Evaluating enzyme activities and structures of DUBs

Jonathan N. Pruneda[a], David Komander[b,*]

[a]Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR, United States
[b]Ubiquitin Signalling Division, The Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia
*Corresponding author: e-mail address: dk@wehi.edu.au

Contents

Abstract

Ubiquitin signaling requires tight control of all aspects of protein ubiquitination, including the timing, locale, extent, and type of modification. Dysregulation of any of these signaling features can lead to severe human disease. One key mode of regulation is through the controlled removal of the ubiquitin signal by dedicated families of proteases, termed deubiquitinases. In light of their key roles in signal regulation, deubiquitinases have become a recent focus for therapeutic intervention as a means to regulate protein abundance. This work and recent discoveries of novel deubiquitinases in humans, viruses, and bacteria, provide the impetus for this chapter on methods for evaluating the activities and structures of deubiquitinases. An array of available deubiquitinase substrates for biochemical characterization are presented and their limitations as standalone tools are discussed. Methods for the determination and analysis of deubiquitinase structure are also presented, with a focus on visualizing recognition of the ubiquitin substrate.

Abbreviations

ABP activity-based probe
DUB deubiquitinase
Ub   ubiquitin
UBL Ub-like protein

1. Introduction

Research from the past several decades has implicated the posttranslational modifier ubiquitin (Ub) in the regulation of nearly all aspects of cellular signaling, including fundamental roles in proteasome-mediated protein degradation, cell cycle progression, and immune responses. The breadth of cellular roles played by Ub stems in part from its ability to be further posttranslationally modified. Modification of Ub can take the form of additional ubiquitination at one or several of its eight possible amide linkage points, creating complex polyUb chains; by modification with a Ub-like modifier (UBL) such as NEDD8 or SUMO; or through the addition of small chemical groups such as phosphorylation or acetylation (Swatek & Komander, 2016). The combinatorial possibilities of these alterations are enormous and give rise to what is sometimes called the "Ub code" (Komander & Rape, 2012). While the significance for many aspects of the Ub code remain to be deciphered, it is clear from the immense body of work at hand that breakdown or dysregulation of Ub signaling can result in severe health-related consequences (Rape, 2018). Therefore, all aspects of the Ub system are under tight control by hundreds of enzymes that together constitute the "writers," "readers," and "erasers" of the Ub code.

Writer enzymes, consisting of the E1 Ub-activating, E2 Ub-conjugating, and E3 Ub-ligating enzymes, regulate the synthesis of defined Ub signals on specific targets. Reader proteins recognize these signals and help elicit the desired cellular outcomes. Eraser enzymes, also known as deubiquitinases (DUBs), are key regulators of the Ub system. Humans encode approximately 100 DUB genes belonging to seven protease families (Haahr et al., 2018; Hermanns et al., 2018; Hewings et al., 2018; Kwasna et al., 2018; Mevissen & Komander, 2017). Additional proteases are specific toward UBL modifiers; herein we collectively refer to all Ub/UBL proteases as DUBs for simplicity. These specialized proteases hydrolyze the isopeptide or peptide linkage at the carboxy-terminus of the Ub/UBL modifier that links it to substrate primary amine groups, usually lysine side chains, thus

reversing the action of the writer enzyme and recycling the Ub/UBL back into the free pool for future rounds of conjugation. DUBs can be exquisitely specific for discrete cellular targets, either by selecting particular forms of the modification (e.g., OTULIN (Keusekotten et al., 2013; Rivkin et al., 2013)), by recognizing the modified substrate (e.g., the SAGA complex (Morgan et al., 2016)), or via regulation of subcellular localization (e.g., USP30 (Bingol et al., 2014)) (reviewed in Mevissen & Komander, 2017). Owing to their roles as key regulators of the Ub signal, DUBs have recently become a popular target for pharmacological intervention (Gavory et al., 2018; Kategaya et al., 2017; Lamberto et al., 2017; Pozhidaeva et al., 2017; Turnbull et al., 2017) and show potential for "drugging the undruggable" (Huang & Dixit, 2016).

Importantly, to have any chance at success, pharmacological efforts must be preceded by a thorough, molecular understanding of the DUB and its target(s). As a key part of this process, one must be able to reconstitute DUB activity in vitro with substrates that have a high likelihood of physiological relevance. Biomedical product catalogues are filled with an array of Ub substrates; choosing those that suit one's needs best and recognizing the intrinsic limitations of such substrates is critical. In the first part of this chapter, we describe several of these substrates and demonstrate their utility in characterizing novel DUB activities. Second, visualization of DUB activity through structural characterization is a prerequisite for understanding the molecular nuts and bolts of the enzyme and its function, as well as identifying any unique properties that distinguish it from related family members. A number of biochemical tools and techniques have emerged in recent years to facilitate such an endeavor, and these will be discussed in the second part of this chapter.

2. Assessing DUB activity

The Ub field has benefited greatly from the past efforts of biochemists and chemical biologists who have reconstituted Ub signals in vitro and generated tools for capturing or measuring DUB activities, many of which can be readily produced or are commercially available (Ekkebus, Flierman, Geurink, & Ovaa, 2014; van Tilburg, Elhebieshy, & Ovaa, 2016). Critically, the nature of the DUB in question and the type of information sought must be considered before choosing a suitable substrate, as each comes with advantages and disadvantages. In the next subsections, we highlight

three classes of Ub substrates and demonstrate their utility in characterizing the activity of a bacterial DUB encoded by *Chlamydia trachomatis*, ChlaDUB1.

2.1 Activity-based probes

A strategy long utilized in the protease field, activity-based probes (ABPs) are substrate mimetics that place an electrophilic "warhead" near the active site nucleophile to produce a stable, covalent adduct. In the case of DUBs, ABPs typically take the form of a single Ub moiety fused to a warhead at its carboxy-terminus. Different warheads are available that tune the strength and selectivity of the reaction, the details of which have been discussed elsewhere (Hewings, Flygare, Bogyo, & Wertz, 2017). Currently, our groups primarily use the propargyl amide warhead (Ekkebus et al., 2013), which offers a high level of reactivity and can easily be made in large quantities using the intein-based method (Borodovsky, Kolli, Gan-Erdene, & Ploegh, 2002). Expanding upon this concept, diUb-based ABPs have also been developed that report on added levels of specificity (Haj-Yahya et al., 2014; Iphöfer et al., 2012; Li, Liang, Gong, Tencer, & Zhuang, 2014; McGouran, Gaertner, Altun, Kramer, & Kessler, 2013; Mulder, El Oualid, ter Beek, & Ovaa, 2014; Weber et al., 2017) (discussed in more detail below). A Ub-ABP can also be coupled with a reporter (e.g., an epitope tag or fluorescent group) at the amino-terminus to allow for measurement of DUB activity in cellular lysates following, e.g., inhibitor treatment (Turnbull et al., 2017), as well as enrichment and identification of novel DUBs (e.g., Abdul Rehman et al., 2016; Hewings et al., 2018; Kwasna et al., 2018; Misaghi et al., 2006). The same approach can also be applied to UBL modifiers, and thus Ub/UBL ABP reactivity can immediately report on substrate specificity. The protocol below describes an Ub/UBL ABP reactivity assay for the bacterial effector ChlaDUB1.

