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1) Leestemaker et al. Methods in Mol. Biology **2017**, *1491*, 113
2) Geurink et al. Methods in Enzymology **2019**, *618*, 357
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Chapter 9

Profiling the Activity of Deubiquitinating Enzymes Using Chemically Synthesized Ubiquitin-Based Probes

Yves Leestemaker, Annemieke de Jong, and Huib Ovaa

Abstract

Deubiquitinating enzymes (DUBs) are of interest as potential new targets for pharmacological intervention. Active-site-directed probes can be used for the accurate profiling of DUB activity as well as the identification of DUBs and DUB inhibitor selectivity. Previously, active-site directed DUB probes have been obtained using intein-based methods that have inherent limitations. Total chemical synthesis of ubiquitin allows for easy incorporation of different tags, such as fluorescent reporters, affinity tags, and cleavable linkers. Here, we describe the total chemical synthesis of a fluorescent active-site directed DUB probe, which facilitates fast, in-gel detection of active DUBs and circumvents the use of Western blot analysis. In addition, an in-gel activity-based DUB profiling assay is described in detail, in which the fluorescent DUB probe is used to visualize active DUBs in cell lysates. Finally, an inhibition assay is described in which the fluorescent probe is used to determine the specificity and potency of a small molecule DUB inhibitor.

Key words Activity-based protein profiling, Deubiquitinating enzymes, Fluorescent probes, Solid-phase synthesis, Ubiquitin

1 Introduction

Ubiquitin (Ub) is a 76-amino acid regulatory protein involved in the regulation of many cellular processes, such as proteasomal protein degradation, DNA repair, and cell cycle regulation. Posttranslational modification of proteins with ubiquitin is performed by the consecutive actions of Ub ligases from three different classes [1]. Mono-ubiquitination or poly-ubiquitination can affect proteins in many different ways, such as tagging proteins for degradation by the proteasome, altering protein localization, affecting protein activity, and promoting or preventing protein–protein interactions [1]. In contrast to ubiquitin ligases, deubiquitinating enzymes (DUBs) remove Ub from substrate proteins. Approximately 100 DUBs are encoded in the human genome and these can be divided into five distinct classes: four classes of cysteine proteases, and one metalloprotease class [2]. DUBs are key

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regulators of important cellular functions [3]. The inhibition of a specific DUB might highly selectively affect the function, localization, or stability of a specific set of proteins, which may be of interest for therapy of human diseases in which ubiquitin-dependent physiological processes are deregulated [4]. Therefore, suitable assay reagents to study DUB activity are valuable research tools and can contribute to identifying DUB inhibitors, which could possibly have future therapeutic applications [5, 6].

Current assay reagents that are commonly used to study DUB activity include activity-based probes [7–11], fluorogenic substrates [12–14] such as Ub-7-amino-4-methylcoumarin and Ub-rhodamine, fluorescence polarization reagents [15–17], ubiquitinated proteins/peptides [16], and hydrolyzable and non-hydrolyzable [18, 19] ubiquitin linkage-specific reagents, such as diubiquitin or polyubiquitin [17, 20, 21]. The advantage of activity-based DUB probes over the other methods is the ability to monitor the activity of multiple DUBs separately in a single experiment. In addition, DUB probes have successfully been used to identify novel DUBs in both eukaryotes and in a wide range of pathogens directly from lysates [7, 14, 22, 23]. Classical activity-based DUB probes are based on the sequence of Ub as the DUB-targeting motif and comprise a reactive C-terminal warhead such as vinyl methyl ester (VME), and an N-terminal epitope tag (Fig. 1a) [7, 24, 25]. These probes react with the active-site cysteine residue that is present in most DUBs, thereby forming a covalent bond between the probe

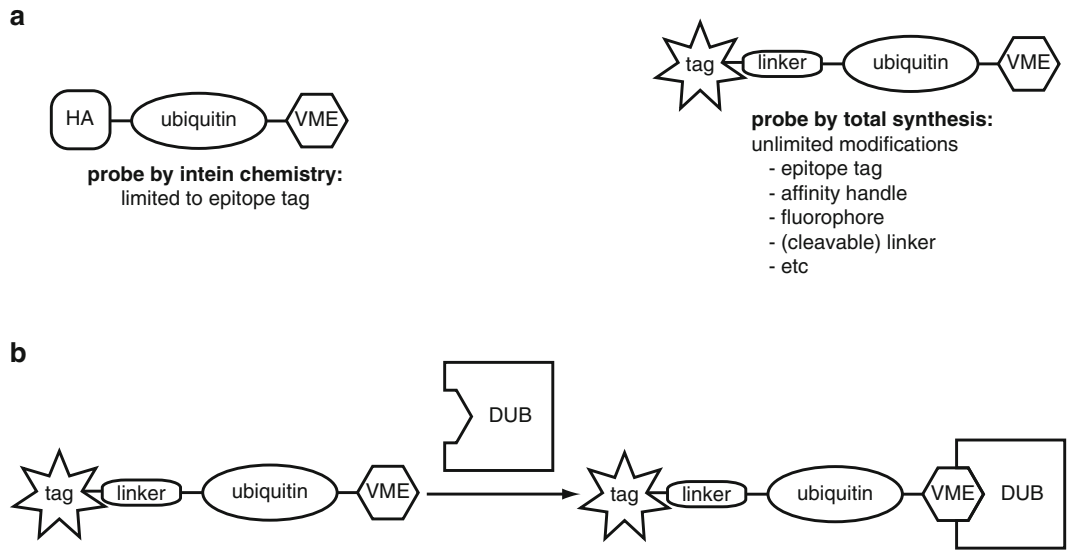

Fig. 1 (**a**) The differences between classically prepared DUB probes versus chemically synthesized probes. Classically prepared probes are largely limited to incorporation of natural amino acids, whereas chemically synthesized probes, a large variety of building blocks can be incorporated. (**b**) Ub-based probes react with the cysteine present in the active site of the majority of DUBs, forming a covalent bond

and the DUB (Fig. 1b). After probe labeling, antibodies against the HA epitope tag are used for the detection of labeled DUBs by western blot analysis or for the immunoprecipitation of labeled DUBs for identification purposes [7]. Despite their usefulness, classical activity-based probes are made using intein-based expression methods, which have inherent limitations, such as the difficulty to obtain the probes in scalable amounts and the limitation to versions with an expressed epitope tag, although recent publications show modification of the Ub-sequence by genetic code expansion methods [26]. In contrast, when Ub-based activity probes are chemically synthesized, these limitations can be overcome. Using the previously reported total linear synthesis of Ub [27], convenient control is allowed over additional moieties that can be incorporated in the Ub protein sequence, such as fluorescent dyes, affinity handles (such as epitope tags or biotin), chemical spacers, and cleavable linkers, while on the C-terminal an active-site directed moiety can be selectively coupled (Figs. 1a and 2).

To demonstrate that DUB probes prepared by total synthesis can be successfully used for labeling DUBs, we chemically synthesized HA-tagged UbVME, making use of the previously reported total linear synthesis of Ub [27]. Subsequently, we incubated EL4 (Murine Thymic Lymphoma) cell extract with increasing concentrations of both chemically synthesized and classically prepared DUB probe HAUbVME. DUB activity was visualized by immunoblotting, showing that the DUB labeling profile for labeling cell extract using chemically synthesized HAUbVME was almost identical to that obtained with the classically prepared probe. This demonstrates that DUB probes prepared by total synthesis can be

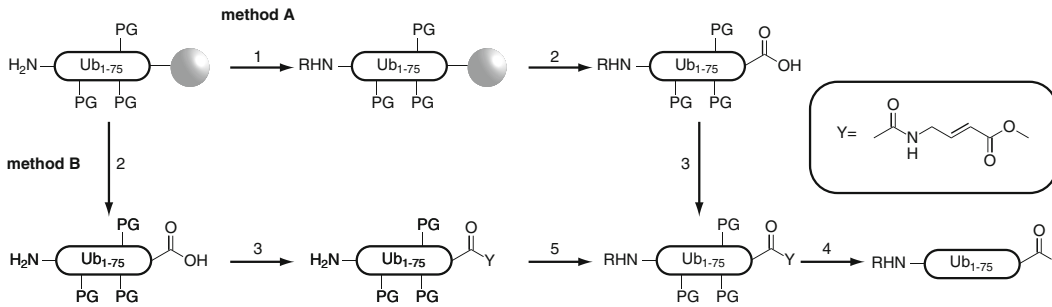

Fig. 2 Synthesis scheme: two reaction pathways to chemically synthesize active-site directed Ub-based DUB probes. The ubiquitin sequence is built up on solid-phase using Fmoc-based solid-phase synthesis. Using method A, building blocks of choice, with side-chains protected, are coupled to the N-terminus of ubiquitin on solid-phase, after which the C-terminal VME warhead is coupled in solution. When the building block to be coupled to the N-terminus of Ub contains an extra free carboxylic acid, as in 5-carboxytetramethylrhodamine (TMR) for the synthesis of TMRUbVME, method B should be followed. Using this method, the VME warhead is coupled to the C-terminus of Ub in solution first, followed by coupling of TMR (or another building block of choice) to the N-terminus of Ub in solution, so that the VME warhead is not coupled to the other carboxylic acid of TMR

successfully used for labeling DUBs in vitro [8, 24]. Subsequent replacement of the HA-epitope tag by the fluorophore 5-carboxytetramethylrhodamine (TMR), yielded the fluorescent DUB activity probe TMRUbVME, allowing for direct in-gel scanning of the SDS-PAGE gel for fluorescence emission of labeled DUBs. Direct fluorescence imaging of SDS-PAGE gels is a more rapid method compared to immunoblotting and is not accompanied with unspecific background labeling signal caused by antibody cross-reactivity. A typical SDS-PAGE-based activity profiling experiment is shown in Fig. 3, in which EL4 cell extract was incubated with increasing concentrations of TMRUbVME and proteins were subsequently resolved by SDS-PAGE. The resulting gel was imaged for fluorescence emission using a fluorescence scanner to visualize fluorescently labeled DUBs. Similar DUB labeling profiles were obtained using the fluorescent probe, compared to the earlier reported HAUbVME probe, demonstrating that similar reactivity towards the DUBs in cell extract was observed for both HAUbVME and TMRUbVME. Compared to HAUbVME, higher resolution results were obtained using TMRUbVME, although resolution could be increased when fluorescent secondary antibodies for western blotting were used. Furthermore, compared to HAUbVME, additional bands were observed for TMRUbVME, indicating the greater sensitivity provided by the latter reagent [8].

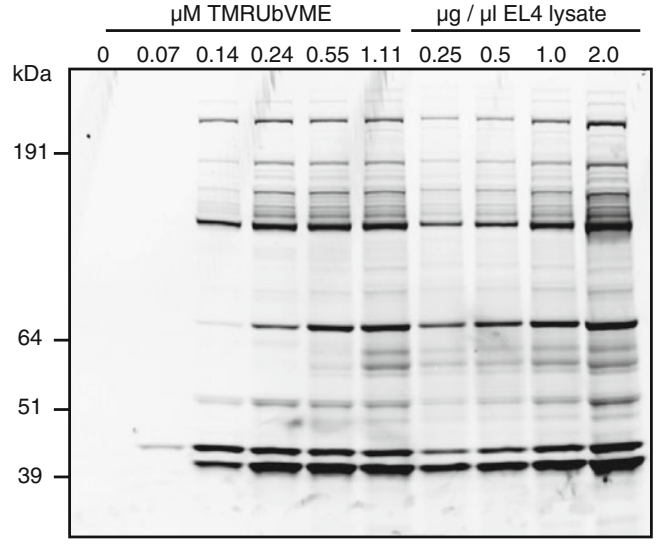

Fig. 3 EL4 lysate was incubated with the indicated concentrations of TMRUbVME (*left*) or different amounts of EL4 lysate were incubated with 1 μM TMRUbVME (*right*). Proteins were separated by SDS-PAGE and the residual DUB activity was visualized by in-gel fluorescence scanning (λ (ex/em) = 550/590). When increasing concentrations of TMRUbVME probe are used, more DUBs are labeled. Using increasing lysate concentrations do not seem to influence the number of labeled DUBs; however, the DUBs that are labeled are better visible

DUB activity profiling can be used to visualize a subset of the most active or abundant DUBs present in a cell extract simultaneously. Therefore, DUB activity probes can be used to test the potency and selectivity of DUB inhibitors [10, 28] in a competition assay, which can find application in the identification of specific DUB inhibitors. A typical DUB inhibitor-profiling assay is shown in Fig. 4. Lysates of EL4 cells were incubated with a series of concentrations of the known DUB inhibitor WP1130 [28, 29] ranging from 1.6 to 200 μM and subsequently incubated with TMRUbVME to label active DUBs. Inhibition of the activity of a DUB results in a disappearance of the respective fluorescent band on the gel, as the probe can no longer bind to this DUB. Since there is a lot of variety between the active sites of DUBs, DUB inhibitors can have different selectivity for the different DUBs present in a cell. Using this probe, the selectivity and potency of DUB inhibitors can be investigated. Cell biological and genetic manipulation of DUBs, such as knockdown or overexpression of specific DUBs, can also be monitored using DUB activity probes [8]. Using western blot analysis of specific DUBs or their tags, the proportion of reacted enzyme can be determined quantitatively In addition, the probe can be used to

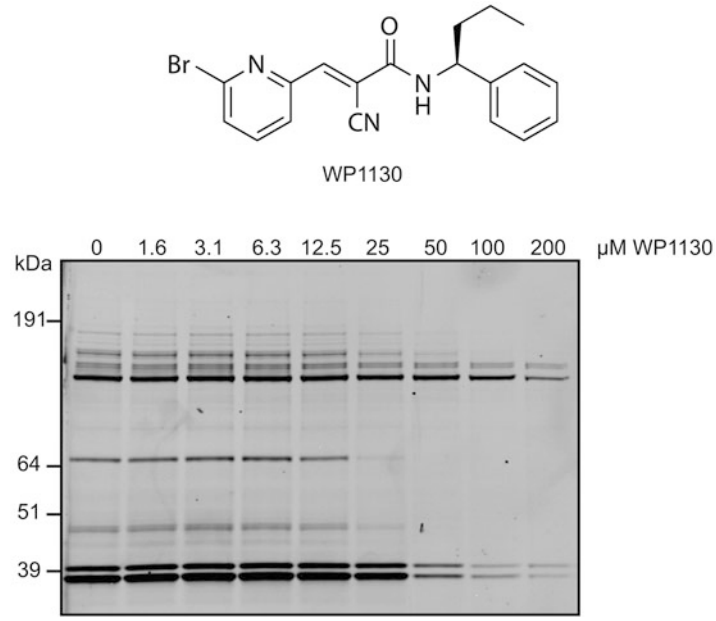

Fig. 4 *Top*: chemical structure of WP1130. *Bottom*: EL4 lysate was incubated with the indicated concentrations of small-molecule DUB inhibitor WP1130 [19, 20]. Subsequently, lysate was labeled with DUB activity probe. Proteins were separated by SDS-PAGE and the residual DUB activity was visualized by in-gel fluorescence scanning (λ (ex/em) = 550/590). Increasing concentrations of WP1130 results in less visible bands, indicating inhibition of these DUBs by WP1130

visualize differential DUB activity profiles in a variety of cell lines, which is very cell line dependent [8]. When an affinity tag, such as an epitope tag, poly-histidine tag or biotin tag, in combination with a cleavable moiety is incorporated in the DUB probe, the resulting probe enables the affinity catch-and-release of DUBs of interest, facilitating identification [8].

