K27-linked diubiquitin inhibits UCHL3 via an unusual kinetic trap
Tilburg, G.B.A. van; Murachelli, A.G.; Fish, A.; Noort, G.J.V. van; Ovaa, H.; Sixma, T.K.

Citation

Version: Publisher's Version
License: Leiden University Non-exclusive license
Downloaded from: https://hdl.handle.net/1887/3234501

Note: To cite this publication please use the final published version (if applicable).
K27-Linked Diubiquitin Inhibits UCHL3 via an Unusual Kinetic Trap

Gabriëlle B.A. van Tilburg,1,4 Andrea G. Murachelli,2,4 Alexander Fish,2 Gerbrand J. van der Heden van Noort,1,* Huib Ovaa,1,3 and Titia K. Sixma2,5,*

1Department of Cell and Chemical Biology and Oncode Institute, Leiden University Medical Center, Einthovenweg 20, 2333 ZC Leiden, the Netherlands
2Department of Biochemistry and Oncode Institute, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands
3In memory of Huib Ovaa, who died too early on May 19, 2020. His curiosity and passion for science will always be an example
4These authors contributed equally
5Lead Contact
*Correspondence: gvanderheden@lumc.nl (G.J.v.d.H.v.N.), t.sixma@nki.nl (T.K.S.)
https://doi.org/10.1016/j.chembiol.2020.11.005

SUMMARY

Functional analysis of lysine 27-linked ubiquitin chains (K27Ub) is difficult due to the inability to make them through enzymatic methods and due to a lack of model tools and substrates. Here we generate a series of ubiquitin (Ub) tools to study how the deubiquitinase UCHL3 responds to K27Ub chains in comparison to lysine 63-linked chains and mono-Ub. From a crystal structure of a complex between UCHL3 and synthetic K27Ub2, we unexpectedly discover that free K27Ub2 and K27Ub2-conjugated substrates are natural inhibitors of UCHL3. Using our Ub tools to profile UCHL3’s activity, we generate a quantitative kinetic model of the inhibitory mechanism and we find that K27Ub2 can inhibit UCHL3 covalently, by binding to its catalytic cysteine, and allosterically, by locking its catalytic loop tightly in place. Based on this inhibition mechanism, we propose that UCHL3 and K27Ub chains likely sense and regulate each other in cells.

INTRODUCTION

Ubiquitination is the addition of ubiquitin (Ub) to a lysine or to the initial methionine of a substrate protein. Because Ub itself contains seven lysines and its initial methionine, various Ub chain types can be constructed on the substrate, which translate to different cellular responses (reviewed in Akutsu et al., 2016; Komander and Rape, 2012). Different chain types require specific enzymes for creating, editing, and removing, and are “read” by specific adaptors/effectors. Enzymes responsible for the removal of Ub are called deubiquitinating enzymes (DUBs).

So far, lysine-27-linked Ub chains (K27 chains) have been relatively less studied, due to a lack of enzymatic tools in vitro. Only recently have enzymes emerged that place K27 chains mainly in the intracellular innate immune response pathway, where they regulate several essential infection sensors, such as STING, cGAS, MAVS, and MDA5, or effectors such as NEMO and Beclin. K27-linked chains have also been implicated in the DNA damage repair response (reviewed in Akutsu et al., 2016; van Huizen and Kikkert, 2020).

Previously, we identified ubiquitin carboxy-terminal hydrolase isozyme 3 (UCHL3) as a tight interactor of lysine-27-linked diubiquitin (K27Ub2) (Zhang et al., 2017). UCHL3 is a DUB belonging to the UCH (ubiquitin carboxy-terminal hydrolase) family, together with UCHL1, UCHL5, and BAP1. UCH family DUBs use a catalytic cysteine as an active nucleophile to attack the (iso)peptide bond between the C-terminal glycine of Ub and the ε-amino group of the substrate lysine or methionine. This leads to the formation of a covalent DUB–Ub thioester intermediate, which is then resolved with a second nucleophilic attack by a water molecule, leading to the release of the substrate (Figure S1A) (Mevissen and Komander, 2017).

To access the catalytic site of UCH DUBs, the ubiquitinated substrate needs to thread underneath a crossover loop (Johnston et al., 1999; Mevissen and Komander, 2017; Misaghi et al., 2005), which closes on top of the C terminus of the conjugated Ub. After catalysis, the loop needs to open again to release the cleaved Ub. The crossover loop of UCHL3 is very short, leading to a specificity for small, preferentially disordered ubiquitinated substrates (Bett et al., 2015; Misaghi et al., 2005; Navarro et al., 2014; Popp et al., 2009; Zhou et al., 2012). In vivo substrates of UCHL3 include the precursor of NEDD8 (Hemelaar et al., 2004; Wada et al., 1998), the ribosomal proteins L40 and RPS27a (Grou et al., 2015; Larsen et al., 1998) (both expressed as N-terminal Ub fusions), and the pathological Ub frameshift mutant UBB+1 (Dennissen et al., 2011).

Functionally, UCHL3 has been implicated in DNA damage repair (Liao et al., 2018; Luo et al., 2016; Nishi et al., 2018), regulation of the interferon response (Zhao et al., 2017), meiosis (Mtango et al., 2012), fertilization (Yi et al., 2007), and osteoblast differentiation (Kim et al., 2011). In addition, UCHL3 knockout mice exhibit neuronal, retinal, and muscular degeneration (Semenova et al., 2003), although the mechanistic reasons are unclear. In vertebrates, the closest homolog of UCHL3 is UCHL1
which has overlapping functions in Ub and NEDD8 processing (Hemelaar et al., 2004). Mouse knockout studies suggest that UCHL1 and UCHL3 might have partially overlapping functions in the nervous system (Kurihara, 2001).

In this article, we generate a set of dedicated Ub tools and substrates to study how K27Ub2 binding affects UCHL3 activity. We find that K27Ub2-conjugated substrates potently inhibit UCHL3 activity. Through kinetic analysis followed by global fitting we elucidate the mechanism whereby, surprisingly, K27Ub2 inhibits UCHL3 via a kinetic trap. This is ruled by stochastic events: when UCHL3 binds K27Ub2-conjugated substrates, it can either rapidly cleave and release the hydrolyzed products or be conformationally trapped into a long-lived inhibited state bound to K27Ub2. We predict that if K27Ub2 substrates are in excess, the majority of the UCHL3 population will be trapped into a long-lived inhibited state. This mechanism is specific for UCHL3 and not UCHL1. Finally, the high affinity of UCHL3 for K27Ub2 opens up the possibility of using UCHL3 as a low-cost and easily obtained high-affinity reagent for the detection of free K27 chains.

RESULTS AND DISCUSSION

UCHL3 is one of the few known cellular proteins that bind tightly and specifically to the poorly studied K27Ub2 (Zhang et al., 2017), but how this affects UCHL3 function is not known. Here we used a combination of chemical synthesis, semi-synthesis, genetics, and enzymatic reactions to produce a series of Ub tools that allow mechanistic insights into UCHL3 activities (Figure 1) and quantitative analysis of UCHL3 activity on substrates conjugated to K27Ub2, K63-linked diubiquitin (K63Ub2), or mono-Ub. Because UCHL3 is reported to remove K63-linked tetra-Ub chains from SUMO2 (Bett et al., 2015), we chose to use the K63 linkage as a comparison to the K27 linkage.

UCHL3 Binds to K27Ub2 Selectively, but Does Not Cleave It

First, we quantified the linkage selectivity of UCHL3 for K27Ub2, K63Ub2, and mono-Ub using surface plasmon resonance (SPR) (Figures 2A and 2B). We obtained a dissociation constant (Kd) of 0.046 ± 0.005 μM for K27Ub2, indicating approximately 100-fold preference over K63Ub2 (3.4 ± 0.45 μM) or mono-Ub (5.2 ± 0.3 μM). In contrast, UCHL1, the closest paralog of UCHL3 (53% identity, Figure S2), displays no linkage preference and binds mono-Ub, K27Ub2, and K63Ub2 with similar affinities (1–5 μM, Figures 2A and 2B).

It is known that UCHL3 cannot cleave any of the diubiquitin chains, regardless of the linkage type (Navarro et al., 2014; Popp et al., 2009; Zhou et al., 2012). We confirmed this for K27Ub2 and K63Ub2; at a very high concentration of DUB (4.5 μM) and long incubation times, no significant cleavage was observed (Figure 2C). Apparently UCHL3 binds, but does not cleave, K27Ub2. Remarkably, however, incubation of UCHL3 with K27Ub2 leads to the appearance of a denaturation-resistant band, which corresponds in size to a UCHL3:K27Ub2 complex (marked with * in Figure 2C).

