Protein Modification

International Edition: DOI: 10.1002/anie.201505456
German Edition: DOI: 10.1002/ange.201505456

# Versatile and Efficient Site-Specific Protein Functionalization by Tubulin Tyrosine Ligase

*Dominik Schumacher, Jonas Helma, Florian A. Mann, Garwin Pichler, Francesco Natale, Eberhard Krause, M. Cristina Cardoso, Christian P. R. Hackenberger,* and Heinrich Leonhardt**

***Abstract:*** *A novel chemoenzymatic approach for simple and fast site-specific protein labeling is reported. Recombinant tubulin tyrosine ligase (TTL) was repurposed to attach various unnatural tyrosine derivatives as small bioorthogonal handles to proteins containing a short tubulin-derived recognition sequence (Tub-tag). This novel strategy enables a broad range of high-yielding and fast chemoselective C-terminal protein modifications on isolated proteins or in cell lysates for applications in biochemistry, cell biology, and beyond, as demonstrated by the site-specific labeling of nanobodies, GFP, and ubiquitin.*

Site-specific functionalization of proteins is crucial for a plethora of applications throughout the life sciences. Fluorescent proteins and self-labeling strategies like SNAP-[1] and HALO-[2] tagging have become indispensable tools for cell biologists to analyze intracellular activity and localize proteins of interest. Genetic fusion with GFP or self-labeling protein tags is straightforward; however, the size and biochemical nature of the attachment may affect the properties and application of the chimeric protein.[3] Protein trans-splicing, expressed protein ligation (EPL), and amber suppression and auxotrophic expression in combination with bioorthogonal labeling are prominent tools that allow the placement of small tags and modifications within the protein sequence.[4] However, low expression yields of genetic encoding systems[5] and challenges associated with impaired protein folding with trans-splicing and EPL[6] can be limiting factors when applying these techniques. As an alternative, chemoenzymatic approaches have attracted increasing attention for the site-specific modification of proteins by using short and specific recognition tags. A variety of enzymes, such as trypsin,[7] Sortase A,[8] phosphopantetheinyl transferase (PPTase),[9] biotin ligase,[10] lipoic acid ligase,[11] AnkX,[12] and formylglycine generating enzyme,[13] open up broad possibilities for subsequent chemoselective and site-specific labeling. However, several chemoenzymatic approaches still need to overcome some limitations, including large and hydrophobic substrates for the enzymatic reaction that may affect the protein of interest.[14] Moreover, the reversibility of some enzymatic reactions and product hydrolysis necessitates extensive enzyme engineering and a high excess of catalyst and substrate.[7,14,15]

Herein, we present a novel and fast method for the site-specific labeling of functional proteins that combines the use of small unnatural amino acids as bioorthogonal handles and the technical advantages of chemoenzymatic labeling. The technique, termed Tub-tag labeling, is based on the enzymatic ligation of easy-to-synthesize small tyrosine derivatives to the C terminus of a fourteen amino acid hydrophilic recognition tag (termed Tub-tag) by tubulin tyrosine ligase (TTL, Figure 1a). In nature, TTL catalyzes the post-translational attachment of tyrosine to the C terminus of α-tubulin, which is involved in the regulation of microtubule homeostasis.[16] Interestingly, it has been shown that TTL also utilizes tyrosine derivatives for the C-terminal modification of tubulin.[17]

We started our investigation by probing whether recombinant TTL can conjugate unnatural tyrosine derivatives to the isolated Tub-tag peptide, which mimics the C terminus of tubulin. For initial Tub-tag labeling experiments, we used 3-$N_3$-L-tyrosine (**1**) and 3-formyl-L-tyrosine (**2**) as substrates and synthesized a *5,6*-carboxyfluorescein-labeled Tub-tag peptide (CF-Tub-tag) by standard solid-phase peptide synthesis (SPPS). The reaction process was analyzed by isocratic HPLC (Figure 1b for **1**, Figure 1c for **2**, and Figure S1 in the Supporting Information). After 120 min of incubation (TTL/peptide 1:200) at 37 °C, we observed a conversion of 90 % and 63 % with **1** and **2**, respectively. The initial reaction rate of azide **1** surpasses that of aldehyde **2**, resulting in an approximately 2.6 times faster formation of the ligation product. However, extending the reaction in the case of aldehyde **2** delivers equal amounts of labeled peptide, thus demonstrating the broad substrate acceptance of tubulin tyrosine ligase.