This chapter covers two methods for the total chemical synthesis of activity-based DUB probes, dependent on the reactive moieties present in the building blocks of choice. In the first method the building blocks of choice are coupled to the N-terminus of Ub on solid phase, after which the VME warhead is coupled to the N-terminus of Ub in solution. When the building block of choice to be coupled to the N-terminus of Ub contains an extra free carboxylic acid, such as in TMR, a different method should be used. In this method both the VME warhead and the desired N-terminal tag are coupled sequentially in solution. In addition, optimized SDS-PAGE procedures for profiling DUB activity in cell lysate using the fluorescent DUB activity probe TMRUbVME are described, including the assessment of small-molecule DUB inhibitor specificities.

2 Materials

2.1 General Materials

All reagents used in this protocol were purchased from Biosolve (Valkenswaard, The Netherlands) or Sigma Aldrich (Zwijndrecht, The Netherlands), unless otherwise indicated, at the highest commercially available grade. All chemicals and solvents were used as received. Peptide building blocks were all l-stereoisomers and purchased from Novabiochem (EMD Millipore).

2.2 Total Chemical Synthesis of Ubiquitin-Based DUB Activity Probes

2.2.1 Synthesis of Ub (1–75) (Scheme 1, See Notes 1 and 2)

1. TentaGel R TRT-Gly Fmoc (Rapp Polymere, Tübingen, Germany); Fmoc-protected and side-chain protected natural amino acids: Fmoc-Ala-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-His(1-Trt)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Met-OH, Fmoc-Asn(Trt)-OH, Fmoc-Pro-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH, Fmoc-Tyr(tBu)-OH; pseudoproline building blocks: Fmoc-L-Leu-L-Thr(ψMe,Mepro)-OH, Fmoc-L-Ile-L-Thr(ψMe, Mepro)-OH, Fmoc-L-Leu-L-Ser(ψMe,Mepro)-OH, Fmoc-L-Ser(tBu)-L-Thr(ψMe,Mepro)-OH; Dmb dipeptides: Fmoc-L-Ala-(Dmb)Gly-OH, Fmoc-L-Asp(OtBu)-(Dmb) Gly-OH; piperidine; *N*-methyl-2-pyrrolidone (NMP); *N*,*N*-diisopropylethylamine (DIPEA); benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop).
2. Diethyl ether.

2.2.2 Method A (Scheme 1, See Note 1)

1. *See* Subheading 2.2.1.
2. Piperidine, NMP, DIPEA, Pybop, peptide building blocks of choice (*see* **Note 2**).
3. Dichloromethane (DCM), 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP).
4. (E)-methyl-4-aminobut-2-enoate (glycine vinyl methyl ester, GlyVME, synthesized according to established procedures [25, 30], PyBOP, triethyl amine, DCM.
5. $KHSO_4$.
6. Na_2SO_4.
7. Trifluoroacetic acid, triisopropylsilane, MilliQ water (MQ).
8. Pentane, diethyl ether.
9. MQ, acetonitrile, acetic acid.

2.2.3 Method B (Scheme 1, See Note 1), for the Synthesis of TMRUbVME

1. *See* Subheading 2.2.1.
2. DCM, HFIP.
3. GlyVME, DCM, PyBOP, triethyl amine.
4. $KHSO_4$.
5. Na_2SO_4.
6. 5-carboxytetramethylrhodamine (TMR, synthesized according to established procedures [31]) or other building block of choice containing free carboxylic acids, PyBOP, DIPEA, DCM.

2.3 Profiling of DUB Activity Using SDS-PAGE Based Assays

2.3.1 Cell Harvesting and Lysis

1. Cell line of choice, cultured in appropriate medium, e.g., RPMI 1640 (Roswell Park Memorial Institute) medium for suspension cell lines and DMEM (Dulbecco's modified Eagle's medium) for adherent cell lines, supplemented with fetal calf serum (FCS).
2. Stock solution of inhibitor of choice, dissolved in dimethyl sulfoxide (DMSO), aqueous buffer or medium, in the appropriate concentration.
3. Phosphate-buffered saline (PBS), trypsin solution (0.05%, Gibco) for adherent cells, appropriate cell culture medium.
4. HR lysis buffer (*see* **Note 3**): 50 mM Tris–HCl (pH 7.4), 5 mM MgCl2, 250 mM sucrose. Optional supplements (*see* **Note 4**): 0.5% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate), 0.1% NP40, 1 mM DTT (added fresh from a 1 M stock solution before use), 2 mM ATP (added fresh from a 0.5 M stock solution before use), protease inhibitors (e.g., cOmplete protease inhibitor cocktail, Roche). Prepare HR buffer without DTT, ATP, or protease inhibitors, filter over a 0.22 mm filter, and store at 4 °C.
5. Sonication equipment (e.g., Bioruptor, Diagenode).
6. Protein concentration determination assay reagents (e.g., Bio-Rad protein assay).

2.3.2 In Vitro Profiling of DUB Activity in Cell Lysates

1. Cell lysate obtained in Subheading 2.3.1, HR lysis buffer (*see* **Notes 4** and **5**).
2. Stock solution of 0.25 mg/mL TMRUbVME probe in 50 mM sodium acetate (pH 4.5, 5% DMSO) (*see* **Note 6**).
3. 50 mM NaOH.
4. 3× reducing sample buffer (*see* **Note 7**): 4× NuPAGE® LDS Sample Buffer, 2-Mercaptoethanol, MQ (75:17.5:7.5). Store at room temperature.

2.3.3 Assessment of DUB Inhibitor Potency in Cell Lysates Using TMRUbVME

1. Cell lysate obtained in Subheading 2.3.1, HR lysis buffer, supplemented with 0.5% CHAPS and 0.1% NP40 (*see* **Notes 4** and **5**). Optional supplements (*see* **Note 4**): 1 mM DTT (added fresh from a 1 M stock solution before use), 2 mM ATP (added fresh from a 0.5 M stock solution before use), protease inhibitors (e.g., cOmplete protease inhibitor cocktail, Roche). Prepare HR buffer without DTT, ATP, or protease inhibitors, filter over a 0.22 mm filter, and store at 4 °C.
2. 20× stock solution of inhibitor of choice, dissolved in DMSO, aqueous buffer or medium.
3. Stock solution of 0.125 mg/mL TMRUbVME probe in 50 mM sodium acetate (pH 4.5, 5% DMSO) (*see* **Note 6**).
4. 50 mM NaOH.
5. 3× reducing sample buffer (*see* **Note 7**).

2.3.4 Gel Electrophoresis and In-Gel Fluorescence Scanning

1. Precast gel system (NuPAGE, Invitrogen), 4–12% NuPAGE® Novex® Bis-Tris precast protein gel (1.0 mm) (Invitrogen).
2. NuPAGE® MOPS SDS Running buffer (Invitrogen).
3. NuPAGE® Antioxidant (Invitrogen).
4. SeeBlue® Pre-Stained Standard (Invitrogen) (*see* **Note 8**).
5. 3× Reducing sample buffer.
6. Power supply, e.g., PowerPac Basic Power Supply (Bio-Rad).
7. ProXPRESS 2D Proteomic imaging system (Perkin Elmer).
8. TotalLab analysis software.

3 Methods

3.1 General Methods

1. Perform solid phase peptide synthesis on a Syro II MultiSyntech Automated Peptide synthesizer.
2. Perform preparative reverse-phase HPLC purifications on a Prominence HPLC system (Shimadzu) equipped with an Atlantis T3 column, using the following mobile phases: A (TFA (0,1%) in water) and B (formic acid (0,1%) in acetonitrile), the following gradient: 0–5 min 5% B, 5–8 min 5–25%

B, 8–30 min 25–60% B, 30–33 min 60–95% B, 33–35 min 95% B, and the following settings: column temperature: 40 °C; flow-rate: 7.5 mL/min; run-time: 35 min.; UV-detection at 230 and 254 nm.

3. Perform analytical HPLC on a 1525EF Binary HPLC pump (Waters) equipped with a 2487 Dual λ Absorbance Detector. Samples were run over an Atlantis DC18 column (6.4 × 50 mm, 10 mm; Waters) with the following two mobile phases: A (TFA (0.05%) in water) and B (TFA (0.05%) in acetonitrile) using the following gradient: 0–1 min 1% B, 1–13 min, 1–90% B, 13–16 min, 90% B; 16–17 min, 90–1% B, 17–25 min, 1% B; or 0–5 min 5% B, 5–30 min 5–95% B, 30–35 min 95% B, 35–40 min 95–5% B, 40–45 min 5% B.
4. Perform LC-MS measurements on a system equipped with an Alliance 2795 Separation Module (Waters), 2996 Photodiode Array Detector (190–750 nm, Waters) and LCT Orthogonal Acceleration Time of Flight Mass Spectrometer. Run samples over a Kinetex C18 column (2.1 × 50 mm, 2.6 μM, Phenomenex, Torrence, CA), at 0.8 mL/min, for 6 min, at a column temperature of 40 °C, using the following two mobile phases: A (acetonitrile(1%) and formic acid (0.1%), in water) and B (water (1%) and formic acid (0.1%) in acetonitrile), and the following gradient: 0–0.5 min 5% B, 0.5–4 min, 5–95% B, 4–5.5 min, 95% B.
5. Perform data processing using Waters MassLynx Mass Spectrometry Software 4.1 (deconvolution with Maxent1 function, Waters).
6. Optional: Perform preparative cation-chromatography using an ÄKTA Unichromat 1500-"PRO" system (15 × 185 mm column packed with Workbeads 40 S) at 4 °C, using the following two mobile phases: A (50 mM NaOAc, pH 4.5) and B (1 M NaCl in 50 mM NaOAc, pH 4.5) using a flow-rate of 5 mL/min.

3.2 Total Chemical Synthesis of Ubiquitin-Based DUB Activity Probes

3.2.1 Synthesis of Ub (1–75) (Scheme 1, See Note 1)

1. Fmoc-Gly functionalized trityl resin (0.14–0.2 mmol/g) is subjected to standard 9-fluorenylmethoxycarbonyl (Fmoc) based solid phase peptide synthesis to synthesize Ub (1–75) using an automated peptide synthesizer at 25 μmol scale.
2. *Cycles 1–30*: Deprotect the Fmoc group with 20% piperidine in NMP (1 × 5 min., 1 × 10 min., 1 × 5 min.) and wash the resin five times with NMP couple the next amino acid of the Ub sequence (counting from the C-terminus) using Fmoc protected amino acid (4 eq., *see* **Note 2**), DIPEA (8 eq.), and PyBop coupling reagent (4 eq.), for 45 min. and repeat the deprotection, and coupling for every next amino acid in the Ub sequence. After each step, wash the resin two times with NMP.

   Cycles 31–61: Extend the coupling time to 60 min.
   Cycles 62–68: Perform double couplings for all amino acids. Decrease coupling time to 30 min.

Use pseudoproline building blocks Fmoc-L-Leu-L-Thr(ψMe,Mepro)-OH, Fmoc-L-Ile-L-Thr(ψMe,Mepro)-OH, Fmoc-L-Leu-L-Ser(ψMe,Mepro)-OH, Fmoc-L-Ser(tBu)-L-Thr(ψMe,Mepro)-OH and Dmb dipeptides Fmoc-L-Ala-(Dmb)Gly-OH and Fmoc-L-Asp(OtBu)-(Dmb)Gly-OH to replace Leu8-Thr9, Ile13-Thr14, Leu56-Ser57, Ser65-Thr66, Ala46-Gly47, and Asp52-Gly53, respectively, and use single couplings for 120 min. to couple these building blocks.

3. Wash resin three times with DCM, three times with diethyl ether and dry the resin under high vacuum. Store in a dry environment.

