

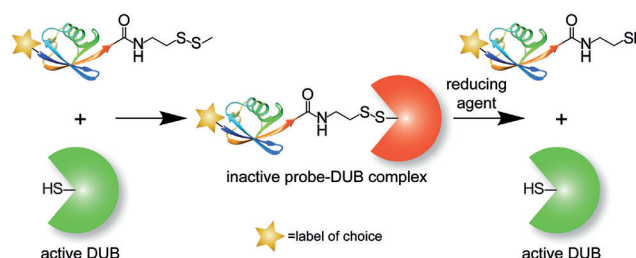
Release of Enzymatically Active Deubiquitinating Enzymes upon Reversible Capture by Disulfide Ubiquitin Reagents

Annemieke de Jong, Katharina Witting[†], Raymond Kooij[†], Dennis Flierman, and Huib Ovaa*

Abstract: Deubiquitinating enzymes (DUBs) catalyze the cleavage of ubiquitin from target proteins. Ubiquitin is post-translationally attached to proteins and serves as an important regulatory signal for key cellular processes. In this study, novel activity-based probes to study DUBs were synthesized that comprise a ubiquitin moiety and a novel disulfide warhead at the C-terminus. These reagents can bind DUBs covalently by forming a disulfide bridge between the active-site cysteine residue and the ubiquitin-based probe. As disulfide bridges can be broken by the addition of a reducing agent, these novel ubiquitin reagents can be used to capture and subsequently release catalytically active DUBs, whereas existing capturing agents bind irreversibly. These novel reagents allow for the study of these enzymes in their active state under various conditions.

The modification of target proteins with ubiquitin (Ub), a process called ubiquitination, is a key post-translational event that regulates a wide range of cellular processes. Ub is a highly conserved 76 amino acid protein, which is conjugated to proteins by the formation of an isopeptide bond between the C-terminus of Ub and a lysine residue or the N-terminus of a target protein. Ub can also be coupled to other Ub moieties to form various polyubiquitin chains. Deubiquitinating enzymes (DUBs) catalyze the cleavage of these isopeptide bonds, thereby regulating the ubiquitination of target proteins. The human genome encodes about 80 active DUBs, which can be classified into five classes of cysteine (Cys) proteases and one class of metalloprotease DUBs.^[1] As the function of many DUBs is still unknown, studying these

enzymes in their active state, either in isolation or as part of a protein complex, is of great importance. Existing reagents to study the activity of DUBs include cleavable Ub-containing substrates^[2] and covalently binding activity-based probes (ABPs)^[3] that react with the active-site Cys residue. The latter have been used in activity profiling, inhibitor screening, and DUB identification.^[3b] However, these ABPs bind irreversibly to the active site of the DUB, rendering them inactive. In this study, we developed a Ub-based ABP with a novel warhead that captures DUBs from cell extracts and allows for their release in an active state (Scheme 1).



Scheme 1. Binding of disulfide probes to DUBs and subsequent release by mild cleavage with a reducing agent.

In our probe design, we intended to use reagents that can reversibly modify thiols as Ub-based ABPs react with the active-site Cys residue. Many such reagents, if not all, are highly reactive, and thus unspecific towards Cys residues in proteins. Therefore, previous methods to modify specific cysteines relied on the mutation of non-target cysteines to prevent their modification. For example, GlcNAc-methylthiosulfonate has been used to modify proteins with GlcNAc moieties on specific cysteines.^[4] In these proteins, undesirable Cys residues were replaced by serine residues. Other known reagents used for the modification of Cys residues are disulfides ($R-S-S-R'$), which are less reactive. The Cys thiolate attacks the disulfide bond, and subsequently a new asymmetric disulfide is formed in a process named thiol-specific exchange. Chatterjee and co-workers have used this approach to generate a ubiquitinated histone complex.^[5] They modified a Cys residue that was introduced in histone 2B by first activating the thiol using 2,2'-dithiobis(5-nitropyridine) to form a disulfide. Subsequently, a Ub moiety carrying a C-terminal aminoethanethiol group was coupled. We exploited the thiol-specific exchange for the design of a reversible warhead for our novel DUB ABP. We postulated that a Ub moiety would direct our disulfide ABP to the active-site cysteine in DUBs. The glycine residue at position 76 of Ub was replaced with methyl disulfide as a warhead (Scheme 2). We hypothesized that this disulfide moiety would

