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Review

Deubiquitinases: From mechanisms to their inhibition by small molecules

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SUMMARY

Deubiquitinases (DUBs) are specialized proteases that remove ubiquitin from substrates or cleave within ubiquitin chains to regulate ubiquitylation and therefore play important roles in eukaryotic biology. Dysregulation of DUBs is implicated in several human diseases, highlighting the importance of DUB function. In addition, many pathogenic bacteria and viruses encode and deploy DUBs to manipulate host immune responses and establish infectious diseases in humans and animals. Hence, therapeutic targeting of DUBs is an increasingly explored area that requires an in-depth mechanistic understanding of human and pathogenic DUBs. In this review, we summarize the multiple layers of regulation that control autoinhibition, activation, and substrate specificity of DUBs. We discuss different strategies to inhibit DUBs and the progress in developing selective small-molecule DUB inhibitors. Finally, we propose a classification system of DUB inhibitors based on their mode of action.

INTRODUCTION

Ubiquitylation is a post-translational modification (PTM) that plays important roles in eukaryotic cells by altering the function, localization, protein-protein interaction, or stability of the modified substrate. Covalent attachment of ubiquitin (Ub) molecules onto target proteins is catalyzed by an enzymatic cascade consisting of E1 activating, E2 conjugating, and E3 ligating enzymes (Streich and Lima, 2014). This cascade typically links the C-terminal glycine residue of Ub to a primary amino group of a lysine residue or the N-terminal amino acid of a target protein or another Ub molecule. Being a reversible PTM, removal of Ub from substrates is catalyzed by Ub-specific proteases called deubiquitinases (DUBs). In humans, there are approximately 100 different DUBs that can be broadly classified into seven structurally distinct superfamilies (Clague et al., 2019). Six of these families are cysteine-based DUBs: Ub C-terminal hydrolases (UCHs), Ub-specific proteases (USPs), Machado-Josephin domain proteases (MJDs), ovarian tumor proteases (OTUs), motif interacting with Ub-containing novel DUB family (MINDY), and zinc-finger-containing Ub peptidase (ZUP1). The Jab1/Mov34/MPN+ protease (JAMM) family members are zinc-binding metalloproteases.

By cleaving Ub attached to substrates or within Ub chains, DUBs play important roles in regulating fundamental cellular processes, functioning either as switches that remove Ub signals or as rheostats to fine-tune the amount and type of ubiquitylation (Clague et al., 2019; Leznicki and Kulathu, 2017). Because DUBs play important regulatory roles in various cellular processes, several mechanisms exist to ensure accurate substrate targeting and specificity and tight control of DUB activity to ensure that they are only functional at a precise time and in a specific subcellular location.

This review will discuss our current understanding of the mechanisms governing autoinhibition and activation of DUBs and how their activity and substrate specificity are tuned by substrate binding, PTMs, and allosteric regulation. Finally, we summarize efforts to develop specific small-molecule inhibitors of human and pathogenic DUBs for use as therapeutic agents and propose a classification system to categorize DUB inhibitors.

THE CHALLENGES FOR A DUB: COMPLEXITY OF SUBSTRATES

DUBs have evolved to differentiate between Ub signals of great complexity that stems from the ability of Ub to build multifarious architectures (Figure 1). The simplest form of ubiquitylation is monoubiquitylation, attachment of a single Ub molecule to a target protein. The extension of monoUb to polyUb chains can occur via eight possible linkages between the C-terminal carboxyl group of a distal Ub and one of the seven Lys or N-terminal Met residues of a proximal Ub. Although homotypic polyUbs contain a single linkage type, heterotypic polyUbs incorporate at least two different linkage types to form mixed and branched chains with a dizzying array of possible Ub chain architectures varying in linkage combinations and length (Haakonsen and Rape, 2019). Additional secondary PTMs on Ub through attachment of Ub-like modifiers, phosphorylation, acetylation, or methylation can further add to the complexity of the Ub signal (Swatek and Komander, 2016). Indeed, multiple modification sites on Ub have been identified and characterized recently, including phosphorylation at Thr12, Ser57, and Ser65 (Hepowit et al., 2021). Adding to this complexity is atypical ubiquitylation, attachment of Ub via ester linkages to Ser and Thr residues in protein substrates and within Ub chains and to non-proteinaceous substrates (Kelsall et al., 2019, 2021; Otten et al., 2021). Although

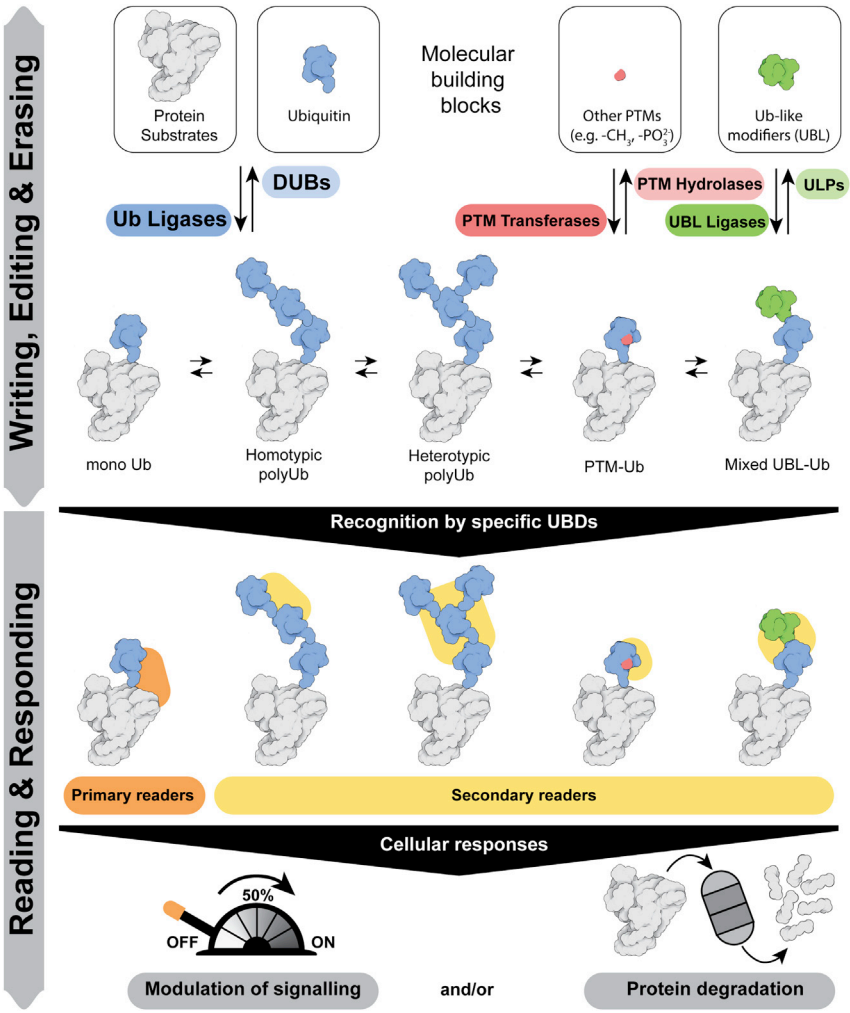

Figure 1. Complexity of Ub signaling

Shown is a schematic of the Ub signaling machinery. Ub signals are generated in the first phase of writing, editing, and erasing: Ub ligases attach Ub molecules to target proteins (writing), and DUBs counteract this reaction through removal of Ub (erasing and editing). Complex Ub architectures can be generated through elongation of monoUb into homotypic and heterotypic polyUb chains and PTM of Ub by, e.g., methylation, phosphorylation, or attachment of Ub-like modifiers (UBLs). The equilibrium of reactions catalyzed by Ub ligases, DUBs, PTM transferases and hydrolases, UBL ligases, and Ub-like specific proteases (ULPs) results in the products of this editing phase. The generated Ub signals are decoded by specific Ub binding domains (UBDs) at the start of the subsequent reading and responding phase: primary readers recognize features of the modified protein and Ub to detect specific ubiquitylated target proteins, and secondary readers detect specific Ub signals independent of the target protein. Most UBDs known to date can be classified as secondary readers, with preference for certain Ub architectures. Primary readers would recognize specific ubiquitylated substrates, analogous to how DUB complexes detect monoubiquitylated histones and FANCD2 (Morgan et al., 2016; Rennie et al., 2021). Binding of UBDs with Ub or polyUb can cause allosteric changes or relocalization events in associated domains or proteins, ultimately triggering a cellular response to the Ub signal, leading to modulation of signaling pathways or degradation of target proteins.

these different modifications make ubiquitylation an extremely information-rich signaling system, it creates a daunting task for the small number of ~100 DUBs identified so far to discriminate between and regulate these distinct signals.