2.1.1 Required materials
- Ub/UBL ABP (purchased from a commercial source or prepared from methods employing intein chemistry, for example, Wilkinson, Gan-Erdene, & Kolli, 2005)—This protocol uses Ub and UBLs modified with a propargyl amide at their carboxy-termini
- Cys-based DUB, purified for Coomassie-based SDS-PAGE readout— In this example, ChlaDUB1 was expressed and purified as described in (Pruneda et al., 2016)
- Activation buffer: 25 mM Tris (pH 7.4), 150 mM NaCl, 10 mM DTT
- Standard SDS-PAGE equipment

2.1.2 Procedure

1. Dilute the DUB to $10\,\mu M$ in Activation buffer, leaving at room temperature for 15 min to fully reduce the catalytic Cys.
2. Prepare Ub/UBL ABP at $2\times$ concentration ($50\,\mu M$ as shown but can be optimized and reduced to conserve ABP) in Activation buffer.
3. Combine DUB and ABP 1:1 and incubate for 1 h (room temperature as shown, but can be optimized based on DUB stability and reactivity). Mix an additional sample with DUB and only buffer as a negative control.
4. Quench the reaction in reducing LDS sample buffer and resolve on SDS-PAGE alongside an untreated control sample. Stain gel with Coomassie Blue or similar.

2.1.3 Interpretation

Reaction with the ABP will form a covalent adduct leading to a higher apparent molecular weight on SDS-PAGE (Fig. 1A). Depending on DUB activity and compatibility with the ABP warhead, reactivity may go to completion or be only marginal. Ub/UBL specificity should be revealed by this simple endpoint assay, but identifying a preference may require optimization of the reaction pH, temperature, time course, or the strength of the electrophilic warhead. An important control to run in parallel is the inactive Cys-to-Ala DUB variant, which should abolish probe reactivity (Fig. 1A). In cases where the active site mutation does not abolish ABP reactivity (e.g., Wang et al., 2009), this may indicate an additional Ub binding site that places the propargyl group in close proximity to a reactive Cys residue.

Limitations of this assay include its application to Cys-based DUBs only (e.g., metalloprotease-based DUBs will not react with the ABP), and even for some select Cys-based DUB mechanisms, reaction with the ABP is precluded. For example, the Met1-specific DUB OTULIN requires substrate-assisted activation from a second ("proximal") Ub moiety, and thus will not react with a monoUb-based ABP (Keusekotten et al., 2013). In another unique case, the foot and mouth disease viral protease Lbpro hydrolyzes the UBL modifier ISG15 two residues short of the carboxy-terminus; conventional ISG15 ABPs place the electrophilic warhead out of register and will not react (Swatek et al., 2018). Although these are special cases of unique DUB mechanisms, they demonstrate the potential for false negative results in this assay. We have also noted false positive reactivity of the NEDD8 ABP with entirely Ub-specific DUB enzymes (Mevissen et al., 2013), due to its sequence similarity to Ub. Therefore, Ub/UBL ABPs provide an excellent first measure of activity but should always be verified through other assays, such as those highlighted in Fig. 1.

| A | Activity-based probes | B | Fluorescent substrates | C | Natural polyUb substrates |

A Activity-based probes **B** Fluorescent substrates **C** Natural polyUb substrates

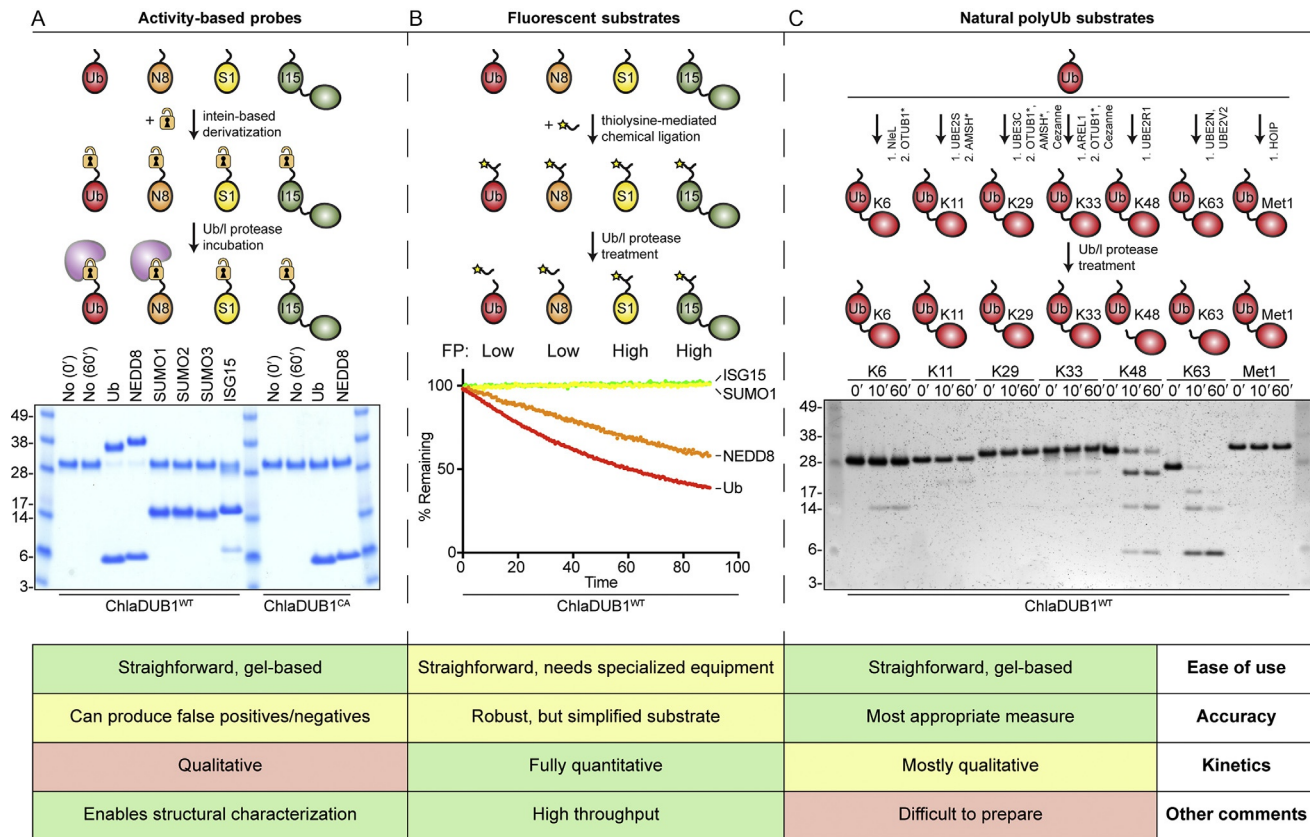| Straighforward, gel-based	Straighforward, needs specialized equipment	Straighforward, gel-based	**Ease of use**
Can produce false positives/negatives	Robust, but simplified substrate	Most appropriate measure	**Accuracy**
Qualitative	Fully quantitative	Mostly qualitative	**Kinetics**
Enables structural characterization	High throughput	Difficult to prepare	**Other comments**

Fig. 1 See legend on opposite page.