3.2.2 Method A (Scheme 1, See Note 1)

1. Synthesize Ub (1–75) as described in Subheading 3.2.1.
2. Perform double couplings to couple the building blocks of choice (e.g., amino acids of the HA tag sequence YPYDVPDYA, 6His-tag, photocleavable or chemocleavable building blocks, fluorophores, and/or biotin) to the N-terminus of the Ub sequence on the resin by using (Fmoc) building block (4 eq. *see* **Note 2**), PyBOP (4 eq.) and DIPEA (8 eq.) in NMP at ambient temperature for 16 h. and similar deprotection reagents and conditions as described in Subheading 3.2.1.
3. Cleave the Ub conjugates from the trityl resin by treating the resin with 5 mL of DCM/HFIP (4:1 v/v) for 30 min and filter the resin. Wash the resin with 3–5 mL DCM, and combine and concentrate the filtrates.
4. Couple GlyVME to the C-terminus of protected Ub conjugate in solution using GlyVME (10 eq.), PyBOP (5 eq.), and triethyl amine (20 eq.) in DCM and stir for 16 h at ambient temperature.
5. Remove excess GlyVME by washing the DCM solution two times with 1 M $KHSO_4$.
6. Dry the organic layer with Na_2SO_4 and concentrate the organic layer to dryness in vacuo.
7. Remove the side chain protecting groups by taking the residue up in 5 mL trifluoroacetic acid–triisopropylsilane–water (95:2.5:2.5) and stir the solution for 3 h. at ambient temperature.
8. Add the mixture to a 50 mL falcon tube containing 40 mL ice-cold pentane/diethyl ether (1:3) to precipitate the Ub conjugate. Isolate the precipitate by centrifugation (1500 × *g*, 6 min. 4 °C) and wash the precipitate by three cycles of resuspension in ice-cold diethyl ether and centrifugation.
9. Take the pellet up in water–acetonitrile–acetic acid (65:25:10), freeze the pellet, and lyophilize the frozen pellet. Purify the activity probe by preparative HPLC. Perform LC-MS measurements to verify the right mass of the activity probe and to check for purity. In Fig. 5, the LC-MS analysis profile is shown for Ub-based DUB probe TMRUbVME.

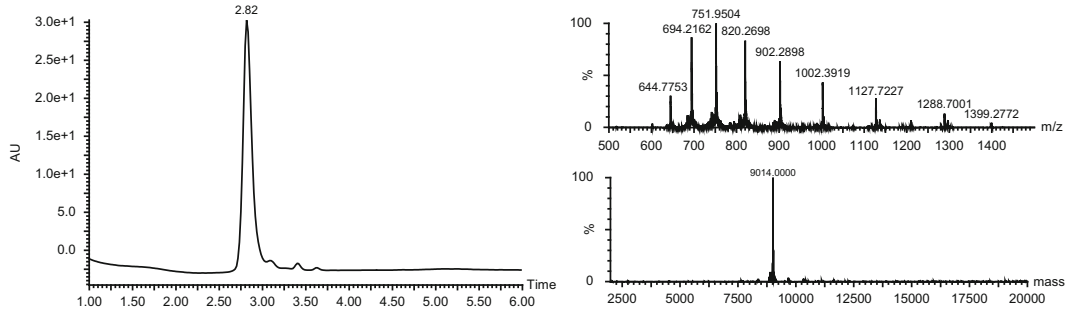

Fig. 5 LC profile of purified TMRUbVME by HPLC (*left*). MS analysis of purified TMRUbVME probe (*top right*) and deconvoluted spectrum (bottom right). Calculated $[M+H]^+$ 9012.8 Da, observed $[M+H]^+$ 9014.0 Da

3.2.3 Method B (Scheme 1, See Note 1), for the Synthesis of TMRUbVME

1. Synthesize Ub (1–75) as described in Subheading 3.2.1.
2. Cleave the Ub conjugates from the trityl resin by treating the resin with 5 mL of DCM/HFIP (4:1 v/v) for 30 min and filter the resin. Wash the resin with 3–5 mL DCM, and combine and concentrate the filtrates.
3. Couple GlyVME to the C-terminus of protected Ub conjugate in solution using GlyVME (10 eq.), PyBOP (5 eq.), and triethyl amine (20 eq.) in DCM and stirred for 16 h at ambient temperature.
4. Remove excess GlyVME by washing the DCM solution two times with 1 M $KHSO_4$.
5. Dry the organic layer with Na_2SO_4 and concentrate the organic layer to dryness in vacuo.
6. Couple TMR (or any other building block of choice containing free carboxylic acids) to the N-terminus of protected Ub conjugate in solution using TMR (4 eq.), PyBOP (4 eq.), and DIPEA (10 eq.) in DCM and stir for 16 h at ambient temperature. Concentrate the organic layer to dryness in vacuo. Proceed with **step 7** of Subheading 3.2.2.

3.3 Profiling of DUB Activity Using SDS-PAGE Based Assays

3.3.1 Cell Harvesting and Lysis

1. Culture cell line of choice in appropriate medium and under appropriate culture conditions. Suspension cells should be cultured until log-phase and adherent cells should be passaged when approximately 80% confluency is reached.
2. Seed cells in a multi well tissue culture plate and allow the cells to attach. Add the compounds to be tested, dissolved in DMSO (or medium if the compounds are water-soluble), in the desired concentrations to the cells. Make sure to have enough wells available to include all of the appropriate controls.
   Critical: the final concentration of DMSO should not exceed 0.5% as this can interfere with the assay.

3. To harvest adherent cells by trypsinization, aspirate the medium, wash cells with PBS and aspirate. Add sufficient trypsin and wait for cells to detach. After the cells have detached, add medium supplemented with 10% FCS to the cells to inactivate the trypsin. Collect cells and pellet by centrifugation at 1300 × *g* for 5 min at 4 °C. To harvest suspension cells, pellet cells by centrifugation at 1300 × *g* for 5 min at 4 °C. Wash cells using 10–20 pellet volumes of PBS and pellet cells again centrifugation at 1300 × *g* for 5 min at 4 °C. Discard the supernatant.
   Pause point: at this time, cell pellets can be snap-frozen in liquid nitrogen and stored at −20 °C until further use (*see* **Note 9**).
4. Resuspend cell pellets in two pellet volumes of cold HR buffer (*see* **Note 3**). Optional (*see* **Note 4**): Supplement the HR lysis buffer with 0.5% CHAPS, 0.1% NP40, 1 mM DTT (add freshly from a 1 M stock solution before use), 2 mM ATP (add freshly from a 0.5 M stock solution before use), and/or protease inhibitors (add freshly, e.g., cOmplete protease inhibitor cocktail, Roche). Keep samples on ice.
5. Lyse cells by sonication using for example a Bioruptor (five cycles of 30 s on and 30 s off).
6. Centrifuge cells at maximum speed for 15 min at 4 °C to remove cell debris. Transfer the supernatant to a fresh Eppendorf tube and determine the protein concentration using for example the Bio-Rad protein assay or a comparable protein assay according to the manufacturer's instructions.
   Pause point: At this point, lysates can be snap-frozen in liquid nitrogen and stored at −20 °C until further use.

To label DUBs directly proceed to Subheading 3.3.2. To determine the effect of DUB inhibitors prior to DUB labeling, proceed to Subheading 3.3.3.

3.3.2 In Vitro Profiling of DUB Activity in Cell Lysates Using Ub-Based DUB Probe TMRUbVME (See **Note 10***)*

1. Add 25 µg of cell lysate to an eppendorf tube and adjust the volume to 22 µL with HR buffer (a final volume of 25 µL and a final protein concentration of 1 mg/mL is obtained after addition of probe/NaOH, **steps 2** and **3**) (*see* **Notes 4** and **5**).
2. Add 1 µL of a 25 µM TMRUbVME solution in sodium acetate buffer (50 mM NaOAc, 5% DMSO, pH 4.5, *see* **Note 6**) to the lysate.
3. Add 2 µL (double the volume compared to volume of probe solution) of 50 mM NaOH solution to adjust for the pH drop after addition of the acidic probe solution to the lysate (*see* **Notes 5** and **6**). Vortex and spin samples briefly. Incubate for 30 min at 37 °C.
4. Add 12,5 µL of a 3× reducing sample buffer (e.g., 4× Invitrogen NuPAGE® LDS Sample Buffer, supplemented with 2-mercaptoethanol and MQ) to the reaction mixture and heat the

samples for 10 min at 70 °C (*see* **Note 7**). Centrifuge at 14,000 × *g* for 1 min at room temperature to spin down condensed water droplets and gently vortex the sample.

Pause point: At this point, reduced and heated samples can be snap-frozen in liquid N2 and stored at −20 °C until further use.

Proceed to Subheading 3.3.4.

3.3.3 *In Vitro Assessment of DUB Inhibitor Potency in Cell Lysates Using TMRUbVME*

1. Add 25 μg of cell lysate to an Eppendorf tube and adjust the volume to 20.75 μL with HR buffer (*see* **Notes 4** and **5**) supplemented with 0,5 % CHAPS, and 0,1 % NP40 (for improved solubility of DUB inhibitors, *see* **Note 4**), so that a final volume of 25 μL and a final protein concentration of 1 mg/mL is obtained after addition of probe/NaOH (**steps 2** and **3**).
   Critical: The presence of 1 mM DTT can improve inhibitory effect. Check beforehand whether the DUB inhibitor of choice is stable in the presence of DTT (*see* **Note 4**).
2. Add 1.25 μL of a 20× stock solution of the desired inhibitor in DMSO. Include a reference sample to which 1.25 μL DMSO, but no inhibitor is added. Vortex and incubate the samples for the desired time period at 37 °C. Typically, samples are incubated for 1 h.
   Critical: The quality of labeling will decrease if more than 5 % DMSO is present in the reaction mixture.
3. Add 1 μL of a 12.5 μM TMRUbVME solution in sodium acetate buffer (50 mM NaOAc, 5 % DMSO, pH 4.5, *see* **Note 6**) to the lysate.
   Critical: When non-covalent inhibitors are used, the use of a lower concentration of TMRUbVME solution and lower probe incubation temperatures are preferred (*see* **Note 11**), compared to standard DUB labeling (Subheading 3.3.2).
4. Add 2 μL (double the volume compared to volume of probe solution) of 50 mM NaOH solution to adjust for the pH drop after addition of the acidic probe solution to the lysate (*see* **Notes 5** and **6**) Vortex and spin samples briefly. Incubate for 5 min at ambient temperature.
5. Add 12.5 μL of a 3× reducing sample buffer to the reaction mixture and heat the samples for 10 min at 70 °C (*see* **Note 7**). Centrifuge at 14,000 × *g* for 1 min at room temperature to spin down condensed water droplets and gently vortex the sample.

   Proceed to Subheading 3.3.4.

3.3.4 *Gel Electrophoresis and In-Gel Fluorescence Scanning*

The following instructions assume the use of the NuPAGE precast gel system and precast protein gels from Invitrogen.

1. Assemble the NuPAGE gel unit using a precast NuPAGE 4–12 % Bis-Tris gel according to the manufacturer's instructions.

2. Add 1× MOPS SDS running buffer to both the inner and outer chamber of the gel unit.
3. Add 125 mL antioxidant to the inner gel chamber to keep the samples in a reduced state.
4. Load 10–30 μL (depending on of the size of the wells) of the reduced and heated samples into the wells of the gel.
   When reduced samples were frozen for storage, heat the samples again for 10 min at 70 °C, centrifuge at 14,000 × *g* for 1 min at room temperature to spin down condensed water droplets, and gently vortex the sample.
   Keep one well free and load this well with 10 μL of prestained protein molecular weight marker (e.g., SeeBlue® Pre-Stained Standard from Invitrogen, *see* **Note 8**).
5. Load 3× reducing sample buffer to remaining empty wells (use a volume of 1/3 of the sample volume).
6. Run the gel at 170–180 V for appropriate time (at least until the blue loading front is no longer visible) using a Power Supply (e.g., PowerPac Basic Power Supply, Bio-Rad).
7. Gently take the gel out from the cassette and image the gel using a fluorescence imager containing appropriate filter settings (λ (ex/em) = 550/590 nm for TMR). For imaging the bands of the protein molecular weight marker SeeBlue® Pre-Stained Standard, image the gel once more using the following filter settings: λ (ex/em) = 625/680 nm.
8. Analyze images using appropriate software.

4 Notes

1. Depending on the reactive moieties present in the building blocks that will be coupled to the N-terminus of Ub, different methods can be used to synthesize Ub-based probes. The Ub sequence is built up from the C-terminus on solid phase using Fmoc-based solid phase synthesis. Method A describes the coupling of building blocks to the N-terminus of Ub directly on solid-phase using Fmoc-based solid phase synthesis, after which the C-terminal warhead is coupled in solution and protecting groups are removed. This applies in case the building blocks that will be coupled to the N-terminus of Ub contain only one free carboxylic acid and other reactive moieties, such as other carboxylic acids and amines, are protected. Method B describes the coupling of the C-terminal warhead to Ub in solution, after which N-terminal building blocks are coupled in solution, followed by deprotection of reactive moieties. Method B applies when the building blocks that will be

coupled to the N-terminus of Ub contain an extra unprotected carboxylic acid. For the synthesis of TMRUbVME method B is used, since 5-carboxytetramethylrhodamine (TMR) contains two unprotected carboxylic acids. If method A would be used, the free-amine containing warhead that should only be coupled to the C-terminus of Ub will be coupled to the other carboxylic acid of TMR as well.

