4D1, Enzo Lifesciences, Cat. # BML-PW0930)
 Anti Mitousin2 (Cell Signaling Technology, Cat. # 9482S)
 Anti POLR2D (Proteintech, Cat. # 16093-1-AP)
 Anti RPB (CTD) (Cell Signaling Technology, Cat. #2629)
 Anti β -actin (Cell Signaling Technology, Cat. # 3700T)
 Anti K48-linkage Specific (Cell Signaling Technology, Cat. # D9D5)
 Goat Anti Mouse IR Dye 800CW (LI-COR Biosciences, Cat. # 926-32210)
 Goat Anti Rabbit IR Dye 680RD (LI-COR Biosciences, Cat. # 926-68071)
 Goat Anti Rabbit IR Dye 800CW (LI-COR Biosciences, Cat. # 926-32211)

Bacterial and Viral Strains

Rosetta 2(DE3)pLysS (EMD Millipore Novagen, Cat. # 71403-3)
 BL21(DE3)pLysS (Promega, Cat. # L1191)
 One Shot™ Stbl3 (Fisher Scientific, Cat. # C737303)

Chemicals, Peptides, and Recombinant Proteins

ProBlock Gold Mammalian Protease Inhibitor Cocktail (Gold Biotechnology, Cat. # GB-331-5)
 Ammonium Chloride (¹⁵N, 99%) (Cambridge Isotope Laboratories, Cat. # NLM-467)
 SYPRO Ruby Stain (Fisher Scientific, Cat. # S12000)

- 1038 Cy5 maleimide (Lumiprobe, Cat. #23080)
- 1039 DBCO-Cy5 (Sigma, Cat. # 777374)
- 1040 Click-iT™ AHA (L-Azidohomoalanine) (Fisher Scientific, Cat. #C10102)
- 1041 Trypsin (Promega, Cat. # V5113)
- 1042 Formic Acid (Sigma-Aldrich, Cat. # 399388)
- 1043 Acetic Acid (Fisher Scientific, Cat. # 351269-4)
- 1044 Creatine phosphate disodium salt (Abcam, Cat. # ab146255)
- 1045 Creatine Kinase (Sigma-Aldrich, Cat. # 10127566001)
- 1046 Adenosine-5'-triphosphate (Goldbio, Cat. # A-081-5)
- 1047 Ub-AMC (Boston Biochem, Cat. # U-550)
- 1048 Suc-LLVY-AMC (Boston Biochem, Cat. # S-280)
- 1049 MG132 (Fisher Scientific, Cat. # 508339)
- 1050 Bortezomib (Selleck Chemicals, Cat. # S1013)
- 1051 Polybrene (Sigma, Cat. # TR-1003-G)
- 1052 Lipofectamine 3000™ (Fisher Scientific, Cat. # L3000008)
- 1053 Doxycycline hyclate (Sigma, Cat. # D9891)
- 1054 L-Methionine (Sigma, Cat. #64319-25G-F)
- 1055 Carfilzomib (PR-171) (Selleck Chemicals, Cat. # S2853)
- 1056 MTS-Alkynyldiazirine (Redbrick Molecular, Cat. # RBM-0000766)
- 1057 Dithiothreitol (Gold Biotechnology, Cat. # DTT10)
- 1058 Dimethyl sulfoxide (Alfa Aesar, Cat. # 42780)
- 1059 Iodoacetamide (Sigma-Aldrich, Cat. # I1149)
- 1060 Acetonitrile (Fisher Scientific, Cat. # A955-4)
- 1061 D-Glucose (Cambridge Isotope Laboratories, Cat. # DLM-2062-10)
- 1062 EZ-Link Iodoacetyl-PEG2-Biotin (Fisher Scientific, Cat. # PI21334)
- 1063
- 1064 **Critical Commercial Assays**
- 1065 pENTR™/SD/D-TOPO™ Cloning Kit (Fisher Scientific, Cat. # K242020)
- 1066 Gateway LR Clonase II Enzyme Mix (Fisher Scientific, Cat. # 11-791-020)
- 1067
- 1068 **Experimental Models: Cell Lines**

1069 HEK293 Expressing Rpn11-HTBH (Applied Biological Materials, T6007)
 1070 HEK293 Expressing Rpn11-HTBH RPN13 KO (Mol Cell)
 1071 HEK293 FT (ATCC, CRL-3216)
 1072 HEK293 FT UCH37^{KO} (Mol Cell)
 1073 HEK293 GFP^u (ATCC, CRL-2794)
 1074 HEK293 GFP^u UCH37^{KO} (Mol Cell)
 1075

1076 ***Recombinant DNA (Source, Identifier)***

1077 pMCSG20: NleL (aa 170-782) (Valkevich et al., 2014, N/A)
 1078 pVP16: UCH37 (DNASU, HsCD00084019)
 1079 pET19: RPN13 or Adrm1 (Addgene, Plasmid #19423)
 1080 pET28b: E1 (Trang et al., 2012; N/A)
 1081 pGEX-4T2: UBE2D3 (Valkevich et al., 2014, N/A)
 1082 pGEX-6P1: UBE2S-UBD (Addgene, Plasmid #66713)
 1083 pGEX-6P1: AMSH (aa 219-424) (Trang et al., 2012, N/A)
 1084 pOPINK: OTUD1 (Addgene, Plasmid #61405)
 1085 pVP16: OTUB1 (Pham et al., 2015, N/A)
 1086 pOPINS: UBE3C (Addgene, Plasmid #66711)
 1087 pDEST17: UBE2R1 (Pham et al., 2015, N/A)
 1088 pST39: UBE2N/UBE2V2 (Pham et al., 2015, N/A)
 1089 pET28b: UBC1 (DNASU, ScCD00009212)
 1090 pOPINK: RSP5 (DNASU, ScCD00008707)
 1091 pOPINS: Titin I27^{V15P} 23-K-35 (Bard et al., 2019, N/A)
 1092 pET22b: Ub and Ub variants (Valkevich et al., 2012, N/A)
 1093 pET22b: GFP (Addgene, Plasmid #11938)
 1094 pMD2.G (Addgene, Plasmid #12259)
 1095 psPAX2 (Addgene, Plasmid #12260)
 1096 pINDUCER21 (Addgene, Plasmid #46948)
 1097 pCW57-MCS1-P2A-MCS2 (RFP) (Addgene, Plasmid #80923)
 1098

