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Catching a DUB in the act: novel ubiquitin-based active site directed probes

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Protein ubiquitylation is an important regulator of protein function, localization and half-life. It plays a key role in most cellular processes including immune signaling. Deregulation of this process is a major causative factor for many diseases. A major advancement in the identification and characterization of the enzymes that remove ubiquitin, deubiquitylases (DUBs) was made by the development of activity-based probes (ABPs). Recent advances in chemical protein synthesis and ligation methodology has yielded novel reagents for use in ubiquitylation research. We describe recent advances and discuss future directions in reagent development for studying DUBs.

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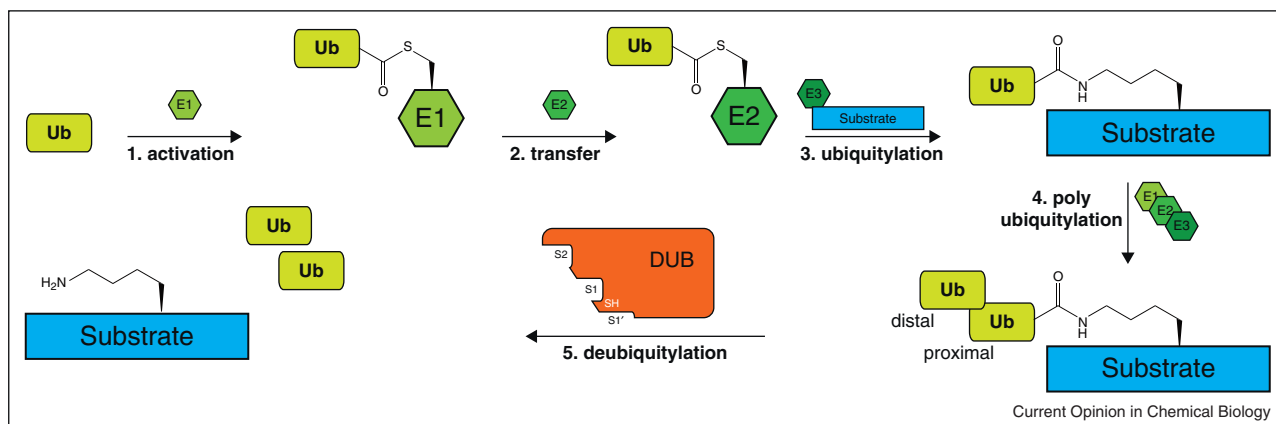
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Introduction

The modification of proteins with the small 76-amino acid protein ubiquitin (Ub) plays a central role in many key cellular processes. Ubiquitylation of a target protein requires the concerted action of a cascade of three enzymes (Figure 1). The ubiquitin-activating enzyme (E1) catalyzes the formation of a reactive thioester bond via its active site cysteine residue and the C-terminal carboxylate of ubiquitin. Ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), which in conjunction with a ubiquitin ligase (E3) confers substrate specificity. In this step the C-terminus of ubiquitin is coupled to the ϵ -amine of a lysine residue in the target protein, forming an isopeptide. Alternatively the C-terminus of ubiquitin is coupled to the N-terminus of ubiquitin resulting in a regular peptide bond, often referred to as linear. The modification of a protein with a single ubiquitin molecule (monoubiquitylation) has been shown to

