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Chemical biology tools to study Deubiquitinases and Ubl proteases

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ABSTRACT

The reversible attachment of ubiquitin (Ub) and ubiquitin like modifiers (Ubls) to proteins are crucial post-translational modifications (PTMs) for many cellular processes. Not only do cells possess hundreds of ligases to mediate substrate specific modification with Ub and Ubls, but they also have a repertoire of more than 100 dedicated enzymes for the specific removal of ubiquitin (Deubiquitinases or DUBs) and Ubl modifications (Ubl-specific proteases or ULPs). Over the past two decades, there has been significant progress in our understanding of how DUBs and ULPs function at a molecular level and many novel DUBs and ULPs, including several new DUB classes, have been identified. Here, the development of chemical tools that can bind and trap active DUBs has played a key role. Since the introduction of the first activity-based probe for DUBs in 1986, several innovations have led to the development of more sophisticated tools to study DUBs and ULPs. In this review we discuss how chemical biology has led to the development of activity-based probes and substrates that have been invaluable to the study of DUBs and ULPs. We summarise our currently available toolbox, highlight the main achievements and give an outlook of how these tools may be applied to gain a better understanding of the regulatory mechanisms of DUBs and ULPs.

1. Introduction

Ubiquitin (Ub) and Ubiquitin-like modifiers (Ubl) are small proteins that despite the lack of sequence similarity share a common β-grasp fold [27]. Post-translational modifications (PTMs) of cellular components with Ub and Ubl have important regulatory functions and have therefore been the focus of intense research over the years. Ub and many of the Ubls are expressed as precursors that cannot be attached to substrates. They must first undergo maturation, which involves cleavage by deubiquitinases (DUBs) or Ubl-specific proteases (ULPs) to produce monomeric Ub or Ubl, respectively, with the correct C-terminal residue for activation and conjugation onto substrates [119]. In addition to Ub/Ubl maturation, DUBs and ULPs also play central roles in regulating these PTMs by removing or editing the modification. They use distinct mechanisms to exert their function and play important regulatory roles in our cells. Over the past two decades, enormous progress has been made in the discovery, mechanistic and functional characterization of these proteases, advances which were aided by the availability of powerful tools such as activity-based probes and substrates. Importantly, the toolbox to investigate these proteases was revolutionized by the application of chemical biology approaches. Here, we provide an overview of the various chemical biology tools available to researchers to study Ub/Ubl proteases and discuss recent advances in this field. With this review, we pay tribute to our colleague Huib Ovaa who led the way with the design of innovative tools, development of synthetic and semi-synthetic methods for Ub and Ubl conjugate synthesis and novel chemistries, which all contributed to significant advances to this field and continue to be invaluable tools.

2. Ubiquitylation

Ub, a well-studied modifier in eukaryotes, is a highly conserved small globular protein of 76 amino acids that consists of a β-grasp fold and a flexible C-terminal tail with a diglycine motif. It can be covalently linked to target proteins through an isopeptide bond between its C-terminus and, in most cases, a lysine residue or through a peptide bond between its C-terminus and the N-terminus of a target protein. More recently, ubiquitylation of Ser and Thr residues and even non-proteinaceous substrates have been described, thus vastly expanding the repertoire of this PTM [19,76,110]. Conjugating Ub to a substrate is a multistep reaction typically [47] catalysed by three types of enzymes: E1 – activating, E2 – conjugating and E3 – ligating enzymes [130] (Fig. 1 **A**). In

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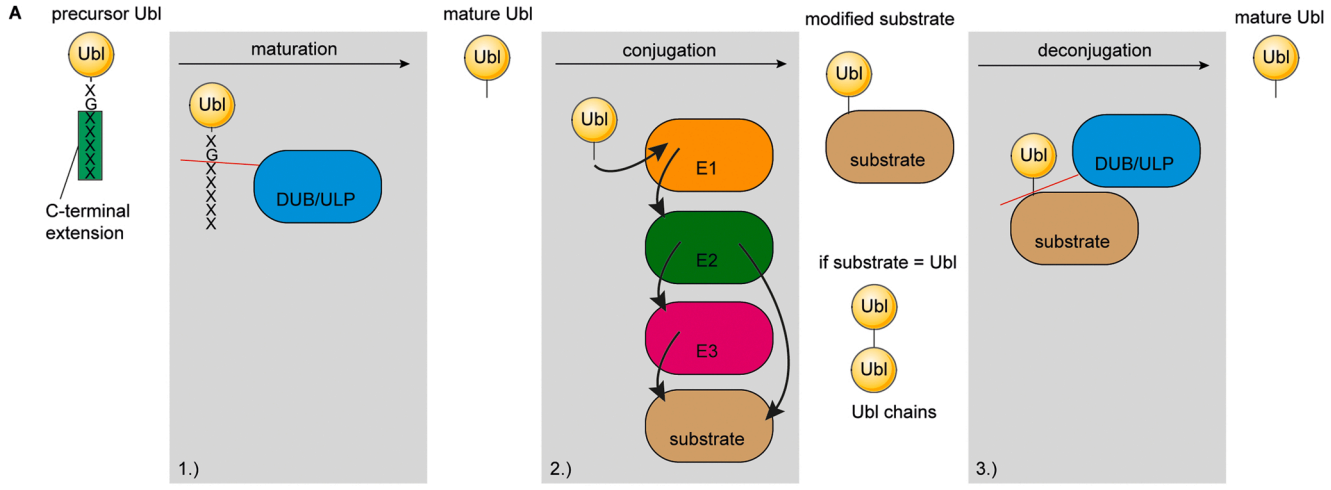
human cells nearly 40 E2s and hundreds of E3s have been identified so far. This leads to a large number of E2-E3 pairs that enables the specific modification of thousands of proteins in cells, which occurs in many flavours: substrates can be modified with a single Ub molecule on a single residue (monoUb), or on multiple residues (multi-monoUb), or by the attachment of further Ub molecules onto monoUb via one of its seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) or the N-terminal methionine (M1) to form polyUb chains (poly-ubiquitylation). These chains can be homogenous (homotypic) with only one linkage type, or have multiple linkages within the same chain in mixed or branched topologies (heterotypic) [79]. Ub can further be modified by phosphorylation and acetylation on Ser, Thr or Tyr providing another layer of regulation and adding to the complexity of the Ub system [134]. The type of Ub modification dictates the type of response produced. For example, attachment of K48-linked Ub chains targets substrates for proteasomal degradation [47,51], whereas attachment of K63-linked Ub chains to specific proteins can activate innate immune signalling and DNA damage response pathways [30, 152]. As a result of the diverse modifications that can be formed, ubiquitylation regulates various processes such as DNA damage response, cellular signalling, membrane trafficking and protein degradation. Defects in these Ub-dependent processes can lead to various pathologies such as neurodegeneration, cancer, and inflammation [21, 31].