1099 ***Software and Algorithms***

1100 Typhoon FLA 9500 (GE Healthcare)
 1101 Odyssey CLx Imager (LICOR)
 1102 Image Studio software (LICOR Biosciences)
 1103 Prism 9 (Graphpad Software)
 1104 Pinpoint 1.4 (Thermo Fisher Scientific)
 1105 Proteome Discoverer 2.3 (Thermo Fisher Scientific)
 1106 FlowJo 10.4 (FlowJo, LLC)
 1107 ImageJ/FIJI (Schindelin et al., 2012)
 1108 ATSAS Software Suite (Manalastas-Canto et al., 2021)
 1109 Primus (Konarev et al. 2003)
 1110 ASTRA Software 6.0 (Wyatt Technology)
 1111 Xcalibur Software (Thermo Fisher Scientific)
 1112 UCSF Chimera (University of California RBVI, San Francisco)
 1113 GROMACS 2018 package (KTH Royal Institute of Technology, Uppsala University,
 1114 University of Groningen)
 1115 HADDOCK 2.2 (Webserver Utrecht University, Bonvin Lab)
 1116 FoXS webserver (University of California, San Francisco, Sali Lab)
 1117 SWISS-MODEL (Swiss Institute of Bioinformatics)
 1118
 1119 ***Other***
 1120 His60 Ni Superflow resin (Clontech, Cat. # 635660)
 1121 Glutathione resin (GenScript, Cat. # L00206)
 1122 Amylose resin (NEB, Cat. # E8021S)
 1123 Streptavidin resin (GenScript, Cat. # L00353)
 1124 Slide-A-lyzer MINI dialysis units (3.5 kDa MWCO) (Thermo Scientific, Cat. # PI69552)
 1125 100mg SEP-PAK C₁₈ column (Waters, Cat. # wat043395)
 1126 C18 StageTips (Thermo Scientific, Cat. # SP301)
 1127 Zeba Spin Desalting Column (Thermo Scientific, Cat. # 89889)
 1128 NuPAGE Novex 12% Bis-Tris Protein Gels (Fisher Scientific, Cat. # NPO343BOX)
 1129 4-20% Mini-PROTEAN Gels (Bio-Rad, Cat. # 4561096)
 1130 Syringe Filters, PES, 0.45 µm (Genesee Scientific, Cat. # 25-246)

PD-10 Desalting Columns (GE Healthcare)
 OmniCure S1500 Spot UV Curing System (Excelitas Technologies)
 NuPAGE™ 12% Bis-Tris Mini Protein Gel (Thermo Scientific)
 Sep-Pak C18 Cartridge (Waters)

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by
 Eric Strieter (estrieter@umass.edu)

Human Cells

HEK293 cells stably expressing His-biotin affinity tagged human RPN11 (RPN11-HTBH) (Wang et al., 2007), HEK293FT, and HEK293 GFP^u cells were cultured at 37°C under 5% CO₂ using high glucose DMEM supplemented with 10% FBS, 1xGlutamax (Gibco), and 1xPen/Strep.

Generation of UCH37 Variants

Human UCH37 (isoform3) in pVP16 plasmid were purchased from DNASU. Mutations in the sequence encoding UCH37 were generated using site-directed mutagenesis following the QuikChange protocols using Phusion polymerases (New England Biolab). The variant of UCH37 with the ULD removed (UCH37^{CD}) was generated using restriction-enzyme free cloning protocols from Integrated DNA Technology using Phusion polymerases (New England Biolab).

Expression and Purification of Ub Conjugation Machinery

E1, UBE2D3, UBE2R1, UBE2N/UBE2V2, UBC1, and UBE3C were purified as previously described with slight modification (Bashore et al., 2015; Deol et al., 2020; Michel et al., 2015; Pham et al., 2016; Trang et al., 2012). Briefly, E1, UBE2D3, UBE2R1, and UBE2N/UBE2V2 constructs were expressed in Rosetta 2(DE3)pLysS *E.coli* cells grown at 37°C in LB media supplemented with 40 µg/mL Ampicillin. Once cells reached an OD₆₀₀ of 0.6-0.8, IPTG (400 µM) was added, and the temperature was

reduced to 20 °C to induce protein expression. Cells were then harvested after 16 h and resuspended in Ni-NTA lysis buffer (50 mM Tris pH 7.5, 300 mM NaCl, 1 mM EDTA and 10 mM imidazole), lysed by sonication, and clarified by centrifugation. Clarified lysate was incubated with Ni-NTA resin for 2 h at 4 °C, washed with Ni-NTA lysis buffer, and eluted into Ni-NTA elution buffer (Ni-NTA lysis buffer plus 300 mM imidazole). NleL (aa 170-782) was purified as previously described (Deol et al., 2020; Valkevich et al., 2014), but with a slight modification. Briefly, NleL was expressed in BL21(DE3)pLysS *E.coli* cells grown at 37°C in LB media supplemented with 40 µg/mL Ampicillin. Once cells reached an OD₆₀₀ of 0.6, IPTG (200 µM) was added, and the temperature was reduced to 20 °C to induce protein expression. Cells were then harvested after 16 h and resuspended in GST lysis buffer (50 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA and 1 mM DTT), lysed by sonication, and clarified by centrifugation. Clarified lysate was then incubated with GST resin for 2 h at 4°C, washed with GST lysis buffer, and eluted into GST elution buffer (GST lysis buffer plus 10 mM reduced glutathione). Eluate was concentrated in TEV protease buffer (50 mM Tris pH 8.0, and 0.5 mM TCEP), cleaved overnight with TEV protease, and further purified using anion exchange chromatography.

Synthesis of Defined Ub Chains

Native homotypic chains were synthesized as previously described (Deol et al., 2020). Briefly, 2 mM Ub, 300 nM E1, 3 µM UBE2R1 (K48) or 3 µM UBE2N/UBE2V2 (K63) were mixed in Ub chain synthesis reaction buffer (20 mM ATP, 10 mM MgCl₂, 40 mM Tris-HCl pH 7.5, 50 mM NaCl, and 6 mM DTT) overnight at 37°C. Native K6/48 branched trimer were synthesized by mixing 2 mM K6/48R Ub, 1 mM UbD77, 300 nM E1, 10 µM UBE2D3, and 1 µM NleL in ubiquitin chain synthesis reaction buffer overnight at 37°C.

All reactions for native chains were quenched by lowering the pH with the addition of 5 M ammonium acetate pH 4.4. Enzymes were then precipitated through multiple freeze thaw cycles and further purified using cation exchange chromatography.