2. Dry all Fmoc-protected amino acid building blocks overnight under high vacuum. Drying removes moisture, as well as traces of acetic acid (or other acids) that are present, which are detrimental for peptide synthesis.
3. Other lysis buffers en methods can be used. Nonetheless, DUB labeling efficiency should be determined experimentally using other lysis buffers and methods.
4. The use of non-supplemented HR lysis buffer in combination with sonication should be sufficient for lysis of the cells. However, the use of additives can increase lysis efficiency and/or labeling efficiency:
   - *Detergents*: Detergents CHAPS and NP40 will improve lysis of cells and increases solubility of DUB inhibitors. However, the use of detergents can decrease the quality of DUB labeling using DUB probe TMRUbVME and should be determined experimentally. Though, the use of 0.5% CHAPS and 0.1% NP40 does not decrease labeling efficiency.
   - *DTT*: DUB inhibitory effect can be increased when DTT is used. However, the effect of DTT on the DUB inhibitor should be examined beforehand. The use of DTT does not affect labeling of DUBs in cell lysates. Add DTT freshly before use, since DTT is not stable in solution.
   - *ATP*: The effect of ATP on labeling is not thoroughly investigated and should be established experimentally.
   - *Protease inhibitors*: The addition of protease inhibitors is recommended, to protect deubiquitinating enzymes in cell lysate from degradation. Labeling efficiency in the presence of protease inhibitors should be tested beforehand. The use of cOmplete protease inhibitor cocktail from Roche did not seem to negatively affect labeling efficiency. Add protease inhibitors freshly, e.g., from a 50× stock solution, before use. Manufacturer's instructions should be checked for storage conditions of stock solutions.
5. Different buffers than HR lysis buffer can be used for the incubation of cell lysate with DUB inhibitors and probe. When stronger incubation buffers are used, possibly the addition of

NaOH, subsequent to the addition of the acidic probe buffer, becomes redundant. The effect of incubation buffers on the inhibition, labeling of DUBs and solubility of DUB inhibitors should be determined experimentally, however. In addition, other reaction volumes and other concentrations can be used. Changing probe and lysate protein concentrations will affect DUB labeling efficiency. We advise to use different conditions at first, to determine optimal labeling conditions.

6. Other buffers can be used to dissolve Ub-based DUB probes. When a buffer of neutral pH is used, the addition of 50 mM NaOH, after the addition of the acidic probe solution, becomes redundant. Probe solubility and labeling efficiency using other buffers should be experimentally established.
7. Heating samples at 70 °C for 10 min is optimal for Invitrogen NuPAGE® LDS Sample Buffer containing buffers. When the NuPAGE precast gel system is used, also NuPAGE LDS Sample buffer (or other recommended buffers) should be used to prepare the 3× reducing sample buffer. The use of a different reducing sample buffer can result in improper running of the gel.
8. Other protein molecular weight marker than SeeBlue® Pre-Stained Standard from Invitrogen can be used. However, molecular weight markers that possess similar fluorescence properties to the dyes used in the DUB probe (in case of TRM: λ (ex/em) = 550/590) could contribute to high signal intensities of these markers during fluorescence imaging.
9. Freezing cell pellets could give rise to differential DUB labeling profiles compared to freshly lysed cells. This should be experimentally determined. Frozen cells cannot be taken into culture again. They will not survive, unless frozen in proper freezing medium.
10. Similar labeling conditions can be used for Ub-based DUB probes containing other dyes (e.g., Cy5) or visualization handles (e.g., HA-tag). Use appropriate fluorescence settings for other dyes or other appropriate visualization methods for other handles (e.g., Western Blotting for HA-tag or Biotin).
11. Since the covalent binding of the TMRUbVME probe to DUBs is very efficient, competition with a non-covalent DUB inhibitor is challenging. When non-covalent inhibitors are used, the use of a lower concentration of TMRUbVME probe, shorter probe incubation times, and lower probe incubation temperatures are preferred, compared to the conditions used for standard DUB labeling (Subheading 3.3.3). In addition, a DUB inhibitor incubation temperature of 37 °C, and long DUB inhibitor incubation times are beneficial.

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CHAPTER SIXTEEN

Profiling DUBs and Ubl-specific proteases with activity-based probes

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Contents

Abstract

Protein (poly-)ubiquitination is a posttranslational modification that plays a key role in almost all cellular processes. It involves the installment of either single ubiquitin (Ub) moieties or one of eight different polyUb linkage types, each giving a distinct cellular outcome. Deubiquitinating enzymes (DUBs) reverse Ub signaling by disassembly of one or multiple poly-Ub chain types and their malfunction is often associated with human disease. The Ub system displays significant crosstalk with structurally homologous ubiquitin-like proteins (Ubls), including SUMO, Nedd8, and ISG15. This can be seen with

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the existence of heterogeneous chains made from Ub–Ubl mixtures as well as the proteolytic cross reactivity displayed by several DUBs toward other Ubl systems. In addition, numerous pathogens have been found to encode Ub(l)-ligases and deconjugating enzymes in order to facilitate infection and fight the host immune response. Studying the activity of DUBs and Ubl-specific proteases, both human as well as pathogen-derived, gives fundamental insights into their physiological roles. Activity-based probes (ABPs) have proven to be valuable tools to achieve this, as they report on enzyme activities by making a (often irreversible) covalent complex, rather than on their relative abundance. In this chapter, we explain the potential of ABPs to assess substrate preferences, structural features, and activity of Ub and Ubl deconjugating enzymes. We further demonstrate the practical use of ABPs to (1) characterize the activity of viral proteases toward Ub and Ubls and (2) to gain more insight in the structural determinants of substrate preference of DUBs.

1. Introduction

Protein ubiquitination is a posttranslational modification that plays a major role in almost all cellular processes in eukaryotes (Hochstrasser, 2009; Komander & Rape, 2012). It involves the covalent attachment of ubiquitin (Ub) via its C-terminal glycine carboxylate to a primary amine of a target protein, generally to a lysine side chain resulting in an isopeptide bond. Ub itself can also be ubiquitinated and as such give rise to polyUb chains. This conjugation occurs at the side chain of one of the seven internal lysine residues (Lys-6, 11, 27, 29, 33, 48, 63), resulting in an isopeptide bond, or at the *N*-terminus (Met-1), resulting in a linear Ub chain, and it has been shown that all eight linkages coexist in cells (Xu et al., 2009).

Counteraction of the build-up of (poly-)ubiquitinated proteins is achieved by a group of deubiquitinating proteases (DUBs) that remove or trim the ubiquitin modification, liberating the substrate protein, recycling Ub and ending the Ub-induced signal. Nearly a hundred genes encoding DUBs have been identified in the human genome, which can be classified in seven distinct families. The subfamilies of ubiquitin-specific proteases (USP), ubiquitin C-terminal hydrolases (UCH), Ovarian TUmor domain proteases (OTU), Machado-Joseph disease proteases (MJD), Motif interacting with ubiquitin-containing novel DUB family (MINDY), and Zinc finger with UFM1-specific peptidase domain protein (ZUFSP) are cysteine proteases, whereas JAB1/MPN/MOV34 proteases (JAMMs) are zinc-dependent metalloproteases (Abdul Rehman et al., 2016; Komander, Clague, & Urbe, 2009; Kwasna et al., 2018; Nijman et al., 2005; Reyes-Turcu, Ventii, & Wilkinson, 2009). As distinct Ub linkages result in distinct biological signals (Komander & Rape, 2012;

Yau & Rape, 2016), the determination of the linkage specificities of DUBs gives fundamental insights into the biological pathways they are involved in. It has been shown that some DUBs, mainly USPs, are able to process all isopeptide linked chains (Faesen et al., 2011) whereas others, especially OTUs, display a preference for one or a few Ub chain types (Mevissen et al., 2013).

Another level of complexity is based on the existence of Ub-like proteins (Ubls) (Kerscher, Felberbaum, & Hochstrasser, 2006). These posttranslational modifiers share structural homology to Ub as well as a highly similar system for conjugation and deconjugation. The most studied examples are the small ubiquitin-like modifiers (SUMO), the neural precursor cell-expressed developmentally downregulated 8 (Nedd8) and interferon-stimulated gene of 15 kDa (ISG15). SUMOylation plays a key role in genome stability, and many of its protein targets are involved in DNA-damage responses (e.g., PCNA and BRCA1) (Flotho & Melchior, 2013). Nedd8 plays an important role in cell cycle control and its main targets are Cullin proteins, which are Ub ligase subunits (Soucy, Smith, & Rolfe, 2009). ISG15 is strongly induced by Type-I interferons as part of the innate immune response to viral and bacterial infections (Zhang & Zhang, 2011). Similar to the Ub system, specific proteases deconjugate Ubls from their targets. These include SENPs acting on SUMO (Hickey, Wilson, & Hochstrasser, 2012), USP18 acting on ISG15 (Malakhov, Malakhova, Kim, Ritchie, & Zhang, 2002) and DENs acting on Nedd8 (Gan-Erdene et al., 2003) and we refer to this group of proteases as Ubl-specific proteases.

Despite their functions in their own respective modification systems, there is growing evidence of crosstalk between Ub and Ubls, increasing the complexity of cellular responses even further. Best studied is the crosstalk between Ub and SUMO signaling, which includes the identification of ubiquitinated SUMO and SUMOylated Ub (Hendriks et al., 2014; Hendriks & Vertegaal, 2016; Nie et al., 2016; Nie & Boddy, 2016). Furthermore, ubiquitinated Nedd8 and crosstalk between Ub and Nedd8 signaling pathways have also been reported (Leidecker, Matic, Mahata, Pion, & Xirodimas, 2012; Singh, Sundar, & Fushman, 2014), as the existence of ISGylated ubiquitin (Fan et al., 2015). However, these so-called heterogeneous chains have so far remained largely unstudied, and their functions remain unknown (Swatek & Komander, 2016). In addition, several DUBs have been found to act on Nedd8 or ISG15 as well (Catic et al., 2007; Gan-Erdene et al., 2003; Geurink, El Oualid, Jonker, Hameed, & Ovaa, 2012; Hjerpe et al., 2012).

Since both the Ub and ISG15 systems are crucial for the innate immune response, many prokaryotic and viral pathogens have evolved ways to hijack

them in order to create a "window-of-opportunity" for efficient replication. Several viral and bacterial proteins have been found to directly target these systems via their deubiquitinating or deISGylating activity (Li, Chai, & Liu, 2016). For example, proteases derived from severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and Crimean-Congo hemorrhagic fever virus (CCHFV) have been shown to deconjugate both Ub and ISG15 in order to suppress innate immune responses (Barretto et al., 2005; Bekes et al., 2016; Frias-Staheli et al., 2007; Lindner et al., 2005; Mielech, Kilianski, Baez-Santos, Mesecar, & Baker, 2014). In contrast to eukaryotic DUBs, proteases encoded by pathogens (bacteria and viruses) often deconjugate more than one type of Ubl. Another example is the CE clan bacterial effector proteases from, for instance, *Rickettsia bellii* and *Chlamydia trachomatis*, which were shown to display both deubiquitinating and deNeddylating activities (Lin & Machner, 2017; Pruneda et al., 2016).

For many human DUBs and Ubl-specific proteases, it has been shown that their malfunction contributes to human disease, including cancer and neurodegenerative disorders (Harrigan, Jacq, Martin, & Jackson, 2018); therefore, tools to study them in detail and on a molecular level are of great interest. Often, proteases are translated as inactive proenzymes, requiring posttranslational activation by their natural regulators. In addition, their activity may be controlled by posttranslational modifications, such as acetylation, phosphorylation, ubiquitination, or methylation. In order to study the role of DUBs in biological processes, it is therefore insufficient to simply monitor the enzyme's abundance by antibody staining, proteomics, or mRNA quantification because this is not necessarily related to a protein's activity (Hewings, Flygare, Bogyo, & Wertz, 2017). A powerful method to visualize enzyme activities in a complex biological setting is the use of Activity-Based Probes (ABPs) (Ovaa, 2007; Verdoes & Verhelst, 2016). ABPs come in many flavors and their design is predominantly determined by their respective protein target(s) and the particular application of the ABP. Generally, ABPs comprise a recognition element, that directs the ABP toward its target, attached to a reactive group (or "warhead") that reacts with the enzyme's active site to form a covalent adduct, either reversible or irreversible, depending on the type of enzyme and reactive group installed (see Fig. 1). The recognition element is designed to resemble structural and functional motifs of the natural substrate of the target, in the form of a short peptide, carbohydrate, nucleoside, or even a small protein. A variety of ABPs has been developed to study the activities of DUBs and these all share

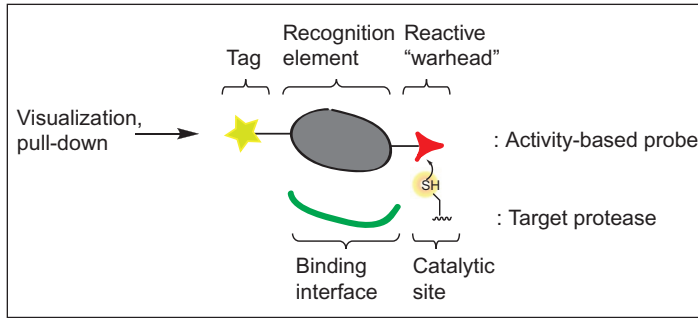

Fig. 1 General design of an activity-based probe (ABP).

a common recognition element derived from full-length Ub. Typically, but not necessarily, an ABP is also equipped with a reporter group, such as a fluorophore, radioactive label or affinity tag, which is used for visualization, purification or identification of the ABP-bound target(s).