Ub is expressed in a precursor form, where multiple copies of Ub are linked together and require cleavage by DUBs before entering the ubiquitylation cascade (Fig. 1**A**). In addition, DUBs are required to reverse Ub modifications, e.g. to recycle Ub when the substrate has engaged with the proteasome. In humans there are approximately 100 different DUBs, which can be broadly classified into seven structurally distinct super-families [87]. Six of these families are cysteine-based enzymes: the Ub C-terminal hydrolases (UCH), Ub-specific proteases (USP), Machado-Josephin domain proteases (MJD), ovarian tumour proteases (OTU), Motif Interacting with Ub-containing novel DUB family (MINDY) and zinc-finger containing Ub peptidase (ZUP1), while the Jab1/Mov34/MPN+ protease (JAMM) family DUBs are zinc-binding



B

Ubl	precursor form	Ubl chains	known DUBs/ULPs	unique features
Ub	fused to ribosomal proteins, linear polyUb chains	yes, different linkages including branched chains	approx. 100	
SUMO	with C-terminal extension	yes, also mixed chains including different paralogues	9	5 paralogues (SUMO1-5)
NEDD8	with C-terminal extension	yes	3	highest sequence similarity with Ub of all Ubls, C-terminal tail almost identical to Ub
ISG15	with C-terminal extension	no (mixed chain with Ub only)	1	diUbl composed of two distinct Ubls, C-terminal tail almost identical to Ub
FAT10	expressed in mature form	no	0	diUbl composed of two distinct Ubls
ATG8	with C-terminal extension	no	1	
ATG12	expressed in mature form	no	1	7 paralogues (3 GABARAPs, 4 LC3s)
UFM1	with C-terminal extension	yes	2	
FUBI	fused to RPS30	no	1	
URM1	expressed in mature form	no	0	no deurmylation events detected so far

Fig. 1. Generalized mechanism of Ubl (de)conjugation. A. 1.) A precursor Ubl is first matured by a DUB/ULP to expose the C-terminal Gly. 2.) The mature Ubl is conjugated to a substrate via a cascade involving an E1 (Ubl activating enzyme), E2 (Ubl conjugating enzyme), and in most cases E3 (Ubl ligating enzyme). The substrate can also be a Ubl molecule, resulting in the formation of di- and polyUbl chains. 3.) Ubls can be removed from substrates by DUBs/ULPs, and the cleaved Ubl can undergo another conjugation reaction. B. List of known eukaryotic Ubls including information about the precursor form, their ability to form chains, the number of identified proteases and notable features.

metalloproteases. DUBs exhibit distinct but overlapping cleavage preferences towards chains of different architecture. For example, OTULIN specializes in cleaving linear chains, AMSH and ZUP1 in K63 chains, while DUBs from MINDY family in K48-linked chains (reviewed in [85]. The activity of DUBs is regulated by multiple mechanisms that ensure activity only at the precise time and location within a cell. In addition to showing preference for the type of linkage cleaved, DUBs can also display specificity for the position in the Ub chain (exo, endo, or base cleavage) where it cleaves. Such linkage selectivity and mode of cleavage is achieved by using additional Ub-binding sites, which can recognize Ub on the distal or proximal side [83].

3. Ubiquitin-like modifications

Besides Ub, there are several ubiquitin-like modifiers (Ubls) that share a similar β-grasp fold and the ability to be conjugated to substrates via their flexible C-termini. Whereas only a few prokaryotic conjugatable Ubls (PUP, SAMP1, SAMP2, SAMP3, TtuB) are known [93], a total of 18 Ubls (5 SUMO paralogues, NEDD8, ISG15, 7 ATG8 paralogues, ATG12, FAT10, UFM1, URM1) have been identified in human cells [27]. The majority of these eukaryotic Ubls follow a similar conjugation mechanism as ubiquitylation involving an enzymatic cascade comprised of E1, E2 and E3 enzymes [17]. While most Ubls have only been observed as mono-modifications, SUMO, UFM1 and NEDD8 have been shown to form chains [136,149,6]. The complexity of SUMOylation is further enhanced by the existence of five paralogues in human cells (SUMO1–5), which share high sequence identity but show significant functional differences. Like Ub chains, SUMO chains of various linkage types have been observed across different paralogues, with K11-linked diSUMO2/3 being the most abundant chain type [65]. The complexity of Ubl modifications is further increased by hybrid Ubl-Ub(l) chains [113], which have been detected for SUMO-Ub [55,137], ISG15-Ub [37], NEDD8-Ub [84] and NEDD8-SUMO [88].

Following similar mechanisms, Ubls can be removed from their target substrates by proteases collectively called Ubl-specific proteases (ULPs). Although some DUBs are, at least in vitro, capable of reversing NEDD8- and ISG15-modifications, Ubls possess distinct sets of ULPs. Besides Ubl deconjugation from modified substrates, these proteases are also responsible for the maturation of Ubls prior to E1-mediated activation since many Ubls are expressed in a precursor form with a C-terminal extension (Fig. 1**B**). The exact role of ULPs differs depending on the characteristics of the corresponding Ubl pathway. For instance, some Ubls (URM1, ATG12, FAT10) are expressed in a mature form, and hence do not require a maturing protease. For FAT10, no protease has been identified to date that can recognize and remove FAT10 from substrates. FAT10, when covalently attached to substrates in an E1-E2-E3-dependent manner, targets the modified protein for proteasomal degradation but unlike Ub, FAT10 is thought to be degraded together with the substrate [118]. This raises the question of whether the modification is irreversible and thus a protease-independent Ubl.

Considering the important roles that DUBs and Ubl proteases play in regulating cellular processes, there is a need for specialized tools to study their activity, characterise their interactome and substrate specificity and potentially identify new (classes of) proteases. In this review, we will discuss currently available approaches to study DUBs and their Ubl-specific equivalents with an emphasis on activity-based probes and substrates, which were made possible by using chemical biology approaches.