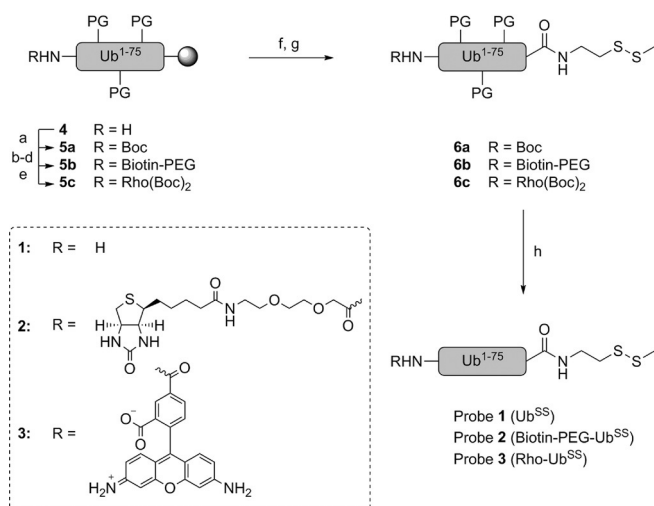
[*] K. Witting,^[†] R. Kooij,^[†] Dr. D. Flierman, Prof. Dr. H. Ovaa
Department of Chemical Immunology
Leiden University Medical Center
Einthovenweg 20, 2333 ZC Leiden (The Netherlands)
E-mail: H.Ovaa@lumc.nl
Homepage: <http://www.ovaalab.nl>

A. de Jong, K. Witting,^[†] R. Kooij,^[†] Dr. D. Flierman, Prof. Dr. H. Ovaa
Department of Cell Biology
The Netherlands Cancer Institute
Plesmanlaan 121, 1066 CX Amsterdam (The Netherlands)

[†] These authors contributed equally to this work.

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.201706738>.

© 2017 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial, and no modifications or adaptations are made.



Scheme 2. Synthesis of disulfide probes **1** (Ub^{SS}), **2** (biotin-PEG-Ub^{SS}), and **3** (Rho-Ub^{SS}). Ubiquitin lacking the C-terminal glycine (Ub¹⁻⁷⁵) was synthesized on resin (**4**). The N-terminus was modified under various conditions (a–e). a) Boc₂O, DiPEA, NMP; b) PyBOP, DiPEA, (8-Fmoc-amino)-3,6-dioxaoctanoic acid, NMP; c) piperidine, NMP; d) biotin, HBTU, HOBT, DiPEA, NMP; e) PyBOP, DiPEA, N,N'-Boc-protected 5-carboxyrhodamine 110 (Rho),^[6] NMP. After cleavage from the resin (f), the C-terminus was modified with an S-SMe group (g). Global deprotection yielded the final probes (h). f) HFIP, DCM; g) PyBOP, Et₃N, 2-(methylidisulfanyl)ethan-1-amine, DCM; h) TFA/H₂O/*i*Pr₃SiH/PhOH. See the Supporting Information for details. DCM = dichloromethane, DiPEA = *N,N*-diisopropylethylamine, HBTU = *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate, HFIP = hexafluoroisopropanol, HOBT = 1-hydroxybenzotriazole, NMP = *N*-methyl-2-pyrrolidone, PyBOP = benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, TFA = trifluoroacetic acid.

undergo thiol-specific exchange with the active-site Cys residue in DUBs, thereby forming a covalent disulfide bond (Scheme 1). The addition of a mild reducing agent would then be able to break this disulfide bond and release enzymatically active DUBs.