be required for internalization of cell surface receptors, such as the EGF receptor, as well as for DNA repair and regulation of transcription through modification of histones [1]. Ubiquitin can also be coupled to itself via any of its seven lysine residues or its N-terminus. Multiple self-conjugations lead to the formation of polyubiquitin chains. These chains can either be linked homotypically through one specific residue in each module or heterotypically through a selection of different residues in each module, including multiple residues per chain forming a branched chain. There are at least 37 E2 and >600 E3 enzymes encoded in the human genome. Their specific pairing determines the type of chain linkage that is formed as well as which protein substrate is modified. Some homotypic chains have been well studied. Lys48-linked chains target proteins to the proteasome for degradation while Lys63-linked chains have functions in DNA repair. Lys11, Lys29 and N-terminally linked (linear) ubiquitin chains play roles in cell cycle control and signaling. The function of other linkage types is less clear, especially when it comes to heterotypic chains [1]. Ubiquitin modification is reversed by the action of deubiquitylating enzymes (DUBs), which cleave the isopeptide bond between the C-terminus of ubiquitin and the side chain of a lysine residue in a substrate or another ubiquitin module. DUBs are divided in five classes: USP, UCH, OTU, Josephin and JAMM/MPN+ proteases. Except for the JAMM/MPN+ class, which are Zn^{2+} metallo-proteases, all DUBs known so far are cysteine proteases. The activity of DUBs is tightly controlled. A large group of DUBs is regulated through reversible oxidation of the catalytic residue [2,3], while the activity of OTUD5 is regulated by its phosphorylation status [4]. Approximately 100 DUBs are encoded in the human genome and for many of them specific functions remain to be determined [5,6]. The OTU class of DUBs shows remarkable selectivity for specific ubiquitin chains and is likely involved in chain editing or recycling of specifically linked chains [7**]. The largest class of DUBs, the USP-class, does not exhibit significant linkage specificity but possibly function in a substrate-specific manner [8,9]. Polyubiquitylation plays an important part in the regulation of both innate and adaptive immunity signaling pathways. The pathways that signal through tumor necrosis factor receptor, IL1 receptor, Toll-like receptor and T cell receptor are modulated by different modes of polyubiquitylation. These pathways effectively regulate the transcription factor NF- κ B, which mediates the transcription of

Figure 1



Protein ubiquitylation. **(1)** Activation of ubiquitin by an E1 enzyme to form a thioester at the expense of ATP. **(2)** Transfer of the activated thioester onto an E2 enzyme, which ubiquitylates **(3)** the substrate protein together with the E3 enzyme. Either by transferring the ubiquitin to the E3 enzyme or using the E3 enzyme as a scaffold. **(4)** Multiple repetitions of this cascade results in polyubiquitylation of the target substrate. The reverse process, deubiquitylation **(5)**, is carried out by deubiquitylating enzymes, which cleave the polyubiquitin chain and recycle it to monomers.

proteins involved in cell survival, proliferation, differentiation, inflammatory response, or apoptosis. Some examples of polyubiquitylation in these pathways are Lys63-polyubiquitylation of the kinase RIP, Met1-linked polyubiquitylation of NEMO, and Lys48-polyubiquitylation of I κ B α and the NF- κ B precursor. All these modifications have different outcomes, which together with the rest of the pathway have been extensively reviewed previously [10–12].

Besides ubiquitin, several ubiquitin-like proteins (Ubl) are known that have an important function in immunity. Ubls are conjugated and deconjugated through mechanisms similar to the ubiquitylation cascade, sometimes sharing components with the ubiquitin pathway. The Ubls SUMO and Nedd8 are also involved in the NF- κ B pathway. Nedd8 modification of the E3 SCF ligase complex is required for the recruitment of E2 and ubiquitylation of I κ B α . On the other hand, SUMO modification of I κ B α counteracts ubiquitylation at the same lysine residue, adding another regulatory step in this complex pathway [13]. ISG15, one of the first identified Ubls, is upregulated by type I interferon stimulation during infection [14–16]. It consists of two Ub-like domains connected by a small linker and plays an important role in the defense against viral and bacterial infections, possibly by modification of viral and host proteins. However, the exact function of ISGylation remains largely unclear [17]. FAT10 is another Ubl implicated in immune defense and also consists of two Ub-like modules. It is expressed in mature dendritic cells and B cells, and expression can be induced by IFN- γ and IFN- α . Mono-FATylated proteins are targeted to the proteasome for degradation [18]. Thus far, no deconjugating enzyme has been identified.

