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Citation

Hameed, D. S., Sapmaz, A., Burggraaff, L., Amore, A., Slingerland, C. J., Westen, G. J. P. van, & Ovaa, H. (2019). Development of Ubiquitin-Based Probe for Metalloprotease Deubiquitinases. *Angewandte Chemie International Edition*, 58(41), 14477-14482.
doi:10.1002/anie.201906790

Version: Publisher's Version

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Article details

Hameed D.S., Sapmaz A., Burggraaff L., Amore A., Slingerland C.J., Westen G.J.P. van & Ovaa H. (2019), Development of Ubiquitin-Based Probe for Metalloprotease Deubiquitinases, *Angewandte Chemie International Edition* 58(41): 14477-14482.

Doi: 10.1002/anie.201906790

Metalloenzymes

International Edition: DOI: 10.1002/anie.201906790
German Edition: DOI: 10.1002/ange.201906790

Development of Ubiquitin-Based Probe for Metalloprotease Deubiquitinases

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Abstract: Deubiquitinases (DUBs) are a family of enzymes that regulate the ubiquitin signaling cascade by removing ubiquitin from specific proteins in response to distinct signals. DUBs that belong to the metalloprotease family (metalloDUBs) contain Zn^{2+} in their active sites and are an integral part of distinct cellular protein complexes. Little is known about these enzymes because of the lack of specific probes. Described here is a Ub-based probe that contains a ubiquitin moiety modified at its C-terminus with a Zn^{2+} chelating group based on 8-mercaptoquinoline, and a modification at the N-terminus with either a fluorescent tag or a pull-down tag. The probe is validated using Rpn11, a metalloDUB found in the 26S proteasome complex. This probe binds to metalloDUBs and efficiently pulled down overexpressed metalloDUBs from a HeLa cell lysate. Such probes may be used to study the mechanism of metalloDUBs in detail and allow better understanding of their biochemical processes.

Ubiquitination is an important post-translational modification that plays a key role in many vital cellular events.^[1] In this process, ubiquitin (Ub) is attached to a substrate protein by the concerted action of an enzyme cascade involving E1, E2, and E3 enzymes, and it is removed by an enzyme family known as deubiquitinases (DUBs).^[2] DUBs are classified into two main families: cysteine proteases and JAMM (JAB1/MPN/MOV34) metalloproteases.

The cysteine protease class of DUBs has a conserved cysteine residue at the active site and it acts as a nucleophile^[2b,3] and forms a covalent intermediate with the carbonyl group of the scissile amide bond in Ub conjugates.^[4] This intermediate has been mimicked by several activity-based probes reported by us and others.^[5]

Unlike cysteine protease DUBs, metalloprotease DUBs (metalloDUBs) do not form a covalent intermediate with their substrate.^[1a,b,6] MetalloDUBs contain a Zn^{2+} ion in their active site and it coordinates two histidine residues and an aspartate residue. During deubiquitination by metalloDUBs, a noncovalent intermediate complex is formed between the active site of the enzyme and the scissile isopeptide bond of Ub.^[7] This step leads to the nucleophilic attack of the amide bond by a water molecule, which is also coordinated to the active site Zn^{2+} ion.^[8] This mechanism of action, lacking a covalent intermediate between substrate and enzyme, presents a challenge to develop a selective activity-based probe for metalloDUBs.^[5d,9] By combining the chemical synthesis of Ub and a small-molecule zinc chelator at the C-terminus of Ub, we developed a Ub-based probe that binds and pulls down metalloDUBs from a cell lysate.

To develop a generic Ub-based probe, we relied on the information on structural similarity in the catalytic domain of known metalloDUBs. The X-ray structures of AMSH, the Rpn11/Rpn8 complex, and the AMSH-LP/Lys63-linked diubiquitin complex reveal a similar active-site configuration with a conserved Ub binding motif (see Figure S1 in the Supporting Information).^[6,10] To validate the inhibition and binding affinity of our Ub-based probe, we used recombinant the *S.cerevisiae* Rpn11/Rpn8 active enzyme complex, which is a part of the 19S proteasome lid (see Figure S2).^[10c] The activity of the recombinant Rpn11/Rpn8 enzyme complex was confirmed using a fluorogenic substrate (Ub-Rho) and a fluorescence polarization (FP) substrate Ub-FP (Ub-TAMRA-K48Ub-peptide).^[11] The rates of the enzyme reaction upon incubation with 1 μ M of Rpn11/Rpn8 were calculated to be 0.075 pmol min⁻¹ using the Ub-Rho substrate and 0.42 pmol min⁻¹ using the Ub-TAMRA-K48Ub-peptide substrate (see Figures S3 and S4).