Generation of High Molecular Weight (HMW) Ub Chains

Native K6/K48 and K48/K63 HMW Ub chains were synthesized as previously described (Deol et al., 2020). Briefly, *K6/K48 Ub chains* were synthesized by mixing 1 mM Ub, 150 nM E1, 5 μ M UBE2D3, and 3 μ M NleL in Ub chain synthesis reaction buffer and *K48/K63 Ub chains* were synthesized using 1 mM Ub, 150 nM E1, 5 μ M UBE2R1, and 5 μ M UBE2N/UBE2V2 in Ub chain synthesis reaction buffer. Reaction mixtures were all incubated at 37°C overnight with shaking. To synthesize biotinylated K6/48 HMW Ub chains, 100 μ M Ub K63C was mixed with 1 mM EZ-link™ iodoacetyl-PEG2-biotin (ThermoFisher Scientific) in 10% DMSO at in biotinylation reaction buffer (50 mM HEPES pH 7.5, 5 mM MgCl₂, 25 mM KCl, and 1 mM TCEP) and incubated at 37°C overnight. All Ub chains were purified using size exclusion chromatography (Superdex 75) to isolate high molecular weight chains >35kDa.

Protein Expression and Purification

Complexes containing UCH37 and RPN13 or RPN13^{DEUBAD} were expressed and purified as previously described (Deol et al., 2020), but with a slight modification. Briefly, UCH37 and RPN13/RPN13^{DEUBAD} constructs were expressed in BL21(DE3)pLysS E.coli cells grown at 37°C in LB media supplemented with 40 μ g/mL ampicillin. Once cells reached an OD₆₀₀ of 0.6, IPTG (400 μ M) was added, and the temperature was reduced to 20°C. Cultures were harvested after 16 h and frozen at -80°C with UCH37•RPN13 or UCH37•RPN13^{DEUBAD} complexes mixed 1:1 (volume) prior to lysis. Cell pellets were resuspended in amylose lysis buffer (50 mM HEPES pH7.4, 150 mM NaCl, 1 mM EDTA, and 1 mM TCEP), lysed by sonication, and clarified by centrifugation. UCH37•RPN13 and UCH37•RPN13^{DEUBAD} were purified following different chromatographic steps. For UCH37•RPN13, clarified lysate was incubated with amylose resin for 2 h at 4°C, washed with amylose lysis buffer, and eluted into amylose elution buffer (lysis buffer plus 10 mM maltose), and incubated overnight with TEV protease at 4°C. Then TEV protease cleaved product was incubated with Ni-NTA resin for 1 h, washed with Ni-NTA lysis buffer, and eluted with Ni-NTA elution buffer (Ni-NTA lysis buffer plus 300 mM imidazole). For UCH37•RPN13^{DEUBAD}, clarified lysate was incubated with Ni-NTA resin for 2 h at 4°C, washed with Ni-NTA lysis buffer, and eluted into Ni-NTA elution buffer, and then incubated overnight with TEV protease. The TEV protease

cleaved product was then incubated with GST resin for 1 h, washed with lysis buffer, and eluted with GST elution buffer (GST lysis buffer plus 10 mM glutathione). Eluate were incubated overnight with 3C protease overnight at 4°C. For both UCH37•RPN13 and UCH37•RPN13^{DEUBAD}, eluate was concentrated and loaded onto a Superdex 200 (GE) gel filtration column in gel filtration buffer (50 mM HEPES pH7.5, 50 mM NaCl, 1 mM EDTA, and 1 mM TCEP). For UCH37 and UCH37deltaULD, clarified lysate was subjected to Ni-NTA chromatography followed by further purification using anion exchange chromatography and Superdex 75 (GE) gel filtration column in gel filtration buffer.

Proteasome (Psm) Purification

Proteasomes (Psm) were purified as previously described (Deol et al., 2020). Wild type and RPN13^{KO} cells stably expressing RPN11-HTBH were grown, harvested and lysed in Psm lysis buffer (40 mM HEPES pH 7.4, 40 mM NaCl, 10 mM MgCl₂, 2 mM ATP, 1 mM DTT, and 10% glycerol). The lysates were clarified at 20,000xg for 20 min and the supernatant was incubated with streptavidin resin overnight with rocking at 4°C. The resin was washed and incubated for 10 min intervals with high salt wash buffer (Psm buffer containing 200 mM NaCl) on ice with rocking. The resin was then resuspended in low salt wash buffer (Psm lysis buffer without DTT) and incubated with TEV protease for 1.5 h at room temperature. Minor modifications were made for the purification of Psm complexes containing variants of UCH37•RPN13, i.e., I216E, F117A, and F121A. Clarified lysates derived from RPN13^{KO} cells were incubated with streptavidin resin overnight with rocking. The resin was pelleted, resuspended in Psm buffer containing 10 μM WT or mutant recombinant UCH37•RPN13 complexes, and further incubated for 4 h with rocking prior to high salt washes. The resulting Psm complexes were characterized by western blot analysis, Ub-AMC, and suc-LLVY-AMC activity.

Purification, Ubiquitination and Fluorescent Labeling of titin I27^{V15P}23-K-35 and UBE2S-UBD

Titin I27^{V15P}23-K-35 was purified as previously described (Deol et al., 2020). Briefly, titin I27^{V15P} 23-K-35 was expressed in BL21(DE3)pLysS *E.coli* cells grown at 30°C in 2xYT media containing 1% glycerol and supplemented with 40 µg/mL ampicillin. When the OD₆₀₀ reached 1.2-1.5, cells were induced with IPTG (400 µM), and grown for an additional 5 h at 30°C. Cultures were harvested, flash frozen, resuspended in titin lysis buffer (60 mM HEPES pH 7.5, 100 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 0.5 mM EDTA, 1 mg/mL lysozyme, 2 mM PMSF, 20 mM imidazole, and 10% glycerol), lysed by sonication, and clarified by centrifugation. Clarified lysate was then incubated with Ni-NTA resin for 2 h at 4 °C, washed with Ni-NTA binding buffer (50 mM HEPES pH 7.5, 150 mM NaCl and 20 mM imidazole), and eluted with elution buffer (binding buffer plus 300 mM imidazole). Eluate was buffer exchanged into Ulp1 protease buffer (50 mM HEPES pH 7.5 and 150 mM NaCl), cleaved overnight with Ulp1, and further purified using a Superdex 200 (GE) gel filtration column in 50 mM HEPES pH7.5 and 5% glycerol. 100 µM purified titin was ubiquitinated by mixing with 5 µM E1, 5 µM Ubc1, 20 µM Rsp5, and 2 mM Ub in labeling buffer (60 mM HEPES pH 7.5, 20 mM NaCl, 20 mM KCl, 10 mM MgCl₂, and 2.5% glycerol) containing 1X ATP regeneration mix for 3 h at room temperature followed by the addition of 5 µM UBE2R1 and incubation overnight at 4°C.

