Weighty synthetic ubiquitin tools to shine a light on the enzymes of the ubiquitin pathway

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CHAPTER 2

Competition or Indifference: Neutron-Encoding to Profile Linkage Selectivity of Deubiquitinating Enzymes

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Abstract

Deubiquitinating enzymes (DUBs) are key regulators in the ubiquitin (Ub) system and an emerging class of drug targets. These proteases disassemble polyUb chains and many DUBs show selectivity for specific polyUb linkages. However, most biochemical insights originate from studies of single diUb linkages in isolation, whereas, in cells, all linkages coexist and are potentially in competition with each other. To address this discrepancy, we developed a multiplexed mass spectrometry (MS)-based DUB assay, probing all Ub linkage types simultaneously, thereby allowing quantification of DUB activity when there is competition between these substrates. For this, all eight native diUbs were generated where each linkage has a distinct molecular weight due to the incorporation of neutron-encoded amino acids. Overall, 22 DUBs were profiled and our results provide a three-dimensional overview of DUB linkage selectivity over time and enzyme concentration, leading to new insights into their activity, such as a specific order of diUb cleavage for several DUBs.

Keywords: Enzyme Selectivity • Linkage Specificity • Mass Spectrometry • Ubiquitin Chains • Deubiquitinating enzymes • Neutron-Encoded Amino Acids • Chemical protein synthesis

Introduction

Ubiquitination is a post-translational modification (PTM) process in which ubiquitin (Ub), a highly stable 76 amino acid long protein[1], is covalently attached to a substrate protein to influence its function or location. Ub signalling is involved in almost all cellular pathways[2] and dysregulation has been observed in various diseases including various types of cancer and neurodegenerative diseases, metabolic disorders, and ageing.[3] Ub is installed via its C-terminal carboxylate, usually onto the amino group of the lysine side chain of a substrate protein, and this process is regulated by an enzymatic cascade involving a ubiquitin-activating (E1)[4], a ubiquitin-conjugating (E2)[5] and a ubiquitin-ligating (E3)[6] enzyme. Ub can also be attached to another Ub resulting in polyUb chains through the formation of an (iso-)peptide bond between the C-terminus of one Ub and the N-terminus (Met1) of the other Ub or one of its lysine sidechains (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63). As such, these polyUb chains come in eight homotypic linkage types (linked via the same (Lys) residue), but many more flavours exist.[7,8] Moreover, each polyUb linkage type has been found to result in different signalling functions.[9] And the installed Ub signals can be effectively antagonized by deubiquitinating enzymes (DUBs), a family of proteases that counteract the ubiquitination process by cleaving Ub from the target substrate protein or trimming polyUb chains[10–12].

Currently, approximately 100 different DUBs have been identified to be encoded in the human genome.[11] They are commonly divided into seven different families; ubiquitin carboxy (C)-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), Machado-Joseph disease protein domain proteases (MJDs), ovarian tumour proteases (OTUs), JAB/MPN/Mov34 metalloenzyme (JAMM), motif interacting with Ub-containing novel DUB family (MINDY) and zinc finger with UFM-1 specific peptidase domain protein/C6orf113/ZUP1 (ZUFSP).[11] DUBs are involved in many different cellular pathways, such as controlling proteasome-mediated protein degradation[2,11,13], DNA damage response[11,14–16], and innate immune signaling[11,17] and are implicated to be involved in different diseases[18–22]. For this reason, and because DUBs have potentially attractive druggable sites, these enzymes are recognized as promising drug targets.[23] Therefore, it is important to study DUB activity and elucidate their catalytic mode of action, efficiency, protein substrate preference, and ubiquitin linkage type selectivity.

As DUBs counteract the signal originating from a certain polyUb chain type, much effort has been dedicated to the determination of the linkage specificity of DUBs in the last decade.[24–27] Advances in synthetic strategies towards diUb[28,29] allowed the design of probes[30–32] and tools[33–35] targeting DUBs and provided insights into their molecular mechanisms. However, analysis mostly relied on incubation of a purified recombinant DUB with one diUb linkage-type at a time with an SDS-PAGE read-out (**Fig. 1, top**). [24,25] Although advances have been made to allow a quantitative read-out by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

(MALDI-TOF-MS) [26] or by fluorescence intensity[34] (**Fig. 1, top**), the element of natural competition between the different linkage types has so far been taken out of the equation.

While all polyUb linkages coexist in cells,[7] it is currently unknown, and so far unaddressed, whether this coexistence influences general DUB activity, cleavage efficiency for each linkage as well as their linkage selectivity in general, as one or more linkage types can potentially influence DUB action on the other linkages. For example, questions arise whether there is direct competition between the linkage types, whether they influence the rate of each other's cleavage, and whether linkages are processed in a specific order when they are all present simultaneously. These are important questions to answer when trying to understand the different deubiquitination pathways in detail. However, the current assays are unable to answer these questions, since it is impossible to distinguish all linkages from each other during the read-out when they are present in a single mixture.

To overcome this, we developed an assay that allows the analysis of all different diUb linkages in a single mixture and with a single measurement. The diUb substrates were modified as little as possible, e.g. avoiding dyes, non-natural amino acids and other unnatural (MS) tags to obtain (near-) native diUb molecules. A complete set was designed that contained all diUb linkage types, seven isopeptide-linked diUb molecules (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63) and the linear diUb (Met1), with each isoform having a distinct mass effected by incorporation of fully ^{13}C and ^{15}N labelled amino acids. Accordingly, these diUb molecules can be measured all at once as MS can distinguish the identity and absolute amount of each linkage type present in the mixture (**Fig. 1, bottom**). Moreover, it allowed us to investigate Ub-linkage specificities of DUBs over time using a single mixture of all eight diUbs. This novel screening method proved to be fast and repeatable and required only small amounts of diUb molecules. As a proof of concept, we assayed the selectivity and activity of 22 human DUBs from different DUB families to obtain more insight into their proteolytic profile. Strikingly, we demonstrate that USP enzymes, while known for their chain type promiscuity, show linkage selectivity at lower enzyme concentrations; a phenomenon commonly observed for OTU family members.[24] In addition, we found that some USPs follow a consecutive cleavage order: they start to process certain diUb linkages only after the consumption of other linkages has come close to completion.

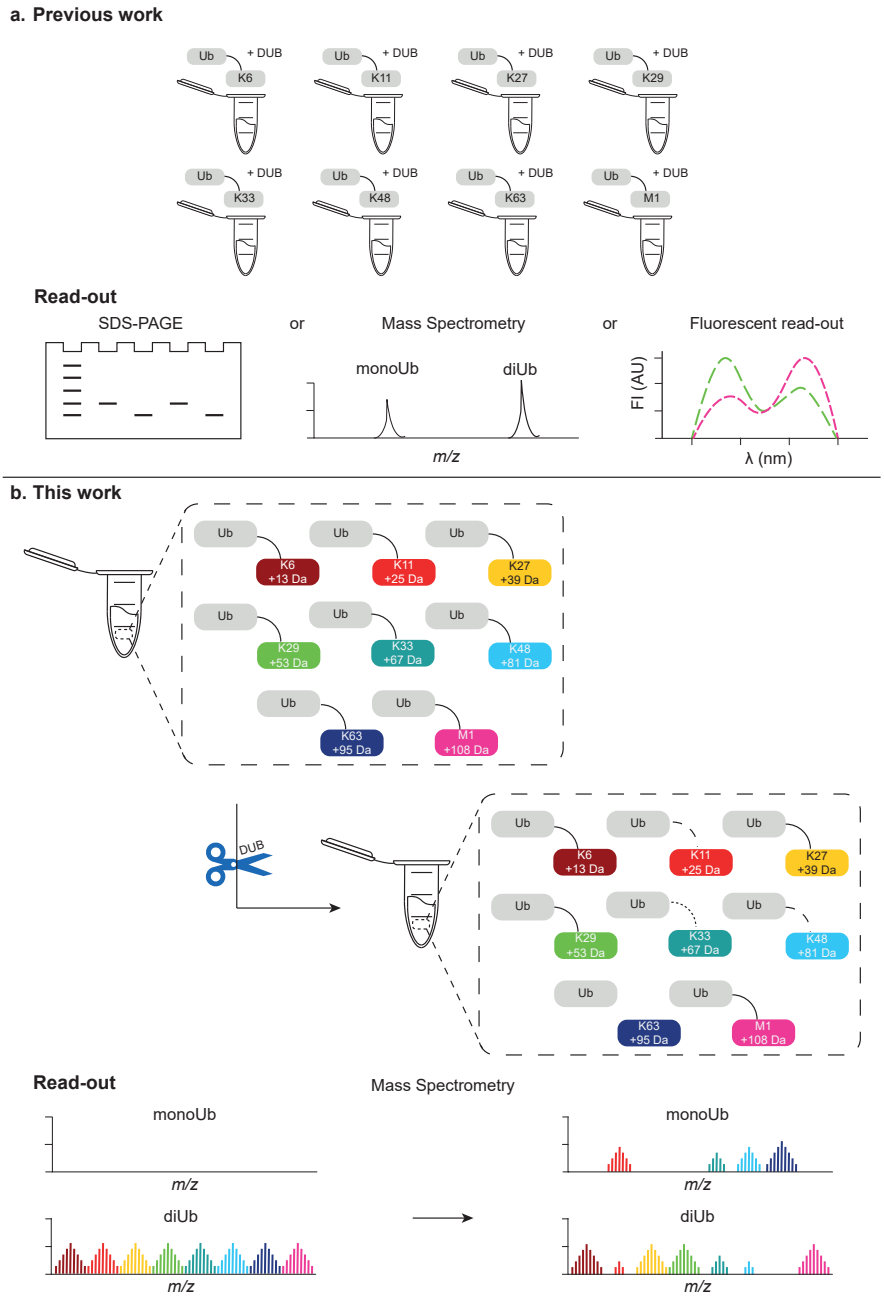


Figure 1| The principle of the neutron-encoded diUb cleavage assay. a, Schematic outline of classical diUb cleavage assays relying on separate incubation of each diUb isoform with a DUB followed by SDS-PAGE, MS or fluorescent intensity read-out for every diUb isoform separately. **b**, Schematic outline of the designed method where a DUB is incubated with a mixture containing all neutron-encoded diUb isoforms followed by MS analysis, allowing the quantification of each linkage from the complex mixture. Upon cleavage of the diUb by a DUB, the diUb signal(s) will disappear and the corresponding monoUb signal(s) will appear in the MS spectrum.

Results

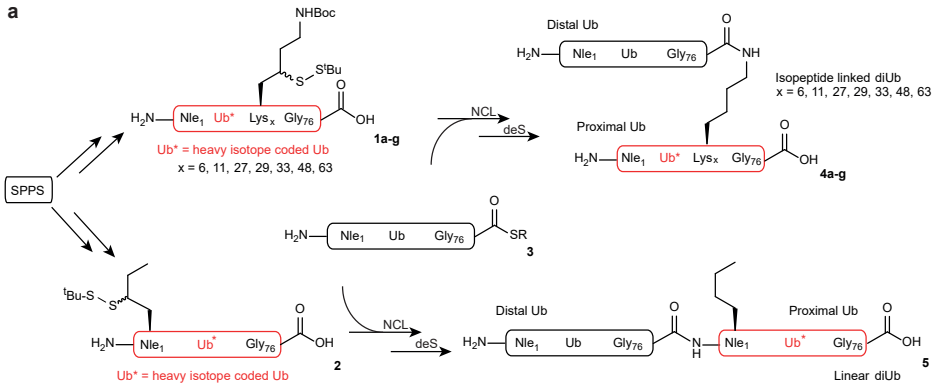
Design and synthesis of the neutron-encoded diUb molecules.

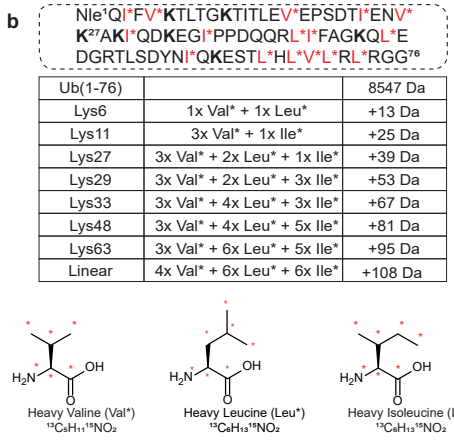
The envisioned MS-based assay (**Fig. 1, bottom**) required the preparation of all eight diUb isoforms equipped with neutron-encoded amino acids to introduce appropriate mass differences (**Fig. 2a** and **b**). To ensure a suitable separation of isotopic patterns during MS analysis, a mass increase of 12, 13, or 14 Da for each consecutive diUb was chosen. Fully ^{13}C and ^{15}N labelled amino acids Val, Leu, and Ile were used to introduce these mass differences because they are abundant in the ubiquitin sequence, introduce a substantial mass difference per amino acid (6 or 7 Da), and are relatively inexpensive. As the diUb linkage type is defined by the proximal Ub's lysine residue that forms the isopeptide bond, all neutron-encoded amino acids were incorporated in the proximal Ub only to directly link the appropriate mass fingerprint, as depicted in **Fig. 2b** and **Supplementary Table 1**, to the linkage type. This way each linkage could be identified by MS in both the diUb (substrate) as well as the monoUb (product) form (*vide infra*). Besides, since the assay relies on intact mass analysis only, the read-out is unaffected by the exact location of heavy-isotope introduction.

All eight diUb molecules were constructed using a native chemical ligation (NCL)-desulfurization (deS) strategy (**Fig. 2a**). First, all eight different proximal Ubs **1a-g** and **2**, harbouring the neutron-encoded amino acids and γ-thioLys [28,36–39] (in **1a-g**) or γ-thioNle [40] (in **2**) NCL handles, and distal Ub thioester **3** were synthesized via traditional Fmoc-based solid-phase peptide synthesis (SPPS) using an optimized version of our protocol (**Fig. 2a** and **Supplementary Table 1**).[28] The NCL[28,34,38] of thiols **1a-g** and **2** with thioester **3** followed by desulfurization under radical conditions[41] (**Fig. 2a** and **Supplementary Scheme 1**) and subsequent purification resulted in the eight neutron-encoded diUb molecules (**4a-g** and **5**) in good overall yield (0.25 – 1.6 mg, 2.5 - 16%). The purity of the final products was analysed by SDS-PAGE (**Fig. 2c** and **Supplementary Figure 1**) and LC-MS (**Fig. 2d**, **Fig. 3a left** and **Supplementary Data; Protein Synthesis - LC-MS spectra**).

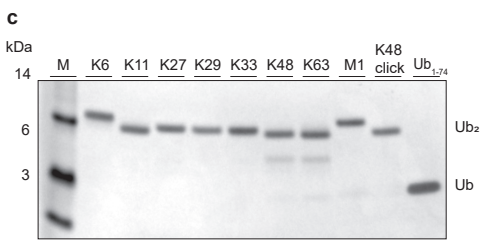
All diUbs can be measured simultaneously and are processed by DUBs.

To show that all eight neutron-encoded diUbs could be detected at the same time, they were mixed in an equimolar ratio and analysed by HPLC-MS. The obtained spectrum clearly showed a distinct isotopic pattern for each of the eight neutron-encoded diUb molecules and displayed a fair separation of all isotopic patterns at all charge states, thereby confirming that the introduced mass difference was sufficient to allow the detection of every single diUb linkage in the mixture (**Fig. 3a right** and **Supplementary Figure 2**). To confirm that all neutron-encoded diUbs are correctly folded, and therefore accepted and processed by DUBs, all eight linkages were separately incubated for 3 hours with non-specific DUB USP21[25] and diUb proteolysis





Ub(1-76)		8547 Da
Lys6	1x Val* + 1x Leu*	+13 Da
Lys11	3x Val* + 1x Ile*	+25 Da
Lys27	3x Val* + 2x Leu* + 1x Ile*	+39 Da
Lys29	3x Val* + 2x Leu* + 3x Ile*	+53 Da
Lys33	3x Val* + 4x Leu* + 3x Ile*	+67 Da
Lys48	3x Val* + 4x Leu* + 5x Ile*	+81 Da
Lys63	3x Val* + 6x Leu* + 5x Ile*	+95 Da
Linear	4x Val* + 6x Leu* + 6x Ile*	+108 Da



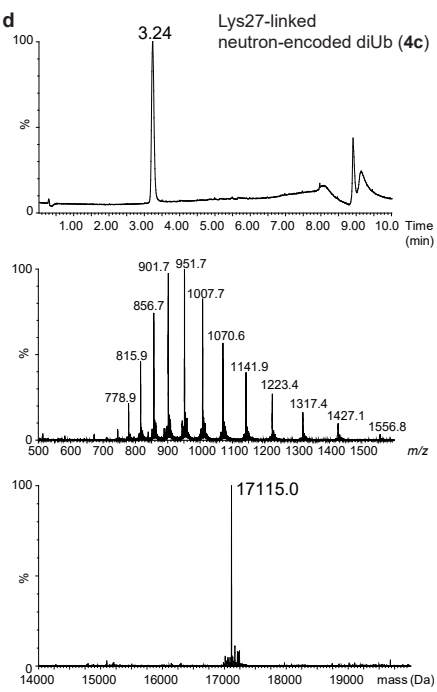


Figure 2| Synthesis of all eight neutron-encoded diUbs. a, Synthesis scheme of neutron-encoded isopeptide-linked and linear diUbs using Solid Phase Peptide Synthesis (SPPS), Native Chemical Ligation (NCL) and radical desulfurisation (deS). Reagents and conditions: Resin liberation and deprotection: 90.5% TFA, 5% H_2O, 2% TIS, 2.5% phenol, 6-40% yield; NCL: 0.1 M TCEP, 0.15 M MPAA, 6 M Gnd·HCl, 0.15 M sodium phosphate, pH 7.5, 37°C; deS: 0.25 M TCEP, 0.1 M GSH, 0.075 M VA-044, 6 M Gnd·HCl, 0.15 M sodium phosphate, pH 7.0, 37°C, 2.5-16% yield (over two steps). (Full synthetic scheme shown in **Supplementary Scheme 1**). **b,** Labelling scheme of the eight diUb isoforms. Potentially heavy-isotope labelled amino acid positions in the proximal Ub are red and marked with an asterisk. Nle = Norleucine. Positions for linkage-dependent lysines to thiolysine replacements are shown in bold. The table shows the number of introduced neutron-encoded amino acids and the introduced mass difference for each linkage. The isotope labelled amino acids used are shown with the ^{13}C and ^{15}N atoms marked with red asterisks. **c,** Coomassie-stained SDS-PAGE analysis of all eight neutron-encoded diUbs and used internal standards. **d,** Representative example of a total ion chromatogram (top), mass- (middle) and deconvoluted mass spectra (bottom) of Lys27-linked neutron encoded diUb (**4c**).

was analysed qualitatively by SDS-PAGE. This resulted in the observation of a cleavage pattern in line with reported data[25] (**Fig. 3b** and **Supplementary Figure 3**). The diUb integrity of our synthetic constructs was confirmed in an assay where we compared the cleavage efficiency of neutron-encoded Lys48-, Lys63- and Met1-linked diUbs with their corresponding enzymatically prepared diUbs side-by-side using OTUB1 (reported Lys48 specific)[24], OTUD1 (reported Lys63 specific)[24] and USP21 (reported unspecific)[25]. DUB mediated hydrolysis was analysed by SDS-PAGE (**Supplementary Figure 4** and **5**), which showed that the enzymatically prepared material and the synthetic material were processed comparably. Next, we investigated whether diUb cleavage and concomitant monoUb formation of different linkages could indeed be visualized by MS and whether (partly) processed and non-processed linkages could be distinguished from each other (**Fig. 3c**). To this end, OTUB2, a DUB known to selectively cleave Lys11, Lys48, and Lys63 linkages,[24] was incubated with an equimolar mixture of the eight diUb molecules, and the reaction was followed over time by MS. As shown in **Fig. 3c**, we observed the disappearance of the expected diUb signals (Lys11, Lys48 and Lys63) over time and the appearance of the corresponding monoUb signals as well as the non-isotope coded distal monoUb (in grey). Interestingly, in contrast to reported findings, we also observed processing of other linkages after prolonged incubation (such as Lys6), which can be attributed to the unique features of our assay as we will address below in our quantitative analyses. The capability to measure all eight diUb molecules simultaneously by MS and the OTUB2 cleavage results, illustrates that DUB selectivity can be measured in a mixture.

Assay set-up and data analysis.

The suitable diUb concentration range for the assay was determined by generating a standard curve of an equimolar mixture of all eight diUb molecules (0 - 2.0 μM). Absolute signals of three independent measurements were normalized to the internal standard non-hydrolysable triazole-linked Lys48 diUb35 (non-hydrolysable clicked Lys48-linked diUb or K48 click, prepared using Copper(I)-catalyzed azide-alkyne cyclodaddition (CuAAC)-chemistry) to calculate the measured concentration of diUb, which was plotted against the theoretical present diUb concentration. A linear response in concentration (and signal height) was well detected by the mass spectrometer between 0.5 μM and 2.0 μM of the diUbs and signals from diUb concentrations below 0.5 μM were less accurate compared to theoretically present diUb (**Supplementary Figure 6**). Therefore, a starting concentration of 1.6 μM of each diUb linkage was chosen to ensure a reliable read-out throughout the entire assay time as the concentration of at least one of the analyte types, either monoUb or diUb, will always be above the linear detection threshold.

In the multiplexed assay, an equimolar mixture of all eight neutron-encoded diUbs (1.6 μM of each linkage) was incubated with recombinantly expressed and purified DUB (0.004 μM – 4.0 μM) at 37 °C. At different time points, small reaction samples were added to and

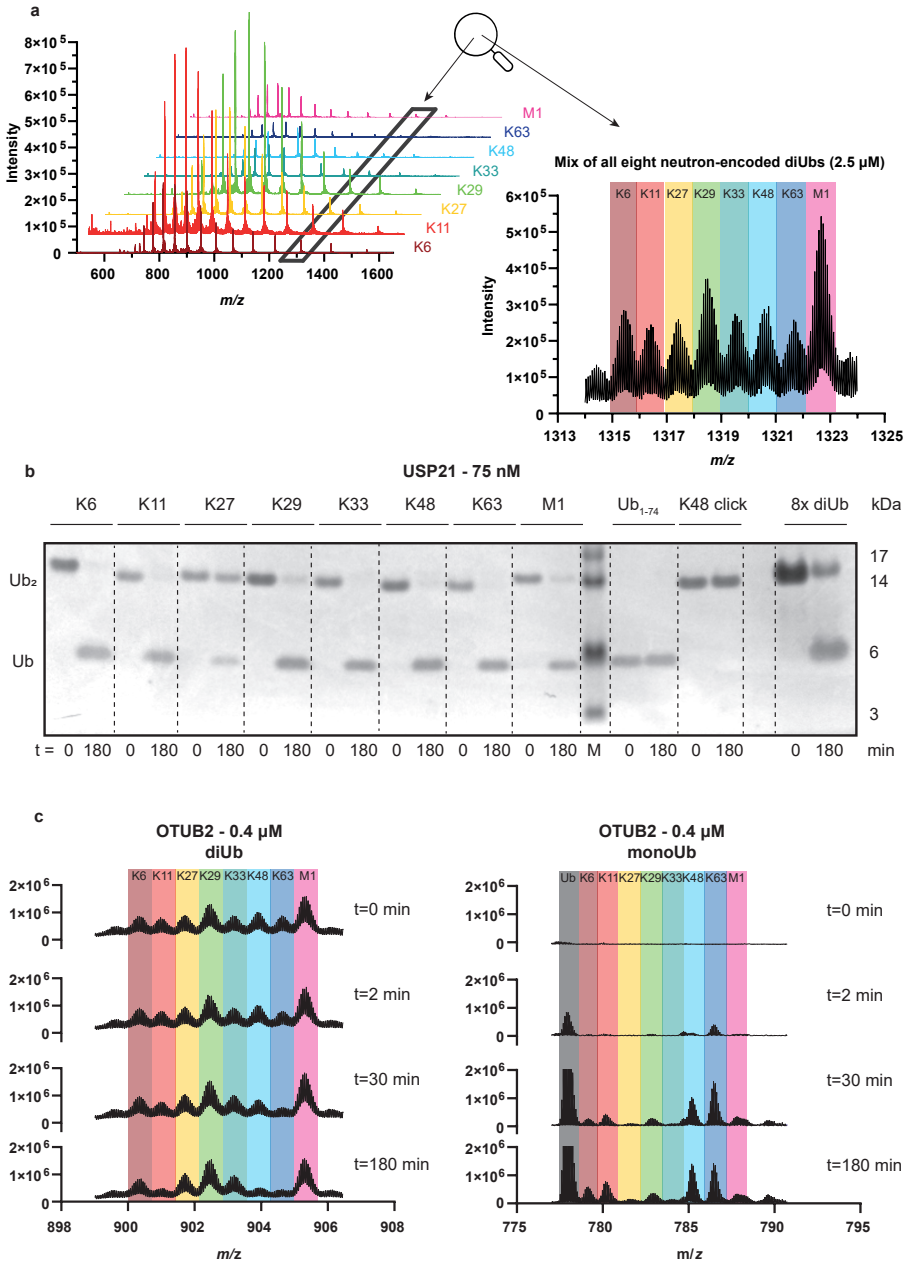


Figure 3| Proof of Principle Assay. a, Overlayed mass spectra of all eight neutron-encoded diUbs (**4a-g** and **5**) measured separately (left panel) and measured in a single analytical LC-MS run in equimolar amounts, magnified at charge state z= 13+ (right panel). **b,** SDS-PAGE analysis of USP21 mediated proteolysis of all eight neutron-encoded diUb. All eight neutron-encoded diUbs (2.5 µM each), internal standards and a mixture of all eight neutron-encoded diUbs (8x 1.6 µM) were incubated with USP21 (75 nM) at 37°C. **c,** OTUB2 (0.4 µM) mediated cleavage of all eight diUbs (1.6 µM each), analysed by MS at indicated time points. Charge states z= 19+ and z= 11+ are shown for diUb and monoUb, respectively. MonoUb signal in grey represents the sum of all released distal monoUbs.

therewith quenched by an acidic solution containing the internal standards and analysed by MS (**Fig. 4a**). Ub_{1-74} and non-hydrolysable clicked Lys48 diUb[35] were chosen as internal standards to control for intrinsic HPLC-MS assay variances, such as injection volume and ionization variability, and to allow normalization and quantification. These controls were selected because their masses lie within the range of, but do not overlap with, the assay products (monoUbs) and substrates (diUbs) respectively.

A big advantage of our assay is the possibility to perform the MS measurements using an intact mass measurement approach, which makes the preparation of the MS samples easy with minimal loss of material. Since each diUb isoform has a distinct ionization pattern, thereby impeding single-charge-state quantification[42–45], we performed quantification over the whole charge state range of the proteins ($z=10^+$ to $z=25^+$ for diUb and $z=5^+$ to $z=13^+$ for monoUb) using a tailor-made version of the open-source software package LaCyTools.[46,47]

To confirm the validity of quantification with LaCyTools, different datasets were explored using the LaCyTools software and compared to a manual analysis (see **Supplementary Figure 7** for details). This revealed that differences between automated and manual analyses were small and the ratio between all analytes within each dataset was comparable.

From the recorded data, diUb disappearance, as well as monoUb appearance, can be quantified, and these values should correspond with each other (**Fig. 4c**). The percentage of consumed diUb substrate and the concentration of formed monoUb were calculated and plotted over time for all measured DUBs at different concentrations (**Supplementary Figure 8a-c**). DUB assay results are summarised in heat maps (**Fig. 5a,b,** and **Fig. 6a**) where the amount of consumed diUb substrates after 180 min is shown for different DUBs and at multiple enzyme concentrations. For data interpretation, both diUb consumption and monoUb appearance were taken into account.

