Review

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Layers of DUB regulation

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Proteolytic enzymes, such as (iso–)peptidases, are potentially hazardous for cells. To neutralize their potential danger, tight control of their activities has evolved. Deubiquitylating enzymes (DUBs) are isopeptidases involved in eukaryotic ubiquitylation. They reverse ubiquitin signals by hydrolyzing ubiquitin adducts, giving them control over all aspects of ubiquitin biology. The importance of DUB function is underscored by their frequent deregulation in human disease, making these enzymes potential drug targets. Here, we review the different layers of DUB enzyme regulation. We discuss how post-translational modification (PTM), regulatory domains within DUBs, and incorporation of DUBs into macromolecular complexes contribute to their activity. We conclude that most DUBs are likely to use a combination of these basic regulatory mechanisms.

DUB regulation: background and overview

Conjugation of ubiquitin and ubiquitin-like molecules (Ubl) (Box 1) to lysines of target proteins represents a major type of PTM that regulates countless processes in eukaryotes [1]. These modifications are catalyzed by an enzymatic cascade involving E1 activating enzymes, E2 conjugating enzymes, and E3 ligases (Box 2). Many different types of Ub/Ubl modification exist, because targets can be monoubiquitylated or modified with a variety of polyubiquitin chains (Box 3) that can each have different signaling outcomes.

Ubiquitin signals have profound cellular effects and, therefore, conjugation events are kept in check by ubiquitin deconjugation. This function is performed by a specialized class of isopeptidases called DUBs, which hydrolyze the isopeptide bond between ubiquitin and the target proteins [2,3]. Five different DUB families have been identified: ubiquitin C-terminal hydrolase (UCH), ubiquitin specific protease (USP), ovarian tumor (OTU), Machado-Joseph disease (MJD); and Jab1/Mpn/Mov34 (JAMM) (Box 4). All of these are cysteine isopeptidases except the JAMM family members, which have metalloisopeptidase activity [3].

Due to their critical role in cellular functions, deregulation of enzymes of the ubiquitin system is important in cancer, infectious, and neurological diseases [4–6]. Hence, there is an increasing interest in targeting these molecules pharmaceutically. Given that E2 conjugating enzymes and most E3 ligases lack distinct catalytic clefts, approaches to therapeutic intervention currently focus on DUBs [7].

In the cell, the activity of degrading enzymes is carefully controlled. This has long been known for peptidases, the distant cousins of DUBs, which are tightly regulated not only through production as inactive enzymes (zymogens), but also through proteinaceous inhibitors and elaborate activation cascades to prevent aberrant proteolysis [8]. This tight control is essential, because unscheduled activation can be disastrous for the cell. It is gradually becoming clear that this is also true for DUB isopeptidases. The need to regulate DUB activity can be explained by the large number of ubiquitin conjugates in cells. Without proper regulation, DUBs could unspecifically hydrolyze any ubiquitin conjugate that they encounter, potentially deregulating cellular physiology.

To cope with this, cells have adopted several strategies to ensure that DUB activity is channeled to the right locations at the right time. Some of this regulation occurs at the transcriptional level, but the proteins themselves are regulated in many different ways. A clear understanding of these processes is important for our knowledge of ubiquitin biology and will assist in the development of therapeutic agents targeting specific DUBs. In recent years, insights into DUBs whose catalytic activity is regulated have steadily increased; through advances in the cellular physiology, biophysics, and structural biology of DUBs, we are starting to elucidate the intricate mechanisms that underlie DUB regulation.

The general roles of DUBs and their target and chain specificity have been discussed elsewhere [3,9,10]. Here, we discuss the emerging themes in regulation of DUBs at the protein level. We distinguish different 'layers' of DUB regulation and describe how they affect activity (Figure 1).

Box 1. Ubiquitin and ubiquitin-like molecules

Eukaryotes have a diverse repertoire of PTMs to fine-tune or alter molecular processes. Among these is ubiquitination, where the small 76-amino acid protein ubiquitin is attached to target proteins [1]. Ubiquitin is characterized by a globular β-grasp fold followed by an extended tail harboring a Gly-Gly motif required for conjugation to target proteins. Over the past few decades, other small proteins that share these characteristics with ubiquitin have been identified. Among these are NEDD8, SUMO-1, SUMO-2, SUMO-3, ATG8, ISG15, and FAT10, but more have been identified that can be conjugated to proteins to alter their fate or function. Moreover, the enzymes responsible for their conjugation and deconjugation are also homologous to the enzymes from the ubiquitin system and follow similar mechanisms. Given these commonalities with ubiquitin, these proteins are collectively called Ubl.

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Box 2. Ubiquitin conjugation and deconjugation

The conjugation of ubiquitin to target proteins proceeds via a conserved enzymatic cascade (Figure I) [1]. This cascade results in the formation of an isopeptide bond between the C terminus of ubiquitin and the ε-amino group of a lysine on the target protein. In the first step, an E1-activating enzyme activates the C-terminal carboxyl of ubiquitin and transfers it to its active site cysteine, after which an E2-conjugating enzyme binds the E1 and is 'charged' with ubiquitin at its active site cysteine. E3 ligases next orchestrate the final formation of the isopeptide bond between ubiquitin and target. DUBs can reverse ubiquitin conjugation by catalyzing the hydrolysis of the isopeptide bond (Figure I) [2]. This allows these enzymes to control all aspects of ubiquitin biology.