UCHL3 Forms a Thioester Intermediate with K27Ub2 in Crystals

To understand the functional consequence of K27Ub2 binding to UCHL3, we solved a crystal structure of the complex (Figure 3A and Table S1). Our structure closely resembles a previously reported UCHL3:K27Ub2 complex (Pan et al., 2019) (root-mean-square deviation [RMSD] 0.45 Å on 378 Cα), with the exception that our K27Ub2 contains the native isopeptide linkage at the K27 conjugation site, whereas the other structure contains a closely related K27 mimic (Lym27). With different crystallization conditions and packing, our structure independently confirms the relevance of the K27Ub2-UCHL3 binding.

Surprisingly, despite the fact that we used wild-type, native K27Ub2 with a free carboxylic acid on the C terminus (Figure S3A),
the complex crystallized in the shape of the Ub-~DUB thioester intermediate expected during UCH catalysis (Figure S1). The Ub moiety of K27Ub2 that would be attached to a substrate (proximal Ub [UbP], Komander and Rape, 2012) occupies the active site of UCHL3 and is linked to UCHL3’s catalytic cysteine via continuous electron density, which indicates a thioester bond (Figure 3B). A similar continuous electron density is also observed in the structure of Pan et al. (2019), although the authors did not model a covalent bond (Figure S3C). The interaction between UbP and the active site is analogous to the previously reported structure of UCH3:Ub-vinyl-methyl-ester (Misaghi et al., 2005) (Figure S3B, RMSD 0.63 Å on 303 Ca). The catalytic triad of UCHL3 (Boudreaux et al., 2012; Komander and Rape, 2012) is correctly aligned for catalysis, including the presence of a water molecule required for the second nucleophilic attack (Figure 3B).

**UCHL3 Can Form a Thioester Intermediate with K27Ub2 and Mono-Ub under Native Conditions**

The observation that UCHL3 can form a stable complex with K27Ub2 under denaturing conditions (Figure 2C) and the occurrence of a thioester bond in our crystal structure prompted us to further investigate the possibility that UCHL3 can form a thioester complex with Ub chains under native conditions. This has been suggested before by Pickart and Rose (Pickart and Rose, 1986). We tested for thioester formation by NaBH4-mediated reduction of mono-Ub or K27Ub2 (58 µM) incubated in presence of reduced Ub/Ub2-aldehyde, whereas the free carboxylic acid of Ub/Ub2 would not (Figure 3C). After 1.5 h incubation at 37°C under mild conditions (Tris-HCl [pH 7.6], 5 mM DTT, 1 mM EDTA), we treated all samples with excess NaBH4. High-resolution mass spectrometry shows the appearance of distinct mass envelopes in the Ub:UCHL3 and K27Ub2:UCHL3 samples that are consistent with the presence of Ub- and K27Ub2-aldehyde, respectively (Figures 3D and 3E), and not Ub- or K27Ub2-alcohol (Figures S3D and S3E). The intensity of both peaks increases with longer NaBH4 incubation (data not shown). The peak observed for K27Ub2-aldehyde is comparably more intense, consistent with the fact that UCHL3 has an ~100-fold higher affinity for K27Ub2 compared with Ub. Thus, UCHL3 can form covalent thioester bonds with Ub and K27Ub2 in solution under physiological buffers.

The affinity between Ub~UCHL3 and K27Ub2~UCHL3 thioester complexes can be captured by mass spectrometry and/or crystallization, which is unexpected, as covalent bond formation between a free carboxyl terminus and a cysteine is energetically disfavored in solution. Indeed, Ub-conjugating enzymes require ATP hydrolysis to form a thioester with Ub in the Ub-conjugation pathway (Komander and Rape, 2012). The influence of mechanical forces on the energy landscape of reactions is widely reported (Bustamante et al., 2004). Upon binding of a proximal Ub, the narrowness of the active channels of UCHL3 seems to disfavor the long-term presence of a free C terminus and active cysteine, pushing toward the formation of a thioester bond. That would suggest that increasing the affinity of UbP for the active site, such as is the case for K27Ub2, would promote covalent bond formation.

**K27Ub2 Restrains the Crossover Loop of UCHL3**

The structure explains the specificity of K27-linkage recognition: the crossover loop of UCHL3 is pinched between the two Ub moieties and adheres to the inner surface created by UbP, the narrow channel of UCHL3 (Figure S2). These differences may prevent UCHL1 from engaging specifically with the distal Ub of K27Ub2. Consequently, these two DUBs must respond differently to K27Ub2 in cells.

**Free, Unconjugated K27Ub2 Inhibits UCHL3**

By pinching the loop, UbP prevents it from opening. With a closed loop, UbP cannot leave the active site, and the entire K27Ub2 molecule is jammed, so to speak, in the jaws of the...
enzyme. This suggests that K27Ub2 should be an inhibitor of UCHL3. We tested whether mono-Ub or any Ub2 would affect cleavage of the minimal substrate Ub-Rho by UCHL3 (Figure 4D) and found that excess K27Ub2 results in almost complete inhibition of UCHL3 activity, with effects comparable to those of a covalent inhibitor of cysteine proteases such as iodoacetamide. The other variants showed limited effect that could be due to “product inhibition.” This strong inhibitory effect of free K27Ub2 is also observed when UCHL3 cleaves other substrates such as Ub-His (Figure 4E).

UCHL3 Prefers Smaller Substrates

The structure predicts that UCHL3 could remove K27 chains from substrates, albeit at the likely cost of being inhibited in the process. We compared UCHL3 activity for substrates conjugated to three different Ub types: mono-Ub, K27Ub2, and K63Ub2 (Figure 1). It is known that UCHL3 prefers smaller substrates that can easily thread underneath the crossover loop (Bett et al., 2015; Navarro et al., 2014; Popp et al., 2009; Zhou et al., 2012). First, we measured the baseline cleavage speed of UCHL3 against differently sized monoubiquitinated substrates.

**Figure 3.** UCHL3 Forms Thioester Bonds with Ub/K27Ub2 in Crystals and in Solution

(A) Structure of UCHL3 in complex with K27Ub2. The proximal Ub of K27Ub2 (UbP, blue) has its C terminus covalently bound to the active site of UCHL3 (gold); the distal Ub (UbD, pink) sits atop the crossover loop of UCHL3 (red).

(B) UCHL3 forms a thioester bond with K27Ub2 in crystals. Continuous density (1.5 σ, blue mesh) connects the catalytic cysteine of UCHL3 (gold) to the C terminus of UbD (blue). The catalytic triad is aligned and the catalytic water is correctly positioned. Dotted lines, hydrogen bonds.

(C) Expected NaBH4-mediated reduction of a non-covalent (path 1) or a thioester (path 2) UCHL3:Ub(n) complex. Ub aldehyde is formed only with a thioester complex.

(D) UCHL3 forms thioester bonds with Ub in solution. UCHL3 only control or Ub:UCHL3 complex was reduced with excess NaBH4 for 30 min, and the reaction was resolved by mass spectrometry. Sections of the m/z spectra for the Ub only control and Ub:UCHL3 complex are plotted. An extra envelope corresponding to Ub-aldehyde appears when treating the Ub:UCHL3 complex with NaBH4, indicating the presence of a thioester complex (right panel) and no reaction on Ub without UCHL3 present (left panel). The y axis is normalized to the height of the free Ub/Ub2 peak.

(E) As in (D), except that K27Ub2 was used instead of Ub, and K27Ub2-aldehyde was detected.
Figure 5C), although the efficiency is much lower than for the smaller substrates, as expected. UCHL3 is, however, unable to remove mono-Ub from the even larger proliferating cell nuclear antigen (PCNA) (Figure 5D). Comparison of cleavage data confirms earlier data: UCHL3 activity decreases with increasing size of the adduct conjugated to the proximal Ub, up to a point where cleavage ceases to happen.

**K27Ub2-Conjugated Substrates Inhibit UCHL3 Activity**

We then tested how UCHL3 deals with the same substrates when they are conjugated to K27Ub2 or to K63Ub2. We found that UCHL3 could deubiquitinate all substrates, except K27Ub2-PCNA (Figures 5E–5H and data not shown). In line with what we observed in Figure 5A, the cleavage speed of diubiquitinated substrates seemed to generally decrease with increased substrate size, with Ub2-AMC being fastest, followed by Ub2-L40-Rho and Ub2-3×SUMO2ΔN (compare incubation times in Figures 5E–5H). K27-ubiquitinated substrates were, however, always processed more slowly than their K63-linked counterparts. Thus, as expected, K27Ub2-conjugated substrates inhibit UCHL3 activity. To quantify the inhibition, we used stopped-flow analysis of DUB activity and compared Ub-AMC, K63Ub2-AMC, and K27Ub2-AMC (Figure 5E). K27Ub2-AMC is relatively slower, and, surprisingly, it becomes even slower with increasing substrate-to-enzyme ratio (Figure 5G). In other words, the inhibitory effect of K27Ub2-AMC increases with increasing substrate turnover by the enzyme. This is also the case for Ub/K27Ub2-AMC conjugated to a larger substrate, L40-Rho (Figure 5F).