SPLITTING BONDS: CATALYTIC MECHANISMS OF DUBs

Active DUBs cycle between substrate-free, substrate-bound, and product-bound states (Figure 2). Most human DUBs are thiol-based proteases and typically possess a catalytic triad consisting of the catalytic cysteine that engages in nucleophilic attack, an adjacent basic histidine that lowers the pKa of the cysteine to make it more nucleophilic, and often a third, acidic residue—usually asparagine, aspartate, or glutamate—to further polarize the basic histidine (Storer and Ménard, 1994). Despite the conserved mechanism, there are notable variations in the active sites of thiol-DUBs. For example, the A20-like subfamily of OTU DUBs appears to use a catalytic dyad where a negatively charged residue is not involved (Licchesi et al., 2011; Mevissen et al., 2016). USP30 employs an unusual Cys-His-Ser triad in which serine positions the basic histidine correctly for engagement with the catalytic cysteine, and sequence analysis suggests that this may be the case in other USP family DUBs, such as USP16 and USP45 (Gersch et al., 2017; Sato et al., 2017). MINDY1/2 utilize a non-canonical mechanism utilizing a water molecule coordinated by a Thr in place of a third acidic residue (Abdul Rehman et al., 2021). Yet another atypical process is found in the active site of the DUB OTULIN, where an acidic residue (E16) of the proximal Ub moiety completes the active site by polarizing the catalytic histidine (Keusekotten et al., 2013; Figure 2B). Intriguingly, the MJD class of DUBs has been shown to function as Ub esterases with high specificity and efficiency for ubiquitylated threonine (De Cesare et al., 2021). These enzymes employ the canonical catalytic triad of thiol-DUBs; however, esterase activity is established by hydrophobic residues in the active site. In contrast to thiol-DUBs, the catalytic architecture of JAMM metalloproteases involves coordination of a zinc ion by two His residues, an Asp, and a water molecule (Figure 2C). A nearby Glu activates the water molecule, which can then engage in nucleophilic attack of the scissile bond, and the tetrahedral intermediate of the reaction is stabilized by a nearby Ser (Ambroggio et al., 2004; Tran et al., 2003).

RULES OF ENGAGEMENT: HOW SUBSTRATE SPECIFICITY IS ACHIEVED

A key determinant of DUB activity is recognition and positioning of ubiquitylated substrates so that the scissile bond is placed

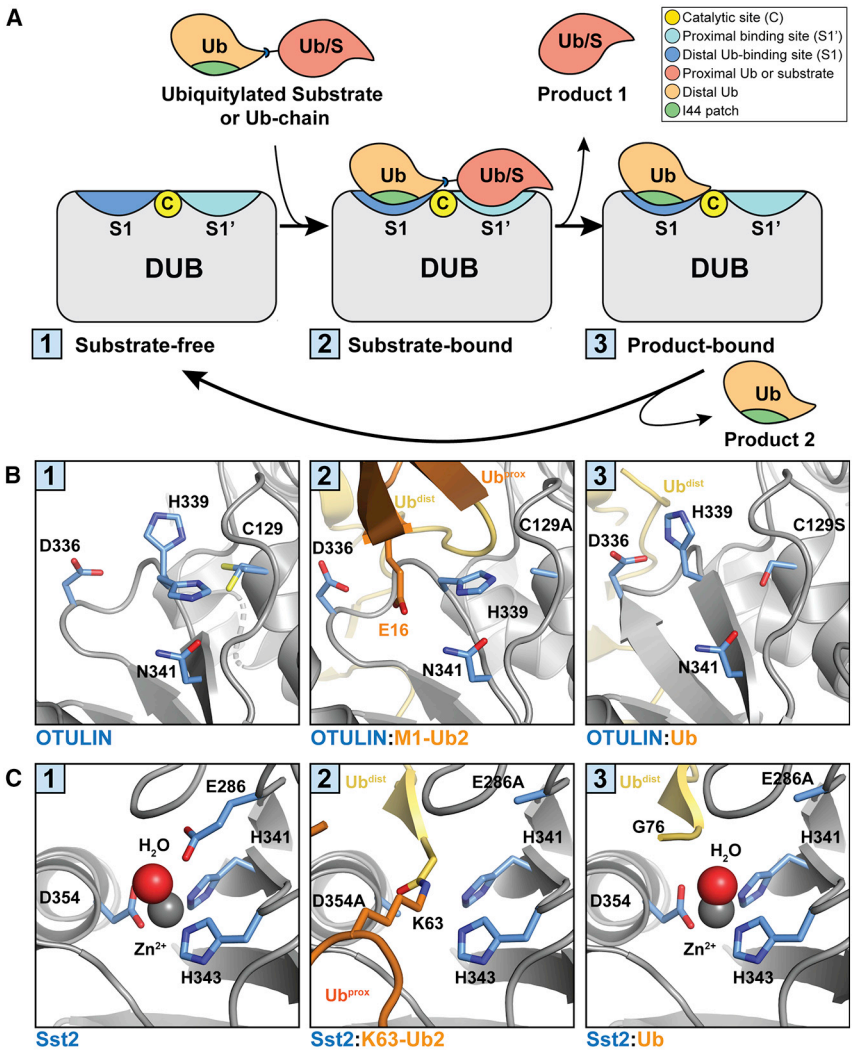

Figure 2. Catalytic cycle of DUBs

(A) Schematic of the three states of a DUB's catalytic cycle. The catalytic center is highlighted in yellow, substrate and Ub moieties in orange and red tones, and Ub- and substrate-binding sites in blue shades. The I44 patch of the distal Ub (green) is a major contributor to S1 site binding.

(B and C) Catalytic mechanisms of thiol- and metallo-protease DUBs captured by crystal structures of wild-type and active-site mutated proteins; cycle states are numbered as in (A). DUBs are shown as blue sticks and gray cartoon models, Ub moieties in yellow/orange, zinc ions as gray spheres, and water molecules as red spheres.

(B) The thiol-DUB OTULIN (PDB: 4KSJ, 4KSL, and 4KSK: Keusekotten et al., 2013; Rivkin et al., 2013) undergoes conformational changes in the catalytic center when it transitions from an auto-inhibited apo state (1), where D336 restrains the catalytic H339, to the substrate-bound form (2), where E16 of the proximal Ub (Ub^{prox}) inserts into the catalytic center to activate H339. After nucleophilic attack on the scissile bond, a tetrahedral intermediate is formed, during which the negative potential on the carbonyl oxygen is stabilized by formation of an oxyanion hole. After cleavage, the proximal Ub departs first, and the distal Ub (Ub^{dist}) forms a short-lived thiol-ester with the catalytic cysteine.

(C) The metallo-DUB Sst2 (PDB: 4JXE, 4NQL, and 4MSM) coordinates the catalytic zinc ion in a tetrahedral geometry with two His, an Asp (or Glu), and a water molecule, which acts as the nucleophile (1). In the substrate-bound stage (2), captured by point mutations that disrupt zinc binding, the isopeptide bond is exposed, and, following cleavage, the C-terminal carboxylate group of Ub is coordinated by a water molecule in the product-bound state prior to Ub departure (3) (Sato et al., 2008; Shrestha et al., 2014).

across the catalytic site for efficient cleavage (Figure 3). This allows a DUB to specify which linkage types or substrates it prefers and often also determines how the substrate is cleaved. In addition, DUBs can show either *endo*-, *exo*-, or *en bloc*-type activity by cleaving within a chain, from one end of the chain, or by removing the whole chain at once, respectively.