Encouraged by these results, we tested whether Tub-tag labeling can be applied to the site-specific modification of proteins and whether the required C-terminal recognition motif influences the protein function. At the outset of our studies, we focused on camel-derived single-domain nano-

[*] D. Schumacher,[+] F. A. Mann, Dr. E. Krause, Prof. Dr. C. P. R. Hackenberger
Chemical Biology, Leibniz-Institut für Molekulare Pharmakologie Berlin (Germany)
E-mail: hackenbe@fmp-berlin.de
Dr. J. Helma,[+] Dr. G. Pichler, Prof. Dr. H. Leonhardt
Department of Biology II, Ludwig Maximilians Universität München and Center for Integrated Protein Science Munich
Martinsried (Germany)
E-mail: h.leonhardt@lmu.de
Dr. F. Natale, Prof. Dr. M. C. Cardoso
Department of Biology, Technische Universität Darmstadt
Darmstadt (Germany)
D. Schumacher,[+] F. A. Mann, Prof. Dr. C. P. R. Hackenberger
Department of Chemistry, Humboldt Universität zu Berlin
Berlin (Germany)
[+] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201505456.

  

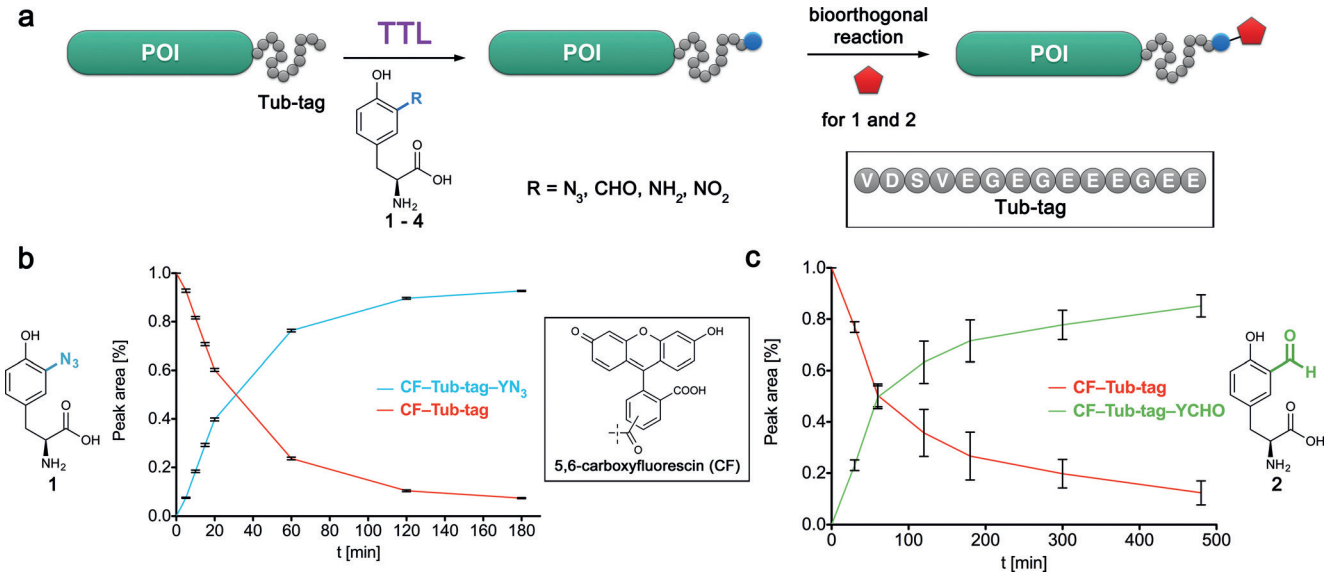


***Figure 1.*** Tub-tag labeling of proteins. a) Chemoenzymatic labeling of proteins by tubulin tyrosine ligase (TTL). Unnatural tyrosine derivatives are ligated to the C terminus of a short recognition tag (Tub-tag) to serve as bioorthogonal handles for the site-specific chemical modification of a protein of interest (POI). b, c) C-terminal addition of b) 3-$N_3$-L-tyrosine (**1**) and c) 3-formyl-L-tyrosine (**2**) to carboxyfluorescein-labeled peptide (CF-Tub-tag). HPLC fluorescence traces were taken at different time points of the TTL reaction and quantitation of substrate and product was performed through peak integration (Figure S1 in the Supporting Information). The red lines represents the consumption of the CF-Tub-tag, the blue and green lines the formation of C-terminally functionalized CF-Tub-tag-$YN_3$/CHO. The mean values and standard deviation (SD) of three replicate reactions are shown.

bodies,[18] which are used as analytical tools in biochemistry as well as for the intracellular recognition and manipulation of antigens in cell biology.[19] In this context, it was our particular goal to access a site-specifically fluorescently-labeled nanobody, since these approximately 15 kDa antigen-recognizing proteins display superior properties in superresolution microscopy owing to their exceptionally small size.[20]