**K27Ub2 Inhibits UCHL3 via a Kinetic Trap**

In the experiments presented so far, we showed that the activity of UCHL3 is influenced by the identity of the Ub chain (K27-linked versus non-K27-linked), by the size of the moiety conjugated to the C terminus of the Ub chain, and by whether free K27Ub2 is present. To fully understand the interplay of these three factors and ultimately how K27Ub2 inhibits UCHL3, we used KinTek Explorer (Johnson et al., 2009a) to generate a global kinetic model of UCHL3 cleavage based on the stopped-flow data in Figure 5F and the binding data in Figures 1A and 1B. The cleavage of Ub-AMC and K63Ub2-AMC is fully explained by a simple product inhibition scheme, depicted in the green portion of the model in Figure 6A (see Figure 6B for a table of kinetic constants, Article 195).
Figure 5F for fit to the data, Figures S5B–S5D for parameter robustness analysis and STAR Methods for details on model fitting). In this model, UCHL3 binds to the Ub(n)-AMC substrate, cleaves and releases the fluorophore, and then releases the (di)Ub and is free for further catalysis. Cleaved (di)Ub may compete with unprocessed substrate for binding to the enzyme, resulting in product inhibition. This is the commonly accepted model for UCH-domain DUB catalysis (Mevissen and Komander, 2017) (Figure S1A). A small discrepancy between measurement and prediction in the terminal phase of each reaction (Figure 5E) is likely due to slow cleavage of contaminant misfolded substrate, since this behavior varied between batches. Cleavage of K27Ub2-AMC, however, cannot be fitted by this model. K27Ub2-AMC provides an inhibitory effect that is dependent on the enzyme turnover (Figure 5G), but product inhibition alone could not explain this. The simplest model that captures this effect requires the addition of an extra reaction pathway, shaded in cyan in Figure 6A. According to the complete model, UCHL3 binds to the substrate and then has two possible reaction ’choices.’ One possibility (the ’fast track’) is the reaction path that is also used for the other Ub chains (green in Figure 6A, estimated probability = 71% ± 13.3%). In this case, the substrate is cleaved, fluorophore and K27Ub2 are released, and the enzyme returns to the active pool. However, there is a second, ’slow track,’ specific for K27Ub2-conjugated substrates (cyan portion of the model in Figure 6B, estimated probability = 29 ± 9.8%). In this path, UCHL3:K27Ub2 must undergo some conformational change before catalysis. Cleavage itself is as fast as in the green path, but after release of the substrate, the reversion of the conformational change to allow release of K27Ub2 is the...
bottleneck of the reaction (Figure 6B, compare the magnitude of $k_{open2}/k_{close2}$ with the other turnover constants). In practice, this means that once UCHL3 takes the slow path, it will be kinetically trapped in a long-lived, stable inhibitory complex with the K27Ub2 product. The probabilistic occurrence of fast and slow pathways explains the observed dependence on catalysis for the inhibition of UCHL3 by K27Ub2-AMC: each round of catalysis increases the cumulative probability that UCHL3 might fall into the inhibitory, kinetic trap.

**UCHL3 Activity Is Quickly and Robustly Blocked by Conjugated and Free K27Ub2 Species**

We have already shown that K27-ubiquitinated substrates inhibit UCHL3. Our model predicts rapid inhibition. If we simulate a reaction in which 100 nM K27Ub2-AMC substrate is given to 1 nM UCHL3 (Figure 6D), we predict that most of the UCHL3 is inhibited within <1 s of coming into contact with the K27Ub2-AMC substrate. The reaction will then slow down, and UCHL3 will stay >95% inhibited as long as the substrate is present (~15 min in this scenario). If, alternatively, we simulate the addition of 100 nM free and unconjugated K27Ub2 (“product-like”) to 1 nM UCHL3, we predict that within 10 s about 80% of the UCHL3 will be inhibited, with 25% of this fraction assuming the long-lived, conformationally inhibited state (Figure 6E). Although the magnitude and speed of inhibition by free K27Ub2-“products” are lower compared with K27Ub2 substrates, in both cases the simulations show that UCHL3 is a sensitive and fast-responding sensor for the presence of K27Ub2 species.

**K27Ub2 Inhibition Is Allosteric and Reversibly Covalent**

Comparison of UCHL3 structures bound to Ub-vinyl-methyl-ester (Misaghi et al., 2005) and free K27Ub2 (PDB: 5UJN, Castaneda et al., 2016) via UbP, in several conformations UbP (orange) does not touch UCHL3, and the crossover loop can close and allow catalysis. NMR models clashing with UCHL3 are not shown. (D) K27Ub2 substrates quickly and strongly inhibit UCHL3 activity. Using the model in (A) we simulated a reaction between 1 nM UCHL3 and 100 nM K27Ub2-AMC (100-fold excess substrate). The curves indicate the concentration of each species over time. Within 1 s, UCHL3;K27Ub2 is kinetically trapped, to be released only after minutes following the disappearance of the substrate. Purple, K27Ub2-AMC; magenta, AMC; gold, free UCHL3; green, open UCHL3;K27Ub2 complex; blue, closed UCHL3;K27Ub2 complex. Time is in log10 scale. (E) Free K27Ub2 inhibits UCHL3 competitively and allosterically. Analogous to (D), but with 100 nM free K27Ub2 and 1 nM UCHL3. Colors as in (D). Binding of K27Ub2 to UCHL3 induces the appearance of the open complex (product inhibition) and of the closed complex (allosteric inhibition). (F) UCHL3 can be used as a tool to detect free K27Ub2. Ub and all possible di-Ubs (Lys- and Met-linked) were western blotted using either anti-Ub antibody (mouse) plus IRdye680-conjugated anti-mouse antibody (red, top) or biotinylated UCHL3 C95A plus IRdye800-conjugated neutravidin (green, middle). Bottom: merged signal, showing that UCHL3 recognizes K27Ub2 specifically.
Fluorescence resonance energy transfer measurements of the distance between UbP and UbD in solution by Pan et al. (Pan et al., 2019) also support this induced-fit model. Overall, the open conformations seem to portray an initial engagement stage, whereas our structure seems to portray a “closed,” long-term stable conformation, with the crossover loop locked in place between the two Ub moieties. Based on these structural considerations, we propose that in the kinetic model the “fast track” represents cleavage in the open conformation. Our structure, on the other hand, corresponds to the kinetically trapped reaction intermediate.

Role of the Covalent Bond

Our kinetic model does not explicitly include a covalent thioester bond, and indeed does not require a covalent bond formation step to fit the data well. However, covalent UCHL3:K27Ub2 complexes exist in solution (Figure 3E) and the model (Figure 6A) does suggest two UCHL3:K27Ub2 complexes coexisting: one with fast exchange rate and one long lived (compare kinetic constants in Figure 6A). It seems likely that the long-lived complex (with a closed K27Ub2 conformation) retains the thioester bond, whereas the open conformation does not. This would explain why K27Ub2-conjugated substrates are better and faster inhibitors than free K27Ub2. K27Ub2-conjugated substrates reach the thioester bond as a natural intermediate in catalysis, and thus can produce the inhibitory complex faster and, at least initially, at higher concentration (compare Figures 6D and 6E). Thus, overall, we speculate that the inhibitory mechanism of K27Ub2 is mediated mainly by the allosteric block of UbD on the crossover loop, with a secondary contribution by the covalent binding of UbD to the catalytic cysteine.

UCHL3 as a Sensor of K27Ub Chains

Because UCHL3 recognizes K27Ub2 with high specificity, we tested if UCHL3 could be a tool to detect K27 chains. We generated a catalytically inactive C95A UCHL3 with a C-terminal biotinylation tag (AviTag, AviD LL), biotinylated it, and used it for detection in western blot. Under these conditions, UCHL3 recognizes K27Ub2 over all other diubiquitin linkages (Figure 6F), similar to the existing K27-specific antibody (data not shown). Similar to other DUBs, mutating the catalytic cysteine to alanine (UCHL3 C95A) increases the affinity of UCHL3 for Ub (Morrow et al., 2018) or, in this case, K27-linked diubiquitin, making it useful as an affinity reagent. Taken together, these results make UCHL3 C95A AviTag an attractive and easily obtained tool to be used as an antibody or affimer to detect free K27Ub2 in western blot experiments.