Ub recognition at the S1 and S1' sites of DUBs

DUBs recognize Ub at their S1 site mainly via extensive hydrophobic interactions with the Ile44 patch of Ub. In addition, most DUBs recognize R72 and R74 in the C-terminal tail of Ub to position the scissile bond within the active site of the DUB for cleavage (Békés et al., 2013; Drag et al., 2008; Sato et al., 2008; Ye et al., 2011). A conserved aromatic residue in thiol DUBs acts as a "gatekeeper" by imposing a size limit permitting only Gly, thus selecting for Ub as substrate (Hermanns et al., 2020). Together, these features enable DUBs to discriminate between Ub and structurally similar Ub-like modifiers (UBLs) (Ye et al., 2011). In general, DUBs with a strong S1 binding site and few or no interactions with the proximal Ub tend to be linkage agnostic, as seen in many USP family DUBs (Faesen et al., 2011; Mevissen et al., 2013; Ritorto et al., 2014).

In contrast to the S1 site, which is present in the catalytic domain, the S1' site may be present within the catalytic domain or provided by auxiliary domains. Importantly, interactions at the S1' site can drive substrate specificity to determine which ubiquitylated protein or polyUb type is specifically recognized. With the Ile44 patch of the distal Ub recognized by DUBs, the conformational restraints within polyUb make only selected interfaces on the proximal Ub available for binding. This is highlighted in the M1-specific DUB OTULIN. Despite the conformational similarity between K63- and M1-linked chains and the proximity of K63 to M1, OTULIN is unable to cleave K63 chains. The extensive binding interface of the S1' site confines Ub, and even the small rotation of the proximal Ub required to bind K63-linked chains is not tolerated (Keusekotten et al., 2013). In contrast, the more promiscuous USP DUB CYLD binds the distal Ub moieties of M1- and K63-linked diubuiquitin (diUb) in a similar manner, whereas the proximal Ub of these linkage types adopt different orientations, which, however, are accommodated by CYLD, allowing cleavage of M1 and K63 linkage types (Sato et al., 2015).

The presence of unique Ub binding interfaces at the S1' site can drive specificity for certain linkage types. In the case of the

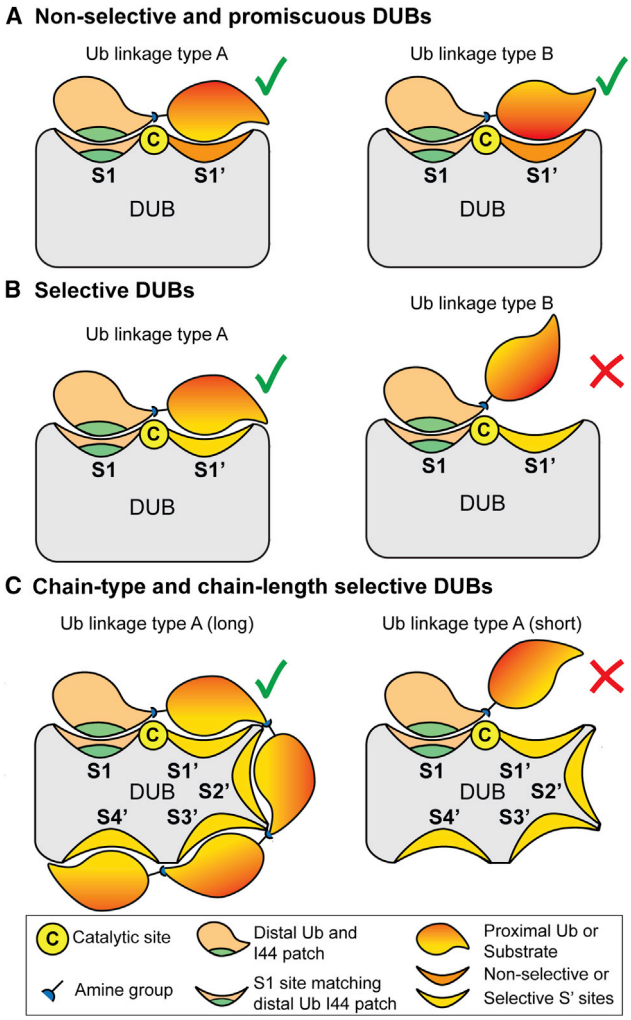

Figure 3. Ub-chain selectivity determination of DUBs

Shown is a schematic of selectivity determination through S1' site binding of Ub moieties or substrates and through additional Ub binding sites.

(A) Non-selective, promiscuous DUB that can bind and cleave different Ub linkage types; for example, USP2.

(B) Chain-type-selective DUB that only accommodates a small subset of linkage types in a catalytically efficient manner; for example, OTULIN.

(C) Chain-type- and chain-length-selective DUB that requires multiple Ub binding sites to engage with and cleave long Ub chains; for example MINDY1/2.

K63-specific JAMM DUB AMSH-LP, the proximal Ub is recognized by two loops in the JAMM core that engage residues surrounding K63 of the proximal Ub and allows only K63-linked polyUb to bind at the S1' site in the correct orientation (Sato et al., 2008). Similarly, the preference for K6-linked polyUb seen in USP30 is provided by unique binding interfaces generated by the so-called palm-thumb subdomains that discriminate toward K6-linked polyUb (Gersch et al., 2017; Sato et al., 2017). A striking example of S1' site binding is illustrated by the K11-specfic DUB Cezanne/OTUD7B, where Ub binding results in a conformational rearrangement leading to the *de novo* formation of an S1' site to accommodate the proximal Ub of a K11-linked chain (Mevissen et al., 2016).

Additional Ub binding sites in DUBs

In addition to being able to discriminate linkage type, some DUBs possess additional Ub-binding sites within their catalytic domain to bind longer chains, making chain length a determinant of substrate recognition, or to impose linkage specificity. OTUD2 and OTUD3, for instance, possess an S2 site (Flierman et al., 2016; Mevissen et al., 2013), whereas MINDY1/2 have at least five Ub binding sites within their catalytic domains (S1 and S1'–S4') (Abdul Rehman et al., 2021), and so these DUBs only efficiently cleave chains containing more than two Ub moieties. In addition, many DUBs possess accessory Ub-binding domains outside of their catalytic domain. MINDY1/2, in addition to the five Ub binding sites of the catalytic domain, possess tandem MIU (motif interacting with ubiquitin) motifs (tMIUs) that further mediate substrate targeting to promote cleavage of long polyUb chains (Abdul Rehman et al., 2021; Kristariyanto et al., 2017). The DUB OTUD1 relies on a UIM (Ub interacting motif) to bind to K63-linked chains, and its removal results in substantial loss of activity and linkage specificity (Mevissen et al., 2013). ATXN3, a member of the Josephin family of DUBs, has three UIMs that bind to K48- and K63-linked polyUb to impart linkage specificity to the DUB (Winborn et al., 2008). A similar phenomenon is observed in TRABID, which contains an ankyrin-repeat Ub-binding domain (AnkUBD) to position Ub chains for cleavage and impose specificity toward K29- and K33-linked chains (Licchesi et al., 2011). The recently discovered DUB ZUP1 binds Ub chains through the ZUP1 helical arm (ZHA), which also contains a MIU motif, and through an additional non-canonical Ub-binding zinc-finger (UBZ) domain, and ZHA and UBZ are required to cleave K63 chains (Hermanns et al., 2018; Kwasna et al., 2018). These features provide an additional layer of substrate selectivity to DUBs by sensing chain length and linkage types.

PATHWAY FUNCTIONALIZATION

Even though a DUB may exhibit preference for certain linkages *in vitro*, additional substrate targeting mechanisms can restrict its activity to particular ubiquitylated proteins *in vivo*, providing a further level of specificity. The M1-specific DUB OTULIN has a PNGase/UBA- or UBX-containing domain (PUB)-interacting motif (PIM) at its N terminus that binds to the PUB domain in HOIP (the catalytic subunit of the LUBAC complex that assembles linear Ub chains in IκB kinase signaling), targeting it to the LUBAC complex, where it regulates LUBAC autoubiquitylation (Elliott et al., 2014; Heger et al., 2018; Schaeffer et al., 2014). CYLD is also recruited to HOIP but requires the adaptor protein SPATA2 to bridge the DUB to HOIP (Schlicher et al., 2017). In addition, CYLD has been linked to ciliogenesis (Yang and Zhou, 2016) and inflammasomes (Mukherjee et al., 2020), but it remains to be elucidated how CYLD is targeted to these pathways.