These chemical tools are designed in such a way that they only bind to active enzymes covalently but do not react with their inactive counterparts. The application of ABPs is widespread. For example, these tools are commonly used in combination with mass spectrometry, to capture, isolate, and identify active enzymes from cells or cell extracts (Cravatt, Wright, & Kozarich, 2008). In addition, ABPs can be applied to determine the active fraction of a recombinantly expressed and purified enzyme or to study the effect of specific enzyme modifications or mutations with respect to the enzyme's activity and its substrate specificities (Mevissen et al., 2013, 2016). ABPs are also very useful tools for gaining insight into the structural characteristics of an enzyme, where an enzyme–ABP complex mimics a certain state of the reaction between the enzyme and its substrate (Basters et al., 2017; van Tilburg, Elhebieshy, & Ovaa, 2016). Also, by designing and testing different structural variants of an ABP (Flierman et al., 2016; Mulder, El Oualid, ter Beek, & Ovaa, 2014), one can identify preferences of a given enzyme for certain structural features (Bekes et al., 2015, 2016; Mevissen et al., 2016). Finally, since only the active fraction of an enzyme is labeled by the ABP, it is possible to check the inhibitory potential of an inhibitor toward one or multiple enzymes in a cell or cell lysate, e.g., by means of an ABP competition assay (Altun et al., 2011; de Jong et al., 2012).

Here, we showcase the toolbox frequently used for the analysis of DUB activity and illustrate its application by profiling pathogen-derived proteases toward Ub and Ubls.

2. Activity-based probes

2.1 Probes based on a monoUb or Ubl recognition element

The first activity-based DUB probe was based on the replacement of the C-terminal Gly76 in Ub with an aldehyde moiety as reactive "warhead" (Ubal) (Pickart & Rose, 1986). Replacement of this reactive C-terminal element with a nitrile moiety (UbCN) (Lam, Xu, DeMartino, & Cohen, 1997), a glycine vinylsulfone (UbVS), or glycine vinylmethylester (UbVME) (Borodovsky et al., 2001, 2002) led to the development of a larger panel of ABPs able to capture the active site cysteine of DUBs. The electron-poor vinyl motifs in UbVME and UbVS act as a Michael acceptor elements that trap the sulfur nucleophile of the active site cysteine under the formation of a covalent, irreversible bond. Later on, the total chemical synthesis of Ub (and mutants thereof) using solid phase peptide synthesis (SPPS) (El Oualid et al., 2010; Kumar, Haj-Yahya, Olschewski, Lashuel, & Brik, 2009; Pasunooti et al., 2009) opened the way to prepare Ub-ABPs carrying fluorescent labels or affinity handles on a large scale (de Jong et al., 2012). Unexpectedly, it was found that the C-terminal amide derivative of Ub_{1-75} with propargylamine (Ub-Prg or Ub-PA) is also able to covalently trap the active site cysteine of DUBs by formation of a stable vinyl thioether (Fig. 2A) (Ekkebus et al., 2013).

In general, specificity of such probes for DUBs originates from the interaction of the Ub-recognition element in the ABP with a Ub-binding interface in the DUB. This so-called S1 pocket in the protease holds the Ub molecule so that its C-terminus with the reactive element is positioned in close proximity to the active site cysteine of the protease. With this positioning, the two reacting partners are optimally aligned to allow formation of a covalent adduct. In a normal reaction with a ubiquitinated species, the DUB would be able to cleave the amide bond between substrate and Ub, resulting in free substrate and Ub, but due to the nature of the warhead, a covalent adduct is formed in the case of ABPs. Along this line, similar ABPs for Ubl-specific proteases have been developed by replacing the C-terminal residue in the respective Ubl for an electrophilic moiety, such as -Prg, -VME, or -VS and these include ABPs based on SUMO1,- 2, −3, Nedd8, ISG15, and UFM1. Currently, many of these Ub and Ub-like ABPs are commercially available but are also readily obtained by chemical synthesis (Basters et al., 2017; Ekkebus et al., 2013; Mulder et al., 2018; Witting et al., 2018), or

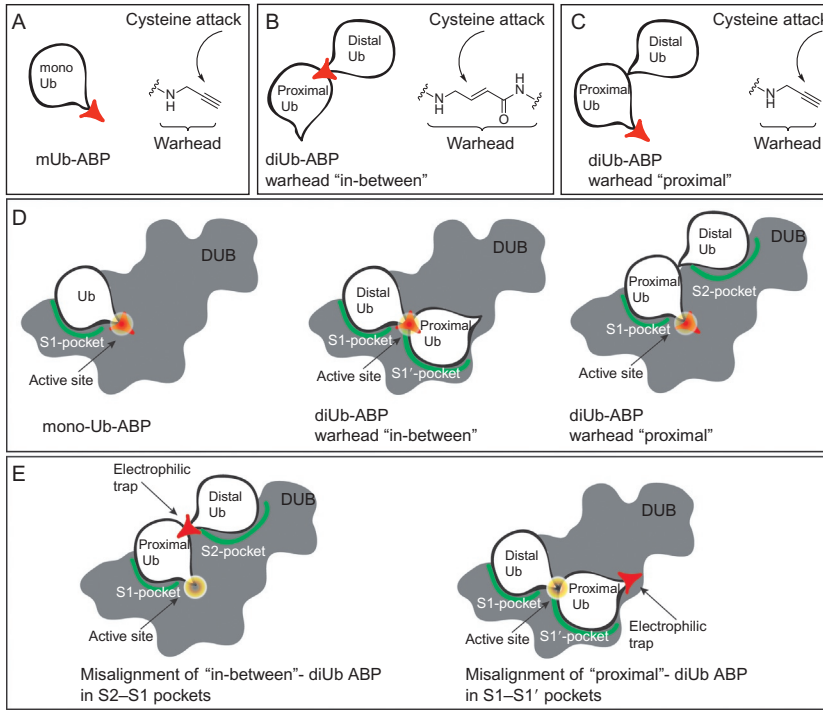

Fig. 2 Activity-based probes to target DUB specificity. (A) monoUb-PA probe. (B) Details of "in-between" diUb probe. (C) Details of "proximal" diUb probe. (D) Different probes targeting S1, S1–S1′ or S1–S2 interactions. (E) Misalignment of probes on DUBS prevents formation of covalent complexes.

semisynthesis, such as the use of intein chemistry for Ub(l)ΔG-thioester formation, followed by reaction of this thioester with an amine nucleophile (Hemelaar et al., 2004).

2.2 Probes based on a diUb recognition element

While monoUb ABPs have greatly increased our understanding of DUB reactivity, these ABPs offer no information on poly-Ub chain recognition and processing. Classically, in order to study possible linkage preferences, a recombinantly expressed and purified DUB is incubated with each of the seven native isopeptide-linked diUb molecules. Cleavage of the diUb molecules is then monitored over time using gel-based analyses. A major limitation of this methodology is that the results are not readily extrapolatable to the substrate preference of a DUB in a more complex environment, such as cell-lysate, which might modulate DUB activity and

preference due to other factors present in such samples. To overcome these issues, ABPs to investigate linkage specific proteolysis of DUBs have been developed, that can be utilized in complex biological systems such as cell lysates. These probes generally consist of two ubiquitin moieties equipped with a Michael acceptor element in the isopeptide linkage region in between the two Ub moieties (often referred to as "in-between" diUb probes). Initial reports show the two Ub regions to be linked together using non-native connections such as a triazole (McGouran, Gaertner, Altun, Kramer, & Kessler, 2013) and a thiolether linkage (Li, Liang, Gong, Tencer, & Zhuang, 2014). Two types of probes, containing either a dehydroalanine (Dha) (Haj-Yahya et al., 2014) or VME-like electrophilic trap (Mulder et al., 2014) (see Fig. 2B), mimic the native lysine–glycine isopeptide linkage the closest.

A panel of all seven isopeptide linked diUb probes can be prepared and used to covalently capture the active site cysteine of DUBs, showing their reactivity and preference toward certain linkage types. When using such probes, the distal Ub molecule will be positioned in the so-called S1-pocket and the proximal Ub molecule in the so-called S1′-pocket, thereby placing the reactive element directly over the reactive cysteine. Due to the geometrical differences between all Lys-linked diUb probes, the DUB will only be able to position the probes mimicking its natural substrates in such a way that the active site cysteine is able to react with the reactive element. Although these covalent vinyl amide probes have allowed more detailed structural investigation of diubiquitin-specific DUB recognition (Mevissen et al., 2016), they do not allow investigation of additional Ub-binding sites.

Some DUBs are able to recognize Ub chain topologies using other binding surfaces positioned further away from the active site, such as the S2-site. A set of probes targeting these S2-binding sites has been developed where a diUb molecule is constructed carrying a reactive element at the proximal C-terminus (Flierman et al., 2016) (see Fig. 2C). These probes are only able to react with DUBs that contain an S2-site that plays a determining role in positioning the diUb molecule in the S2- and S1-sites, thereby placing the reactive warhead directly over the active site cysteine (see Fig. 2D). Noteworthy is that the isopeptide linkage between proximal and distal Ub has been substituted for a protease-stable triazole linkage, prohibiting the protease of interest from degrading the probe during the assays.

If a DUB recognizes such a "proximal" diUb probe using its S1- and S1′-sites, the reactive warhead will not be in the vicinity of the active

site cysteine and no covalent adduct will be formed. Conversely, if a "in-between" diUb probe will react with a DUB recognizing the diUb moiety using its S2- and S1-sites, no reaction will occur either since the reactive element will not be optimally aligned with the reactive cysteine (see Fig. 2E). Having access to both "proximal' and "in-between" diUb probes offers an exciting combination to investigate the binding interfaces that play a role in determining binding preferences of DUBs and cast a light on their molecular mechanisms of action as showcased in the next section.

3. Characterization of coronavirus-encoded DUBs with activity-based probes

To study the activity of specific DUBs, investigators frequently take advantage of the ~10 kDa (monoUb or Ubl ABP) or ~20 kDa (DiUb ABP) increase in MW on probe labeling. SDS-PAGE analysis or blotting for an individual DUB, and comparing the intensities of the larger (labeled) band to the smaller (unlabeled) band, which allows the reactivity of the DUB toward the probe to be inferred. Here, we demonstrate how a typical experiment can be performed, by showing analysis of the MERS-CoV-encoded papain like cysteine protease (abbreviated PLpro), a viral DUB, upon incubation with our panel of ABPs. Additionally, having access to both "proximal" and "in-between" diUb probes we will demonstrate their use in the investigation of the binding interfaces that play a role in the specificity of the coronavirus-encoded DUBs MERS-CoV PLpro and SARS-CoV PLpro.

3.1 Probes based on a monoUb or Ubl recognition element

We first demonstrate how a typical ABP labeling experiment can be performed using a panel of Ub-Prg and Ubl-Prg probes in combination with their known proteases. The panel of ABPs consists of untagged constructs of human Ub, Nedd8, SUMO1, SUMO2, SUMO3, ISG15, and the C-terminal domain of ISG15 (see Table 1). The C-terminal glycine is replaced by propargylamine in all ABP reagents.

The reaction of a DUB or Ub-like protease with an ABP can be confirmed by incubation of the enzyme with the ABP followed by SDS-PAGE analysis. Fig. 3 shows the image of a typical ABP labeling experiment in which the ABPs were incubated with three proteases known to act on them: The DUB UCH-L3 is known to process Ub and Nedd8 (Gan-Erdene et al., 2003), SUMO protease SENP1 is active on all three SUMO proteins

Table 1 Overview of Ub and Ubl ABPs used in this study

Protein	Abbreviation	UniProt ID	Residues[a]	MW (kDa)
Ubiquitin	Ub	P0CG47[b]	1–75	8.5
Nedd8	N8	Q15843	1–75	8.5
SUMO1	S1	P63165	1–96	11.1
SUMO2	S2	P61956	1–92	10.6
SUMO3	S3	P55854	1–91	10.5
ISG15 C-domain	I15c	P05161	79–156	8.9
ISG15 (C78S)[c]	I15	P05161	1–156	17.1

[a]The C-terminal Gly residue is not included in this list.
[b]Ub is only listed as a polyubiquitin in UniProt; the UniProt ID refers to polyubiquitin-B (UBB).
[c]The C78S-mutation was introduced to solubilize the ISG15-protein.

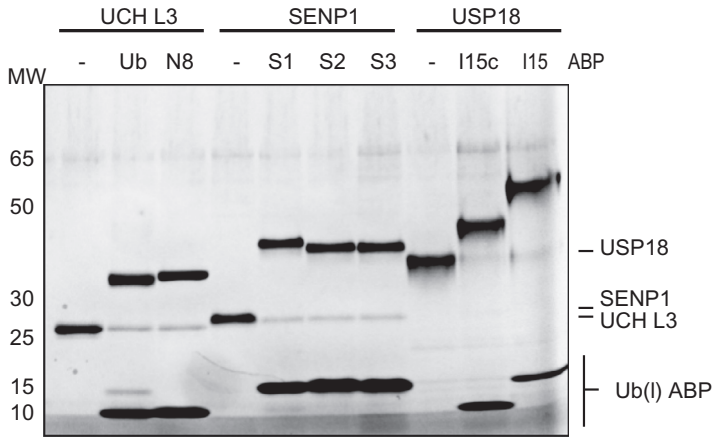

Fig. 3 Profiling of proteases UCH-L3, SENP1, and USP18 against Ub(l)-Prg ABPs.

(Gong, Millas, Maul, & Yeh, 2000), and deISGylase USP18 targets ISG15 (Malakhov et al., 2002). The reaction between the enzyme and an ABP becomes apparent from the shift of a protein band to a higher molecular weight equal to the total mass of enzyme plus ABP. Here, the UCH-L3 band around 25 kDa shifts to a ~35 kDa band with either the Ub- or Nedd8 ABP. Similarly, the SENP1 band shifts from ~26 to ~40 kDa when incubated with either of the SUMO probes. It is to note here that SUMO proteins run somewhat higher than what would be expected from their mass. Finally, the USP18 corresponding band shifts from ~38 to ~43 kDa and ~50 kDa upon incubation with the

truncated ISG15c ABP or the full-length ISG15, ABP respectively. From this result it follows that USP18 does not require full-length ISG15 for proper binding but that the C-terminal domain alone is enough, which corroborates earlier published results (Basters et al., 2017).