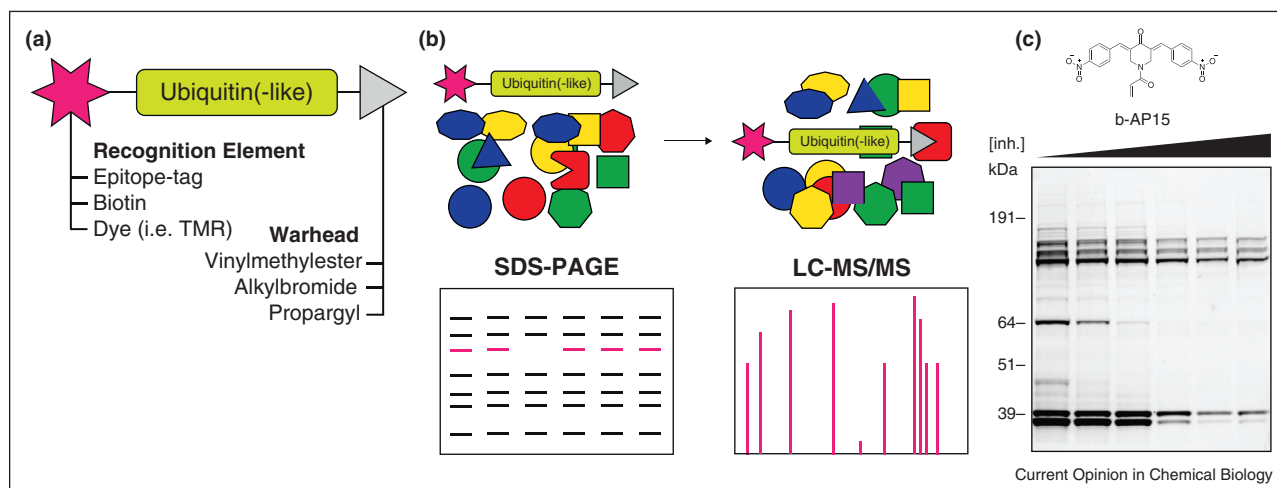
Interestingly, pathogens have evolved highly effective mechanisms to modulate host immune signaling pathways through Ub/Ubl remodeling to secure replication, infection and pathogenesis. For example, the Herpes Simplex Virus-1 (HSV-1) tegument protein UL36 possesses deubiquitylating activity [19], which is conserved in homologs found in murine cytomegalovirus and Epstein Barr Virus [20]. The SARS coronavirus PLpro processing protease acts on a broad range of ubiquitylated and ISG15ylated host proteins and is required for viral replication [21,22]. Crimean-Congo hemorrhagic fever (CCHF) virus harbors an OTU-like DUB which acts as both a deubiquitylase and a deISGylase [23–25]. In addition, *Yersinia* virulence factor YopJ has deubiquitylating activity that results in deubiquitylation of I κ B α and hence inhibition of NF- κ B signaling [26,27]. Moreover, *Chlamydia trachomatis* expresses two DUB-like proteases that possess deubiquitylating and deneddylating activity [28].

To date only few deconjugating enzymes have been characterized at a molecular level. However, in recent years the importance of DUBs has been recognized and research has focused on characterizing DUBs and implicating them in fundamental biological processes. In addition, this class of enzymes has attracted attention as a target for drug development.

Synthesis of ubiquitin and Ubl reagents

The development of the first ubiquitin-based assay reagents relied on transpeptidation reactions. Often trypsin is used in these reactions, which removes the last two glycine residues of ubiquitin under native conditions [29]. During the cleavage reaction, an intermediate ester is

Figure 2



(A) Schematic representation of an activity-based probe. **(B)** Schematic overview of a typical ABP-experiment. A probe is added to a cell lysate where it labels its target enzymes. The recognition elements allow for visualization of the bound targets after SDS-PAGE by fluorescence scanning or Western blot analysis. Proteomics can be used to identify and quantify proteins from a pull-down experiment. **(C)** A typical result of an inhibitor competition assay. An EL4 cell lysate is treated with an inhibitor after which an ABP is used to label and visualize residual enzyme activities [42].

formed with the active-site serine residue. This intermediate ester can undergo a transesterification reaction resulting in a new peptide bond with an amine nucleophile of choice, if a large amount of nucleophile is present. The fluorogenic substrate Ub-AMC has initially been synthesized using this method [30]. Another method uses intein chemistry for a more convenient preparation of reactive ubiquitin thioester intermediates [31]. These intermediates can be chemically converted to introduce a reactive group in the synthesis of ubiquitin-based active site-directed probes. Intein chemistry is generally preferred over transesterification methods as it is more generally applicable [32–34].

Advances in the total chemical synthesis of ubiquitin have enabled the efficient synthesis of new and improved ubiquitin-based reagents. Using an optimized linear synthesis, ubiquitin can now be easily obtained in high yield and purity [35]. Using this synthetic methodology, ubiquitin can be functionalized with any reactive group, dye or label that is compatible with standard peptide synthesis procedures at any specific position.