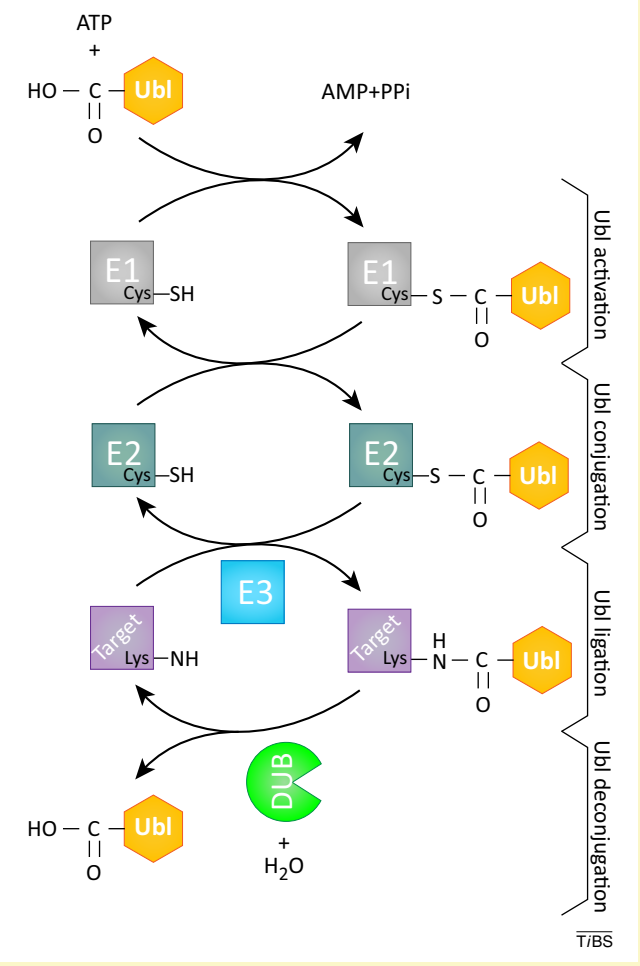

Figure I. Ubl conjugation and deconjugation cascade. Abbreviation: Ubl, ubiquitin-like molecule.

Box 3. Ubiquitin signals

Ubiquitin signals come in many flavors, some of which are schematically depicted in Figure I. Target proteins can be conjugated with a single ubiquitin or multiple ubiquitins. Furthermore, ubiquitin can also be conjugated to itself in eight different ways because it has seven lysine and one free amino-group of the Met1 amino acid that all can serve as targets. Thus, different ubiquitin chains are possible. These chains have different structural properties and are associated with different cellular processes [93]. For example, proteins modified with polyubiquitin chains linked through lysine 48 mark them for proteasomal destruction, whereas lysine 63 and 'linear' Met1-linked chains have roles in signaling pathways. Mixed chains, containing several different linkages in the same polyubiquitin molecule, have also been reported [94]. Ubl-deconjugating enzymes can hydrolyze polyubiquitin chains to single ubiquitin moieties with, in some cases, remarkable specificity. For example, the DUB OTULIN can only hydrolyze linear Met1-linked ubiquitin chains [33], while the DUB AMSH specifically cleaves Lys63-linked polyubiquitin chains [55].

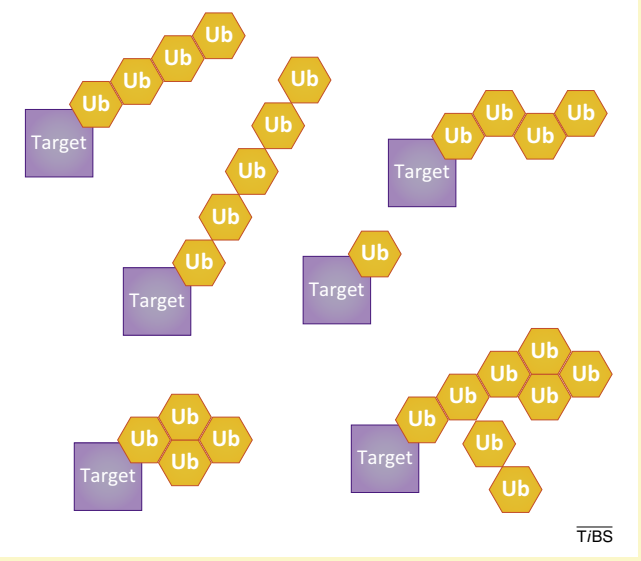

Figure I. Different ubiquitin chain types result in different signals.

After examination of the individual layers, we analyze how these different mechanisms can cooperate. Although our list of examples is not exhaustive (Table 1), it provides a good basis for discussing the different layers of DUB regulation.

Cellular and target recruitment

In Figure 1, we present a simplified classification of the different layers of DUB regulation. The first layer we discuss is that of DUB recruitment factors. Guiding the almost 100 DUBs encoded in the human genome to their relevant substrates and pathways is crucial for cellular physiology because it insulates DUBs from unwanted interactions and the cell from spurious activity. It can be mediated by distinct regions within the enzyme or by external factors: For instance, the Ubl domain of ubiquitin-specific protease 14 (USP14) recruits it to the proteasome, where its activity is increased 500-fold [11]. The endosomal protein signal transducing adaptor molecule (STAM) recruits the DUBs AMSH (associated molecule with an Src3 homology domain of STAM) and USP8 to the endosome pathway by interacting with an SRC homology 3 (SH3)-binding motif or MIT domain (microtubule interacting and transport), respectively [12,13].

Another pathway that requires proper DUB recruitment is the DNA damage response (DDR). After ultraviolet (UV)-induced DNA damage, monoubiquitylated proliferating cell nuclear antigen (PCNA) mediates signaling that leads to repair. The DUB complex USP1/USP1-associated factor 1 (UAF1) deubiquitylates PCNA after the complex is recruited to the substrate by recruitment factor human ELG1 [14]. BRCA1/BRCA2-containing complex, subunit 36 (BRCC36) is another DUB in the DDR, where it deubiquitylates several proteins as a catalytic subunit of the BRCA1-A complex. In this complex, specialized ubiquitin and small ubiquitin-like modifier (SUMO)-binding domains recruit BRCC36 to sites of

Box 4. Deubiquitylating enzymes

DUBs deconjugate ubiquitin from targets. They are subdivided into five families based on the structures of their CDs (Figure I): UCH, USP, OTU, MJD, and JAMM. In addition, the SENP family deconjugates the Ubl molecule SUMO from target proteins. All of these families, with the exception of JAMM, are cysteine proteases that are predicted to have a catalytic mechanism similar to papain-like proteases [3]. This mechanism depends on a so-called 'catalytic triad', where a cysteine, histidine, and asparagine or aspartic acid are present in a specific configuration and cooperate to break the bond between Ubl and target. The JAMM enzymes are metal-dependent proteases and are predicted to share their catalytic mechanism with other metalloproteases [3]. These enzymes are in general specific for a certain Ubl, although there is some divergence. Most JAMM members, for instance, are DUBs, but the JAMM member CSN5 has NEDD8-deconjugating activity [34]. Likewise, most SENP members are specific for the Ubl SUMO, but member DEN1 is specific for the Ubl NEDD8 [95].