Determining DUB specificity during linkage competition

We applied our MS DUB assay (**Fig. 4a**) to determine the linkage specificities of 22 recombinant human DUBs, divided over three groups (**Fig. 4b**) under linkage competition conditions. The enzymatic reactions were performed using four different DUB concentrations. We started with seven well-known and well-studied DUBs from the panel used for Ubiquitin Chain Restriction analysis (UbiCRest), OTUB1, OTUB2, OTUD2, OTUD3, OTULIN, Cezanne and USP21, to check whether the data produced in our assay corresponds to literature.[48] These seven DUBs are reported to be specific towards one linkage or a subset of linkages. Using our assay we were able to confirm their known specificities (**Fig. 5a** and **Supplementary Figure 8a**). OTUB1 showed a preference for Lys48-linked diUb after 180 minutes of processing at different concentrations (**Fig. 5a**), which is its reported preferred linkage.[24,48] However, the full cleavage profile (**Supplementary Figure 8a**) also showed processing of Lys63-linked chains at 0.4 and 4.0 μM and monoUb products from Met1-linked chains were

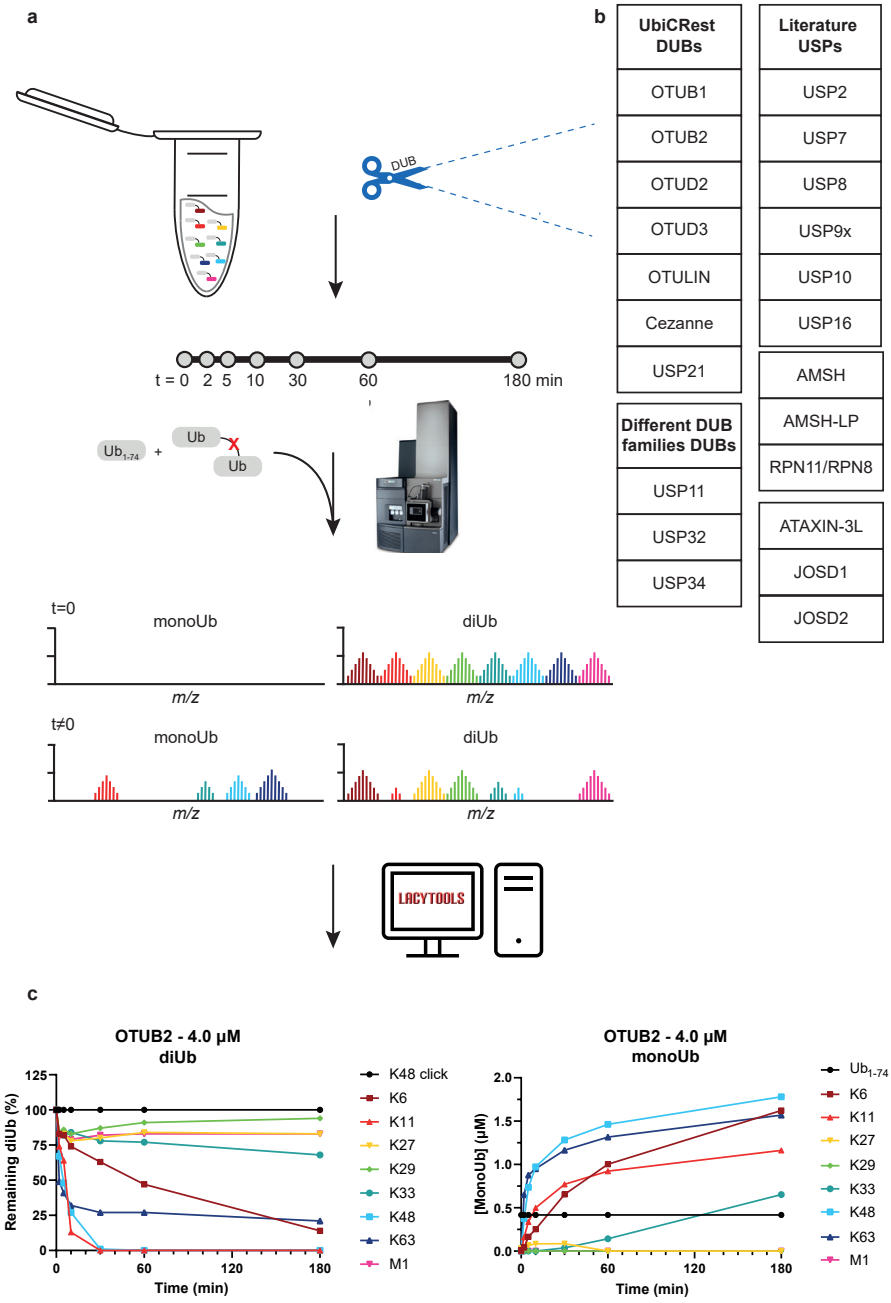


Figure 4| Workflow of the neutron-encoded diUb cleavage assay. a, Schematic representation of the DUB assay and MS analysis. A DUB is incubated with a mixture containing all eight diUb molecules. At the indicated time points, a sample of the reaction mixture was taken and quenched by diluting in an acidic solution containing the internal standards. Subsequently, the samples were analysed by LC-MS and the data was processed using LaCyTools software. **b,** The 22 selected DUBs that were analyzed with the assay. **c,** Quantification of OTUB2 mediated diUb cleavage in a mixture containing all eight diUbs (1.6 µM each), represented by diUb disappearance (left) and monoUb appearance (right).

observed at high concentration (4.0 μM). OTUB2 at low concentration (0.04 μM) cleaved the preferred Lys63 linkage but broadened its preference to include Lys11 and Lys48 at moderate enzyme concentration (0.4 μM).[24] Notably, at 4.0 μM the specificity further broadened to include cleavage of Lys6 diUb, a so-far unreported observation.[24,26,27] OTUD2 only showed activity at 4.0 μM with a main preference for Lys11 diUb (**Fig. 5a**). OTUD3 specifically cleaved Lys6 and Lys11 diUb at 0.4 μM, confirming literature observations[24,48,49] but interestingly also showed nearly full conversion of Lys48 and Lys63 diUb at 4.0 μM. For OTULIN we observed an exclusive consumption of the Met1-linked diUb even at the highest enzyme concentration, corroborating that this is an M1 specific protease.[48,50] Cezanne specifically cleaved Lys11-linked diUbs at lower concentrations, but loses this specificity at higher enzyme concentrations,[24] as it processed Lys6-, Lys48- and Lys63-linked diUbs at 0.4 μM and 4.0 μM as well. USP21 is reported to cleave all diUb linkages[48,51], which is indeed confirmed by our data. Of note, generally Lys27-linked diUb is often not (fully) processed. Our data confirms that this is not due to incorrect folding of Lys27-linked diUb, since proper processing of this linkage was observed at a high concentration of USP21 (4.0 μM).

Furthermore, we investigated six well-studied DUBs from the USP family, which are known for their chain-type promiscuity (USP2, USP7, USP8, USP9x, USP16, and USP10) (**Fig. 5b** and **Supplementary Figure 8b**). We found that USP2 mainly cleaved Lys11 diUb at low enzyme concentration (0.04 μM) (**Fig. 5b**) and consumption of this linkage was also faster than other linkages at 0.4 μM of USP2 (**Fig. 5c**). USP7 showed a preference for Lys6, Lys11, Lys33, Lys48, and Lys63 diUb although processing of Lys33 diUb was slower compared to the other linkages (**Supplementary Figure 8b**). At 0.4 μM USP8 showed a similar specificity pattern as USP7 at 0.04 μM. USP9x cleaved Lys6, Lys11, Lys48, and Lys63 diUb at 0.4 μM, but at a higher concentration cleavage of Lys29, Lys33, and Met1 diUb was also observed (**Fig. 5b**). Strikingly, USP9x only started the consumption of Lys29 and Lys33 chains after Lys6, Lys11, Lys48 and Lys63 were almost fully processed (**Fig. 5c** in combination with **Supplementary Figure 8b**). USP16 showed a similar pattern as USP21, with apparent linkage-type promiscuity. USP10 showed a preference for Lys11 and Lys48 diUb and to a lesser extent for Lys6 and Lys63 diUb at lower DUB concentrations (0.4 μM). At 2.0 μM Lys33 diUb was also fully converted but processing of this chain appeared to start only once Lys11 and Lys48 diUb processing had come close to completion (**Fig. 5c** in combination with **Supplementary Figure 8**).

Next, we investigated the specificity of nine DUBs from different DUB families (**Fig. 6a**) at a single DUB concentration of 4.0 μM. Some of these DUBs were taken along in reported specificity screens and usually, results were analysed qualitatively at only one timepoint using SDS-PAGE analysis. So, we set out to complement their selectivity profiles in a quantitative manner using our assay. USP11 processed Lys6, Lys11, Lys33, Lys48, and Lys63 diUb (almost) completely after 180 min, while Lys29 diUb was only partially processed (**Fig. 6a,b,** and

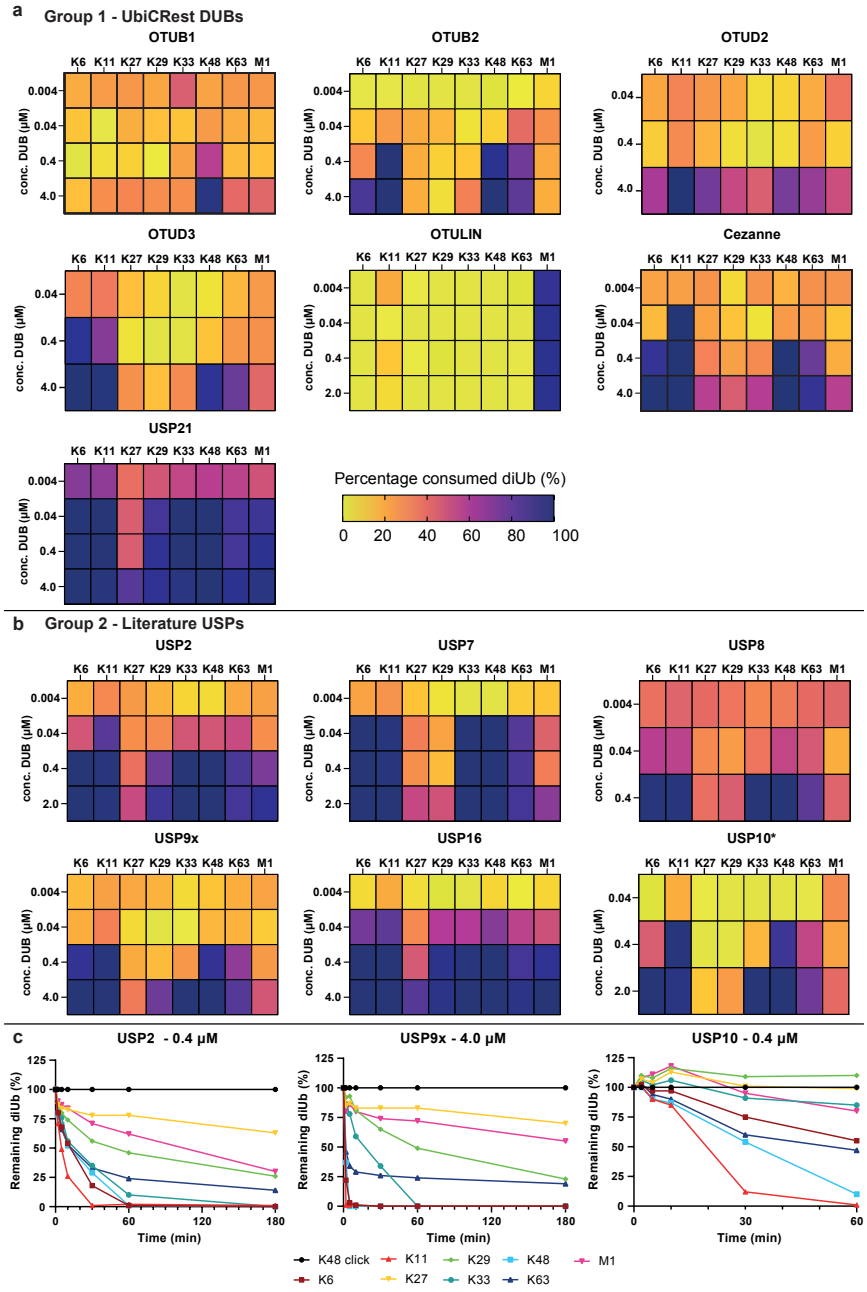


Figure 5| Linkage specificity profiles of well-studied OTU and USP DUBs. Heatmaps showing the percentage of consumed diUb isoforms per DUB of **a,** UbiCRest panel or **b,** USP family at different concentrations after 180 min. Asterisk (*) indicates that the heatmap of USP10 was constructed with diUb concentration after 60 min **c,** Full quantification profiles of diUb disappearance over time for USP2 (0.4 μM), USP9x (4.0 μM) and USP10 (0.4 μM). An equimolar mixture (8 x 1.6 μM), containing all neutron-encoded diUb isoforms, was incubated with the DUB at 37 °C, samples were taken at the indicated time points and analysed by LC-MS. For full DUB characterization profiles see **Supplementary Figures 8a** and **b.**

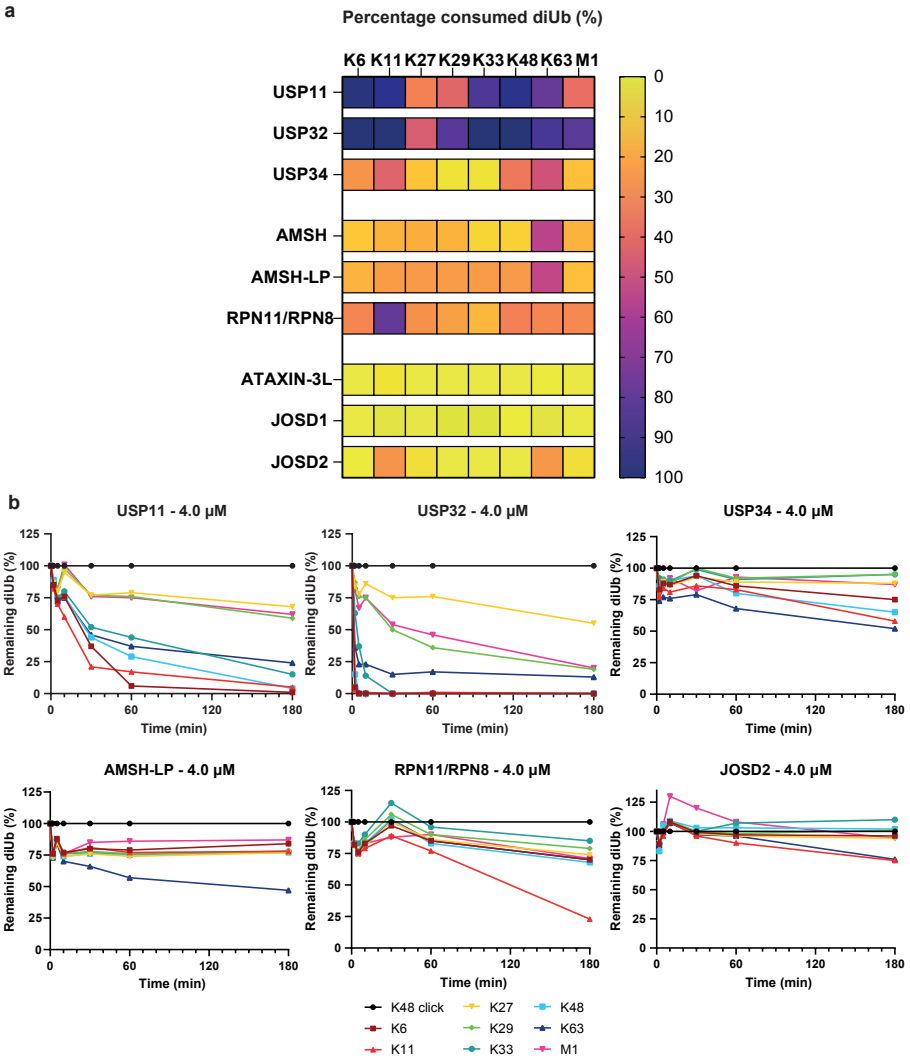


Figure 6 | Linkage specificity profiles of DUBs from different DUB families. a, Heatmap showing the percentage of consumed diUb isoforms by 9 different DUBs (4.0 µM) from different DUB families after 180 min incubation. **b,** Full quantification profiles of diUb disappearance over time for USP11, USP32, USP34, AMSH-LP, RPN11/RPN8 and JOSD2. An equimolar mixture (8 x 1.6 µM), containing all neutron-encoded diUb isoforms, was incubated with the DUB at 37 °C, samples were taken at the indicated time points and analysed by LC-MS. For full DUB characterization profiles see **Supplementary Figure 8c.**

Supplementary Figure 8c). Similar results were obtained for USP32, although Lys29 was processed to a higher extent after prolonged incubation times, and also Lys27 and Met1 processing was observed. USP34 showed low activity with a slight preference for Lys63 chains.

The metallo-DUBs AMSH and AMSH-LP were moderately active at 4.0 µM and specifically processed Lys63 diUb as expected.[26,52,53] The yeast RPN11/RPN8 complex, which is comparable

to the human PSMD14/PSMD7 complex, has previously been reported to hydrolyse all isopeptide-linked diubiquitins[54]. In our assay we found a main preference for Lys11 diUb with almost full proteolysis after 3 h, although other linkages are also processed to lesser extent.

The three enzymes from the MJD family (ATAXIN-3L, JOSD1, and JOSD2) displayed low activity, even at 4.0 μM, which made it difficult to properly profile these DUBs with our assay. No clear substrate conversion could be observed in the diUb channel, except for JOSD2, the most active DUB from the family, that processed some Lys11 and Lys63 chains (**Fig. 6a,b,** and **Supplementary Figure 8c**), which is in line with earlier findings.[55,56]

Discussion

Given the critical roles of DUBs in a lot of cellular processes, there is a growing interest to exploit them as targets for drug development[57]. Further establishing insights into their functioning at a specific location in the cell or their activity under certain circumstances are important additional steps to couple DUB activity with therapeutic intervention. Profiling their linkage selectivity and likewise, their preference when a variety of substrates, for example, different polyUb linkages, are at their disposal is important in such efforts. Our fast, adaptable and quantifiable assay to monitor a DUB's proteolytic preference, activity and selectivity *in vitro,* when all eight natural diUb linkages compete with each other, adds to the growing toolbox of assays and dedicated reagents to decipher part of the Ub network. The key conceptual advantage of our MS assay lies in its ability to analyse DUB specificity in the presence of all diUb linkage types in a single assay. This was achieved by synthesizing a set of all eight native diUbs, in which each linkage type has its own fully ^{13}C and ^{15}N labelled amino acid fingerprint, resulting in eight diUb isoforms with a distinguishable molecular weight. Altering the substrate or enzyme concentration, or potentially the influence of an allosteric binder (e.g. another Ub linkage) can be easily measured and quantified on a mass spectrometer in a matter of minutes, compared to longer periods when several SDS-PAGE assays need to be performed side by side. [24,25]

Isotope coding is a commonly used technique in MS-based analysis methods, such as tandem mass tag (TMT) labelling[58], stable isotope labelling by amino acids in cell culture (SILAC)[59] or the absolute quantification strategy (AQUA)[60], to identify or comparatively quantify specific proteins in a complex biological sample. However, none of these methods can (easily) be applied in a straightforward and fast DUB assay, without having to compromise on the structural integrity of the Ub chains. Since by using conventional techniques the differential labelling of all eight diUb linkages can only be achieved by the instalment of artificial MS tags in the substrates. These small but intrinsic differences between the resulting diUb linkage types might impact DUB recognition and/or processing parameters. Furthermore, most isotope

coding techniques require substantial sample preparation, e.g. tryptic digestion or peptide enrichment, which is time-consuming and can result in sample loss. Many of these methods require tandem MS analysis, which can be problematic with analytes that have an identical retention time. Our method on the other hand, in which we take full advantage of chemical protein synthesis in combination with isotopically labelled amino acids, bypasses these issues. The sophisticated stable-isotope code we installed, allows for proper baseline separation in the mass spectra and hence discrimination between each of the eight diUb analytes and nine monoUb analytes, without complex sample processing or modifying any of the biophysical properties of the diUbs apart from their molecular weight. The only alteration we made was the replacement of Met1 by its bioisostere norleucine (Nle) to avoid the notorious oxidation of the Met1 thioether moiety, leading to different molecular weights (M, M+16Da and M+32Da) for a single type of Ub protein, which is detrimental to MS analysis and quantification. This generally accepted Met1Nle substitution typically does not affect recognition by DUBs.[40,61]

In this study, we demonstrated that, by using an intermediate resolution mass spectrometer, we can already achieve sufficient resolution and sensitivity which makes it an attractive workflow that could be incorporated in other (bio)chemical laboratories. Moreover, we expect that the sensitivity of our assay will even be further improved with the continuous development of new technologies in the field of mass spectrometry, that will drive the limit of detection and improvement of resolution to an even higher level in the future. Eventually, this would lead to lower amounts of required substrates to be detected, thereby making it easier to switch from equimolar ratios between diUb linkages to ratios that are found in cells, which will bring the assay one step closer to *in vivo* circumstances. The possibility to detect lower concentrations could even allow for Michaelis-Menten kinetic measurements in a linkage competitive setting.

Data analysis is an important part of our assay and although it is possible to perform manually, it is a laborious process that is prone to errors. By adjusting LaCyTools, an open-source software package that was initially developed in-house to analyse glycopeptides[46,47], we managed to streamline our data analysis. LaCyTools automatically aligns, calibrates, and integrates LC-MS data with the appropriate quality control (e.g. ppm error, S/N and isotopic pattern comparison), and in addition allows to fully define the atomic composition of analytes, including our neutron-encoded (di)Ubs. To further simplify data analysis we implemented two essential features in our assay. First, the proteins were eluted using a shallow HPLC gradient to separate monoUb and diUb at baseline level, which prevents an undesired overlap of the monoUb and diUb MS signals. Second, we make use of two internal standards, $Ub_{1\text{-}74}$ and non-hydrolysable Lys48 diUb, which have a molecular weight in the same mass range as the neutron-encoded monoUbs and diUbs, respectively, to account for ionisation efficiency differences due to protein size. This allowed us to properly quantify both the diUb consumption as well as the monoUb formation, which led to some important observations detailed below.

In some cases, the observed diUb consumption does not match the monoUb formation. Illustrative of this is the data for USP2 (0.4 μM) (**Supplementary Figure 8b**), which has a clear preference for K11 diUb, followed by K6, K33, K48 and K63 diUb in the diUb channel, whereas the monoUb channel shows the highest formation of K29-derived monoUb. A concomitant observation is that upon full conversion in the diUb channel, the end-point signal in the monoUb differs for each linkage, sometimes reaching values above the initial substrate concentration of 1.6 μM, which is for example clearly shown for USP16 at 4.0 μM (**Supplementary Figure 8b**). A possible explanation lies with the equilibration of the diUb mixture, which was based on quantification of the protein bands after SDS-PAGE analysis and Coomassie staining (like **Fig. 2c**) as an orthogonal method with respect to MS. Although this is a generally accepted method, the actual diUb concentrations can slightly differ, which will eventually affect the calculated concentration of formed monoUb. Another important point is the data processing by LaCyTools, where we applied very stringent quality control and background subtraction to our MS data. This can affect the quantification and is most pronounced for lower MS signals, which is well in line with our determination of the optimal substrate concentration to be above 0.8 μM. Overall, the most accurate linkage specificity data is obtained by considering both the diUb and monoUb channels, which can easily be done from a single measurement in our assay.

Overall, the results obtained with our neutron encoded diUb substrates were in good agreement with literature[24,25,54–56,26,27,48–53] but also provided some interesting new insights into DUB selectivity. OTUB1 has previously been annotated as Lys48 specific[24], but we also observed cleavage of Lys63- and Met1-linked chains (**Supplementary Figure 8a**). Although the processing of Lys63-linked linkages has previously been observed,[62] the processing of Met1-linked chains was only recently described for OsOTUB1, a homologue of human OTUB1.[63] We found OTUB2 to also process Lys6 diUb but only at elevated enzyme concentration and upon almost full consumption of its known target linkages Lys63, Lys48, and Lys11 (**Fig. 5a** and **Supplementary Figure 8a**). The OTU domain of OTUD3 beautifully illustrates a dose-dependent specificity change (**Fig. 5a** and **Supplementary Figure 8a**), where at low enzyme concentration it preferentially cleaved Lys6 followed by Lys11 chains but at elevated enzyme concentration, its specificity is broadened to include Lys48 and Lys63 diUb after (almost full) consumption of Lys6 and Lys11 linkages. The observation that the linkage specificity changes with DUB concentration has been well documented for the OTU DUB family[24], but this has so far not been observed for USP DUBs. We here demonstrated for the first time a concentration-dependent linkage specificity for some members of the USP family. For example, USP2 which initially processes Lys11 diUb at 0.04 μM, additionally processes Lys6, Lys29, Lys33, Lys48, Lys63, and Met1 diUb at 0.4 μM and cleaves all diUbs except the Lys27 linkage equally fast at 4.0 μM (**Fig. 5b** and **Supplementary Figure 8b**). USP7 also showed a pronounced linkage selectivity pattern at intermediate enzyme concentrations

which became less prominent at high enzyme concentrations (**Fig. 5b** and **Supplementary Figure 8b**). Furthermore, USP9x and USP10 cleavage patterns pointed towards a preference in cleavage order for the different processed diUbs; they only start processing certain linkage types after other linkage types are almost fully consumed by the enzyme first (**Supplementary Figure 8b**). These results showcase the potential of our assay, where these USPs were reported to lack a clear linkage specificity, our data showed that under linkage competition conditions their assumed chain type promiscuity is aberrant. Notably, since the reaction progress is being monitored at several timepoints (**Fig. 4a**), it is possible to deduce information on the cleavage rates of each diUb linkage type relative to each other. This provided some interesting observations that differ from literature findings. USP9x was earlier shown to faster process Lys63- compared to Lys11-linked diUb[64], whereas our data showed the exact opposite effect (**Supplementary Figure 8b**). Kinetic analysis of USP7 previously revealed a 1.5 times higher catalytic efficiency towards Lys33- over Lys11- and Lys63-linked diUb[25], which contrasts our finding that Lys33 is processed more slowly compared to Lys63 and Lys11 (**Supplementary figure 8b**). Also, our data revealed an similar processing rate of Lys48- and Lys63-linked diUb by Cezanne (**Supplementary figure 8a**), where a fourfold higher catalytic effiency towards Lys63- over Lys48-linked diUb was reported previously.[65] These observed differences in hydrolysis rates between those reported for individual chains and the ones we found in the mixture containing all linkages may indicate that the presence of certain diUb linkages can lead to a change in hydrolysis rates of other linkages.

Another interesting finding is the high preference of the metallo-DUB RPN11/RPN8 complex for Lys11 diUb. The RPN11/RPN8 complex is reported to process all linkages, however the catalytic efficiency for the processing of Lys11-linked diUb is two times higher as for Lys48-linked diUb and four times higher as for Lys63-linked diUb.[54] In accordance with the reported catalytic efficiencies, we observed efficient processing of Lys11-linked diUb after 180 minutes, while only half of the amount of Lys48-related monoUb was formed and even less Lys63-related monoUb (**Fig. 6** and **Supplementary figure 8c**).

We identified several cleavage patterns of DUBs that were not consistent with those obtained from conventional cleavage assays. Since our assay possesses the element of linkage competition, which is also present in cells, the selectivity pattern of DUBs can potentially be influenced by the presence of one or more diUb linkages. Our data will therefore provide further insights into the physiological role of these DUBs.

We believe that our method provides a major progress in the field of characterizing Ub linkage specificties of DUBs but at the same time the method in its current state is only limited to diUb linkages. Although the use of diUb tools has been widely considered a valid approach, it does not address the full complexity of Ub biology. In cells, more complex poly-Ub chains, including higher order homotypic and heterotypic chains, branched chains and

hybrid chains of Ub with Ub-like proteins exist, and these chains are often conjugated to substrate proteins. DUB activity can be affected by the existence of such chains as is for example well illustrated by the finding that the DUB MINDY-1 only processes tetraUb or longer Lys48-linked chains.[67] The ability to include more complex Ub chains beyond diUb would therefore be a valuable extension of our method. The nature of our assay, the widely available chemical preparations of Ub conjugates, as well as our MS-based read-out, should in principle allow for its application in the analysis of more complex Ub chains. The main determinant is the possibility to chemically introduce sufficient mass differences to discriminate between each individual Ub species. Current synthetic procedures already allow for the preparation of (branched) triUb, tetra-, penta-, hexaUb and ubiquitinated peptides in a controlled way starting from SPPS.[36,68–70] Our MS-based assay set-up can easily be multiplexed further beyond the currently used 8-plex format. For example, with Ub trimers a total mass difference of 600 Da can be introduced before the charge states begin to overlap. With the 12 Da mass difference for every consecutive Ub, an up to 50-plex MS assay would theoretically be possible. The appropriate mass differences can be achieved by the introduction of more and different neutron-encoded amino acids via SPPS and this is possible on nearly any location in the Ub sequence since all fully ^{13}C,^{15}N-labelled amino acids are commercially available. Ongoing developments in Ub synthesis may enable the preparation of even more complex Ub chains in the future, but with the currently available methods we anticipate that our assay, can be extended to all tri- and tetraUb isoforms, which will already mean a substantial step forward towards complex Ub chains present in cells.

In summary, we present a novel MS-based assay to profile the Ub linkage specificities of deubiquitinating enzymes (DUBs) when all eight existing ubiquitin linkage types are present in a single mixture. This was achieved by synthesizing a neutron-encoded set of eight native diUb isoforms, each having a distinguishable molecular weight. Using our assay we profiled the linkage specificity of 22 DUBs, which led to novel insights into their mode of action that could not have been acquired with conventional methods. Our data revealed that direct competition between different diUb linkages can alter a DUBs Ub chain specificity pattern and we showed a first indication that different diUb linkages are sometimes processed in a specific order. The assay has several advantages over existing methods: it makes use of the isotopologues of native diUb substrates, is quantitative, requires low amounts of material, the read-out can be done in a mid-throughput fashion, and competition between linkages is taken into account. The straightforward nature of the experimental setup makes the assay easily adaptable and custom-tailored for a biochemical lab having access to a mass spectrometer. We hence expect our new neutron-encoded diUb assay to become invaluable in analysing DUB linkage specificity and anticipate its application in different studies under different circumstances to help shine a light on the intricacies of deubiquitination.

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Author contributions

The concept of this study was conceived by P.P.G. and H.O. Ub analogues were designed by P.P.G. Synthesis of Ub analogues was executed by B.D.M.v.T. with technical assistance from C.T.O. and P.H. under supervision of G.J.v.d.H.v.N. and P.P.G. Biochemical assays were performed by B.D.M.v.T. Data analysis was performed by B.D.M.v.T. with the assistance of G.S.M.L.K. Data analysis software was adjusted by B.C.J. DUBs were expressed by R.Q.K., A.M. and A.S. Plasmid design and DUB expression by D.K. Mass spectrometry support and gradient design were done by B.R.v.D. The manuscript was prepared by B.D.M.v.T., G.J.v.d.H.v.N. and P.P.G. with input from all authors. The project was supervised by G.J.v.d.H.v.N., M.W., H.O. and P.P.G.