2.2 Fluorescent substrates

While ABPs are indispensable for their ease of use and their applications in DUB discovery and structural characterization (see below), they often fall short of providing the kinetic information necessary for understanding enzyme mechanism and regulation. Fluorescent Ub substrates allow for the direct, time-resolved measurement of DUB activity in a plate format. Historically, the most widely used substrates have been Ub-7-amido-4-methyl coumarin (Ub-AMC) and Ub-Rhodamine, which fluoresce upon DUB-mediated hydrolysis and have proven amenable to high-throughput inhibitor screens (e.g., Turnbull et al., 2017). Though their fluorogenic nature makes Ub-AMC and Ub-Rhodamine substrates simple to use, chemically, they are not true mimetics of a Ub modification as they do not contain an isopeptide linkage. In recent years, improved reporter substrates have been developed. These improved substrates utilize other biophysical methods, such as fluorescence polarization (FP) or Förster resonance energy transfer (FRET), that allow the fluorescent reporter(s) to be located away from the chemistry of the hydrolysis reaction. Hydrolysis of a diUb chain can be monitored by these methods, for example by measuring FP of a fluorescein arsenical hairpin (FlAsH)-labeled diUb (Keusekotten et al., 2013; Ye et al., 2011) or by measuring energy transfer across a diUb labeled with a FRET pair (Geurink et al., 2016). As a monoUb-based substrate, we typically prefer to use the recently developed Ub-KG(TAMRA) substrate (Geurink, El Oualid, Jonker, Hameed, & Ovaa, 2012), which

Fig. 1 Assessing DUB activity. *Upper*, cartoon schematic illustrating the setup and performance of biochemical assays assessing DUB activity. *Center*, example data from assays performed on the *C. trachomatis* effector protein ChlaDUB1. *Lower*, pros and cons of each type of biochemical assay. (A) Ub/UBL ABP assay monitoring reactivity of Cys-based DUBs. ABPs with a propargyl amide "warhead" can be purchased commercially or prepared using intein technology (Borodovsky et al., 2002); reactivity is monitored by a molecular weight shift in SDS-PAGE following Coomassie staining. ChlaDUB1 reacts irreversibly with ABPs based on Ub and NEDD8, but not SUMO or ISG15. (B) Ub/UBL-KG(TAMRA) fluorescence-based activity assay. Fluorescent substrates can be purchased commercially or prepared using native chemical ligation (Geurink et al., 2012), and monitored by change in fluorescence polarization. As indicated by the ABP assay, ChlaDUB1 cleaves Ub and NEDD8 substrates, but not SUMO or ISG15. (C) PolyUb chain specificity assay. Purified tetraUb chains for all linkages except K27 can be purchased commercially or prepared enzymatically by the investigator (Michel, Komander, & Elliott, 2018), and cleavage monitored by a molecular weight shift in SDS-PAGE following silver staining. Among the seven polyUb chain types tested, ChlaDUB1 prefers to cleave K63- and (to a lesser extent) K48-linked chains.

consists of a Ub isopeptide-linked to a short tetramethylrhodamine (TAMRA)-labeled Lys-Gly peptide. With this substrate, one can monitor DUB activity by FP following release of the small KG(TAMRA) peptide. The Ub moiety can be exchanged for UBL modifiers, enabling determination of a kinetic preference among Ub/UBL substrates, as described below for ChlaDUB1.

2.2.1 Required materials

- Ub/UBL fluorescent substrate (purchased from a commercial source or prepared from methods employing intein chemistry, for example, Wilkinson et al., 2005)—This protocol uses Ub/UBL-KG(TAMRA) substrates
- Purified DUB—In this case ChlaDUB1 was expressed and purified as described in Pruneda et al. (2016)
- Dilution buffer: 25 mM Tris (pH 7.4), 100 mM NaCl, 5 mM ß-mercaptoethanol, 0.1 mg/mL BSA, filtered through a 0.44 μm syringe filter
- Black microplate (e.g., 384-well low volume)
- Microplate reader equipped for fluorescence polarization measurements at suitable wavelengths (e.g., BMG Labtech PHERAstar equipped with an FP 540590590 optic module)

2.2.2 Procedure

1. Prepare a small dilution series of purified DUB in Dilution buffer at 2× final concentration. Performing the initial assay with a dilution series allows for a measure of enzyme concentration dependence and provides a range of activities for use and optimization in future assays. ChlaDUB1 is shown at a final concentration of 1 nM. Allow the enzyme to be fully reduced by incubation at room temperature for 15 min.
2. Dilute Ub/UBL-KG(TAMRA) substrates to 300 nM in Dilution buffer (2× final concentration). Also prepare a KG(TAMRA) positive control sample at 50 nM. Allow to equilibrate at room temperature.
3. Pipette 10 μL of Ub/UBL-KG(TAMRA) into microplate in triplicate. Also include buffer-only (blank), Ub/UBL-KG(TAMRA)-only (negative control), and KG(TAMRA)-only (positive control) samples. Avoid bubbles, and centrifuge the plate if necessary to drive liquid droplets to the bottom.
4. Place microplate into the reader and optimize gain and focal length parameters.

5. Remove plate and quickly add 10 μL of DUB dilutions to all wells (except control samples). Mix by pipetting two to three times, avoid bubbles and/or centrifuge the plate.
6. Return the microplate to the reader and begin data collection. Allow to continue for ∼60 min with readings every ∼1 min.
7. Normalize FP values to the positive and negative control samples (creating a % substrate remaining curve) to account for sample drift due to evaporation.

2.2.3 Interpretation

Following normalization to the positive and negative control samples, one can plot the percentage of substrate remaining over time for each Ub/UBL fluorescent substrate (Fig. 1B). Activity and specificity can be assessed qualitatively, or curves can be fitted to an exponential rate decay to obtain substrate half-lives. Parameters such as pH, enzyme concentration, and temperature can be optimized, though evaporation will become an issue at higher temperatures. The assay is quite robust and amenable to high-throughput methodologies, but false negatives may arise for examples such as OTULIN (discussed above) that require more complex substrate mimetics.

2.3 Natural substrates

Though all of the substrates discussed above come with certain advantages for assessing DUB activity, none can reliably serve as a suitable replacement for the bona fide ubiquitinated physiological substrate. Obtaining site-specifically ubiquitinated substrate is not an easy feat. Various strategies exist currently to chemically ubiquitinate an unnatural amino acid target introduced into a protein backbone either through orthogonal genetic coding or total peptide synthesis (Gopinath, Ohayon, Nawatha, & Brik, 2016; van Tilburg et al., 2016). The resulting ubiquitinated substrate can be used biochemically to assess DUB activity (Bavikar et al., 2011) or structurally to understand enzyme-substrate interactions (Morgan et al., 2016). Some DUBs display remarkable specificity for both the length and type of polyUb modification (Mevissen et al., 2013) and may therefore selectively edit the Ub signal attached to a protein substrate. For the study of these polyUb-targeted DUBs, developing tools to assemble all eight possible Ub chain types to be used as in vitro substrates has been a major achievement of the last decade. The synthesis of K27-linked Ub chains still requires chemical assembly, but for the remaining chain types, biochemical strategies to prepare large quantities have now been developed using

linkage-specific writer enzymes (Michel et al., 2018). These breakthroughs have not only provided additional structural insights, but also provided a full panel of natural polyUb substrates with which DUB specificity can be tested, as outlined below for ChlaDUB1.