To chelate Zn^{2+} to the active site of metalloDUBs, we first used a hydroxamate moiety, that is known as a general metalloprotease inhibitor,^[9,12] linked at the C-terminus of Ub. It has also been proposed that an Ub-containing N-hydroxy isopeptide at the C-terminal glycine may inhibit metalloDUBs.^[13] We found that Ub-hydroxamic acid derivatives failed to inhibit Rpn11 (see Figure S5). This finding necessitated a different approach for developing a probe for metalloDUBs.

Many molecules have been reported as zinc-chelating agents, and among them, 8-mercaptoquinoline (8-MQ; **6**) was reported as an efficient chelator (Scheme 1B).^[14] Furthermore, 8-MQ and its derivatives were also reported as a specific inhibitors for metalloDUBs like Rpn11 and AMSH.^[14b,15] In addition, it is known that a modification at position 3 of **6** will

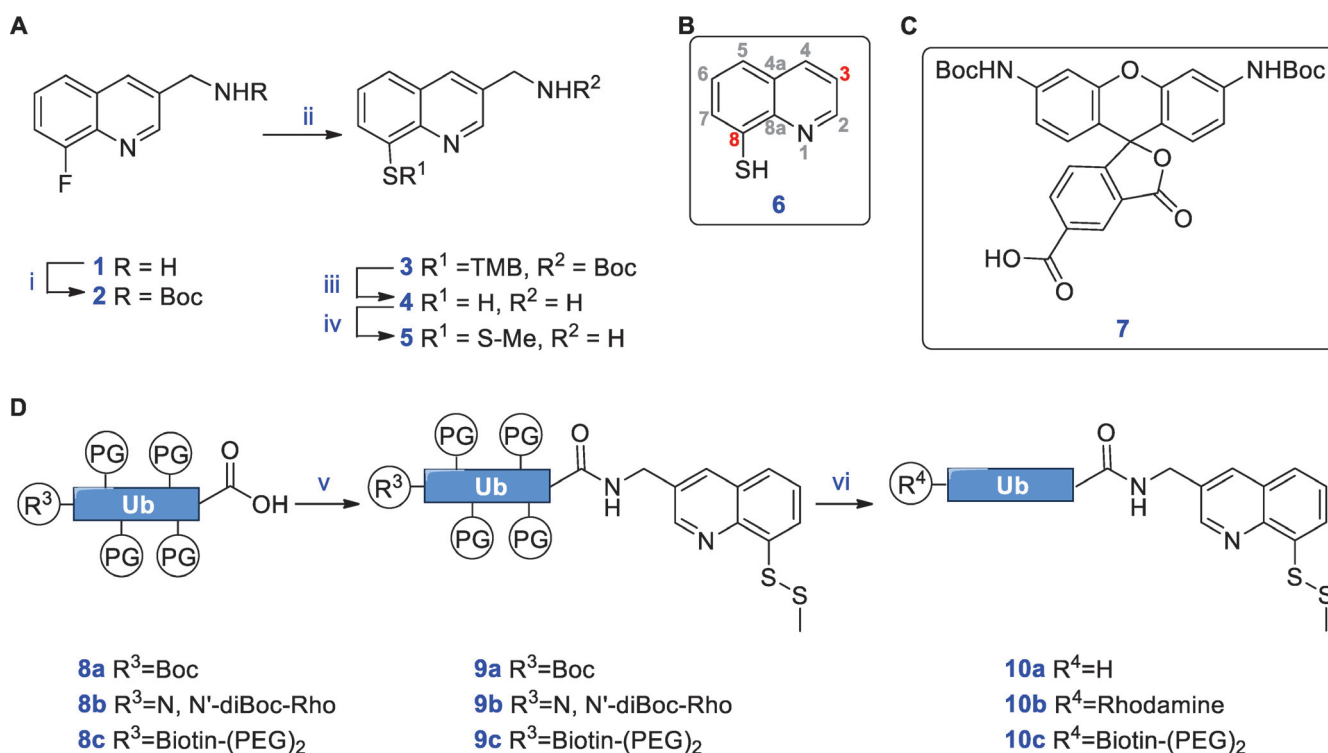
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Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under:
<https://doi.org/10.1002/anie.201906790>.



Scheme 1. A) i) Di-tert-butyl dicarbonate, DIPEA, CH₃CN, room temperature; ii) TMB-thiol, NaH, THF, 60 °C; iii) TFA, TES, H₂O; iv) MMTS, MeOH. B) Chemical structure of 8-quinoliniumthiolate (**6**) and its carbon positions are indicated. C) *N,N'*-diBoc-protected rhodamine (**7**) used in Fmoc-SPPS of rhodamine-labeled Ub derivatives. D) v) **5**, HBTU, HOBT, DIPEA, room temperature; vi) TFA, TIPS, phenol, H₂O. DIPEA=diisopropylethylamine, HBTU=O-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate, HOBT=1-hydroxybenzotriazole, MMTS=S-methyl methanethiosulfonate, PG=protecting group, TES=triethylsilyl, TFA=trifluoroacetic acid, THF=tetrahydrofuran, TIPS=triisopropylsilyl.

not diminish its inhibitory potential.^[16] Hence, an 8-MQ derivative modified with an amino group at position 3 was subsequently synthesized as a zinc-binding group (ZBG).