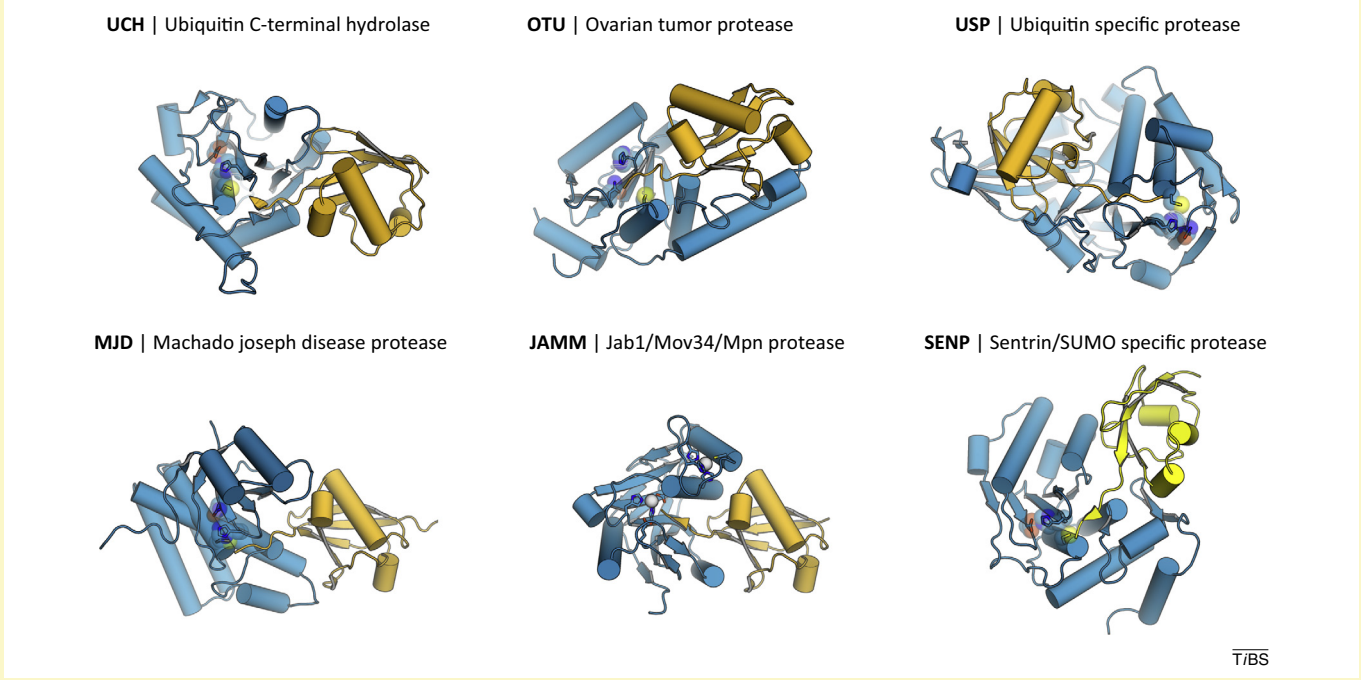

Figure I. Structural classification of Ubl isopeptidases.

damage [15–19]. Finally, also in the nucleus, transcription factor forkhead box K2 (FOXK2) targets the UCH class DUB BAP1 (BRCA1-associated protein 1) to chromatin to facilitate histone H2A deubiquitylation [20].

In NF-κB signaling, ubiquitin conjugation has multiple roles. Different components of the pathway recruit the DUBs USP10 and CYLD (cylindromatosis). The protein MCPIP-1 (monocyte chemotactic protein induced protein 1) recruits USP10, whereas CYLD is recruited by the E3 ligase HOIP (HOIL interacting protein) (Figure 1). Moreover, CYLD contains a B-box domain that promotes its cytoplasmic localization [21,22].

The previous examples illustrated how external proteins can recruit DUBs to relevant pathways or substrates. While DUBs in general can recognize the ubiquitin part of a substrate via their catalytic domains (CDs), sometimes extra specificity is achieved by specialized domains that are present in DUBs themselves. One of the best-studied examples is the DUB USP7. Here, the N-terminal TRAF domain of USP7 binds small peptide motifs in its targets EBNA-1 (Epstein–Barr nuclear antigen 1), p53 and MDM2 (Mouse double minute 2 homolog) to facilitate their deubiquitylation (Figure 1) [23,24]. USP15 uses its DUSP-Ubl domain to recruit and deubiquitylate the E3 ligase BRCA1-associated protein (BRAP) [25], while the H2A deubiquitinase USP3 requires its intact Zinc finger domain to bind H2A [26].

Thus, specialized domains within DUBs and external proteins can guide DUB activity to the correct pathways and substrates by functioning as recruitment factors.

Substrate-mediated regulation

Besides recruitment, some DUBs require further activation. Surprisingly, the cognate Ubl itself can affect the activity of the DUB or ubiquitin-like protease by rearranging the catalytic triad. Early structural studies on the CD of USP7 revealed that its catalytic triad can exist in an inactive configuration [27]. Binding of a ubiquitin derivative 'realigned' the catalytic triad towards an active configuration and also changed the conformation of the 'switching loop', a surface loop close to the active site that is important for activation [27,28]. These effects suggest that USP7 CD is only active when ubiquitin is correctly bound. In UCH-L1 (Figure 1), ubiquitin binding at an 'exosite' (i.e., distant from the active site), also induces a cascade of conformational changes that rearranges the catalytic triad [29]. This type of allosteric activation also occurs in the sentrin-specific protease (SENP) class of SUMO proteases, where it has been elegantly quantified. When SENPs are incubated with the tail-less SUMO β-grasp domain, this increases the catalytic turnover against a model peptide substrate [30–32]. However, this type of ubiquitin/Ubl-induced rearrangement is not generically present, because other inactive DUBs contain correctly