4. Model substrates

The use of suitable substrates such as Ub/Ubl chains, modified peptides and proteins is essential to assess DUB/ULP activity and can provide both qualitative and quantitative information on enzyme activity. For DUBs, the use of Ub chains of all 8 different linkage types can provide information on the linkage selectivity of the enzyme and identify the appropriate substrate for in vitro biochemical and biophysical assays. However, as recent as a decade ago, no methods were available to enzymatically make the majority of these linkage types, limiting analyses to K48- and K63-linked chains, the only two chain types that could be enzymatically assembled in large quantities. Chemical biology efforts from several labs using different strategies made diUb chains of other linkage types available for biochemical and structural studies [49,60]. This revealed linkage preferences of many DUBs for the first time and enabled detailed characterization [36,99]. More recently, mixed and branched Ub/Ubl chains have been made using non-enzymatic strategies, which expands the toolbox available to probe DUB/ULP activity [20,28,32,113,135]. Fluorescent substrates come in handy to monitor DUB and ULP activity, kinetics and substrate preference. These generally consist of a recognition sequence, usually a natural substrate (one or more Ub or Ubl moieties), and a reporter fluorophore that emits a quantifiable signal [117]. Upon binding to an active DUB, the fluorogenic group is cleaved off so that a fluorescent signal can be detected, and the turnover of a substrate can be measured [24,61]. Alternatively, a substrate can be flanked by a fluorophore on one side and a quencher on the other side where cleavage events releasing the quencher would lead to increase in fluorescence signal, thereby reporting on DUB/ULP activity [14]. Such fluorescence-based detection can be easily adapted for multi plate readout format and high-throughput screening, and measurements in cells and non-invasive measurements in vivo can also be performed. In contrast to activity-based probes, which covalently bind and inhibit DUBs/ULPs (see below), fluorescent substrates are turned over by the proteases leaving the enzymes active and capable to continue processing substrate molecules, leading to signal amplification [121].

Ub/Ubl-AMC (7-amido-4-methylcoumarin) and Ub/Ubl-Rho (rhodamine) are common fluorogenic substrates to monitor enzyme activity. However, as the fluorogenic moiety is not linked to the Ub/Ubl via an (iso)peptide linkage, they don't mimic the physiological modification [117]. The development of monoUb-based substrates such as Ub-Lys-TAMRA, using chemical biology means, overcomes this limitation. Here, the TAMRA (tetramethylrhodamine, sometimes referred to as TMR)-labelled dipeptide (LysGly) is linked to the C-terminus of Ub via an isopeptide bond and this short peptide is released upon cleavage [42]. Of note, a variant, Ub-Thr-TAMRA was recently used as a substrate to screen for DUBs that have esterase activity and led to the identification of some DUBs with preferential esterase activity [25]. This kind of cleavage with an improved substrate can be measured with florescence polarization (FP) or Förster resonance energy transfer (FRET) methods. For instance, a set of diUb FRET pairs of all seven isopeptide-linkages was constructed by native chemical ligation (NCL), which provides an additional method to assess DUB-mediated cleavage of chains and quantify linkage specificity [43]. The assays using fluorescent substrates described here can also be scaled into high-throughput format to screen small molecule libraries to identify selective DUB/ULP inhibitors [140].

Another major limitation in the study of how DUBs/ULPs are targeted to their substrate is the inability to enzymatically generate substrates that are modified at specific residues with Ub/Ubl. Chemical biology approaches have again contributed significantly to this area by synthesizing site-specifically modified substrates for biochemical and structural studies. These include the generation of substrates such as ubiquitylated histones [94,138,132,104] and their use to reveal how DUBs recognize ubiquitylated substrate [105]. More recently, an exciting method was developed to modify substrates by the site-specific attachment of Ub and Ubls in cells providing a powerful way to analyse activity of DUBs/ULPs in living cells [39]. However, one limitation of this approach at present is the introduction of two point mutants at the C-terminus of Ub, R72A and R74T, for sortase recognition and it is unclear how these mutations may impact recognition by DUBs.

5. Activity-based probes

Activity-based probes (ABPs) are chemical tools which are frequently employed to profile the enzymatic activity of related enzymes. ABPs resemble a substrate for an enzyme, but instead of being processed they are coupled to the active site. They are versatile tools that can target enzymes of interest as purified recombinant proteins and in cell lysates. Furthermore, cell-permeable ABPs have been developed to target enzymes within live cells [52,58,91,92] and introducing ABPs into living tissues and whole animals can transform investigation of DUB/ULP activity. ABPs are typically composed of three elements – a reactive group ('warhead'), a recognition element (targeting group), and a reporter tag (label) [67] (Fig. 2**A**). We first list the properties of these 3 elements that are key for the function and specificity of ABPs.

The choice of warhead group installed at the C-terminus of the Ub/Ubl determines the reactivity and selectivity of the ABP. Typically an electrophile, the warhead, reacts covalently with the nucleophilic catalytic Cys of DUBs and ULPs [121]. Mechanistically, the reaction between the catalytic Cys and the electrophilic warhead occurs via direct addition (e.g., Ub-aldehyde (Ubal), Ub-nitrile (Ub-CN)), conjugate addition (e.g., Ub-vinyl methyl sulfone (VS)), or nucleophilic substitution (e.g., Ub-bromoethyl (Ub-Br2), Ub-bromopropyl (Ub-Br3), Ub-chloroethyl (Ub-Cl)) [12]. In addition, photoreactive groups that label proximal residues in enzyme active sites after UV irradiation can also be used as reactive groups [131]. The warhead chemistry together with the binding affinity of the ABP to the DUB/ULP determines target selectivity and efficiency of the ABP.

The first ABPs developed for capturing DUBs were based on aldehyde (Ubal) and nitrile (Ub-CN) chemistries [114]; Lam et al., 1997) that react with the catalytic Cys through 1,2-addition and are potent and specific inhibitors of USPs. A major limitation of these probes stems from the reversibility of the reactions especially in reducing conditions making these first generation probes unsuitable for many experiments. The first ABP that overcame these limitations was created by Hidde Ploegh and colleagues who introduced vinyl methyl sulfone (VS) as a warhead at the C-terminus of Ub that irreversibly modified UCHs and USPs [11]. Following this breakthrough, the Ploegh lab expanded the number of ABPs available by establishing a facile method using intein-based chemical ligation to generate Ub-derived ABPs with seven different C-terminal electrophilic traps [12]. These new probes led to the identification of the ovarian tumor (OTU) domain family of DUBs [12]. Importantly, the simplified chemistry and methods to make ABPs no longer restricted their generation to chemistry labs and drove DUB research widely in labs making it possible to not only identify new DUBs and Ubl proteases but also to investigate their mechanisms in various species including bacteria and viruses [90,71,34,126].

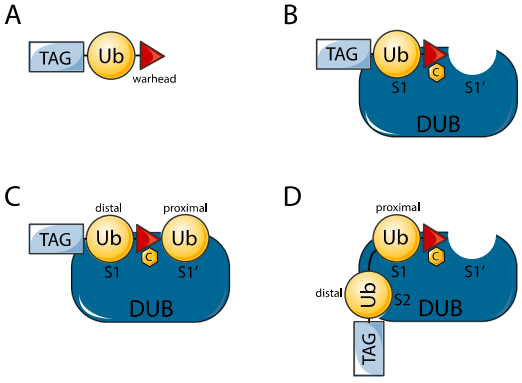


Fig. 2. Design of probes reflecting substrate recognition in DUBs. A. A DUB ABP contains a reporter tag (TAG), which is usually located at its N- terminus, a Ub molecule as a recognition element and a reactive group (warhead). B. MonoUb probe with a C-terminal warhead positioned at the catalytic Cys (labelled as C). C. DiUb probe capturing S1-S1′ interaction. D. DiUb probe capturing a DUB targeting S1-S2 interactions.