We prepared the 8-MQ derivative **5** (Scheme 1 A) starting from commercially available (8-fluoroquinolin-3-yl) methanamine (**1**). After *tert*-butoxycarbonyl (Boc) protection, the fluorine atom was substituted with trimethoxybenzyl (TMB) thiol and afforded the protected thiol **3**. After acid deprotection of the thiol, **4** was obtained. We observed that **4** could dimerize as a disulfide, leading to difficulties during purification. Hence, we protected the free thiol as a thio-methyl disulfide (**5**), which was then purified and used in further peptide-coupling reactions.

To precisely accommodate our ZBG at the C-terminus of Ub, we performed molecular-docking analysis. For this purpose, we used the known X-ray crystal structure of Rpn11/Rpn8 (PDB: 4O8X) and Ub (PDB: 1UBQ).^[10c,17] Among all the models tested, we arrived at optimal binding when our ZBG was attached at the C-terminus of Arg74 of Ub (Figure 1 A; see Figure S6). Therefore, we designed our metalloDUB probe containing an N-terminally modified Ub (1–74) attached to 3-aminomethyl 8-mercapto quinoline at its C-terminus (Figure 1 B).

We synthesized fully protected Ub74 containing either protected rhodamine (**7**, Scheme 1 C) or Biotin-(PEG)₂, or a Boc protecting group at its N-terminus, by using Fmoc-based solid-phase peptide synthesis (SPPS) on a 2-chlorotrityl resin.^[18] After selective C-terminal cleavage using 20 %

hexafluoroisopropanol (HFIP) in dichloromethane (DCM), we obtained fully protected Ub containing a free C-terminal carboxylate group and either a bis-Boc-rhodamine or Biotin-(PEG)₂ or a Boc protecting group on the N-terminus (**8a–c**, Scheme 1 C). The amino group of **5** was then coupled to the C-terminal carboxylate of Ub74 to yield **9a–c**. After global deprotection, the final products were purified using reversed-phase HPLC followed by size-exclusion chromatography to afford either labelled or unlabeled versions of the Ub-8MQ reagents **10a–c**. The disulfide-protected Ub-8MQ was reduced using TCEP prior to use and we observed only a slight decrease in the activity of Rpn11/Rpn8 upon the addition of TCEP (see Figure S7 and S8). Commercially available 8-quinoliniumthiolate (**6**, Scheme 1 B) was used as a positive control in our assay.

Inhibition of Rpn11/Rpn8 by **10a** was then tested using both Ub-rhodamine and Ub-fluorescence polarization (Ub-FP) assay reagents as substrates.^[10c] We observed that both **6** and **10a** were able to inhibit Rpn11/Rpn8 with an IC₅₀ value of about 2 μM (Figure 2; see Figure S9). To discern whether this inhibition is reversible or irreversible, we followed two different approaches. First, we performed a time-course preincubation assay using EDTA as a control. EDTA is known to strip Zn²⁺ from many proteins and therefore acts as an irreversible inhibitor.^[19] The activity assay showed that inhibition by EDTA increased with longer pre-incubation times. In contrast, **10a** initially decreased the enzyme activity and then maintained the inhibition irrespective of longer pre-