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Materials and Methods

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SDS-PAGE analysis:

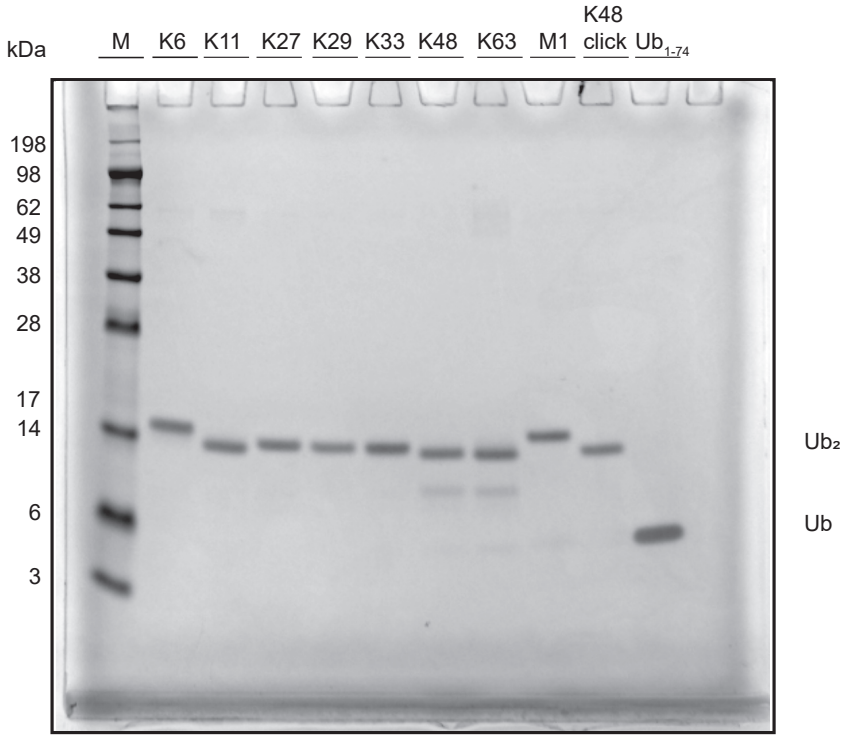
- *Characterization of all eight neutron-encoded diUb isoforms, K48 click diUb and* Ub_{1-74}
- *USP21 mediated hydrolysis of neutron-encoded diUb*
- *OTUB1, OTUD1 and USP21 mediated hydrolysis of synthetic neutron-encoded and enzymatically prepared Lys48-, Lys63- and Met1-linked diUb*

In vitro DUB assays with mass spectrometry read-out

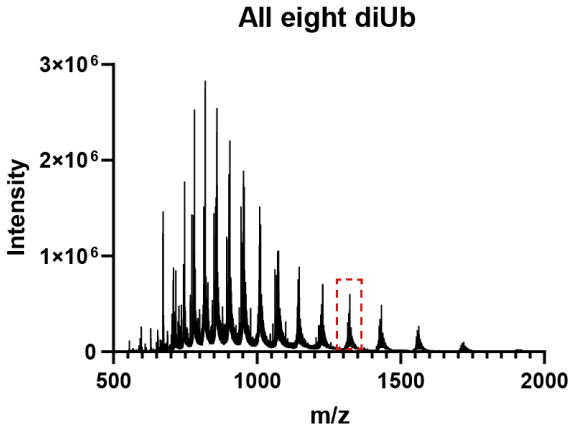
Assay window and linearity determination

References

1. Supplementary Figures, Schemes and Tables

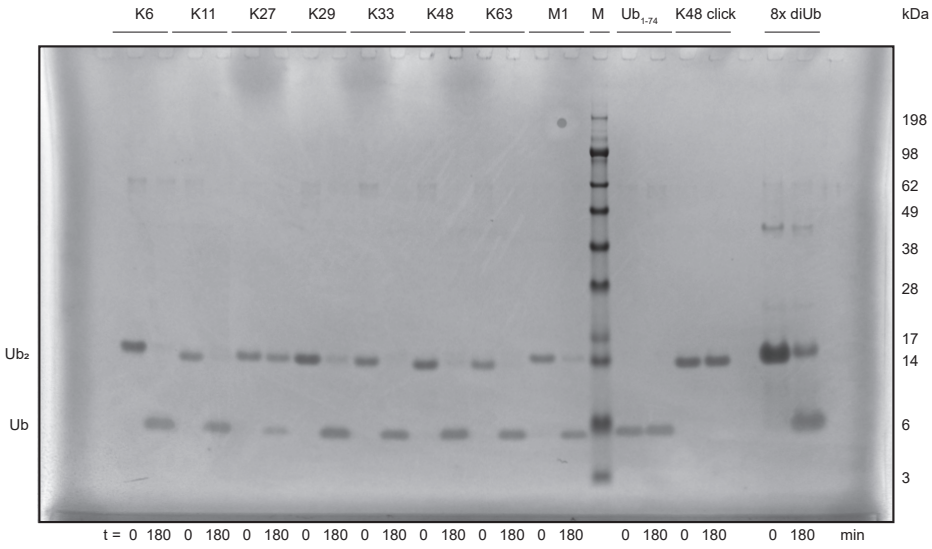


Supplementary Figure 1| Full gel image of all eight neutron-encoded diUbs (4a-g and 5), non-hydrolysable clicked Lys48 diUb (23) and Ub_{1-74} on 12% Bis-Tris gel related to Figure 2c in the main paper. Protein marker = SeeBlue™ Plus2 Pre-stained Protein Standard. Loading: ~0.5 µg/lane. *Method*; Stock solutions of all Ubs were diluted in a buffer containing 50 mM TRIS and 100 mM NaCl to a concentration of 3.5 µM. 20 µL of sample buffer was added to 40 µL of diluted stock solution. The samples were boiled for 5 min at 95°C and 15 µL was loaded on a precast 12%Bis-Tris gel (Invitrogen). The samples were resolved by gel electrophoresis with MES running buffer and the gel was stained with InstantBlue™ staining.



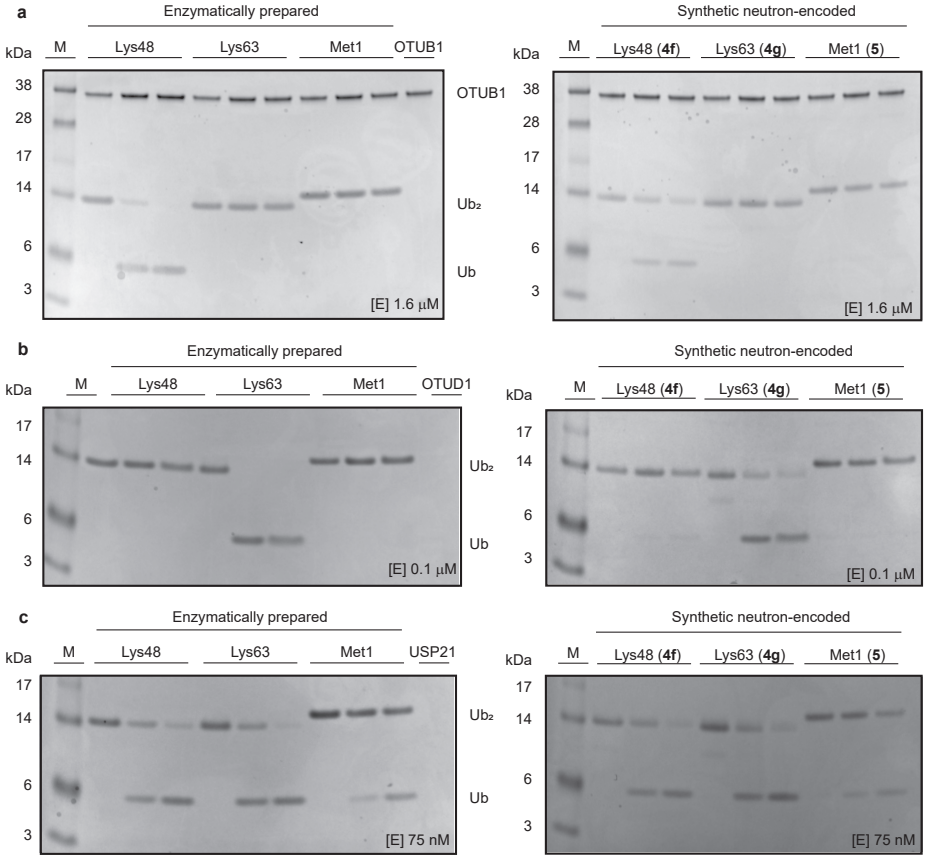
Supplementary Figure 2| Sum spectrum of the main peak of the chromatogram of the mix of all eight neutron-encoded diUbs (2.5 µM each). Full mass range of sum spectrum of the right panel of Figure 3a in the main paper.

Method; All eight neutron-encoded diUbs were mixed in equimolar amount (Final concentration = 2.5 µM for each diUb). This mixture was 16 times diluted with 0.1% FA in MQ and 8 µL of this solution was injected for a HPLC-MS run (LC-MS – System 2 – Gradient 3). Spectra 115:228 were combined for the sum spectrum.



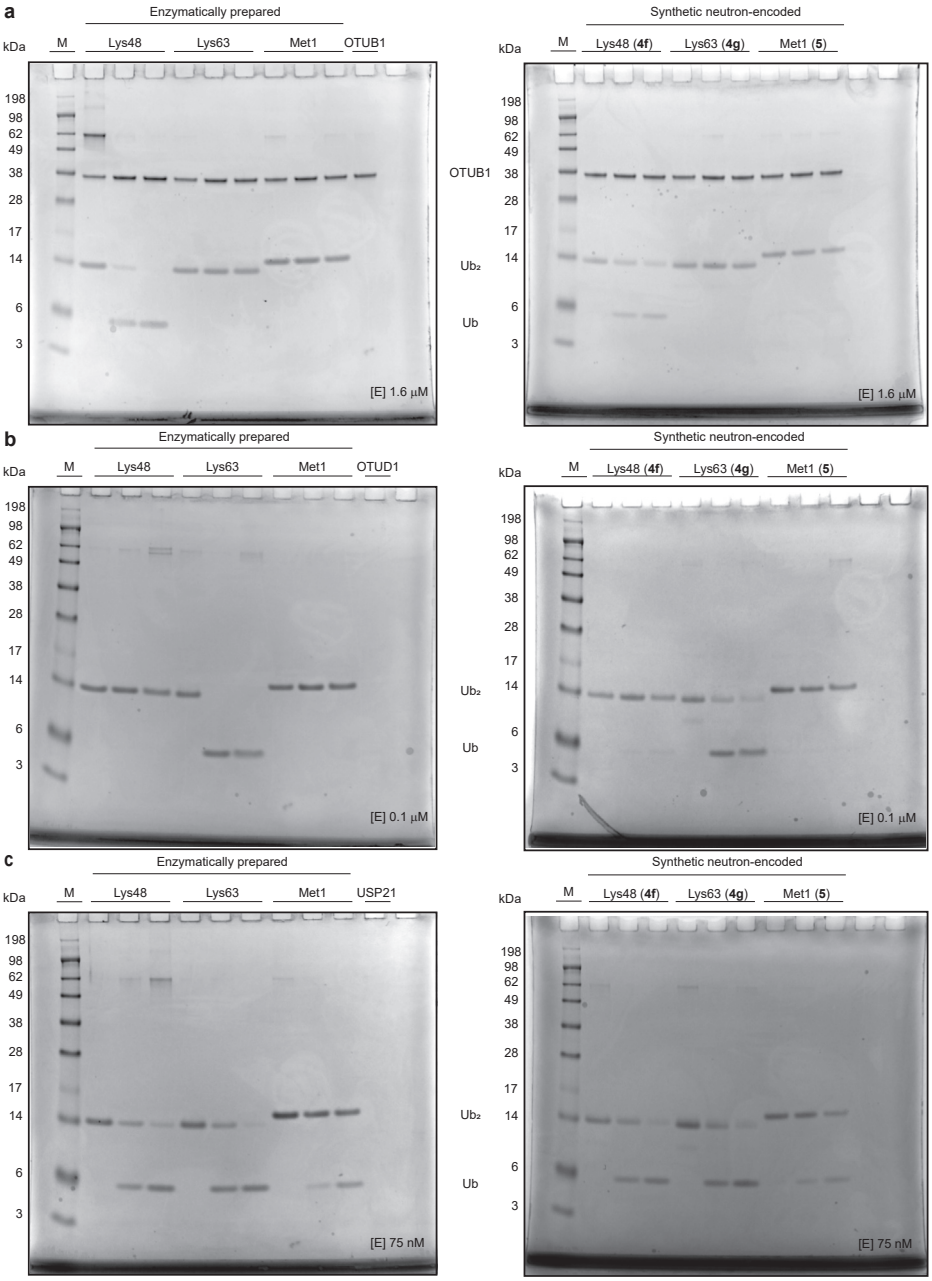
Supplementary Figure 3| USP21 mediated hydrolysis of all eight neutron-encoded diUb molecules (4a-g and 5) analysed by SDS-PAGE related to Figure 3b in the main paper.

Protein marker = SeeBlue™ Plus2 Pre-stained Protein Standard. Loading: 10 µL reaction mixture. *Method*; Quenched samples of the reaction mixtures were boiled for 5 min. at 95°C and 15 µL was loaded on gel. The samples were resolved by gel electrophoresis on a 12% Bis-Tris gel (Invitrogen) with MES running buffer and the gel was stained with InstantBlue™ staining.

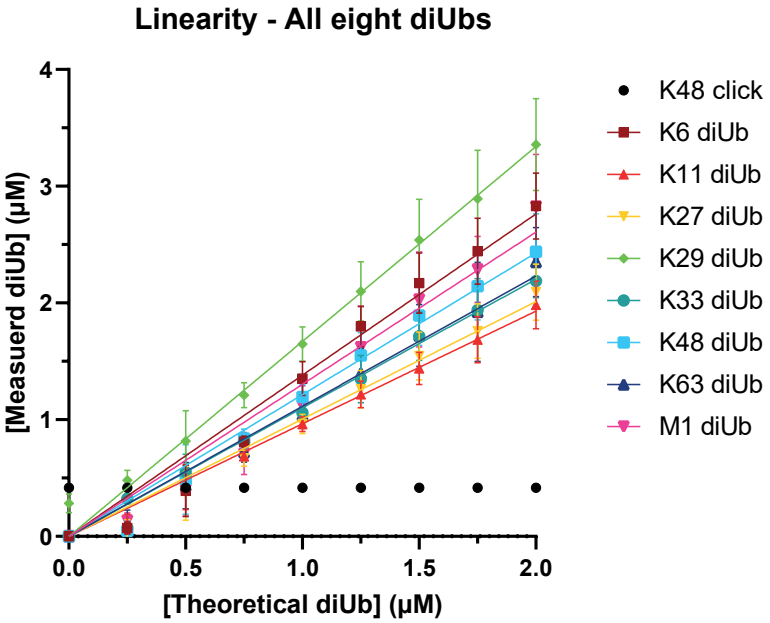


Supplementary Figure 4| DUB mediated hydrolysis of synthetic K48-, K63- and Met1-linked diUb molecules (4f, 4g and 5) and enzymatically prepared K48-, K63- and Met1-linked diUb molecules analysed by SDS-PAGE. a, OTUB1**. b,** OTUD1**. c,** USP21**.**

Protein marker = SeeBlue™ Plus2 Pre-stained Protein Standard. Loading: 5 μL reaction mixture. *Method;* Quenched samples of the reaction mixtures were boiled for 5 min. at 95°C and 7.5 μL was loaded on gel. The samples were resolved by gel electrophoresis on a 12% Bis-Tris gel (Invitrogen) with MES running buffer and the gel was stained with InstantBlue™ staining.

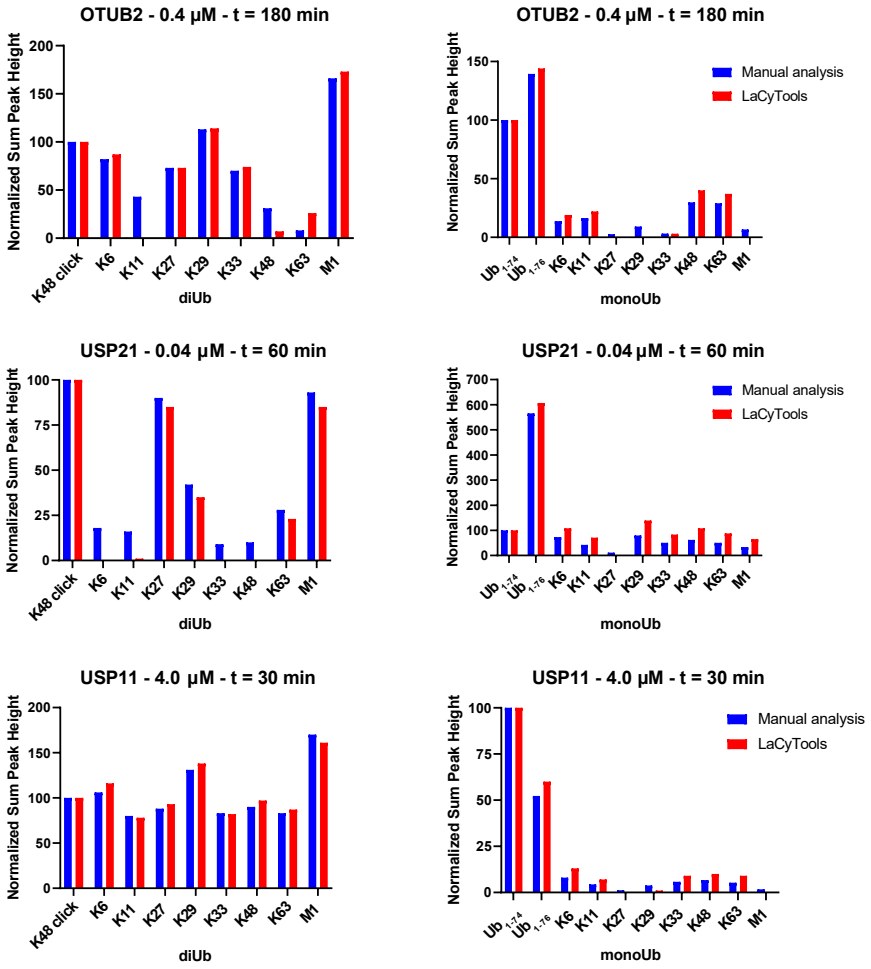


Supplementary Figure 5| Full gel images of DUB mediated hydrolysis of K48-, K63- and Met1-linked diUb molecules (4f, 4g and 5) and enzymatically prepared K48-, K63- and Met1-linked diUb molecules analysed by SDS-PAGE related to Supplementary Figure 4. a, OTUB1**. b,** OTUD1**. c,** USP21**.**
Protein marker = SeeBlue™ Plus2 Pre-stained Protein Standard. Loading: 5 µL reaction mixture. *Method*; Quenched samples of the reaction mixtures were boiled for 5 min. at 95°C and 7.5 µL was loaded on gel. The samples were resolved by gel electrophoresis on a 12% Bis-Tris gel (Invitrogen) with MES running buffer and the gel was stained with InstantBlue™ staining.



Supplementary Figure 6| Assay window and linearity determination. The linearity of the measured signal (signal height vs concentration) and the assay window were analysed by measuring standard curves of all eight neutron-encoded diUbs over the concentration range of 0.0-2.0 µM in the presence of non-hydrolysable clicked Lys48 diUb as internal standard in three separately performed experiments on three different days. Showing a reliable assay and measurement window between 0.5 and 2.0 µM. Concentrations of below 0.5 µM were divergent from the theoretically present amount of diUb, meaning these concentrations cannot be determined accurately.

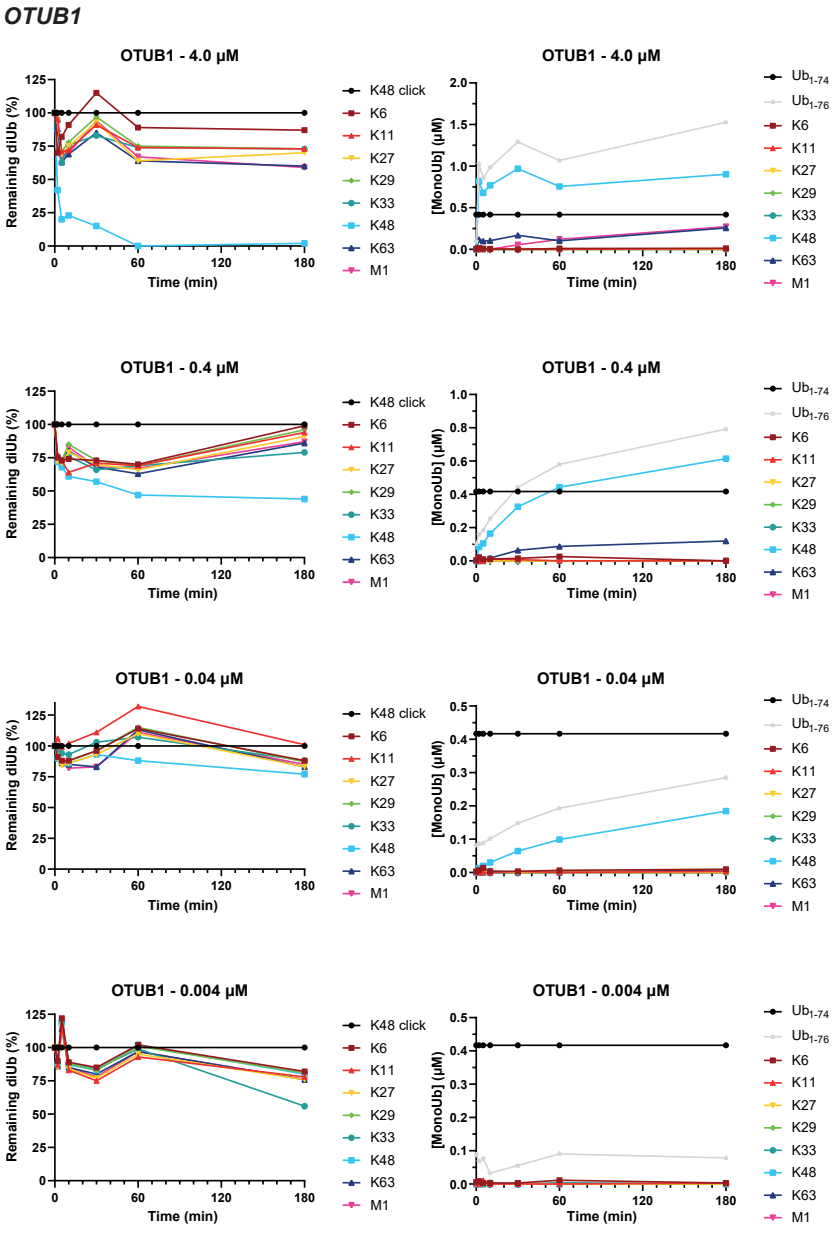
Method; All eight diUb solutions were mixed to obtain a solution containing equimolar amounts of these diUbs over a range of 0.0 – 2.0 µM diUbs (0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00 µM) in a buffer containing 50 mM TRIS and 20 mM NaCl, pH 7.55. These solution (2 µL) was diluted using a mixture containing 0.4µL 10% TFA in MQ, 1.6 µL 0.1% FA in MQ and 12 µL of the internal standard solution (1 µL of 5.0 µM internal standard diluted with 11 µL of 0.1% FA in MQ) and 8 µL of these solutions were injected for an HPLC-MS run. The obtained data was quantified using LaCyTools and the standard data analysis protocol. The area under the curve for the internal standard was linked to the concentration of non-hydrolysable K48 present in the analysed solution. The measured concentrations of diUb analytes were calculated using their areas under the curve and plotted against the theoretical amount of diUb present in the mixture



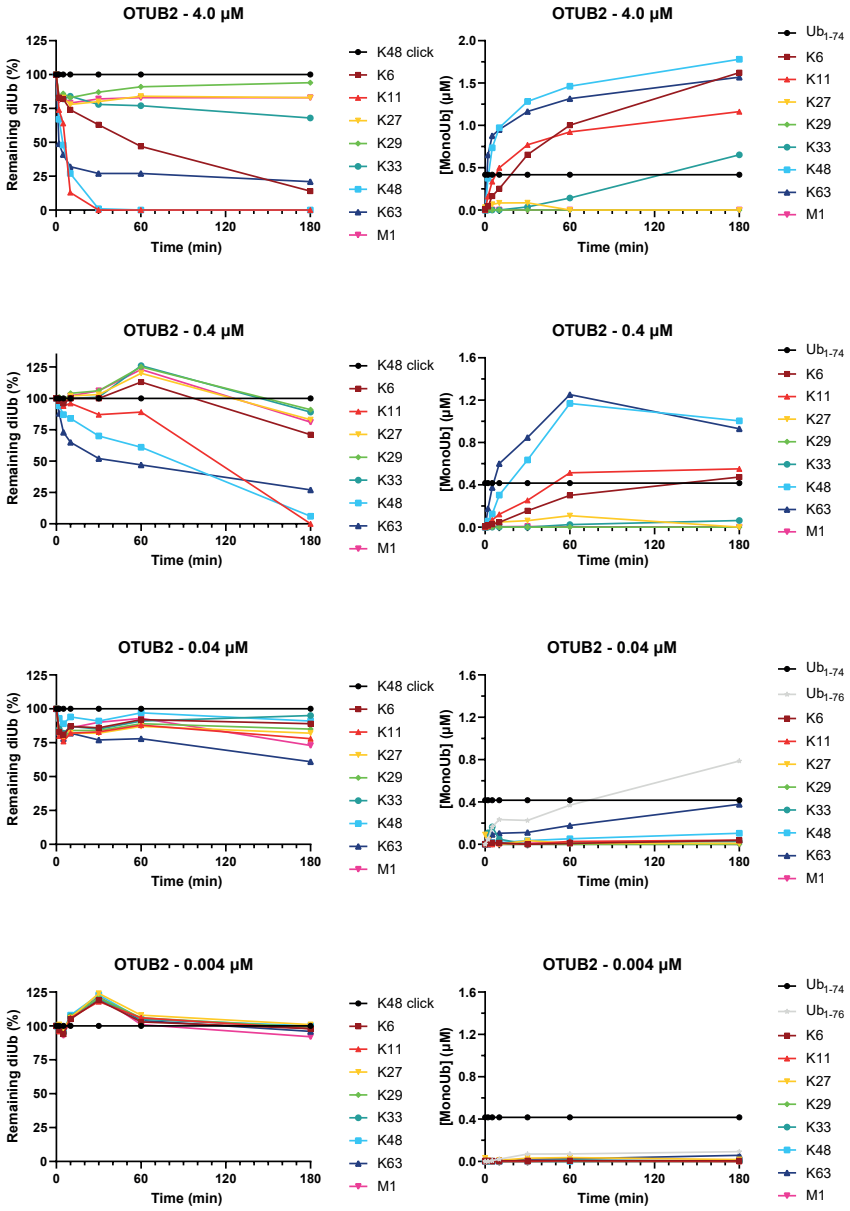
Supplementary Figure 7| Comparison between LaCyTools analysis versus manual data analysis. Three MS spectra from different DUBs cleavage assays, at different enzyme concentrations and different time-points were selected to be analysed using LaCyTools analysis and manually. LaCyTools calculates the area under the curve of the relevant charge states. The areas under the curve of all relevant charge states (diUb $z=10^+$ to $z=25^+$ and monoUb $z=5^+$ to $z=13^+$) with the correct quality parameters were summed and normalized to the sum area of the internal standard. For the manual analysis the TIC count of the highest peak from an isotope pattern distribution was selected to serve as value for the peak height. The peak height of all relevant charge states (diUb $z=10^+$ to $z=25^+$ and monoUb $z=5^+$ to $z=13^+$) were summed and normalized to the sum peak height of the internal standard. For the manual analysis no quality control or background subtraction was performed.

Supplementary Figure 8| Determination of the linkage specificities of 22 DUBs. The quantified assay results of the DUBs belonging to **a**, the UbiCRest DUBs **b**, literature USPs and **c**, different DUB families DUBs are shown in two or three different graphs. The first graph shows the remaining % of diUb normalized to the internal standard non-hydrolysable Lys48 linked diUb and calculated starting from 100% remaining at t=0. The second graph shows the amount of monoUb formed (µM), normalized to the internal standard Ub_{1-74} and calculated using the concentration of the internal standard as reference.