In 2013, Huib Ovaa and colleagues made the serendipitous discovery that the chemically inert alkyne moiety, propargylamine (Prg, sometimes also referred to as PA) is highly reactive towards the catalytic Cys of DUBs but not towards unrelated Cys-proteases when present at the C-terminus of Ub (Ub-Prg) [33]. The exact mechanism was unclear and was thought to occur via a proximity-driven thiol–alkyne addition that involves direct nucleophilic attack of the catalytic cysteine thiol [33, 129]. A detailed analysis of this thiol-alkyne addition reaction was explored recently and identified the alkyne intermediate as the reactive species, but the role of proximity of the catalytic Cys thiol to the alkyne moiety in driving nucleophilic addition is still unclear [103]. Nevertheless, this Prg probe reacts with all classes of thiol DUBs known to date [33,1,81]. These superior properties make this alkyne moiety, propargylamine, the warhead of choice in ABPs for Ub/Ubl proteases [33]. Furthermore, ABPs incorporating Prg can be produced easily and in large scale with intein-based reactions. The warheads mentioned above only target cysteines and are hence only suitable for thiol proteases. A major unmet need in the field is for warheads that can target metalloproteases, which will enable the development of ABPs for this class of DUBs. The recent development of a metalloDUB probe where a Ub molecule was modified with a Zn^{2+} chelating group at its C-terminus may herald the further development of novel and more potent ABPs of metalloDUBs [59].

ABPs are also equipped with a reporter tag (e. g. epitope tag or a fluorescent group; most often at the N-terminus of the probe) to allow for DUB detection, measurement of its activity in cell lysates and enrichment of labelled enzymes. Common epitope tags like HA, 6xHis or biotin are widely used as reporter tags. More recently, tags such as HaloTag have been used to enable capture and mass spectrometric analyses of DUBs from cell lysates [81]. The incorporation of fluorophores such as Cy5, fluorescein or TAMRA allows for direct in-gel detection of ABP-modified proteases, often with increased sensitivity compared to traditional immunoblotting-based approaches [73,95]. The choice of reporter tag determines the readouts that can be employed to follow ABP labelling of DUBs/ULPs. The simplest and most widely used readout is SDS-PAGE followed by staining such as Coomassie or silver staining or using the reporter tag in immunoblotting or in-gel fluorescence scanning where probe labelling introduces an observable shift in the electrophoretic mobility of the ABP-modified DUB/ULP. Mass spectrometry-based approaches provide an alternative method to identify and monitor ABP-modified DUBs and ULPs. When combined with quantitative methods such as tandem mass tag (TMT) labelling, probe labelling can be compared under different conditions such as signalling pathway activation or when cells are treated with small molecule inhibitors to determine the fraction of active DUB/ULP.

The recognition element, also known as targeting group, is the element that confers selectivity towards a particular protease or protease class. It can be a short peptide, full-length protein or a small-molecule inhibitor. For instance, DUB-substrate interactions involve a large surface area of Ub and so ABPs for DUBs often require a full ubiquitin molecule as a recognition element for optimal binding. In contrast, the substrate specificity of SENPs derives from the C-terminal residues of the corresponding SUMO paralogue, allowing recognition elements that are made up of shorter peptide sequences [29]. Indeed, peptide vinyl sulfones corresponding to the C-terminus of Ub and Ubls (NEDD8, SUMO1, FAT10, ATG12) are able to modify and inhibit DUBs and ULPs. Such truncated versions of the recognition element are useful to understand whether enzyme specificity is restricted to a smaller area of the substrate [13,64,63].

A refinement that can be introduced to tune the specificity of ABPs towards only a given DUB is the application of Ub variants (UbVs) as recognition elements. These variants can be identified by screening phage display libraries of UbVs generated by mutating Ub residues known to interact with the binding sites of DUBs [35]. The application of UbVs led to the generation of specific and tight protein-based inhibitors

targeting USP2, USP7, USP8, USP21, OTUB1, JAB1 and STAMBP [151, 50,54]. These tweaked Ub molecules can provide higher affinity and specificity of ABPs for DUBs which are inherently weak binders of their substrates. Based on this strategy, Gjonaj et al. developed tailored ABPs incorporating UbVs into their design to recognise specific DUBs. Of note, the UbVs generated by phage display could not be readily translated into active ABPs and one possible explanation could be that binding mode of the phage display UbVs on the DUB could misalign the C-terminus of the UbV with respect to the active site, thus making the ABP poorly reactive. However, by incorporating additional mutations, Huib Ovaa and colleagues developed ABPs that are selective towards USP7 and USP16 and can be used to determine active levels of these DUBs in cells [45,46]. A similar approach utilizes the incorporation of unnatural amino acids within the C-terminal tail of Ub to engineer highly selective probes (chemistry of these modifications are described in [120,138,142].

As mentioned above, post-translational modifications of Ub expand the signalling capability of ubiquitylation. Of note, diUb chains of different linkage types containing phosphorylated Ub (phosphoUb) have been generated using chemical biology approaches. Interestingly, DUBs exhibit altered activity towards polyUb containing phosphoUb [10,144, 70]. Hence, phosphorylated variants of Ub have also been implemented into ABPs and could be utilised to probe for DUBs selective for this modification [92].

While monoUb/Ubl probes have been widely used, a refinement of this strategy was the development of more sophisticated probes, which bind to different substrate binding sites on a DUB/ULP (Fig. 2). This advance was made possible by introducing an internally positioned electrophile between the two Ub moieties of a diUb chain that binds to the S1 and S1′ sites of a DUB, leading to covalent modification of the catalytic Cys. Such probes are very useful as they can uncover S1-S1′ preferences and linkage specificity of DUBs [96] (Fig. 2**C**). Further, non-hydrolyzable diUb ABPs with a C-terminal warhead have been developed that can reveal the contribution of the S2 site to linkage specificity [38] (Fig. 2**D**). Hence, design or choice of the recognition element and placement of the warhead can provide tailored ABPs that target DUBs and ULPs in specific ways.

6. Applications of ABPs to study DUBs

In the following section we discuss the various applications that ABPs have been used for to reveal important insights into the mechanisms and biology of DUBs (illustrated in Fig. 3).