We fused the Tub-tag sequence to the C terminus of a GFP-specific nanobody (GBP4[21]) and performed TTL-mediated labeling with a TTL/GBP4 ratio of 1:5 at 37 °C for 24 h. Tryptic digest followed by HPLC–MS/MS experiments showed the successful C-terminal addition of tyrosine, 3-$N_3$-L-tyrosine (**1**), 3-formyl-L-tyrosine (**2**), 3-$NH_2$-L-tyrosine (**3**), and 3-$NO_2$-L-tyrosine (**4**; Figure S3 in the Supporting Information). Next, we combined the incorporation of **1** with subsequent strain-promoted azide–alkyne cycloaddition (SPAAC)[22] for the conjugation of a dibenzocyclooctyne (DBCO)-biotin derivative (Figure 2a). For this, ligations were performed using different ratios of TTL/GBP4 and the reactions stopped by cooling down fast to 4 °C after one or three hours. A subsequent dialysis step was used to remove excess tyrosine derivative **1**, followed by SPAAC to DBCO-biotin. The yields were determined by SDS-PAGE analysis. Using a TTL/GBP4 ratio of 1:5 at 37 °C, we found that 82 % of the GBP4 was converted after one hour, whereas a TTL/GBP4 ratio of 1:10 delivered 71 % C-terminally modified GBP4. Extending the ligation time to three hours resulted in 99 % and 88 % conversions at TTL/GBP4 ratios of 1:5 and 1:10, respectively. The incorporation of 3-formyl-L-tyrosine (**2**) could be achieved with similar efficiency. To further validate the modularity of the Tub-tag labeling concept, we performed fluorescence labeling by SPAAC (Figure S7 in the Supporting Information) and employed a variety of well-established bioorthogonal reactions, including Staudinger ligation[23] (Figure S8 in the Supporting Information) and the Staudinger-phosphite reaction[24] (Figure S9 in the Supporting Information), to 3-$N_3$-L-tyrosine. In addition, hydrazone- (Figure S10 in the Supporting Information) and oxime- (Figure S11 in the Supporting Information) forming reactions[3c] were applied on site-specifically incorporated 3-formyl-L-tyrosine (**2**). This allowed us to incorporate different functional modules like biotin and fluorophores, and even enabled the branched PEGylation of GBP4 (Figure S9 in the Supporting Information). To demonstrate the broad applicability of this chemoenzymatic modification, we additionally applied it to the site-specific functionalization of GFP, ubiquitin, and another GFP-specific nanobody (GBP1[21]). SDS-PAGE analysis showed similar yields to the modification of GBP4 (Figure S12 and S13 in the Supporting Information). Moreover, the presence of the Tub-tag sequence did not affect the fluorescence and fluorescence-modifying properties[21] of GFP or GFP-specific nanobodies (Figures S4, S5 in the Supporting Information).