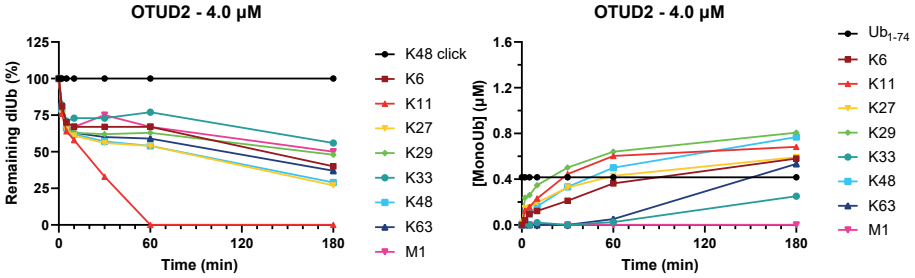
a, the UbiCRest DUBs

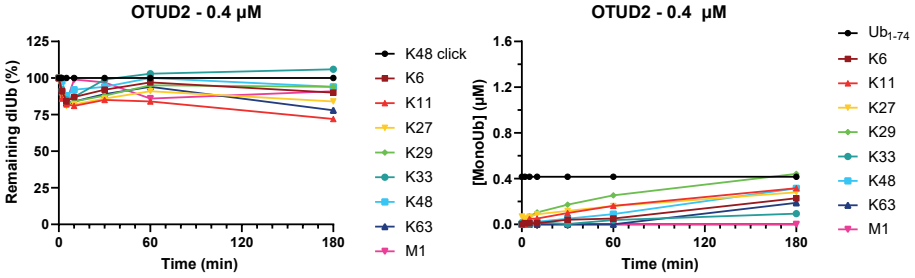


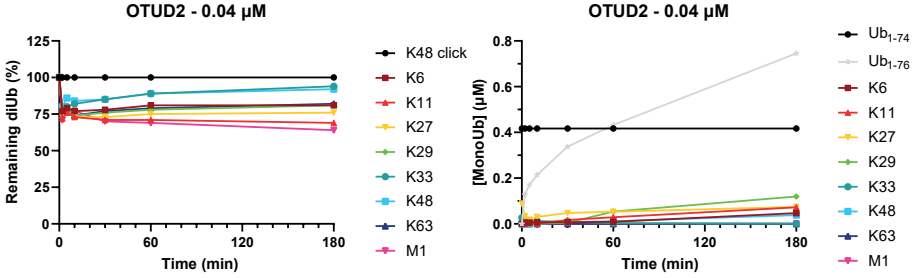
OTUB2


OTUB2 - 4.0 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTUB2 - 4.0 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1
OTUB2 - 0.4 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTUB2 - 0.4 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1
OTUB2 - 0.04 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTUB2 - 0.04 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1
OTUB2 - 0.004 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTUB2 - 0.004 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1

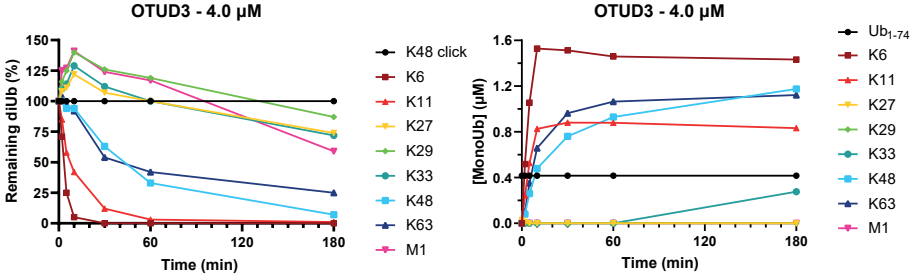
OTUD2

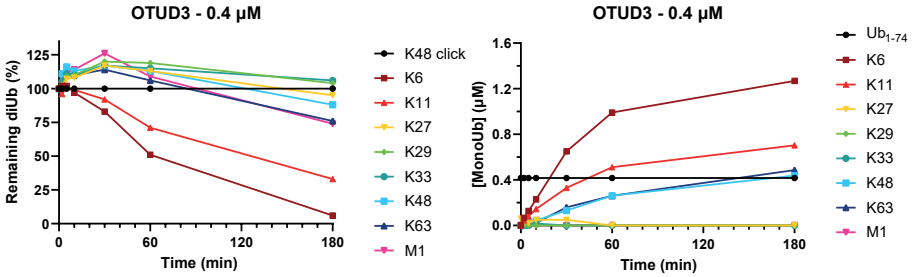

OTUD2 - 4.0 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTUD2 - 4.0 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

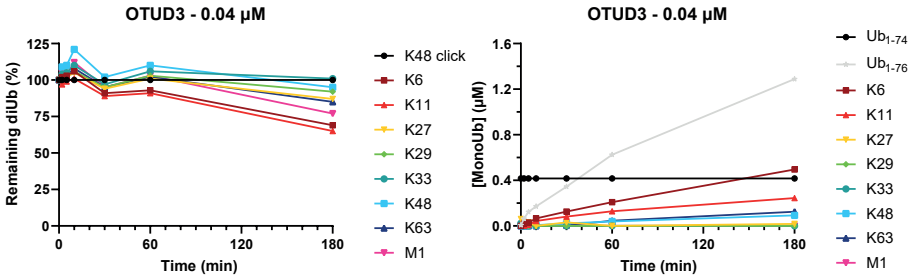

OTUD2 - 0.4 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTUD2 - 0.4 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1


OTUD2 - 0.04 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTUD2 - 0.04 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1

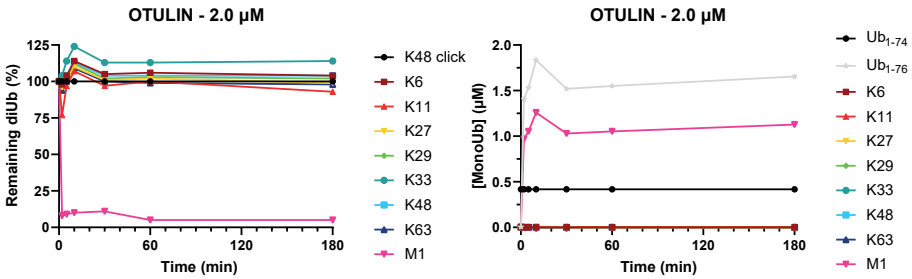
OTUD3

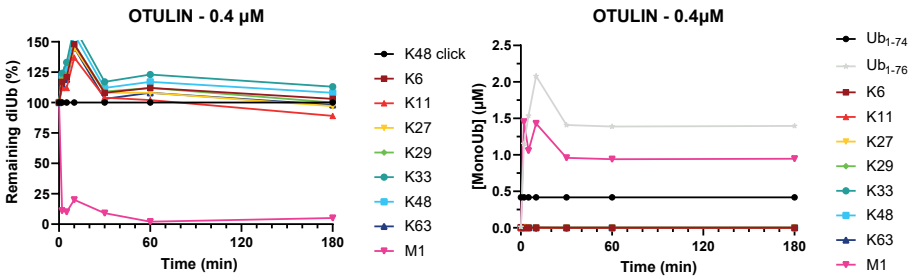

OTUD3 - 4.0 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTUD3 - 4.0 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1


OTUD3 - 0.4 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTUD3 - 0.4 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1


OTUD3 - 0.04 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTUD3 - 0.04 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1

OTULIN


OTULIN - 2.0 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTULIN - 2.0 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1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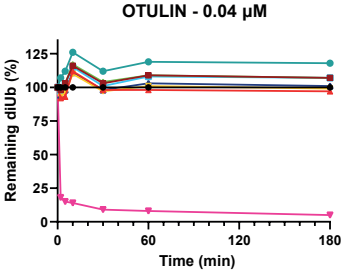
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
OTULIN - 0.4µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1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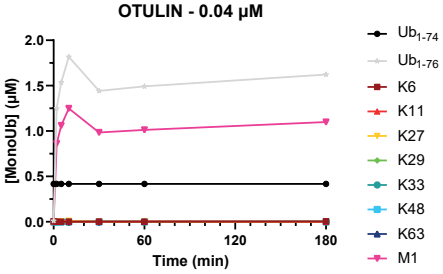
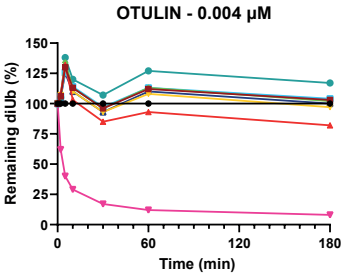
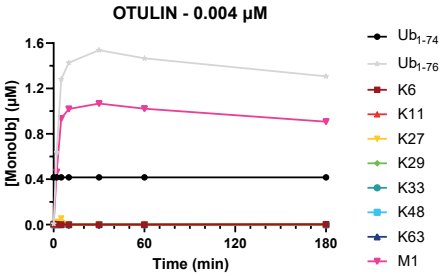
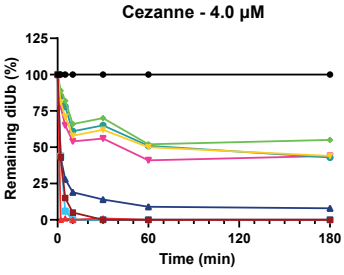
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1

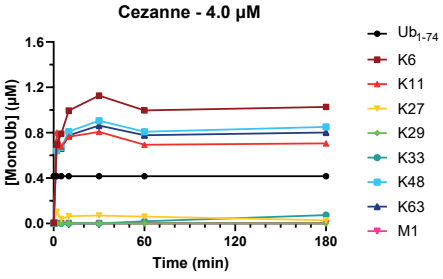

OTULIN - 0.04 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1

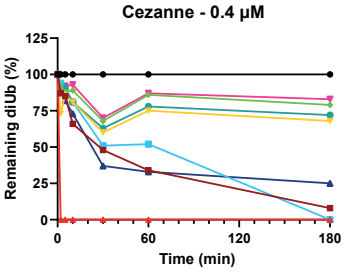

OTULIN - 0.004 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1


OTULIN - 0.004 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1

Cezanne


Cezanne - 4.0 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1


Cezanne - 4.0 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1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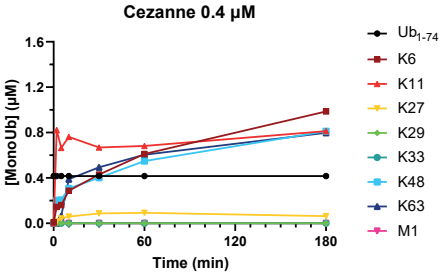
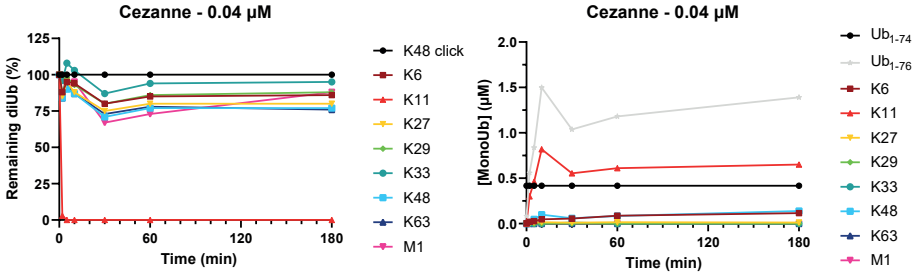
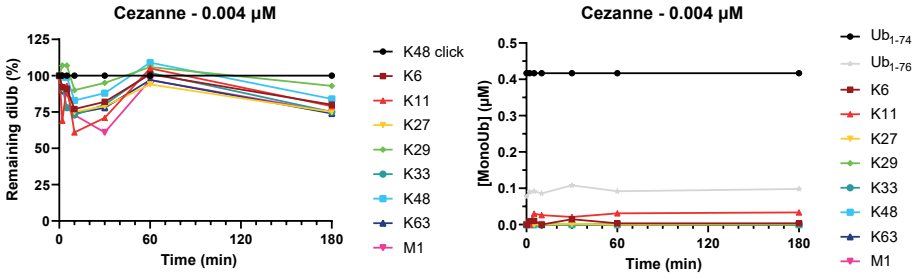
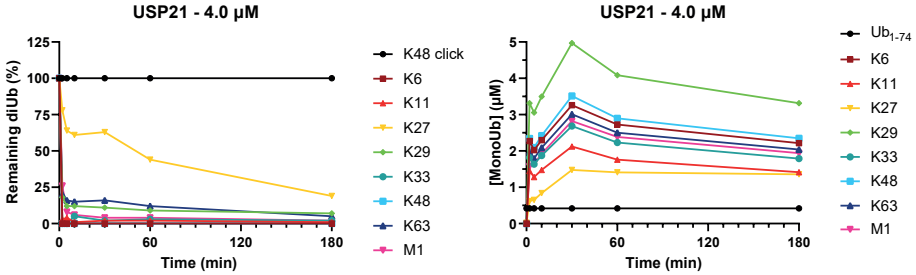
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1

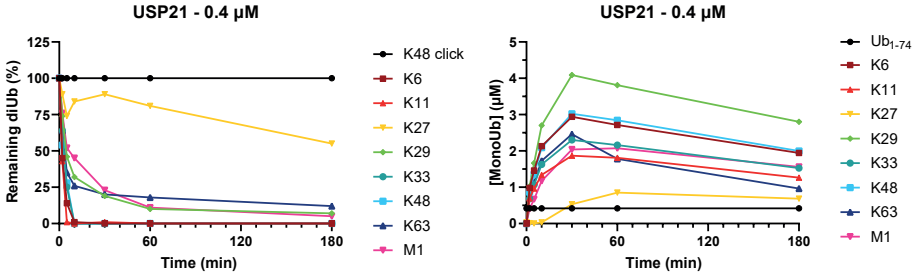

Cezanne 0.4 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

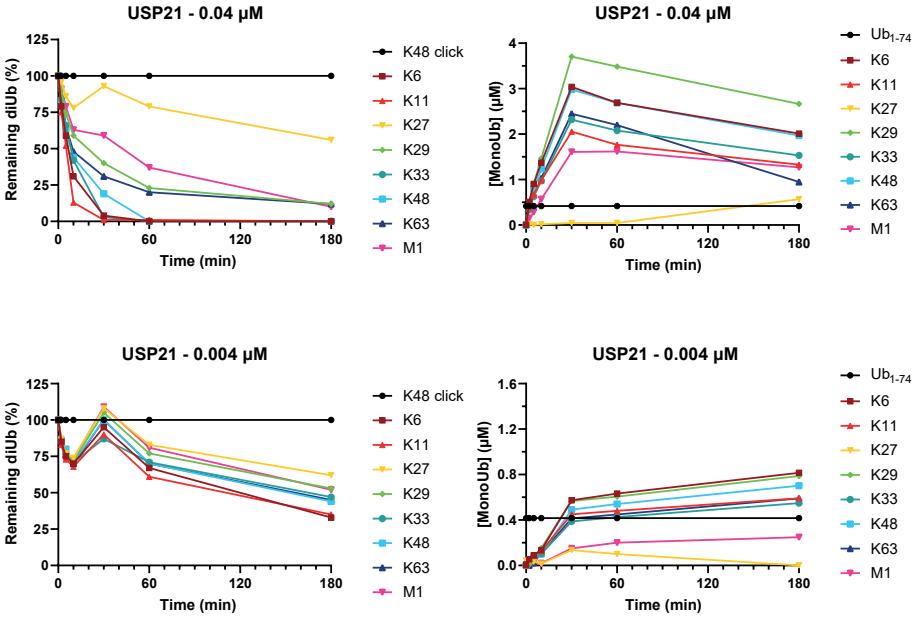

Cezanne - 0.04 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
Cezanne - 0.04 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1


Cezanne - 0.004 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
Cezanne - 0.004 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1

USP21

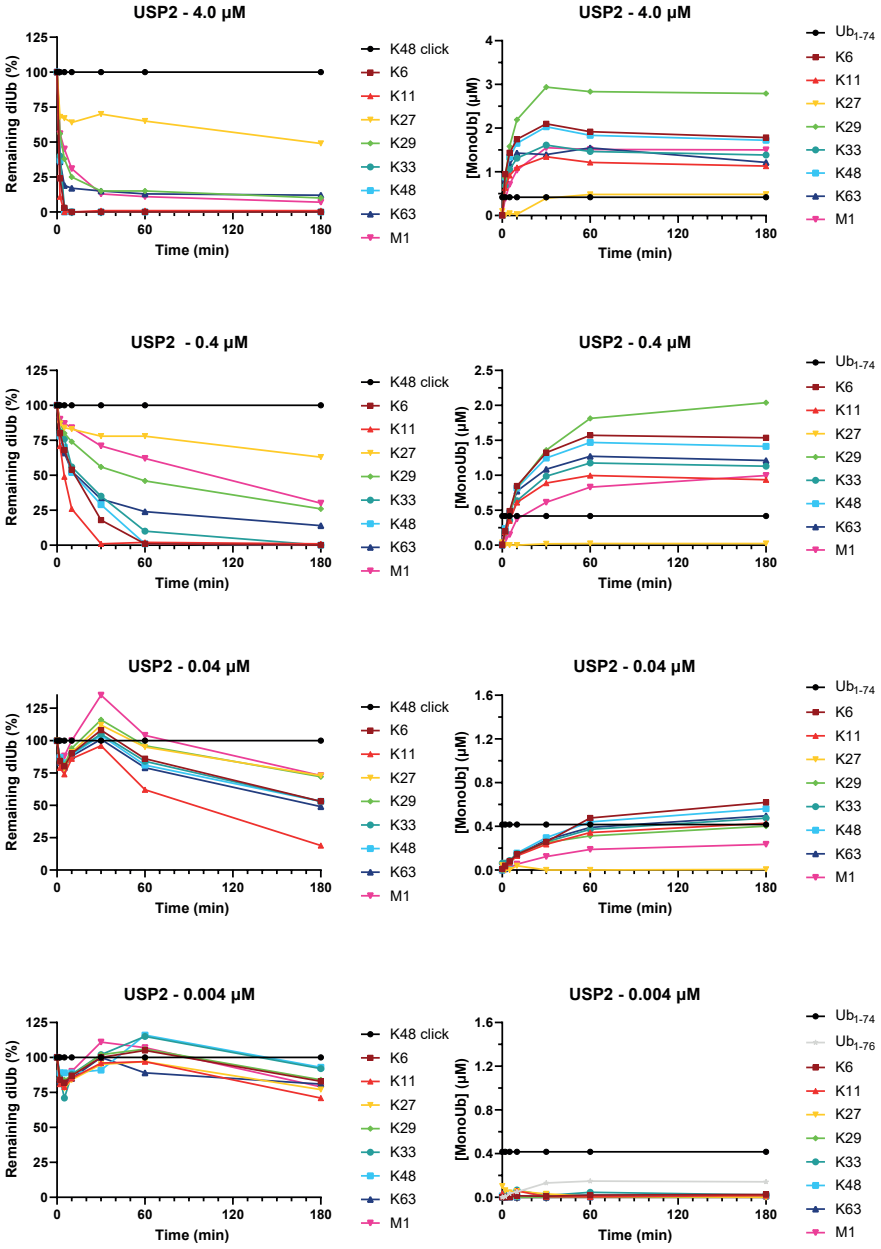

USP21 - 4.0 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
USP21 - 4.0 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1


USP21 - 0.4 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
USP21 - 0.4 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

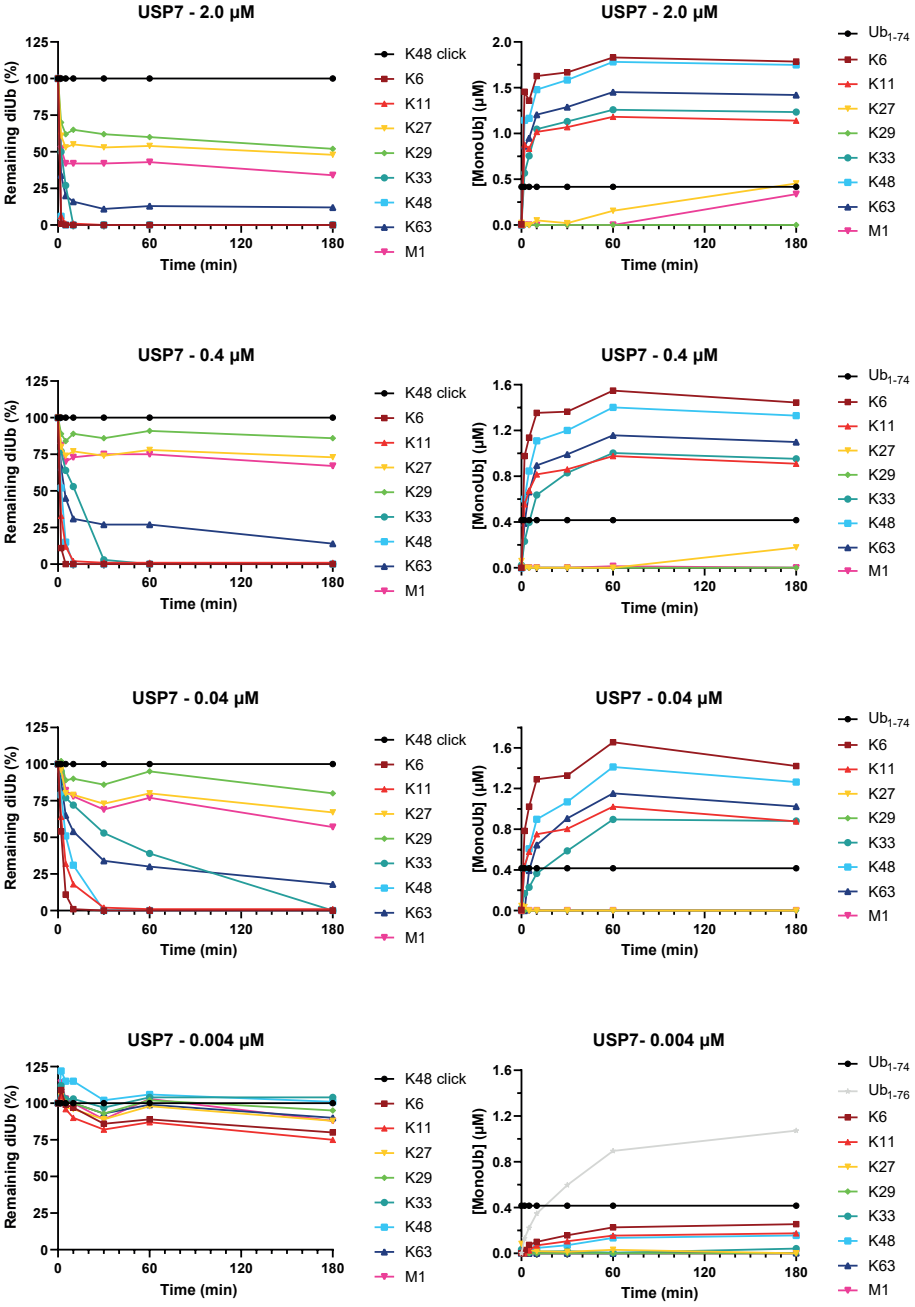

USP21 - 0.04 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
USP21 - 0.04 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1
USP21 - 0.004 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
USP21 - 0.004 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

b, literature USPs

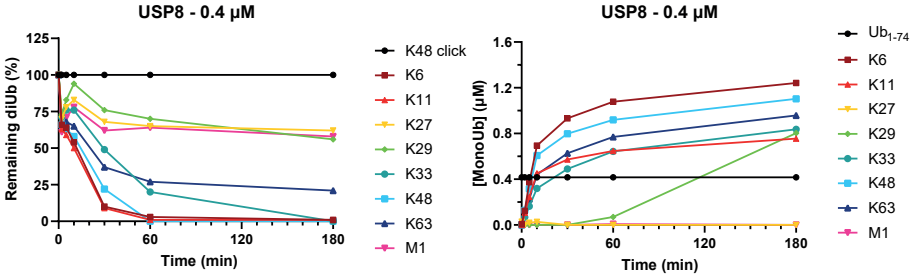
USP2

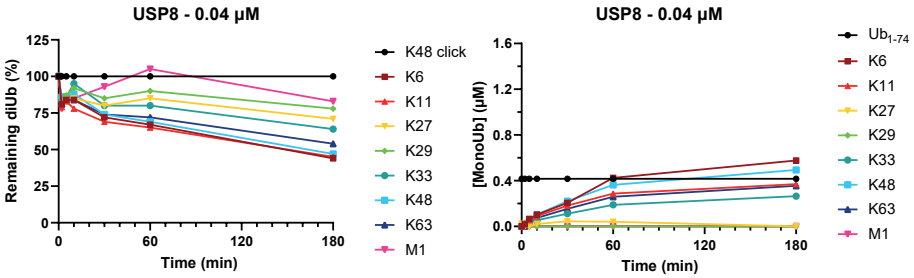


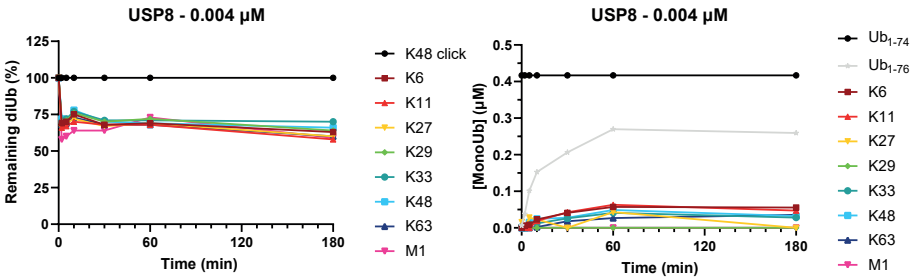
USP7


USP7 - 2.0 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
USP7 - 2.0 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1
USP7 - 0.4 µM
Remaining diUb (%)
Time (min)
USP7 - 0.4 µM
[MonoUb] (µM)
Time (min)
USP7 - 0.04 µM
Remaining diUb (%)
Time (min)
USP7 - 0.04 µM
[MonoUb] (µM)
Time (min)
USP7 - 0.004 µM
Remaining diUb (%)
Time (min)
USP7- 0.004 µM
[MonoUb] (µM)
Time (min)
Ub1-76

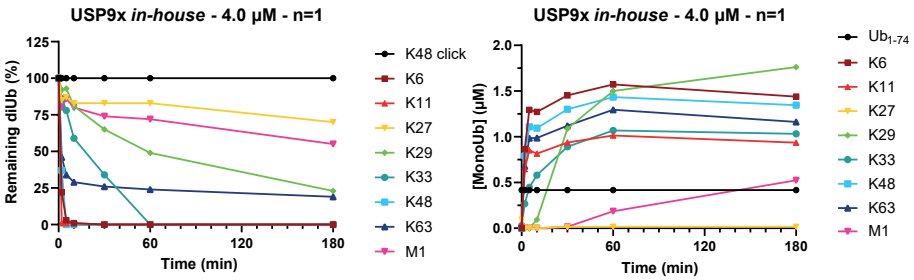
USP8

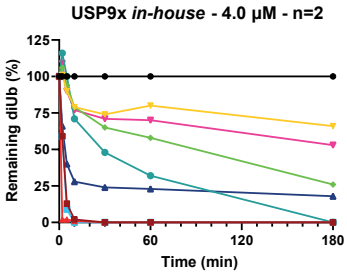

USP8 - 0.4 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
USP8 - 0.4 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

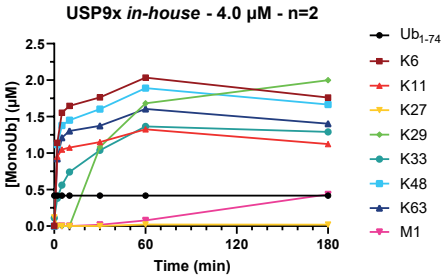

USP8 - 0.04 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
USP8 - 0.04 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

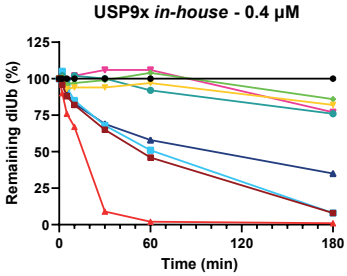

USP8 - 0.004 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
USP8 - 0.004 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1

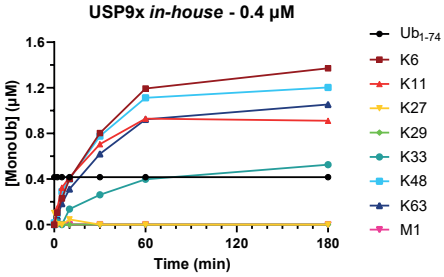
USP9x

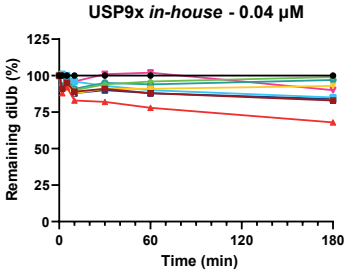

USP9x in-house - 4.0 µM - n=1
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
USP9x in-house - 4.0 µM - n=1
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

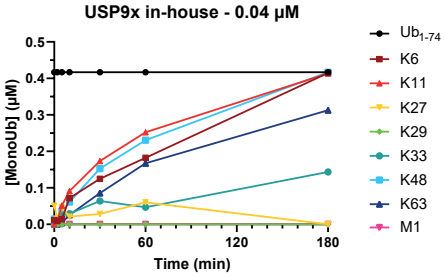

USP9x *in-house* - 4.0 µM - n=2
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1

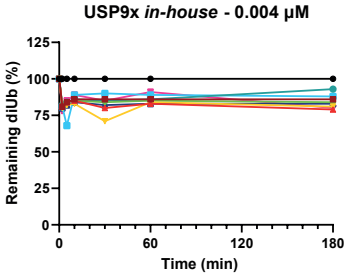

USP9x *in-house* - 4.0 µM - n=2
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

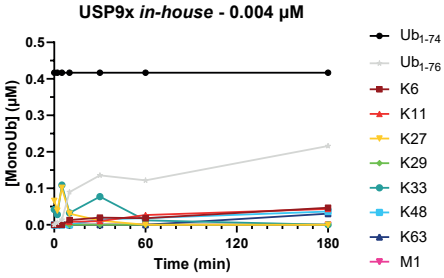

USP9x *in-house* - 0.4 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1

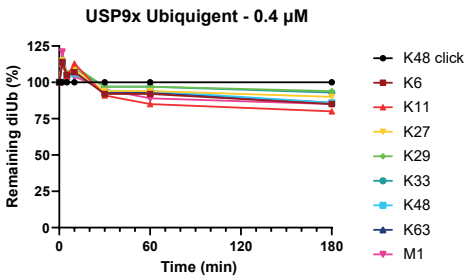

USP9x *in-house* - 0.4 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

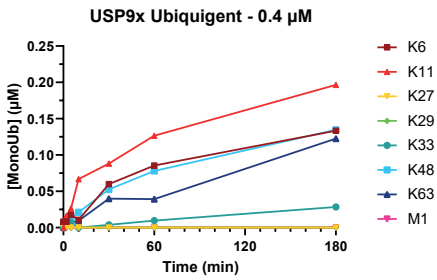

USP9x *in-house* - 0.04 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1

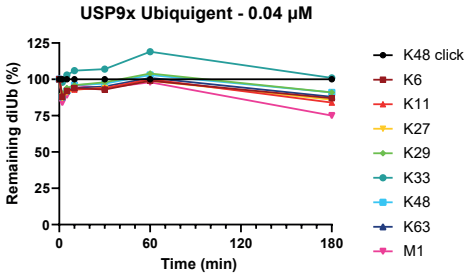

USP9x in-house - 0.04 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

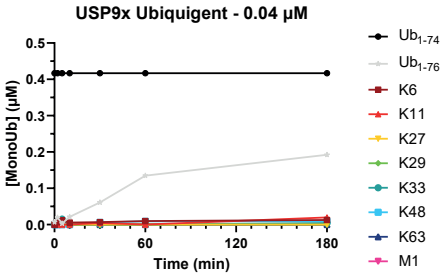

USP9x *in-house* - 0.004 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1


USP9x *in-house* - 0.004 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1

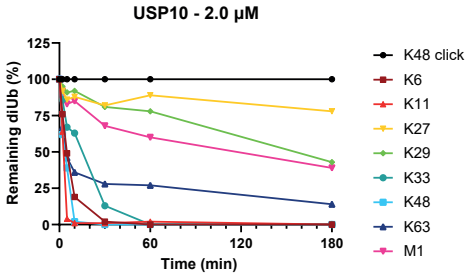

USP9x Ubiquigent - 0.4 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1

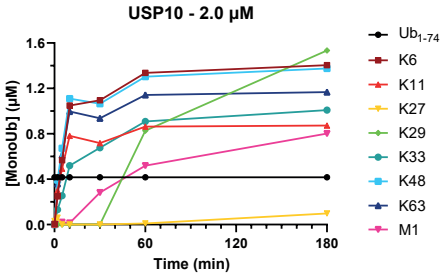

USP9x Ubiquigent - 0.4 µM
[MonoUb] (µM)
Time (min)
K6
K11
K27
K29
K33
K48
K63
M1

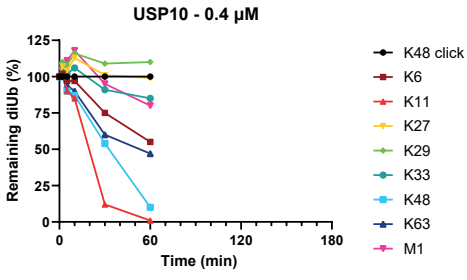

USP9x Ubiquigent - 0.04 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1

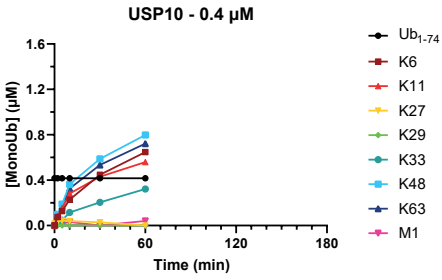

USP9x Ubiquigent - 0.04 µM
[MonoUb] (µM)
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1