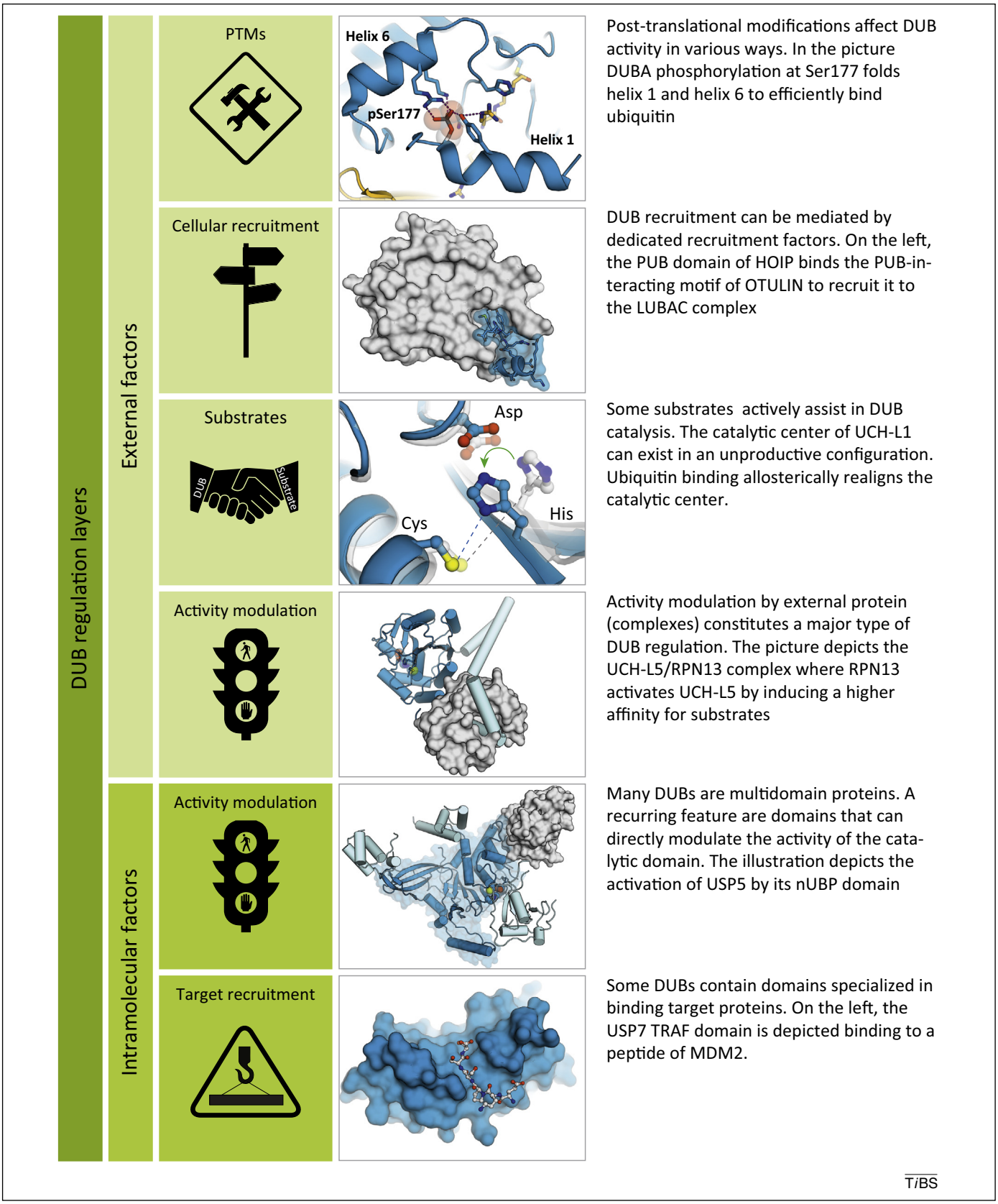

Figure 1. Classification of regulatory layers for deubiquitylating enzymes (DUBs). Two major divisions are intramolecular factors, focused on domains within DUBs, and external factors focused on protein partners or modifications. Each of these two divisions contains additional layers of DUB regulation. DUBs are colored blue or cyan. Regulatory elements are colored in light gray. For definitions of abbreviations, please see the main text.

Table 1. Summary of known DUB regulatory mechanisms

Family	DUB	Intramolecular factors		External factors				Refs
		Activity modulation	Target recruitment	Activity modulation	PTMs	Cellular recruitment	Substrate assistance	
UCH	UCH-L1				Ubiquitylation of K157 decreases activity; inhibited by ROS		Ubiquitin binding to an exosite aligns catalytic site to active configuration	[29,81]
	UCH-L5			RPN13 increases while INO80G decreases ubiquitin binding		Recruited to proteasome by RPN13 and to INO80 complexes by INO80G		[62–66]
	BAP1			ASXL1 promotes activity	Ubiquitylation of the NLS mislocalizes BAP1	Targeted to genomic loci by several protein partners		[20,61]
USP	USP1	UV-induced autocleavage impairs activity		UAF1 increases k_{cat}	Phosphorylation of Ser313 promotes UAF1 binding; inhibited by ROS	ELG1 recruits USP1/UAF1 complex to PCNA, while Spartan prevents it		[14,48,50, 51,71,72, 75,89]
	USP3					USP3 ZnF domain recruits target H2A		[26]
	USP4	DUSP-Ubl domain promotes ubiquitin release			Phosphorylation by AKT alters localization			[45,85]
	USP5	nUBP allosterically activates while other UBDs assist in substrate binding					Ubiquitin moiety can bind to ZnF UBP domain and allosterically activate USP5	[43,44]
	USP7	HUBL domain activates CD	USP7 TRAF domain recruits targets	GMPS increases k_{cat}			Ubiquitin binding realigns active site	[23,24,27, 28,47]
	USP8					STAM binds USP8 MIT domain to recruit it to endosomes		[12]
	USP12			Activated by UAF1 and hyperactivated by WDR20				[52,54]
	USP10				Phosphorylation translocates USP10 to nucleus	MCPIP-1 recruits USP10 to NEMO		[22,86]
	USP13			Activated by Beclin-1				[56]
	USP15		USP15 DUSP-Ubl domain recruits target BRAP					[25]
	USP14/yUbp6			Integration into proteasome actives enzyme		Proteasome regulatory particle binds USP14/ Ubp6 Ubl domain		[11,96]
	USP25		UBDs promote ubiquitin chain hydrolysis		Sumoylation at K99 decreases chain hydrolysis activity			[80]
	CYLD		B-Box domain promotes cytoplasmic localization		Sumoylated and phosphorylated			[21,78,79]
	USP37							[74]