Possible Roles for UCHL3 and K27Ub2 in Cell Signaling

In vitro, both free K27Ub2 and K27Ub2-conjugated substrates quickly and potently inhibit UCHL3. Conversely, UCHL3 can specifically detect free K27Ub chains over other linkages. The abundance of free K27-linked chains in cells is unknown, but K27Ub chains are hallmark signals of the innate immune response and are conjugated to virtually all of the sensors and effectors of the intracellular pathogen response pathway (van Huizen and Kikkert, 2020). In addition, K27-linked chains are involved in the regulation of the intracellular DNA damage response (Akutsu et al., 2016). It is possible that UCHL3 acts as a sensor for free or conjugated K27-linked chains in these pathways or, conversely, that K27-linked chains regulate UCHL3’s activity. Corroborating this idea, UCHL3 is necessary for the orderly progression of both the immune response and the DNA damage pathway, although the mechanism is still unclear. However, because UCHL3 prefers to cleave smaller substrates (Figures 5A–5D and Navarro et al., 2014; Popp et al., 2009), we further speculate that K27Ub2-conjugated substrates that affect UCHL3 are likely to be small and/or disordered proteins. Recent work has shown the existence and importance of a large number of functional and abundant microproteins (10–100 residues) (Chen et al., 2020; Yin et al., 2019) that, if appropriately K27-ubiquitinated, could be viable targets for UCHL3. Our data showed an unusual kinetic response of UCHL3 to K27 chains. Further research will have to elucidate if and how this relationship affects host-pathogen interactions.

SIGNIFICANCE

K27-linked ubiquitin chains are a poorly understood type of ubiquitin modification, owing in part to the fact that they cannot be made enzymatically. Although not largely present in cells under normal conditions, they are upregulated during pathogenic infections and interact with virtually all players of the immune response pathway.

UCHL3 is one of only three deubiquitinating enzymes that show a strong preference for K27-linked diubiquitin. However, the relation between UCHL3 and K27 is not a simple preference for a substrate. Rather, using a series of specifically crafted ubiquitin tools, we show that K27Ub2 strongly inhibits UCHL3 activity, both when free and when conjugated to a substrate.

The mechanism of inhibition is unusual. K27Ub2 can form a thioester bond with UCHL3’s catalytic cysteine and can adopt an inhibitory conformation that restrains UCHL3’s crossover loop. At each cleavage cycle, there is a finite probability that K27Ub2 might adopt the inhibitory conformation, trapping UCHL3. Thus, the more K27-ubiquitinated substrates UCHL3 cleaves, the higher is the chance of it becoming inhibited. We dubbed this phenomenon a “kinetic trap.”

The fact that the kinetic trap is specific for the K27 linkage and the observation that the levels of inhibition depend on the ratio of substrate to UCHL3 suggest that K27Ub2 and UCHL3 act as a signal–response pair. UCHL3 could be sensing K27-ubiquitinated substrate levels in the cell, or K27-ubiquitinated substrates could be dampeners of UCHL3 activity. Either way, since both UCHL3 and K27-linked ubiquitin chains play roles in the immune response, it seems possible that this mechanism is required for a proper response during innate immune reactions.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:
We thank M. Bower and MASSIF-1/ID30A staff for data collection; X. Guo for experimental reagents. G.B.A.v.T. and A.M. contributed equally to this work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: September 17, 2020
Revised: October 25, 2020
Accepted: November 4, 2020
Published: November 24, 2020

SUPPORTING CITATIONS

The following reference appears in the supplemental information:


REFERENCES

UCHL3 regulates topoisomerase-induced chromosomal break repair by con-
3358–3368.
200 Cell Chemical Biology
Hibbert, R.G., and Sixma, T.K. (2012). Intrinsic flexibility of ubiquitin on prolif-
Anal. Biochem.
algorithm to evaluate multidimensional parameter space in fitting kinetic data.
tions convert deubiquitinases into high-affinity ubiquitin-binding proteins.
Pickart, C.M., and Rose, I.A. (1986). Mechanism of ubiquitin carboxyl-terminal hydrolase. Borohydrate and hydroxylamine inactivate in the presence of ubiqui-


## STAR★METHODS

### KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibodies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti SUMO2 (rabbit polyclonal)</td>
<td>Life Technologies</td>
<td>Cat#519100</td>
</tr>
<tr>
<td>Anti Ubiquitin (mouse)</td>
<td>Santa Cruz</td>
<td>Cat#P4D1</td>
</tr>
<tr>
<td>neutravidin DyLight800</td>
<td>Thermo Fisher Scientific</td>
<td>Cat#22853</td>
</tr>
<tr>
<td>donkey-α-rabbit IRDye®680</td>
<td>LI-COR</td>
<td>Cat# 926-68073; RRID: AB_10954442</td>
</tr>
<tr>
<td>goat-α-mouse IRDye®800</td>
<td>LI-COR</td>
<td>Cat#926-32210; RRID: AB_621842</td>
</tr>
<tr>
<td>Goat Anti-Mouse IgG (H + L)-HRP Conjugate</td>
<td>Bio Rad</td>
<td>Cat#170-6516; RRID: AB_11125547</td>
</tr>
<tr>
<td>Goat Anti-Rabbit IgG (H + L)-HRP Conjugate</td>
<td>Bio Rad</td>
<td>Cat#1706515; RRID: AB_2617112</td>
</tr>
<tr>
<td><strong>Bacterial and Virus Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli BL21(DE3) T1 phage resistant</td>
<td>New England Biosciences</td>
<td>Cat#C2527H</td>
</tr>
<tr>
<td><strong>Chemicals, Peptides, and Recombinant Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K27Ub2</td>
<td>El Oualid et al., 2010</td>
<td>N/A</td>
</tr>
<tr>
<td>K63Ub2</td>
<td>El Oualid et al., 2010</td>
<td>N/A</td>
</tr>
<tr>
<td>Ub-Rho</td>
<td>El Oualid et al., 2010</td>
<td>N/A</td>
</tr>
<tr>
<td>K27Ub2-Rho</td>
<td>Flierman et al., 2016</td>
<td>N/A</td>
</tr>
<tr>
<td>K63Ub2-Rho</td>
<td>Flierman et al., 2016</td>
<td>N/A</td>
</tr>
<tr>
<td>Ub-L40-Rho</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>K27Ub2-L40-Rho</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Ub-His</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Ub-SUMO2</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>3xSUMO2aN11</td>
<td>Eifler et al., 2018</td>
<td>N/A</td>
</tr>
<tr>
<td>UCHL3</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>UCHL3 E158R</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>UCHL3 E158A</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>UCHL3 C95S</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>UCHL3 C95A</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>His-SUMO-UCHL3</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>His-SUMO-UCHL3 C95A</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>His-SUMO-UCHL3 C95S</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Biotinylated UCHL3 C95A</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Ub-PCNA</td>
<td>Hibbert and Sixma, 2012</td>
<td>N/A</td>
</tr>
<tr>
<td>Ub(n)-3xSUMO2aN</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Critical Commercial Assays</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA chip Biacore</td>
<td>Cytiva Life Sciences</td>
<td>Cat#29104992</td>
</tr>
<tr>
<td><strong>Deposited Data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCHL3</td>
<td>Johnston et al., 1997</td>
<td>PDB: 1UCH</td>
</tr>
<tr>
<td>UCHL3-UbVME</td>
<td>Misaghi et al.</td>
<td>PDB: 1XD3</td>
</tr>
<tr>
<td>UCHL3-Lym27Ub2</td>
<td>Pan et al., 2019</td>
<td>PDB: 6ISU</td>
</tr>
<tr>
<td>K6-linked diubiquitin</td>
<td>Virdee et al., 2010</td>
<td>PDB: 2XK5</td>
</tr>
<tr>
<td>K11-linked diubiquitin</td>
<td>Bremm et al., 2010</td>
<td>PDB: 2XEW</td>
</tr>
<tr>
<td>K11-linked di-ubiquitin</td>
<td>Matsumoto et al., 2010</td>
<td>PDB: 3NOB</td>
</tr>
<tr>
<td>TRABID NZF1- K29 linked di-Ubiquitin</td>
<td>Kristariyanto et al., 2015</td>
<td>PDB: 4S1Z</td>
</tr>
<tr>
<td>K29 linked di-Ubiquitin</td>
<td>Kristariyanto et al., 2015</td>
<td>PDB: 4S22</td>
</tr>
</tbody>
</table>

(Continued on next page)
RESOURCE AVAILABILITY

**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Prof. Titia Sixma (t.sixma@nki.nl).

**Materials Availability**

**Plasmids**
The pETNKI vectors (Luna-Vargas et al., 2011) are available through Addgene; all constructs generated are available on request or through Addgene.