6.1. *Discovery tool*

Ubiquitin ABPs have been significant for the identification of new DUBs [12] and new DUB families as illustrated by the discovery of several OTUs [7] and the ZUP1 class of DUBs [68,81]. The discovery of new DUBs was facilitated by activity-based protein profiling (ABPP), the combination of ABPs with proteomic methods, which allows for a broad study of the interactions of mechanistically related classes of enzymes with the wide range of ABPs [23]. Since the discovery of ZUP1 was made possible by the development of new alkyne warheads that had not been used in ABPs before, we suggest that developing ABPs for metalloenzymes has the potential to uncover hitherto unknown metalloDUBs.

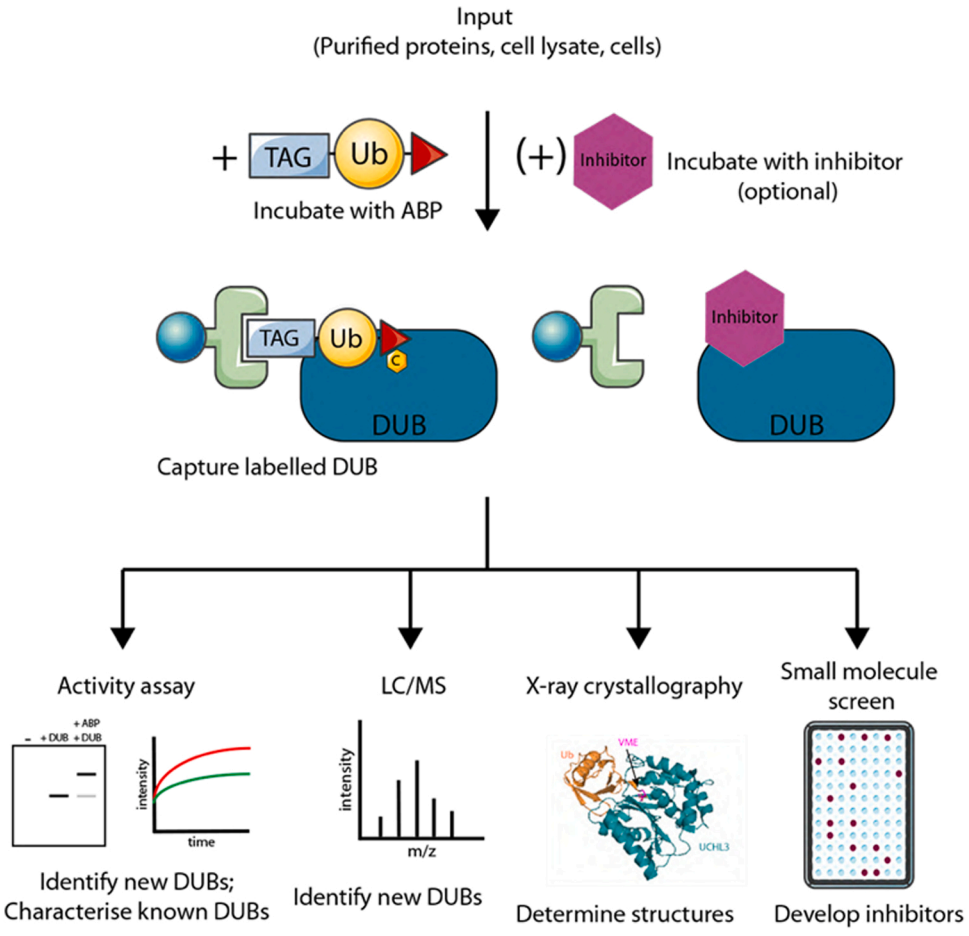


Fig. 3. Pipeline of ABP applications. This schematic depicts the various applications of ABPs to study DUBs.

6.2. ABPs reveal DUB mechanisms

DUB-substrate interactions are dynamic and hence not suitable for structural studies. Here, ABPs can be used to covalently trap the DUB in distinct states in their catalytic cycle, namely DUB-substrate state, and DUB-product intermediate state. These ABPs have been invaluable tools to capture DUB complexes for structural studies and have provided important mechanistic insights over the years (Table 1). Of note, for application in crystallographic studies there is no need for a reporter tag on the probe as its presence could prevent crystallization and a bulky tag could interfere with substrate conformation.

Ub-ABP:DUB complex structures have been used to establish how Ub is recognized by the DUB at the S1 site and to ascertain the active site residues. For instance, structures of such complexes revealed these key details for newly discovered DUBs such as MINDY and ZUP1 [1,66,81]. Schubert et al. confirmed several bacterial proteases to be OTU DUBs by resolving their crystal structure in complex with a Ub-C2Br probe [123].

Table 1
Overview of crystal structures for DUBs and Ubl peptidases with ABPs including PDB entry and year of publication.

Warhead	Peptidase	Ub (l)	Other proteins	PDB link	Year
Ubal	Yuh1	Ub		1CMX	1999
	USP7	Ub		1NBF	2002
	USP14	Ub		2AYO	2005
	SAGA	Ub		3MHS	2010
	USP21	M1-Ub2		2Y5B	2011
	OTUB5	Ub		3TMP	2012
	OTUB1	Ub		4DHZ	2012
	OTUB1	Ub	Ubc13-Ub	4DHJ	2012
	OTUB1	Ub	Ubch5B-Ub	4LDT	2013
	USP37	Ub		4I6N	2013
	SARSPlPro	Ub		4MM3	2014
	Ubp6	Ub	26 S proteasome	5A5B[EM]	2015
VME	UCHL3	Ub		1XD3	2005
	M48USP	Ub		2J7Q	2007
	UCHL1	Ub		3KW5	2010
	UCHL3	Ub		2WDT	2010
	SdeA	Ub		5CRA	2015
	USP46	Ub		5L8H	2016
VS	vOTU	Ub		3PHW	2011
	vOTU	ISG15c		3PHX	2011
PA/PRG	vOTU	Ub		3ZNH	2013
	UCHL5	Ub	Rpn13	4UEL	2015
	UCHL5	Ub	Ino80G	4UF6	2015
	SARSPlPro	K48-Ub2		5E6J	2016
	ERVV vOTU	ISG15c		5JZE	2016
	A20 OTU	Ub		5LRX	2016
	USP18	ISG15		5CHV	2017
	USP30	Ub		5OHK, 5OHN	2017
	SARSPlPro	ISG15c		5TL6, 5TL7	2017
	MERSPlPro	ISG15c		5W8U	2017
	LbPro	ISG15c		6FFA	2018
	Lem27	Ub		7BU0	2020
	PlPro	ISG15c		6XA9	2020
	PlPro	Ub		6XAA	2020
	Dub2	Ub		6OAM	2020
	MERSPlPro	ISG15		6BI8	2020
	PedvPlPro	Ub		7MC9	2021
Br2	OTUB2	Ub		4FJV	2015
	Cezanne	K11-Ub2		5LRV	2016
	Cezanne	Ub		5LRW	2016
Br3	vOTU	Ub		3PRP	2011
	CCHFV-OTU	ISG15		3PSE	2011
	CCHFV-OTU	Ub		3PT2	2011
	vOTU	Ub		4HXD	2013
Dha	OTULIN	M1-Ub2		5OE7	2017
C2Br	wMelOTU	Ub		6W9R	2020

The crystal structure of one of the most extensively studied DUBs, USP7 was determined in complex with Ubal [69], which revealed not only how Ub is recognized by the DUB but also conformational changes in the active site upon Ubal binding. The crystal structure of a yeast member of UCH family in complex with Ubal indicated that upon binding with Ub the active-site cleft of Yuh1 is rearranged and becomes open allowing for Ub to be processed [72]. This study provided key insights into the substrate specificity of UCH family DUBs. Further studies on the UCH family DUB, UCHL3, used the Ub-VME probe and suggested alternative conformations of the active site allowing to accommodate and hydrolyse larger substrates [101].