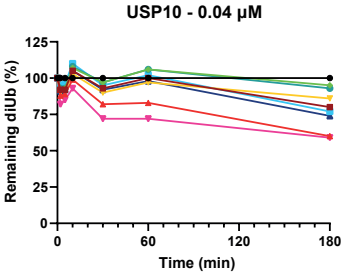
USP10

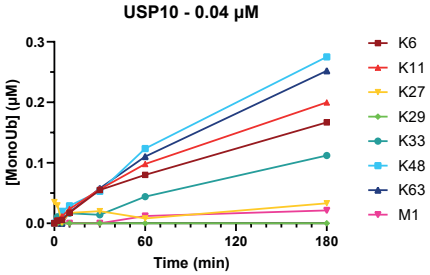

USP10 - 2.0 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1


USP10 - 2.0 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

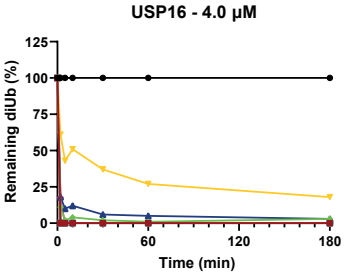

USP10 - 0.4 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1

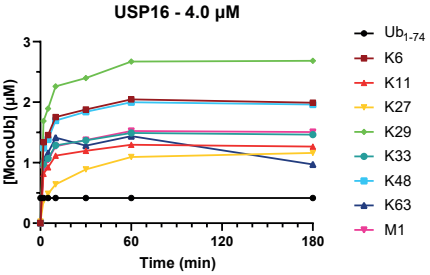

USP10 - 0.4 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

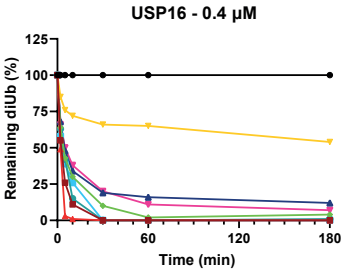

USP10 - 0.04 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1

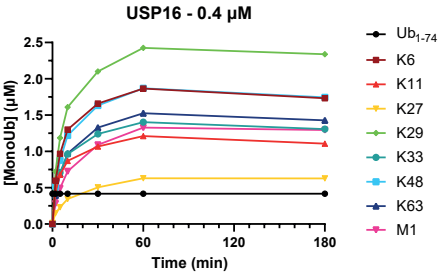

USP10 - 0.04 µM
[MonoUb] (µM)
Time (min)
K6
K11
K27
K29
K33
K48
K63
M1

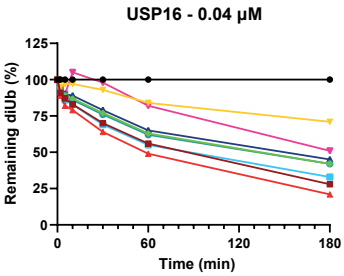
USP16

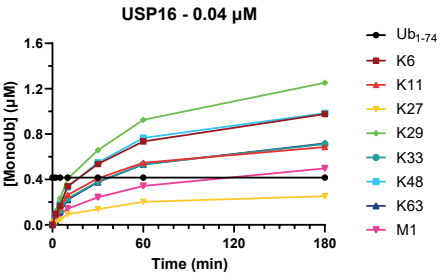

USP16 - 4.0 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1

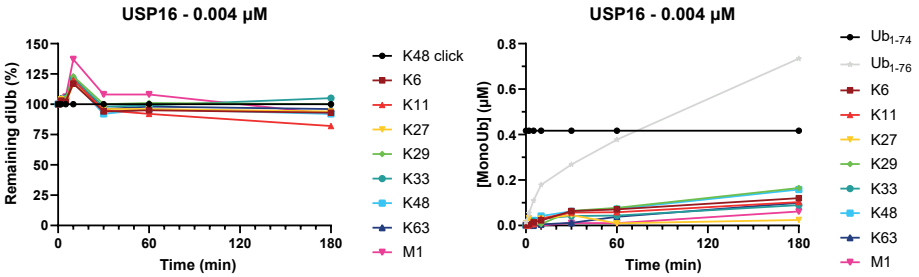

USP16 - 4.0 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1


USP16 - 0.4 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1


USP16 - 0.4 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

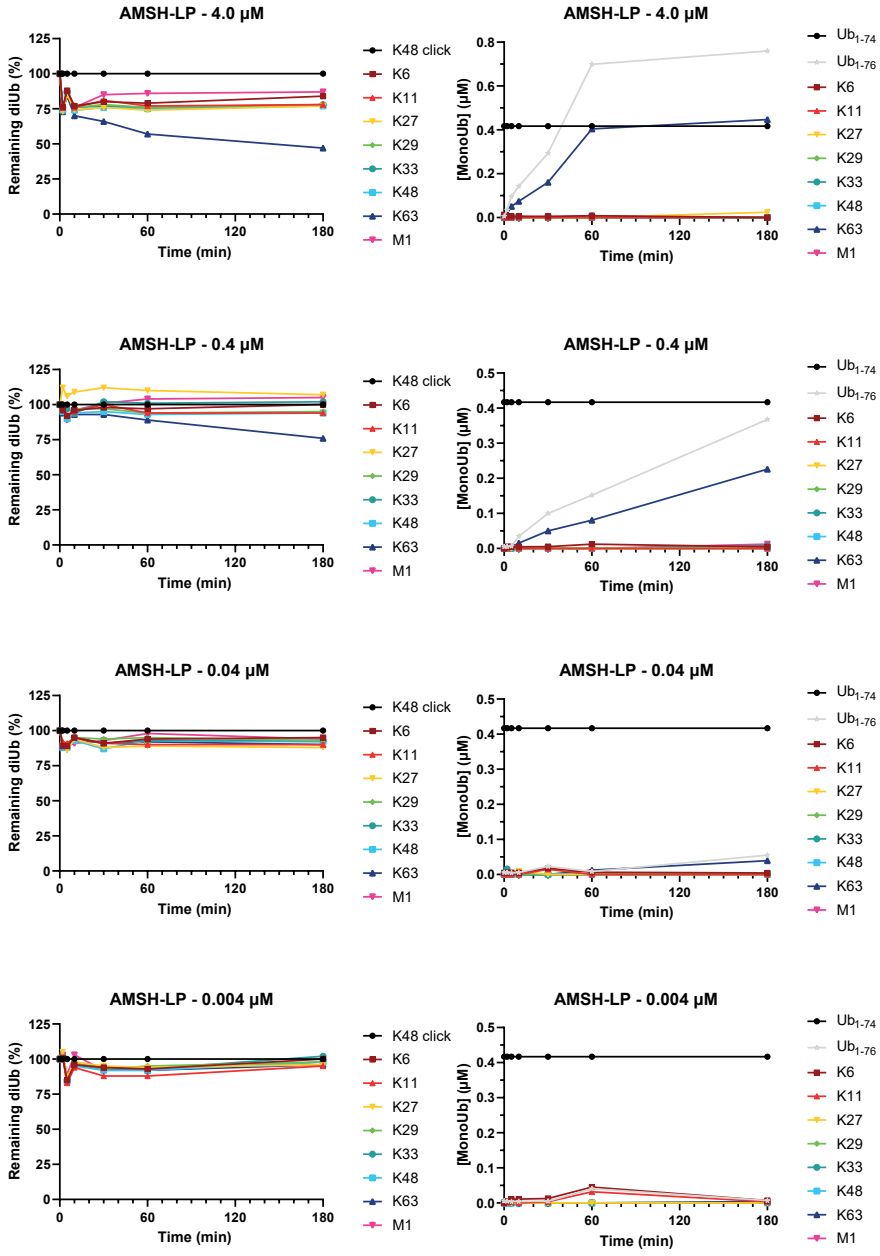

USP16 - 0.04 µM
Remaining diUb (%)
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1


USP16 - 0.04 µM
[MonoUb] (µM)
Time (min)
Ub1-74
K6
K11
K27
K29
K33
K48
K63
M1

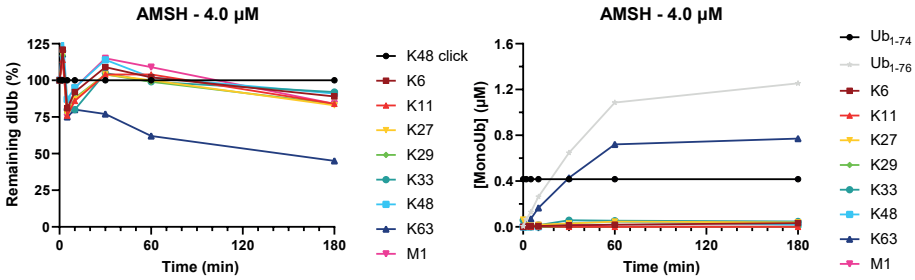

USP16 - 0.004 µM
Remaining diUb (%)
150
125
100
75
50
25
0
0
60
120
180
Time (min)
K48 click
K6
K11
K27
K29
K33
K48
K63
M1
USP16 - 0.004 µM
[MonoUb] (µM)
0.8
0.6
0.4
0.2
0.0
0
60
120
180
Time (min)
Ub1-74
Ub1-76
K6
K11
K27
K29
K33
K48
K63
M1

c, different DUB families DUBs

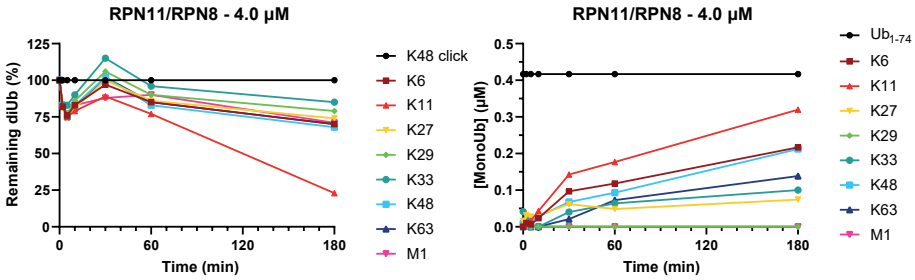
AMSH-LP



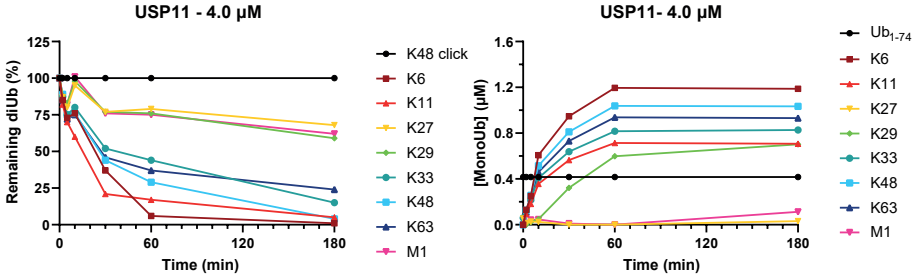
AMSH



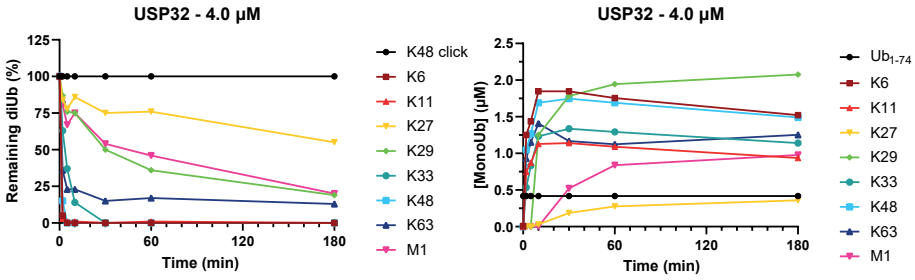
RPN11/RPN8



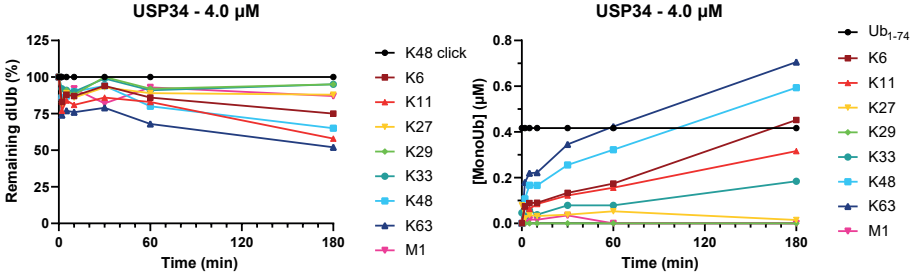
USP11



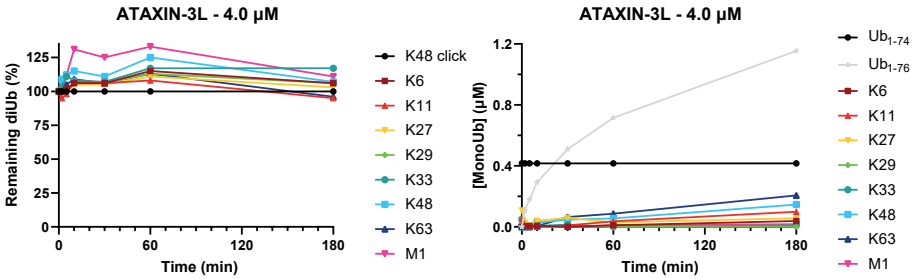
USP32



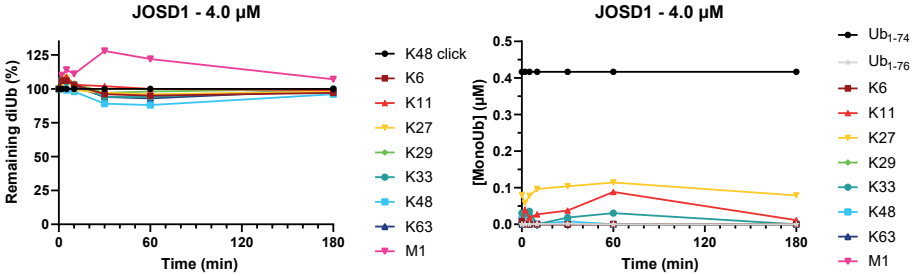
USP34



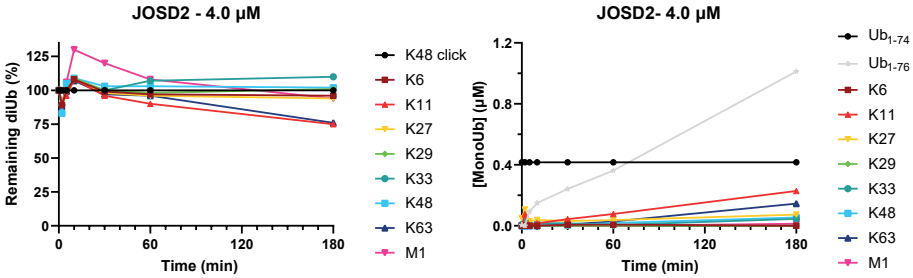
ATAXIN-L3

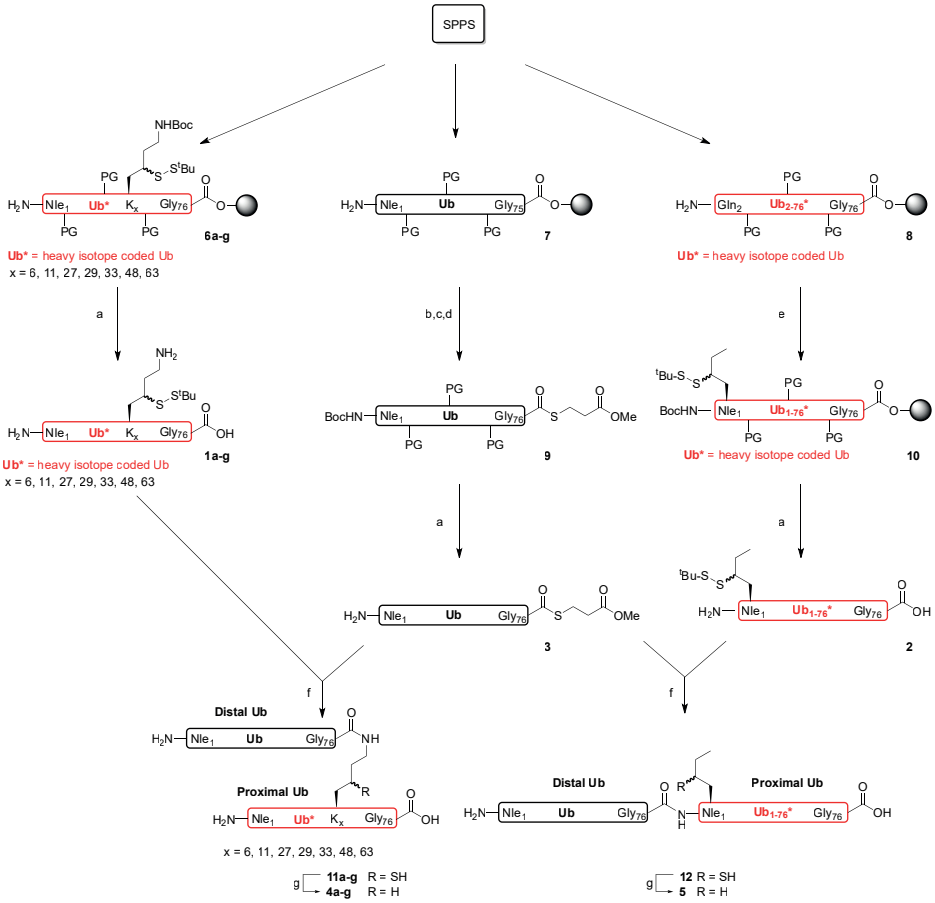


JOSD1

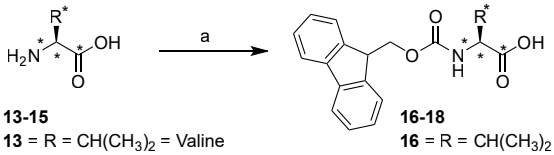


JOSD2





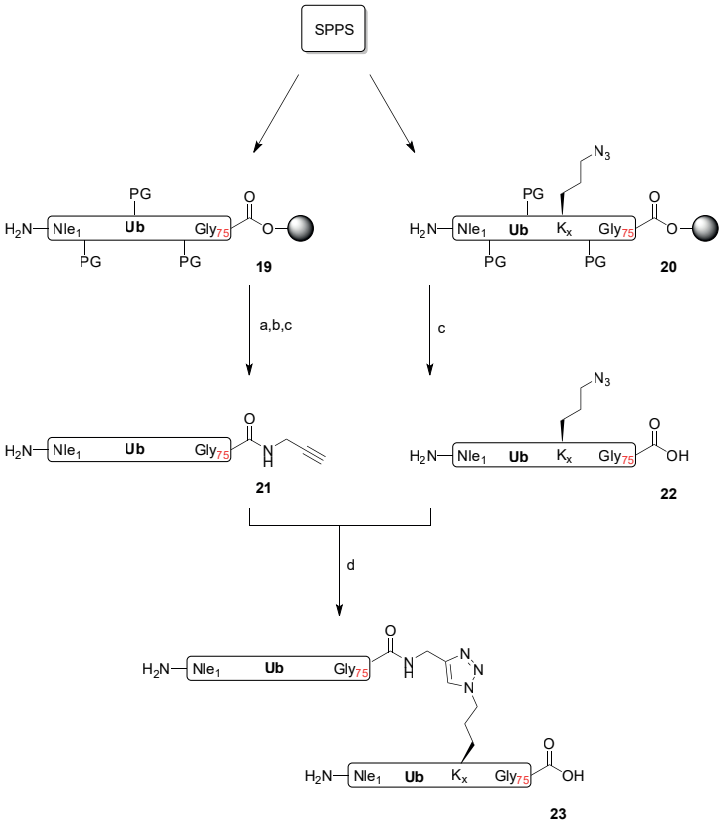
Supplementary Scheme 1| Synthesis of all eight neutron-encoded diUbs. Reagents and conditions: (a) TFA/H_2O/PhOH/*i*Pr_3SiH (90.5/5/2.5/2); (b) Boc_2O, DiPEA, DCM (c) 20% HFIP/DCM; (d) HCl·H-Gly-$S(CH_2)_2CO_2Me$, EDC, HOBt, DCM; (e) S^{t}Bu γ-thioNle[1], PyBOP, DiPEA, NMP; (f) 0.10 M TCEP, 0.25 M MPAA, 6 M Gnd·HCl, 0.15 M sodium phosphate, pH 7.6, 37°C; (g) 75 mM VA-044, 0.25 M TCEP, 100 mM GSH, 6 M Gnd·HCl, 0.15 M sodium phosphate, pH 7.0, 37°C. Monoubiquitins **6a-g**, **7** and **8** on resin were synthesized using solid phase peptide synthesis (SPPS). Monoubiquitins **6a-g** were liberated from the resin and deprotected using 90% TFA, yielding neutron-encoded Ub_{1-76} containing γ-thioLys **1a-g**. The N-terminus of monoubiquitin **7** was protected with a Boc-protection group whereafter the protein was liberated from the resin using mild acidic conditions (20% HFIP/DCM), while protecting groups on the amino acid side chains remain intact. Methyl-3-(glycylthio)-propionate (**26**) was coupled to the liberated C-terminal glycine yielding **9**. Acid-mediated deprotection yielded Ub_{1-76}-thioester **3.** Resin-bound monoubiquitin **8** was elongated with γ-thioNle using the SPPS coupling conditions resulting in resin-bound monoubiquitin **10**. Neutron-encoded Ub_{1-76} containing γ-thioNle **2** could be obtained after resin liberation and amino acid side chain deprotection using 90% TFA. Native chemical ligation (NCL) reactions between Ub_{1-76}-thioester **3** and neutron-encoded Ub_{1-76} containing γ-thioLys **1a-g** or neutron-encoded Ub_{1-76} containing γ-thioNle **2** yielded neutron-encoded diUb **11a-g** and **12**. Finally, the remaining sulfur atom was removed using desulfurization under radical conditions[2] to obtain the native diUb sequences **4a-g** and **5**.



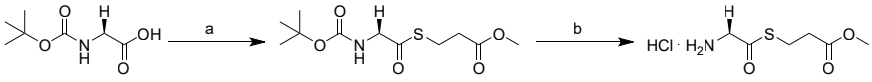
13-15
13 = R = $CH(CH_3)_2$ = Valine
14 = R = $CH_2CH(CH_3)_2$ = Leucine
15 = R = $CH_2(CH_3)(CH_2CH_3)$ = Isoleucine

16-18
16 = R = $CH(CH_3)_2$ = Fmoc -Val
17 = R = $CH_2CH(CH_3)_2$ = Fmoc-Leu
18 = R = $CH_2(CH_3)(CH_2CH_3)$ = Fmoc-Ile

Supplementary Scheme 2| Fmoc-protection of neutron-encoded Val, Leu and Ile. Atoms marked with an asterisk (*) are ^{13}C or ^{15}N. Reagents and conditions: (a) 10% Na_2CO_3 in H_2O (11 mL/mmol AA) and 1.2 eq. FmocOSu in 1,4-dioxane (7 mL/mmol FmocOSu). Neutron-encoded Val, Leu and Ile were protected with an Fmoc-protection group using FmocOSu in an alkaline solution.



Supplementary Scheme 3| Synthesis of internal standard non-hydrolyzable clicked Lys48 diUb. Reagents and conditions: (a) 20% HFIP/DCM; (b) Propargylamine (PA), PyBOP, DiPEA, DCM; (c) TFA/H_2O/PhOH/$i$$Pr_3SiH$ (90.5/5/2.5/2); (d) $CuSO_4 \cdot 5H_2O$; Sodium Ascorbate; TBTA-analogue[3], 8M Urea, 100 mM phosphate buffer, pH 7. Monoubiquitins **19** and **20** on resin were synthesized using linear solid phase peptide synthesis (SPPS). Monoubiquitin **19** was liberated from the resin using mild acidic conditions (20% HFIP/DCM), while protecting groups on the amino acid side chains remain intact. Propargylamine was coupled to the liberated C-terminal glycine followed by acid-mediated deprotection yielded Ub_{1-75}-PA **21**. Monoubiquitin **20** was liberated from the resin and deprotected using 90% TFA, yielding Ub_{1-75} (K48= L-azido-ornithine) **22**. Subsequent CuAAC of alkyne **21** and azide **22** yields final K48 click diUb **23**.



24 **25** **26**

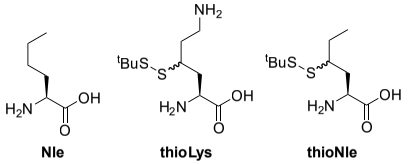
Supplementary Scheme 4| Synthesis of methyl-3-(glycylthio)-propionate. Reagents and conditions: (a) Methyl 3-mercaptopropionate, EDC, DMAP, DCM; (b) 4M HCl in dioxane. Boc-protected glycine **24** was converted into Boc-protected methyl-3-(glycylthio)-propionate **25** using methyl 3-mercaptopropionate together with the carboxyl activating carbodiimide EDC, and the nucleophilic catalyst DMAP. Hydrochloride salt of glycine thioester **26** was obtained by treatment of Boc-protected methyl-3-(glycylthio)-propionate **25** with hydrochloric acid in dioxane.

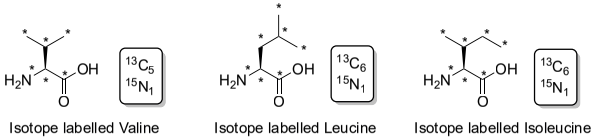
Supplementary Table 1| Amino acid sequence of designed proximal ubiquitins.

Sites of neutron-encoded amino acids, pseudoproline building blocks, dipeptides, thioLys and thioNle incorporation are indicated and relevant structures are shown below

Diubiquitin Linkage	Protein sequence for synthesizer	Amount of neutron-encoded amino acids	Mass difference in comparison with unlabeled ubiquitin	Mass difference in comparison with previous ubiquitin
K6	NleQIFV**K**TLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLV**L**RL**R**LRGG	1x**V** + 1x**L**	+13 Da	13 Da
K11	NleQIFVKTLTG**K**TITLE**V**EPSDTIEN**V**KAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN**I**QKESTLHLV**L**RLRGG	3x**V** + 1x**I**	+25 Da	12 Da
K27	NleQIFVKTLTGKTITLE**V**EPSDTIEN**V**K**A**KIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYN**I**QKESTLHLV**L**RL**R**LRGG	3x**V** + 2x**L** + 1x**I**	+39 Da	14 Da
K29	NleQIFVKTLTGKTITLE**V**EPSDTIEN**V**KA**K**IQDKEG**I**PPDQQRL**I**FAGKQLEDGRTLSDYN**I**QKESTLHLV**L**RL**R**LRGG	3x**V** + 2x**L** + 3x**I**	+53 Da	14 Da
K33	NleQIFVKTLTGKTITLE**V**EPSDTIEN**V**KAKIQD**K**EG**I**PPDQQRL**I**FAGKQLEDGRTLSDYN**I**QKESTL**H**LV**L**RL**R**GG	3x**V** + 4x**L** + 3x**I**	+67 Da	14 Da
K48	NleQIFVKTLTGKTITLE**V**EPSDT**I**EN**V**KAK**I**QDKEG**I**PPDQQRL**I**FAG**K**QLEDGRTLSDYN**I**QKESTL**H**LV**L**RL**R**GG	3x**V** + 4x**L** + 5x**I**	+81 Da	14 Da
K63	NleQIFVKTLTGKTITLE**V**EPSDT**I**EN**V**KAK**I**QDKEG**I**PPDQQR**L**I**F**AGKQ**L**EDGRTLSDYN**I**Q**K**EST**L**H**L**V**L**RL**R**GG	3x**V** + 6x**L** + 5x**I**	+95 Da	14 Da
M1	**Nle**Q**I**F**V**KTLTGKTITLE**V**EPSDT**I**EN**V**KAK**I**QDKEG**I**PPDQQR**L**I**F**AGKQ**L**EDGRTLSDYN**I**Q**K**EST**L**H**L**V**L**RL**R**GG	4x**V** + 6x**L** + 6x**I**	+108 Da	13 Da

K = *thioLys* *Nle* = *thioNle* *XX* = *dipeptide building block* **X** = *neutron-encoded amino acid*





Supplementary Table 2| Purified recombinant DUBs used in this work.