**Synthetic Proteins**
Protocols to generate synthetic diUb-reagents and recombinant proteins are included in the Materials & Methods section. Native isopeptide linked diubiquitin reagents are available via Boston Biochem. Other synthetic and semi-synthetic substrates are custom made and questions regarding assistance with synthesis can be addressed to gvanderheden@lumc.nl.

**Data and Code Availability**
The accession number for structure factors and model coordinates for the UCHL3_K27Ub2 crystal structure reported in this paper is PDB: 6QML.

Further details and raw data for the Kintek model are available on request.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial Strains
All recombinant proteins were expressed in Escherichia coli strain BL21 (DE3), T1 Phage resistant (New England Biosciences Cat#C2527H). Details of culture and expression conditions for each recombinant construct are specified in Method Details.

METHOD DETAILS

Capture of the Thioester UCHL3~K27Ub2 Bond by NaBH4
Mono Ub (10 μg/mL) and UCHL3 (10 μg/mL) or K27Ub2 (20 μg/mL) and UCHL3 (10 μg/mL) were incubated for 90 minutes at 37 °C in 200 μL buffer (50 mM Tris-HCl pH 7.6, 5 mM DTT, 1 mM EDTA). Subsequently, 1 mM of NaBH4 was added 6 times in total with an interval of 5 minutes between each new addition (total incubation time 30 minutes, final concentration 6 mM NaBH4). 1 M formic acid was added for 5 minutes before final measurement on a Waters Xevo-G2 XS Q-TOF mass spectrometer equipped with an electrospray ion source in positive mode (source voltage 3.0 kV, desolvation gas flow 900L/h, temperature 250°C) with resolution R = 22000 (mass range m/z = 50-2000) and 200 pg/μl Leu-Enk (m/z = 556.2771) as “lock mass”). Samples were run according to the following protocol: 0 - 4 minutes in 2% B (1% H2O and 0.1% formic acid in CH3CN), a gradient from 2% to 100% B over 7.5 minutes (4 - 11.5 minutes), 0.5 minute at 100% B (11.5 - 12 minutes) and a reduction to 2% B and 98% A (H2O with 0.1% formic acid) in 2 minutes (12.10 - 14.00 minutes). Reaction products were separated on an Acquity UPLC Protein BEH C4 300 Angstrom column (1.7 μm, 2.1 mm x 50 mm) at 60 °C using a flow rate of 0.5 mL/min. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1.

Synthesis of Ubiquitin and Diubiquitin Species
Solid phase peptide synthesis (SPPS) was performed on a Syro II MultiSyntech Automated Peptide synthesizer using standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide chemistry at 25 μmol scale, using fourfold excess of amino acids relative to pre-loaded Fmoc amino acid trityl resin (0.2 mmol/g, Rapp Polymere GmbH). All synthetic products were assayed for purity by high resolution mass spectrometry on a Waters Acquity H-class UPLC withXEVO-G2 XS Q-TOF mass spectrometer and by SDS PAGE analysis.

Ub-Rho
Ub-Rho was prepared using the methodology described in (El Oualid et al., 2010). Briefly, Ub1-75 was synthesized using SPPS and liberated from the resin using 20% v/v HFIP in DCM while leaving all protective groups in place. After concentration of the crude material, subsequent activation of the C-terminal carboxylate of Gly75 using HBTU/HOBt allowed coupling of bis-glycine-rhodamine (Ub-Rho) synthesis. DiUb products were purified by RP-HPLC (detailed below) and size exclusion (S75 16/60 PG, GE healthcare) in 20 mM Tris-HCl pH 7.6, 150 mM NaCl.

Ub-AMC, K27Ub2-AMC, K63Ub2-AMC
Ub-AMC was prepared as described in (El Oualid et al., 2010). Briefly, Ub1-75 was synthesized using SPPS and liberated from the resin using 20% v/v HFIP in DCM while leaving all protective groups in place. After concentration of the crude material, subsequent activation of the C-terminal carboxylate of Gly75 using HBTU/HOBt allowed coupling of amine-ACMC. Treatment with 95% TFA removed all protective groups. Precipitation from Et3O/Pentane 1:1, v/v followed by RP-HPLC purification and lyophilization of the appropriate fractions yielded the Ub-AMC product.

K63Ub2-AMC and K27Ub2-AMC were prepared as described in (Flierman et al., 2016). Briefly, Ub-propargylamide was coupled to Ub-AMC, containing azido-ornithine at the respective lysine position. Products were purified by RP-HPLC (detailed below) and size exclusion (S75 16/60 PG, GE healthcare) in 20 mM Tris-HCl pH 7.6, 150 mM NaCl.

K27Ub2 (K27Ub2Met1NLE)
K27Ub2 was prepared similarly to the protocol described in (El Oualid et al., 2010). We substituted methionine for norleucine on position 1 of the proximal ubiquitin to avoid oxidation during synthesis; the norleucine residue has no contact with UCHL3, and the construct behaves like wild type Ub2. Met1 on the distal ubiquitin is not substituted. In brief, tert-butylthio (StBu)-protected Met1NLE Lys27 γ-thiolysine Ub (16.5 mg) was dissolved in 100 μL DMSO and added to 350 μL of 8 M Guanidium-HCl (Gdn.HCl)/100 mM phosphate...
buffer at pH 7.6 supplemented with 50 μL 1 M tris(2-carboxy-ethyl)phosphine (TCEP) and reacted at 37 °C. After LC-MS analysis revealed complete deprotection of the thiolysine, 1 equivalent of Ub-thioester dissolved in 100 μL DMSO and 400 μL 8 M Gdn.HCl/100 mM phosphate buffer at pH 7.6 and 100 μL 1 M 4-Mercaptophenylacetic acid (MPAA) were added. The pH was adjusted to 7.6 and the mixture reacted for 16 hours at 37 °C. RP-HPLC purification was followed by lyophilization of the appropriate fractions. The lyophilized \( ^{27} \text{KUb}_2 (\sim 30 \text{ mg}) \) was desulfurized by dissolving in 200 μL dimethyl sulfoxide (DMSO) and subsequent dilution into 6 mL 8 M Gdn.HCl/100 mM phosphate buffer at pH 7.6 and 1 mL 1M TCEP. 2,2′-Azobis[2-(2-midazolin-2-yl)propane] Dihydrochloride (VA044) (25 mg/mL) and reduced glutathione (GSH, 25 mg/mL) were added, pH was adjusted to 7.0 and the reaction was agitated for 16 hours at 37 °C. RP-HPLC purification was followed by lyophilization of the appropriate fractions. The lyophilized \( ^{27} \text{KUb}_2 \) was dissolved in 200 μL DMSO and diluted into 1800 μL 20 mM TRIS, 150 mM NaCl at pH 7.6, purified on a S75 16/600 Superdex size exclusion column (GE healthcare) and concentrated to 7.2 mg/mL by spin filtration. The aliquots were snap frozen and stored at -80 °C until further use. Diubiquitins of other linkages (including \( ^{K63} \text{Ub}_2 \)) were prepared similarly using tert-butyl thiol (StBu)-protected Met1NLE, Lys(γ)-thiolysine Ub as the proximal ubiquitin, where X is the lysine on the proximal ubiquitin to be linked.

**Ub-L40-Rho**

Ub-L40 (ubiquitin fused to the decapeptide IIEPSLRQLA with rhodamine on the C-terminus) was prepared on trityl resin using automated synthesis and its N-terminus was protected with a BOC group by treatment with Boc2O (5 equivalents) and DiPEA (15 equivalents) in DCM for 2 hours. Subsequently the resin was washed with DCM and the peptide liberated from the resin while leaving all protective groups in place by reacting the resin 2 times for 20 minutes with 20 % v/v HFIP/DCM. The collected filtrates were concentrated, co-evaporated with dichloroethane three times and put under high vacuum for 30 minutes before further use. Amino-propane modified rhodamine (3 equivalents, synthesis described below) was coupled to the C-terminus of the peptide using HBTU (3 equivalents), HOBt (6 eq., 1.44 mmol, 1.44 mL of a 1M solution in THF) and the reaction was stirred for 15 hours after which TLC analysis indicated a complete conversion after 30 min. The mixture was diluted with 20 mL DCM and extracted with 0.1 M HClaq (2x), saturated aqueous NaHCO3 (2x) and brine and dried over MgSO4. Celite was added and the mixture was concentrated to dryness under reduced pressure. The compound bound to Celite was loaded onto a 24g silica column and purified on a BUCHI automated column chromatography system using a 10 → 100% v/v ethyl acetate/n-heptane gradient. The product was obtained as a pale yellow solid (yield: 160 mg, 0.24 mmol, 68%).