Upon closer examination of the gel image in Fig. 3, it can be seen that in most cases where the enzyme is incubated with the ABP, a small protein band remains at the molecular weight corresponding to the unbound enzyme. This indicates that not all enzyme reacted with the ABP and that most likely the enzyme is not 100% active. Quantification of the band intensities will give an estimate of the active fraction of the enzyme.

An experiment as shown in Fig. 3 can also be used to validate the properties of an ABP that was constructed and purified, by incubation of the ABP with its known protease target. An appropriately folded and active ABP will result in a proper reaction with its protease, which can be checked and quantified by SDS-PAGE analysis.

3.2 Profiling of MERS-CoV PLpro using monoUb and Ubl ABPs

These Ub and Ubl ABPs are also particularly well suited to profile the activity of pathogen-derived proteases toward Ub and Ubls. We here present how a typical experiment can be performed, by showing analysis of a cysteine protease encoded by MERS-CoV, named MERS-CoV PLpro (Mielech et al., 2014), upon incubation with our panel of ABPs. The experiment is similar to the one described above and the result is shown in Fig. 4. Unbound MERS-CoV PLpro gives a band around 32 kDa (outer left lane) and incubation with Ub, Nedd8 or either of the ISG15 ABPs shows a

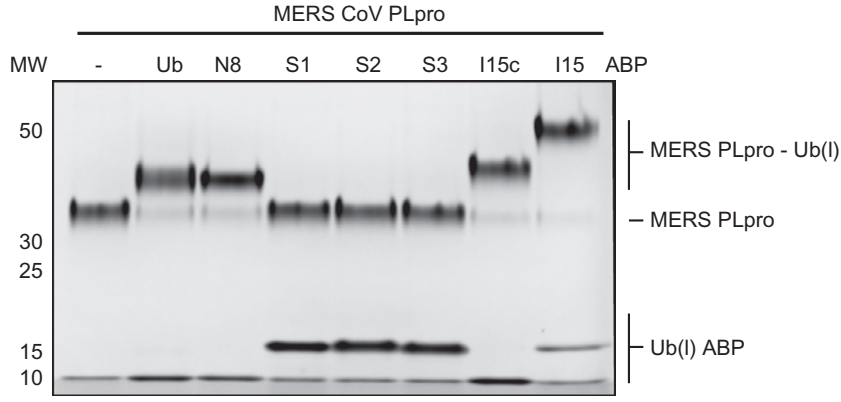

Fig. 4 Profiling of MERS PLpro against Ub(l)-Prg ABPs.

clear shift to a higher molecular weight band, whereas no change is observed with any of the three SUMO ABPs. It also becomes apparent that, like USP18, MERS PLpro only requires the C-terminal domain of ISG15 for proper binding, which is consistent with previously described results (Daczkowski, Goodwin, Dzimianski, Farhat, & Pegan, 2017). In all cases where a reaction takes place with the Ub(l)-ABP it shows almost full conversion, meaning that the enzyme preparation is close to 100% active.

3.3 DiUb ABPs to characterize MERS CoV PLpro and SARS CoV PLpro activity

Virus-encoded DUBs such as MERS-CoV PLpro and SARS-CoV PLpro (both papain-like proteases) have been shown to counteract the host cell's ubiquitination machinery. The SARS-CoV and MERS-CoV PLpro enzymes both can bind and cleave K48-linked Ub chains, however they do this in distinctive manners. It was shown that MERS-CoV PLpro has a monodistributive mechanism, meaning it nibbles one Ub-molecule off the K48-polyUb chain at a time (Bekes et al., 2015). The SARS-CoV PLpro on the other hand was shown to have a di-distributive mechanism, as it recognizes a diUb molecule and cleaves this off the polyUb chain as one unit (Bekes et al., 2016). ABPs with a diUb recognition element (Flierman et al., 2016; Mulder et al., 2014) were used to study this interesting difference between SARS-CoV- and MERS-CoV PLpro as demonstrated in Fig. 5.

SARS-CoV PLpro has a strong preference for the K48-linked "proximal"-probe as full complex formation is observed within 1 min of reaction time (see Fig. 5A, lanes 6–7). In contrast, the monoUb-probe does react with the protease but more slowly, showing a similar extend of labeling only after 15 min of incubation time (see Fig. 5A, lanes 2–3). The protease shows little reactivity toward the K48-linked "in-between"-probe, showing only marginal complex formation after 15 min (see Fig. 5A, lanes 4–5). This experiment shows the strong dependency of this DUB on an S2-site to bind diUb-molecules or longer Ub chains (Bekes et al., 2016). These results are in line with previous findings showing SARS-CoV PLpro to have a di-distributive mode of action (Bekes et al., 2015). A crystal structure of the K48-diUb "proximal"-probe in complex with the enzyme has allowed a detailed look at this S2-site and pin-pointed important interactions contributing to this strong S2 dependency in the DUB's proteolytic profile (Bekes et al., 2016).

Reactivity of MERS-CoV PLpro toward monoUb-, diUb "in-between"- and diUb "proximal"-probes shows a distinct profile compared to SARS-CoV

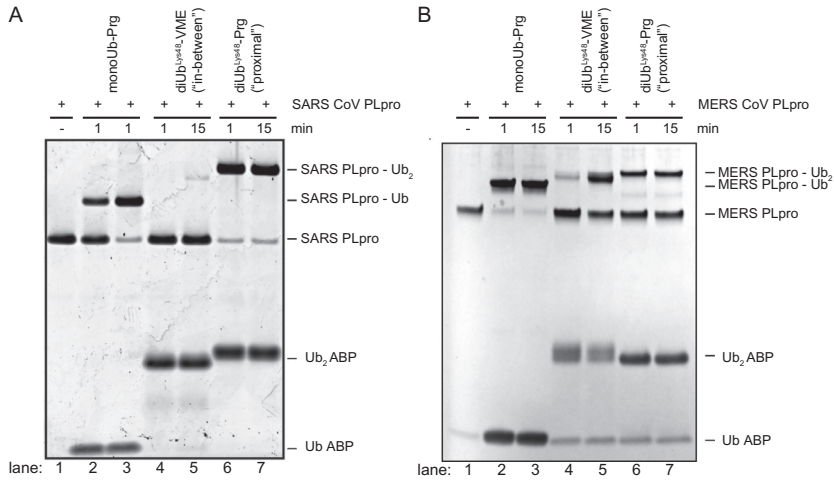

Fig. 5 SARS PLpro and MERS PLpro profiling using monoUb-, K48 linked diUb "in-between"-, and K48-linked diUb "proximal"-ABPs. (A) SDS-PAGE analysis of SARS PLpro reactivity toward the three types of probes. (B) SDS-PAGE analysis of MERS PLpro reactivity toward the three types of probes. *Panel A: Adapted from Bekes, 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*(4), 572–585. doi:10.1016/j.molcel.2016.04.016.*

PLpro (see Fig. 5A and B). The MERS-CoV DUB reacts fast with the monoUb-probe showing complete labeling within 1 min (Fig. 5B, lanes 2–3). Both diUb-probes are also processed by the DUB, but more slowly as roughly 50% of the protease is labeled after 15 min (Fig. 5B, lanes 5 and 7). When looking at the "in-between" probe a notable increase in labeling is observed when comparing the two time points (Fig. 5B, lanes 4 and 5), whereas for the "proximal" probe no increase in labeling is observed after 1 min (Fig. 5B, lanes 6 and 7). These results might indicate that recognition of the Ub-substrate by MERS-CoV PLpro primarily occurs using the S1-site and additional substrate context either on the proximal or distal site of this recognized Ub-moiety is tolerated, however slowing down the proteolysis event.

When using ABPs to study DUB substrate preferences, finding the correct reaction time window is crucial because measurements at different times may result in different outcomes. The SARS-CoV PLpro for instance (Fig. 5A) shows a preference for the K48-linked diUb "proximal" probe (lane 6) over the monoUb-Prg probe (lane 2) at short incubation times, whereas at longer incubation times the amount of labeling for both seems to be identical (lanes 3 and 7). The same holds true for MERS PLpro

(Fig. 5B), where at 15 min incubation the difference between "in-between"-probe and "proximal"-probe (lanes 5 and 7) is less pronounced then at short incubation times (lanes 4 and 6). One way to overcome making assumptions based on such time-dependent snapshots of enzyme activity is to measure full kinetic parameters of the DUBs. Michaelis–Menten kinetics can be measured based on fluorogenic (di)Ub substrates that start to emit a fluorescent signal upon DUB activity. Rather than forming a covalent complex with the DUB, these substrates are processed by the DUB in a normal way, liberating a fluorescent molecule. As such, the appearance of the fluorescent signal is a direct measure of DUB activity, that can be followed in real-time. Both "proximal" diUb and monoUb substrates have been generated and compliment the toolbox of ABPs to study DUBs (Dang, Melandri, & Stein, 1998; Flierman et al., 2016).

4. Methods

4.1 Preparation of Ub-like-PRG probes using intein chemistry

4.1.1 Equipment

- Incubator
- Vortex mixer
- Centrifuge
- Sonicator (we used Fisher Scientific FB120, 120 W, 20 kHz with a CL-18 tip)
- Sealable column
- RP-HPLC system (we used a Waters HPLC system equipped with a Waters XBridge Prep C18 5-μm OBD column (30 × 150 mm)
- LC–MS system (we used a Waters 2795 Separation Module (Alliance HT) using a Phenomenex Kinetex C18-column (2.1 × 50, 2.6 μm), Waters 2996 Photodiode Array Detector (190–750 nm) and LCT™ ESI-Mass Spectrometer
- Spinfilter (3000-Da cutoff)
- Gel filtration system (FPLC) (we used a BioRAD NGC)
- Hi-Load Superdex75 16/600 (GE-Healthcare) size-exclusion chromatography column
- Nanodrop
- Cold room (4°C)
- 50 mL Falcon tubes
- Lyophilizer

4.1.2 Buffers and reagents

- Lysis buffer: 50 m*M* HEPES, 100 m*M* NaOAc, pH 6.5
- SEC buffer: 50 m*M* MES, pH 6.5, 100 m*M* NaCl
- Chitin resin, stored in EtOH (New England BioLabs, catalog number S6651S)
- Protease inhibitor cocktail (Complete, Roche)
- β-mercaptoethanesulfonic acid sodium salt (MesNa)
- Propargylamine (Sigma Aldrich, catalog number P50900)
- DMSO
- Acetic acid
- Deionized water

4.1.3 Procedure

Expression of Ubl-intein-chitin-binding domain fusion proteins can be performed in *Escherichia coli* BL21 cells as reported elsewhere (Basters et al., 2017; Hemelaar et al., 2004). The Ubl-PRG probes can be prepared from the bacterial cell pellet as follows:

1. Resuspend the bacterial cell pellet from a 2.5 L culture in 80 mL lysis buffer (+ protease-inhibitor cocktail) by vigorous vortexing.
2. Lyse the cells by sonication: 6 × (30 s ON, 45 s OFF, amplitude 50%).
3. Centrifuge for 10 min at 3500 rpm at 4°C.
4. Collect the supernatant by decantation.
5. Prepare a 30 mL chitin-bead column, remove the EtOH and flush the column with 120 mL lysis buffer.
6. Load the supernatant onto the chitin-bead column at a flow rate of 0.5 mL/min.
7. Wash the column with 120 mL lysis buffer, followed by 60 mL lysis buffer containing 50 m*M* MesNa.
8. Add 30 mL lysis buffer containing 50 m*M* MesNa to the chitin beads, seal the column tube and incubate for 15 h at 37°C.
9. Collect the 30 mL elution (this contains the protein-MesNa thioester) and wash the beads with another 25 mL lysis buffer containing 50 m*M* MesNa and collect this as well.
10. Pool the fractions and concentrate them to a concentration of ~5 mg/mL by ultrafiltration using 3000 Da cutoff centrifugal filter units.
11. Prepare a solution of 2 *M* propargylamine in lysis buffer and add this to the protein-MesNa thioester such that the final concentration of propargylamine becomes 225 m*M*.

12. Check the pH. It should be around pH 8.5, otherwise adjust the pH accordingly by addition of 1 *M* HCl or 1 *M* NaOH.
13. Incubate the mixture at room temperature and follow the reaction by LC–MS analysis. A typical reaction time is 90 min to achieve complete conversion.
14. Acidify the mixture to pH 4.5 by addition of acetic acid.
15. Purify the Ub-like-PRG protein by RP-HPLC purification: 20%–60% CH_3CN in MQ with 0.1% TFA over 15 min at a flow rate of 37.5 mL/min.
16. Combine and lyophilize the fractions containing pure Ub-like-PRG protein.
17. Dissolve the dried protein in DMSO to a concentration of 10 m*M*.
18. Slowly dilute this into SEC buffer to a concentration of 1 m*M*.
19. Load the protein solution onto a size-exclusion chromatography Superdex 75 (16/600) column equilibrated with SEC buffer and elute in the same buffer.
20. Combine pure fractions and concentrate them where necessary by ultrafiltration.
21. The pure Ub-like PRG protein solution can be stored at −80°C and remain stable for more than a year.

4.1.4 Notes

1. Unless noted otherwise, keep everything on ice or in a cold room at 4°C.
2. It is important to predissolve propargylamine in the lysis buffer and adjust the pH before adding it to the Ubl-MesNa thioester solution to prevent local pH increases.