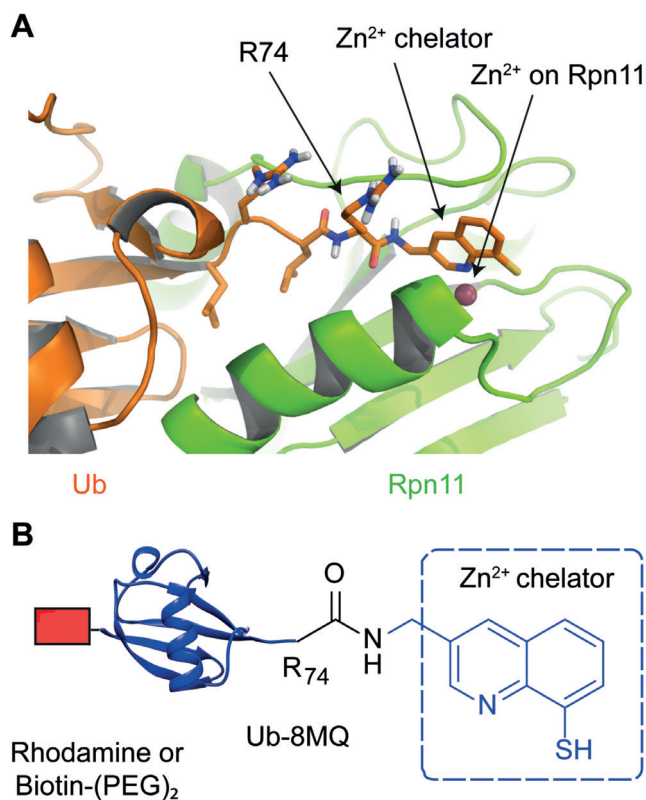


Figure 1. A) Molecular docking using Prime showing Ub (1–74) coupled to our Zinc chelator at the C-terminus, which coordinated with Zn²⁺ on the active site of Rpn11 (PDB: 1UBQ, orange and 4O8X, green). B) The metalloDUB probe consists of an N-terminal dye or a tag, Ub (1–74) and the modified 8-MQ attached at the C-terminus of Arg74 of Ub.

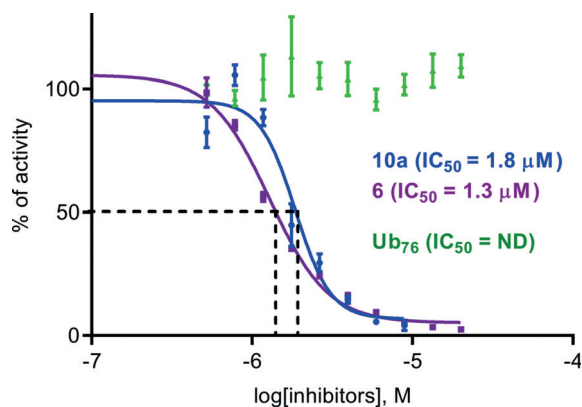


Figure 2. Rpn11 inhibition by **6**, **10a** and Ub₇₆. IC₅₀ curve of Ub-Rho hydrolysis in a typical fluorogenic assay. In this assay, compound **6** was also taken along, which shows an IC₅₀ of about 1.3 μM while **10a** shows an IC₅₀ of 2 μM. Full length Ub did not inhibit the enzyme.

incubation times (see Figure S10), suggesting that unlike EDTA, the reagent **10a** reversibly binds by coordinating to the active site of the Rpn11/Rpn8 enzyme.

Secondly, we carried out an activity recovery assay using ZnSO₄. After pre-incubating Rpn11/Rpn8 with either **6** or **10a**, we added an excess of ZnSO₄ to the assay buffer and incubated further. Then, we tested the activity of the enzyme

using an FP assay. We observed that the enzyme Rpn11/Rpn8, pre-incubated with **6** and **10a**, completely recovered its activity after adding ZnSO₄ to the buffer (see Figure S11), implying that the chelating groups in **6** and **10a** bind to Zn²⁺ in a reversible manner.

To establish the binding affinity of **10a** with Rpn11/Rpn8, we used the fluorescently labelled derivative **10b** as a FP probe.^[20] We measured the binding of unlabeled Rpn11/Rpn8 using both **10b** and rhodamine-labelled Ub₇₆ in a typical FP binding experiment. In our assays, **10b** showed a binding affinity of 8.5 μM. In contrast, full-length Ub binds to Rpn11/Rpn8 with a K_d of 38 μM (Figure 3). This data shows that **10b** can tightly bind to the Rpn11/Rpn8 complex as compared to wild-type Ub.

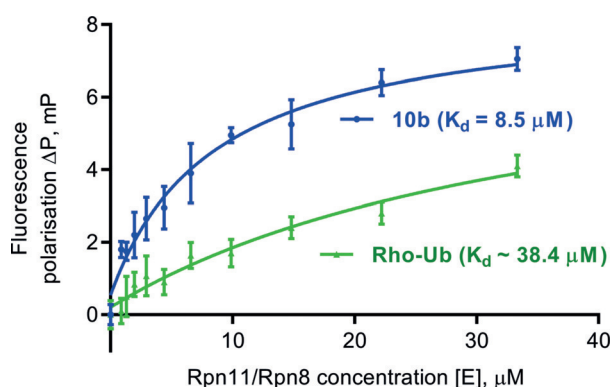


Figure 3. Fluorescence polarization based binding assay between **10b** and Rpn11/Rpn8. A typical FP-based binding between Rho-Ub and **10b** was measured against an unlabelled Rpn11/Rpn8. **10b** has an almost 5 times higher affinity compared to full length Ub.

Next, we tested the formation of a stable complex between our probe and Rpn11/Rpn8 in a retention-time shift assay on a size exclusion chromatography (SEC) column. To visualize the coelution of Rpn11/Rpn8 with our probe, we used **10b** and rhodamine-labelled Ub₇₆ such that the absorption signal of rhodamine was followed. In addition, the fractions from the SEC column were visualized by SDS-PAGE both by in-gel fluorescence imaging and by Coomassie staining.