**Synthesis of Rho[Boc]2-\( \text{NH}(\text{CH}_2)_{3}\text{N}_3 \)**

\( \text{N}^{\prime} \)-diBoc-5-carboxyrhodamine (206 mg, 0.36 mmol) was dissolved in DCM (10 mL), DiPEA (4.5 eq., 1.62 mmol, 285 μL), HCTU (1.5 eq., 0.54 mmol, 223 mg) and 3-azido-1-propanamine (1.5 eq., 0.54 mmol, 53 μL) were added and the mixture was stirred until TLC analysis indicated a complete conversion after 30 min. The mixture was diluted with 20 mL DCM and extracted with 0.1 M HClaq (2x), saturated aqueous NaHCO3 (2x) and brine and dried over MgSO4. Celite was added and the mixture was concentrated to dryness under reduced pressure. The compound bound to Celite was loaded onto a 24g silica column and purified on a BUCHI automated column chromatography system using a 10 → 100% v/v ethyl acetate/n-heptane gradient. The product was obtained as a pale yellow solid (yield: 150 mg, 0.23 mmol, 98%).

**Ub-L40-Rho**

\( ^{27} \text{KUb}_2 \)-L40-Rho was prepared analogously to Ub-L40-Rho, except StBu-protected K27-γ-thiolysine-Ub-L40 was used as starting material to obtain K27-γ-thiolysine-Ub-L40-Rho. Native chemical ligation and desulfurization as described for \( ^{27} \text{KUb}_2 \) was then performed to provide the target \( ^{27} \text{KUb}_2 \)-L40-Rho.

**Reverse Phase, High Performance Liquid Chromatography (RP-HPLC) Purifications**

RP-HPLC purification were carried out on a Waters preparative RP-HPLC system equipped with a Waters C18-Xbridge 5 μm OBD (10 x 150 mm) column at a flowrate of 37.5 mL/min. using 3 mobile phases: A: MQ, B: CH₃CN and C: 1% TFA in MQ. Prep-HPLC program: Gradient: 0 → 5 min: 5% B, 5% C; 5 → 7 min: 5 → 20% B, 5% C; 7 → 18 min: 20 → 45% B, 5% C; on a Waters C18-Xbridge 5 μm OBD (30 x 150 mm) column at a flow rate of 37.5 mL/min. Pure fractions were pooled and lyophilized.

**Recombinant Protein Cloning, Expression and Purification**

*Escherichia coli* strain BL21 (DE3), T1 Phage resistant, was transformed according to standard protocol. After transformation, a single colony was amplified overnight in Luria Bertani broth (LB) at 37 °C and used as inoculum for large scale expression. Protein expression was carried out using 500 ml of medium in 3.5-liter, baffled Fernbach flasks, with 50 μL Antifoam 204 (Sigma-Aldrich) added to avoid foam. Flasks were shaken at 120 rpm during growth. Induction details vary for each construct and are indicated under each subheading. Pellets were harvested by centrifugation and used fresh, or frozen in liquid nitrogen and stored at -20 °C.

**Ub-His**

Human ubiquitin was slabcasted in a PET3a vector derivative encoding for a C-terminal His tag, resulting in the final protein sequence (Ubiquitin1-76)-DHHHHHHH. Protein expression was carried out in Terrific Broth (TB). Cells were grown at 37 °C to an optical density of 1.5, then induced with 0.5 mM isopropyl-thiogalactoside (IPTG) for 3 hours. Pellets were harvested and resuspended in lysis buffer (300 mM NaCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.0) and lysed with two passes through an Avestin emulsiflex C5 (Avestin) at 200 kPa of pressure. The lysate was clarified by centrifugation (75000 g, 45 minutes, Avanti JXN26 centrifuge and JA25.50 rotor, Beckman Coulter) and applied with a peristaltic pump (flow 2 ml/min) to 10 ml of chelating sepharose resin.
SUMO2

A pHis-TEV30a encoding a decahistidine-tagged 3xSUMO2 was concentrated, aliquoted, flash frozen and stored at -80°C. All steps after lysis were carried out at 4°C or on ice.

Ub-SUMO2

The Ub-SUMO2 construct encodes a single copy of ubiquitin (Uniprot ID: P0CG48, residues 1-76) N-terminally fused to SUMO2 (Uniprot ID: P61956) and was synthesized as a gene block (IDT DNA) and subcloned in the pET-NKI-1.1-His3C-Kan vector (encoding for a 3C protease-cleavable hexahistidine tag, addgene #108703, Luna-Vargas et al., 2011) using ligation independent cloning (Scholz et al., 2013). Expression and purification followed the same protocol as Ub-His, but the hexahistidine tag was removed by 3C protease cleavage (NIKI protein facility) during overnight dialysis prior to gel filtration.

3xSUMO2.ΔN11

A pHis-TEV30a encoding a decahistidine-tagged 3xSUMO2ΔN11 construct was a kind gift from Prof. A. Vertegaal. For purification, we used a protocol adapted from (Eifler et al., 2018). Briefly, His10-tagged 3xSUMO2 was expressed in BL21 cells using 500 mL LB media in 2.5 liter baffled shaking flasks. Protein expression was induced at OD600 = 0.6 with 0.5 mM IPTG and overnight incubation at 18°C. Cells were lysed through sonication on ice in 20 mM Tris-HCl pH 7.6, 150 mM NaCl, 25 mM MgCl2, 5 mM dithiothreitol (DTT), 15 μg/mL DNase I (Roche), 10 mM imidazole and 1x Complete protease inhibitor without EDTA (Roche). The protein was captured by affinity purification using 2 mL Ni-NTA beads (Qiagen) in a Poly-Prep Chromatography column (BioRad) and eluted two times at 4°C in 5 mL 500 mM imidazole, 20 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM DTT. After elution, the N-terminal His10 tag was removed by TEV protease and a final size exclusion step (S75 16/60 PG, GE healthcare) was performed in 20 mM Tris pH 7.6, 150 mM NaCl, 5 mM DTT.

Wild type UCHL3, UCHL3 E158A, UCHL3 E158R

Human UCHL3 was cloned in pRP265, a pGEX-2T derivative, using IVA cloning (Garcia-Nafria et al., 2016) and expressed in E. coli BL21(DE3) as N-terminal Glutathione-S-transferase (GST) fusion. Cells were grown in baffled shaking flasks containing LB medium and grown to an absorbance of 0.8 at 600 nm before induction with 0.5 mM IPTG. Cells were pelleted by centrifugation and resuspended in 40 mL lysis buffer (50 mM Tris pH 7.5, 10 mM DTT, 50 μM PMSF (Sigma), 1 mM EDTA, 10 mM MgCl2). Lysozyme was added to 10,000 U/mL and incubated for 30 min. at 4°C, after which the suspension was sonicated. The lysate was incubated with 2 mL glutathione sepharose 4B (GE healthcare) O/N at 4°C, while rolling in a 50 mL Falcon tube. The next day, beads were washed 5 times with 50 mL buffer A (50 mM Tris pH 7.5, 0.5 mM EDTA, 5 mM DTT) and UCHL3 was cleaved from the beads with thrombin protease (3 hours, RT), leaving a Gly-Ser remnant at the N-terminus. After a reverse affinity purification step to remove residual GST traces, the protein was gel filtered in buffer A (S75 16/60 PG, GE healthcare). Purified protein fractions were analyzed by SDS-PAGE and LC-MS (LCT™ Orthogonal Acceleration Time of Flight Mass spectrometer, Micromass). The appropriate fractions were concentrated, aliquoted, flash frozen and stored at -80°C.

His-SUMO-UCHL3

Human UCHL3 was cloned in pETNKI-1.10-HisSUMO-Kan vector (Luna-Vargas et al., 2011), encoding for a N-terminal hexahistidine SUMO2 tag. Cells were grown in shaking, baffled Fernbach flasks (3L, Schott) using 500 ml of terrific broth (TB) medium per flask until optical density of 1.5 and protein expression was induced by addition of 0.5 mM isopropyl-thiogalactoside (IPTG) for 16 hours at 25°C. Pellets were resuspended in lysis buffer (20 mM NaCl, 20 mM HEPEs, pH 8.0) and lysed with two passes through an Avestin emulsiflex C5 (Avestin) at 200 kPa of pressure. The lysate was cleared by centrifugation (75000 g, 45 minutes, Avanti JXN26 centrifuge and JA25.50 rotor, Beckman Coulter) and applied with a peristaltic pump (flow 2 ml/min) to 10 ml of chelating sepharose resin (GE healthcare) preloaded with Ni2+, pre-equilibrated in lysis buffer and packed in an HK-16/30 column holder (GE healthcare). The column was then washed with 1M NaCl, 20 mM HEPEs pH 8.2, transferred to an AKTA or DuoLogic Bioflow liquid chromatography system (GE healthcare / Biorad, respectively), washed further with 300 mM NaCl, 50 mM imidazole pH 8.0, 20 mM HEPEs. The protein was eluted using gel filtration buffer (100 mM NaCl, 20 mM HEPEs pH 8.2) supplemented with 250 mM imidazole pH 8.0, dialysed overnight against gel filtration buffer + 5 mM dithiothreitol (DTT), concentrated by ultrafiltration (Amicon ultra, 3000 MWCO) and further purified by size exclusion chromatography (Superdex 75 16/60, GE healthcare or ENrich SEC 70 10/300, Biorad). The resulting pure protein was concentrated, aliquoted, flash frozen and stored at -80°C. All steps after lysis were carried out at 4°C or on ice.