UBE2S-UBD was purified as previously described. Briefly, UBE2S-UBD constructs were expressed in Rosetta 2(DE3)pLysS *E.coli* cells grown at 37°C in LB media supplemented with 40 µg/mL ampicillin. Once the OD₆₀₀ reached 0.6-0.8, IPTG (400 µM) was added, and the temperature was reduced to 16°C. After 16 h of expression, cultures were harvested, resuspended in UBE2S lysis buffer (270 mM sucrose, 50 mM Tris pH8.0, 50 mM NaF, and 1 mM DTT), lysed by sonication, and clarified by centrifugation. Clarified lysate was then incubated with GST resin for 2 h at 4 °C, washed with washing buffer (25 mM Tris pH 8.0, 150 mM NaCl, and 5 mM DTT), and eluted in elution buffer (washing buffer plus 10 mM glutathione). Eluate was buffer exchanged into 3C protease buffer (50 mM Tris pH8.0 and 150 mM NaCl) and cleavage was performed with HRV 3C protease overnight, followed by further purification by size exclusion chromatography. UBE2S-UBD was ubiquitinated by mixing 0.6 µM Ub, 150

nM E1, and 5 μ M UBE2S-UBD in reaction buffer (10 mM ATP, 10 mM $MgCl_2$, 40 mM Tris pH 8.5, 100 mM NaCl, 0.6 mM DTT, and 10% (v/v) glycerol) for 3 h at 37°C, followed by addition of 3 μ M AMSH and 0.5 μ M OTUD1. The resulting mixture was incubated overnight at 37°C. An additional amount of AMSH and OTUD1 was added, and the mixture was incubated for 3 h at 37°C prior to the purification by size exclusion chromatography to isolate products with a molecular weight >35 kDa. Purified HMW K11-linked chains were then mixed with 0.6 μ M Ub, 150 nM E1, and 3 μ M UBE2R1 in reaction buffer (10 mM ATP, 10 mM $MgCl_2$, 40 mM Tris pH 8.5, 100 mM NaCl, 0.6 mM DTT, and 10% (v/v) glycerol) at 37°C overnight, followed by purification using size exclusion chromatography to collect >35 kDa products.

2 mg/mL ubiquitinated titin or UBE2S-UBD were fluorescently labeled using Cy5-maleimide at pH 7.2 for 2 h at room temperature and quenched with excess DTT. Free dye was separated from the substrate using a Zeba spin desalting column and buffer exchanged into labeling buffer (60 mM HEPES pH 7.5, 20 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, and 2.5% glycerol).

Ub Chain Debranching Assay and Ptsm-Mediated Degradation

The debranching and degradation assays were performed as previously described (Deol et al., 2020), but with slight modifications. For the debranching assays, a stock solution of recombinant UCH37 or replenished Ptsms were warmed to 37°C in DUB buffer or Ptsm buffer along with either unlabeled HMW Ub chains (0.2 mg/mL, for UCH37) or biotinylated HMW Ub chains (0.25 mg/mL, for Ptsm). Reactions were initiated by the addition of UCH37 or Ptsm and quenched with 6x Laemmli loading buffer after 1 or 2 h. Samples were then separated on a 15% SDS-PAGE gel. Gels were stained with SYPRO® Ruby to image unlabeled HMW Ub chains. Biotinylated HMW Ub chains were imaged by western blot analysis using streptavidin Alexa-Fluorophore 647.

For the degradation assays, ubiquitinated substrates (250 ng/ μ L) and Ptsms (200 ng/ μ L) were warmed to 37 °C in Ptsm degradation reaction buffer (40 mM HEPES pH 7.4, 150 mM NaCl, $MgCl_2$) supplemented with 1X ATP-regeneration mix. Reactions

were incubated for 1 h at 37°C and quenched with 6x Laemmli loading buffer. Samples were loaded onto 10-16% tricine gels, separated using SDS-PAGE, and imaged using Cy5 fluorescence on a Typhoon FLA 9500 (GE) set at a pixel density of 50 µm/pixel.

Fluorescence Polarization Assay

Catalytically inactive UCH37^{C88A}•RPN13 was serially diluted in fluorescence polarization (FP) buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 0.01% Brij-35) and loaded into a 384-well, black nonbinding polystyrene plate (Corning). A fixed concentration of Ub-fluorescein (50 nM) was then added to each well. After incubating for 1 h at 25 °C, FP was measured using a plate reader (Biotek Snergy 2) with polarized filters and optical modules for fluorescein ($\lambda_{\text{ex}} = 480 \text{ nm}$, $\lambda_{\text{em}} = 535 \text{ nm}$). FP values (mP) were calculated from raw parallel and perpendicular fluorescence intensities. Graphpad Prism 8.0 was used to fit the data and the equilibrium dissociation constant K_d was calculated as previously described.

Steady-state Kinetic analysis with Defined Ub Chains

Stock solutions of enzymes and Ub chains were prepared in DUB buffer (50 mM HEPES pH7.5, 50 mM NaCl, and 2 mM DTT). All reactions were performed at 37 °C. Each sample along with a Ub and di-Ub standard were then separated on a 15% SDS-PAGE gel and stained with SYPRO[®] Ruby. Gels were visualized on a Typhoon FLA 9500 (GE) and densitometry was performed using Image Studio[™]. Initial velocities of Ub and di-Ub formation were converted to concentration per minute (µM/min). These values were then fit to the Michealis-Menten equation using nonlinear regression in Graphpad Prism 8.0. Error bars represent the standard deviation of three biological replicates for each reaction performed using UCH37•RPN13 complexes.

Size Exclusion Chromatography Coupled with Multi-Angle Light Scattering (SEC-MALS)

The molecular masses of UCH37 complexes with Ub variants were determined using on-line SEC-MALS. Samples (1 mg/mL) were injected in a total volume of 100 µl onto a 7.8 mm I.D. x 30 cm, 5µm TSKgel column (TOSOH) equilibrated in 20 mM HEPES pH

7.4 and 10% isopropanol, at a flow rate of 0.5 ml/min. The weight-averaged molecular mass of material contained in chromatographic peaks was calculated using the ASTRA software version 6.0 (Wyatt Technology Corp). An overall average molecular mass and polydispersity term for each species was calculated by combining and averaging the results from the individual measurements at each 0.5 s time interval of the selected peak.

Isothermal Titration Calorimetry (ITC) Analysis

ITC measurements were performed on a MicroCal Auto-ITC200 (Malvern) at 25°C with a setting of 20 × 2 µL injections. UCH37^{C88A}•RPN13 and Ub chains were all dialyzed into dialysis buffer (50 mM HEPES pH7.4, 150 mM NaCl, and 500 µM TCEP). For all measurements, the syringe contained Ub or Ub chains at a concentration of 45–100 µM, and the cell contained UCH37^{C88A}•RPN13 at a concentration of 3–10 µM. The heats of dilution for diluting Ub chains into measurement buffer were subtracted from binding experiments before curve fitting. Manufactured supplied Origin software (OriginLab 7 SR4) was used to fit the data to a single-site binding model and to determine the stoichiometry (N), ΔH, ΔS, and the association constant K_a . The dissociation constant, K_d , was calculated from K_a .