					Phosphorylation at Ser628 activates enzyme			
	USP46			Activated by UAF1 and hyperactivated by WDR20				[52,54]
	yUbp15	N-terminal TRAF domain and C terminus stimulate catalytic domain						[46]
	yDoa4			C terminus Bro1 stimulates activity; Rfu1 inhibits activity		Bro1 domain of Bro1 recruits it to endosomes		[69,70]
	yUbp8			Activated in SAGA DUB module				[39]
	USP28				Sumoylation decreases activity			[97]
OTU	DUBA				Phosphorylation of Ser177 promotes ubiquitin binding			[73]
	OTULIN				Tyr56 phosphorylation decreases LUBAC association	HOIP PUB domain recruits OTULIN to LUBAC	Substrate-aided catalysis by linear polyubiquitin	[33,76,77]
	OTUB1			UbcH5B promotes K48 polyubiquitin binding				[59]
MJD	ATXN3				Ubiquitylation activates chain hydrolysis activity			[82]
	JosD1				Ubiquitylation activates chain hydrolysis activity			[83]
JAMM	AMSH			STAM activates chain hydrolysis		SH3 binding motif in AMSH recruits to ESCRT complexes		[13,55]
	CSN5	Autoinhibited by Ins1 loop		Only active in COP9 signalosome			Neddylated CRLs activate COP9 catalysis	[34,35]
	BRCC36			Activated in BRCA1-A and BRISC complexes		Recruited to DNA damage foci by repair protein through UIMs and SIMs in BRCA1-A complex		[15,16,38]
	RPN11			Activated in proteasomal 19S particle				[36,37]
SENP	SENP1,2 and SENP5–8						Globular SUMO domain activates catalysis	[30–32]

aligned active sites, even in the absence of ubiquitin. When present, this regulation by a Ubl itself cannot give much specificity. Thus, it is not surprising that regulation of USP7 for example, has further layers of complexity, as discussed later.

Substrates can give rise to more complex types of activation, generating high specificity. In OTULIN (OTU domain-containing deubiquitinase with linear linkage specificity), a member of the OTU class DUBs, the substrate actively assists in catalysis. OTULIN regulates NF-κB signaling by its exclusive ability to disassemble linear ubiquitin chains [33]. In these chains, ubiquitin moieties are linked via the amino terminus at Met1 instead of via one of its lysines. They are made by the HOIP E3 ligase in the linear chain assembly (LUBAC) complex. Interestingly, OTULIN uses this unique linkage to sense its substrate. Normally, OTULIN Asp336 functions as an autoinhibitory element that favors an unproductive catalytic triad conformation, but Glu16 of the proximal ubiquitin (the target ubiquitin) in linear chains reorganizes the catalytic triad towards an active state. Mutation of this substrate Glu16 reduces the *k*cat 240 times but hardly affects the binding, indicating that actively it promotes catalysis. Of all ubiquitin chain types, only a linear chain can bind such that the Glu16 in the proximal ubiquitin is correctly positioned to assist in catalysis, explaining how OTULIN activity is specifically restricted to cellular pathways that feature linear polyubiquitin signaling.

A different example of substrate-dependent activation is seen in CSN5, a JAMM-type DUB found in the eight subunit COP9 signalosome (CSN) [34]. The CSN deconjugates NEDD8 (neural precursor cell expressed developmentally downregulated 8) from Cullin Ring ubiquitin E3 ligases (CRLs). Through this activity, CSN decreases the ubiquitin E3 ligase activity of CRLs. A recent structural analysis of CSN rationalized how interaction between DUB and its neddylated CRL substrate leads to activation [35]. The catalytic subunit CSN5 forms a subcomplex with CSN6 and CNS4. In absence of substrate, a loop in CSN5, Ins1, occludes the active site, leading to autoinhibition. In the presence of neddylated CRLs, the CSN4 subunit undergoes a large conformational change to bind the substrate, at the expense of its interaction with the so-called 'Ins-2' loop of CSN6. These substrate-induced conformational changes alleviate the autoinhibition and prime CSN for deconjugation. Point mutations in the Ins1 and Ins2 loops can activate CSN even in the absence of neddylated CRLs, confirming that they act as autoinhibitory elements [35]. The ability of CSN4 to sense neddylated CRLs ensures that activation only occurs in the presence of the substrate.

The examples above illustrate an important safeguard mechanism for unwanted proteolytic activity by only allowing enzymatic activity to take place in the presence of the correct substrates.

DUB regulation by intramolecular and external factors

The next layer of regulation is the direct regulation of DUB catalytic activity. In this mode of regulation, the CD of DUBs can be viewed as core units whose catalytic activity is modulated by interaction with other protein modules, either external, or within the DUB itself [2].

A common form of regulation of this type is that given by a large molecular machine. There are several examples of DUBs that only attain optimal activity and localization within the structural integrity of such multisubunit molecular machines, such as the COP9 signalosome. Other notable examples of this type include USP14 and RPN11 in the proteasome [11,36,37], BRCC36 within the BRCA1-A and BRISC complexes [15,16,38] and Ubp8 in the yeast SAGA DUB module [39–41]. In these cases, the DUBs display low activity in isolation, but are robustly activated within the complex. For Ubp8, complex formation likely stabilizes the active site and the ubiquitin-binding surface [40,42]. In general, the exact molecular mechanism of activation in these large complexes remains poorly understood, but may depend on a combination of regulatory influences.

Apart from these examples where DUBs are activated as part of large macromolecular assemblies, many well-studied examples exist where simple domains, within the DUB or from outside, specifically modulate the activity of CDs. USP5 contains several ubiquitin binding domains (UBDs) that assist in the disassembly of polyubiquitin chains of a variety of linkages [43]. The crystal structure of full-length USP5 revealed a previously unpredicted domain, named nUBP, that packs tightly against the CD and allosterically activates it 1000-fold (Figure 1) [44]. Moreover, addition of free ubiquitin to USP5 can further stimulate USP5 activity through binding to the ZnF-UBP domain via an as yet unknown mechanism [43].