Active-site directed probes and their applications

The discovery and study of DUBs and Ubl deconjugating enzymes has been greatly accelerated by the development of activity-based probes (ABPs). These ubiquitin-based probes are suicide substrates that specifically react with the active site cysteine nucleophile of DUBs in an activity-based manner. Their covalent nature can be used

to visualize and purify an entire family of active proteins simultaneously. ABPs contain three essential elements (Figure 2A). The *targeting element* confers specificity for the desired enzyme targets. In the case of DUB probes, the targeting element is ubiquitin. Secondly, the *reactive group* or *warhead*, which reacts with the active site of the target enzyme after the targeting element has bound. Finally, a *recognition* or *retrieval element* is incorporated to allow for the selective retrieval or visualization of the enzyme-ABP complex [34]. The first of these ubiquitin ABPs was ubiquitin-nitrile, which allowed the labeling of a proteasome-bound cysteine dependent DUB [36]. Many improvements to probe design and synthesis have been reported since [32–34,37**]. For instance, these developments led to the discovery that OTU class enzymes act as deubiquitylases [38].

Last year, our lab reported the discovery of a novel warhead for use in activity-based profiling and purification of active deubiquitylating enzymes [37**]. The functionality of this warhead was nicely confirmed and complemented in work reported by Sommer *et al.* [39**]. The introduction of a chemically inert terminal alkyne moiety at the C-terminus of ubiquitin (Ub-Prg) renders it highly reactive toward deubiquitylating enzymes. It was shown that this novel Ub-Prg probe reacts with all classes of cysteine DUBs. An advantage of this alkyne-based probe is that it is very reactive toward DUBs but does not react with unrelated Cys-proteases under the same conditions. Further reviews on general ABP chemistry can be found elsewhere [40].

DUB specificity profiling

Ubiquitin-based ABPs can be used to identify known and putative deubiquitylating enzymes from cell lysates by mass spectrometry. In addition, active DUBs in cell lysates can be directly visualized after SDS-PAGE separation using western blotting or fluorescence scanning (Figure 2B). This direct visualization can be used to assay novel DUB inhibitors for putative enzyme specificity (Figure 2C), recently described by Altun *et al.* [41]. Using the same principle, De Jong *et al.* [42] demonstrated that the DUB inhibitor b-AP15 has little selectivity among DUBs (Figure 2C). While inhibitor selectivity profiling is feasible, these experiments are not trivial to perform due to the covalent nature of the ABPs and the high affinity for targets. Careful timing and elevated inhibitor concentrations in these experiments are crucial.

Native chemical ligation to synthesize ubiquitin chain specific probes

A number of non-enzymatic ways to obtain ubiquitin conjugates have been reported in recent years [43]. An important technology enabling the synthesis of natively isopeptide-linked assay reagents is the use of native chemical ligation (NCL) as depicted in Figure 3A. In an NCL a native peptide bond is formed between two peptides, one containing a C-terminal thioester and another containing an N-terminal cysteine residue [44]. To generate diubiquitin modules, which are normally linked through an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ϵ -amine of lysine, this technology needed to be adapted. By introducing a thiolysine residue (Figure 3B) that harbors a thiol moiety positioned either at the δ - or γ -position of a lysine residue (Figure 3C) in the sequence of ubiquitin, an acceptor ubiquitin is created onto which a ubiquitin molecule can

be ligated [35,45–47]. With this technology, all isopeptide-linked ubiquitin chain topologies can be synthesized chemically.