Ub-AMC and Suc-LLVY-AMC Assays

Ub-AMC and suc-LLVY-AMC were used to report on the Ub hydrolysis and peptidase activity, respectively, of UCH37 variants and replenished Ptsms. Assays were performed in black-well, clear-bottom 96-well plates (Corning). Mixtures containing UCH37•RPN13^{DEUBAD}, UCH37•RPN13 (20 nM), or Ptsms (1 µg) in their respective assay buffer (DUB buffer for UCH37•RPN13^{DEUBAD} and UCH37•RPN13 and Ptsm reaction buffer for Ptsms) were incubated at 37°C for 20 min. The fluorogenic reagent (50 nM for Ub-AMC or 50 µM suc-LLVY-AMC) was then added to the appropriate wells and hydrolysis was monitored continuously for 30 min at 37°C on a fluorescence plate reader (BioTek Synergy 2, λ_{ex} = 360nm, λ_{em} = 460nm).

Ub Chain Pulldown Assay with UCH37 Variants

6xHis-tagged UCH37 variants (5 nmol) were incubated with Ub chains composed of different linkages (50 pmol) and Ni-NTA resin (20 μ L) in pulldown buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.1% IGEPAL; 100 μ L) for 2 h at room temperature. The resin was washed 3x with pulldown buffer before captured Ub chains were eluted with 6X Laemmli loading buffer, separated using SDS-PAGE, and visualized by Coomassie staining.

K6-Linkage-Specific Affimer Pulldown

The indicated cells were grown to about 60% confluency and treated with H₂O₂ for 1 h. The medium was then replaced with medium containing 10 mM MG132 and the cells were grown for additional 4 h. Emetine (25 μ M) was then added to the medium and the cells were harvested at indicated time points in lysis buffer (50 mM HEPES pH 7.5, 50 mM NaCl, 1X protease inhibitor cocktail). Cells were lysed by one freeze-thaw cycle followed by sonication. The resulting lysate was clarified by centrifugation at 20,000xg for 10 min. In parallel, the Halo-tagged K6-linkage-specific affimer was immobilized on Halo resin by incubating 500 μ L of a 500 μ M stock solution of Halo-tagged affimer with 300 μ L of Halo beads in pulldown buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.1% IGEPAL) for 2 h at 4°C. The immobilized affimer was then washed 3x with wash buffer and incubated with 10 mg of cell lysate overnight at 4 °C. The resin was then washed 5x with pulldown buffer prior to elution with 6X Laemmli loading buffer or treatment with different DUBs (e.g., 1 μ M of OTUB1 or 2 μ M UCH37•RPN13) for 1 h at 37 °C. The eluted proteins were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane and immunoblotted with an α Mfn2 antibody and α K48-linkage-specific Ub antibody.

SEC-SAXS Measurements and Data Processing

SEC-SAXS (size-exclusion chromatography-small angle X-ray scattering) experiments were performed at Lawrence Berkeley lab (SIBYLS Beamline 12.3.1, Advanced Light Source). To ensure sample monodispersity, analytes were first separated by size-exclusion chromatography (SEC) using an Agilent 1260 series HPLC with a PROTEIN KW-802.5 analytical column (Shodex) at a flow rate of 0.5 ml/min. The column outlet

was directly connected to the small-angle X-ray scattering (SAXS) sample cell. Samples were then analyzed by SAXS as they emerge from the column with $\lambda=1.03 \text{ \AA}$ incident light at a sample to detector distance of 1.5 m. The q-range sampled was from 0.013 to $0.5 \text{ \AA}^{-1}$, where $q = 4\pi\sin\theta/\lambda$ and 2θ is the measured scattering angle. Three second exposures are collected for each frame over the course of 40 min during the SEC run. SEC-SAXS chromatographs are generated and initial SAXS curves are analyzed using ScÅtter. Samples were analyzed at room temperature in 50 mM HEPES buffer pH 7.4, 50 mM NaCl, and 0.5 mM TCEP. Data were corrected for background scattering by subtracting the buffer curve from protein plus buffer curves. P(r) functions and R_g values were determined from the scattering data using PRIMUS from the ATSAS software package.

Hydrogen Deuterium Exchange Mass Spectrometry (HDX-MS)

Sample preparation and data collection

Complexes between Ub variants and UCH37·RPN13^{DEUBAD} were generated by incubating UCH37·RPN13^{DEUBAD} (60 μM) with the Ub variant (180 μM) for 1 h on ice in dilution buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, and 1 mM TCEP). Next, 4 μL of protein complex was diluted into either 96 μL of H₂O buffer (20 mM sodium phosphate, pH 7.0) for reference mapping or 96 μL of D₂O buffer (20 mM sodium phosphate, pD 7.0 in D₂O) to initiate deuterium labeling. After 1, 10, 30, 60, and 120 min exposure to the deuterated buffer at 15 °C, 50 μL labeling reaction was quenched by mixing with 50 μL quenching buffer (0.8M Guanidine-HCl, 1% acetic acid, pH 2.2) at 4 °C. Quenched samples were immediately injected into the HDX platform. Upon injection, samples were passed through an immobilized pepsin column (2.1 x 30 mm) (Waters Corp.) at 150 $\mu\text{L}/\text{min}$ for inline digestion. Following digestion, the resulting peptides were desalted on a 1.8 μm C8 trap column (Waters Corp.) and separated on a 1.0 mm x 5 cm C18 column (Accuity) with linear gradient of 80% B (A is 0.1% formic acid in H₂O and B is 0.1% formic acid in acetonitrile) over 12 min and peptides were identified using ion mobility MS through a Synapt mass spectrometer (Waters Corp.).

Peptide Identification

Product ion spectra were acquired in data-dependent mode with a one second duty cycle, which means that the most abundant ions selected for the product ion analysis by higher-energy collisional dissociation between survey scans occurs once per second. The resulting MS data files were submitted to ProteinLynx Global Server (PLGS) 3.0 software (Waters Corp.) to identify the peptic peptides in the undeuterated protein samples. Peptides included in the HDX analysis peptide set had a PLGS score greater than 7 and the MS spectra were verified by manual inspection.