UCHL3 C95A, C95S

C95A and C95S mutants of His-SUMO-UCHL3 were generated by Quikchange site-directed mutagenesis (Agilent). Expression, lysis and affinity purification were carried out as for His-SUMO-UCHL3, but during dialysis, the SUMO-His tag was cleaved by digestion with SENP2 protease (NIK protein facility). UCHL3 was separated from the cleaved tag using a 10 ml nickel column, with UCHL3 collected from the flowthrough. The protein was then further gel filtered as detailed in His-SUMO-UCHL3.

UCHL3 K27Ub2 Complex

To obtain the UCHL3 K27Ub2 complex, UCHL3 and K27Ub2 were mixed in 1 to 1.1 ratio and incubated for 10’ on ice; the excess of ubiquitin was removed by size exclusion chromatography (Superdex 75 10/300, GE healthcare) in 100 mM NaCl, 20 mM HEPEs pH 8.1, and the protein complex was concentrated to 17 mg/ml by ultrafiltration after addition of 5 mM tris(2-carboxyethyl)phosphine (TCEP).
**Biotinylated UCHL3 C95A**

The biotinylated UCHL3 C95A-AviTag, used as anti-K27Ub2 western blotting reagent (Figure 1D), was obtained by fusing the AviTag sequence GLNDIFEAQKIEWHE (Daiss et al., n.d.) directly after the UCHL3 gene. Expression was performed as described for wild type UCHL3, but the bacterial pellet was resuspended in biotinylation buffer I (25 mM Hepes pH 8.3, 10 mM Mg(OAc)2, 10 mM CaCl2, 50 µM PMSF (Sigma), 15 µg/mL DNase I). The pellet was lysed by sonication, after which 10 mM ATP and 1 mM biotin were added to the suspension. Biotinylation was performed in total lysate with 50 µg/mL BirA ligase for 4 hours at 30 °C. The biotinylated UCHL3 C95A-AviTag protein was further purified as described above for wild type UCHL3.

**Protein Ubiquitination**

Ubiquitinated PCNA was produced according to the protocol described in (Hibbert and Sixma, 2012). Briefly, human PCNA was expressed and purified from E. coli, then ubiquitinated using 100 nM E1 (UBA1), 30 µM mutant E2 (UBCH5C S22R) and wild type Ub, K63Ub2 or K27Ub2. The reaction was carried out at 25 °C in 100 mM NaCl, 5 mM MgCl2, 5 mM ATP, 50 mM malic acid MES Tris (MMT) buffer, pH 9. Reaction progression was monitored by SDS PAGE.

(d)Ubiquitinated 3xSUMO2ΔN was produced by mixing 65 µM 3xSUMO2ΔN with 0.5 µM UBA1 (E1), 0.5 µM UBE2W (E2), 0.5 µM RNF4 and 2 µM synthetic Ub or Ub2 in ubiquitination buffer (100 mM NaCl, 20 mM HEPES pH 7.5, 5 mM ATP, 5 mM MgCl2). The mixture was incubated for 3 hours at 37 °C, aliquoted and stored at -80 °C.

**Deubiquitination Reactions**

### Ub-Rho, Ub-His, Ub-L40-Rho, Ub-SUMO2

20 µM (9 µl) of each substrate were mixed with a serial dilution of UCHL3 (45 µM → 45 mM, 2 µl) in deubiquitination buffer (NaCl 100 mM, 20 mM HEPES pH 7.5, 5 mM DTT) and incubated at 37 °C in a PCR machine for the indicated time. Reactions were quenched by boiling SDS loading buffer and resolved by SDS PAGE. All reactions were pipetted with a multichannel pipet to minimize time errors. Rhodaminated substrate bands were revealed using UV light in a Chemidoc XRS+ imager (Biorad Inc.); non-rhodaminated errors. Rhodaminated substrate bands were revealed using UV light in a Chemidoc XRS+ imager (Biorad Inc.); non-rhodaminated substrates were stained with Coomassie blue. The black and white Chemidoc gel images were colorized to magenta and blue in Adobe Photoshop for visualization purposes.

**Ub-PCNA**

Ubiquitinated PCNA (10 µM final concentration) was mixed with either UCHL3 (45 µM final) or recombinant USP7 (Kim et al., 2019) (1 µM final) in deubiquitination buffer and incubated for the indicated times at 37 °C in a PCR machine. At each time point, aliquots were taken, immediately boiled for 5’ in SDS loading buffer, and analyzed by SDS PAGE.

**Ub(n)-3xSUMO2ΔN**

Similar amounts (~1 µg, assessed by SDS PAGE) of Ub 3xSUMO2ΔN, K63Ub2-SUMO2ΔN and K27Ub2-SUMO2ΔN were incubated with 22 µM UCHL3 in deubiquitination buffer for the indicated time at 37 °C. The reaction was aliquoted at the indicated time points and resolved by SDS PAGE, followed by western blot against SUMO2 (anti-SUMO2, Life Technologies 51-9100).

**Crystallization and Structure Solution**

Sitting drop crystallization screens were set up in 96-well format using a Mosquito crystallization robot (TTP Labtech), with 100 + 100 nl (protein + precipitant) drops. Initial crystals (plates + needles) were observed in multiple conditions containing polyethyleneglycol (PEG) 3350 and PEG 2000 mono methyl ether (20-30% w/v concentration range), with 0.1 M 2,2-Bis(hydroxymethyl)-2,2’-nitrilotriethanol (Bis-Tris) pH <6.0 as buffer and various potassium salts or ammonium sulphate (0.1-0.2 M range). After optimization, the best diffracting crystals were grown in 21% PEG 3350, 0.15M KBr, 0.1M Bis-Tris pH 5.5 and were cryoprotected by stepwise addition of 30% ethylene glycol prior to data collection. Data were collected by the Massively Automated Sample Selection Integrated Facility (MASSIF-1) (Bowler et al., 2016) at beamline ID30A-1 of the European Synchrotron Radiation Facility (ESRF) at a wavelength of 0.966 nm. The data were indexed, integrated and merged using XDS (Kabsch, 2010) and Aimless (Evans and Murshudov, 2013) in spacegroup P21/c, to a maximum resolution of 2.1 Å. Phasing was done by molecular replacement using Phaser (McCoy et al., 2007) with ubiquitin (PDB: 1UBQ,Vijay-kumar et al., 1987) and apo-UCHL3 (PDB: 1UCH,Johnston et al., 1997) as search models, followed by cycles of manual building and refinement with Coot (Emsley et al., 2010) and REFMAC (Murshudov et al., 2011) within the CCP4 suite (Winn et al., 2011). The final model was polished using the latest version of PDB_REDO (https://pdb-redo.eu, Joosten et al., 2014). Data collection and refinement statistics are in Table S1. Coordinates of the model were deposited under PDB: 6QML. The difference density in Figure S2C was generated by molecular replacement of the final model (minus Gly75,76 of the proximal ubiquitin) into the original data. All structural figures were generated using CCP4MG (McNicholas et al., 2011), with aesthetic fog, blur or colorization effects optionally added in Adobe Photoshop (Adobe Inc.). Structural superposition and root mean square deviation (rmsd) were calculated using the secondary structure matching (SSM) method (Krissinel and Henrick, 2004) in CCP4MG.