	DUB family	DUB	Domain/ Length / Fragment	Tag	UniProt accession number	Species / Origin / Organism	Expression system / Host / Source	Stock concentration	Source or reference
1	Ubiquitin-specific proteases (USPs)	USP2	Full length (1-369)	GST-tagged	O75604-4	Human	E.coli	0.5 mg/mL ~73 kDa 6.9 µM	Obtained from Ubiquigent. #64-0014-050
2		USP7	Full length (1-1102)	GST-tagged, clvd	Q93009	Human	E.coli BL21(DE3) Rosetta2	10.13 µM	*In-house.* [Kim, 2019][4]
3		USP8	Full length (1-1118)	untagged	P40818-1	Human	E.coli	0.5 mg/mL ~128 kDa 3.9 µM	Obtained from Ubiquigent. # 64-0053-050
4		USP9x	CD (1554-1995)	GST-tagged	Q93008	Human	E.coli	0.5 mg/mL ~79 kDa 6.35 µM	Obtained from Ubiquigent # 64-0017-050
5		USP9x	CD(1551-1970)	His-tagged, clvd	Q93008	Human	E.coli BL21(DE3) Rosetta2	228 µM	*In-house.* [Paudel, 2019][5]
6		USP10	Full length (1-798)	C-terminal 6-His tag	Q14694-1	Human	Sf21 Insect cells (baculovirus)	~ 88 kDa 10 µM	Obtained from BioTechne, # E-592-050
7		USP11 Isoform 2	FL(1-920) Canocical (43-963)	N-terminal His-tag, clvd	G5E9A6 (P51784-2)	Human	E.coli	13.5 mg/mL 136 µM	*In-house*, [Luna-Vargas 2011][6]
8		USP16FL isoform 3	FL (22-823) Q141H, EY480DN	N-terminal His-tag	Q9Y5T5-3	Human	Sf9 Insect cells (baculovirus)	~ 12 µM	In-house, [Mons, 2021][7]
9		USP21	CD(196-565)	N-terminal His-SUMO, clvd	Q9UK80	Human	E.coli BL21(DE3) Rosetta2	79.95 µM	*In-house*, [Ye, 2011][8]
10		USP32	Full-length (1-1604)	6-His tag N-terminus	Q8NFA0	Human	Sf9 Insect cells (baculovirus)	182 kDa 26 µM	*In-house*, [Sapmaz, 2019][9]
11		USP34	CD(1892-2241)	6-His tag	Q70CQ2	Human	E. coli	42 kDa 12.9 µM	Obtained from LifeSensors, #DB506

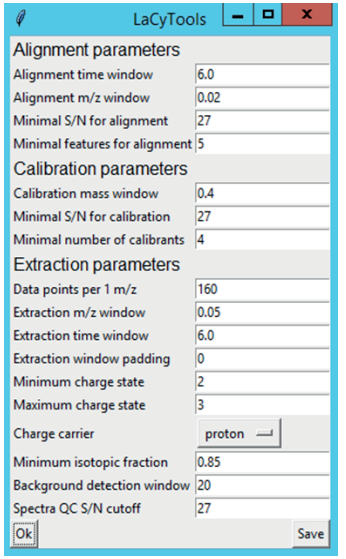
	DUB family	DUB	Domain/ Length / Fragment	Tag	UniProt accession number	Species / Origin / Organism	Expression system / Host / Source	Stock concentration	Source or reference
12	Ovarian tumour proteases (OTUs)	Cezanne	CD (129-438)	C-terminal 6-His tag and N-terminal GST tag, clvd	Q6GQQ6	Human	E.coli Rosetta2 (DE3) pLacI	362 µM	Gift from David Komander, [Mevissen, 2013][10]
13		OTULIN	Full length (1-352)	GST-tagged	Q96BN8-1	Human	E. coli	0.5 mg/mL ~67 kDa 7.46 µM	Obtained from Ubiquigent # 64-0017-050
14		OTUB1	Full length (1-271)	N-terminal His6-GST tag, clvd	Q96FW1	Human	E.coli Rosetta2 pLacI	943 µM	Gift from David Komander, [Mevissen, 2013][10]
15		OTUB2	Full length (1-234)	6-His tag, clvd	Q96DC9	Human	E.coli BL21(DE3)	2.4 mg/mL 81 µM	*In house*, [Nanao, 2004][11]
16		OTUD1	CD (287-481)	N-terminal His6-GST tag, clvd	Q5VV17	Human	E.coli Rosetta2 pLacI	223 µM	Gift from David Komander, [Mevissen, 2013][10]
17		OTUD2	Full length (1-348)	N-terminal His6-GST tag, clvd	Q5VVQ6	Human	E. coli Rosetta2 pLacI	10 mg/mL 274 µM	Gift from David Komander, [Mevissen, 2013][10]
18		OTUD3	CD (52-209)	N-terminal His6-GST tag, clvd	Q5T2D3	Human	E. coli Rosetta2 pLacI	220 µM	Gift from David Komander, [Mevissen, 2013][10]
19	MJD+	JOSD1	1-202	6-His tagged	Q15040	Human	E.coli	0.5 mg/mL – 26 kDa 19 µM	Ubiquigent, # 67-0006-001
20		JOSD2	1-188	6-His tagged	Q8TAC2-1	Human	E.coli	0.5 mg/mL ~23 kDa 21.7 µM	Ubiquigent, # 67-0006-001
21		ATAXN3L	Full length (1-355)	6-His tagged	Q9H3M9	Human	E.coli	0.5 mg/mL 11.6 µM ~43 kDa	Ubiquigent, # 67-0006-001
22	JAB/MPN/Mov34 metalloenzyme (JAMM)	AMSH	Full length (1-424)	GST-tagged, clvd	O95630	Human	E.coli	33 µM	*In-house*, This work

	DUB family	DUB	Domain/ Length / Fragment	Tag	UniProt accession number	Species / Origin / Organism	Expression system / Host / Source	Stock concentration	Source or reference
23		AMSH-LP	Full length (1-436)	6-His tagged	Q96FJ0	Human	E.coli	0.5 mg/mL, 52 kDa 9.6 µM	Obtained from Abcam, # ab139776
24		RPN11/ RPN8	RPN11(2-239)/ RPN8(1-179)	N-terminal 6-Hig tagged C-terminal StrepII tagged, both clvd	P43588/ Q08723	Saccharomyces cerevisiae	E.coli BL21 (DE3)	88 µM	*In-house*, [Hameed, 2019][12]

Supplementary Table 3| Alignment file/table

m/z	Seconds
599.3889	200
619.6895	197
767.6107	217
777.8871	218
803.0705	217
901.7223	230
905.3077	238
947.0356	226

Supplementary Table 4| LaCyTools settings



Supplementary Table 5| Analytes and calibrants – LaCyTools

Peak	RT	Mass Window	Time Window	Minimal charge state	Maximum charge state	Calibrant
_C756_H1255_N211_O233	227			10	25	X
_C747_hC11_H1260_N208_hN2_O235	230			10	25	X
_C737_hC21_H1260_N206_hN4_O235	230			10	25	X
_C725_hC33_H1260_N204_hN6_O235	230			10	25	X
_C713_hC45_H1260_N202_hN8_O235	230			10	25	X
_C701_hC57_H1260_N200_hN10_O235	230			10	25	X
_C689_hC69_H1260_N198_hN12_O235	230			10	25	X
_C677_hC81_H1260_N196_hN14_O235	230			10	25	X
_C666_hC92_H1260_N194_hN16_O235	237			10	25	X
_C375_H625_N103_O116	218			5	13	X
_C379_H631_N105_O118	218			5	13	X
_C368_hC11_H631_N103_hN2_O118	218			5	13	X
_C358_hC21_H631_N101_hN4_O118	218			5	13	X
_C346_hC33_H631_N99_hN6_O118	218			5	13	X
_C334_hC45_H631_N97_hN8_O118	218			5	13	X
_C322_hC57_H631_N95_hN10_O118	218			5	13	X
_C310_hC69_H631_N93_hN12_O118	218			5	13	X
_C298_hC81_H631_N91_hN14_O118	218			5	13	X
_C287_hC92_H631_N89_hN16_O118	218			5	13	X

_C = carbon atom = 12.00000

_hC = heavy carbon atom = 13.0033550

_H = hydrogen atom = 1.007825

_N = nitrogen atom = 14.003074

_hN = heavy nitrogen atom = 15.000109

_O = oxygen atom = 15.994915

Supplementary Table 6| AMSH expression: PCR reaction

PCR Reaction Component	Volume (µL)	Final Concentration
ThermoPol Reaction Buffer (10X)	2.5 µL	1X
Deoxynucleotide (dNTP) Solution Mix (10 mM)	1 µL	200 µM
Forward Primer (10 µM stock)	0.6 µL	0.24 µM
Reverse Primer (10 µM stock)	0.6 µL	0.24 µM
DNA Template (MelJuSo cDNA)	1 µL	N/A
Vent DNA Polymerase (New England Biolabs)	0.3 µL	0.6 unit
$MgCl_2$	0.6 µL	2 mM
Nuclease-free water	Bring reaction to a final volume of 30 µL	

Supplementary Table 7| AMSH expression: PCR cycle condition

Steps	Temperature	Time	# of cycle
Initial Denaturation	95 °C	2 min	34
Denaturation	95 °C	30 sec	
Annealing	56 °C	30 sec	
Elongation	72 °C	1 min 30sec	
Final Elongation	72 °C	5 min	

Supplementary Table 8| Sequence of the cloning primers for AMSH

Name	Sequence
AMSH Forward	cagggacccggtATGTCTGACCATGGAGATGTGAGCC
AMSH Reverse	cgaggagaagcccggttaTCGAAGGTCTGTGATGGTCACTGC

Materials and Methods; Chemical Synthesis

General. All commercially available reagents and solvents were purchased from various suppliers (listed in Supplementary Table 6) and used as received.

Supplementary Table 6| Building blocks, reagents and solvents for chemical synthesis.

Compound	Abbreviation	CAS#	Source or reference
Building blocks			
L -Valine 13C5,15N1	Val $^{13}C_5$ $^{15}N_1$	202407-30-5	Cortecnet #CCN5000P01
L -Leucine 13C6,15N1	Leu $^{13}C_6$ $^{15}N_1$	202406-52-8	Cortecnet #CCN1600P01
L -Isoleucine 13C6,15N1	Ile $^{13}C_6$ $^{15}N_1$	202468-35-7	Cortecnet #CCN1300P01
N-(tert-Butoxycarbonyl)glycine	Boc-Gly-OH	4530-20-5	Sigma Aldrich #15420
Chemicals			
Sodium carbonate	Na_2CO_3	497-19-8	Acros Organics #424280025
***N*-(9-Fluorenylmethoxycarbonyloxy) succinimide**	FmocOSu	82911-69-1	Chem-Impex #00147
Hydrochloric acid, 37%	HCl	7647-01-0	Sigma Aldrich #258148
Acetic acid	AcOH	64-19-7	Sigma Aldrich #27225
Sodium Chloride	NaCl	7647-14-5	Sigma Aldrich #S9625
Sodium sulfate	Na_2SO_4	7757-82-6	Sigma Aldrich #239313
Methyl 3-mercaptopropionate		2935-90-2	Sigma Aldrich #108987
4-(Dimethylamino)pyridine	DMAP	1122-58-3	ChemImpex #00120
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride	EDC	25952-53-8	Combi blocks #SS-7536
Sodium bicarbonate	$NaHCO_3$	144-55-8	Acros #123360050
Magnesium sulfate	$MgSO_4$	22189-08-8	Acros # 196850010
Hydrogen chloride, 4N solution in 1,4-dioxane	4M HCl in 1,4-dioxane	7647-01-0	Acros # 388368000
Solvents			
1,4-dioxane (AR)		123-91-1	Biosolve #4240501
Diethyl ether (AR)	Et_2O	60-29-7	Biosolve #5280501
Ethyl acetate (AR)	EtOAc	141-78-6	VWR # 23880324
n-Heptane (AR)	Hept	142-82-5	VWR #24551324
Dichloromethane (AR)	DCM	75-09-2	VWR #23 366 327

Compound	Abbreviation	CAS#	Source or reference
Dimethyl sulfoxide (AR)	DMSO(AR)	67-68-5	Biosolve #4470501
Acetonitrile (ULC-MS)	CH_3CN (ULC-MS)	75-05-8	Biosolve #1204102
Formic Acid (ULC-MS)	FA (ULC-MS)	64-18-6	Biosolve #6914143
Methanol-d4	MeOD- d_4	811-98-3	Cortecnet #D024H
DMSO-d6	DMSO- d_6	2206-27-1	Cortecnet #D010H

Thin Layer Chromatography (TLC) analysis was performed on TLC plates from Merck (aluminum sheets precoated with Silica (SiO_2) Kieselgel 60 F_{254} neutral) and compounds were visualized by UV absorption (254 nm) and/or by using a solution of ninhydrin (15 g/L) and acetic acid (30 mL/L) in ethanol or a solution of $KMnO_4$ (7.5 g/L) and K_2CO_3 (50 g/L) in water followed by charring.

Flash column chromatography (FCC) purifications were purified by a Büchi Sepacore automatic flash chromatography system X10/X50. The Büchi Sepacore system was equipped with two Büchi Pump Modules C-605, a Büchi Control Unit C-620, a Büchi Fraction Collector C-660 and a Büchi UV Photometer C-640. The silica columns were purchased at BUCHI® (FlashPure EcoFlex Silica) and were packed with silica with an irregular particle size (40-63 µm) and pore size (55-75 Å). FCC purifications were performed with the indicated eluent.

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker UltraShield™ Avance II™ 300 (300 MHz for ^1H, 75 MHz for ^{13}C) at 298 K using the residual solvent as internal standard (^1H: δ 7.26 ppm for $CDCl_3$, 3.31 ppm for MeOD and 2.50 ppm for DMSO-d_6. ^{13}C: δ 77.16 ppm for $CDCl_3$, 49.00 ppm for MeOD and 39.52 ppm for DMSO-d_6). Chemical shifts (δ) are given in ppm and coupling constants (*J*) are quoted in hertz (Hz). Multiplicities are reported as a s (singlet), d (doublet), t (triplet), q (quartet), b (broad) and m (multiplet) or combinations thereof.

LC-MS analysis of crude reaction mixtures and pure products were performed on a Waters ACQUITY UPLC H-class System equipped with Waters ACQUITY Quaternary Solvent Manager (QSM), Waters ACQUITY UPLC Photodiode Array (PDA) eλ Detector (λ = 210-800 nm) and Waters ACQUITY UPLC Protein BEH C18 column (1.7 µm, 2.1 x 50 mm) (Column Temp = 40 °C) and LCT Premier Orthogonal Acceleration Time of Flight Mass Spectrometer (*m/z* = 100-1600) in ES+ mode. Samples were run with a 1.6 minute gradient (run time 3 min) using three mobile phases: 100% H_2O, 100% CH_3CN and 50% H_2O + 50% CH_3CN + 2.5% FA (flow rate = 0.5 mL/min).

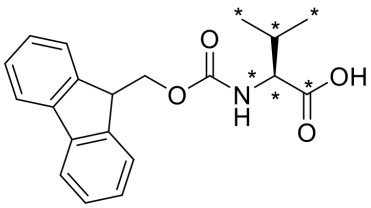
Time (min)	100% H_2O (%)	100% CH_3CN (%)	50% H_2O + 50% CH_3CN + 2.5% FA(%)
0.00	94.0	2.0	4.0
0.20	94.0	2.0	4.0
1.80	0.0	96.0	4.0
2.15	0.0	96.0	4.0
2.20	94.0	2.0	4.0
3.00	94.0	2.0	4.0

Pure products were run with a 7 minute gradient (run time 10 min) using three mobile phases: 100% H_2O, 100% CH_3CN and 50% H_2O + 50% CH_3CN + 2.5% FA (flow rate = 0.5 mL/min).

Time (min)	100% H_2O (%)	100% CH_3CN (%)	50% H_2O + 50% CH_3CN + 2.5% FA(%)
0.00	94.0	2.0	4.0
0.50	94.0	2.0	4.0
7.50	0.0	96.0	4.0
8.00	0.0	96.0	4.0
8.10	94.0	2.0	4.0
10.00	94.0	2.0	4.0

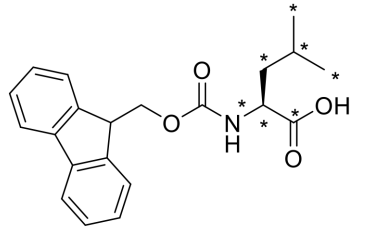
General procedure A: Fmoc protection of neutron-encoded amino acid

AA ($^{13}C_x$, $^{15}N_1$) (1 eq.) was dissolved in 10% Na_2CO_3 in H_2O (11 mL/mmol AA) and the solution was cooled to 0 °C. FmocOSu (1.2 eq.) was dissolved in 1,4-dioxane (7 mL/mmol FmocOSu). The FmocOSu solution was added dropwise to the cooled amino acid solution over the course of 2h. The reaction mixture was allowed to warm to room temperature and was stirred for 16 hours. H_2O (11 mL/mmol AA) was added to the reaction mixture resulting in a clear solution. The reaction solution was washed with Et_2O (3x 30 mL/mmol AA). The aqueous layer was acidified to pH ~1 with conc. HCl and extracted with EtOAc (2x 16mL/mmol AA and 1x 30 mL/mmol AA). The combined organic phase was washed with BRINE (2x 25 mL/ mmol AA), dried over Na_2SO_4 and concentrated *in vacuo*. The resulting residue was purified by Büchi flash column chromatography (100% n-Hept → 100% EtOAc) to yield the pure Fmoc-protected neutron-encoded amino acid.



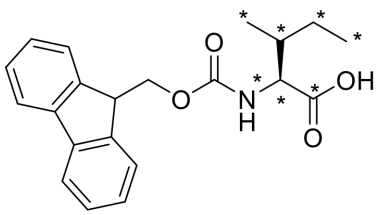
*(((9H-fluoren-9-yl)methoxy)carbonyl)-L-valine-$^{13}C_5{}^{15}N_1$ (**16**)*

Prepared according to general procedure A, starting from L-valine-$^{13}C_5{}^{15}N_1$ (250 mg; 2.03 mmol). The product was obtained as a white powder. (Yield: 576.9 mg, 1.67 mmol, 82.3%). TLC R_f =0.65 (60:40 EtOAc/n-Hept + 1 drop AcOH). ^{1}H-NMR (300 MHz, Methanol-*d4*): δ = 7.80 (d, *J* = 7.4 Hz, 2H, CH_{arom} Fmoc), 7.69 (dd, *J* = 7.5, 4.6 Hz, 2H, CH_{arom} Fmoc), 7.39 (t, *J* = 7.4 Hz, 2H, CH_{arom} Fmoc), 7.31 (td, *J* = 7.4, 1.2 Hz, 2H, CH_{arom} Fmoc), 4.36 (d, *J* = 7.4 Hz, 2H, CH_2 Fmoc), 4.24 (t, *J* = 7.0 Hz, 1H, CH Fmoc), 4.06 (d, *J* = 140.3 Hz, 1H, CαH), 2.16 (d, *J* =130.3 Hz, 1H, CβH), 1.23 – 0.58 (m, 6H, $C\gamma H_3$) ppm. ^{13}C-NMR (75 MHz, Methanol-*d4*): δ = 175.41 (d, *J* = 58.8 Hz, C=O), 145.38 (C_q), 145.20 (C_q), 142.59 (C_q), 128.77 (CH_{arom} Fmoc), 128.17 (CH_{arom} Fmoc), 128.14 (CH_{arom} Fmoc), 126.27 (CH_{arom} Fmoc), 120.90 (CH_{arom} Fmoc), 67.98 (CH_2 Fmoc), 61.01 (ddd, *J* = 58.8, 34.3, 12.6 Hz, Cα), 31.71 (q, *J* = 35.0 Hz, Cβ), 19.64 (dd, *J* = 35.5, 2.6 Hz, $C\gamma H_3$), 18.24 (d, *J* = 35.2 Hz, $C\gamma H_3$) ppm. LC-MS (4 → 98% ACN/H_2O); R_t = 5.09 min; calculated for [M+H$^+$] 346.11 ; found 346.15.



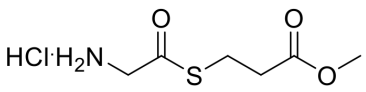
*(((9H-fluoren-9-yl)methoxy)carbonyl)-L-leucine-$^{13}C_6{}^{15}N_1$ (**17**)*

Prepared according to general procedure A, starting from L-Leucine-$^{13}C_6{}^{15}N_1$ (300 mg; 2.17 mmol). The product was obtained as a white powder. (Yield: 721.2 mg, 2.00 mmol, 92.2%). TLC R_f =0.68 (60:40 EtOAc/n-Hept + 1 drop AcOH). ^{1}H-NMR (300 MHz, Methanol-*d4*): δ = 7.80 (d, *J* = 7.4 Hz, 2H, CH_{arom} Fmoc), 7.68 (t, *J* = 6.5 Hz, 2H, CH_{arom} Fmoc), 7.39 (t, *J* =7.5 Hz, 2H, CH_{arom} Fmoc), 7.31 (t, *J* = 7.3 Hz, 2H, CH_{arom} Fmoc), 4.36 (d, *J* = 6.5 Hz, 2H, CH_2 Fmoc), 4.23 (t, *J* = 7.0 Hz, 1H, CH Fmoc), 4.18 (d, 2H, Cα+ …), 1.71 (d, *J* = 124.4 Hz, 1H, CγH), 1.61 (d, *J* = 127.4 Hz, 2H, $C\beta H_2$ 1.23-0.58 (m, 6H, $C\delta H_3$) ppm. ^{13}C-NMR (75 MHz, Methanol-d4): δ = 176.67 (dd, J = 59.1, 3.2 Hz, C=O), 145.39 (C_q), 145.17 (C_q), 142.59 (C_q), 128.75 (CH_{arom} Fmoc), 128.15 (CH_{arom} Fmoc), 128.12 (CH_{arom} Fmoc), 126.26 (CH_{arom} Fmoc), 120.88 (CH_{arom} Fmoc), 67.88 (CH_2 Fmoc), 53.77 (ddd, *J* = 58.9, 34.7, 12.5 Hz, Cα), 41.67 (t, *J* = 34.7 Hz, Cβ), 26.00 (qd, *J* = 34.8, 3.1 Hz, Cγ), 23.41 (dd, *J* = 35.0, 3.5 Hz, $C\delta H_3$), 21.69 (dd, *J* = 34.9, 2.1 Hz, $C\delta H_3$) ppm. LC-MS (4 → 98% ACN/H_2O); R_t = 5.38 min; calculated for [M+H$^+$] 361.12; found 361.17.



*(((9H-fluoren-9-yl)methoxy)carbonyl)-L-isoleucine-$^{13}C_6{}^{15}N_1$ (**18**)*

Prepared according to general procedure A, starting from L-Isoleucine-$^{13}C_6{}^{15}N_1$ (200 mg; 1.45 mmol). The product was obtained as a white powder. (Yield: 493.7 mg, 1.37 mmol, 94.5%). TLC R_f =0.70 (60:40 EtOAc/n-Hept + 1 drop AcOH).^{1}H-NMR (300 MHz, MeOD): δ = 7.80 (d, *J* = 7.5 Hz, 2H, CH_{arom} Fmoc), 7.68 (dd, *J* = 7.4, 4.8 Hz, 2H, CH_{arom} Fmoc), 7.39 (t, *J* = 7.4 Hz, 2H, CH_{arom} Fmoc), 7.30 (td, J = 7.5, 1.3 Hz, 2H, CH_{arom} Fmoc), 4.36 (d, *J* = 7.0 Hz, 2H, CH_2 Fmoc), 4.23 (t, *J* = 7.0 Hz, 1H, CH Fmoc), 4.16 (d, *J* = 172.9 Hz, 1H, Cα), 1.77 (d, *J* = 191.4 Hz, 1H, CβH), 1.78-1.23 (m, 2H, CγH_2), 1.23 – 0.56 (m, 6H, Cγ+δH_3) ppm. ^{13}C-NMR (75 MHz, Methanol-*d4*): δ = 175.41 (d, *J* = 58.9 Hz), 145.38 (C_q), 145.19 (C_q), 142.59 (C_q), 128.76 (CH_{aro} Fmoc), 128.16 (CH_{arom} Fmoc), 128.13 (CH_{arom} Fmoc), 126.26 (CH_{arom} Fmoc), 120.89 (CH_{arom} Fmoc), 67.95 (CH_2 Fmoc), 60.12 (dddd, *J* = 58.9, 34.5, 12.5, 3.4 Hz, Cα), 38.34 (q, *J* = 35.0 Hz, Cβ), 26.13 (t, *J* = 34.9 Hz, CγH_2), 16.09 (d, *J* = 35.5 Hz, CγH_3), 11.81 (dt, *J* = 35.0, 2.5 Hz, CδH_3) ppm. LC-MS (4 → 98% ACN/H_2O); R_t = 5.38 min; calculated for [M+H$^+$] 361.12; found 361.18.



*Methyl-3-(glycylthio)-propionate hydrochloride (**26**)*

BocGlyOH (1.1 eq., 11 mmol, 1.93 g) was dissolved in DCM (50mL). Methyl 3-mercaptopropionate (1 eq, 10 mmol, 1.1mL), DMAP (0.1 eq., 1 mmol, 122 mg) and EDC (1.4 eq., 14 mmol, 2.6 g) were added and the mixture was stirred at RT overnight. EtOAc (100 mL) was added to the reaction mixture and washed with 1M HCl (aq.) (2x 100 mL), sat. $NaHCO_3$ (2x 100 mL) and BRINE (100 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was obtained as a white powder. (Crude yield: 2,9 g, 10.46 mmol, 105%). The crude was dissolved in 4M HCl in dioxane (20mL) and stirred for 1h at RT. Et_2O (20 mL) was added to the reaction mixture and Gly-S(CH_2)CO_2Me precipitated from the solution. The product was dissolved in H_2O/ACN (50/50; v/v; 5 mL) and lyophilized. The product was obtained as a white solid. (Yield: 1.69 g, 7,9 mmol, 79%). ^{1}H-NMR (300 MHz, DMSO-*d6*): δ = 8.63 (s, 2H, NH_2), 4.04 (d, *J* = 1.0 Hz, 2H, CαH_2), 3.36 (s, 3H, OCH_3), 3.15 (t, *J* = 6.8 Hz, 2H, CH_2), 2.66 (t, *J*=6.8 Hz, 2H, CH_2) ppm.

Analytical LC-MS data (UV traces, total ion chromatograms and mass spectra) for all for neutron-encoded Fmoc-labelled AA's are available in the online version of the supplementary information of this paper. Top panel: UV traces (λ = 210-800 nm). Middle panel; Total ion chromatogram (*m/z* =100-1600). Bottom panel; Sum spectrum of mass spectra from main peak in chromatogram.

Materials and Methods; Protein Synthesis

General. Solvents and reagents for peptide synthesis were purchased from various suppliers (listed in Table 7) and were used as received. Linear solid phase peptide synthesis of Ub was performed on resin according to an established method described by our group.[13] LC-MS data processing was performed using Waters MassLynx Mass Spectrometry Software 4.2. Deconvoluted mass was obtained from the electrospray ionization mass spectrum envelope (average isotopes) with Maxent1 function. The calculated mass of Ub (derivatives) was obtained with ChemDraw Professional 20.0 (PerkinElmer Informatics, Inc.) by calculating the molecular weight of the complete structure.

Supplementary Table 7| Building blocks, reagents and solvents for diubiquitin synthesis.*

Compound	Abbreviation	CAS#	Source or reference
Building blocks			
Fmoc Gly TentaGel® R Trt resin	Gly-Trt	-	Rapp Polymere GmbH #RA1213
Fmoc Gly TentaGel® R HMPA resin	Gly-HMPA	-	Rapp Polymere GmbH #RA1513
Fmoc Arg(pbf) TentaGel® R HMPA resin	Arg-HMPA	-	Rapp Polymere GmbH #RA1502
Fmoc-L-Valine-OH 13C5,15N1	Fmoc-L-Val $^{13}C_5$ $^{15}N_1$	-	In-house synthesis, this work
Fmoc-L-Leucine-OH 13C6,15N1	Fmoc-L-Leu $^{13}C_6$ $^{15}N_1$	-	In-house synthesis, this work
Fmoc-L-Isoleucine-OH 13C6,15N1	Fmoc-L-Ile $^{13}C_6$ $^{15}N_1$	-	In-house synthesis, this work
Fmoc- L-*S*^{t}Bu γ-thiolysine(Boc)-OH	γ-thioLys	-	In-house synthesis, [Van der Heden van Noort, 2017][14]
Boc-L-*S*^{t}Bu γ-thionorleucine-OH	thioNle	-	In-house synthesis, [Xin, 2018][1]
Methyl-3-(glycylthio)-propionate hydrochloride	HCl·H-Gly-S$(CH_2)_2CO_2$Me	-	In-house synthesis, this work
Chemicals			
Piperidine	-	110-89-4	Carlo Erba Reagents #P0663516
(Benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate)	PyBOP	128625-52-5	NovaBiochem #851009
***N,N*-Diisopropylethylamine**	DiPEA	7087-68-5	VWR #84574.290

Compound	Abbreviation	CAS#	Source or reference
Trifluoroacetic acid	TFA	76-05-1	Biosolve #20233320
2,2′-(Ethylenedioxy)diethanethiol	DODt	14970-87-7	SigmaAldrich #465178
Triisopropylsilane	$i\mathrm{Pr}_3\mathrm{SiH}$	6485-79-6	SigmaAldrich #233781
Phenol	PhOH	108-95-2	SigmaAldrich #328111
Di-*tert*-butyldicarbonate	$\mathrm{Boc}_2\mathrm{O}$	24424-99-5	Chem-Impex #00128-100G
1,1,1,3,3,3-hexafluoroisopropylalcohol	HFIP	920-66-1	Chem-Impex #00080
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride	EDC	25952-53-8	Combi blocks #SS-7536
1-hydroxybenzotriazol hydradrate	$\mathrm{HOBt{\cdot}H_2O}$	123333-53-9	Biosolve #081233
Guanidine hydrochloride	Gnd·HCl	50-01-1	Sigma Aldrich #50950-2.5 KG
Sodium phosphate dibasic dihydrate	$\mathrm{Na_2HPO_4}$	10028-24-7	Fluka #71645-1 KG
Tris(2-carboxyethyl)phosphine hydrochloride	TCEP·HCl	51805-45-9	Combi blocks #OR-5119
4-Mercaptophenylacetic acid	MPAA	39161-84-7	Chem-Impex #28402
Glutathione (reduced)	GSH	70-18-8	Chem-Impex #00159
2,2'-Azobis[2-(2-imidazolin-2-yl) propane] dihydrochloride	VA-044	2997-92-4	Combi blocks #QC-7454
Propargylamine	PA	2450-71-7	SigmaAldrich #P50900
Triethylamine	TEA	121-44-8	SigmaAldrich #471283
Urea		57-13-6	Bio-Connect, #SC-29114B
Copper(II) sulfate pentahydrate	$\mathrm{CuSO_4{\cdot}5H_2O}$	7758-99-8	SigmaAldrich #61245
Sodiumascorbate		134-03-2	ChemImpex #01436
2-amino-2-(hydroxymethyl)propaan-1,3-diol	TRIS	77-86-1	Biosolve #20097789
Sodium Chloride	NaCl	7647-14-5	Sigma Aldrich #S9625
Solvents			
Dichloromethane (AR)	DCM	75-09-2	VWR #23 366 327
***N*-Methyl-pyrrolidone (PEPTIDE)**	NMP	872-50-4	VWR #84 572 320

Compound	Abbreviation	CAS#	Source or reference
Diethyl ether (AR)	Et_2O	60-29-7	Biosolve #5280501
***n*-Pentane**	Pentane	109-66-0	Biosolve #16050502
Dimethyl sulfoxide (AR)	DMSO(AR)	67-68-5	Biosolve #4470501
Acetonitrile (ULC-MS)	CH_3CN (ULC-MS)	75-05-8	Biosolve #1204102
Formic Acid (ULC-MS)	FA (ULC-MS)	64-18-6	Biosolve #6914143
Acetonitrile (HPLC-R)	CH_3CN (AR)	75-05-8	VWR #83 639 320
Formic Acid (AR)	FA (AR)	64-18-6	Fisher Scientific #147932500
Methanol	MeOH		VWR #20847307
1,2-Dichloroethane	DCE	107-06-2	Biosolve #4050602

[*] *Peptide building blocks are listed in Supplementary Table 8.*

Solid Phase Peptide Synthesis (SPPS)

SPPS was performed on a Syro II Multisyntech Automated Peptide synthesizer (SYRO robot; Part Nr: S002PS002; MultiSyntech GmbH, Germany) under inert gas (N_2) application, using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry on a 10 or 25 µmol scale. A fourfold excess of amino acids relative to pre-loaded Fmoc amino acid trityl resin or HMPA resin (between 0.17 and 0.20 mmol/g, Rapp Polymere, Germany) was used. Ubiquitin variants on resin were prepared by linear SPPS as described previously[13]. Some optimizations were made in the synthesis protocol. Optimization of the procedure led to discarding of the capping step. Optimization also led to the equalizing of all cycles (equivalents reagents, time etc.) except for the coupling cycles of isotope-labeled amino acids. Briefly, Fmoc-glycine-loaded TentaGel® R trityl resin (Rapp Polymere, Germany, #RA1213), Fmoc-glycine-loaded TentaGel® R HMPA resin (Rapp Polymere, Germany, #RA1513) or Fmoc-Arg(Pbf)-loaded TentaGel® R HMPA resin (Rapp Polymere, Germany, #RA1502) was washed with DCM (1x 5 mL) and swelled with NMP (1x 1250 µL) for 5 minutes prior to further modifications. Fmoc-protecting groups were removed by incubating three times with 20% piperidine/NMP (v/v) for 2, 2 and 5 minutes. Resin was washed with NMP (5x 1100 µL). Fmoc-protected amino acids to-be-coupled (4 eq.) were preactivated with PyBOP (4 eq.) and DIPEA (8 eq.) in NMP. Deprotected resin was incubated twice for 25 minutes with the preactivated mixture, washed with NMP (3x 1100 µL) after the second coupling step and Fmoc removal was performed as described above. This procedure was repeated for each amino acid coupling cycle, with a total of 69, 68 or 67 cycles. Coupling cycles with isotope-labelled amino acids were extended to two times 60 minutes and only three equivalents of Fmoc-protected amino acid was used, PyBOP (4eq.) and DIPEA (8eq.) stayed unchanged. Details on acid-labile side chain protecting groups (PG) and coupling of Fmoc-protected (di)peptide building blocks are provided in Supplementary Table 7 and Supplementary Table 8.