Three variants of the Ub-based disulfide probe with different N-terminal modifications were synthesized; one with a free N-terminus (**1**, Ub^{SS}), one with an N-terminal biotin affinity tag linked via a poly(ethylene glycol) (PEG) spacer (**2**, biotin-PEG-Ub^{SS}), and one with the fluorescent dye 5-carboxyrhodamine 110 (**3**, Rho-Ub^{SS}; Scheme 2; see also the Supporting Information, Figure S1 a). Ub¹⁻⁷⁵ was synthesized by total chemical solid-phase synthesis.^[3c,7] The N-terminus of Ub¹⁻⁷⁵ (**4**) was subsequently modified with a Boc group (**5a**), modified with a PEG spacer and a biotin moiety (**5b**), or

modified with N,N'-Boc-protected 5-carboxyrhodamine 110^[6,8] (**5c**). The peptides were then cleaved from the resin using hexafluoroisopropanol (HFIP). 2-(Methylidisulfanyl)ethan-1-amine was coupled to the C-terminus of Ub¹⁻⁷⁵ as the warhead (**6a–6c**). 2-(Methylidisulfanyl)ethan-1-amine was synthesized as described previously.^[9] TFA-mediated deprotection and purification yielded probes **1**, **2**, and **3** (Scheme 2 and Figure S1 a). The probes were analyzed by SDS-PAGE and LC-MS (Figure S1).

To test whether our novel Ub-based DUB probes containing the disulfide warhead are able to bind to DUBs *in vitro*, we incubated the DUB UCHL3 with the probes Ub^{SS} or biotin-PEG-Ub^{SS} and followed the reactions by LC-MS and gel electrophoresis (Figures 1 a, b and S2). We observed an increase in the mass of UCHL3 corresponding to the binding of one probe moiety, which confirmed that these probes are indeed able to react covalently. At higher concentrations of Ub^{SS} and biotin-PEG-Ub^{SS}, additional bands were observed on the gel, which may represent binding of the probes to other Cys residues in UCHL3 although labeling is minimal as shown by LC-MS analysis. To determine whether the formed probe–DUB complex could be reduced, we incubated the reaction mixture with 10 mM dithiothreitol (DTT). As shown in Figures 1 a, b and S2, the probe–UCHL3 complexes are completely reduced, yielding unlabeled UCHL3 and reduced probe (Ub^{SH}). To examine whether Ub-based disulfide probes can bind to DUBs from other classes, we incubated fluorescently labeled Ub^{SS} (**3**, Rho-Ub^{SS}) with UCHL3, OTUB2, or USP7 TRAF/catalytic domain (USP7¹⁻⁵⁶⁰), and monitored the labeling by fluorescence scanning of the non-reducing SDS-PAGE gel (Fig-