**Surface Plasmon Resonance**

100 ± 10 responsive units (RU's) of N-terminally biotinylated K27Ub2, K63Ub2 or Ub were immobilized on lanes 2-4 of a single streptavidin chip (SA chip, GE healthcare). Increasing concentrations of wild type or mutant UCHL3 (range 60 PM - 65.5μM) or UCHL1 (60 PM - 35.5 µM) were flowed over the chip at a speed of 30 µl/min and binding levels to each ligand was compared to the empty lane 1. The buffer used was 100 mM NaCl, 20 mM HEPES pH 7.5, 5 mM DTT, 0.05% TWEEN-20, supplemented with 10 mg/ml bovine serum albumin.
The probabilities derived with this method are essentially identical to those derived by looking at the ratio of \( k_{\text{open}} \) and \( k_{\text{close}} \) in (green + cyan paths). When fitting this model to K27Ub2-AMC cleavage, the \( k_{\text{cut}} \) converged to 38.8 s\(^{-1}\), a value similar to that calculated transferred to a 0.2 µm nitrocellulose membrane using a Trans-Blot Turbo transfer system (BioRad). The membrane was blocked for albumin (Sigma Aldrich), 1 mg/ml CM-Dextran (Sigma Aldrich) to reduce aspecific interactions. To account for different sized analytes, response units (RUs) of all experiments were normalized dividing by \( k = RU_{\text{immob}} \times MW_{\text{analyte}} / MW_{\text{ligand}} \), where \( RU_{\text{immob}} \) are the units of analyte on chip, and \( MW_{\text{analyte}} \) and \( MW_{\text{ligand}} \) are the molecular weight of analyte and ligand. Binding data were fitted with a one site specific binding model in GraphPad Prism 7.0 (www.graphpad.com). Binding affinities are reported as \( K_d \pm \) standard error. Plots were generated in Graphpad Prism and adapted for illustration using Adobe Illustrator (Adobe Inc.).

**Diubiquitin Inhibition Experiments**

**Ub-Rho Cleavage**

UCHL3 (10 µM final concentration) was pre-incubated for 30 minutes at room temperature with diubiquitin (5 nM final concentration), monoubiquitin (10 nM final concentration), buffer (positive control) or iodoacetamide (negative control, 10 mM final concentration). Then, Ub-Rho (500 nM final concentration) was added and fluorescence intensity (\( \lambda_{\text{ex}} = 487 - 14 \) nm, \( \lambda_{\text{em}} = 535 - 30 \) nm) was measured every 20 seconds for 60 minutes on a Clariostar (BMG Labtech) microplate reader. Each condition was measured in quadruplicate using a volume of 8 µl per well. Buffer conditions: 50 mM Tris (pH 7.6), 50 mM NaCl, 2 mM 1,4-Dithiothreitol (DTT), 0.05% Tween-20. The data were plotted using GraphPad Prism 7.

**Ub-His Cleavage**

Ub-His (100 µM final concentration), UCHL3 (50 nM) and either buffer (reference control) or the appropriate diubiquitin (0.5 µM) were mixed in 150 mM NaCl, 20 mM HEPES pH 8.1, 5 mM DTT. The reactions were incubated at 37 °C and aliquots were taken at the indicated time points. The samples were resolved on a 4-20% Bolt gradient gel (Invitrogen) using MES-SDS running buffer and stained with Coomassie blue.

**Ub-SUMO2 Cleavage**

Reactions were performed as described above, in presence or absence of the appropriate diubiquitin (2.5, 5 or 10 µM). The sample was separated by SDS-PAGE and the bands were revealed by western blot. Antibodies: α-ubiquitin (mouse, 1:2000 (P4D1, Santa Cruz)); α-SUMO2/3 (rabbit, Vertegaal et al., 2004 (Vertegaal et al., 2004)); donkey-α-rabbit IRDye®680 (LI-COR, cat. no. 926-68073); goat-α-mouse IRDye®800 (LI-COR, cat. no. 926-32210). Images were read out on an Odyssey Classic imager (LI-COR).

**Stopped Flow Measurements**

Equal amounts of a stock solution of ubiquitin substrate (100 nM for Ub-AMC, K63Ub2-AMC, K27Ub2-AMC; 50 nM for Ub-L40-Rho and K27Ub2-L40-Rho) and of UCHL3 (ranging in concentration from 0.025 to 128 nM) were mixed in a SF-61DX2 stopped flow fluorimeter system (TgK scientific Ltd, UK) with R10699 photomultipliers (Hamamatsu Photonics K.K., Japan). Measurements were carried out for 10s, with each measurement repeated 5 times and averaged for the final analysis. The plots of the stopped flow data and their residuals in Figures 5E, 5F, and S5 are displayed as percentage of response on the Y axis to facilitate visual comparison. Percentage of response was calculated by normalizing each dataset to the interval (0,100) using Graphpad Prism 7.

**Kintek Modelling**

Kintek Explorer version 8.0 (Kintek Corporation, Johnson et al., 2009a) was used to fit the reaction mechanism. Binding data from Figure 2B (Ub, K63Ub2, K27Ub2) and cleavage data from Figure 4B (Ub-AMC, K63Ub2-AMC, K27Ub2-AMC) were used for the fitting. The fitting presented here is based on all data, but the model was built in stages: first the cleavage data for Ub-AMC and K63Ub2-AMC was fitted to a product inhibition model (green part of model in Figure 6A and see text). In this model, \( k_{\text{cut}} \) and \( k_{-1} \) converged independently to very similar values (values Ub(kcut) = 47.8 s\(^{-1}\); Ub(k-1) = 285 s\(^{-1}\); K63Ub2(kcut) = 49.5 s\(^{-1}\); K63Ub2(k-1) = 295 s\(^{-1}\)). The cleavage of K27Ub2-AMC was then considered. The simplest model that could account for the observed kinetics is the entire model in 6a (green + cyan paths). When fitting this model to K27Ub2-AMC cleavage, the \( k_{\text{cut}} \) converged to 38.8 s\(^{-1}\), a value similar to that calculated for Ub-AMC and K63Ub2-AMC. We then fitted all three substrates (Ub-AMC, K27Ub2-AMC, K63Ub2-AMC) with the entire model in 6a.

Given the independent convergence of \( k_{\text{cut}} \) an \( k_{-1} \) from the different substrates, we imposed the conditions: (1) \( P_{\text{open}} / P_{\text{close}} = [K27Ub2-AMC:UCHL3]_{\text{open}} / [K27Ub2-AMC:UCHL3]_{\text{closed}} \) and (2) \( P_{\text{open}} = 1 - P_{\text{close}} \). To estimate the probability of choice between the open and closed cleavage pathways, we used the general model to simulate the equilibrium ratio between the open and closed K27Ub2-AMC:UCHL3 complexes in conditions when the rate of K27Ub2-AMC cleavage was set to zero. Then we assumed: (1) \( P_{\text{open}} / P_{\text{close}} = [K27Ub2-AMC:UCHL3]_{\text{open}} / [K27Ub2-AMC:UCHL3]_{\text{closed}} \) and (2) \( P_{\text{open}} = 1 - P_{\text{close}} \), where \( P_{\text{open}} \) and \( P_{\text{close}} \) are the probabilities of open and closed cleavage, and \( [K27Ub2-AMC:UCHL3]_{\text{open}} \) and \( [K27Ub2-AMC:UCHL3]_{\text{closed}} \) are the concentration of the open and closed substrate:enzyme complex at equilibrium reported by the simulation. The probabilities derived with this method are essentially identical to those derived by looking at the ratio of \( k_{\text{open}} \) and \( k_{\text{close}} \) in the model. Standard error on the estimate was calculated using error propagation from the errors of the kinetic constants.

**Detection of K27Ub2 with Biotinylated UCHL3**

Equal amounts of mono and diubiquitin (500 ng) were run on a 4-12% Bis-Tris gel in MES buffer (NuPAGE, Life Technologies) and transferred to a 0.2 µm nitrocellulose membrane using a Trans-Blot Turbo transfer system (BioRad). The membrane was blocked for...
30 minutes with 5% milk (skim milk powder, LP0031, OXOID) in 0.2% PBS-Tween20 (PBST) and incubated with primary α-ubiquitin (mouse monoclonal, 1:2000 (P4D1, Santa Cruz) antibody or biotinylated UCHL3 C95A-AviTag (0.1 μg/mL) for 1 hour at room temperature. After washing, three times 15 minutes in 0.2% PBST, the blot was incubated with the secondary antibody goat-anti-mouse IRDye®680 (LI-COR, cat. no. 926-68070) or neutravidin DyLight800 (Thermofisher Scientific, cat. no. 22853). Images were read out on an Odyssey Classic imager (LI-COR).

**Experimental Design**

Unless otherwise specified in the relevant section, all experiments were repeated at least two times with comparable results. No blinding was applied. Randomization of samples is not applicable.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Assumptions and procedure used for kinetic model fitting are discussed in Method Details, Kintek modeling. Residuals of model fitting and chi²/chi² simulations are shown in Figures S5B–S5D and were calculated using FitSpace within the Kintek software suite.

SPR measurements treatment, normalization and fitting are discussed in Method Details, Surface Plasmon Resonance. Binding affinity fitting was performed in GraphPad Prism 7.0 using a single, specific binding model.

Data and model quality of the crystallographic structure are summarized in Table S1 and were calculated automatically by the appropriate software within the CCP4 suite. Extended data and model quality analysis were calculated by the Protein Data Bank upon deposition and can be found at the entry page (PDB id 6QML).