2.3.1 Required materials

- Panel of polyUb chains (purchased from a commercial source or self-prepared (Michel et al., 2018))—This protocol uses enzymatically prepared tetraUb chains of all types except K27-linked
- Purified DUB—In this case ChlaDUB1 was expressed and purified as described in Pruneda et al. (2016)
- Activation buffer: 25 mM Tris (pH 7.4), 150 mM NaCl, 10 mM DTT
- 10 × Assay buffer: 500 mM Tris (pH 7.4), 500 mM NaCl, 50 mM DTT
- Standard SDS-PAGE equipment

2.3.2 Procedure

1. Dilute DUB in Activation buffer to 2 × final concentration (this will need optimization; demonstrated here with 5 nM ChlaDUB1 final concentration). Allow full enzyme Cys reduction at room temperature for 15 min.
2. Prepare tetraUb chains at 2.5 μM in Assay buffer (2 × final concentration of both tetraUb and Assay buffer).
3. Equilibrate DUB and tetraUb samples to 37°C.
4. Mix DUB and tetraUb 1:1, 7 μL of each, and incubate at 37°C.
5. Prepare a "time zero" sample by mixing 2.5 μL each of DUB and tetraUb directly in reducing LDS sample buffer.
6. Collect 5 μL at each reaction time point (shown with 10- and 60-min time points) and quench in reducing LDS sample buffer. These samples can be directly carried forward to SDS-PAGE analysis (do not boil).
7. Resolve samples by SDS-PAGE and silver stain for higher sensitivity. Western blotting is not recommended unless the primary antibody has been shown to detect all Ub chain types equally (many do not).

2.3.3 Interpretation

PolyUb substrates offer multiple advantages over other DUB substrates. First, if parameters such as enzyme concentration, reaction temperature, and time are adjusted such that the fastest reaction is at or near completion at the end of the time course, substrate specificity over the other chain types can be estimated qualitatively. DUBs can display no, some, or in several cases

absolute specificity for a single chain linkage type, and determining this requires repeated and careful analyses at multiple enzyme concentrations (Mevissen et al., 2013). ChlaDUB1 demonstrates a preference for K63–linked chains and (to a lesser extent) K48-linked chains (Fig. 1C), but at higher concentration or longer incubations will cleave other chain types as well. An additional advantage over other substrates is that the behavior of polyUb cleavage can be telling of added layers of specificity. Stochastic cleavage of all Ub chain lengths, as observed for ChlaDUB1 (Fig. 1C), indicates recognition of diUb as a minimal unit, whereas rapid cleavage of longer chains down to diUb would indicate additional Ub binding sites that favor increased chain length (see below).

3. Understanding DUB structure

3.1 Anatomy of a DUB

Some DUBs display multiple layers of specificity, each contributed by a distinct substrate binding site on the surface of the enzyme. Description of these binding sites follows classic protease nomenclature, with the exception that separate polypeptides on either side of the scissile bond are considered specificity determinants as opposed to individual protein residues. The "S1 site" determines Ub and/or UBL specificity and orients the carboxy-terminus of the distal moiety into the active site (Fig. 2). This site typically contributes the bulk of the binding energy to the ubiquitinated substrate and
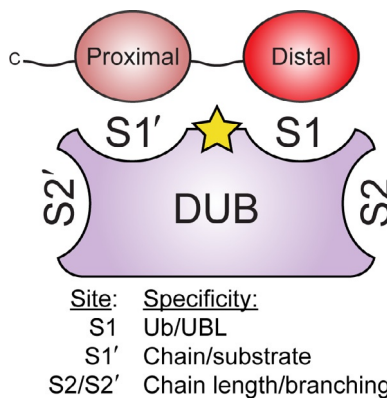

Fig. 2 Anatomy of a DUB. Cartoon schematic representing the multiple Ub binding sites a DUB may have and the layers of specificity they would impart. The substrate, in this case a diUb chain (*red*), sits above the DUB (*purple*) straddling the active site (*yellow star*).

therefore is the most susceptible to manipulation through point mutation (e.g., Gersch et al., 2017; Keusekotten et al., 2013; Mevissen et al., 2016; Pruneda et al., 2016). Characterization of S1 site specificity can be accomplished using Ub/UBL ABPs or fluorescent substrates, as discussed above.

The S1′ site encodes an additional layer of substrate specificity through binding to the ubiquitinated target, be it a second Ub molecule or another protein (Fig. 2). In the case of a polyUb chain, recognition of the proximal Ub moiety at the S1′ site determines linkage specificity by orienting the ubiquitinated Lys residue into the active site, which can be manipulated through point mutations (e.g., Gersch et al., 2017; Keusekotten et al., 2013; Mevissen et al., 2016; Pruneda et al., 2016). Identification of an S1′ site that introduces chain specificity can be performed using the panel of polyUb chains as discussed above.

Additional substrate binding sites on either end of the S1 and S1′ sites are called S2 and S2′ sites, respectively, and are by and large relevant only for polyUb–targeted DUBs (Fig. 2). Examples of DUBs containing these sites are fewer, but they determine the context of the minimal diUb unit recognized across the active site. For example, S2/S2′ sites can introduce a preference toward longer polyUb chain length or toward a heterotypic or branched chain architecture (Békés et al., 2015; Mevissen et al., 2013; Ye et al., 2011). The existence of an S2/S2′ site that introduces a preference for chain length can be determined by closely monitoring the cleavage of tetraUb chains (as discussed above); enzymes that possess these sites will rapidly cleave the longer chain down to the less preferred tri or diUb length, while enzymes that lack S2/S2′ sites will cleave tetraUb more stochastically. DiUb fluorescent substrates and ABPs have also been developed to characterize the existence and specificity of S2 sites (Békés et al., 2016; Flierman et al., 2016; Ye et al., 2011).

3.2 Visualizing DUB activity

Rigorous biochemical characterization of DUB specificities, as described above, helps clarify the opportunities available for trapping a substrate-bound DUB complex for structural analysis. Beyond the information obtained on target recognition, substrate binding can stabilize the enzyme fold, making it more amenable for biophysical characterization through crystallography or NMR. For a full picture of DUB activity and mechanism, multiple substrate- and product-bound states can be characterized alongside the apo enzyme to visualize the entire catalytic cycle, as has been performed with the K11-specific DUB Cezanne (Mevissen et al., 2016).

3.2.1 Trapping substrate-bound DUB complexes

Choosing the optimal method to trap a substrate-bound DUB complex requires careful groundwork to define the ideal substrate and enzyme conditions. Identification of the preferred substrate that satisfies all binding sites presented by the enzyme (see Section 3.1) increases both the relevance and likelihood of obtaining useful complexes. Likewise, many factors can contribute to DUB activity in vitro, and optimizing these beforehand is key. Beyond the biochemical parameters of pH and salt concentrations, some DUB activities are highly sensitive to expression construct boundaries (e.g., XopD (Pruneda et al., 2016)), phosphorylation status (e.g., OTUD5/DUBA (Huang et al., 2012)), or binding partners (e.g., UCH37 (Yao et al., 2008)). Thus, establishing these parameters upfront is a prerequisite to any structural biology endeavor.