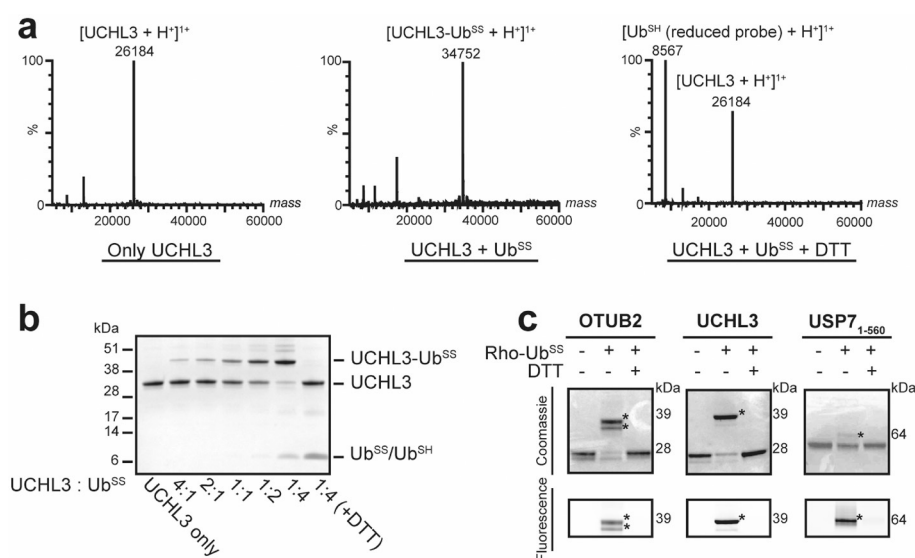


Figure 1. a) Binding of Ub^{SS} to UCHL3 monitored by mass spectrometry at a molar ratio of UCHL3/Ub^{SS} = 1:4. Treatment with DTT shows complete reduction of the complex. Unlabeled UCHL3 is shown as a reference. Additional peaks are detailed in Figure S2. b) Binding of Ub^{SS} to UCHL3 at different UCHL3/Ub^{SS} molar ratios. Treatment with DTT releases reduced probe (Ub^{SH}) from the Ub^{SS}-UCHL3 complex. Analysis was done by SDS-PAGE and Coomassie Brilliant Blue staining. c) Binding of the fluorescent probe Rho-Ub^{SS} to OTUB2, UCHL3, and the TRAF/catalytic domain of USP7 (USP7¹⁻⁵⁶⁰). Analysis was done by SDS-PAGE and Coomassie Brilliant Blue staining or in-gel fluorescence scanning. DUBs labeled with Rho-Ub^{SS} are indicated with an asterisk (*).

ure 1c). Rho-Ub^{SS} bound to all three DUBs as fluorescent bands with the expected molecular weights were observed after labeling. In addition, upwards shifts were observed after staining the gel with Coomassie Brilliant Blue, corresponding to the binding of one probe moiety to the DUBs. DTT treatment of the probe-DUB complex resulted in complete reduction and release of the reduced probe Rho-Ub^{SH} from the DUB.

To determine whether the activity of a DUB could be restored after reduction, we first reacted UCHL3 with Ub^{SS} and purified the complex by anion exchange chromatography (Figure 2a). Unlabeled UCHL3 was purified using the same procedure (Figure 2b). The Ub^{SS}-UCHL3 complex was then either left as is or treated with DTT, after which the enzymatic activity was monitored using a fluorogenic activity assay. In this assay, Ub-Rhodamine110 (Ub-Rho110) is cleaved, which releases fluorescent Rho110. In the absence of DTT, no cleavage was observed (Figure 2c). However, upon addition of DTT, Ub-Rho110 was cleaved with similar kinetics as observed with unlabeled UCHL3, indicating that the disulfide bond in the Ub^{SS}-UCHL3 complex was reduced, and the activity could be completely restored. The activity of UCHL3 was also monitored using the fluorescent Ub-propargylamide (PA) DUB ABP Rho-Ub-PA.^[3d] Fluorescent bands corresponding to UCHL3 bound to Rho-Ub-PA were observed for both unlabeled UCHL3 and DTT-treated Ub^{SS}-UCHL3