Although many USP family DUBs are not Ub linkage specific and will cleave multiple linkage types, most are thought to recognize specific ubiquitylated substrates *in vivo*. One of the best-studied examples is the SAGA (Spt-Ada-Gcn5 acetyltransferase) coactivator complex that acts on monoubiquitylated histone H2B to erase this mark of transcriptional activation (Henry et al., 2003). The minimal domain with DUB activity is known

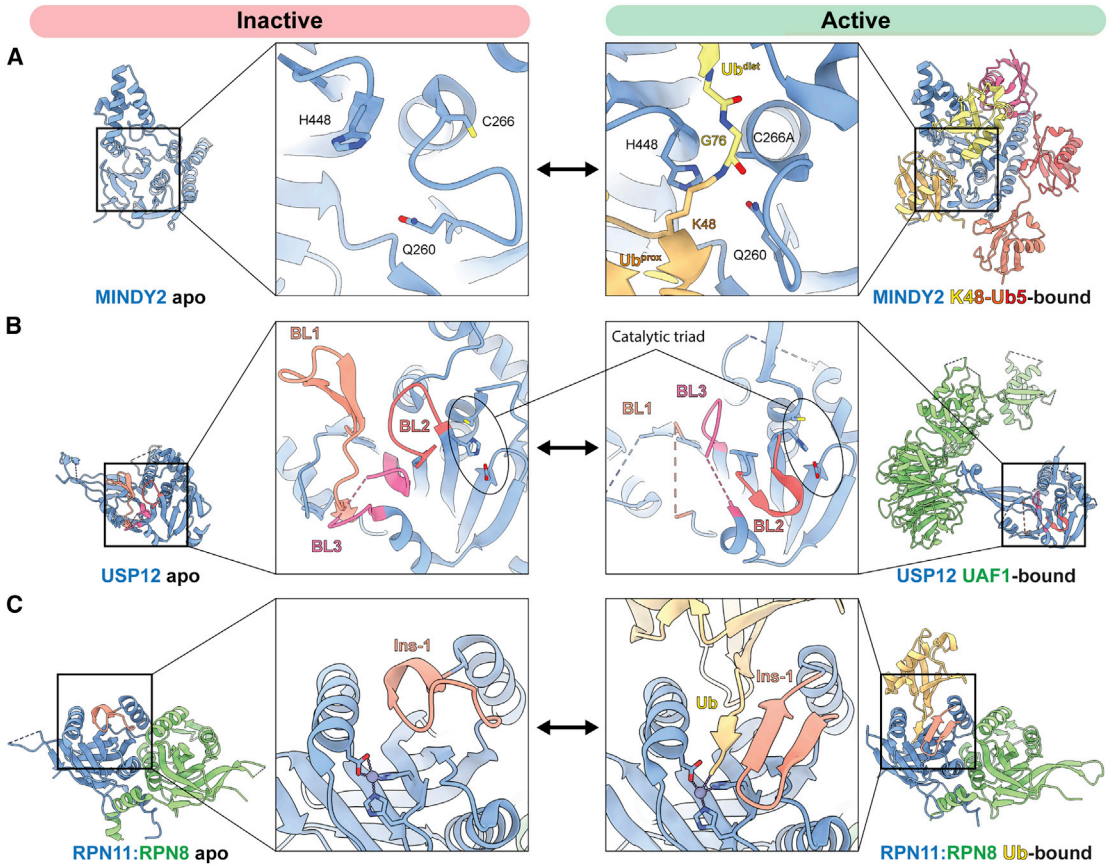

Figure 4. Control of DUB activity through conformational changes

(A) Reorganization of the MINDY2 active site from the inactive apo to active substrate-bound states (PDB: 6Z49 and 7NPI).

(B) Movement of UFSP12 blocking loop 1 (BL1)–BL3 upon UAF1-binding (PDBs 5K16 and 5K1A). Residues of the catalytic triads are shown as stick models; black circles indicate catalytic triad of USP12.

(C) Conformational transition of the Ins-1 loop of RPN11 upon binding of Ub (PDB: 4O8X and 5U4P). The catalytic zinc atoms are shown as gray spheres and zinc-coordinating residues as stick models.

as the SAGA DUB module, which, in yeast, consists of the DUB Ubp8 and three accessory proteins (Sgf11, Sus1, and Sgf73), which hold the DUB in an active conformation (Köhler et al., 2010; Samara et al., 2010; Yan and Wolberger, 2015). Although Ubp8 makes contacts with H2B and the attached Ub, the zinc-finger domain of Sgf11 plays an important role in targeting the DUB module to ubiquitylated histones by binding a conserved acidic patch on H2A/H2B. Together, these devices ensure specificity for ubiquitylated H2B (Morgan et al., 2016). In the following section, we discuss how some of these substrate targeting modules also play additional allosteric roles to activate the DUB.

REGULATION OF DUB ACTIVITY

In the absence of substrate, many DUBs exist in an inhibited state where the catalytic residues are misaligned, or structural elements impede substrate binding. Indeed, DUBs such as USP7, USP14, and MINDY1/2 undergo conformational changes to accommodate substrate binding, during which the catalytic residues are repositioned into an active conformation (Figure 4A). Residues in the substrate can also play an active role in driving these conformational changes, as observed in OTULIN. Upon binding of M1-linked polyUb, the carbonyl group of the M1 residue of the proximal Ub repositions the catalytic His from an autoinhibited conformation to a productive conformation. In addition, E16 of the proximal Ub inserts itself into the active site to stabilize the catalytic residues in an active conformation (Keusekotten et al., 2013). Ub binding at the proximal site thereby forces the DUB into a catalytically competent state, and catalysis occurs in a substrate-assisted manner.

Regulatory loops

A common theme in many DUBs is the presence of active-site-blocking loops that hinder Ub access to the catalytic cleft. This blockage is often observed in apo structures of DUBs, and the blocking loop is remodeled in crystal structures of complexes with Ub suicide probes to permit Ub binding. This has led to the idea that these flexible loops simply move to accommodate substrate. However, it is increasingly appreciated that these loops may serve important inhibitory roles to permit allosteric activation only under the right conditions. This concept is best

illustrated by the related DUBs USP1, USP12, and USP46, which are inhibited by blocking loop 1 (BL1) and BL2 (Figure 4B). UAF1 (WDR48) and WDR20 are β-propeller proteins that bind to and allosterically activate the enzyme activity of USP12 and USP46 (Li et al., 2016; Yin et al., 2015; Zhu et al., 2019). UAF1 plays a dual role in substrate recruitment and in allosterically activating USP12 into a partially active conformation by remodeling BL1 and by stabilizing the C terminus of Ub in the catalytic cleft. Further activation is mediated by WDR20 binding, which remodels the catalytic cleft and BL2 to fully activate USP12 for catalysis. Similarly, USP1, which plays an important role in DNA repair by specifically deubiquitylating FANCI/FANCD2 (I-D2), relies on UAF1 for allosteric activation and substrate recognition (Cohn et al., 2007). A challenge encountered by USP1 is that ubiquitylated I-D2 adopts a closed conformation, with FANCI binding to and making the Ub inaccessible to the DUB (Lemonidis et al., 2021). Elegant work by Rennie et al. (2021) revealed that, when USP1-UAF1 binds, the I-D2-Ub complex is remodeled to a more open conformation, allowing USP1 to extract Ub from the complex and enclose it within its palm and thumb subdomains, positioning the isopeptide bond between K561 of FANCD2 and Ub across the active site for cleavage. In addition, the L1 insert of USP1 has been shown to be critical for specific substrate-mediated activation of USP1 by DNA-loaded PCNA (Dharadhar et al., 2021). Hence, in a well-coordinated process, substrate recognition by UAF1 and conformational changes in the substrate drive site-specific deubiquitylation by USP1.