The simplest method to trap a substrate-bound DUB complex is to inactivate the enzyme by point mutation and form a noncovalent complex with substrate. In the case of Cys-based DUBs, this means mutating the active site Cys to Ala, which has been shown for a variety of tested examples to artificially enhance binding affinity to ubiquitinated substrates (Morrow et al., 2018). In DUBs where a defined S1′ site dictates a strong preference for a particular polyUb chain type, this method has enabled crystallization of DUB-diUb complexes (Gersch et al., 2017; Keusekotten et al., 2013; Rivkin et al., 2013; Sato et al., 2015, 2017).

More often, the enzyme-substrate interaction is too transient for structural studies, and in these cases ABPs can be used to covalently trap the complex. This strategy has proven successful on numerous occasions, most of which take advantage of a monoUb/UBL ABP to study substrate binding to the S1 site. However, particularly for those DUBs that specifically target polyUb chains, a monoUb-bound complex does not tell the full story of substrate encounter. For these cases, nonhydrolyzable diUb-based ABPs were developed that place the electrophilic warhead between the two Ub moieties (Haj-Yahya et al., 2014; Iphöfer et al., 2012; Li et al., 2014; McGouran et al., 2013; Mulder et al., 2014; Weber et al., 2017). These ABPs trap the substrate–enzyme complex, and have proven effective for structure determination of the K11-specific DUB Cezanne (Mevissen et al., 2016) and the Met1-specific DUB OTULIN (Weber et al., 2017). A second diUb-based ABP, in which the warhead is placed at the carboxy-terminus of the proximal Ub, can trap a substrate bound into the S1 and S2 sites (Békés et al., 2016; Ye et al., 2011). In all cases, buffer conditions, reaction parameters, and enzyme:ABP stoichiometry are optimized to push the reaction as close to completion as possible. Final purification of

the trapped complex can be achieved through ion exchange and/or size exclusion chromatography, or through the use of an ABP bearing an affinity tag.

3.2.2 Visualizing DUB activity

As with any structural biology project, it is difficult to provide a certain recipe for success. Instead, we will discuss the techniques and examples that have proven successful in the past in order to provide a framework for what can be done with the tools available in the field. Well-defined substrate-bound DUB complexes can be studied with solution and crystallographic structural methods. Depending on size and behavior, NMR can be a useful tool for understanding DUB dynamics and regulation in solution, as shown for the DUBs AMSH and Cezanne (Hologne et al., 2016; Mevissen et al., 2016) as well as the UBL protease SENP1 (Ambaye, Chen, Khanna, Li, & Chen, 2018). Hydrogen-deuterium exchange mass spectrometry can also be used to monitor conformational changes associated with substrate recognition (Gersch et al., 2017; Mevissen et al., 2016). Finally, single-molecule techniques can inform on more global structural parameters, such as polyUb chain conformation following DUB binding (Ye et al., 2012). Crystallography and potentially cryo-electron microscopy can provide the highest-resolution information on substrate recognition and combined with covalent ABPs can offer the fastest route to understanding DUB specificity and mechanism.

Ub ABPs have enabled structural characterization and mechanistic understanding of multiple layers of DUB specificity. We have used mono-oUb/UBL ABPs extensively to characterize the role of the S1 site in substrate recognition. Cross-specific DUBs that possess Ub and UBL protease activities are particularly interesting cases, and ABPs have allowed us to explain the Ub/ISG15 cross-reactivity of the Crimean Congo hemorrhagic fever virus vOTU (Akutsu, Ye, Virdee, Chin, & Komander, 2011; James et al., 2011), as well as the Ub/tomato SUMO (tSUMO) cross-reactivity of *Xanthomonas campestris* XopD (Pruneda et al., 2016). In the case of XopD, we found that the S1 site is malleable, allowing it to recognize the structurally similar but sequence-divergent Ub and tSUMO substrates (Fig. 3A). The propargyl amide warhead was used for both substrates in this case, and provided a nice mimetic in the XopD active site (Fig. 3B). S1–S1′ diUb ABPs have also successfully trapped and allowed the crystallization of the Cezanne-K11 diUb complex (Mevissen et al., 2016) and the OTULIN-Met1 diUb complex (Weber et al., 2017). In the case of OTULIN, the

Fig. 3 See legend on next page.
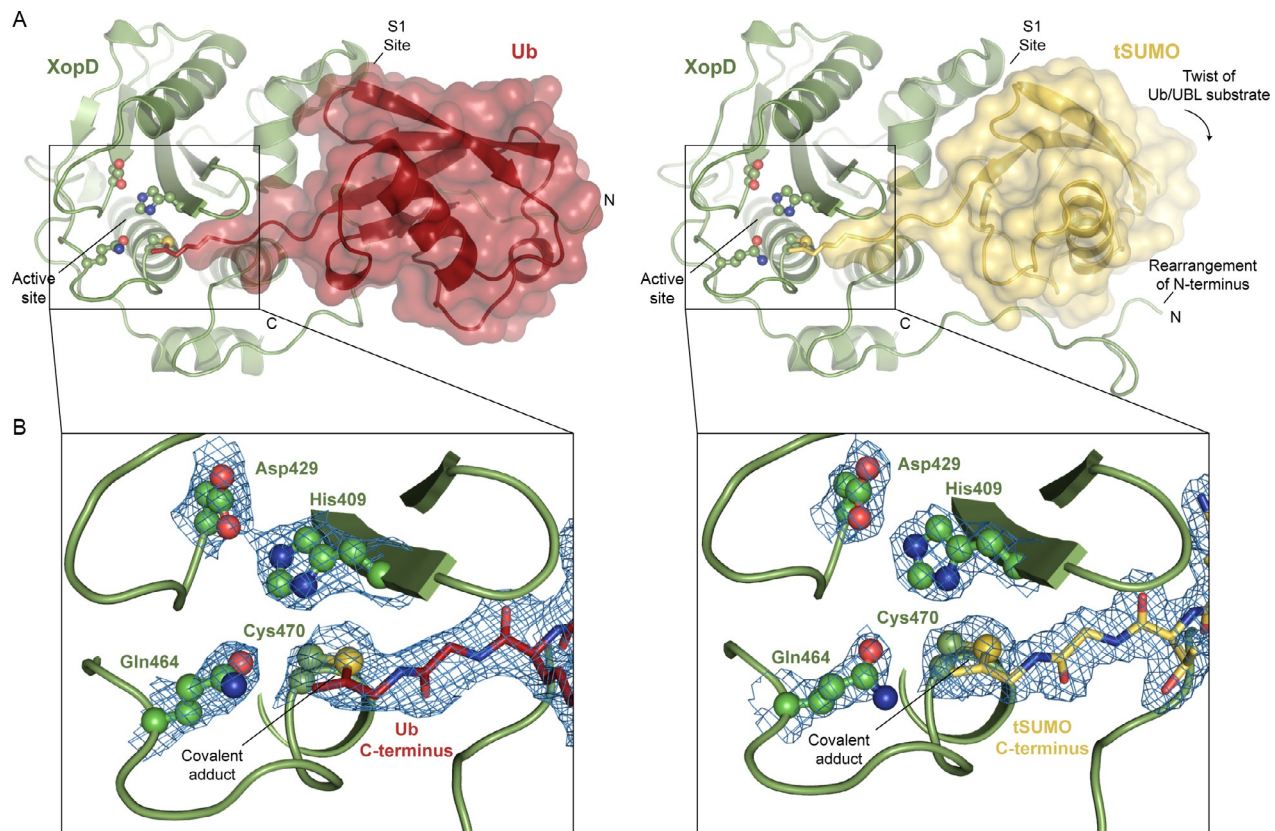
noncovalent complex with the inactive Cys-to-Ala variant DUB had been crystallized previously (Keusekotten et al., 2013; Rivkin et al., 2013), and the diUb ABP could be confirmed as a suitable mimetic (Weber et al., 2017). The S1–S2 diUb ABP has also proven effective in the crystallization of the SARS coronavirus papain-like protease, explaining its di-distributive behavior of cleaving K48-linked polyUb (Békés et al., 2016).