Having established the retention times of Rpn11/Rpn8, rhodamine-labelled Ub₇₆, and **10b** separately (see Figures S12A–C), we incubated Rpn11/Rpn8 with an excess of either rhodamine-labelled Ub₇₆ or **10b** and used SEC to determine whether a complex is formed between them. We observed that rhodamine-labelled Ub₇₆ does not coelute with the Rpn11/Rpn8 (Figure 4A). Interestingly, incubating **10b** with Rpn11/Rpn8 resulted in coelution, implying that the Ub74-8MQ forms a tight complex with the enzyme (Figure 4B). This shows that the Ub-8MQ reagent can effectively bind Rpn11/Rpn8.

After determining that the Ub-8MQ probe was able to bind efficiently with Rpn11/Rpn8 in our in vitro assays, we examined whether metalloDUBs in the cell lysate could be captured by our probe. For this purpose, we synthesized a biotinylated Ub-8MQ probe (**10c**, Scheme 1D) and used

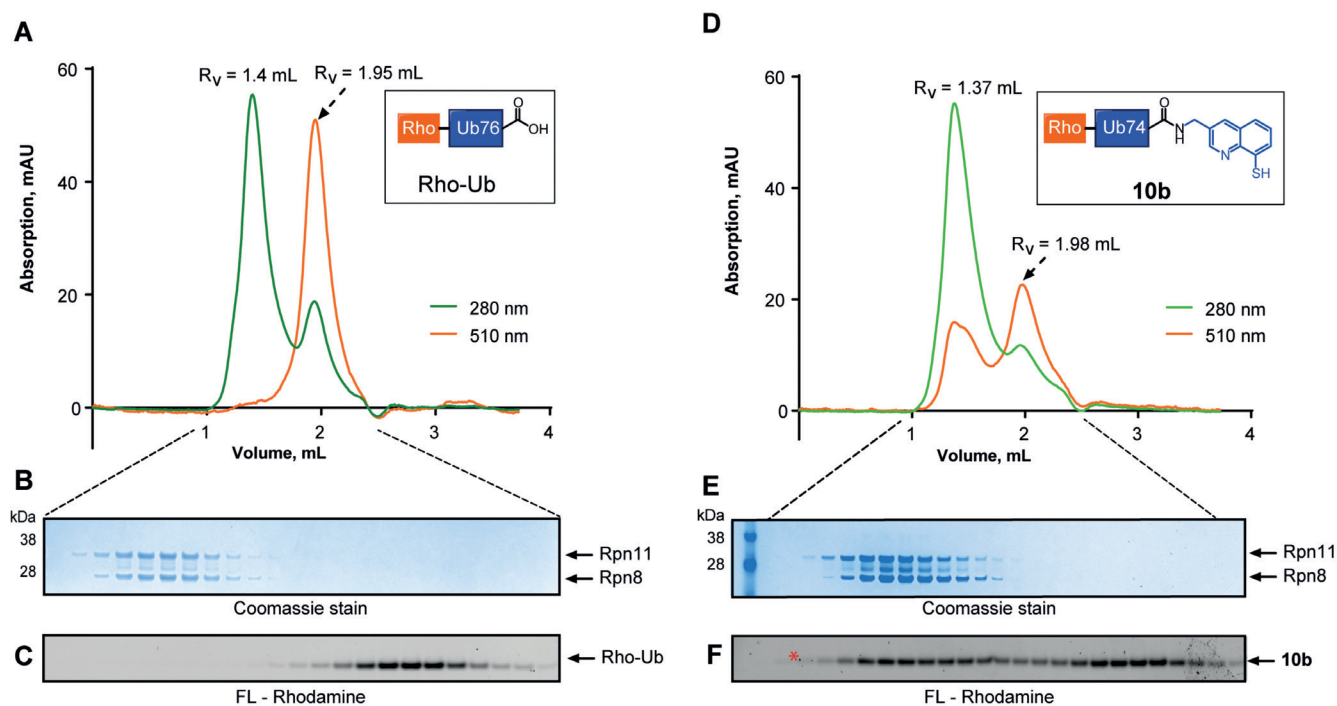


Figure 4. Co-elution assay. A) Co-elution pattern of RhoUb (inset) with an Rpn11/Rpn8 complex by SEC. B) The fractions from the column in A were used for analysis by SDS-PAGE (coomassie stain). C) The same gel (B) analyzed using fluorescence at the emission wavelength of rhodamine. D) Coelution pattern of **10b** (inset) with Rpn11/Rpn8 by SEC. E) The fractions from the column in D were used in a SDS-PAGE and analyzed by coomassie stain. F) The same gel (E) analyzed using fluorescence at the emission wavelength of rhodamine. The red asterisk marks the presence of rhodamine signal from the **10b** probe in the fractions from D.