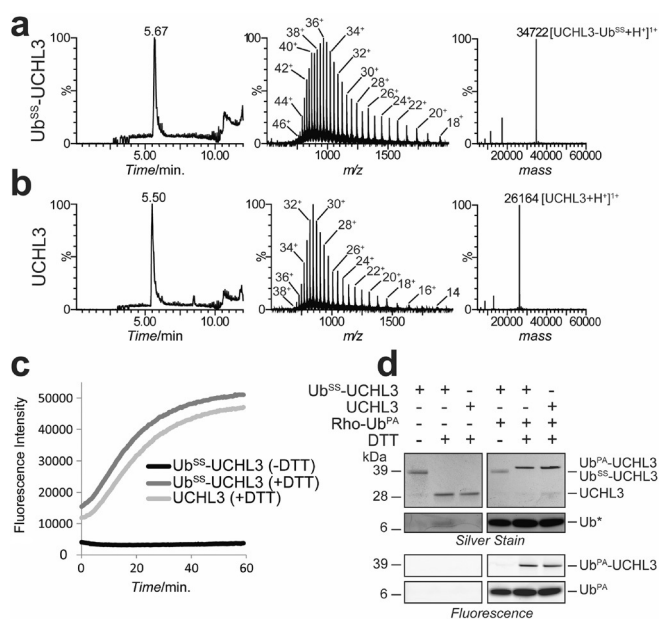


Figure 2. Analytical LC-MS profile of a) Ub^{SS}-UCHL3 complex and b) untreated UCHL3, purified by anion exchange chromatography. Additional peaks are detailed in Figure S3. c) The enzymatic activity of untreated and DTT-treated purified Ub^{SS}-UCHL3 complex and purified unlabeled UCHL3 was monitored using a Ub-Rho110 fluorogenic DUB activity assay. d) The enzymatic activity of untreated and DTT-treated purified Ub^{SS}-UCHL3 complex and purified unlabeled UCHL3 was monitored using the fluorescent DUB probe Rho-Ub-PA.^[3d] Proteins were resolved by non-reducing SDS-PAGE and visualized by fluorescence scanning or silver staining. Ub* indicates reduced disulfide probe Ub^{SH} (left panel) or Rho-Ub-PA and Ub^{SH} (right panel).

complexes, but not for untreated Ub^{SS}-UCHL3 complexes (Figure 2d). Correspondingly, upward shifts in the silver-stained gel were observed for both UCHL3 and DTT-treated Ub^{SS}-UCHL3 complex. UCHL3 released from the Ub^{SS}-UCHL3 complex after reduction showed similar labeling with Rho-Ub-PA as UCHL3 alone, suggesting that released UCHL3 regains full activity.

To validate that disulfide probes can also bind to DUBs in cell extract, we tested whether Ub^{SS} can inhibit the binding of the fluorescent DUB ABP TMR-Ub-PA.^[3d] Cell extracts from HeLa cells were pre-incubated with 1 μM of Ub^{SS}, followed by incubation with 1 μM of TMR-Ub-PA. As shown in Figure 3a, Ub^{SS} almost completely inhibited the labeling of DUBs by TMR-Ub-PA. The presence of DTT in the assay did allow for the labeling of DUBs with the TMR-Ub-PA probe, which suggests that Ub^{SS}-DUB complexes are readily released or not formed in the presence of reducing agent. To visualize the binding of Ub^{SS} to specific DUBs in cell extract, we overexpressed GFP fusions of the DUBs OTUB1 (Figures 3b and S4a), OTUB2 (Figure S4b), and USP8 (Figure S4c). Cell extracts were made and incubated with Ub^{SS} or Ub-PA as a positive control. We visualized labeling by non-reducing SDS-PAGE followed by anti-GFP immuno-

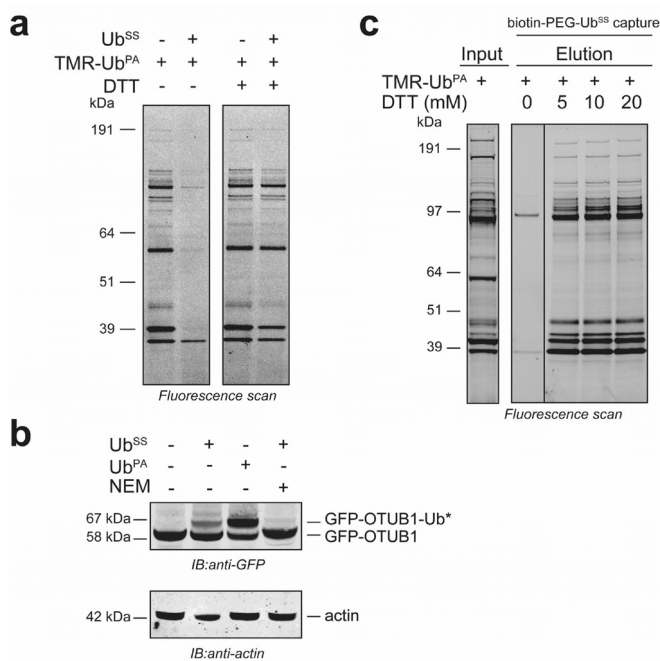


Figure 3. a) Competition experiment in which HeLa cell extract was incubated with Ub^{SS}, followed by DUB labeling with fluorescent TMR-Ub-PA^[3d] in the presence or absence of DTT. Labeled proteins were visualized using in-gel fluorescence scanning. b) Binding of Ub^{SS} or Ub-PA to overexpressed GFP-OTUB1. GFP-OTUB1-Ub* indicates GFP-OTUB1 bound to either Ub^{SS} (lane 2) or Ub-PA probe (lane 3). Preincubation with *N*-methyl maleimide (NMM) was used as a negative control (lane 4). GFP-OTUB1 was visualized using anti-GFP immunoblotting. Actin immunoblotting was used as a loading control. c) A HeLa cell extract was incubated with biotin-labeled Ub^{SS}, and reacted DUBs were captured on neutravidin resin. DUBs were released from the resin using DTT. Eluted DUBs and DUBs in the input cell extract were labeled with the fluorescently labeled ABP TMR-Ub-PA^[3d] and visualized using in-gel fluorescence scanning.