Beyond polyUb chains, some DUBs preferentially recognize the most proximal, substrate-attached Ub linkage for hydrolysis. DUBs encoding this level of substrate- and site-specificity are likely few in number (as the number of ubiquitination sites outweighs the number of regulatory DUBs by ~500 fold) but critical for regulating fundamental cellular processes. Proteases responsible for regulating the UBL modifier SUMO must not only recognize polySUMO chains but also process the precursor SUMO translation product and remove SUMO from target proteins. The latter two processes have been captured in noncovalent complexes and crystallized to reveal the details of how both peptide- and isopeptide-linked SUMO are coordinated into the active site for hydrolysis (Reverter & Lima, 2006). The major role of the UBL modifier NEDD8 is in the regulation of cullin–RING Ub ligases, and this modification is in turn regulated by a dedicated ~350 kDa complex termed the COP9 signalosome (Lingaraju et al., 2014). While higher-resolution studies are eagerly awaited, low-resolution electron microscopy studies show large conformational changes associated with NEDDylated cullin binding to the COP9 signalosome, placing the NEDD8 carboxy-terminus near the catalytic subunit, CSN5 (Lingaraju et al., 2014). Lastly, a recent crystal structure has captured how a module from the SAGA transcriptional coactivator complex deubiquitinates monoubiquitinated histone H2B (Morgan et al., 2016). Each of these studies needed to overcome major obstacles in both enzyme and substrate preparation with the reported structures revealing remarkable insights into DUB mechanism and biology.

Fig. 3 Visualizing DUB activity. Covalent complexes of the Ub (*left*) and tomato SUMO (tSUMO, *right*) carboxy-termini linked to the active site of *X. campestris* effector protein XopD. Ub and tSUMO ABPs were prepared from intein constructs (Borodovsky et al., 2002) with the propargyl amide warhead (Ekkebus et al., 2013). (A) Crystal structures of Ub (*red*) and tSUMO (*yellow*) bound in the XopD (*green*) S1 site, with the carboxy-termini threading into the active site. Conformational changes that accommodate the two substrates are highlighted. (B) Zoom-in of the XopD active site showing the full catalytic triad (Cys, His, Asp), the oxyanion hole (Gln), and the covalent linkage to substrate. $2|F_o|-|F_c|$ electron density contoured at 1σ is shown for the relevant components of the active site.

4. Summary and outlook

Advances in understanding DUB activity and structure have progressed hand-in-hand with developments in synthesizing suitable substrate molecules. Ub ABPs, fluorescent substrates, and natural substrates together offer a large repertoire of tools for in vitro studies. Though each comes with advantages and disadvantages (Fig. 1), together they offer rich information into many levels of DUB specificity and structure (see Section 3.1 and Fig. 2). However, pharmacological efforts targeting DUB activity demand high-throughput, and the ideal solution to this requirement remains to be seen. Advances in fluorescent substrates are producing more and more suitable mimetics to the natural substrate (Geurink et al., 2012). On the flip side, novel approaches to monitoring hydrolysis of natural substrates, such as MALDI-TOF mass spectrometry (Ritorto et al., 2014), may allow for high-throughput screening with physiologically relevant substrates.

Recent years have seen major in-roads in our understanding of DUB mechanism and structure, but with every answer come many new questions as to how the complexity of Ub signaling is controlled. As our appreciation of the in vivo Ub code expands, so must our toolset for biochemical and structural studies in vitro. We now know that Ub can be coated by additional posttranslational modifications such as UBLs, phosphorylation, or acetylation (Swatek & Komander, 2016) and that Ub modification of other proteins is not limited to their Lys residues but can also be attached through the amino-terminus or Cys and Ser residue side chains (McDowell & Philpott, 2013). DUB substrates with Ub linked to the target protein in these ways are becoming available (Huguenin-Dezot et al., 2016; Sun, Meledin, Mali, & Brik, 2018) or are on the horizon, and these will undoubtedly reveal fascinating new details of DUB specificity and function.

References

Abdul Rehman, S. A., Kristariyanto, Y. A., Choi, S.-Y., Nkosi, P. J., Weidlich, S., Labib, K., et al. (2016). MINDY-1 Is a member of an evolutionarily conserved and structurally distinct new family of deubiquitinating enzymes. *Molecular Cell*, *63*, 146–155.

Akutsu, M., Ye, Y., Virdee, S., Chin, J. W., & Komander, D. (2011). Molecular basis for ubiquitin and ISG15 cross-reactivity in viral ovarian tumor domains. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 2228–2233.

Ambaye, N., Chen, C.-H., Khanna, S., Li, Y.-J., & Chen, Y. (2018). Streptonigrin inhibits SENP1 and reduces the protein level of hypoxia-inducible factor 1α (HIF1α) in cells. *Biochemistry*, *57*, 1807–1813.

Bavikar, S. N., Spasser, L., Haj-Yahya, M., Karthikeyan, S. V., Moyal, T., Ajish Kumar, K. S., et al. (2011). Chemical synthesis of ubiquitinated peptides with varying lengths and types of ubiquitin chains to explore the activity of deubiquitinases. *Angewandte Chemie*, *124*, 782–787.

Békés, M., Rut, W., Kasperkiewicz, P., Mulder, M. P. C., Ovaa, H., Drag, M., et al. (2015). SARS hCoV papain-like protease is a unique Lys48 linkage-specific di-distributive deubiquitinating enzyme. *The Biochemical Journal*, *468*, 215–226.

Békés, M., van der Heden van Noort, G. J., Ekkebus, R., Ovaa, H., Huang, T. T., & Lima, C. D. (2016). Recognition of Lys48-linked Di-ubiquitin and deubiquitinating activities of the SARS coronavirus papain-like protease. *Molecular Cell*, *62*, 572–585.

Bingol, B., Tea, J. S., Phu, L., Reichelt, M., Bakalarski, C. E., Song, Q., et al. (2014). The mitochondrial deubiquitinase USP30 opposes parkin-mediated mitophagy. *Nature*, *510*, 370–375.

Borodovsky, A., Kolli, N., Gan-Erdene, T., & Ploegh, H. L. (2002). Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family. *Chemistry & Biology*, *9*, 1149–1159.