biotin-Ub and biotin-8MQ as controls (Figure 5A). Cell lysate prepared from HeLa cells overexpressing GFP-POH1 (human homologue of Rpn11) was incubated with **10c**, along with biotin-Ub and biotin-8MQ, and streptavidin beads were

used to pull-down GFP-POH1 bound to our probe. As expected, **10c** was able to pull down GFP-POH1 with higher efficiency than either biotin-Ub or biotin-8MQ alone (Figure 5B). Even though the IC_{50} values of **10a** and 8MQ are

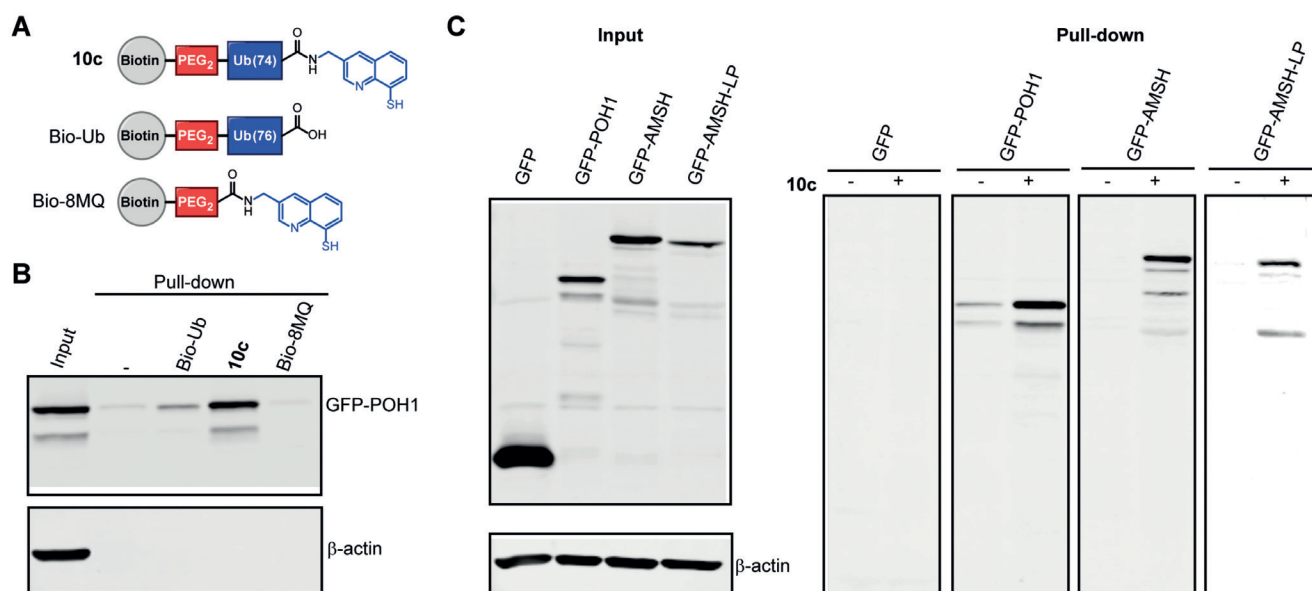


Figure 5. Pull-down using **10c**. A) Schematic representation of pull-down reagents used in the assay. B) Western Blot analysis of pull-down from cell lysate of HeLa cells overexpressing GFP-POH1. **10c** efficiently pulled-down GFP-POH1 compared with Bio-Ub or Bio-8MQ alone. C) Using **10c**, other metalloDUBs like GFP-AMSH and GFP-AMSHLP were also pulled-down from overexpressing HeLa cell lysate.

similar (Figure 2), our pull-down experiments show that the Ub handle is indispensable to tightly interact with metalloDUBs.

To determine whether this is true for other known metalloDUBs, we overexpressed GFP-POH1, GFP-AMSH, and GFP-AMSHLP in HeLa cells and carried out a pull-down assay using **10c**. As expected, **10c** was able to pull-down metalloDUBs from cell lysate (Figure 5C). This data shows that our probe can also be used to pull-down other metalloDUBs from complex mixtures in human cells.

In conclusion, a zinc chelator was synthesized and coupled to the C-terminal end of Ub to generate a first-generation metalloDUB probe. In our assays, the Ub₇₄-8MQ reagent inhibited the activity of Rpn11/Rpn8 better than full-length Ub alone. In addition, the Ub₇₄-8MQ reagent can form a tight complex with Rpn11/Rpn8. A biotinylated metalloDUB probe was also able to pull-down POH1, AMSH, and AMSH-LP from HeLa cell lysates. The metalloprotease DUBs, in general, share similar structural features in the active site of the enzyme and exist as a part of the multi-molecular protein complex.^[2c,21] Thus, reagents like the one described here may be used as a probe to detect the activity of metalloDUBs and their associated proteins.