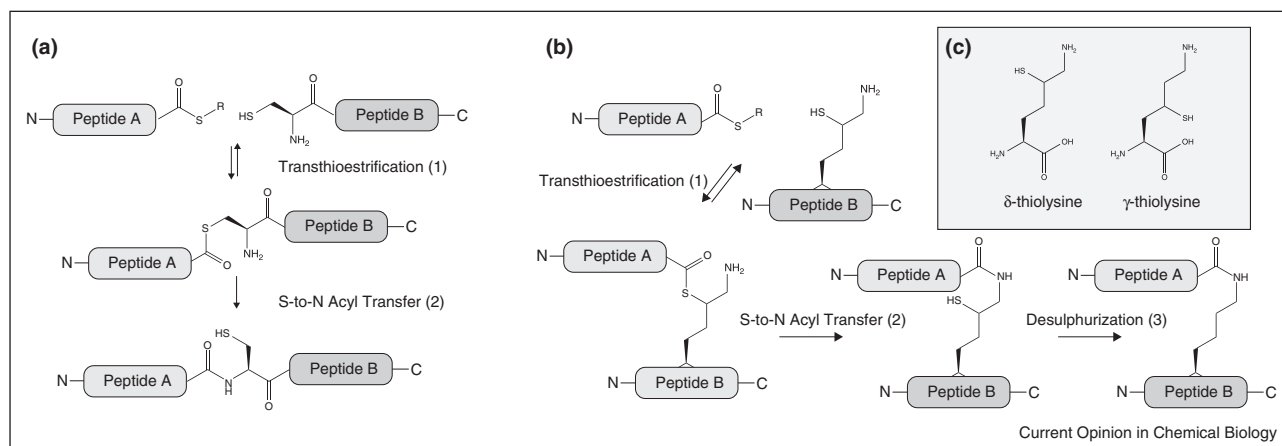
Chain specific ubiquitin probes

An important question in understanding the biological outcome of ubiquitin signaling is the elucidation of the mechanism by which DUBs confer substrate and ubiquitin linkage specificity. There are at least two ways DUBs can exert their deubiquitylating activity on polyubiquitylated substrates. Firstly, they can cleave between the most proximal ubiquitin in the chain and the substrate, resulting in complete removal of the entire ubiquitin chain. Secondly, cleavage can occur between any two ubiquitin moieties, leaving part of the ubiquitin chain on the substrate available for receptor recognition, chain editing by ligases or further processing by other DUBs.

The conventional way of assessing linkage specificity is by measuring turnover rates of different diubiquitin molecules as substrates. Although this method has been applied successfully in several studies [7,8] it is limited to isolated DUBs, whereas linkage specificities may also arise from yet unknown interactions as part of larger protein complexes [48]. ABPs on the other hand are not limited to purified enzymes and can be used to study DUBs in a complex biological setting. Recently, diubiquitin-based ABPs bearing a warhead between the ubiquitin modules were developed to allow study of linkage specificities in a relevant context, that is, cell lysates.

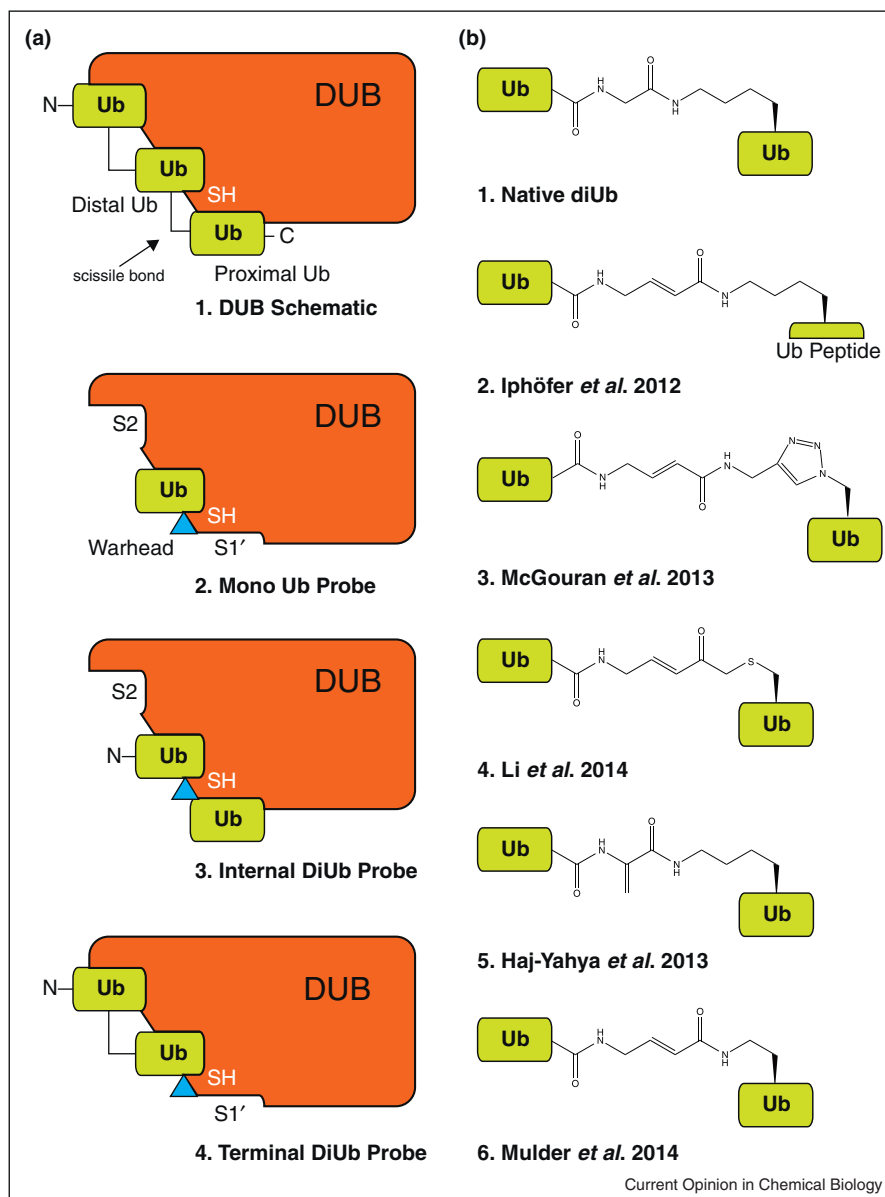
One of the main factors that determine DUB linkage specificity is the presence of one or multiple ubiquitin-binding pockets in DUBs (Figure 4A, top panel). These