Data Processing

Data from peptic peptides generated from PLGS were imported into DynamX 3.0 (Waters Corp.), with peptide quality thresholds of MS1 signal intensity greater than 5,000, maximum mass error of 1 ppm, minimum products per amino acid greater than 0.3 and PLGS score greater than 7. Following the automated peptide search and ion detection, each spectrum was manually inspected to ensure the corresponding m/z and isotopic distributions at various charge states were properly assigned to the appropriate peptic peptide. The relative deuterium incorporation plot and overall deuterium uptake heat map for each peptic peptide were generated by DynamX 3.0. DynamX 3.0 calculated the relative deuterium incorporation by subtracting the weight-averaged centroid mass of the isotopic distribution of peptic peptides of undeuterated sample from that of samples exposed to deuterated buffer at various time points. All HDX-MS experiments were performed in triplicate, with single preparations of each purified protein complex. Since all differential HDX-MS experiments were conducted under the same conditions, the differences in the back exchange for each experiment would be negligible. Thus, the reported deuterium uptake levels are relative, neglecting the effect of the back exchange. The percent relative deuterium uptake was calculated by dividing the relative deuterium uptake of each peptic peptide by its theoretical maximum uptake. To generate heat maps of individual residues, the HDX data from all overlapping peptides were consolidated to individual amino acid values by averaging each residue. Briefly, for each residue, the deuterium incorporation values were calculated by dividing the total relative deuterium uptake of the peptic peptide by the peptide length. The differential deuterium uptake plot was generated by subtracting the relative deuterium

uptake of peptic peptide of one complex by that of another. Statistical significance for the differential HDX data is determined by t-test for each time point, and a 98% confidence limit for the uncertainty of the mean relative deuterium uptake of ± 0.6 Da was calculated as described. Differences in deuterium uptake between two states that exceed 0.6 Da is considered significant.

Lentivirus Packaging, Infection and Cell Line Creation

Lentiviral packaging and target cell transductions were performed by the Cell Culture Core Facility at UMASS Amherst's Institute for Applied Life Sciences (Amherst, MA). Briefly, Lenti-X 293T cells (Takara Bio) were transfected with packaging and envelope plasmids psPAX2 and pMD2.G, respectively (gifts from Didier Trono, Addgene), and transfer plasmid pInducer21-UCH37-F117A, or pInducer21-UCH37-I216E. Lipofectamine2000 reagent (Invitrogen) was used to deliver DNA to the cells. Transfection reagent was removed 6 h after transfection and replaced with fresh EMEM (Lonza) supplemented with 10% FBS (Corning). Lentiviral supernatants were collected 18 h later, passed through a 0.45 μ m filter and stored at -80°C.

HEK293FT UCH37^{KO} cells were transduced with lentivirus in a 6-well plate when cells were roughly 80% confluent. A 3-fold serial dilution of lentivirus was prepared in OptiMEM medium (Gibco) supplemented with 8 μ g/mL polybrene (Millipore). Growth medium was removed from target cells and replaced with 2 mL dilution per well. Transduction plate was centrifuged at 2,000 rpm for 2 h at room temperature. Plate was incubated at 37 °C/5% CO₂ for an additional 6 h. Transduction medium was removed and replaced with fresh growth medium. Plates were incubated for an additional 40 h before flow cytometry sorting.

Emetine Chase Using GFP^u Reporter System

WT and UCH37^{KO} GFP^u cells were grown in 12-well plates until cells reached ~50-60% confluency and then treated with 36 μ M emetine to shutoff translation for the indicated times. Cells were harvested in cold PBS containing 1 mM EDTA, followed by centrifuging at 1,000xg for 2 min. Complementation experiments were performed by

transfecting UCH37^{KO} GFP^u cells with plasmids expressing RFP under a constitutively expressed promoter and either UCH37^{F117A} or UCH37^{I216E} under a doxycycline inducible promoter. The transfected cells were grown in DMEM containing 0.1 µg/ml doxycycline for 48 h in 12-well plates until ~40-50% confluency. Cells were then treated with 36 µM emetine for the indicated times, washed, harvested, and resuspended in FACS buffer (1 mM EDTA and 0.1% BSA in cold PBS) for flow cytometry. Data acquisition was performed on a BD LSR Fortessa X20 with 488 nm (530/30 band-pass collection filter), equipped with 488 nm and 561 nm lasers for excitation of GFP and RFP, respectively. Data analysis was performed using FlowJo version 10 (FlowJo, LLC). Statistical analysis was performed using Prism 8 and are represented as the mean fluorescence in BL1-A with standard deviation of three independent experiments. ****P*<0.0005 (ANOVA two-way comparison).

Pulse-Chase Experiments with L-Azidohomoalanine (AHA) Labeling

UCH37^{KO} cells expressing either UCH37^{F117A} or UCH37^{I216E} were seeded in 6-well plates and grown to ~60% confluency. Cells were then washed once with PBS and incubated for 1 h in methionine free medium supplemented with or without 25 µM AHA. Cells were then either harvested or chased in complete DMEM supplemented with 2 mM excess methionine for indicated time points up to 4 h. Negative controls were not treated with AHA and were harvested without chase. Carfilzomib controls were treated with 1 µM carfilzomib during both AHA incubation and 4 h chase. Cells were harvested in cold PBS containing 1 mM EDTA and resuspended in RIPA buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5% Triton X-100, 140 mM NaCl, 0.1% SDS, and 0.1% sodium deoxycholate supplemented with 1X protease inhibitor cocktail) before flash freezing on dry ice and storing at -80°C. Pellets were lysed via 3x freeze-thaw cycles and sonicated for 3 min before centrifugation at 20,000xg for 10 min at 4°C. Total protein concentration was quantified using BCA assay. AHA-containing proteins were then labeled with 10 µM DBCO-Cy5 for 30 min at RT. Labeling was quenched with 6x Laemmli loading buffer without DTT and samples were separated by SDS-PAGE on 4-20% Mini-PROTEAN gels. Cy5 fluorescence and total protein staining using SYPRO[®] Ruby was measured on a Typhoon FLA 9500 (GE). For UCH37^{KO} cells expressing either WT or UCH37^{C88S},

cells were seeded in medium containing 0.1 µg/mL doxycycline for 48 h. Doxycycline remained present for the rescue cell lines after each medium change.

¹³C-Methyl ILV Labeled Protein Expression

Ub monomers (Ub^{D77} and Ub^{K6R/K48R}) were expressed in M9 minimal medium supplemented with [1,2,3,4,5,6,6-D7] D-glucose (2 g/L) in D₂O. Once cells reached an OD₆₀₀ ~1.0, [3-methyl-¹³C, 3,4,4,4-D4] α-ketoisovaleric acid (120 mg/L) and [methyl-¹³C, 3,3-D2] α-ketobutyric acid (70mg/L) were added (Tugarinov et al., 2006). After an hour, 400 µM IPTG was added to induce expression. Purification of labeled Ub was performed as described above for unlabeled Ub.