Acknowledgements

We would like to thank Henk Hilkmann and Dris el Atmioui for peptide synthesis; Remco Merckx for providing TMB-thiol reagent; Andreas Martin for providing the plasmid for Rpn11/Rpn8 heterodimer expression; Ilana Berlin for providing us with the plasmid constructs for GFP-POH1, GFP-AMSH and GFP-AMSH-LP; Paul Geurink for providing bis-Boc-protected rhodamine, Ub-FP assay reagent and for critical reading of the manuscript. Work in the H.O lab is funded by an NWO (VICI grant 724.013.002) to H.O and an ITN fellowship to A.S.

Conflict of interest

Huib Ovaa is a shareholder of UbiquBio B.V.

Keywords: biological activity · chelates · metalloenzymes · ubiquitin · zinc

How to cite: *Angew. Chem. Int. Ed.* **2019**, *58*, 14477–14482
Angew. Chem. **2019**, *131*, 14619–14624

- [1] a) X. I. Ambroggio, D. C. Rees, R. J. Deshaies, *PLoS Biol.* **2004**, *2*, 113–119; b) K. Kikuchi, N. Ishii, H. Asao, K. Sugamura, *Biochem. Biophys. Res. Commun.* **2003**, *306*, 637–643; c) R. Verma, L. Aravind, R. Oania, W. H. McDonald, J. R. Yates III, E. V. Koonin, R. J. Deshaies, *Science* **2002**, *298*, 611–615.
- [2] a) A. Hershko, A. Ciechanover, *Annu. Rev. Biochem.* **1998**, *67*, 425–479; b) D. Komander, *Subcell. Biochem.* **2010**, *54*, 69–87; c) D. Komander, M. J. Clague, S. Urbe, *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 550–563.
- [3] S. A. Abdul Rehman, Y. A. Kristariyanto, S. Y. Choi, P. J. Nkosi, S. Weidlich, K. Labib, K. Hofmann, Y. Kulathu, *Mol. Cell* **2016**, *63*, 146–155.
- [4] a) M. Hu, P. Li, M. Li, W. Li, T. Yao, J. W. Wu, W. Gu, R. E. Cohen, Y. Shi, *Cell* **2002**, *111*, 1041–1054; b) B. M. Kessler, *Expert Rev. Proteomics* **2006**, *3*, 213–221; c) D. Komander, D. Barford, *Biochem. J.* **2008**, *409*, 77–85.
- [5] a) D. S. Hameed, A. Sapmaz, H. Ovaa, *Bioconjugate Chem.* **2016**, *28*, 3; b) R. Ekkebus, S. I. van Kasteren, Y. Kulathu, A. Scholten, I. Berlin, P. P. Geurink, A. de Jong, S. Goerdal, J. Neeffjes, A. J. Heck, D. Komander, H. Ovaa, *J. Am. Chem. Soc.* **2013**, *135*, 2867–2870; c) S. Sommer, N. D. Weikart, U. Linne, H. D. Mootz, *Bioorg. Med. Chem.* **2013**, *21*, 2511–2517; d) D. S. Hewings, J. A. Flygare, M. Bogyo, I. E. Wertz, *FEBS J.* **2017**, *284*, 1555–1576; e) P. Gopinath, S. Ohayon, M. Nawatha, A. Brik, *Chem. Soc. Rev.* **2016**, *45*, 4171–4198; f) A. Borodovsky, H. Ovaa, N. Kolli, T. Gan-Erdene, K. D. Wilkinson, H. L. Ploegh, B. M. Kessler, *Chem. Biol.* **2002**, *9*, 1149–1159; g) Y. A. Lam, W. Xu, G. N. DeMartino, R. E. Cohen, *Nature* **1997**, *385*, 737–740; h) R. Meledin, S. M. Mali, O. Kleinfeld, A. Brik, *Angew. Chem. Int. Ed.* **2018**, *57*, 5645–5649; *Angew. Chem.* **2018**, *130*, 5747–5751.
- [6] C. W. Davies, L. N. Paul, M. I. Kim, C. Das, *J. Mol. Biol.* **2011**, *413*, 416–429.
- [7] M. J. Clague, I. Barsukov, J. M. Coulson, H. Liu, D. J. Rigden, S. Urbe, *Physiol. Rev.* **2013**, *93*, 1289–1315.
- [8] H. J. Tran, M. D. Allen, J. Lowe, M. Bycroft, *Biochemistry* **2003**, *42*, 11460–11465.
- [9] A. Saghatelian, N. Jessani, A. Joseph, M. Humphrey, B. F. Cravatt, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 10000–10005.
- [10] a) G. R. Pathare, I. Nagy, P. Sledz, D. J. Anderson, H. J. Zhou, E. Pardon, J. Steyaert, F. Forster, A. Bracher, W. Baumeister, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 2984–2989; b) Y. Sato, A. Yoshikawa, A. Yamagata, H. Mimura, M. Yamashita, K. Ookata, O. Nureki, K. Iwai, M. Komada, S. Fukai, *Nature* **2008**, *455*, 358–362; c) E. J. Worden, C. Padovani, A. Martin, *Nat. Struct. Mol. Biol.* **2014**, *21*, 220–227.
- [11] a) U. Hassiepen, U. Eidhoff, G. Meder, J. F. Bulber, A. Hein, U. Bodendorf, E. Lorthiois, B. Martoglio, *Anal. Biochem.* **2007**, *371*, 201–207; b) P. P. Geurink, F. El Oualid, A. Jonker, D. S. Hameed, H. Ovaa, *ChemBioChem* **2012**, *13*, 293–297.
- [12] a) M. Morell, T. Nguyen Duc, A. L. Willis, S. Syed, J. Lee, E. Deu, Y. Deng, J. Xiao, B. E. Turk, J. R. Jessen, S. J. Weiss, M. Bogyo, *J. Am. Chem. Soc.* **2013**, *135*, 9139–9148; b) S. A. Sieber, S. Niessen, H. S. Hoover, B. F. Cravatt, *Nat. Chem. Biol.* **2006**, *2*, 274–281.
- [13] S. K. Maity, S. Ohayon, A. Brik, *Org. Lett.* **2015**, *17*, 3438–3441.
- [14] a) K. Kawai, N. Nagata, *Eur. J. Med. Chem.* **2012**, *51*, 271–276; b) C. Perez, J. Li, F. Parlati, M. Roufflet, Y. Ma, A. L. Mackinnon, T. F. Chou, R. J. Deshaies, S. M. Cohen, *J. Med. Chem.* **2017**, *60*, 0.
- [15] J. Li, T. Yakushi, F. Parlati, A. L. Mackinnon, C. Perez, Y. Ma, K. P. Carter, S. Colayco, G. Magnuson, B. Brown, K. Nguyen, S. Vasile, E. Suyama, L. H. Smith, E. Sergienko, A. B. Pinkerton, T. D. Y. Chung, A. E. Palmer, I. Pass, S. Hess, S. M. Cohen, R. J. Deshaies, *Nat. Chem. Biol.* **2017**, *13*, 486–493.
- [16] H. Zhou, F. Parlati, M. Roufflet, E. Emberley, R. Deshaies, S. Cohen, *WO/2012/158435*, **2012**.
- [17] S. Vijay-Kumar, C. E. Bugg, W. J. Cook, *J. Mol. Biol.* **1987**, *194*, 531–544.
- [18] a) F. El Oualid, R. Merckx, R. Ekkebus, D. S. Hameed, J. J. Smit, A. de Jong, H. Hilkmann, T. K. Sixma, H. Ovaa, *Angew. Chem. Int. Ed.* **2010**, *49*, 10149–10153; *Angew. Chem.* **2010**, *122*, 10347–10351; b) A. Shanmugham, A. Fish, M. P. Luna-Vargas, A. C. Faesen, F. El Oualid, T. K. Sixma, H. Ovaa, *J. Am. Chem. Soc.* **2010**, *132*, 8834–8835; c) P. P. Geurink, B. D. van Tol, D. van Dalen, P. J. Brundel, T. E. Mevissen, J. N. Pruneda, P. R.