Ekkebus, R., Flierman, D., Geurink, P. P., & Ovaa, H. (2014). Catching a DUB in the act: Novel ubiquitin-based active site directed probes. *Current Opinion in Chemical Biology*, *23*, 63–70.

Ekkebus, R., van Kasteren, S. I., Kulathu, Y., Scholten, A., Berlin, I., Geurink, P. P., et al. (2013). On terminal alkynes that can react with active-site cysteine nucleophiles in proteases. *Journal of the American Chemical Society*, *135*, 2867–2870.

Flierman, D., van der Heden van Noort, G. J., Ekkebus, R., Geurink, P. P., Mevissen, T. E. T., Hospenthal, M. K., et al. (2016). Non-hydrolyzable diubiquitin probes reveal linkage-specific reactivity of deubiquitylating enzymes mediated by S2 pockets. *Cell Chemical Biology*, *23*, 472–482.

Gavory, G., O'Dowd, C. R., Helm, M. D., Flasz, J., Arkoudis, E., Dossang, A., et al. (2018). Discovery and characterization of highly potent and selective allosteric USP7 inhibitors. *Nature Chemical Biology*, *14*, 118–125.

Gersch, M., Gladkova, C., Schubert, A. F., Michel, M. A., Maslen, S., & Komander, D. (2017). Mechanism and regulation of the Lys6-selective deubiquitinase USP30. *Nature Structural & Molecular Biology*, *24*, 920–930.

Geurink, P. P., El Oualid, F., Jonker, A., Hameed, D. S., & Ovaa, H. (2012). A general chemical ligation approach towards isopeptide-linked ubiquitin and ubiquitin-like assay reagents. *Chembiochem: A European Journal of Chemical Biology*, *13*, 293–297.

Geurink, P. P., van Tol, B. D. M., van Dalen, D., Brundel, P. J. G., Mevissen, T. E. T., Pruneda, J. N., et al. (2016). Development of diubiquitin-based FRET probes to quantify ubiquitin linkage specificity of deubiquitinating enzymes. *Chembiochem: A European Journal of Chemical Biology*, *17*, 816–820.

Gopinath, P., Ohayon, S., Nawatha, M., & Brik, A. (2016). Chemical and semisynthetic approaches to study and target deubiquitinases. *Chemical Society Reviews*, *45*, 4171–4198.

Haahr, P., Borgermann, N., Guo, X., Typas, D., Achuthankutty, D., Hoffmann, S., et al. (2018). ZUFSP deubiquitylates K63-linked polyubiquitin chains to promote genome stability. *Molecular Cell*, *70*, 165–174.e6.

Haj-Yahya, N., Hemantha, H. P., Meledin, R., Bondalapati, S., Seenaiah, M., & Brik, A. (2014). Dehydroalanine-based diubiquitin activity probes. *Organic Letters*, *16*, 540–543.

Hermanns, T., Pichlo, C., Woiwode, I., Klopffleisch, K., Witting, K. F., Ovaa, H., et al. (2018). A family of unconventional deubiquitinases with modular chain specificity determinants. *Nature Communications*, *9*, 799.

Hewings, D. S., Flygare, J. A., Bogyo, M., & Wertz, I. E. (2017). Activity-based probes for the ubiquitin conjugation-deconjugation machinery: New chemistries, new tools, and new insights. *The FEBS Journal*, *284*, 1555–1576.

Hewings, D. S., Heideker, J., Ma, T. P., AhYoung, A. P., El Oualid, F., Amore, A., et al. (2018). Reactive-site-centric chemoproteomics identifies a distinct class of deubiquitinase enzymes. *Nature Communications*, *9*, 1162.

Hologne, M., Cantrelle, F.-X., Riviere, G., Guillière, F., Trivelli, X., & Walker, O. (2016). NMR reveals the interplay among the AMSH SH3 binding motif, STAM2, and Lys63-linked diubiquitin. *Journal of Molecular Biology*, *428*, 4544–4558.

Huang, X., & Dixit, V. M. (2016). Drugging the undruggables: Exploring the ubiquitin system for drug development. *Cell Research*, *26*, 484–498.

Huang, O. W., Ma, X., Yin, J., Flinders, J., Maurer, T., Kayagaki, N., et al. (2012). Phosphorylation-dependent activity of the deubiquitinase DUBA. *Nature Structural & Molecular Biology*, *19*, 171–175.

Huguenin-Dezot, N., De Cesare, V., Peltier, J., Knebel, A., Kristaryianto, Y. A., Rogerson, D. T., et al. (2016). Synthesis of isomeric phosphoubiquitin chains reveals that phosphorylation controls deubiquitinase activity and specificity. *Cell Reports*, *16*, 1180–1193.

Iphöfer, A., Kummer, A., Nimtz, M., Ritter, A., Arnold, T., Frank, R., et al. (2012). Profiling ubiquitin linkage specificities of deubiquitinating enzymes with branched ubiquitin isopeptide probes. *Chembiochem: A European Journal of Chemical Biology*, *13*, 1416–1420.

James, T. W., Frias-Staheli, N., Bacik, J.-P., Levingston Macleod, J. M., Khajehpour, M., García-Sastre, A., et al. (2011). Structural basis for the removal of ubiquitin and interferon-stimulated gene 15 by a viral ovarian tumor domain-containing protease. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 2222–2227.

Kategaya, L., Di Lello, P., Rougé, L., Pastor, R., Clark, K. R., Drummond, J., et al. (2017). USP7 small-molecule inhibitors interfere with ubiquitin binding. *Nature*, *550*, 534–538.

Keusekotten, K., Elliott, P. R., Glockner, L., Fiil, B. K., Damgaard, R. B., Kulathu, Y., et al. (2013). OTULIN antagonizes LUBAC signaling by specifically hydrolyzing Met1-linked polyubiquitin. *Cell*, *153*, 1312–1326.

Komander, D., & Rape, M. (2012). The ubiquitin code. *Annual Review of Biochemistry*, *81*, 203–229.

Kwasna, D., Abdul Rehman, S. A., Natarajan, J., Matthews, S., Madden, R., De Cesare, V., et al. (2018). Discovery and characterization of ZUFSP/ZUP1, a distinct deubiquitinase class important for genome stability. *Molecular Cell*, *70*, 150–164.e6.

Lamberto, I., Liu, X., Seo, H.-S., Schauer, N. J., Iacob, R. E., Hu, W., et al. (2017). Structure-guided development of a potent and selective non-covalent active-site inhibitor of USP7. *Cell Chemical Biology*, *24*, 1490–1500.e11.

Li, G., Liang, Q., Gong, P., Tencer, A. H., & Zhuang, Z. (2014). Activity-based diubiquitin probes for elucidating the linkage specificity of deubiquitinating enzymes. *Chemical Communications*, *50*, 216–218.

Lingaraju, G. M., Bunker, R. D., Cavadini, S., Hess, D., Hassiepen, U., Renatus, M., et al. (2014). Crystal structure of the human COP9 signalosome. *Nature*, *512*, 161–165.

McDowell, G. S., & Philpott, A. (2013). Non-canonical ubiquitylation: Mechanisms and consequences. *The International Journal of Biochemistry & Cell Biology*, *45*, 1833–1842.