Synthesis of Labeled Ub Chains

The protocol for synthesizing Ub chains was modified slightly to generate labeled chains. Briefly, 1 mM Ub^{D77}, 1 mM Ub^{K6R/K48R}, 800 nM E1 and 4 µM UBE2R1 (K48-linkage-specific) were mixed in Ub chain synthesis reaction buffer (10 mM ATP, 10 mM MgCl₂, 50 mM Tris-HCl pH 8.0 and 1 mM DTT) overnight at 37°C. Selective labeling of proximal and distal subunit of K48 di-Ub was achieved using either [¹³C-methyl ILV] Ub^{D77} or [¹³C-Methyl ILV] Ub^{K6R/K48R}. Labeled K48 di-Ub was purified using the same procedure as unlabeled dimers.

NMR Spectroscopy

NMR spectra were collected on a Bruker Avance III HD spectrometer operating at 900 MHz (¹H) and equipped with a cryogenic probe. The temperature of the samples was regulated at 25°C throughout data collection. NMR samples were prepared in 99.9% D₂O, 20 mM potassium phosphate buffer (pH 6.8), and 0.1% (v/w) NaN₃. All NMR samples contained 30 µM mono-Ub or di-Ub [¹³C-Methyl ILV]-labeled at the appropriate subunit. Titration experiments were performed with individually prepared samples at molar ratios of 1:0.5, 1:1, 1:1.5 for mono-Ub and 1:1, 1:1.5 for di-Ub. Two-dimensional (2D) ¹H,¹³C-SOFAST-HMQC spectra were collected with 1024 x 256 complex points in the direct ¹H and indirect ¹³C dimensions, respectively. The power and pulse width for the selective 90° and 180° shaped pulses on the methyl ¹Hs, PC9 and Reburp,

respectively, were calculated on-the-fly within the pulse program from the 4.0ppm bandwidth, while the pulse offset was set to 0.5ppm. A recycle delay of 1 sec was used and 64 scans were accumulated for each FID for a total acquisition time of 10 h. In addition, 2D ^1H , ^{13}C -ZZ-exchange experiments were performed on mono-Ub and K48 di-Ub labeled at either proximal or distal subunit. With the 1:1 UCH37^{C88A}•RPN13:mono-Ub complex, ZZ-exchange spectra were recorded using mixing times of 100, 200, 400 and 800 ms. With the proximal subunit of K48 di-Ub, ZZ-exchanged spectra were collected using mixing times of 100, 200 and 400 ms, while with the distal subunit labeled, mixing times of 100 and 200 ms were used. All ZZ-exchange spectra were recorded using 1024 x 128 complex points in the direct ^1H and indirect ^{13}C dimensions, respectively. The recycle delay was set to 1 sec and 512 scans were accumulated for each FID for a total acquisition time of approximately 2 days. All 2D spectra, SOFAST and ZZ-exchange, were recorded using a ^1H spectral window of 16.3 ppm, while the ^1H offset was set on water at 4.7 ppm. For the ^{13}C dimension, the spectral window was set to 24.5 ppm and the offset was set to the middle of the methyl region at 16.6ppm. All NMR data were processed with NMRPipe and analyzed using NMRFAM-Sparky.

Molecular Dynamics

MD simulations were performed on UCH37•RPN13^{DEUBAD} using a homology model based on PDB ID: 4UEL. Missing N- and C-terminal residues of UCH37 were added using UCSF-Chimera (Pettersen et al., 2004) and homology modelling was done by the Swiss-Model webserver (Schwede et al., 2003) to fill in missing loops. Missing N- and C-terminal segments of RPN13^{DEUBAD} were added from PDB ID:2KR0 and backbone dihedral angles adjusted to avoid clashes with UCH37. MD simulations were carried out with GROMACS-2018 using CHARMM36 force field (Huang et al., 2016). The complex was solvated in a TIP3P periodic dodecahedron box with a minimal distance of 9.5 Å to the box edge using counterions. The system was minimized using 5000 steps of steepest descent and heated to 310 K and equilibrated for 300 ps using an isothermal-isochoric ensemble (NVT). This was followed by 500 ps at the same temperature and 1 bar pressure using an isothermal isobaric ensemble (NPT). A total of 8 independent simulations were performed for 50 ns each. To ensure proper geometries and bond

lengths during the simulation, the LINCS algorithm was used with a 2 fs integration step and a cutoff of 12 Å for non-bonded interactions. Structures were clustered using the Jarvis Patrick algorithm with an RMSD cutoff of 3 Å and the structure with the lowest RMSD to the average structure was used for further analysis.

Docking Simulations and Fitting to SAXS Data

Docking was performed using the HADDOCK2.2 webserver (van Zundert et al., 2016) to generate a structural model for UCH37•RPN13^{DEUBAD} in complex with K48 di-Ub. Starting structures for UCH37•RPN13^{DEUBAD} were selected from the best scoring multistate model to the SAXS data according to the FoXS webserver (Schneidman-Duhovny et al., 2016). The best scoring multistate model identified three conformations of UCH37•RPN13^{DEUBAD}, an additional two models were selected with lower scores but considerably different conformations to increase structural variability. Each conformation within the five-member ensemble was used in a separate docking simulation. Active residues on these structures were identified based the HDX-MS and crosslinking data. Residues F117 and F121 were selected based on their effect on di-Ub binding and K154 due to the K_m effect. Passive residues were selected based on HDX-MS. Residues that are protected upon K48 di-Ub binding on the catalytic domain and are solvent exposed were set as passive. N- and C-terminal segments of RPN13^{DEUBAD} were left fully flexible during docking. Starting coordinates for K48 di-Ub were obtained from PDB ID: 1UBQ and used during multibody docking as proximal and distal subunits. Active residues for ubiquitin subunits were determined based on NMR titration experiments. Residues that belong to the I44 patch with identified exchange peaks were selected as active. Passive residues were identified within 4.5 Å cutoff of active residues and included other exchanging residues that were identified by NMR. Residues 72-74 and K48 of the proximal subunit were left fully flexible during all stages of docking. Unambiguous restraints were introduced between the ubiquitin subunits to preserve distances characteristic to isopeptide linkages (Fushman and Walker, 2010). Docking was performed using the standard HADDOCK protocol with minor adjustments. Rigid body docking was performed using 10000 structures and the best 500 structures were selected for semi-flexible refinement according to AIR energies. The resulting 500

structures were analyzed and clustered according to Fraction of Common Contacts (FCC) with a cutoff of 0.6 and minimal cluster size of 4. All structures obtained from clustering of MD simulations and docking were used to back calculate theoretical SAXS profiles and fitted against experimental data using the FoXS webserver. The calculation of SAXS profiles was performed with standard settings using on offset and background adjustment.