- Elliott, G. B. van Tilburg, D. Komander, H. Ovaas, *ChemBioChem* **2016**, *17*, 816–820.
- [19] J. K. Nyborg, O. B. Peersen, *Biochem. J.* **2004**, *381*, e3–4.
- [20] R. Buchli, R. S. VanGundy, H. D. Hickman-Miller, C. F. Giberson, W. Bardet, W. H. Hildebrand, *Biochemistry* **2004**, *43*, 14852–14863.
- [21] a) E. M. Cooper, C. Cutcliffe, T. Z. Kristiansen, A. Pandey, C. M. Pickart, R. E. Cohen, *EMBO J.* **2009**, *28*, 621–631;
b) G. A. Cope, G. S. Suh, L. Aravind, S. E. Schwarz, S. L. Zipursky, E. V. Koonin, R. J. Deshaies, *Science* **2002**, *298*, 608–611; c) J. McCullough, P. E. Row, O. Lorenzo, M. Doherty, R. Beynon, M. J. Clague, S. Urbe, *Curr. Biol.* **2006**, *16*, 160–165;
d) T. Yao, R. E. Cohen, *Nature* **2002**, *419*, 403–407.
- Manuscript received: May 31, 2019
Revised manuscript received: August 1, 2019
Accepted manuscript online: August 5, 2019
Version of record online: August 28, 2019
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