blotting. Labeling with one Ub^{SS} moiety was observed for all three GFP-fused DUBs, illustrated by a shift in migration on gel. Moreover, catalytic cysteine mutants showed minimal labeling (Figure S4), which corroborated that the active-site cysteine is the main site for modification.

To determine whether we can capture and release active DUBs from cell extract on resin, we incubated HeLa cell extracts with biotin-PEG-Ub^{SS}. The mixture was then allowed to bind to neutravidin resin. After washing of the resin, bound DUBs were eluted with the reducing agents DTT, TCEP, β -mercaptoethanol, or Cys (Figures 3c and S5). TMR-Ub-PA was added to the eluted fractions to visualize active DUBs by in-gel fluorescence scanning. The majority of DUBs that could be labeled in cell extract could also be labeled after elution with reducing agent. Notably, the 56 kDa DUB USP14 (65 kDa after binding of TMR-Ub-PA)^[3a,c,f,10] was not retrieved or not active after elution. This is likely due to the absence of proteasome after washing the resin. USP14 is known to be activated upon association with the proteasome.^[3a,11] Almost no active DUBs were eluted when no reducing agents were used. Thus active DUBs can be caught using our biotin-PEG-Ub^{SS} probe and specifically released under mild reducing conditions.

In conclusion, we have developed a novel type of Ub-based activity-based probe containing a methyl disulfide warhead that binds to DUBs by means of active-site-specific disulfide exchange. We have shown that we can capture DUBs from cell extract on resin, and elute them by using reducing agents. The importance of this probe lies in its ability to isolate active DUBs from their natural environment as cell-specific post-translational modifications may be present that regulate DUB activity. Our reversible probe can thus be used to isolate active DUBs or DUB-protein complexes from cells to study the activity of specific DUBs in in vitro deubiquitination assays. To the best of our knowledge, the DUB probes presented here are the first probes that can capture a specific family of enzymes and release active enzymes under mild conditions.

Acknowledgements

This work was funded by a VICI grant (to H.O.) from The Netherlands Foundation for Scientific Research (NWO). We thank Dris el Atmioui and Henk Hilkmann for the solid-phase synthesis of ubiquitin, the NKI protein facility for the expression and purification of OTUB2, Alexander Fish from the NKI protein facility for help with anion-exchange purification of Ub^{SS}-UCHL3 and UCHL3, and Paul Geurink and Titia Sixma for advice. We thank Robbert Kim for providing USP7¹⁻⁵⁶⁰, Remco Merckx for providing Rho-Ub-PA and UCHL3, Gerbrand van der Heden-van Noort for providing Ub-PA and TMR-Ub-PA, and Ilana Berlin for providing the GFP-fused DUB constructs.

Conflict of interest

The authors declare no conflict of interest.

Keywords: activity-based protein profiling · disulfide exchange · enzyme activity · post-translational modifications · ubiquitin

How to cite: *Angew. Chem. Int. Ed.* **2017**, *56*, 12967–12970
Angew. Chem. **2017**, *129*, 13147–13150