6.3. Studying chain-specific DUBs

MonoUb ABPs on its own are not sufficient to fully understand how DUBs achieve linkage specificity or substrate specificity. To get a more complete picture of the recognition mechanism adapted by DUBs, longer, diUb probes are needed. They reflect a substrate context better than monoUb probes. A major advance was made when ABPs mimicking the native Ub isopeptide linkages of all seven lysine-linked chain topologies (i.e., K6, K11, K27, K29, K33, K48, and K63) were generated with the use of several chemical ligation strategies [86,106]. These diUb ABPs capture DUBs in a linkage-specific manner as binding of both the distal and proximal Ub (in the S1 and S1′ binding sites, respectively) of the DUB occurs only with the appropriate linkage type. Of note, this is a prerequisite to position the reactive warhead, which is located in the linkage between the Ub molecules, close to the catalytic Cys for covalent modification. Such probes can be used to profile linkage specificity of DUBs from cell lysates and reveal mechanistic insights [96,106]. The availability of such a probe was key to trapping an enzyme-substrate complex, which shed light on the mechanism driving linkage specificity in the K11-specifc DUB, Cezanne [100]. Fluorescent versions of diUb ABPs can be used to visualize labelled DUBs with in-gel fluorescence scanning [106]. Similar activity-based di- and triUb probes of different linkages were developed to interrogate Ub chain binding, linkage preference and endo vs exo cleavage modes in USP DUBs [112].

The OTU DUB OTUD2 is more active towards longer chains and shows an additional requirement for Ub binding at the S2 site. Hence, the Ovaa lab developed a non-hydrolyzable diUb probe that binds at the S1 and S2 sites and is equipped with a warhead at its C-terminus [38]. This designer ABP helped to trap OTUD2:diUb complexes and the crystal structure revealed the mechanism that targets OTUD2 to longer Ub chains [38,99]. Such probes have also helped to reveal the mechanism of the papain protease encoded by SARS-COV and MERS viruses [9].

OTULIN is a unique DUB as it is highly specific for Met1-linked Ub chains. However, the diUb ABP based on Met1-linked diUb did not react with OTULIN suggesting that the design of a warhead that mimics the G76-M1 linkage of Met1-linked Ub chains requires alternative chemistries [96]. A modified linear Ub_{G76Dha}-Ub_{G76} ABP was developed, which incorporated an electrophilic dehydroalanine (Dha) warhead between the two Ub moieties [145]. This probe successfully labelled OTULIN and was highly specific, leading to the application of this probe for structural studies and monitoring OTULIN activity and interactions in cells.

While Ub-based ABPs specifically modify the catalytic Cys, additional non-catalytic cysteines have been observed to be modified in rare instances. This was first observed when two different Ub probes were incubated simultaneously with OTU1 [143], or when diUb probes were incubated with USP9X [112]. Interestingly, in both cases this reactivity was driven by the presence of additional Ub binding sites on the DUB. These reactive non-catalytic Cys can be exploited for the development of specific covalent inhibitors that inhibit activity by blocking Ub binding.

6.4. ABPs assays to screen DUB inhibitor selectivity

ABPs can be used to profile the selectivity and potency of inhibitors

for DUBs and ULPs. The underlying rationale of this approach is that blocking an active site of a DUB with an inhibitor prevents it from being labelled by the ABP. In such assays, cells are first pre-treated with candidate small molecule DUB inhibitors, followed by incubation of cell lysates with an ABP such as HA-Ub-Br2 or HA-Ub-VME and subsequent anti-HA immunoprecipitation. The active DUBs captured this way can be analysed quantitatively using immunoblotting and mass spectrometry approaches to establish what fraction of the target DUB was inhibited. Further, these activity-based chemical proteomics experiments also reveal off-target effects of the candidate inhibitor, and such approaches have been used to establish the broad-range effect of PR-619 and the selectivity of USP7 inhibitors [2,75,82,115,139]. Indeed, validation of candidate inhibitors using quantitative activity-based protein profiling with mass spectrometry are routinely being incorporated into pipelines to identify and stringently validate specificity of DUB inhibitors [140].

6.5. ABPs for ULPs

Since the functional concepts of ubiquitylation and modification with other Ubls show a lot of similarities, the design principles of the chemical tools to study DUBs can also be applied to Ubl specific proteases. While for some Ubls, numerous proteases have been identified (SUMO, NEDD8), others seem to be regulated by a single protease (ISG15) and yet others exist for which deconjugating enzymes have not been identified to date (URM1, FAT10). Hence, the purpose of chemical tools for each Ubl can vary greatly from their application to determine precise enzyme kinetics to the identification of the first protease for a given Ubl.

6.5.1. SUMO

To date, there are nine known deSUMOylases (SENP1, SENP2, SENP3, SENP5, SENP6, SENP7, DeSI-1, DeSI-2 and USPL1) that are responsible for the maturation of precursor-SUMO and/or deSUMOylation from substrates [80]. Impaired SUMOylation has been associated with tumor development, making SUMO and regulators of SUMOylation an attractive target for small-molecule drug design [15]. Here, SUMO isopeptidases have been of interest since low SUMOylation levels can also be a consequence of hyperactive deSUMOylation activity. Hence probing the activity of deSUMOylases using ABPs is of interest. In 2004, Hemelaar et al. developed the first series of Ubl-based ABPs including a SUMO1-based ABP that was equipped with a VS-warhead. This probe was able to modify the catalytic Cys of SENP2, demonstrating that ABPs were also suitable for ULPs [64,63]. SUMO1-VS and SUMO2-VS probes were subsequently used to shed light on the specificity and preference of the SENP family members for the different SUMO paralogues [78]. Analyses using SUMO ABPs and fluorescent SUMO substrates revealed that SENP5, 6 and 7 have a preference for SUMO2 whereas SENP1, 2, and 3 reacted similarly with both SUMO1 and SUMO2. To screen and identify novel SUMO proteases, the Melchior lab used HA-SUMO-VME as a probe in HeLa cell lysates using pull-downs followed by immunoprecipitation and mass spectrometry analysis. Using serial capture with SUMO1-VME and SUMO3-VME probes from large scale cell cultures led to the identification of an entirely new SUMO-protease, USPL1 [124]. As discovered for Ub by the Ovaa lab [33], Sommer et al. identified that the thiol-resistant propargylamine warhead appended at the C-terminus of SUMO was able to covalently modify SENP1 [129]. The subsequent development of a protocol for the linear synthesis of SUMO enabled the chemical generation of SUMO1, SUMO2, SUMO3 and K11-diSUMO2, which simplifies the synthesis of probes with different warheads [107]. Like Ub-based probes, SUMO-based probes have also been designed using a C-terminal peptide of SUMO only [13,29].