Supplementary Table 8| Fmoc protected L-amino acids and dipeptides

AA	Reagent	CAS#	Cat# *
A	Fmoc-L-Ala-OH	35661-39-3	852003
R	Fmoc-L-Arg(Pbf)-OH	154445-77-9	852067
N	Fmoc-L-Asn(Trt)-OH	132388-59-1	852044
D	Fmoc-L-Asp(OtBu)-OH	71989-14-5	852005
Q	Fmoc-L-Gln(Trt)-OH	132327-80-1	852045
E	Fmoc-L-Glu(OtBu)-OH	71989-18-9	852009
G	Fmoc-Gly-OH	29022-11-5	852001
H	Fmoc-L-His(Trt)-OH	109425-51-6	852032
I	Fmoc-L-Ile-OH	71989-23-6	852010
L	Fmoc-L-Leu-OH	35661-60-0	852011
K	Fmoc-L-Lys(Boc)-OH	71989-26-9	852012
Nle	Fmoc-L-Nle-OH	77284-32-3	852014
F	Fmoc-L-Phe-OH	35661-40-6	852016
P	Fmoc-L-Pro-OH	71989-31-6	852017
S	Fmoc-L-Ser(tBu)-OH	71989-33-8	852019
T	Fmoc-L-Thr(tBu)-OH	71989-35-0	852000
Y	Fmoc-L-Tyr(tBu)-OH	71989-38-3	852020
V	Fmoc-L-Val-OH	68858-20-8	852021
AG	Fmoc-L-Ala-(Dmb)Gly-OH	-	852108
DG	Fmoc-L-Asp(OtBu)- (Dmb)Gly-OH	900152-72-9	852115
IT	Fmoc-L-Ile-L-Thr($\Psi^{Me,Me}$pro)-OH	957780-52-8	852193
LS	Fmoc-L-Leu-L-Ser($\Psi^{Me,Me}$pro)-OH	339531-50-9	852179
LT	Fmoc-L-Leu-L-Thr($\Psi^{Me,Me}$pro)-OH	955048-89-2	852184
ST	Fmoc-L-Ser(tBu)-L-Thr($\Psi^{Me,Me}$pro)-OH	-	852192

** Brand: Novabiochem. Supplier: Merck*

General procedure for trial cleavage

A small amount of resin was parted from the reaction mixture and washed with DCM and Et_2O. The resin was air-dried, and incubated with 'fast' trial cleavage mix (TFA/H_2O/DODT/*i*Pr_3SiH; 92.5/2.5/2.5/2.5; v/v/v/v; 100 µL) and shaken for 30 minutes at 37 °C. Samples were transferred to a filter tip and filtered. The reaction mixture (filtrate) was collected in cold Et_2O/*n*-pentane (3/1; v/v; 1.5 mL) to precipitate the product. The suspension was centrifuged and the supernatant was decanted. The precipitate was resuspended twice in cold Et_2O, centrifuged and Et_2O was decanted. The remaining Et_2O was removed by submitting to a gentle air flow. The solid

material was dissolved in DMSO (50 µL), the DMSO solution (2 µL) was diluted in 0.1% aqueous formic acid (80 µL) and reaction progress was analysed by LC-MS. *System 1 – Gradient 1*.

LC-MS analysis of trial cleavages, crude reaction mixtures and purification fractions

LC-MS analysis of crude reaction mixtures and purification fractions were performed on a Waters ACQUITY UPLC H-class+ System equipped with Waters ACQUITY Quaternary Solvent Manager (QSM), Waters ACQUITY FTN-H AutoSampler and Waters ACQUITY UPLC Photodiode Array (PDA) eλ Detector (λ = 210-800 nm), Waters ACQUITY UPLC Protein BEH C4 Column (300 Å, 1.7 µm, 2.1 x 50 mm) and LCT Premier Orthogonal Acceleration Time of Flight Mass Spectrometer (*m/z* = 100-1600) in ES+ mode (System 1). Samples were run with a 1.6 minute gradient (run time 3 min) using three mobile phases; 100% H_2O, 100% CH_3CN and 50% H_2O + 50% CH_3CN + 2.5% FA (flow rate = 0.5 mL/min).

LC-MS - System 1 - Gradient 1:

Time (min)	100% H_2O (%)	100% CH_3CN (%)	50% H_2O + 50% CH_3CN + 2.5% FA(%)
0.00	94.0	2.0	4.0
0.20	94.0	2.0	4.0
1.80	0.0	96.0	4.0
2.15	0.0	96.0	4.0
2.20	94.0	2.0	4.0
3.00	94.0	2.0	4.0

Pure products were run with a 7 minute gradient (run time 10 min) using three mobile phases: 100% H_2O, 100% CH_3CN and 50% H_2O + 50% CH_3CN + 2.5% FA (flow rate = 0.5 mL/min).

LC-MS – System 1 - Gradient 2:

Time (min)	100% H_2O (%)	100% CH_3CN (%)	50% H_2O + 50% CH_3CN + 2.5% FA(%)
0.00	94.0	2.0	4.0
0.50	94.0	2.0	4.0
7.50	0.0	96.0	4.0
8.00	0.0	96.0	4.0
8.10	94.0	2.0	4.0
10.00	94.0	2.0	4.0

LC-MS analysis of purified mono- and diubiquitins

LC-MS analysis of purified mono- and diubiquitins and the described assay were performed on a Waters Acquitiy H-Class UPLC system equipped with a Waters ACQUITY Quaternary Solvent Manager (QSM) and Waters ACQUITY FTN AutoSampler. Separation was achieved on a Waters Acquity UPLC Protein BEH C4 column, 300Å, 1,7 uM (2.1 x 50 mm); flow rate = 0.6 mL/min , runtime = 4.55 min, column T = 60°C using 2 mobile phases: A = 0,1% formic acid in water and B = 0,1% formic acid in CH_3CN. Mono- and diubiquitins were separated at baseline level and eluted using a shallow gradient focused from 26% 30% B over 1 minute. The products were analysed by intact MS analysis (MS1) and masses were detected in a range from 550-2000 Da from 2.51 - 4.50 min and were recorded on a Waters XEVO-G2 XS Q-Tof mass spectrometer equipped with an electrospray ion source in positive mode (Capillary Voltage: 0.5 kV, desolvation gas flow: 900 L/h, desolvation gas temperature: 500°C, source temperature: 130 °C, probe angle: 9.5) with a resolution of *R* = 22,000 (System 2).

LC-MS – System 2 - Gradient 1: diubiquitin gradient

Time (min)	Flowrate (mL/min)	100% H_2O (%) +0.1% FA	100% CH_3CN (%) +0.1% FA
0.00	0.6	98.0	2.0
0.20	0.6	98.0	2.0
0.70	1.0	98.0	2.0
1.80	1.0	98.0	2.0
2.30	0.6	98.0	2.0
2.50	0.6	74.0	26.0
2.83	0.6	74.0	26.0
3.83	0.6	70.0	30.0
3.90	0.6	0.0	100.0
4.30	0.6	0.0	100.0
4.35	0.6	98.0	2.0
4.55	0.6	98.0	2.0

In between assay runs, the column was washed using a run with the same gradient without the column flush with a 1 minute gradient focused from 26% → 30% B (run time 2.20 min) using two mobile phases: 100% H_2O + 0.1% FA and 100% CH_3CN + 0.1% FA (flow rate = 0.6 mL/min) and masses were detected in a range from 550-2000 Da during the entire run time.

LC-MS – System 2 - Gradient 2: diubiquitin wash run

Time (min)	Flowrate (mL/min)	100% H_2O (%) +0.1% FA	100% CH_3CN (%) +0.1% FA
0.00	0.6	98.0	2.0
0.05	0.6	74.0	26.0
0.33	0.6	74.0	26.0
1.33	0.6	70.0	30.0
1.40	0.6	0.0	100.0
1.80	0.6	0.0	100.0
1.85	0.6	98	2.0
2.20	0.6	98	2.0

Pure products were run with a 7 minute gradient (run time 10 min) using three mobile phases two mobile phases: 100% H_2O + 0.1% FA and 100% CH_3CN + 0.1% FA (flow rate = 0.5 mL/min).

LC-MS – System 2 - Gradient 3: Analytical run

Time (min)	Flowrate (mL/min)	100% H_2O (%) +0.1% FA	100% CH_3CN (%) +0.1% FA
0.00	0.5	96.0	4.0
0.50	0.5	96.0	4.0
7.50	0.5	2.0	98.0
8.00	0.5	2.0	98.0
8.10	0.5	96.0	4.0
10.00	0.5	96.0	4.0

RP-HPLC purification

System 1. RP-HPLC purifications (max. 20 mL/run) were performed on a Waters HPLC equipped with a Waters 2489 UV/Vis detector, Waters fraction collector III and Waters XBridge BEH C18 OBD Prep Column (130 Å, 5 µm, 30 × 150 mm). Samples were run with a 13 or 23 minutes gradient detailed below (run time 25 or 35 minutes) at a flowrate = 37.5 mL/min. Mobile phase: A = H_2O, B = CH_3CN and C = 1% TFA in H_2O. Fraction collection was triggered by UV intensity (λ = 210 nm).

RP-HPLC – System 1 - Gradient 1:

Time (min)	H_2O (%)	CH_3CN (%)	1% TFA in H_2O (%)
0.00	90.0	5.0	5.0
5.00	90.0	5.0	5.0
7.00	75.0	20.0	5.0
18.00	50.0	45.0	5.0

Time (min)	H_2O (%)	CH_3CN (%)	1% TFA in H_2O (%)
18.50	0.0	95.0	5.0
21.50	0.0	95.0	5.0
21.60	90.0	5.0	5.0
25.00	90.0	5.0	5.0

RP-HPLC – System 1 - Gradient 2 (optimized):

Time (min)	H_2O (%)	CH_3CN (%)	1% TFA in H_2O (%)
0.00	90.0	5.0	5.0
6.00	90.0	5.0	5.0
7.00	85.0	10.0	5.0
10.00	70.0	25.0	5.0
22.00	60.0	35.0	5.0
30.00	25.0	70.0	5.0
30.10	0.0	95.0	5.0
32.00	0.0	95.0	5.0
32.10	90.0	5.0	5.0
35.00	90.0	5.0	5.0

System 2. RP-HPLC purifications (max. 5 mL/run) were performed on a Shimadzu LC-20AT HPLC system equipped with a Shimadzu SPD-20A UV/Vis detector, a Shimadzu FRC-10A fraction collector and a Waters XBridge BEH C18 OBD Prep Column (130 Å, 5 µm, 10 × 150 mm) was used. Samples were run with a 15 or 23 minute gradient detailed below (run time 25 or 35 minutes) at a flowrate = 4.00 or 6.50 mL/min. Mobile phase: A = 0.05% TFA in H_2O and B = 0.05% TFA in CH_3CN. T = 40 °C.

RP-HPLC – System 2 - Gradient 1:

Time (min)	0.05% TFA in H_2O (%)	0.05% TFA in CH_3CN (%)	Flow rate (mL/min)
0.00	95.0	5.0	4.00
6.00	95.0	5.0	4.00
7.00	90.0	10.0	6.50
10.00	75.0	25.0	6.50
22.00	50.0	50.0	6.50
22.10	5.0	95.0	6.50
24.00	5.0	95.0	6.50
24.10	95.0	5.0	6.50
25.00	95.0	5.0	6.50

RP-HPLC – System 2 - Gradient 2:

Time (min)	0.05% TFA in H_2O (%)	0.05% TFA in CH_3CN (%)	Flow rate (mL/min)
0.00	95.0	5.0	4.00
6.00	95.0	5.0	4.00
7.00	90.0	10.0	6.50
10.00	75.0	25.0	6.50
22.00	65.0	35.0	6.50
30.00	30.0	70.0	6.50
30.10	5.0	95.0	6.50
32.00	5.0	95.0	6.50
32.10	95.0	5.0	6.50
35.00	95.0	5.0	6.50

Synthesis of Ub(1-76, Nle_1, γ-thioLysxx, xx V*, xx L*, xx I*) (**1 a-g**)

Step 1. SPPS

See SPPS procedure with extended coupling times.

Sequences neutron-encoded Ub1-76 ^{Met1Nle} (Kxx = γ-thioLys)

See table Supplementary Table 1.

69 cycles on Fmoc-Gly-loaded TentaGel® R HMPA resin (Rapp Polymere, Germany, #RA RA1513) on 10 µmol scale. To check the quality of the SPPS product a trial cleavage was performed.

Step 2. Global deprotection

Global deprotection was performed as described previously.[15] The resin-bound polypeptide Ub(1-76, Nle_1, γ-thioLysxx, xx V*, xx L*, xx I*)(PG) **6a-g** was deprotected and detached from the resin by treatment with TFA/H_2O/Phenol/iPr_3SiH (90.5/5/2.5/2; v/v/v/v; 7.5 mL) for 2.5-3.5 hours at room temperature under gentle shaking. The reaction mixture was filtered directly into ice-cold Et_2O/*n*-pentane (3/1; v/v; 35 mL) and the resin was washed with TFA (2x 4mL). The mixture of Et_2O/*n*-pentane and filtrate was centrifuged (1500 rpm, 5 min, 4 °C) and the supernatant was decanted. The pellet was washed three times by resuspension in Et_2O (20 mL), spinning down by centrifuge (1500 rpm, 5 min, 4 °C) and removal of the supernatant. The pellet was dissolved in H_2O/CH_3CN/formic acid (65/35/10; v/v/v; 15 mL) and lyophilized. The protein was subsequently purified using RP-HPLC.

RP-HPLC purification

The crude monoubiquitin was properly dissolved in a minimal amount of DMSO (max. 10 vol% of the final volume) while heated carefully. The DMSO was added dropwise into H_2O

(10 to 20 mL). The pH was checked and should be below 7. The mixture was centrifuged (5 min @3800 rpm). The supernatant was filtered and purified by RP-HPLC.
See *RP-HPLC – System 2 – Gradient 1.*
Pure fractions (checked by LC-MS) were pooled and lyophilized to obtain the product as a white powder.

Ub(1-76, Nle1, γ-thioLysxx, xx V*, xx L*, xx I*) (**1 a-g**)
The products were obtained as white solids. LC-MS analysis using *System 2 - Gradient 3*.

Yields:
Ub(1-76, Nle_1, γ-thioLys6, 1x V*, 1x L*) **1a** = 34.36 mg, 3.96 µmol, 39.6%. LC-MS: R_t 3.18 min: MS ES+ (amu) calculated: 8679,96 Da[M]; found: 8680 Da.
Ub(1-76, Nle_1, γ-thioLys11, 3x V*, 1x I*) **1b** = 21.51 mg, 2.47 µmol, 16.5%. LC-MS: R_t 3.18 min: MS ES+ (amu) calculated: 8691.87 Da[M]; found 8692 Da.
Ub(1-76, Nle_1, γ-thioLys27, 3x V*, 2x L*, 1x I*) **1c** = 17.99 mg, 2.07 µmol, 13.8%. LC-MS: R_t 3.28 min: MS ES+ (amu) calculated: 8705.77 Da[M]; found 8706 Da.
Ub(1-76, Nle_1, γ-thioLys29, 3x V*, 2x L*, 3x I*) **1d** = 22.28 mg, 2.55 µmol, 17.0%. LC-MS: R_t 3.25 min: MS ES+ (amu) calculated: 8719.66 Da[M]; found 8720 Da.
Ub(1-76, Nle_1, γ-thioLys33, 3x V*, 4x L*, 3x I*) **1e** = 23.31 mg, 2.67 µmol, 17.8%. LC-MS: R_t 3.22 min: MS ES+ (amu) calculated: 8766.56 Da[M]; found 8734 Da.
Ub(1-76, Nle_1, γ-thioLys48, 3x V*, 4x L*, 5x I*) **1f** = 23.5 mg, 3.53 µmol, 23.5%. LC-MS: R_t 3.19 min: MS ES+ (amu) calculated: 8747.45 Da[M]; found 8747 Da.
Ub(1-76, Nle_1, γ-thioLys63, 3x V*, 6x L*, 5x I*) **1g** = 27.90 mg, 3.18 µmol, 21.2%. LC-MS: R_t 3.22 min: MS ES+ (amu) calculated: 8761.34 Da[M]; found 8761 Da.

Analytical LC-MS data (Total ion chromatogram and mass spectra) for all synthetic mono-ubiquitins are available in the online version of the supplementary information of this paper. Top panel: Total ion chromatogram (*m/z* =550-2000). Middle panel; mass spectra. Bottom panel; deconvoluted mass spectra.

Synthesis of Ub(1-76, thioNle_1, 4x V*, 6x L*, 6x I*) (**2**)
Step 1. SPPS
See SPPS procedure with extended coupling times.
Sequences neutron-encoded Ub2-76
See Supplementary table 1.
68 cycles on Fmoc-Gly-loaded TentaGel® R HMPA resin (Rapp Polymere, Germany, #RA RA1513) on 10 µmol scale. To check the quality of the SPPS product a trial cleavage was performed.

Step 2. ThioNle coupling

PyBOP (4 eq., 20.9 mg, 40.12 µmol) was dissolved in NMP (100 µL). ThioNle (1.4 eq., ~14 mg, 39.82 µmol) was dissolved in NMP (200 µL). Both solutions were added to resin-bound Ub (2-76, 4x V*, 6x L*, 6x I*) **8**. DiPEA (8 eq., 10.3 mg, 79.79 µmol, 13.9 µL) was dissolved in NMP (60 µL) and this solution was also added to the resin. The reaction mixture was shaken overnight. To check the reaction progress by LC-MS a trial cleavage was performed. The reaction solution was filtered from the resin and the resin was washed three times with DCM and MeOH alternately, three times with DCM and Et_2O alternately and three times with Et_2O. The resin was either resubmitted to coupling conditions until reaction completion was achieved or submitted to global deprotection conditions.

Step 3. Global deprotection

The resin-bound polypeptide Ub(1-76, ThioNle1, 4x V*, 6x L*, 6x I*)(PG) **10** was deprotected and detached from the resin by treatment with TFA/H_2O/Phenol/iPr3SiH (90.5/5/2.5/2; v/v/v/v; 2 mL) for 2.5-3.5 hours at room temperature under gentle shaking. The reaction mixture was filtered directly into ice-cold Et_2O/*n*-pentane (3/1; v/v; 15 mL) and the resin was washed with TFA (2x 2 mL).The mixture of Et_2O/*n*-pentane and filtrate was centrifuged (1500 rpm, 5 min, 4 °C) and the supernatant was removed. The pellet was washed with Et_2O (3 x 15 mL), the solution was vortexed, the suspension was centrifuged and the Et_2O was removed. Wash step was repeated twice. The pellet was dissolved in H_2O/CH_3CN/formic acid (65/25/10; v/v/v; 5 mL) and lyophilized. The protein was subsequently purified using RP-HPLC.

RP-HPLC purification

The crude monoubiquitin was properly dissolved in a minimal amount of DMSO (max. 10 vol% of the final volume) under careful heating. The DMSO was added dropwise into H_2O (10 to 20 mL). The pH was checked and should be below 7. The mixture was centrifuged (5 min @3800 rpm). The supernatant was filtered and purified by RP-HPLC.

RP-HPLC – System 2 – Gradient 1.

Pure fractions (checked by LC-MS) were pooled and lyophilized to obtain the product as a white powder.

<u>Ub(1-76, ThioNle1, 4x V*, 6x L*, 6x I*) (**2**)</u>

The product was obtained as white solid. LC-MS analysis using *System 2 - Gradient 3*.

Yield:

Ub(1-76, ThioNle1, 4x V*, 6x L*, 6x I*) **2 =** 5.52 mg, 0.63 µmol, 6.3%. LC-MS: R_t 3.24 min: MS ES+ (amu) calculated: 8774.246 Da[M]; found 8774 Da.

Analytical LC-MS data (Total ion chromatogram and mass spectra) for all synthetic mono-ubiquitins are available in the online version of the supplementary information of this paper. Top panel: Total ion chromatogram (*m/z* =550-2000). Middle panel; mass spectra. Bottom panel; deconvoluted mass spectra.

Synthesis of Ub(1-76, Nle_1)-$S(CH_2)_2CO_2Me$ (**3**)

Step 1. SPPS

See SPPS procedure.

Sequence Ub1-75^{Met1Nle}

(Nle)QIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRG

68 cycles on Fmoc-Gly-loaded TentaGel® R trityl resin (Rapp Polymere, Germany, #RA RA1213) on 25 µmol scale. To check the quality of the SPPS product a trial cleavage was performed.

Step 2. Boc-protection

Resin-bound H-Ub(1-75, Nle_1)(PG) **7** was washed with DCM (3x 5 mL) and NMP (3x 5 mL) alternately and with DCM (3x 5 mL). Boc2O (4 eq., 21.83 mg, 100 µmol, 23 µL) and DiPEA (15 eq., 48.47 mg, 375 µmol, 65.3 µL) were dissolved in DCM (1 mL). This solution was added to the resin-bound H-Ub(1-75, Nle_1) and the mixture was shaken for 3 hours at room temperature. After 3 hours, the liquid was removed and the resin was washed with NMP (3x 5 mL) and DCM (3x 5 mL) alternately followed by washing with DCM (3x 5 mL) and MeOH (3x 5 mL) alternately.

Step 3. Cleavage from resin

Resin cleavage was performed as described previously.[13] Resin-bound Boc-Ub(1-75, Nle_1)(PG) was cleaved from the trityl resin using a solution of hexafluoroisopropanol (HFIP) in DCM (1/4; v/v; 2.5 mL). The resin was treated twice for 20 minutes at room temperature and the filtrate was collected. The resin was rinsed two times with DCM in between HFIP treatments. All combined filtrates were concentrated under reduced pressure. Residual HFIP was removed by co-evaporation with DCE (3x 12 mL) (to prevent formation of HFIP ester in next steps) and the product lyophilized overnight.

Step 4. Gly-thioester coupling

The protected protein was dissolved in DCM (4 mL) and EDC (3 eq., 14.4 mg, 75 µmol), HOBt (3 eq., 10.1 mg, 75 µmol) and HCl·H-Gly-$S(CH_2)_2CO_2Me$ (3 eq., 16 mg, 75 µmol) were added to the solution. The reaction mixture was stirred for 16 hours. To evaluate the reaction progress by LC-MS, a trial cleavage was performed. The reaction mixture was concentrated

under reduced pressure and the residue was either resubmitted to coupling conditions until reaction completion was achieved or submitted to global deprotection conditions.

Step 5. Global deprotection

Global deprotection was performed as described previously.[15] Ub(1-76, Nle_1)(PG)-$S(CH_2)_2CO_2Me$ **9** was treated with a freshly prepared solution of TFA/H_2O/Phenol/iPr_3SiH (90.5/5/2.5/2; v/v/v/v; 5 mL) for 2.5-3.5 hours at room temperature to remove all protecting groups from all amino acid sidechains. The cleavage mixture was added dropwise to an ice-cold Et_2O/*n*-pentane (3/1; v/v; 20 mL) mixture to precipitate the protein. The suspension was centrifuged (1500 rpm, 5 min, 4 °C) and the Et_2O/ *n*-pentane (supernatant) was removed. The pellet was resuspended in Et_2O (20 mL), the suspension was vortexed, centrifuged (1500 rpm, 5 min, 4 °C) and the Et_2O was removed. The wash step was repeated twice. The remaining solvent was removed by a gentle N_2 flow over the pellet. The solid crude material was dissolved in H_2O/CH_3CN/formic acid (65/25/10; v/v/v; 10 mL) and lyophilized. The crude material was subsequently purified using preparative RP-HPLC.

RP-HPLC purification

The crude monoubiquitin was properly dissolved in a minimal amount of DMSO (max. 10 vol% of the final volume) under careful heating. The DMSO solution was added dropwise into H_2O (10 to 20 mL). The pH was checked and should be below 7. The mixture was centrifuged (5 min @3800 rpm). The supernatant was filtered and purified by RP-HPLC.
See *RP-HPLC – System 1 - Gradient 1.*
Pure fractions (>95%, checked by LC-MS) were pooled and lyophilized to obtain the product as a white powder.

Ub(1-76, Nle_1)-$S(CH_2)_2CO_2Me$ (**3**)

The product was obtained as white solid. LC-MS analysis using *System 2 - Gradient 3*.

Yield:
Ub(1-76, Nle_1)-$S(CH_2)_2CO_2Me$ **3** = 57.53 mg, 6.65 µmol, 26.6%. LC-MS: R_t 3.13 min: MS ES+ (amu) calculated: 8649.0 Da[M]; found 8649 Da.

Analytical LC-MS data (Total ion chromatogram and mass spectra) for all synthetic monoubiquitins are available in the online version of the supplementary information of this paper. Top panel: Total ion chromatogram (*m/z* =550-2000). Middle panel; mass spectra. Bottom panel; deconvoluted mass spectra.

Native Chemical Ligation of Ub(1-76, Nle_1)-$S(CH_2)_2CO_2Me$ (**3**) and Ub(1-76, Nle_1, γ-thioLysxx, xx V*, xx L*, xx I*) (**1a-g**)

Native Chemical Ligation

Ub(1-76, Nle_1, γ-thioLysxx, xx V*, xx L*, xx I*) (1 a-g, 1 eq., 10.0 mg, 1.10 μmol) was dissolved in DMSO (50 μL) and added dropwise to aqueous 8 M Gnd·HCl and 0.2 M Na_2HPO_4 pH 7.55 (175 μL). 1 M aqueous TCEP solution pH 7.0 (25 μL) was added. This solution was pre-incubated for 180 min. and the disulfide bond cleavage was monitored by LC-MS analysis (Program 1). Ub(1-76, Nle_1)-$S(CH_2)_2CO_2Me$ (3, 1.5 eq., 15.0 mg, 1.73 μmol) was dissolved in DMSO (37.5 μL) and added dropwise to aqueous 8 M GndHCl and 0.2 M Na_2HPO_4 pH 7.55 (130 μL). 1 M aqueous MPAA solution pH 7.0 (137.5 μL) was added. The solution was pre-incubated for 5 minutes. The thioLys 1a-g and the thioester 3 containing solutions were properly mixed and the pH of the reaction mixture was adjusted to pH 7.33 by the addition of 35 μL 10% Na_2CO_3 in H_2O solution. The reaction mixture was flushed with argon and shaken for 16h at 37 °C. The reaction progress was checked by LC-MS analysis (Program 1). The formed diUb was purified by RP-HPLC.

RP-HPLC purification

To prepare the sample, the reaction mixture was added dropwise to 3.76 mL aqueous buffer containing 6.0 M Gnd·HCl and 0.15 M Na_2HPO_4. This solution was diluted with water to 14 mL. 1 M aqueous TCEP solution pH 7.0 (250 μL) was added. The pH was checked and adjusted below 7 with 1 M aqueous HCl solution. The mixture was centrifuged (5 min @ 3800 rpm), filtered and purified by RP-HPLC.

See *RP-HPLC – System 2 - Gradient 2.*

Pure fractions (>95%, checked by LC-MS) were pooled and lyophilized, dissolved in H_2O/CH_3CN/formic acid (65/25/10; v/v/v; 15 mL) and lyophilized again. The product was obtained as white powder.

Native Chemical Ligation of Ub(1-76, Nle_1)-$S(CH_2)_2CO_2Me$ (**3**) and Ub(1-76, ThioNle1, 4x V*, 6x L*, 6x I*) (**2**)

Native Chemical Ligation

The Native Chemical Ligation reaction to create a linear diUb molecule was described previously.[1] Briefly, Ub(1-76, ThioNle1, 4x V*, 6x L*, 6x I*) (2) (5 mg) was dissolved in an aqueous buffer containing 8 M Gnd·HCl and 0.2 M Na_2HPO_4 pH 7.55 (100 μL). 1 M aqueous TCEP solution pH 7.0 (12.5 μL) was added to the solution to reduce the disulfide bond on the ThioNle side chain. This solution was pre-incubated for 1h and disulfide bond reduction was monitored by LC-MS. Ub(1-76, Nle_1)-S-$(CH_2)_2CO_2Me$ (3) (1.5 eq., 7.5 mg) was dissolved in 151.9 μL of aqueous 8 M Gnd·HCl and 0.2 M Na_2HPO_4 pH 7.55 and 46.9 μL of 1 M MPAA solution

was added. This solution was pre-incubated for 5 minutes. Both solutions were properly mixed and the pH of the reaction mixture was adjust to pH 7.45 by the addition of 22 µL 10% Na_2CO_3 in H_2O solution. The reaction mixture was shaken overnight at 37 °C. The progress of the reaction was checked by LC-MS analysis. The formed diUb was purified by RP-HPLC.