Having established the modular and high-yielding TTL-based modification method, we investigated the selectivity of the tyrosine transfer reaction within complex protein mixtures. *E. coli* cell lysate containing overexpressed GFP-Tub-tag was incubated with 3-$N_3$-L-tyrosine (**1**) and TTL for four hours. A subsequent dialysis was used to remove excess azide, and SPAAC to DBCO-biotin showed selective C-terminal functionalization of GFP (Figure 3a, b and Figure S14 in the Supporting Information). Next, we probed the specificity and applicability of the site-specifically modified nanobody GBP1 for the enrichment and isolation of overexpressed GFP from

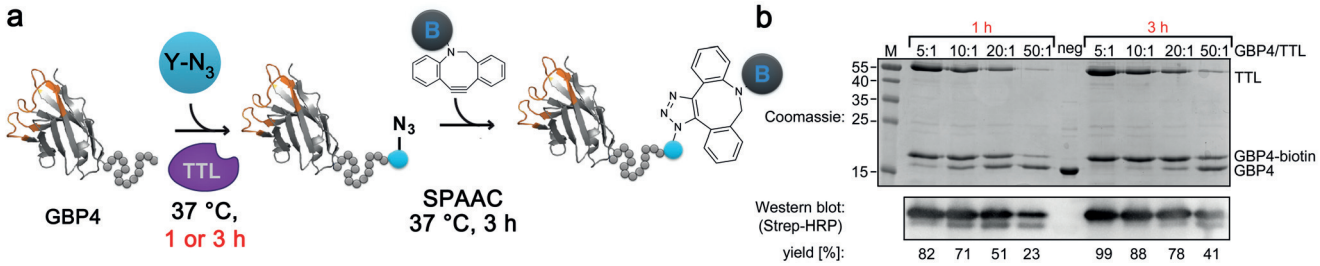


***Figure 2.*** Principle and efficiency of TTL-mediated functionalization a) Illustration of TTL-mediated incorporation of azide **1** followed by strain-promoted azide–alkyne cycloaddition (SPAAC) with DBCO-biotin. Dark gray circle = biotin. b) Incorporation of azide **1** into the C terminus of GBP4 by using different ratios of GBP4/TTL and reaction times (one and three hours), followed by SPAAC to a DBCO-biotin derivative. SDS-PAGE and western blot (Strep Ab-HRP) show efficient biotin labeling of GBP4 within one hour.