A second example where additional domains are important is USP4, a DUB with roles in TGF-β (transforming growth factor beta) signaling and splicing. The isolated USP4 CD was found to have an unusually high affinity for ubiquitin (low nanomolar range), suggesting that it could be constitutively product inhibited in cells, where the ubiquitin concentration is in the range of 4–20 μM [45]. However, the N-terminal DUSP-Ubl domain present in full-length USP4 can allosterically promote product release, thereby increasing *k*cat of USP4. This effect involves the 'switching loop', a loop close to the catalytic triad.

This 'switching loop' also has a role in the activity of USP7 or its yeast homolog Ubp15. The C-terminal HUBL domain of USP7 can dynamically fold back onto the CD to allow contact of a C-terminal peptide at the end of the HUBL domain with the 'switching loop'. This intramolecular interaction increases both *k*cat and *KM* of the CD [28]. Unlike USP7, the N-terminal TRAF domain of Ubp15 also affects intrinsic activity [46]. Apparently, the 'switching loop' has a conserved regulatory function in multiple USPs, although the details of the activation differ.

External factors, such as protein partners, can also activate DUBs by either reinforcing the stimulatory effects of intramolecular factors or by other means. The intramolecular activation of the USP7 CD by its HUBL domain is allosterically potentiated by external factor GMP synthase (GMPS), which consolidates the active state leading to an additional increase in *k*cat [28,47].

One of the best-studied examples of DUB activation by external factors is USP1. This DUB controls DNA repair signaling by deubiquitylating FANCD2 (Fanconi anemia group D2) and PCNA [48,49] after DNA cross-linking and

UV damage, respectively. USP1 is regulated by autocleavage [48] and its *k*cat is strongly stimulated by the WD40 repeat protein UAF-1 [50]. UAF-1 achieves this by increasing the basicity of the histidine general base in the USP1 catalytic triad [51]. Two closely related DUBs, USP12 and USP46, are similarly activated by UAF-1, but unlike USP1, these can be hyperactivated by another WD40 repeat protein, named WDR20 [52–54].

DUB activation also occurs in endocytosis and autophagy pathways. The JAMM class enzyme AMSH is recruited to endosomes by the adaptor protein STAM [55]. Besides recruitment, STAM can also directly activate AMSH hydrolysis of polyubiquitin chains on endosome-targeted proteins. The exact mechanism of this activation is unclear, but both *k*cat and *KM* effects were suggested [13,55]. In autophagy, the activity of USP10 and USP13 is modulated by Beclin-1, which is a subunit of the essential Vps34 complexes that have a role in phagosome nucleation. These complexes can be rapidly degraded by ubiquitylation of Beclin-1. However, Beclin-1 prevents this by binding USP10 and USP13, and stimulating these DUBs to remove ubiquitin from itself, both in cells and *in vitro* [56].

A particularly interesting example of DUB regulation by external proteins involves stimulation of OTUB1 activity by ubiquitin E2 enzymes. OTUB1 can noncatalytically interfere with polyubiquitin synthesis by specifically inhibiting E2 enzyme Ubc13 linked to ubiquitin at its active site (charged E2) [57,58]. Conversely, a subset of charged and uncharged E2s, including UbcH5B, stimulate Lys48-linked polyubiquitin hydrolysis by OTUB1 by increasing substrate affinity [59]. Crystal structures indicate that UbcH5B achieves this increased affinity by stabilizing a ubiquitin-binding site on OTUB1. Whether charged E2s stimulate OTUB1 activity, or whether OTUB1 inhibits polyubiquitin synthesis of the E2 depends on the relative concentrations of charged E2 and free ubiquitin. Therefore, the authors suggested an elegant model wherein OTUB1-E2 complexes dynamically regulate the level of polyubiquitin chains in cells by either activating chain hydrolysis or inhibiting E2-mediated chain synthesis.

The activities of the UCH family DUBs UCH-L5 and BAP1 are regulated by related deubiquitinase adaptor (DEUBAD) domains [60]. BAP1 is a tumor suppressor that is activated ASX (additional sex combs) to deubiquitylate H2A in Polycomb gene repression [61]. In the proteasome, the DEUBAD domain of RPN13 activates UCH-L5 by increasing the affinity for substrates [62–66]. This occurs through a combination of mild effects, including allosteric stabilization of the so-called 'active site crossover loop' and restriction of the inhibitory mobility of the C-terminal ULD domain of UCH-L5 [65,66].

These examples illustrate how domains within DUBs or external proteins can activate the CDs of DUBs through a variety of different mechanisms.

Negative regulation of DUBs

While all DUBs discussed so far are activated by intramolecular domains or external proteins, in a limited number of rare cases DUBs are directly inhibited by other proteins. The first example of this type is UCH-L5 inhibition by INO80G. INO80G is a metazoan-specific subunit of INO80 chromatin-remodeling complexes and is associated with UCH-L5 during DNA repair [67,68]. This interaction strongly inhibits UCH-L5 [67]. Similar to the UCH-L5 activator RPN13 (discussed earlier), INO80G contains a DEUBAD domain [60] and recent structural analyses revealed that it inhibits UCH-L5 by occupying the ubiquitin-docking site on the enzyme through an unique hairpin structure that is absent in the DEUBAD domain of activator RPN13 [65,66]. While the DEUBAD domain of RPN13 activates UCH-L5 by increasing its affinity for substrates, in INO80G it does the opposite and dramatically decreases the affinity for substrates. Thus, the regulation of UCH-L5 is achieved at the level of substrate affinity, leading to a change in *KM* rather than *k*cat, as observed in USP1 regulation for instance [50]. The dual mode of regulation of UCH-L5 suggests that strict spatial and temporal control should exist to ensure the right activity at the right place. For example, the inhibition of UCH-L5 by INO80G must be alleviated during DNA repair because the catalytic activity of UCH-L5 is required in this pathway [68].