Figure 3



(A) Schematic overview of native chemical ligation (NCL). (1) The first step is a transthioesterification reaction between the thioester of peptide A and the thiol in peptide B. (2) This is followed by a S-to-N acyl transfer in which the amide bond is formed. (B) NCL between the C-terminal thioester of peptide A and peptide B functionalized with a thiolysine residue functioning as the NCL acceptor. The thiol moiety is removed by a radical desulphurization procedure after NCL (3) (C) γ -thiolysine and δ -thiolysine residues used for native chemical ligation at the ϵ -amine of lysine.

Figure 4



(A) (1) Schematic representation of a DUB binding three ubiquitin modules in three different binding sites: The S1 and S2 sites bind the ubiquitin modules N-terminal to the scissile bond and the C-terminal ubiquitin binds in the S1' site. **(2) A** mono-ubiquitin probe is equipped with a C-terminal warhead and only binds the S1 site **(3) A** diubiquitin probe with the warhead positioned between the proximal and distal ubiquitin, which in this model bind in the S1' and S1 sites respectively. **(4) A** diubiquitin probe equipped with a C-terminal warhead binding the S1 and S2 sites. **(B)** Overview of all reported diubiquitin probes. The top structure shows a native diubiquitin isopeptide linkage.

allow for selective recognition of different ubiquitin chain topologies and hence the correct positioning of a polyubiquitylated substrate. The proposed S1 and S1' sites position a polyubiquitin chain across the active-site to allow cleavage between ubiquitin molecules in a chain. The S1' site binds the ubiquitin moiety C-terminal to the scissile bond and the distal ubiquitin moiety in the chain will bind the S1 site. For cleavage of polyubiquitin chains

from a substrate, S1 and S2 binding pockets are likely used. The most proximal ubiquitin in the chain binds to the S1 site and the more distal ubiquitin binds to the S2 site. In this manner the chain is positioned such that the bond between chain and substrate can be cleaved. The previously described monoubiquitin-based probes only bind the S1 site (Figure 4A, second panel) and therefore provide no information on linkage specificity. The

recently developed diubiquitin-based probes are all designed to bind the S1 and S1' sites (Figure 4A, third panel), after which the DUB active site thiol reacts with the internal warhead.

Initial steps toward diubiquitin probes were published by Iphöfer *et al.* [49]. They developed the first chain-specific ubiquitin isopeptide probes in which HA-tagged ubiquitin is linked through its C-terminus to a lysine sidechain in a 13-amino acid peptide derived from ubiquitin. The C-terminal glycine residue of ubiquitin was replaced by a vinyl amide warhead (Figure 4B-2). Both the Lys48 and Lys63 diubiquitin mimics were synthesized and used to label purified DUBs. In addition, they were able to capture and identify 22 DUBs from a cell lysate and to quantify their relative binding efficiencies for the Lys48- and Lys63-derived probes.