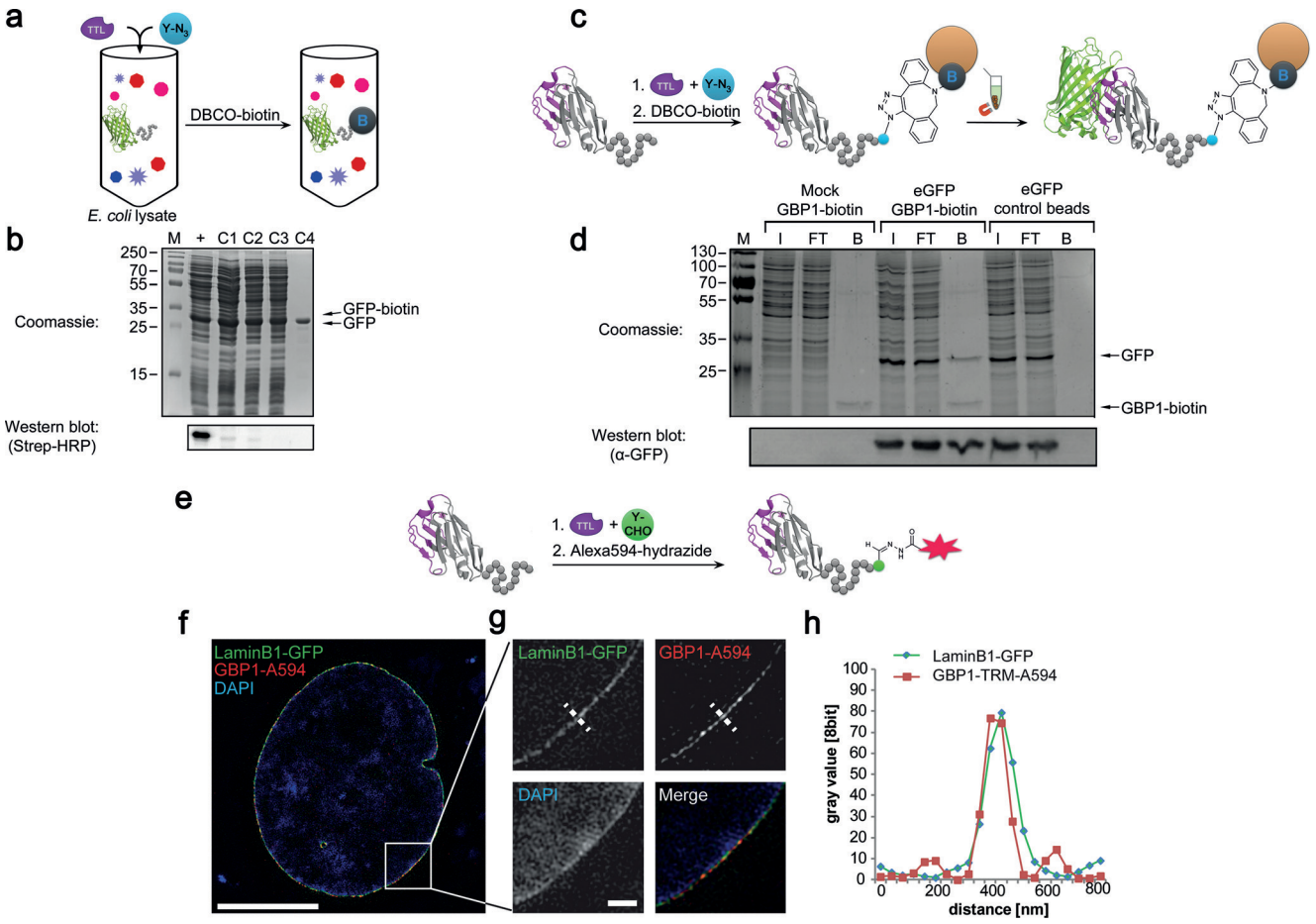


***Figure 3.*** Lysate labeling and the application of chemoenzymatically functionalized nanobodies to protein enrichment and superresolution microscopy. a) Schematic outline of Tub-tag labeling of GFP in complex protein mixtures (*E. coli* lysate). b) Coomassie staining and western blot analysis showing the high selectivity of the tyrosine ligation and subsequent biotinylation. (+: lysate treated with TTL, 3-$N_3$-L-tyrosine (**1**) and DBCO-biotin; C1: no 3-$N_3$-L-tyrosine (**1**) added; C2: lysate treated with DBCO-biotin; C3: lysate; C4: purified GFP). c) Outline of the site-specific biotinylation of the GFP-binding nanobody GBP1 and subsequent immunoprecipitation of GFP. d) Coomassie staining and western blot analysis showing efficient and specific GFP pulldown (Mock GBP1-biotin: lysate lacking overexpressed GFP; GFP GBP1-biotin: beads loaded with GBP1 and lysate containing GFP; GFP control beads: beads without immobilized GBP1, I: input to streptavidin beads; FT: flowthrough; B: beads). e) Outline of the site-specific labeling of GBP1 with Alexa594 f) Immunofluorescence with GBP1-Alexa594. Shown is a fixed HeLa cell nucleus with the lamina colabeled with LaminB1-GFP and GBP1-Alexa594. Scale bar:10 μm. g) expansion of the region highlighted in (d). h) Fluorescence intensity profile along the dotted line shown in (g) demonstrates high colocalization accuracy at subdiffraction resolution.

cell lysate. Following 3-$N_3$-L-tyrosine (**1**) incorporation via TTL, GBP1 was biotinylated with DBCO-biotin as described above and immobilized on Streptavidin-coated magnetic beads (Figure 3c). These beads were then used for the immunoprecipitation of GFP from HEK cell lysates. Subsequent western blot analysis demonstrated specific GFP

  

pulldown compared to controls with mock-transfected cell lysate and non-functionalized beads (Figure 3d).