McGouran, J. F., Gaertner, S. R., Altun, M., Kramer, H. B., & Kessler, B. M. (2013). Deubiquitinating enzyme specificity for ubiquitin chain topology profiled by di-ubiquitin activity probes. *Chemistry & Biology*, *20*, 1447–1455.

Mevissen, T. E. T., Hospenthal, M. K., Geurink, P. P., Elliott, P. R., Akutsu, M., Arnaudo, N., et al. (2013). OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis. *Cell*, *154*, 169–184.

Mevissen, T. E. T., & Komander, D. (2017). Mechanisms of deubiquitinase specificity and regulation. *Annual Review of Biochemistry*, *86*, 159–192.

Mevissen, T. E. T., Kulathu, Y., Mulder, M. P. C., Geurink, P. P., maslen, S. L., Gersch, M., et al. (2016). Molecular basis of Lys11-polyubiquitin specificity in the deubiquitinase Cezanne. *Nature*, *538*, 402–405.

Michel, M. A., Komander, D., & Elliott, P. R. (2018). Enzymatic assembly of ubiquitin chains. *Methods in Molecular Biology*, *1844*, 73–84.

Misaghi, S., Balsara, Z. R., Catic, A., Spooner, E., Ploegh, H. L., & Starnbach, M. N. (2006). Chlamydia trachomatis-derived deubiquitinating enzymes in mammalian cells during infection. *Molecular Microbiology*, *61*, 142–150.

Morgan, M. T., Haj-Yahya, M., Ringel, A. E., Bandi, P., Brik, A., & Wolberger, C. (2016). Structural basis for histone H2B deubiquitination by the SAGA DUB module. *Science*, *351*, 725–728.

Morrow, M. E., Morgan, M. T., Clerici, M., Growkova, K., Yan, M., Komander, D., et al. (2018). Active site alanine mutations convert deubiquitinases into high-affinity ubiquitin-binding proteins. *EMBO Reports*, *19,* e45680.

Mulder, M. P. C., El Oualid, F., ter Beek, J., & Ovaa, H. (2014). A native chemical ligation handle that enables the synthesis of advanced activity-based probes: Diubiquitin as a case study. *Chembiochem: A European Journal of Chemical Biology*, *15*, 946–949.

Pozhidaeva, A., Valles, G., Wang, F., Wu, J., Sterner, D. E., Nguyen, P., et al. (2017). USP7-specific inhibitors target and modify the enzyme's active site via distinct chemical mechanisms. *Cell Chemical Biology*, *24*, 1501–1512.e5.

Pruneda, J. N., Durkin, C. H., Geurink, P. P., Ovaa, H., Santhanam, B., Holden, D. W., et al. (2016). The molecular basis for ubiquitin and ubiquitin-like specificities in bacterial effector proteases. *Molecular Cell*, *63*, 261–276.

Rape, M. (2018). Ubiquitylation at the crossroads of development and disease. *Nature Reviews. Molecular Cell Biology*, *19*, 59–70.

Reverter, D., & Lima, C. D. (2006). Structural basis for SENP2 protease interactions with SUMO precursors and conjugated substrates. *Nature Structural & Molecular Biology*, *13*, 1060–1068.

Ritorto, M. S., Ewan, R., Perez-Oliva, A. B., Knebel, A., Buhrlage, S. J., Wightman, M., et al. (2014). Screening of DUB activity and specificity by MALDI-TOF mass spectrometry. *Nature Communications*, *5*, 4763.

Rivkin, E., Almeida, S. M., Ceccarelli, D. F., Juang, Y.-C., MacLean, T. A., Srikumar, T., et al. (2013). The linear ubiquitin-specific deubiquitinase gumby regulates angiogenesis. *Nature*, *498*, 318–324.

Sato, Y., Goto, E., Shibata, Y., Kubota, Y., Yamagata, A., Goto-Ito, S., et al. (2015). Structures of CYLD USP with Met1- or Lys63-linked diubiquitin reveal mechanisms for dual specificity. *Nature Structural & Molecular Biology*, *22*, 222–229.

Sato, Y., Okatsu, K., Saeki, Y., Yamano, K., Matsuda, N., Kaiho, A., et al. (2017). Structural basis for specific cleavage of Lys6-linked polyubiquitin chains by USP30. *Nature Structural & Molecular Biology*, *24*, 911–919.

Sun, H., Meledin, R., Mali, S. M., & Brik, A. (2018). Total chemical synthesis of ester-linked ubiquitinated proteins unravels their behavior with deubiquitinases. *Chemical Science*, *9*, 1661–1665.

Swatek, K. N., Aumayr, M., Pruneda, J. N., Visser, L. J., Berryman, S., Kueck, A. F., et al. (2018). Irreversible inactivation of ISG15 by a viral leader protease enables alternative infection detection strategies. *Proceedings of the National Academy of Sciences of the United States of America*, *115*, 2371–2376.

Swatek, K. N., & Komander, D. (2016). Ubiquitin modifications. *Cell Research*, *26*, 399–422.

Turnbull, A. P., Ioannidis, S., Krajewski, W. W., Pinto-Fernandez, A., Heride, C., Martin, A. C. L., et al. (2017). Molecular basis of USP7 inhibition by selective small-molecule inhibitors. *Nature*, *550*, 481–486.

van Tilburg, G. B., Elhebieshy, A. F., & Ovaa, H. (2016). Synthetic and semi-synthetic strategies to study ubiquitin signaling. *Current Opinion in Structural Biology*, *38*, 92–101.

Wang, T., Yin, L., Cooper, E. M., Lai, M.-Y., Dickey, S., Pickart, C. M., et al. (2009). Evidence for bidentate substrate binding as the basis for the K48 linkage specificity of otubain 1. *Journal of Molecular Biology*, *386*, 1011–1023.

Weber, A., Elliott, P. R., Pinto-Fernandez, A., Bonham, S., Kessler, B. M., Komander, D., et al. (2017). A linear diubiquitin-based probe for efficient and selective detection of the deubiquitinating enzyme OTULIN. *Cell Chemical Biology*, *24*, 1299–1313.e7.

Wilkinson, K. D., Gan-Erdene, T., & Kolli, N. (2005). Derivitization of the C-terminus of ubiquitin and ubiquitin-like proteins using intein chemistry: Methods and uses. *Methods in Enzymology*, *399*, 37–51.

Yao, T., Jin, J., Cai, Y., Takahashi, H., Swanson, S. K., Washburn, M. P., et al. (2008). Distinct modes of regulation of the Uch37 deubiquitinating enzyme in the proteasome and in the Ino80 chromatin-remodeling complex. *Molecular Cell*, *31*, 909–917.

Ye, Y., Akutsu, M., Reyes-Turcu, F., Enchev, R. I., Wilkinson, K. D., & Komander, D. (2011). Polyubiquitin binding and cross-reactivity in the USP domain deubiquitinase USP21. *EMBO Reports*, *12*, 350–357.

Ye, Y., Blaser, G., Horrocks, M. H., Ruedas-Rama, M. J., Ibrahim, S., Zhukov, A. A., et al. (2012). Ubiquitin chain conformation regulates recognition and activity of interacting proteins. *Nature*, *492*, 266–270.