- [1] a) F. E. Reyes-Turcu, K. H. Ventii, K. D. Wilkinson, *Annu. Rev. Biochem.* **2009**, *78*, 363–397; b) M. J. Clague, C. Heride, S. Urbe, *Trends Cell Biol.* **2015**, *25*, 417–426.
- [2] a) P. P. Geurink, F. El Oualid, A. Jonker, D. S. Hameed, H. Ovaa, *ChemBioChem* **2012**, *13*, 293–297; b) T. E. Mevissen, M. K. Hospenthal, P. P. Geurink, P. R. Elliott, M. Akutsu, N. Arnaudo, R. Ekkebus, Y. Kulathu, T. Wauer, F. El Oualid, S. M. Freund, H. Ovaa, D. Komander, *Cell* **2013**, *154*, 169–184; c) L. C. Dang, F. D. Melandri, R. L. Stein, *Biochemistry* **1998**, *37*, 1868–1879; d) U. Hassiepen, U. Eidhoff, G. Meder, J. F. Bulber, A. Hein, U. Bodendorf, E. Lorthiois, B. Martoglio, *Anal. Biochem.* **2007**, *371*, 201–207.
- [3] a) A. Borodovsky, B. M. Kessler, R. Casagrande, H. S. Overkleeft, K. D. Wilkinson, H. L. Ploegh, *EMBO J.* **2001**, *20*, 5187–5196; b) A. Borodovsky, H. Ovaa, N. Kolli, T. Gan-Erdene, K. D. Wilkinson, H. L. Ploegh, B. M. Kessler, *Chem. Biol.* **2002**, *9*, 1149–1159; c) A. de Jong, R. Merckx, I. Berlin, B. Rodenko, R. H. Wijdeven, D. El Atmioui, Z. Yalcin, C. N. Robson, J. J. Neeffjes, H. Ovaa, *ChemBioChem* **2012**, *13*, 2251–2258; d) R. Ekkebus, S. I. van Kasteren, Y. Kulathu, A. Scholten, I. Berlin, P. P. Geurink, A. de Jong, S. Goerdalay, J. Neeffjes, A. J. Heck, D. Komander, H. Ovaa, *J. Am. Chem. Soc.* **2013**, *135*, 2867–2870; e) J. A. Ward, L. McLellan, M. Stockley, K. R. Gibson, G. A. Whitlock, C. Knights, J. A. Harrigan, X. Jacq, E. W. Tate, *ACS Chem. Biol.* **2016**, *11*, 3268–3272; f) D. Flierman, G. J. van der Heden van Noort, R. Ekkebus, P. P. Geurink, T. E. Mevissen, M. K. Hospenthal, D. Komander, H. Ovaa, *Cell Chem. Biol.* **2016**, *23*, 472–482.
- [4] S. I. van Kasteren, H. B. Kramer, H. H. Jensen, S. J. Campbell, J. Kirkpatrick, N. J. Oldham, D. C. Anthony, B. G. Davis, *Nature* **2007**, *446*, 1105–1109.
- [5] C. Chatterjee, R. K. McGinty, B. Fierz, T. W. Muir, *Nat. Chem. Biol.* **2010**, *6*, 267–269.
- [6] 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. Ovaa, *ChemBioChem* **2016**, *17*, 816–820.
- [7] 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.
- [8] D. Bonnet, B. Ilien, J. L. Galzi, S. Riche, C. Antheaune, M. Hibert, *Bioconjugate Chem.* **2006**, *17*, 1618–1623.
- [9] L. z. Borg, D. Lee, J. Lim, W. K. Bae, M. Park, S. Lee, C. Lee, K. Char, R. Zentel, *J. Mater. Chem. C* **2013**, *1*, 1722–1726.
- [10] M. Altun, H. B. Kramer, L. I. Willems, J. L. McDermott, C. A. Leach, S. J. Goldenberg, K. G. Kumar, R. Konietzny, R. Fischer, E. Kogan, M. M. Mackeen, J. McGouran, S. V. Khoronenkova, J. L. Parsons, G. L. Dianov, B. Nicholson, B. M. Kessler, *Chem. Biol.* **2011**, *18*, 1401–1412.
- [11] B. H. Lee, M. J. Lee, S. Park, D. C. Oh, S. Elsasser, P. C. Chen, C. Gartner, N. Dimova, J. Hanna, S. P. Gygi, S. M. Wilson, R. W. King, D. Finley, *Nature* **2010**, *467*, 179–184.

Manuscript received: July 3, 2017

Accepted manuscript online: August 25, 2017

Version of record online: September 12, 2017