The DUB USP14 is similarly kept inactive by an inhibitory network of BLs that impede access of the C terminus of Ub to the active site (Hu et al., 2005). When USP14 binds to the proteasome, allosteric changes remodel the BLs to activate USP14 (Huang et al., 2016; Hung et al., 2021). The same theme is found in UCHL5, a UCH family DUB whose activity is also enhanced by binding to the proteasome. A hallmark of UCH DUBs is a crossover loop near the active site that closes over the C-terminal tail of the conjugated Ub. Importantly, the length of this crossover loop can restrict the size of the ubiquitinated substrate the DUB can accommodate (Bett et al., 2015; Zhou et al., 2012). When the proteasome subunit RPN13, which has a deubiquitylase adaptor domain (DEUBAD), binds UCHL5, it stabilizes the flexible active site crossover loop in a catalytically productive conformation to relieve autoinhibition and activate the DUB (Vander Linden et al., 2015; Sahtoe et al., 2015). Interestingly, UCHL5 is also found in chromatin remodeling complexes, where it binds and is inhibited by another DEUBAD domain containing protein INO80. Here, INO80 binding causes conformational changes that lower the substrate affinity of UCHL5 and disrupt the active-site geometry. Another UCH family DUB regulated in a related manner is the tumor suppressor BRCA1-associated protein-1 (BAP1), which is activated upon binding to the DEUBAD domains of ASXL1 to deubiquitylate histone H2A on nucleosomes (Masclef et al., 2021). In a mechanism of activation similar to that of UCHL5 and RPN13, the DEUBADs correctly position the crossover loop of BAP1 and anchor the UCH37-like domain (ULD) to the catalytic domain (De et al., 2019; Sahtoe et al., 2016).

The metalloDUB RPN11, the last DUB encountered by substrates at the proteasome, sits directly above the central pore of the proteasome (Bard et al., 2018). RPN11 is also inhibited by a flexible loop, Ins-1, which, in the inactive state, blocks access of the C terminus of Ub to the active site (Figure 4C). When the ubiquitylated substrate translocates into the core of the proteasome, Ins-1 undergoes a conformational transition to a β-hairpin to form one side of the active site groove, and the C terminus of Ub adopts a β strand as it reaches into the active site to form a β sheet with the remodeled Ins-1, properly positioning the scissile bond for cleavage (Worden et al., 2017). These examples highlight how regulatory loop remodeling is not a passive process but a key feature controlling DUB activity. We suggest that studying how these loops are remodeled has the potential to reveal regulatory principles in other DUBs.

INTRAMOLECULAR REGULATION

Many DUBs are modular and contain additional domains that can function as intrinsic regulatory elements to influence DUB activity. USP7 is one such modular DUB with an additional TRAF domain, 5 Ubl domains, and a highly conserved C-terminal tail. In the absence of substrate, USP7 exists in an equilibrium of inactive states where Ubl4-5 can fold back over the catalytic domain. The TRAF domain recruits ubiquitylated substrates and, in a coordinated process, Ubl4-5 promote Ub binding, whereas the C-terminal tail inserts into the catalytic site to stabilize the active state (Hu et al., 2002; Kim et al., 2019; Rougé et al., 2016; Figure 5A). USP7 therefore provides an example of multiple levels of regulation within a single DUB.

INTERMOLECULAR REGULATION

Interactome studies have shown DUBs to associate with other DUBs, suggesting cooperativity and possible intermolecular regulation (Sowa et al., 2009). In addition, DUBs also self-associate to form higher-order complexes that can alter DUB activity. Upon association with the ABRAXAS pseudo-DUBs, the JAMM metalloprotease BRCC36 can form higher-order complexes that are essential for DUB activity (Cooper et al., 2009; Dong et al., 2003; Zeqiraj et al., 2015). In the BRCC36-Abraxas2 (BRISC) complex, the catalytic residues sit in a competent formation, whereas in an inactive homodimer of BRCC36, the catalytic residues are misaligned. However, it is unknown whether the BRCC36 homodimer may be an *in vitro* artifact. BRISC also interacts with the serine hydroxymethyltransferase SHMT2, which assists in targeting the complex to type I interferon receptors (Zheng et al., 2013). Recent studies revealed dual roles of SHMT2 in inhibiting BRISC to prevent non-specific activity and to target the DUB complex to substrates. An SHMT2 dimer binds to BRISC and acts as a bridge between the two constituent dimers of a BRISC superdimer while also blocking the active site of BRCC36 (Rabl et al., 2019; Walden et al., 2019). Interestingly, SHMT2 itself is regulated by oligomerization, where it transitions from an inactive dimer to an enzymatically active tetramer in the presence of PLP (the active form of vitamin B6) (Giardina et al., 2015). Only the inactive SHMT2 dimer binds and inhibits BRISC, revealing how a metabolite can regulate DUB activity (Figure 5B; Walden et al., 2019).

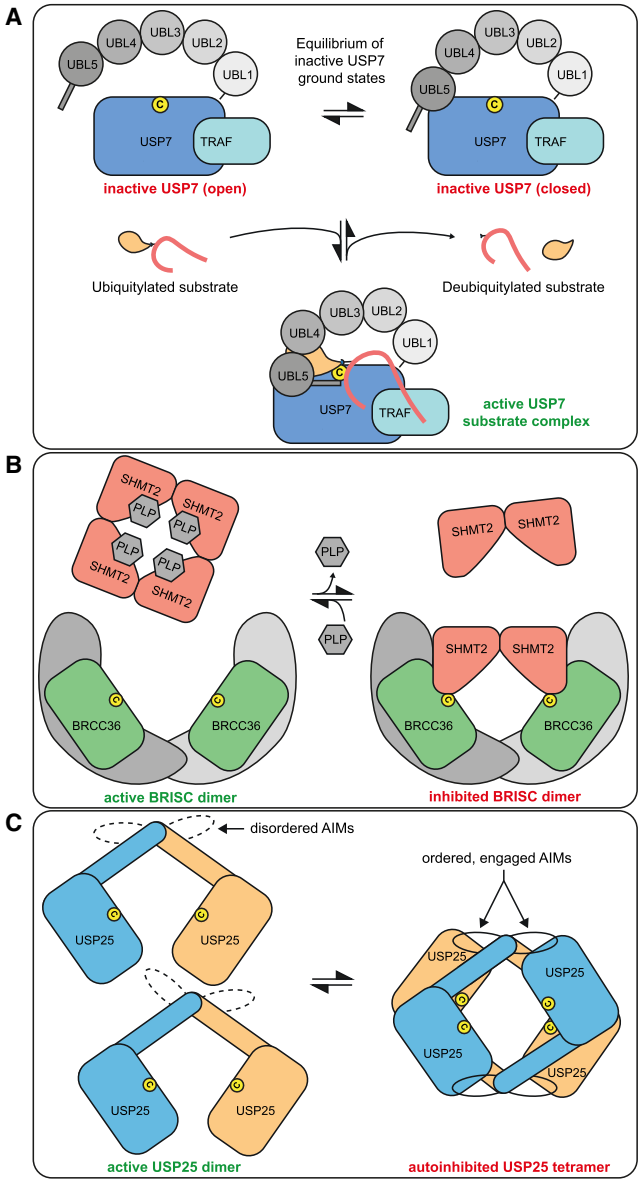

Figure 5. Intra- and intermolecular activity regulation of DUBs
(A) USP7 exists in an equilibrium of inactive, open, and closed conformations. The TRAF domain recruits ubiquitylated substrates, and Ub binding releases the inhibited state. Binding of UBL4/5 and the C-terminal tail of UBL5 further stabilizes the active conformation.
(B) The DUB modules BRCC36 of the dimeric BRISC complex are inhibited by dimeric SHMT2 but not by pyridoxal-5'-phosphate (PLP)-bound tetrameric SHMT2.
(C) Active USP25 dimers can assemble to an autoinhibited tetrameric configuration. The autoinhibitory motifs (AIMs) are disordered in the dimeric state but become ordered and engage with the neighboring USP25 molecules in the tetramer to block Ub binding.