4.2 Preparation of synthetic ubiquitin probes

4.2.1 Equipment

- Rotation film evaporator
- Round bottom flasks
- Fritted syringes 5 mL
- Magnetic stirrer
- Multitech Syro II peptide synthesizer
- LC–MS system for analysis
- RP-HPLC system with C18 column
- Lyophilizer

- Eppendorf tubes 1.5 mL
- Gel filtration system (FPLC) equipped with a Superdex 75 16/600 size-exclusion chromatography column

4.2.2 Reagents

- Preloaded trityl resin TentaGel® R TRT-Gly Fmoc (Rapp Polymere GmbH; RA1213)
- 1,1,1,3,3,3-hexafluoro-2-propanol
- 1,2-Dichloroethane
- Dichloromethane
- TFA cleavage cocktail: 90% trifluoroacetic acid, 5% H_2O, 2.5% triisopropylsilane, 2.5% phenol (v/v/v/v)
- Diethylether
- *n*-Pentane
- PyBOP: (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
- Triethylamine
- Propargylamine
- Sodium ascorbate
- TBTA analog (prepared according to (Zhou & Fahrni, 2004))
- Cu(II)SO_4
- EDTA: ethylenediaminetetraacetic acid
- MPAA: 4-mercaptophenylacetic acid
- TCEP: tris(2-carboxyethyl)phosphine
- Chaotropic buffers: 8 *M* Urea containing 100 m*M* sodium phosphate pH 7 or 6 *M* Guanidinium·HCl containing 150 m*M* sodium phosphate pH 7
- 2,5-dibromohexandiamide

4.2.3 Procedure

Ub-mutants were synthesized as reported elsewhere (El Oualid et al., 2010) on an automated solid phase peptide synthesizer from Multitech Syro II on 25 μm scale. Preloaded trityl resin TentaGel® R TRT-Gly Fmoc (Rapp Polymere GmbH; RA1213) was used to allow mild acidic release of the final peptide from the resin without removing all side chain functionality protective groups. After automated synthesis the crude Ub-mutants were processed as follows:

4.2.3.1 Ub-Prg probe synthesis

1. React $Ub_{1–75}$ resin with 4 mL 20% v/v hexafluoro-2-propanol in dichloromethane for 20 min in a fritted syringe while gently shaking at room temperature.
2. Collect the filtrate in a 25 mL round bottom flask and concentrate using a rotation film evaporator.
3. Repeat the treatment of the resin with 4 mL 20% v/v hexafluoro-2-propanol in dichloromethane for 20 min and concentrate the combined filtrates. Coevaporate with 1,2-dichloroethane three times to remove all traces of hexafluoro-2-propanol.
4. Dissolve the partially protected peptide in a round bottom flask in 5 mL dichloromethane and add 5 eq. PyBOP, 5 eq. triethylamine and 10 eq. propargylamine. React for 16 h at room temperature while stirring with a magnetic stirrer.
5. Concentrate the reaction mixture using a rotation film evaporator and redissolve in 5 mL TFA cleavage cocktail and react for 2.5 h at room temperature while stirring with a magnetic stirrer.
6. Add the reaction mixture to chilled (−20°C) 3:1 v/v diethylether: pentane and centrifuge for 10 min at 3500 rpm.
7. Collect the precipitate and remove traces of diethylether:pentane using a N_2 flow for 5 min.
8. Dissolve the crude peptide in 3 mL warm DMSO and add this solution to 27 mL warm MilliQ, by pipetting the DMSO stock up and down quickly when adding it to the MilliQ, to avoid precipitation.
9. Filter the sample over a 0.2 μm filter and purify the product on a RP-HPLC system. (We used a Waters XBridge OBD (150 × 30) C18 column with a linear gradient between 20% and 45% B over 25 min (A = 95/5/0.05 v/v/v H_2O/acetonitrile/trifluoroacetic acid; B = 5/95/0.05 v/v/v H_2O/acetonitrile/trifluoroacetic acid).
10. Check purity of fractions using LC–MS analysis and pool and lyophilize pure fractions (>95%).

Mutants containing azido-ornithine or alloc-diaminopropionic acid at respective lysine positions were prepared using SPPS as reported elsewhere (Flierman et al., 2016; Mulder et al., 2014) and used to construct diUb probes as described below.

4.2.3.2 Proximal diUb-Prg probe synthesis

1. Dissolve $Ub_{1–75}$Prg and K48 Azido-ornithine $Ub_{1–75}$-thioester in DMSO (50 mg/mL final concentration) and dilute in 8 *M* Urea

chaotropic buffer to reach a final concentration of 5 mg/mL in an Eppendorf tube.

2. Add 150 μL catalyst solution containing 25 mg/mL Cu(II)SO_4 in MilliQ, 120 mg/mL sodium ascorbate in MilliQ and 52 mg/mL TBTA-analog (Chan, Hilgraf, Sharpless, & Fokin, 2004) in acetonitrile (1:1:1, v/v/v) to the Ub mutants solutions followed by a short vortex, repeat this addition two times in total in 15 min intervals.
3. Perform LC–MS analysis by diluting 1 μL of reaction mixture in 48 μL of MilliQ and 1 μL of 0.5 *M* EDTA (to quench the copper source).
4. After the reaction is finished, quench the reaction by the addition of 100 μL of 0.5 *M* EDTA, pH 7.0 and purify by RP-HPLC.
5. Check the purity of fractions using LC–MS analysis and pool and lyophilize pure fractions (>95%). Dissolve the lyophilized fractions in 10% final concentration DMSO and add to 150 m*M* NaCl, 20 m*M* Tris–HCl pH 7.6, and purify further over a Superdex 75 pg 16/600 column (GE).
6. Collect appropriate fractions and concentrate using Amicon (10 MWCO) spinfilters. Snap freeze in liquid N_2 and store at −80°C until used in labeling experiments.

4.2.3.3 In-between diUb probe synthesis

1. Dissolve $Ub_{1–75}$SEt and $Ub_{1–76}$ K48 mutant in 10% final concentration of DMSO and dilute into 6 *M* Guanidinium chaotropic buffer containing 250 m*M* MPAA (50 mg/mL final concentration) in an Eppendorf tube and shake the mixture overnight at 37°C.
2. Follow the reaction progress by LC–MS analysis by diluting 1 μL of reaction mixture in 48 μL of MilliQ and 1 μL of 1 *M* TCEP (to reduce the MPAA disulfide).
3. After the reaction is finished, add TCEP and the dilute the guanidinium concentration to a maximum of 2 *M* with MilliQ.
4. Purify the obtained mixture by RP-HPLC.
5. Pool and lyophilize pure fractions (>95%), as judged by LC–MS analysis.
6. Dissolve the K48-linked precursor in 10% final concentration of DMSO and dilute into 50 m*M* sodium phosphate buffer (pH 8, 0.5–1 mg/mL final concentration).
7. Add 2,5-dibromohexandiamide (100 eq.) and react the mixture while shaking overnight at 37°C.

8. Spin down the reaction to remove insoluble dibromide and perform LC–MS analysis.
9. After the reaction is finished, purify the reaction using RP-HPLC.
10. Pool and lyophilize pure fractions (>95%), as judged by LC–MS analysis.
11. Dissolve the lyophilized fractions in 10% final concentration DMSO and add to 150 m*M* NaCl, 20 m*M* Tris–HCl pH 7.6, and purify further over a Superdex 75 pg 16/600 column (GE).
12. Collect appropriate fractions and concentrated using Amicon (10 MWCO) spinfilters. Snap freeze in liquid N_2 and store at −80°C until used in labeling experiments.

4.3 Batch-purification of His-tagged MERS-CoV PLpro

4.3.1 Equipment

- Eppendorf centrifuge
- Table centrifuge for 50 mL Falcon tubes
- Falcon tubes (50 mL), Eppendorf tubes (1.5 mL), screw-cap tubes (2 mL)
- Erlenmeyer flasks (for *E. coli* growth)
- Sonicator (we used MSE Soniprep 150)
- SDS-PAGE gel electrophoresis equipment (we used Bio-Rad Mini-PROTEAN^{R} gel kit)
- Roller bench
- End-over-end rotator

4.3.2 Buffers and reagents

- *E. coli* (strain C2523/pCG1; expressing ubiquitin-specific protease Ubp1) transformed with pASK3 plasmid encoding His-tagged MERS-CoV PLpro (Bailey-Elkin et al., 2014)
- Standard LB *E. coli* growth medium, ampicillin, chloramphenicol
- Talon beads (GE Healthcare)
- Lysis buffer: 20 m*M* HEPES, pH 7.0, 200 m*M* NaCl, 10% glycerol (vol/vol), 0.1 mg/mL lysozyme
- Anhydrotetracycline
- Imidazole
- Dialysis buffer: 20 m*M* HEPES, pH 7.0, 100 m*M* NaCl, 50% glycerol (vol/vol), 2 m*M* DTT

4.3.3 Procedure

4.3.3.1 Preparation of Talon beads

1. Take 400 µL resuspended Talon beads (stored in ethanol), add 10 mL of water for washing, centrifuge at 1000 × *g* for 2 min, wash beads two times in lysis buffer (centrifugation at 1000 × *g* for 2 min).

4.3.3.2 *E. coli* culture

1. Inoculate 50 mL LB + ampicillin (pASK resistance) + chloramphenicol (pCG1 resistance) with transformed *E. coli* colony from fresh plate or sample from glycerol stock.
2. Grow bacteria at 37°C, 210 rpm until OD_{600} ~0.7.
3. Cool the culture to RT.
4. Induce protein expression with anhydrotetracycline (200 µg/mL final).
5. Incubate for protein expression at 20°C, 190 rpm, overnight.
6. Harvest cells by centrifugation at 3000 × *g*, 20 min, 4°C.

4.3.3.3 Lysis

1. Freeze–thaw cell pellet at 20°C once to ease lysis.
2. Resuspend pellet in 5 mL lysis buffer (1 mL per 10 mL culture) + 0.1 mg/mL lysozyme.
3. Perform enzymatic lysis in 50 mL Falcon tube for 1 h at 4°C.
4. Perform subsequent mechanical lysis by sonication (10 times 10 s with cooling on ice in between).
5. Clarify suspension by centrifugation at 20,000 × *g*, 20 min, 4°C.
6. Take a sample of the supernatant and the pellet for SDS-PAGE analysis.

4.3.3.4 Purification

1. Incubate the clarified supernatant with Talon beads for 1–2 h at 4°C on the roller bench.
2. Centrifuge at 1000 × *g*, 2 min, 4°C.
3. Discard supernatant (take a sample of this supernatant for SDS-PAGE analysis).
4. Add 10 mL lysis buffer + 20 m*M* imidazole to beads.
5. Incubate 15 min at 4°C on the roller bench.
6. Repeat this washing step 3 more times (with the same buffer).
7. Transfer beads to 2 mL tube after last wash step.
8. Elute protein from beads with 300 µL lysis buffer + 250 m*M* imidazole for 15 min at 4°C with end-over-end rotation.
9. Collect supernatant (take sample for SDS-PAGE gel analysis).
10. Repeat elution with another 300 µL buffer (take sample for SDS-PAGE gel analysis).
11. Pool both supernatants and dialyze overnight at 4°C against dialysis buffer.

4.4 Procedure for labeling of enzymes with Ub-Prg and Ubl-Prg probes

4.4.1 Equipment

- Micropipettes (2, 20, 200 μL) with appropriate tips
- Vortex mixer
- 0.5 mL and 1.5 mL Eppendorf tubes
- 15 mL Falcon tubes
- Table top mini centrifuge (6 × 1.5 mL tubes)
- ThermoMixer with thermoblock (we used: Eppendorf Thermomixer C equipped with a Smartblock 24 × 1.5 mL) or incubator at 37°C
- Heating block
- SDS-PAGE gel tank (we used: Invitrogen Mini Gel Tank, catalog number A25977, coupled to a Biorad PowerPac™ Basic Power Supply)
- Precast gels (we used: Invitrogen NuPAGE™ 4%–12% Bis-Tris Gel, 1.0 mm, 10 and 12 wells)
- Plastic container to store the gel
- Reciprocating shaker (we used GFL reciprocating shaker 3018)
- Gel scanner (we used: GE Amersham Imager 600 RGB)

4.4.2 Buffers and reagents

- Reaction buffer: 50 m*M* Tris–HCl, pH 7.6, 100 m*M* NaCl, 5 m*M* dithiothreitol (DTT), 1 mg/mL 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)
- Sample buffer: 900 μL Invitrogen NuPage® LDS Sample Buffer (4 ×) (cat. number NP0007), 210 μL Milli-Q® deionized water, 90 μL β-mercaptoethanol
- Protein ladder (we used: ThermoFisher PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa, cat. number 26619)
- SDS-PAGE running buffer: Novex NuPage® MOPS SDS Running Buffer (cat. number NP0001)
- Expedeon InstantBlue™ protein staining solution or other Coomassie-based protein staining solution
- Deionized water
- monoUb(l) ABPs (see Table 1): Ub-Prg (Ekkebus et al., 2013) and ISG15c-PRG (Basters et al., 2017) were prepared by SPPS as reported. Nedd8-Prg, SUMO1-Prg, SUMO2-Prg, SUMO3-Prg and ISG15-Prg (Basters et al., 2017) were prepared with intein chemistry using a method similar to a reported procedure (Hemelaar et al., 2004)

- diUb ABPs. K48-linked diUb "in-between"-probe was prepared as reported by (Mulder et al., 2014); K48 linked diUb "proximal"-probe was prepared as reported by Flierman et al. (2016)
- Enzymes: human UCH-L3, expressed and purified according to Larsen, Price, and Wilkinson (1996), SENP1, expressed and purified according to Mikolajczyk et al. (2007) and USP18, expressed and purified according to Basters et al. (2017), viral MERS-CoV PLpro (see protocol under Section 4.3) and SARS-CoV PLpro, expressed and purified according to Bekes et al. (2015)

4.4.3 Procedure for labeling of enzymes with Ub-Prg and Ubl-Prg probes

1. Dilute all enzymes to a concentration of 2 μ*M* in reaction buffer and incubate them for 10 min on ice.
2. Dilute all ABPs to a concentration of 10 μ*M* in reaction buffer.
3. Transfer 8 μL of the enzyme solutions to an empty 1.5 mL tube.
4. Add 8 μL of the ABP solutions to the appropriate enzyme solutions and pipette the solutions up and down a few times for proper mixing.
5. Spin down the samples for a few seconds.
6. Incubate the samples at 37°C for 45 min.
7. Add 8 μL sample buffer to each sample.
8. Vortex and spin down the samples a few seconds.
9. Put the samples in a preheated heating block at 95°C for 10 min.
10. Prepare the gel tank with the gel and SDS-PAGE running buffer.
11. Load the samples (whole sample) onto the gel.
12. Run the gel for 40 min at 200 V.
13. Remove the wet gel slab from the cassette and put it into the plastic container that is half-filled with water.
14. Decant the water and wash the gel three times with water.
15. Add InstantBlue™ staining solution and stain the gel under gently shaking until clear bands appear.
16. Decant the InstantBlue™ solution and wash the gel three times with water.
17. Add water to the gel and let it shake gently until proper background destaining has been achieved.
18. Transfer the wet gel slab to the gel scanner and capture the gel image using the colorimetric settings.