6.5.2. NEDD8

With a sequence similarity of 80%, NEDD8 is the closest relative of Ub within the Ubl family. Full-length NEDD8 possesses a C-terminal extension of five residues following the double glycine motif, which is removed by the denNEDDylase NEDP1 to generate mature NEDD8 with an exposed diglycine motif. NEDP1 also removes NEDD8 from modified substrates [97]. In addition to sharing an almost indistinguishable three-dimensional structure with Ub, there is remarkable sequence conservation of the C-terminal tail between NEDD8 (LHLVL**A**LRGG) and Ub (LHLVL**R**LRGG) leading to cross-reactivity of the proteases. Importantly, this single residue difference drives specificity of NEDP1 for NEDD8 and recognition of Ub R72 is a factor which determines specificity of several DUBs for Ub [72,125,148]. In addition to NEDP1, the COP9 signalosome (CSN) is a deNEDDylase that primarily removes NEDD8 from cullin-RING ligases (CRLs) [22].

In 2003, the first NEDD8-based ABP (NEDD8-VS) was generated, which when incubated with mouse fibroblast lysates led to the identification of NEDP1 (also known as DEN1 and SENP8) as a specific NEDD8-maturating protease [40]. The same probe also binds and reacts with the DUBs UCHL1 and UCHL3 demonstrating the cross-reactivity of DUBs for NEDD8 [64,63]. When the co-crystal structure of USP21 with its substrate, M1-diUb, was determined with the help of a diUb-aldehyde probe, NEDD8-C2Cl probes were used as a structure validation tool by mutating key residues in NEDD8 to the corresponding residues in Ub (A72R and K4F, E12T, E14T, A72R) to demonstrate USP21′s selectivity for Ub over NEDD8 [148]. A similar strategy was used to validate crystal structures of bacterial proteases SseL and XopD in complex with Ub. Here, Ub's R72 was a key recognition site for the bacterial proteases and NEDD8 A72R- and Ub R72A-PA probes could direct their selectivity towards NEDD8 [116]. NEDD8-based probes have been used to test the selectivity of a number of DUBs in pathogens [4,5,102,74] and viruses [41,44].

6.5.3. ISG15

ISG15 is a diUbl containing a tandem repeat of Ubl folds. It is expressed in a precursor form and requires maturation by proteases to expose its C-terminal double-glycine motif, which is required for subsequent conjugation to the lysine residue of its target. Like NEDD8, ISG15 shows remarkable sequence conservation with the C-terminal tail of Ub (the last six residues are identical), potentially allowing a high degree of cross-reactivity between Ub- and ISG15- deconjugation. Despite this similarity, USP18 is considered to be the main peptidase for ISG15 and is responsible for both ISG15 maturation and deconjugation from modified substrates. The first ISG15 ABP, HA-ISG15-VS, was used to screen for ISG15-processing proteases in EL-4 cell lysates and identified USP5 as a new deISGylase by immunoprecipitation and subsequent mass spectrometry analysis [64,63]. The same probe was used in a subsequent study where a panel of 22 DUBs was screened for cross-reactivity for ISG15 and showed that USP2, USP13 and USP14 also possessed deISGylase activity [18]. In a more extended, microarray-based screen ISG15-VME was used as a probe for 35 deconjugating enzymes (DUBs, deSUMOylases and deISGylases) and unveiled deISGylating activity of the DUBs USP28 and, to a lesser extent, USP5 and USP51 [89]. ISG15-probes have been used to explore the recognition site of ISG15 for USP18. Intriguingly, both full-length ISG15-Prg, containing both Ubls, and a probe containing only the C-terminal Ubl reacted with USP18 suggesting that the C-terminal UBL alone is sufficient for ISG15 binding [8]. Using ISG15 ABPs, crystal structures of USP18:ISG15 complexes were determined which confirmed the biochemical observations that the C-terminal UBL is the main recognition element. ISG15-based ABP have also played an important role to characterise viral proteases. In particular, propargylated ISG15 was used to measure activity of the Ub/ISG15 cross-reactive SARS-CoV [9] and MERS-CoV [44] PLPro. More recently, it also helped to understand the preference of SARS-CoV2 PLPro for ISG15 over Ub [77,127]. Future studies with ISG15-based probes will benefit from the fact that full-length ISG15 can now be chemically synthesised, which allows the incorporation of unnatural amino acids and simplifies the conjugation of various warheads and reporter tags [147].

6.6. ATG8 and 12

In human cells there are at least 7 ATG8 paralogues (GABARAP, GABARAPL1, GABARAPL2, LC3A, LC3B1, LC3B2, LC3C) and a single ATG12 paralogue [27]. ATG12 is expressed in a mature form and can directly be conjugated to target (ATG5) lysines, whereas ATG8 is expressed as a precursor. After protease-mediated maturation it is, in contrast to other Ubls, conjugated to membrane lipids instead of lysine residues. The only known protease for ATG Ubls is ATG4 and has been shown to mature ATG8 and cleave all ATG related family members from their conjugation targets. The first ABP for ATG8/12 has been designed in 2003 when Hemelaar et al. used different ATG8-VS (GABARAP, GABARAPL1, GABARAPL2, LC3B) probes to identify their corresponding proteases in mouse thymoma cell lysate. They trapped a single protease with all four probes, which they identified to be ATG4 by using an HA-tagged probe followed by immunoprecipitation and mass spectrometry analysis [62]. In addition to ABPs, a C-terminal peptide of LC3B with a C-terminal AMC tag was applied to measure the activity of ATG4 by the release of the fluorophore [128,141]. In other approaches, full-length ATG8 was expressed with C-terminal tags which could be used for a readout upon cleavage. Here, LC3 with a phospholipase A2-tag [128] and FRET-LC3B [108] have been used.