An intriguing example of DUB inhibition in *trans* is seen in the related DUBs USP25 and USP28, which are active in their dimeric state. USP25 can also form tetramers, which are inactive because an autoinhibitory motif (AIM) slots into the catalytic domain of a neighboring USP25 molecule to block Ub binding, leaving all subunits of the tetramer in an autoinhibited state (Figure 5C). Thus, USP25 provides a novel paradigm of how DUBs can regulate themselves in *trans* (Gersch et al., 2019; Liu et al., 2018; Sauer et al., 2019). How the transition between inactive tetramers to active dimers is regulated is unclear, but cancer mutations in USP25 favor the dimeric state.

In contrast, some DUBs can also be inhibited by their products, as shown for UCHL3, a DUB that binds K27-linked diUb chains and preferably cleaves non-K27 linkages (van Tilburg et al., 2021). UCHL3 is inhibited by free K27-diUb chains and by substrates modified with K27-Ub chains through covalent and allosteric mechanisms. Covalent product inhibition occurs when the C-terminal tail of the proximal Ub of a free K27-linked diUb chain forms a thioester bond with the catalytic cysteine of UCHL3. Further, allosteric inhibition of UCHL3 is mediated by an inhibitory conformation of K27-diUb chains that restrains the crossover loop of UCHL3, producing a long-lived complex. UCHL3 has therefore been proposed to act as a sensor of K27-linked substrates in cells and provides a captivating example of how DUB activity can be regulated by polyUb chains themselves.

REGULATION BY PTMs

Additional layers of regulation can be imposed by PTMs of DUBs and substrates, leading to cross-talk between ubiquitylation and other PTMs. Phosphorylation of OTUD5 at S177 is an absolute requirement for DUB activity because pS177 acts as a lynchpin that locks together two helices that are disordered in the unphosphorylated state. This allows the helices to envelop the C-terminal tail of Ub to promote catalysis (Huang et al., 2012). S177 is phosphorylated by casein kinase II (CK2), a constitutively active kinase, making it likely that changes in the activity of the protein phosphatase that dephosphorylates OTUD5 controls its activity. Another phospho-switch is observed in A20, where phosphorylation at S381 activates K63-polyUb cleavage capability and therefore plays a key role in its anti-inflammatory function (Wertz et al., 2015). Mechanistically, it remains unclear how phosphorylation of a residue located outside of the catalytic domain of A20 switches its linkage specificity. Phosphorylation of DUBs can also be inhibitory, as seen in USP8, where phosphorylation of S680 enhances binding to 14-3-3 proteins, decreasing catalytic activity toward Ub chains *in vitro* (Mizuno et al., 2007).

In addition to phosphorylation, DUBs are also regulated by other PTMs. For instance, hydroxylation of the UBA domain of OTUD7B (Cezanne) by factor inhibiting HIF1 (FIH1) prevents binding to Ub (Mader et al., 2020). DUBs can also be regulated by ubiquitylation and addition of other UBLs (Leznicki and Kulathu, 2017). In addition, reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) can oxidize the catalytic cysteine of DUBs like A20, inhibiting their activity (Cotto-Rios et al., 2012; Kulathu et al., 2013).

We have outlined the multiple layers of regulation that prevent DUBs from being constitutively active enzymes and how their activity can be switched on and off and targeted to specific substrates and pathways to ensure integrity of Ub signals and maintain their downstream effects. These layers of regulation and the plasticity of DUBs provide opportunities to exploit using small molecules to inhibit DUBs.

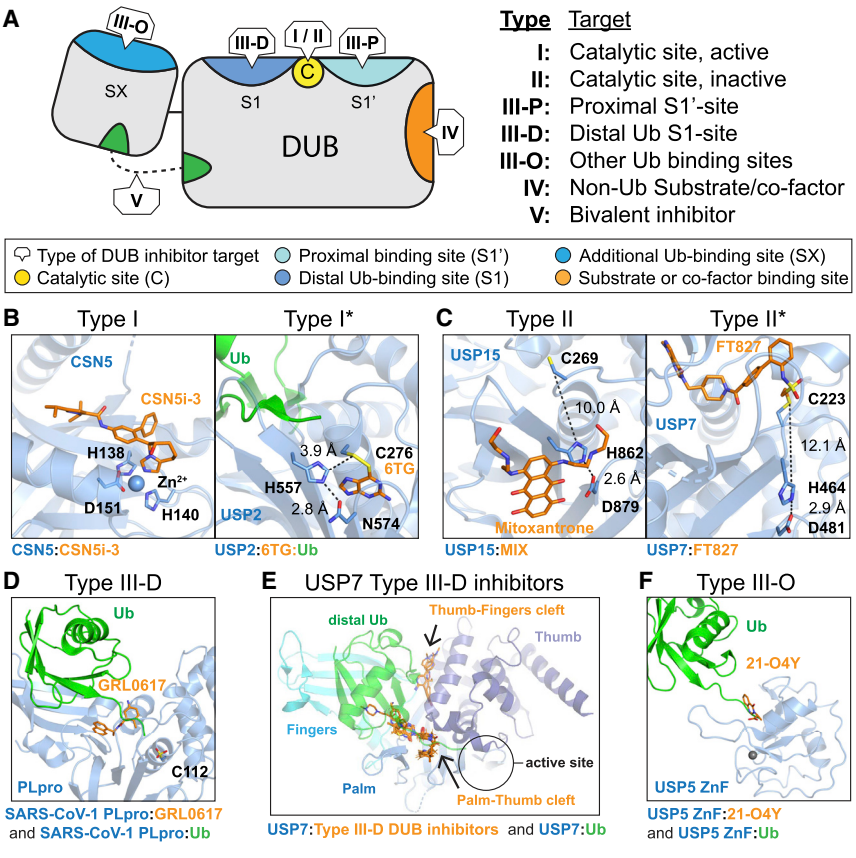

Figure 6. Classification of DUB inhibitors

(A) Schematic of a DUB with DUB inhibitor binding sites indicated.

(B–D) Examples of DUB:inhibitor complexes found in the PDB.

(B) Type I, CSN5:CSN5i-3 (PDB: 5JOG); type I*, USP2:6TG:Ub (PDB: 5XU8).

(C) Type II, USP15:MIX (PDB: 6GH9); type II*, USP7:FT827 (PDB: 5NGF).

(D) Type III-D, SARS-CoV PLpro:GRL0617 and Ub (PDB: 3E9S and 5E6J).

(E) Superposition of USP7 (cyan/blue/purple) with type III-D DUB inhibitors (orange) bound to the thumb-finger or palm-thumb clefts (PDB: 5NGE, 5N9R, 5N9T, 6F5H, 5WHC, 5VSB, 5VSK, 5VS6, 5UQV, and 5UQX) and Ub (PDB: 1NBF).

(F) Type III-O, USP5 ZnF:21-O4Y and Ub (PDB: 6P9G and 2G45).

(B–F) DUBs are shown in blue, inhibitors in orange, and Ub in green.

DUBs: DRUGGING THE UNDRUGGABLE

Extensive research over the past two decades to unravel the biology of DUBs has resulted in their emergence as novel targets for therapeutic intervention in cancer, neurodegenerative diseases, and various immune disorders. A range of excellent reviews covers the plethora of identified biological roles of individual DUBs, their dysregulation in disease, and their potential as drug targets (Basar et al., 2021; Cohen and Tcherpakov, 2010; Harrigan et al., 2018; Huang and Dixit, 2016; Leznicki and Kulathu, 2017; Schauer et al., 2020; Wertz and Murray, 2019). Further, bacteria and viruses express pathogenic DUBs that target host pathways dependent on Ub and UBLs to dampen the host immune response to promote pathogen survival and replication (Bailey-Elkin et al., 2017; Hermanns and Hofmann, 2019). A relevant example is the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus, the causative agent of the ongoing coronavirus disease 2019 (COVID-19) pandemic that expresses a papain-like protease (PLpro), which is essential for cleavage of viral polypeptides to produce mature non-structural proteins. In addition, PLpro can remove K48-linked polyUb and the UBL interferon-stimulated gene 15 (ISG15) from host cell proteins, dysregulating the host immune defense mechanisms against the virus, including the interferon response (Shin et al., 2020). Indeed, there is intense ongoing research to develop potent and specific inhibitors to PLpro for use as antiviral agents (Armstrong et al., 2021; Lim et al., 2021; Rut et al., 2020).