4.4.4 Procedure for labeling of viral enzymes with diUb probes

1. Dilute all enzymes to a concentration of 2 μ*M* in reaction buffer and incubate them for 10 min on ice.
2. Dilute all ABPs to a concentration of 10 μ*M* in reaction buffer.
3. Transfer 12.5 μL of the enzyme solutions to an empty 0.5 mL tube.
4. Add 12.5 μL of the ABP solutions to the appropriate enzyme solutions and pipette the solutions up and down a few times for proper mixing.
5. Spin down the samples for a few seconds.
6. Incubate the samples at 37°C while gently shaking (~500 rpm.) for indicated amount of time.
7. Take 10 μL sample and add it to 5 μL sample buffer and 5 μL distilled water.
8. Vortex and spin down the samples a few seconds.
9. Prepare the gel tank with the gel and SDS-PAGE running buffer.
10. Load the samples (17.5 μL) onto the gel.
11. Run the gel for 45 min at 190 V.
12. Remove the wet gel slab from the cassette and put it into the plastic container that is half-filled with water.
13. Decant the water and wash the gel three times with water.
14. Add InstantBlue™ staining solution and stain the gel under gently shaking until clear bands appear.
15. Decant the InstantBlue™ solution and wash the gel three times with water.
16. Add water to the gel and let it gently shake until proper background destaining has been achieved.
17. Transfer the wet gel slab to the gel scanner and capture the gel image using the colorimetric settings.

4.4.5 Notes

1. Keep all enzyme and ABP solutions on ice until needed.
2. Addition of CHAPS detergent to the reaction buffer is not always required but in some cases (here in the case of USP18) gives more pronounced protein bands.
3. The 10 min incubation of the enzymes with reaction buffer helps to reduce the possibly oxidized active site cysteine of the enzymes by DTT.
4. The type of gel, type of running buffer and running time is determined by the type of proteins used and must be chosen as such to achieve maximum separation between the unbound enzyme and ABP-bound enzyme.
5. To achieve optimal protein staining the InstantBlue™ staining and destaining were performed for 12 h each.

5. Conclusions and outlook

The advent of numerous ABPs has aided greatly in unraveling the complex and highly sophisticated ubiquitination system. Since the first ABP targeting DUBs, the field has brought forth an assortment of tools enabling profound insights into the structural, biochemical, and biological role of these enzymes. Although these advancements have helped gain insights into the functions of DUBs, it is becoming increasingly clear that these ABPs require innovation to address outstanding questions. The generation of tools specifically designed for dissecting the proteolytic processing of ubiquitin chains have revealed profound differences among these proteases in their specificity. Adding to this complexity, the discovery of heterotypic and hybrid Ub chains warrants the development of customized tools in order to understand the regulatory roles of DUBs in this context. Other outstanding questions include the development of ABPs capable of capturing metalloprotease DUBs, ABPs targeting a single DUB-type specifically and optimization of cell delivery methodologies for ABPs to enable in-cell enzymology.

The introduction of ABPs into living cells permit visualization and in-cell enzymology in a spatial, temporal, and substrate context, allowing study of the intrinsic regulation by cellular signaling events such as phosphorylation of DUBs to enhance their proteolytic activity as highlighted by the necessity of serine phosphorylation of OTUD5/DUBA (Huang et al., 2012). Most ABP profiling experiments are currently performed using either recombinant enzymes or cell lysates, although several methods allowing their biochemical study in a functional cellular environment are emerging, such as electroporation (Mulder et al., 2016) or the use of cell-penetrating peptides (Gui et al., 2018; Shahul Hameed, Sapmaz, Gjonaj, Merkx, & Ovaa, 2018).

Given the intrinsic role of Ub in the pathogenesis of a variety of diseases, enzymes involved in this system are emerging drug targets. Without a doubt the next generation of Ub-based tools will help increase our knowledge, ultimately leading to new diagnostic tools or therapeutics making it to the clinic.

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CHAPTER FOURTEEN

Evaluating enzyme activities and structures of DUBs

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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.

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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 m*M* Tris (pH 7.4), 150 m*M* NaCl, 10 m*M* DTT
- Standard SDS-PAGE equipment

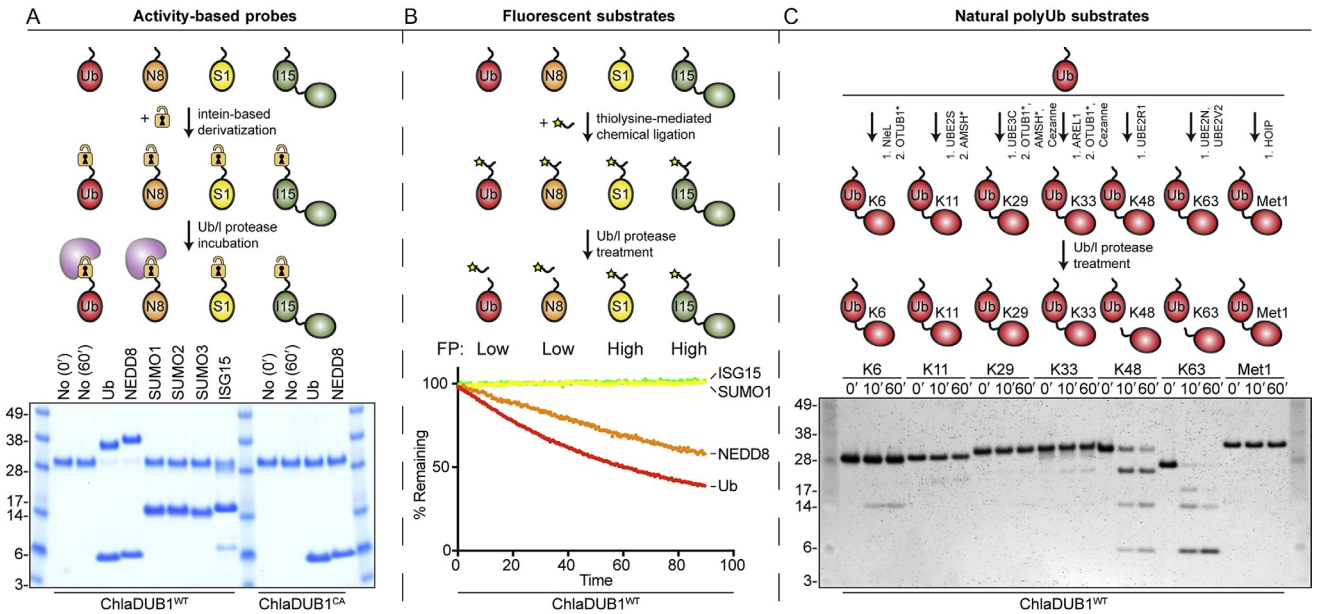
2.1.2 Procedure

1. Dilute the DUB to 10 μ*M* in Activation buffer, leaving at room temperature for 15 min to fully reduce the catalytic Cys.
2. Prepare Ub/UBL ABP at 2 × concentration (50 μ*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.



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 m*M* Tris (pH 7.4), 100 m*M* NaCl, 5 m*M* 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 n*M*. Allow the enzyme to be fully reduced by incubation at room temperature for 15 min.
2. Dilute Ub/UBL-KG(TAMRA) substrates to 300 n*M* in Dilution buffer (2 × final concentration). Also prepare a KG(TAMRA) positive control sample at 50 n*M*. 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 m*M* Tris (pH 7.4), 150 m*M* NaCl, 10 m*M* DTT
- 10 × Assay buffer: 500 m*M* Tris (pH 7.4), 500 m*M* NaCl, 50 m*M* 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 n*M* ChlaDUB1 final concentration). Allow full enzyme Cys reduction at room temperature for 15 min.
2. Prepare tetraUb chains at 2.5 μ*M* 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

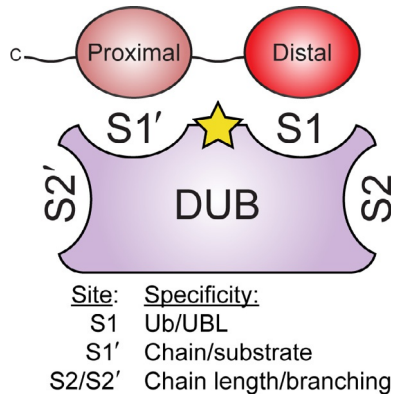


Fig. 2 Anatomy of a DUB. Cartoon schematic representing the multiple Ub binding sites a DUB may have and the layers of specificity they would impart. The substrate, in this case a diUb chain (*red*), sits above the DUB (*purple*) straddling the active site (*yellow star*).

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 monoUb/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

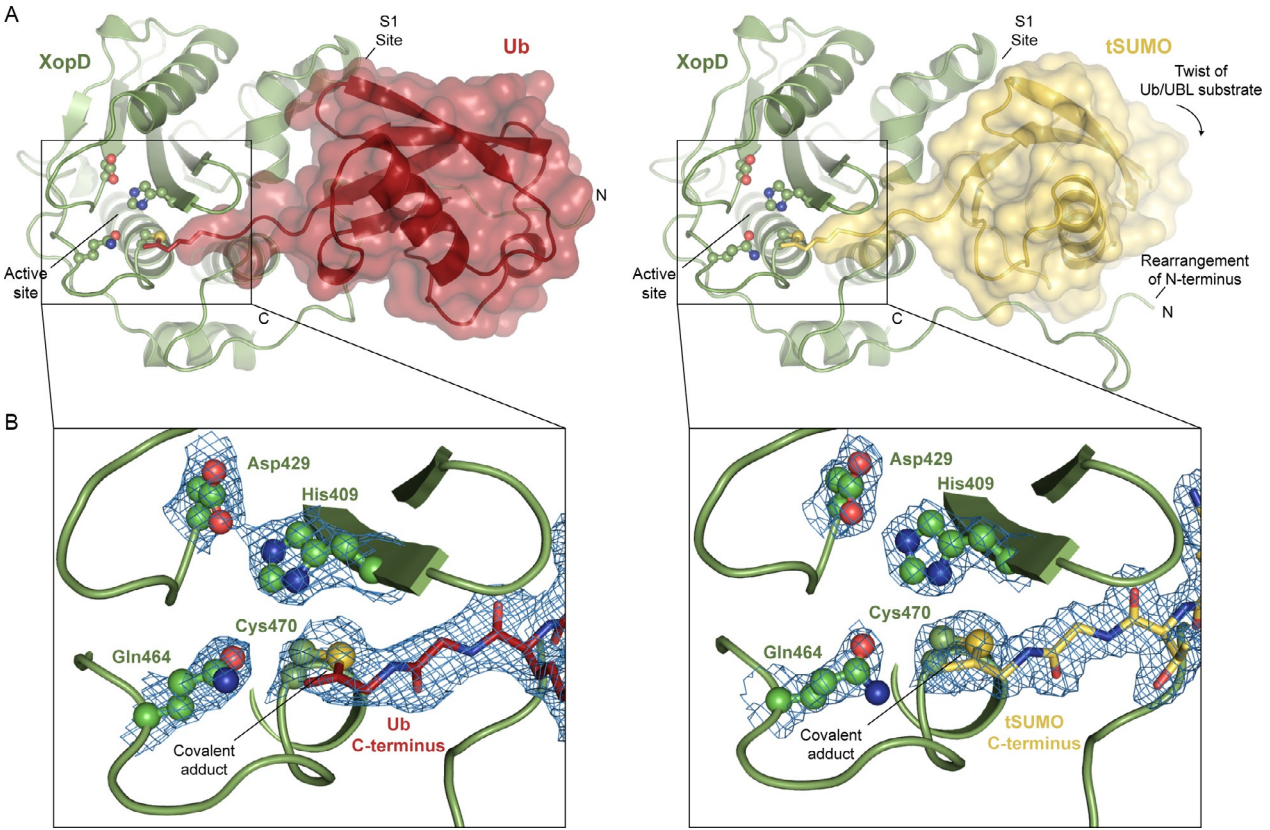


Fig. 3 See legend on next page.

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.

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