6.7. Emerging Ubls

The UBLs UFM1 and FUBI are poorly understood, and recent efforts are beginning to shed light on these enigmatic UBLs. UFM1 is expressed as a precursor that requires protease-mediated maturation to expose a C-terminal glycine before it can be conjugated to a lysine residue of its target [17]. In 2006, the deUFMylases UFSP1 and UFSP2 were identified with a FLAG-UFM1-VME probe, which was incubated with mouse tissue extract followed by immunoprecipitation and mass spectrometry analysis [133]. Although only the presence of UFSP1 was determined using the ABP, the existence of UFSP2 was unveiled by a BLAST search and the protease was able to bind the UFM1 ABP [133]. Intriguingly, humans only express UFSP2 while UFSP1 is annotated as an inactive enzyme. The use of UFM1 probes was also instrumental in understanding the catalytic mechanism of UFSP2 [16,56,57]. The development of UFM1-based probes has been simplified by successful attempts to synthesize UFM1 chemically. While the first published synthesis using a-Ketoacid-Hydroxylamine (KAHA) ligations involved many steps [109], UFM1 was subsequently synthesized in one step by NCL [146]. Akin to ubiquitylation, polyUFM1 chains linked via K69 can be assembled in cells [149]. However, the ability and selectivity of the two UFSPs to cleave polyUFM1 chains is not known since methods to make such chains are lacking. Hence, the development of K69-linked diUFM1 based ABPs incorporating a warhead between the two UFM1 moieties will be useful tools.

The ribosomal protein RPS30 (eS30) is expressed as a fusion protein with the Ubl FUBI (also known as MNSFβ or UBIM) in many species. Research on FUBI and its cellular roles is in its infancy as it was unknown how the RPS30-FUBI fusion was cleaved to release mature FUBI with a C-terminal diglycine motif, which is essential in Ub for conjugation onto substrates and removal by DUBs [27]. Recent work has identified the DUB USP36 to cleave the RPS30-FUBI fusion [26], and the development of chemical biology tools and probes for FUBI will likely accelerate research in this field.

6.8. Ancient Ubls

URM1 is considered to be an ancestral Ubl owing to its two distinct functions: first, it can transfer sulfur from its E1, UBA4, to cytosolic tRNAs, similar to the prokaryotic Ubls, MoaD and ThiF; second, it can, like most eukaryotic Ubls, be conjugated to lysine residues of its substrates, including Ahp1, after E1-mediated activation [48,111]. It shares a similar β-grasp fold with other Ubls and has a C-terminal tail including a diglycine motif. However, no related deURMylating enzymes have been identified so far, raising the question whether URM1 can be deconjugated from its substrates. Although Anjum et al. observed that an ancestral URM1 homolog underwent proteasomal degradation in the archeon *Sulfolobus acidocaldarius* [3], this does not rule out the existence of deURMylating enzymes. An URM1-based ABP (URM1-VME) has been generated and could identify physiological binding partners, in particular the tRNA binding proteins ATPB3 and UPF0432 [122]. Given that the known prokaryotic isopeptidases lack a catalytic cysteine it might not have been a surprise that the ABP was unable to identify deURMylating enzymes. For instance, the pathogen *Mycobacterium tuberculosis* possesses the Ubl Pup, which can be covalently conjugated to substrate lysines and subsequently deconjugated by the depupylase DOP (Burns et al., 2010). However, the isopeptide linkage is established via the sidechain of the C-terminal glutamate residue, in contrast to all other known Ubls where the C-terminal residue is glycine. Nevertheless, a fluorescent ABP could be developed to study Dop, where the AMC warhead conjugation mimics a glutamate-lysine linkage [98]. Interestingly, this probe failed to identify the dual role of the Pup ligase PafA as a second depupylase [150]. A possible explanation could be that PafA requires additional recognition elements provided by the substrate in order to act as a depupylase.

The zinc metallopeptidase HvJAMM1 has been identified as a peptidase for the Ubl SAMP in *Haloferax volcanii* (Hepowit et al., 2012) and its homolog Ttc1133 is involved in the deconjugation of the Ubl Ttub in *Thermus thermophilus* (Shigi, 2012). Our understanding of proteases involved in the regulation of prokaryotic Ubls is still very limited, and the design and application of ABPs to characterise these peptidases can prove informative.

7. Conclusions / challenges

The development and application of ABPs have advanced our understanding of DUBs and ULPs. One limitation of the present approaches is that ABPs have mainly be designed to profile activity of DUBs and ULPs either as purified proteins or in cell extracts. Their feasibility for use in *in cellulo* assays is often hampered by the large recognition element of ABPs, which prevents them from freely entering cells. Being able to monitor the activity of DUBs and ULPs in living cells and tissues will reveal important insights. Attempts to enable labelling of proteases in their physiological environment, which preserves their native regulatory network, include application of pore-forming toxins (Claessen et al., 2013), electroporation (Mulder et al., 2016), cell-penetrating peptides (CPPs) [52] and small-molecule based cell permeable ABPs (Ward et al., 2020; Kooji et al., 2020). Since ABPs with smaller recognition elements like C-terminal Ub peptides lack affinity for DUBs, novel strategies for introducing these bulkier ABPs into cells will transform our understanding of DUBs. Recently, the use of cyclic polyarginine peptides (cR_{10}) led to an increased cellular uptake of full-length Ub-based ABPs [53,91]. Systematic activity-based profiling using ABPs incorporating PTMs such as phosphorylation, methylation and acetylation would complement the set of tools available and shed light on the roles of Ub/Ubl PTMs in modulating recognition by DUBs and ULPs. A key challenge in DUB/ULP research is that the cellular substrates for many still remain unknown and hence the biology and cellular processes regulated by several DUBs and ULPs are still poorly understood. Here, we anticipate the development of chemical biology based tools for facile capture of substrates will accelerate progress in this area.

As outlined in this review, a number of warheads with different electrophilic elements/groups are available to probe thiol DUBs and ULPs. However, none of these warheads is able to react with metalloDUBs and metalloULPs. Although a first metalloDUB-targeting ABP has recently been reported [59], metalloDUB-probe design is still at an early stage and will likely require the development of novel strategies both in the design of the warhead and the recognition element. For instance, affixing metalloprotease inhibitors to the C-terminus of Ub and

Ubls can improve the selectivity and reactivity towards this class of DUBs and ULPs. Such ABPs will also enable the discovery of hitherto unknown metalloenzymes modulating Ub and Ubl deconjugation. We envision that similar approaches where Ser/Thr or Asp/Glu protease inhibitors are appended to Ub/Ubls may also reveal if Ser/Thr and eukaryotic Asp/Glu proteases exist that function on Ub and Ubls. Such studies will significantly expand our understanding of how the Ub and Ubl systems are regulated. Furthermore, the emergence of atypical Ub-modifications of Ser, Thr and non-proteinaceous substrates warrants the development of novel ABPs and tools and we envision chemical biology approaches will play an important role.

Declaration of Competing Interest

None.

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