With most DUBs being Cys proteases, a prevailing belief in the field was that developing covalent inhibitors that specifically target a given DUB would be challenging, if not impossible, because many early DUB inhibitors showed poor specificity (Ritorto et al., 2014). A further challenge is that several DUBs are closely related, with similar catalytic pockets, making it difficult to identify compounds that show selectivity. However, the distinct mechanisms, substrate specificity determinants, and layers of regulation outlined above suggest that DUBs can be targeted selectively. For instance, despite the S1 site being the binding site of Ub, a range of synthetic Ub variants have been developed that bind a given DUB with high affinity and specificity to inhibit them (Ernst et al., 2013). This highlights how subtle differences can be exploited to target DUBs. Indeed, a flurry of activity in recent years has seen development of potent and specific small-molecule inhibitors of DUBs, establishing that they are indeed druggable. In addition to their potential to treat diseases, cell-permeable specific DUB inhibitors will be powerful research tools to elucidate physiological roles of DUBs.

CLASSIFICATION OF DUB INHIBITORS BY THEIR MECHANISM OF ACTION

Several co-crystal structures of DUB inhibitors bound to their targets that have emerged over the last decade allow us to review these inhibitors by their mode of inhibition. Here we conceptualize five distinct DUB inhibitor types based on existing structural and biochemical data and propose ways in which we foresee DUBs to be targeted (Figure 6A; Table 1).

Type I: Bind within the active site and stabilize the active DUB conformation

Inhibitors that interact directly with catalytic-site residues and stabilize the target DUB in an active conformation, with the active

Table 1. Members of DUB inhibitor classes with structural examples

Type of DUB inhibitor	Characteristics of DUB inhibitors	Molecules	PDB code (reference)	Target DUBs or candidates
I	binding within active site and stabilized active conformation	CSN5i-1b, CSN5i-3, CSN5i-6	5JOH, 5JOG (Schlierf et al., 2016); 5M5Q (Altmann et al., 2017)	CSN5
I*	covalent binding within active site and stabilized active conformation	6TG	5XU8 (Chuang et al., 2018)	USP2
II	binding within active site and stabilized inactive conformation	mitoxantrone	6GH9 (Ward et al., 2018)	USP15
II*	covalent binding within active site and stabilized inactive conformation	FT827	5NGF (Turnbull et al., 2017)	USP7
		HBX19818	(Reverdy et al., 2012)	
III	P: allosteric binding in proximal site (S1')	NA		OTULIN
	D: allosteric binding in distal Ub site (S1)	GRL0617	3E9S (Ratia et al., 2008)	SARS-CoV PLpro (palm-thumb cleft)
		15 g, 3k, 3j	3MJ5 (Ghosh et al., 2010) 4OW0, 4OVZ (Báez-Santos et al., 2014)	
		ALM2, ALM5, ALM45	5N9R, 5N9T (Gavory et al., 2018), 6F5H (O'Dowd et al., 2018)	USP7 (palm-thumb cleft)
		FT671	5NGE (Turnbull et al., 2017)	
		Cpd1, Cpd8, XL188	5VSB, 5VSK, 5VS6 (Lamberto et al., 2017)	
		GNE6640, GNE6776	5UQV, 5UQX (Kategaya et al., 2017)	USP7 (thumb-fingers cleft)
		Cpd2	5WHC (Di Lello et al., 2017)	
		IU1, IU1-47, IU1-206, IU1-248	6IIK, 6IIL, 6IIM, 6IIN (Wang et al., 2018)	USP14 (palm-thumb cleft)
	O: allosteric binding in other Ub site	1 (HHY), 5 (KKG), 7 (HH4), 21 (O4Y)	6DXT, 6NFT, 6DXH, 6P9G (Mann et al., 2019)	USP5 (ZnF-UBD)
IV	allosteric binding outside Ub binding and active sites	N/A		USP1:UAF1
		N/A		USP14:RPN1
		N/A		UCHL5:RPN13
		N/A		USP7
V	bivalent DUB inhibitors	N/A		USP25
		N/A		USP28

Shown is a list of DUB inhibitor categories and small-molecule members with available structural data. To our knowledge, no small molecules have been developed to date that are III-P, IV, or V, for which we have listed DUBs that might be promising candidates for future development. III-P: OTULIN requires specific interaction of the proximal Ub with the S1' site for activity, and small molecules targeting the proximal binding site could be efficient inhibitors. IV: DUBs that require binding of inter- or intramolecular co-factors for activation are promising candidates for allosteric inhibition by molecules that disrupt these interactions. USP1, USP14, and UCHL5 are examples of DUBs that are activated by binding partners, whereas USP7 is activated by binding of its UBL domains. V: USP25 and USP28 are potential candidates for small molecules that link two dimers to stabilize the inhibited tetrameric DUB complexes.

site residues aligned for catalysis, are "type I" DUB inhibitors. For type I and type II inhibitors, we imposed maximum cutoff distances of 3.5 Å for polar and 4.5 Å for non-polar interaction of inhibitors with catalytic residues (i.e., catalytic Cys, catalytic His, acidic residue, etc.). Examples of non-covalent type I inhibitors are a series of small molecules developed by Novartis that target the metalloprotease DUB constitutive photomorphogenic-9 signalosome subunit 5 (CSN5) (Schlierf et al., 2016). CSN5 is part of the COP9 signalosome and plays a central role in remodeling of cullin-RING E3 ligases (CRLs) by removing the UBL NEDD8. The inhibitor CSN5i-3 coordinates to the Zn^{2+} ion of the active site and extends into the substrate binding site of CSN5 to inhibit its deneddylation activity (Figure 6B).

Type I* ("star") inhibitors are covalent modifiers like 6-thioguanine (6TG), which forms a disulfide bond with the catalytic cysteine of USP2 (C276) while the positions of the other catalytic

triad residues H557 and N574 remain unaffected (Chuang et al., 2018; Figure 6).

Type II: Bind and stabilize the inactive DUB conformation

Inhibitors that interact directly with catalytic-site residues and stabilize the target DUB in an inactive conformation, with misaligned active-site residues, are "type II" DUB inhibitors. The US Food and Drug Administration (FDA)-approved antineoplastic drug mitoxantrone (MIX) has multiple cellular targets, including USP11 and USP15 (Burkhart et al., 2013; Ward et al., 2018). The co-crystal structure with USP15 revealed that MIX binds non-covalently to its catalytic histidine H862 while keeping the catalytic cysteine C269 10 Å apart (Figure 6C). An example of a type II* inhibitor is the small molecule FT827, which forms a covalent bond with the catalytic cysteine of USP7 (Turnbull et al., 2017). FT827 extends into the binding pocket for the C-terminal end of the distal Ub and stabilizes USP7 in a catalytically misaligned conformation (Figure 6C). Many type I* and II* inhibitors against thiol DUBs rely on reactive warheads that form reversible or irreversible covalent adducts with the catalytic Cys to inhibit it. A limitation of such orthosteric inhibitors is that the warheads are commonly oxidative or alkylating in nature, which can lead to toxic off-target effects.

Type III: Allosteric inhibitors in Ub-binding sites of a DUB

Type III DUB inhibitors are competitive molecules that bind to the Ub-binding sites of DUBs and block substrate recognition but do not interact directly with catalytic residues. We subdivide type III DUB inhibitors into molecules that target the S1' site, blocking proximal Ub binding (type III-P); the S1-binding pocket, blocking the distal Ub from binding (type III-D); or other Ub-binding sites of the DUB (type III-O) (Figure 6).

Type III-D inhibitors are currently the largest class of DUB inhibitors with the most potent and specific binders reported to date. Interestingly, most compounds are targeted at DUBs that contain a USP-like fold with the characteristic right-hand architecture, where the S1 site is typically located between the palm, fingers, and thumb. The first reported crystal structure of a type III-D inhibitor was the small molecule GRL0617 in complex with the PLpro domain of SARS-CoV (Ratia et al., 2008). GRL0617 occupies the binding cleft for the distal Ub's C-terminal residues, located between the palm and thumb domains, and sterically blocks Ub access to the active site (Figure 6D). This palm-thumb cleft is conserved in other USP-fold DUBs and is targeted by the majority of type III-D inhibitors, as observed in co-crystal structures with SARS-CoV PLpro, USP7, and USP14 (Figure 6E; Báez-Santos et al., 2014; Gavory et al., 2018; Ghosh et al., 2010; Lamberto et al., 2017; O'Dowd et al., 2018; Turnbull et al., 2017; Wang et al., 2018).