RP-HPLC purification

To prepare the sample, the reaction mixture was added dropwise to 2.5 mL aqueous buffer containing 6 M Gnd·HCl and 0.15 M Na_2HPO_4. This solution was diluted with water to 10 mL. 1 M aqueous TCEP solution pH 7.0 (125 µL) was added. The pH was checked and adjusted below 7. The mixture was centrifuged (5 min @ 3800 rpm), filtered and purified by RP-HPLC on the Shimadzu. Pure fractions (checked by LC-MS) were pooled and lyophilized, dissolved in H_2O/CH_3CN/formic acid (65/25/10; v/v/v; 15 mL) and lyophilized again. The product was obtained as white powder.

See *RP-HPLC – System 2 - Gradient 2.*

Thiol-containing linear diUb (4x V*, 6x L*, 6x I*) (**12**)

The product was obtained as white solid. LC-MS analysis using *System 1 - Gradient 1*.

Yield:

Thiol-containing linear diUb (4x V*, 6x L*, 6x I*) **12** = 3.89 mg, 0.22 µmol, 38.9%. LC-MS: R_t 2.00 min: MS ES+ (amu) calculated: 17215 Da[M]; found 17214 Da.

Desulfurization

Desulfurization

The desulfurization reaction was described previously.[1,15] Briefly, thiol-containing diUb was dissolved in aqueous buffer containing 6 M Gnd·HCl, 0.15 M Na_2HPO_4 and 0.25 M TCEP at pH 7.0 to a concentration of 1 mg/mL protein. Reduced glutathione (GSH) was added to the solution to a concentration of 100 mM. The pH of the solution was adjust to 7.20 by the addition of 400 µL of 10% Na_2CO_3 solution. VA-044 was added to the solution to a final concentration of 75 mM. The reaction mixture was flushed with argon and shaken overnight at 37 °C. The progress of the reaction was checked by LC-MS analysis (Program 1). The desulfurized diUb was purified by RP-HPLC.

RP-HPLC purification

The reaction mixture was diluted with water (same amount as the reaction volume) and 1 M NaOAc/AcOH buffer (40 vol% of the reaction volume). The sample diluted with MQ to 10 mL, the pH was checked and adjusted below 7, the sample was centrifuged (5 min @ 3800 rpm)

and filtered, before it was purified by RP-HPLC on the Shimadzu HPLC. Pure fractions (checked by LC-MS) were pooled and lyophilized, dissolved in H_2O/CH_3CN/formic acid (65/25/10; v/v/v; 15 mL) and lyophilized again. The product was obtained as white powder.
See *RP-HPLC – System 2 - Gradient 2.*

Size exclusion

The products were purified by gel filtration using a Biorad NGC Chromotography system on a size exclusion HiLoad® 16/600 Superdex® 75 pg GE healthcare column with a volume bed of 120 mL and 3-70 kDa separation range using a filtered aqueous buffer containing 50 mM TRIS·HCl and 20 mM NaCl at pH 7.55 at a flowrate of 1 mL/min. The sample was prepared by dissolving the product in DMSO (250 µL), dropwise addition of this solution to MilliQ (2450 µL) and dropwise addition of 10x TRIS buffer (300 µL). The mixture was centrifuged for 5 min @3500 rpm. The fractions were analysed by SDS-PAGE analysis and LC-MS and pure fractions were pooled. The products were obtained as colorless solutions containing 50 mM TRIS·HCl and 20 mM NaCl buffer at pH 7.55. LC-MS analysis (*System 2 - Gradient 3*) was done to check the purity. Pure fractions were combined and concentrated using 3 kDa MWCO Amicon Ultra spin filters.

Concentration determination

To determine the concentration of the solutions (and the yield), these solutions were together with a concentration series of monoubiquitin (0.5 µg, 1 µg, 2 µg, 4 µg per lane), resolved by gel electrophoreses, stained with InstantBlue™ Staining and scanned. The concentration of the solution was determined by quantification of the bands using a GE Healthcare Amersham Imager 600 with ImageQuant TL 8.1 GE Healthcare lifesciences software.

Protein	Stock concentration (mg/mL)	Stock concentration (µM)	Amount
Neutron-encoded K6 diUb (**4a**)	0.44	25.75	1.5 mL
Neutron-encoded K11 diUb (**4b**)	0.45	26.32	1.6 mL
Neutron-encoded K27 diUb (**4c**)	0.94	54.92	1.65 mL
Neutron-encoded K29 diUb (**4d**)	0.88	51.38	1.85 mL
Neutron-encoded K33 diUb (**4e**)	0.36	21.00	1.65 mL
Neutron-encoded K48 diUb (**4f**)	0.26	15.15	1.95 mL
Neutron-encoded K63 diUb (**4g**)	0.20	11.65	1.5 mL
Neutron-encoded M1 diUb (**5**)	0.46	26.77	0.55 mL

Isopeptide-linked diUb (xx V*, xx L*, xx I*) (**4a-g**)

The products were obtained as solutions. LC-MS analysis using System 2 – *Gradient 3*.

Yields:
Lys6 linked neutron-encoded diUb (1x V*, 1x L*) **4a** = 0.66 mg, 0.039 µmol, 3.3% (over two steps) . LC-MS: R_t 3.22 min: MS ES+ (amu) calculated: 17089 Da[M]; found 17089 Da.
Lys11 linked neutron-encoded diUb (3x V*, 1x I*) **4b** = 0.72 mg, 0.042 µmol, 3.6% (over two steps). LC-MS: R_t 3.25 min: MS ES+ (amu) calculated: 17100 Da[M]; found 17100 Da.
Lys27 linked neutron-encoded diUb (3x V*, 2x L*, 1x I*) **4c** = 1.55 mg, 0.090 µmol, 7.75% (over two steps). LC-MS: R_t 3.24 min: MS ES+ (amu) calculated: 17115 Da[M]; found 17115 Da.
Lys29 linked neutron-encoded diUb (3x V*, 2x L*, 3x I*) **4d** = 1.63 mg, 0.095 µmol, 8.15% (over two steps). LC-MS: R_t 3.23 min: MS ES+ (amu) calculated: 17129 Da[M]; found 17129 Da.
Lys33 linked neutron-encoded diUb (3x V*, 4x L*, 3x I*) **4e** = 0.59 mg, 0.034 µmol, 2.95% (over two steps). LC-MS: R_t 3.23 min: MS ES+ (amu) calculated: 17143 Da[M]; found 17143 Da.
Lys48 linked neutron-encoded diUb (3x V*, 4x L*, 5x I*) **4f** = 0.51 mg, 0.029 µmol, 2.55% (over two steps). LC-MS: R_t 3.21 min: MS ES+ (amu) calculated: 17157 Da[M]; found 17156 Da.
Lys63 linked neutron-encoded diUb (3x V*, 6x L*, 5x I*) **4g** = 0.3 mg, 0.017 µmol, 1.5% (over two steps). LC-MS: R_t 3.22 min: MS ES+ (amu) calculated: 17171 Da[M]; found 17171 Da.

Analytical LC-MS data (Total ion chromatogram and mass spectra) for all synthetic diubiquitins are available in the online version of the supplementary information of this paper. Top panel: Total ion chromatogram (*m/z* =550-2000). Middle panel; mass spectra. Bottom panel; deconvoluted mass spectra.

Linear diUb (4x V*, 6x L*, 6x I*) (**5**)
The product was obtained as a solution. LC-MS analysis using System 2 – *Gradient 3*.

Yield:
Linear neutron-encoded diUb (4x V*, 6x L*, 6x I*) **5** = 0.25 mg, 0.15 µmol, 11.8%. LC-MS: R_t 3.32 min: MS ES+ (amu) calculated: 17171 Da[M]; found 17184 Da.

Extra attention was given to obtaining a good purity of the diUb molecules since impurities will have a detrimental effect on the MS-based quantification during the envisioned assay. Based on the LC-MS data, the purity of all linkages was sufficient for the designed assay.

Analytical LC-MS data (Total ion chromatogram and mass spectra) for all synthetic diubiquitins are available in the online version of the supplementary information of this paper. Top panel: Total ion chromatogram (*m/z* =550-2000). Middle panel; mass spectra. Bottom panel; deconvoluted mass spectra.

Synthesis of Ub(1-74, Nle_1)

Step 1. SPPS

See SPPS procedure.

Sequence Ub1-74^{Met1Nle}

(Nle)QIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLR

67 cycles on Fmoc-Arg(Pbf)-loaded TentaGel® R HMPA resin (Rapp Polymere, Germany, #RA1502)

25 µmol scale – ~12.5 µmol used for next step. To check the quality of the SPPS product a trial cleavage was performed.

Step 2. Global deprotection

Global deprotection was performed as described previously.[15] The resin-bound polypeptide Ub(1-74, Nle_1) was deprotected and detached from the resin by treatment with a freshly prepared solution of TFA/H_2O/Phenol/iPr_3SiH (90.5/5/2.5/2; v/v/v/v; 2.5 mL) for 2.5-3.5 hours at room temperature to remove all protecting groups from all amino acid sidechains. The reaction mixture was filtered directly into ice-cold Et_2O/*n*-pentane (3/1; v/v; 10 mL) and the resin was washed with TFA (2x 4mL). The mixture of Et_2O/*n*-pentane and filtrate was centrifuged (1500 rpm, 5 min, 4 °C) and the Et_2O/ *n*-pentane (supernatant) was removed. The pellet was resuspended in Et_2O (12 mL), the suspension was vortexed, centrifuged (1500 rpm, 5 min, 4 °C) and the Et_2O was removed. The wash step was repeated twice. The remaining solvent was removed by a N_2 flow over the pellet. The solid crude material was dissolved in H_2O/CH_3CN/ formic acid (65/25/10; v/v/v; 15 mL) and lyophilized. The crude material was subsequently purified using preparative RP-HPLC (*RP-HPLC – System 1 – Gradient 1).* Pure fractions (>95%, checked by LC-MS) were pooled and lyophilized to obtain the product as a white powder.

Ub(1-74, Nle_1)

The product was obtained as white solid. LC-MS analysis using Program 2.

Yield:

Ub(1-74, Nle_1) = 32.85 mg, 3.89 µmol, 31.1%. LC-MS: R_t 3.13 min: MS ES+ (amu) calculated: 8432.7 Da[M]; found 8433 Da.

The products was purified by gel filtration using a Biorad NGC Chromotography system on a size exclusion HiLoad® 16/600 Superdex® 75 pg GE healthcare column. See **Size Exclusion** (above). The product was obtained as colorless solution containing 50 mM TRIS·HCl and 20 mM NaCl buffer at pH 7.55. LC-MS analysis (LC-MS – System 2 – Gradient 2) was done

to check the purity. Pure fractions were combined and concentrated using a 3 kDa MWCO Amicon Ultra spin filters. Yielding a stock concentration of 2.5 mg/mL or 296.5 µM.

Synthesis of Ub(1-75, Nle_1)-PA (**21**)

Step 1. SPPS

See SPPS procedure.

Sequence Ub1-75^{Met1Nle}

(Nle)QIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRG

68 cycles on Fmoc-Gly-loaded TentaGel® R trityl resin (Rapp Polymere, Germany, #RA RA1213) 25 µmol scale. To check the quality of the SPPS product a trial cleavage was performed.

Step 2,3 and 4. Cleavage from resin, PA coupling and Global deprotection

Ub(1-75, Nle_1)-PA (**21**) was prepared as described previously.[16] Briefly, after SPPS and release from the resin using HFIP/DCM (1/4; v/v) the protected polypeptide Ub(1-75, Nle_1) (25 µmol) was dissolved in DCM (1 mL/ 5 µmol), and PyBOP (5 eq., 125 µmol), triethylamine (5 eq., 125 µmol) and propargylamine (10 eq., 250 µmol) were added to the solution. The reaction was stirred for 16 hours at RT. The reaction mixture was concentrated and deprotected using TFA/H_2O/Phenol/iPr_3SiH (90.5/5/2.5/2; v/v/v/v) for 2.5 hours. The crude polypeptide was collected after precipitation from ice-cold Et_2O/*n*-pentane (3/1; v/v), centrifugation (1500 rpm, 5 min, 4 °C) and the Et_2O/ *n*-pentane (supernatant) was removed. The pellet was resuspended in Et_2O (20 mL), the suspension was vortexed, centrifuged (1500 rpm, 5 min, 4 °C) and the Et_2O was removed. The crude polypeptide was purified using preparative RP-HPLC (Method A; Gradient 1). Pure fractions (> 95%, checked by LC-MS) were pooled and lyophilized to obtain the product as a white powder.

Synthesis of Ub(1-75, Nle_1, L-azido-ornithine$_{48}$) (**22**)

Step 1. SPPS

See SPPS procedure.

Sequence Ub1-75 ^{Met1Nle} (K48 = L-azido-ornithine)

(Nle)QIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAG(L-azido-ornithine)QL EDGRTLSDYN IQKESTLHLV LRLRG

Step 2. Global deprotection

Global deprotection was performed as described previously.[15] The resin-bound polypeptide Ub(1-75, Nle_1, L-azido-ornithine$_{48}$) was deprotected and detached from the resin by treatment with a freshly prepared solution of TFA/H_2O/Phenol/iPr_3SiH (90.5/5/2.5/2; v/v/v/v; 5 mL)

for 2.5-3.5 hours at room temperature to remove all protecting groups from all amino acid sidechains. The reaction mixture was filtered directly into ice-cold Et_2O/*n*-pentane (3/1; v/v; 35 mL) and the resin was washed with TFA (2x 4mL). The mixture of Et_2O/*n*-pentane and filtrate was centrifuged (1500 rpm, 5 min, 4 °C) and the Et_2O/*n*-pentane (supernatant) was removed. The pellet was resuspended in Et_2O (20 mL), the suspension was vortexed, centrifuged (1500 rpm, 5 min, 4 °C) and the Et_2O was removed. The wash step was repeated twice. The remaining solvent was removed by a gentle N_2 flow over the pellet. The solid crude material was dissolved in H_2O/CH_3CN/formic acid (65/25/10; v/v/v; 15 mL) and lyophilized. The crude material was subsequently purified using preparative RP-HPLC (*RP-HPLC – System 2 – Gradient 1*). Pure fractions (checked by LC-MS) were pooled and lyophilized to obtain the product as a white powder.

Synthesis of non-hydrolysable clicked Lys48 diubiquitin (**23**)

Copper-catalysed alkyne-azide cycloaddition (CuAAC) – "Click reaction"

The click reaction was performed as described previously.[17] Briefly, the CuAAC reaction was performed under denaturing conditions in 8 M Urea, 100 mM phosphate buffer pH 7. Ub(1-75, Nle_1)-PA (**21**) (11.5 mg) was dissolved in warm DMSO (100 µL) and Ub(1-75, Nle_1, L-azido-ornithine$_{48}$) (**22**) (10.25 mg) was dissolved in warm DMSO (100 µL). Both DMSO solutions were added to an aqueous buffer containing 8 M Urea and 100 mM phosphate, pH 7 (2 mL). To the solution 210 µL of catalyst solution containing 25 mg/mL $CuSO_4 5H_2O$ in H_2O, 120 mg/mL sodium ascorbate in H_2O and 52 mg/mL TBTA-analogue[3] in CH_3CN (1/1/1; v/v/v) was added. The reaction was gently shaken at room temperature. Extra catalyst solution was added after 1h (90 µL) and fresh catalyst solution was added after 16h (210 µL). After reaction was finished, as judged by LC-MS (~ 24 hour), the reaction was quenched by the addition of 34 µL of 0.5 M EDTA (1 eq. compared to $CuSO_4 \cdot 5H_2O$), pH 7.0. The crude material was subsequently purified using preparative RP-HPLC (*RP-HPLC – System 2 – Gradient 2*). Pure fractions (checked by LC-MS) were pooled and lyophilized to obtain the product as a white powder.

The products were purified by gel filtration using a Biorad NGC Chromotography system on a size exclusion HiLoad® 16/600 Superdex® 75 pg GE healthcare column. See **Size Exclusion** (above). The product was obtained as colorless solution containing 50 mM TRIS·HCl and 20 mM NaCl buffer at pH 7.55. LC-MS analysis (LC-MS – System 2 – Gradient 2) was done to check the purity. Pure fractions were combined and concentrated using a 3 kDa MWCO Amicon Ultra spin filters. Yielding a stock concentration of 1.52 mg/mL or 89.25 µM.

Analytical LC-MS data (total ion chromatograms, sum spectra of mass spectra of main peak in chromatograms and deconvoluted mass spectra) for all synthetic mono- and diubiquitins are available in the online version of the supplementary information of this paper. Top panel: Total ion chromatogram (*m/z* = 500-1600). Middle panel; sum spectrum of mass spectra from main peak in top panel. Bottom panel; deconvoluted mass spectra.

Materials and Methods; Biochemistry

Recombinant protein expression and purification of AMSH.

Protein expression constructs. cDNA generated from MelJuSo cells was used as a PCR template for the cloning of AMSH. AMSH DNA was amplified by PCR reaction described in Supplementary Table 6 and 7 using the primers in Supplementary Table 8 and cloned into the pGEXNKI-GSThis3C-LIC vector using ligation-independent cloning[71]. In brief, a PCR fragment of AMSH flanked with specific LIC sequences was generated, and the pGEXNKI-GSThis3C-LIC vector was cleaved with KpnI enzyme, followed by agarose gel extraction. Extracted PCR fragment and the vector were treated with T4 DNA polymerase in the presence of dATP and dTTP, respectively. The insert ligation into the vector was performed by mixing them in 2:1 (insert: vector) dilution and incubating them at room temperature for 5 min. Ligated DNA was transformed into DH5α E. coli strain. The expression construct was purified and confirmed by DNA sequencing.

Expression and purification protocol of AMSH. GST-tagged AMSH construct was transformed into Rosetta *E. coli* strain. A single colony was grown in LB media containing 100 µg/µL ampicillin and 34 µg/µL chloramphenicol at 37 °C overnight as a starter culture. The starter culture was diluted in LB media containing 100 µg/µL ampicillin and 34 µg/µL chloramphenicol for a large-scale protein expression. The bacterial culture was incubated at 37 °C until A600 reached 0.6-0.8. Further, the culture was incubated at 30 °C for 4 hours after adding a 500 µM final concentration of IPTG (Isopropyl β-D-1-thiogalactopyranoside). Cells were lysed with lysis buffer 3 (20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, and EDTA-Free complete protease inhibitor cocktail (Roche) and sonication. The lysates were centrifuged at 21,000 × *g* for 30 min at 4 °C. The supernatants were incubated with prewashed Glutathione Sepharose™ 4 Fast Flow (GE Healthcare, Cat. 17-5132-03) for 1 hour at 4 °C under gentle rotation and the beads were then washed with wash buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol). Protein was eluted with elution buffer containing 20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, and 20 mM Glutathione. After elution, the GST tag was removed using 3C protease under dialysis against the buffer containing 20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol and further purified on a size exclusion column (S200 16/60 column) using an ÅktaPrime (GE Healthcare) purifier. All proteins were aliquoted and stored at −80 °C.

General method SDS-PAGE analysis

After indicated reaction time, the reaction was quenched by addition of 3x reducing sample buffer (SB) (containing 900 µL 4x LDS sample buffer (NuPAGE, Invitrogen) diluted with 210 µL water and 90 µL β-mercaptoethanol) and heated to 95°C for 5 min. (denaturing conditions). Samples were

loaded on precast 12% NuPAGE® Novex® Bis-Tris Mini Gels (Invitrogen) and resolved by SDS-PAGE gel electrophoresis using MES running buffer (NuPAGE MES SDS running buffer 20X, Novex by Life Technologies). Reference protein standard/ladder: SeeBlue™ Plus2 Pre-stained Protein Standard (Invitrogen, cat# LC5925). Proteins were visualized by InstantBlue™ (Expedeon Protein Solutions, #ISB1L), and stained gels were scanned using a GE Healthcare Amersham Imager 600.

Characterization of all eight neutron-encoded diUb isoforms, K48 click diUb and Ub_{1-74} by SDS-PAGE analysis

All eight neutron-encoded diUb isoforms, K48 click diUb and Ub_{1-74} stock solutions were diluted to ~3.5 μM in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.55. To 40 μL of these solutions 20 μL 3x SB was added, samples were boiled and loaded on gel (15 μL/lane) and separated by gel electrophoresis. (**Fig. 2d** and **Supplementary Figure 1**).

SDS-PAGE analysis of USP21 mediated hydrolysis of neutron-encoded diUb

All neutron-encoded diUb, K48 click diUb and Ub_{1-74} stocks were separately diluted to 2x final concentration (5 μM) in a buffer containing 50 mM Tris·HCl, 20 mM NaCl, pH 7.55, 5 mM DTT. USP21[196-565] was diluted to 2x final concentration (150 nM) in a buffer containing 50 mM Tris·HCl, 20 mM NaCl, pH 7.55, 5 mM DTT. Subsequently, 15 μL of diUb solution (5.0 μM) was mixed with 15 μL of enzyme solution (150 nM) Hydrolysis reactions were incubated for 3 hours at 37°C. Samples for timepoint 0 min. were quenched before hydrolysis reaction started by mixing a diUb solution (2x final concentration, 5 μL) with 3x SB (5 μL) and subsequent addition of DUB (2x final concentration, 5μL).

Next to that, all neutron-encoded diUb stocks were mixed to yield a solution containing 2.5 μM of all eight neutron-encoded diUbs (1.5x final concentration) in a buffer containing 50 mM Tris·HCl, 20 mM NaCl, pH 7.55. USP21[196-565] was diluted to 3x final concentration (1200 nM) in a buffer containing 50 mM Tris·HCl, 20 mM NaCl, pH 7.55, 5 mM DTT. Subsequently, 15 μL of the 8x neutron-encoded diUb solution (8x 2.5 μM) was mixed with 7.5 μL of enzyme solution (1200 nM). Samples for timepoint 0 minutes were quenched before hydrolysis reaction started by mixing a diUb solution (1.5x final concentration, 5 μL) with 3x SB (5 μL) and subsequent addition of DUB (2x final concentration, 2.5μL).

Samples from the reaction mixture (10 μL) were taken after 180 min. and the reaction was quenched 3x SB (5 μL) and analyzed according to the general method for SDS-PAGE analysis (**Fig. 3b** and **Supplementary Figure 3**).

SDS-PAGE analysis of OTUB1, OTUD1 and USP21 mediated hydrolysis of synthetic neutron-encoded and enzymatically prepared Lys48-, Lys63- and Met1-linked diUb

Enzymatically prepared Lys48- and Lys63-linked diUb were obtained from Ubiquigent #60-

0106-050 and #60-0107-010. Enzymatically prepared Met1-linked diUb was prepared *in house*.[40]

Synthetic neutron-encoded and enzymatically prepared Lys48-, Lys63- and Met1-linked diUb stocks were diluted to 2x final concentration (approx. 10 μM, diUb conc. equalized by SDS-PAGE and InstantBlue™ staining) in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.55, 10 mM DTT. OTUB1 (FL), OTUD1[287-481] and USP21[196-565] were diluted to 2x final concentration (3.2 μM, 0.2 μM and 150 nM respectively) in a buffer containing 50 mM Tris·HCl, 100 mM NaCl, pH 7.55, 10 mM DTT. Subsequently, 20 μL of diUb solution (approx. 10 μM) was mixed with 20 μL of enzyme solution (3.2 μM, 0.2 μM or 150 nM). Reactions were incubated for 30 minutes at 37°C. Samples for timepoint 0 min. were quenched before hydrolysis reaction started by mixing a diUb solution (2x final concentration, 5 μL) with 3x SB (5 μL) and subsequent addition of DUB (2x final concentration, 5μL). Samples from the reaction mixture (10 μL) were taken after 10 and 30 min. and the reaction was quenched with 3x SB (5 μL) and analyzed according to the general method for SDS-PAGE analysis. (**Supplementary Figure 4** and **5**).

In vitro DUB assays with mass spectrometry read-out

The assays were performed in a 1.5 mL Eppendorf tube at 37°C in a buffer containing 50 mM Tris·HCl, 20 mM NaCl, pH 7.6 and 5.0 mM final concentration of DTT or TCEP.

All eight neutron-encoded diubiquitins were mixed in an equimolar amount (8x 2.5 μM; 1.5x final concentration). Neutron-encoded K6 (145.5 μL), K11 (142.5 μL), K27 (68.25 μL), K29 (72.25 μL), K33 (178.5 μL), K48 (247.5 μL), K63 (321.75 μL) and M1 (139.5 μL) stocks were mixed and diluted with buffer (50 mM Tris·HCl, 20 mM NaCl, pH 7.6; 183.75 μL). Recombinant purified DUBs were obtained from commercial sources, received as a gift, or expressed and purified according to reported procedures (details provided in **Supplementary Table 2**). Recombinant purified DUBs were diluted to 3x final concentration (12 or 6 μM, 1,2 μM, 0,12 μM and 0,012 μM respectively) in a buffer containing 50 mM Tris·HCl, 20 mM NaCl, pH 7.6 and **15** mM DTT or TCEP. The diUb mixture (15 μL) was added to the Eppendorf tube and 1.33 μL was taken for timepoint 0. Subsequently, the enzyme (6.84 μL, 3x final concentration) was added to the remaining diUb mixture (13.67 μL, 1.5x final concentration). The reaction mixtures were incubated at 37°C for 180 min. After 2, 5, 10, 30, 60 and 180 min a sample was taken from the reaction mixture for analysis.

Sample preparation. After indicated incubation time, 2 μL of the reaction mixture was taken. The reaction was quenched by acidification of the mixture and internal standards for MS analysis were directly added. Therefore, 2 μL of the reaction mixture was quenched, spiked and diluted with a mixture containing 0.4μL 10% TFA in MQ, 1.6 μL 0.1% FA in MQ and 12 μL of the internal standard solution (1 μL of 5.0 μM internal standard diluted with 11 μL of 0.1% FA in MQ).

Samples were collected in a 96-well plate and measured by LC-MS analysis. 8 μL of the sample was injected onto the column.

Data acquisition. The samples were separated by an Acquity H-class UPLC system using a BEH C4 column (300Å, 1.7 µM (2.1 x 50 mm)), column T =60°C. For the first 2.5 min, the flow was diverted from the detector to flush the column with 2% ACN in H_2O and 0.1% FA at 1 mL/min to elute most of the buffer components and salt. After 2.5 min. proteins were eluted using a shallow gradient that ranged from 26% up to 30% ACN in H_2O with 0.1% FA over 1 min using a flow rate of 0.6 mL/min, which was able to separate monoUb and diUb products (baseline level). Products were analysed by intact MS analysis on a XEVO G2 XS Q-TOF in reflector positive ion mode with a resolution of *R* = 22,000 using positive electrospray ionization (ESI) (Cap. V. = 0.5 kV) and a detection range of *m/z* 550-2000. The system check of the detector voltage, lock mass accuracy (of LeuEnk) and calibration using NaI solution were performed daily prior to analysis. Lock mass correction was applied during each analysis, to correct for possible mass shifts during the course of the assay.

Data analysis. Proteowizard 3.0.20274 was used to convert raw data files to the mzXML file format.[72] mzXML files were further processed using LaCyTools version 22.04.29. The alignment was performed using theoretical *m/z* values of various charge states from the internal standard and the expected elution time (**Supplementary Table 3**). The extraction parameters are specified in **Supplementary Table 4**. Atom compositions were determined of all eight diUb molecules, all nine monoUbs that can be formed, Ub_{1-74} as well as the non-hydrolysable clicked K48 diUb and listed as analytes (**Supplementary Table 5**). From all analytes, their retention time was predicted and the charge states that should be taken along during quantification are specified (**Supplementary Table 5**). The LaCyTools output file (Summary.txt) was further processed in Microsoft Excel, where boundaries for quality control parameters (Mass Accuracy < 15 ppm; IPQ < 0.25; S/N > 9) were set and the areas of all *m/z* peaks, within the quality control boundaries, from the same analyte were summed.

The absolute area for each analyte was normalized at each timepoint using the internal standard, non-hydrolysable clicked Lys48 diUb. The normalized area under the curve for each analyte was set to 100% remaining diUb at t=0 and percentage remaining diUb at each timepoint was calculated relative to this, to account for variations in ionization of different diUb isoforms. Percentage remaining diUb was plotted against time using GraphPadPrism 9.3.0.

For the monoUb signals, the total absolute area under the curve was normalized at each timepoint using the internal standard Ub_{1-74}. The concentration of monoUb present at each timepoint was calculated using the theoretical concentration of present Ub_{1-74} in the analysed mixture. Concentration monoUb was plotted against time using GraphPadPrism 9.3.0.

Assay window and linearity determination

The assay concentration window is considered to be valid at concentrations for which the amount of loaded diUb corresponds to the experimentally determined amount. Linearity curves (theoretical amount of diUb plotted against determined amount) were constructed for all eight diUbs over the concentration range of 0.0-2.0 µM in a mixture containing all diUbs and K48 click as internal standard in three separately performed experiments on different days.

All eight diUb solutions were mixed (final concentration 2.0 µM of all linkages) and a serial dilution of 0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00 µM was made in buffer (50 mM Tris, 20 mM NaCl). Each sample (2 µL) was diluted with 14 µL of a mixture containing 0.4µL 10% TFA in MQ, 1.6 µL 0.1% FA in MQ and 12 µL of the internal standard solution (1 µL of 5.0 µM internal standard diluted with 11 µL of 0.1% FA in MQ). A total of 8 µL of these sample mixtures were loaded and analyzed in an identical way as during the DUB assays. The data was quantified with LaCyTools. Non-hydrolysable clicked K48 click area was linked to the K48 click diUb concentration present in the mixture, using the measured area of all eight diUbs, their measured concentration was calculated. The theoretical amount of all eight diUbs present was plotted versus the measured and calculated amount of all eight diUbs. The concentration window was determined to be valid between 0.5 and 2.0 µM (**Supplementary Figure 6**).

For the assay, a starting concentration of 1.6 µM of each diUb linkage was chosen. Assuming the linear detection threshold of monoUb and diUb are similar, it was presumed that as soon as the concentration of present diUb molecules dropped below the linear detection range, the concentration of present monoUb isoforms reached the linear detection threshold. Resulting in a reliable read-out of at least one of the analytes types during the assay. A higher starting concentration was avoided to prevent potential detector crowding or overloading of the LC column.

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

Material availability. The synthetic neutron-encoded diUb reagents and internal standards will be made available by the authors upon request.

Data availability. The experiment data that support the findings of this study are available from the corresponding author upon reasonable request. All MS datasets generated and/or analysed during the current study have been deposited with the ProteomeXchange Consortium via the MassIVE partner repository with data set identifier MSV000090455.

The synthetic neutron-encoded diUb reagents and internal standards will be made available by the authors upon request.

Code availability. LaCyTools is freely available for download at *https://github.com/Tarskin/LaCyTools*.

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