Another example of negative regulation is the inhibition of the yeast endosome-associated DUB Doa4 by Rfu1 (free ubiquitin chains 1). In a yeast genetics screen, deletion of Rfu1 was serendipitously found to alter global ubiquitin levels [69]. DUBs can regulate these levels by liberating conjugated ubiquitin from targets. Changes in global ubiquitin levels are often associated with cellular stress responses [69]. Rfu1 was shown to directly inhibit Doa4 activity through a mechanism that is unknown on the molecular level, suggesting that the Doa4/Rfu1 system contributes to the regulation of global ubiquitin levels [69]. Interestingly, Doa4 can also be activated by the endosome-associated protein Bro1, indicating that, similar to UCH-L5, Doa4 is subject to both positive and negative regulation [70].

A final example of negative regulation is the deubiquitylation of monoubiquitylated PCNA (PCNA-Ub) by the USP1/UAF1 complex. During replication stress, the protein Spartan binds to monoubiquitylated PCNA [71,72]. This binding event was suggested to protect PCNA-Ub from deubiquitylation by the USP1/UAF1 complex to drive the stress response that is dependent on the ubiquitin signal [71]. This is different from UCH-L5/INO80G, where INO80G directly prevents substrate docking onto the DUB; instead, Spartan blocks the DUB-binding site on the substrate, protecting it from deubiquitylation.

Thus, although less common than the stimulation of DUB activity, inhibition of DUBs by external proteins constitutes another type of DUB regulation. However, activity can also be regulated by direct covalent modification of DUBs through PTMs.

Post-translational modifications

PTMs, such as sumoylation, ubiquitylation, and phosphorylation, are a convenient way for cells to further fine-tune DUB activity. An example is DUBA, an OTU class enzyme that has an important role in the immune system. DUBA is only active when it is phosphorylated at Ser177 (pSer177) [73] and crystal structures demonstrated that phosphorylation refolds part of the protein that assists in ubiquitin binding, explaining the importance of the modification

(Figure 1). This is highlighted in antigen-stimulated macrophages, in which pSer177 DUBA levels are increased to regulate the immune response. Activation by phosphorylation also takes place during the cell cycle, where USP37 is modified by CDK-2 to directly stimulate DUB activity [74]. In an analogous manner, USP1 phosphorylation was suggested to be required for complex formation with the activator UAF-1 [75].

Phosphorylation can also negatively affect DUB activity. The DUB OTULIN is recruited to the NF-κB pathway by binding the HOIP PUB domain with its PUB Interacting Motif (PIM) [76,77], thus connecting the E3 ligase (HOIP in the LUBAC complex) and the DUB for linear ubiquitin chains. Phosphorylation of Tyr56 within the PIM abrogates this interaction and the ability of OTULIN to antagonize NF-κB signaling.

The activity of the NF-κB-associated DUB CYLD is negatively affected by both phosphorylation and sumoylation [78,79], while sumoylation also impedes USP25 activity. This multidomain DUB contains UBDs that are required for efficient polyubiquitin hydrolysis. Sumoylation at one of these UBDs decreases USP25 chain hydrolysis activity [80].

In addition to sumoylation, ubiquitylation of DUBs has also been reported to regulate activity. UCH-L1 monoubiquitylation at Lys157 of the active site cross-over loop decreased its activity [81]. By contrast, ubiquitylation of the MJD class DUBs ATXN3 and JosD1 stimulates their polyubiquitin chain hydrolysis activities [82,83].

In some cases, PTMs can alter DUB subcellular localization. Ubiquitylation of BAP1 near its nuclear localization sequence negatively regulates its activity by excluding BAP1 from the nucleus, where most of its targets reside [84]. Similarly, phosphorylation of USP4 by AKT also leads to its redistribution from the nucleus to the cytoplasm, where it ultimately reaches the cell membrane to deubiquitylate the TFG-β receptor I [85]. Conversely, during DNA damage, the predominantly cytoplasmic DUB USP10 is translocated to the nucleus after phosphorylation to deubiquitylate p53 [86].

More unusual modifications also regulate DUBs. Recent reports have illustrated how reactive oxygen species (ROS)