A set of type III-D DUB inhibitors developed by Genentech penetrate a different cleft of the S1 site of USP7, located between the thumb and fingers domains (Kategaya et al., 2017; Di Lello et al., 2017), and prohibit binding of the distal Ub by sterically blocking the β3-β4 loop of Ub (Figure 6E).

Compounds that block Ub binding in sites other than the S1 and S1' sites belong to the group of type III-O DUB inhibitors, as seen in the recently reported small molecule that targets the USP5-zinc finger (ZnF) UBD, which occupies the binding cleft that recognizes the C-terminal Gly-Gly motif of Ub, inhibiting DUB activity (Mann et al., 2019; Figure 6F).

To our knowledge, no type III-P DUB inhibitors that exclusively target the S1' site without contacting the active site directly have been reported to date. However, several DUBs, such as OTULIN, Cezanne, and USP30, rely on proximal Ub recognition for full catalytic activity and might therefore be promising targets for such inhibitors.

Type IV: Allosteric inhibitors outside of Ub-binding sites

As discussed in the previous sections, several DUBs exist in an inactive state until activated by mechanisms, such as structural changes induced by substrate binding or through interactions with specific co-factors. The regulatory mechanisms and the plasticity of DUB catalytic domains suggest that there is huge potential to develop type IV inhibitors that would bind DUBs outside of the active site or substrate-binding sites to prevent activation. A major advantage of such inhibitors is that they would be very target specific because they would exploit a natural regulatory mechanism unique to a given DUB. To date, no type IV inhibitors have been identified because of several challenges in their development, primary among them identification of suitable regulatory sites. However, recent successes in identifying allosteric regulators in other enzymes suggest that such strategies could be amenable to identifying allosteric binding sites and allosteric networks in DUBs (Keedy et al., 2018; Kessler et al., 2019). Developing type-III and type-IV allosteric inhibitors is an attractive strategy for DUBs whose active sites are closely related and therefore difficult to target with high specificity.

Type V

We envisage type V DUB inhibitors to be bivalent molecules that simultaneously bind two regions of a DUB; for example, tethering the catalytic domain to an adjacent inhibitory domain, locking the enzyme in an inhibited conformation. A potential advantage of these bivalent molecules would be increased target specificity with inhibitors directed against unique non-catalytic regions of a DUB. Although bivalent inhibitors to DUBs do not exist to date, such inhibitors have been developed against multiple protein kinases and phosphatases, like c-Jun N-terminal kinase 1 (JNK1) (Stebbins et al., 2011), SHP2 (Chen et al., 2016), and protein tyrosine phosphatase 1B (PTP1B) (Stebbins et al., 2011). Because many DUBs are modular, and the non-catalytic domains can exert autoinhibitory effects, developing such bivalent type V compounds should be possible.

PROTACs and DUBTACs

Several DUBs have additional non-catalytic functions, like scaffolding, which is highlighted by discrepancies in the phenotypes observed in knockout models versus knockin approaches expressing catalytically dead DUBs, and catalytic inhibition might therefore not have the desired effect. Further, inhibition could potentially convert a DUB into a strong Ub binder (Morrow et al., 2018), which may have unpredictable consequences. Hence, one attractive strategy would be to use proteolysis-targeting chimera (PROTAC) approaches, linking DUB inhibitors to E3 ligases, to degrade DUBs (Figure 7). In addition, molecular

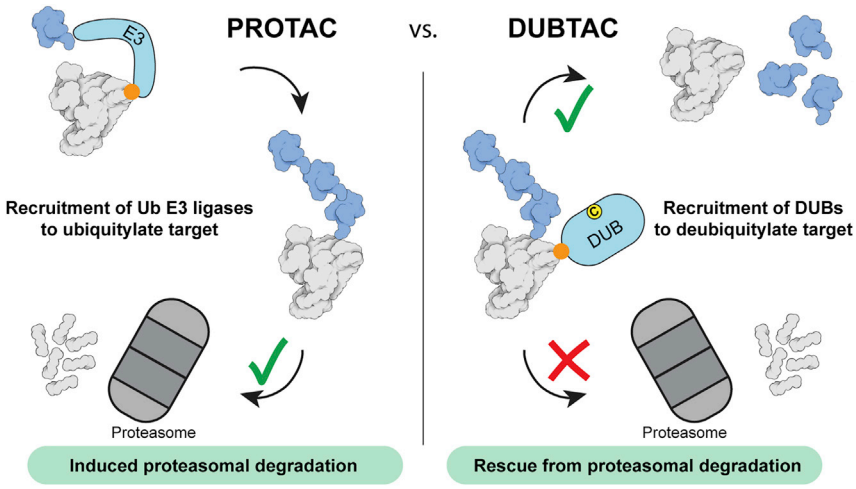

Figure 7. Schematic comparison of PROTAC and DUBTAC principles

Left: PROTACs induce proteasomal degradation of target proteins by recruiting Ub E3 ligases that ubiquitylate the target. Right: DUBTACs rescue target proteins from proteasomal degradation by recruiting DUBs that remove Ub chains from the target. Target proteins are shown in light gray, PROTAC and DUBTAC as orange circles, E3 and DUB enzymes in light blue, Ub molecules in dark blue, and proteasomes in dark gray.

glues designed to bind to the interface of a DUB and an E3 ligase can also be a way to selectively degrade a DUB. Naturally, a potential challenge here is that a DUB may counter the E3 activity by deubiquitylating itself.

An interesting application of DUBs as an adaptation of PROTAC technology is to target DUBs to neosubstrates. One can imagine employing such DUB-targeting chimera (DUBTACs) approaches to stabilize or rescue proteins that are ubiquitylated and degraded in disease. Indeed, a recent study demonstrates how a heterobifunctional DUBTAC bridges the K48-specific DUB OTUB1 to ΔF508-CFTR to stabilize the mutant CFTR protein in cells, opening a new possibility to treat cystic fibrosis (Henning et al., 2021). Developing DUBTACs will require identification of ligands that bind DUBs at non-catalytic sites so that they do not interfere with catalytic activity. In addition, the choice of DUB will be critical in terms of substrate specificity (i.e., promiscuous versus linkage-specific DUBs) as well as their cleavage mode (i.e., *exo*-, *endo*-, or *en bloc*-type activity); all of these factors offer avenues to fine-tune the activity of future DUBTACs.

FUTURE PERSPECTIVES

To accelerate our understanding of DUB biology, we need better approaches to define cellular substrates of DUBs. Indeed, one of the biggest challenges in the field is identification of DUB substrates. With the growing availability of selective DUB inhibitors, application of chemical genomic approaches will facilitate identification of DUB substrates and novel DUB biology (Magin et al., 2021). Most *in vitro* screens commonly use activity-based probes or simplified substrates such as Ub-AMC (7-amino-4-methylcoumarin) or diUb chains (Pruneda and Komander, 2019). Comprehensive screening of DUBs against more complex modifications, such as branched Ub chains, ester-linked protein, and non-protein substrates will also reveal new insights. Such screens may identify substrates of DUBs, such as OTUD6B and ALG13, that do not cleave diUb substrates and reveal the biology of poorly understood DUBs. Improved substrates and assays will also help inhibitor development efforts for screening as well as validation of compounds. Recent discoveries of new DUBs suggest that there may be more awaiting discovery for which new tools and approaches are required. Development of innovative Ub probes that can target metalloproteases will reveal whether there are still unknown metallo-DUBs. Similarly, it remains to be established whether there are DUBs that use Ser/Thr or Asp protease mechanisms. DUBs play key roles in modulating the Ub system and maintaining homeostasis, which provides the rationale and impetus for continued research into their mechanisms and biology and offers the potential for novel discoveries with exciting translational implications.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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