Photo-crosslinking of Ubiquitin Chains and UCH37

A photocrosslinker was installed on different subunits of K48 di-Ub and K6/K48 tri-Ub using a Ub variant in which A46 is replaced with Cys (A46C). The A46C bearing Ub chains were incubated with 500 μ M MTS-alkynyldiazirine (Redbrick molecular) in 25 mM Tris, pH 8, 10% DMSO) at room temperature for 30 min. Excess photocrosslinker was removed with Zeba spin column (Thermo Fisher scientific). The modified Ub chains (15 μ M) were incubated with UCH37^{C88A}•RPN13^{DEUBAD} (45 μ M) in crosslink reaction buffer (50 mM HEPES, pH 7.4, 50 mM NaCl) on ice for 1 h. The mixture was then irradiated with a UV lamp (Omnicure, 320 – 500 nm) for 5 min on ice, followed by separation with SDS-PAGE and Coomassie staining. The gel slice containing crosslinked UCH37-Ub chain conjugate was cut out and reduced with 10 mM DTT (Ub peptide is released from the crosslinked peptide, but UCH37 peptide is still modified with the crosslinker) and then alkylated with 55 mM iodoacetamide. In-gel digestion was done with trypsin at 37 °C overnight.

Crosslinked Peptide Detection by LC-MS/MS

Extracted tryptic peptides were desalted and separated by a homemade fused silica capillary column (75 μ m x 150 mm) packed with C-18 resin (120 Å, 1.9 μ m, Dr. Maisch HPLC GmbH). Peptides were eluted by applying a 90 min gradient elution (solvent A: 0.1% formic acid (FA) in water/solvent B: 0.1% FA in acetonitrile) on the EASY-nLC 1000 nano-HPLC system (Thermo Fisher Scientific), coupled with the Orbitrap Fusion Tribrid mass spectrometer. Full MS scans were acquired at a resolution of 60000 between 350-2000 m/z. Collision-induced dissociation (CID) was induced on the ten most abundant ions per full MS scan using an isolation width of 20 ppm. Fragmented

precursor ions were allowed for one repeated MS/MS analysis and then excluded for 15 s. LC-MS/MS Data were searched against the manual database containing the UCH37^{C88A}•RPN13^{DEUBAD} and Ub sequence by using the Proteome Discoverer 2.4 with the SEQUEST HT search engine. Peptide's mass and MS/MS tolerance are set to 10 ppm and 0.6 Da. Methionine oxidation, N- terminal acetylation, cysteine carbamidomethylation and a manually added photocrosslinker modification at any residue was used as a variable modification.

Global Proteomics Analysis Using Tandem Mass Tag (TMT) Labeling

UCH37^{KO} HEK293 FT cells transduced with WT UCH37, UCH37^{F117A} and UCH37^{I216E} were grown in DMEM supplemented with 10% FBS, 2 mM glutamine and 100 units/ml penicillin/streptomycin. Cells were plated in 6-well plate and allowed to grow 12 h before treating them with doxycycline to induce the expression of UCH37. After 12 h doxycycline treatment, all cells were washed twice with cold PBS, and harvested with 1 mM EDTA in cold PBS. To induce the oxidative stress, cells were treated with 250 μ M H₂O₂ for 1 h prior to harvesting. Cells were then suspended in lysis buffer (8 M urea, 50 mM HEPES pH 7.4, 75 mM NaCl, and 1x protease inhibitor cocktail) and lysed by freeze-thaw cycles and sonication. Clarified lysate were then diluted in 100 mM TEAB buffer, followed by reduction (20 mM TECP pH 7.5), alkylation (37.5 mM iodoacetamide) and precipitated by cold acetone overnight. Precipitated proteins were resuspended in 50 mM TEAB buffer and digested by trypsin overnight at 37°C. Then 20 μ L of 20 ng/ μ L 10-plex TMT reagents (Thermo Scientific) dissolved in anhydrous acetonitrile were added to 50 μ g of digested peptide samples. Following incubation at RT for 1 h, the labeling reaction was quenched with hydroxylamine to a final concentration of 0.5% (v/v) for 15 min, and the equal volume of TMT labeled samples were pooled together. Pooled samples were dried using vacuum centrifuge and dried samples was dissolved in 10 mM NH₄HCO₃ pH 8.0 and fractionated by high pH reverse-phase HPLC as previously described 39-40. Samples were fractionated into 96 fractions through an aeris peptide xb-c18 column (Phenomenex; 250 mm x 3.6 mm) with mobile phase A containing 5% acetonitrile and 10 mM NH₄HCO₃ in H₂O (pH 8.5), and mobile phase B containing 90% acetonitrile and 10 mM NH₄HCO₃ in H₂O (pH 8.5). The 96

resulting fractions were then pooled in a non-continuous manner into 8 fractions for subsequent mass spectrometry analysis. Samples were dried using vacuum centrifuge and resuspended in 0.1% formic acid in H₂O for LC-MS/MS analysis. Mass spectrometry data were collected using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) coupled to a Proxeon EASY-nLC1200 liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were separated on a 75 µm x 15 cm, nanoViper C18, 2 µm, 100 Å column (Thermo Fisher Scientific) with a gradient of 5% (0-10 min), 5%–35% (10-150 min), 35%–85% (150-160 min) Solvent B (0.1% formic acid in acetonitrile) in Solvent A (0.1% formic acid in H₂O) over a total 165 min run at 500 nL/min. 1-2 µg peptide from each fraction was injected onto the column for analysis. The scan sequence began with an MS1 spectrum (Orbitrap analysis; resolution 120,000; mass range 400-1400 m/z; automatic gain control (AGC) target 5x10⁵; maximum injection time 100 ms). MS2 analysis consisted of collision-induced dissociation with 35% energy (AGC 5x10³; isolation window 0.7 Th; maximum injection time 150 ms; activation time 10ms). Monoisotopic peak assignment was used, and previously interrogated precursors were excluded using a dynamic window (150 s ± 7 ppm) and dependent scans were performed on a single charge state per precursor. Following acquisition of each MS2 spectrum, a synchronous-precursor-selection (SPS) MS3 scan was collected on the top 10 most intense ions in the MS2 spectrum. MS3 precursors were fragmented by high energy collision-induced dissociation (HCD) with 55% energy and analyzed using the Orbitrap (AGC 5x10⁴; maximum injection time 150 ms, resolution was 10,000 at 2 Th isolation window).

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