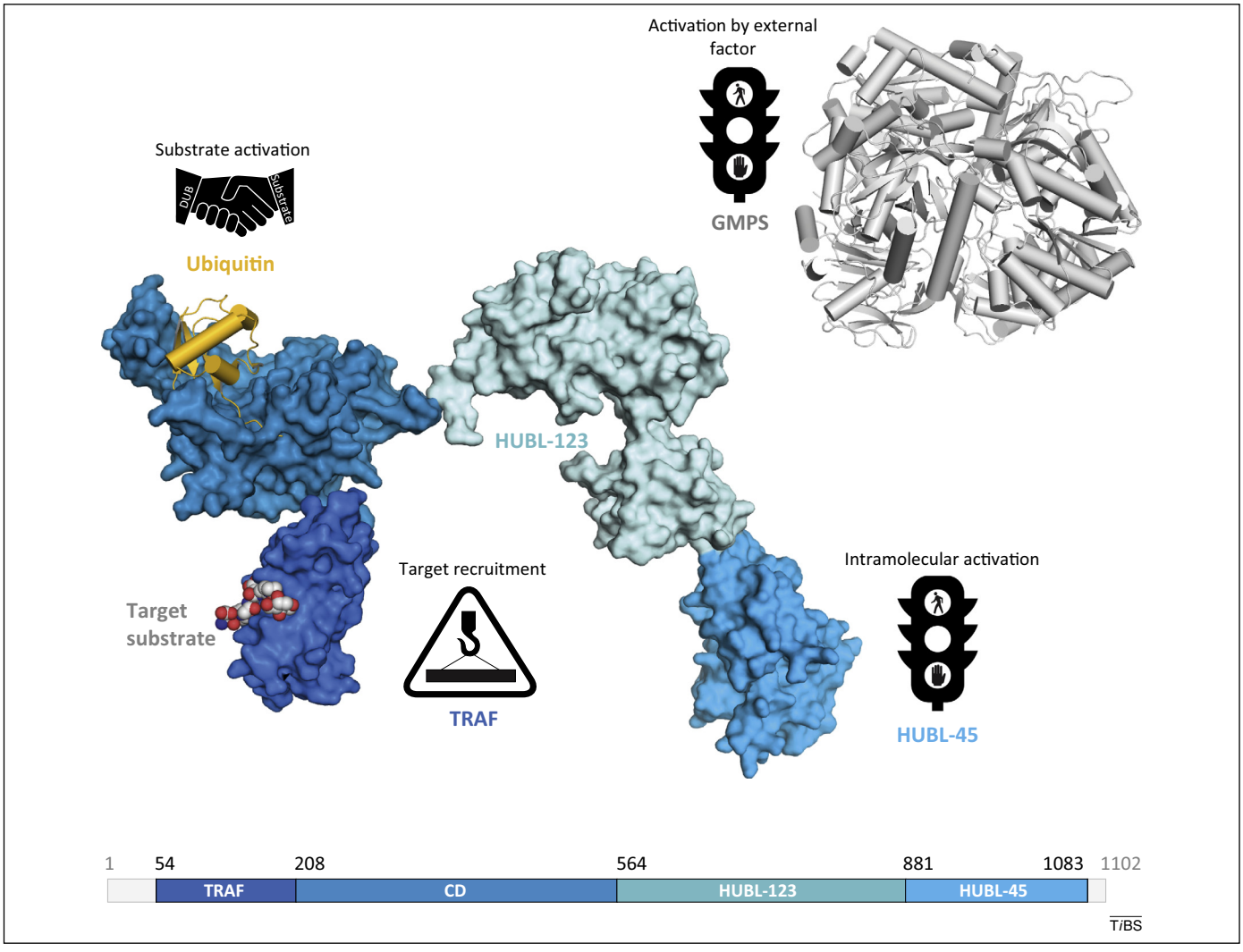

Figure 2. Combining the layers of deubiquitylating enzyme (DUB) regulation. The different layers of DUB regulation can coexist in a single DUB. In the figure, a model of USP7 (blue surfaces) is presented based on existing structures (1nbf, 2ylm, and 1yy6) where ubiquitin (part of the substrate) in yellow itself can activate the catalytic domain of USP7. This activation is reinforced intramolecularly by the HUBL-45 subdomain of USP7 and from outside by GMPS (gray cartoon). The USP7 N-terminal TRAF domain can furthermore recruit targets (sphere representation), such as p53, MDM2, or EBNA-1. This multilayered regulation likely exists for many more DUBs. For definitions of abbreviations, please see the main text.

can regulate DUB activity [87–90]. ROS can serve as potent signaling molecules by reacting with active site cysteines of tyrosine phosphatases and some cysteine peptidases to form reversible sulfenic acid adducts or irreversible sulfinic or sulfonic acid adducts [91]. Oxidation of active site cysteines to sulfenic acid appears to be widespread in the OTU, USP, and UCH classes of DUBs, where it reduces DUB activity. Functionally, the modification may have important roles in cells, as exemplified by the oxidative inactivation of the PCNA deubiquitinase USP1 [89]. This results in the accumulation of monoubiquitinated PCNA, a mark of cellular stress responses [88,89].

In summary, several types of PTM of DUBs have been described that can have a variety of effects on DUBs and thereby further fine-tune DUB activity.

Multiple layers of regulation

In the previous sections, we discussed separately for illustrative purposes how certain types of regulation impinge on DUB activity. In practice, however, many regulatory mechanisms coexist. This situation sometimes even occurs within a single domain. The RPN13 DEUBAD domain, for example, is responsible for both the activation of UCH-L5 and recruiting the enzyme to the proteasome [62–64]. We expect that more factors exist that have multiple regulatory roles that occur simultaneously.

The concept of multiple regulatory layers can best be exemplified by considering USP7 (Figure 2 and Table 1). The USP7 CD can exist in a catalytically incompetent state that can be activated by ubiquitin binding and USP7 HUBL-45 [27,28]. This active state can be further reinforced allosterically by the external factor GMPS, which binds to HUBL-123 [28,47] and target recruitment is promoted by its N-terminal TRAF domain [23,24]. Another example of multiple layers of regulation impinging on a single protein is the tumor suppressor BAP1. This enzyme is activated by ASXL1 to deubiquitylate H2A and can be targeted to certain genomic loci by its association with transcriptional regulators [20,61,92]. BAP1 can furthermore be spatially separated from its targets by ubiquitylation of its C-terminal nuclear localization signal (NLS) by UBE2O, causing mislocalization to the cytoplasm [84]. This type of multilayered regulation is likely a feature of many DUBs and multiprotein DUB complexes, and contributes to the tight control of deubiquitylation.

Concluding remarks

Research on the mechanisms of DUB regulation has advanced significantly over the past few years. The regulation takes place at different layers and a notable feature is the diversity of the known mechanisms. This variety is also present at the biochemical level: the regulatory mechanisms range from solely impinging on catalytic activity ($k$cat) to primarily substrate interaction (KM), to combinations of both. The accumulated regulatory effects of the layers determine DUB activity and, ultimately, the fate of ubiquitylated substrates.

Even though we understand some aspects of DUB mechanism, there are important outstanding questions (Box 5), such as how DUB activity is regulated within the large macromolecular complexes. For example, recently determined UCH-L5/RPN13 structures give insights into the basic activation mechanisms of UCH-L5 [65,66], but do not explain the polyubiquitin hydrolysis activity of UCH-L5 as part of the proteasome. Similar challenges exist for other DUBs and can only be addressed by studying large holo-enzyme complexes over different activation states.

As more details of DUB regulator systems become available, an important task will be to identify possible common elements in DUB regulation that underlie the apparent diversity. This information may allow a more guided design of potential therapeutic compounds. That many DUBs are allosterically regulated raises the interesting prospect of developing therapeutic agents that target allosteric and exo sites rather than active sites. This type of targeting may allow for greater specificity towards certain functions of DUBs because they may have multiple substrates. A lack of information about DUB substrates poses a major hurdle for targeted therapy and one that future studies will need to address.

Box 5. Outstanding questions

- How is DUB regulation achieved temporally?
- Do DUBs dynamically cycle between different complexes to meet functional requirements or are they stably associated in separate complexes?
- Are DUBs merely catalytic modules or do they also perform scaffolding or adaptor functions in large molecular assemblies?
- How many different targets do DUBs generally have and how specific are they for these targets?
- Is target specificity intrinsic to a certain DUB or is it achieved through formation of multiprotein complexes?

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