The first full-length diubiquitin probes were reported by McGouran *et al.* [50]. They developed probes that mimic all eight natural occurring diubiquitin linkages. The distal HA-tagged ubiquitin containing the vinyl amide warhead and a terminal alkyne moiety was linked to the proximal ubiquitin containing an azidohomoalanine residue by means of a bio-orthogonal 'click reaction' (Figure 4B-3). In a quantitative proteomics analysis of HEK293T cell lysate, they quantified the relative selectivity of 28 DUBs for the various ubiquitin linkages. While reported data from diubiquitin turnover assays shows overlap with the apparent linkage preferences observed, there are some major differences. These differences likely arise from the difference between isolated proteins versus DUBs in their cognate environment or simply the difference in detection methods (e.g. kinetics in a cleavage assay versus end-point in probe labeling). These differences are often observed and an important point of further investigation.

Li *et al.* [51] reported the development of a diubiquitin probe in which the native diubiquitin linkage is more accurately resembled (Figure 4B-4). From a structural point of view this probe has the correct linker length (Figure 4B-1). The authors mutated Lys48 and Lys63 from the HA-tagged proximal ubiquitin module into a cysteine residue, which they reacted to the distal ubiquitin containing the vinyl amide warhead and a bromide. Similar to the probes described above it was shown that the probes could label isolated DUBs as well as DUBs in a cell lysate with distinct profiles.

Haj-Yahya *et al.* [52] applied native chemical ligation for the construction of diubiquitin probes. They attached a cysteine residue to the lysine side chain of the proximal ubiquitin and ligated the distal module using the C-terminal thioester of ubiquitin. Elimination of the sulfur atom resulted in the formation of a dehydroalanine warhead (Figure 4B-5). Lys48, Lys63-linked and linear

diubiquitin probes were constructed and used for labeling of six purified DUBs. Notably, this probe is cleaved by DUBs, which can be expected on the basis of the positioning of the vinyl amide moiety.

Mulder *et al.* recently developed a dedicated ligation handle designed to construct diubiquitin probes via native chemical ligation. Subsequent thiol elimination yields the warhead in the final step of synthesis (Figure 4B-6) [53]. These diubiquitin probes have the appropriate linker length and warhead correctly positioned with respect to the native diubiquitin isopeptide linkage. All seven diubiquitin isopeptide linkages were created and shown to label USP7. The Lys11 and Lys48 probes were fluorescently tagged and used to label DUBs in EL-4 cell lysate, which revealed distinct labeling patterns between these two linkages and monoubiquitin probe.

Notably, five papers concerning diubiquitin(-mimic) probes have been published in the last two years, which underscores the importance of this topic in DUB research. Significant advances have been made in terms of probe design and preparation. These probes will ultimately shed some light on the function and relevance of the 8 different ubiquitin signals.

Directions in ubiquitin probe research

Ubiquitin-based probes have proven to be valuable tools for structural studies that aim to understand substrate recognition by DUBs on a molecular level using X-ray crystallography. This was predominantly achieved using pan-DUB probes but it will now become key to design probes that target one DUB or a family of DUBs selectively. Recent developments in this direction involve tuning of the affinity for ubiquitin by creating ubiquitin mutants that act as rather selective DUB inhibitors [54,55]. The ubiquitin linkage specificity of DUBs remains an intriguing field of research and many studies have focused on the involvement of the S1 and S1' ubiquitin binding pockets. However, for certain DUBs it has been shown that there is a significant contribution of the S1 and S2 pockets [7]. A specific probe that targets these sites remains to be reported (Figure 4A bottom panel).

Finally, the class of metallo-DUBs remains difficult to target by using ubiquitin probes. Their catalytic mechanism does not involve a covalent DUB-substrate intermediate, which makes it impossible to capture this DUB family using any currently known warhead. Studying these DUBs using ABPs will require a new probe design.

Conflict of interests

R.E., P.G., and H.O. are inventors on patent applications on active-site reactive molecules and entitled to royalties

resulting from technology licensing. H.O. owns shares in the company UbiQ.

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