We subsequently studied whether site-specifically TTL-labeled GBP1 can be used to immunostain cellular structures for superresolution microscopy. The higher resolution of superresolution microscopy imposes new requirements on detection reagents, and using the smallest possible immunofluorescent binding reagents is thus important for unleashing the full potential of superresolution microscopy.[20b]

Following 3-formyl-L-tyrosine (**2**) incorporation via TTL, GBP1 was labeled with the Alexa594 dye by using oxime formation (Figure 3e). In addition to the microscopy application, it was shown that both the azide and the aldehyde tyrosine derivatives could be used to obtain functional protein conjugates. HeLa cells expressing GFP-LaminB1, which localizes at the interior of the nuclear envelope and forms the nuclear lamina, were fixed and stained with GBP1-Alexa594 (ca. 1 μg per object slide). 3D-SIM superresolution microscopy revealed laminar colocalization of the GBP1 staining reagent and the GFP-LaminB1 at high resolution, thereby indicating functional binding to GFP in this cellular context (Figure 3f–h). Similar results were obtained with GFP-PCNA and the detection of subnuclear DNA replication sites (Figure S15 in the Supporting Information).

These studies demonstrate the applicability of Tub-tag-labeled nanobodies for functional studies in biochemistry and cell biology.

In summary, we introduce Tub-tag labeling for the simple, site-specific modification of functional and antigen-recognizing proteins. The TTL-mediated chemoenzymatic ligation of unnatural tyrosine derivatives like 3-$N_3$-L-tyrosine (**1**) and 3-formyl-L-tyrosine (**2**) enabled bioorthogonal functionalization in a total yield of up to 99% when using moderate enzyme concentrations and short reaction times. These modified tyrosine residues then serve as bioorthogonal handles for a variety of well-established chemoselective labeling reactions. The overall labeling efficiency under mild reaction conditions yields homogeneously modified and functional proteins, as demonstrated by the site-specific labeling of nanobodies for immunoprecipitation and superresolution microscopy. In summary, Tub-tag labeling endows recombinant antibodies—and proteins in general—with novel properties for exploring and manipulating cellular functions, with possible applications in biotechnology and medicine.

## Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SPP1623) to M.C.C. (CA 198/8-1), C.P.R.H. (HA 4468/9-1) and H.L. (LE 721/13-1), the Nanosystems Initiative Munich (NIM) to H.L., the Einstein Foundation Berlin (Leibniz-Humboldt Professorship) and the Boehringer-Ingelheim Foundation (Plus 3 award) to C.P.R.H. and the Fonds der Chemischen Industrie (FCI) to C.P.R.H. and to D.S. (Kekulé-scholarship). We thank Ralf Jacob for providing a plasmid with the TTL coding sequence.[25] We are grateful to Kristin Kemnitz-Hassanin, Heike Stephanowitz, and Andreas Maiser for technical support and to the late Bijàn Mir-Montazeri for help in the initial phase of this project. The authors declare competing financial interest: The technology described in the manuscript is part of a pending patent application.

**Keywords:** antibodies · chemoenzymatic labeling · nanobodies · site-specific functionalization · tubulin tyrosine ligase

**How to cite:** *Angew. Chem. Int. Ed.* **2015**, *54*, 13787–13791
*Angew. Chem.* **2015**, *127*, 13992–13996

[1] A. Keppler, S. Gendreizig, T. Gronemeyer, H. Pick, H. Vogel, K. Johnsson, *Nat. Biotechnol.* **2003**, *21*, 86–89.
[2] G. V. Los, L. P. Encell, M. G. McDougall, D. D. Hartzell, N. Karassina, C. Zimprich, M. G. Wood, R. Learish, R. F. Ohana, M. Urh, D. Simpson, J. Mendez, K. Zimmerman, P. Otto, G. Vidugiris, J. Zhu, A. Darzins, D. H. Klaubert, R. F. Bulleit, K. V. Wood, *ACS Chem. Biol.* **2008**, *3*, 373–382.
[3] a) D. Schumacher, C. P. Hackenberger, *Curr. Opin. Chem. Biol.* **2014**, *18*, 62–69; b) K. Lang, J. W. Chin, *Chem. Rev.* **2014**, *114*, 4764–4806; c) E. M. Sletten, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2009**, *48*, 6974–6998; *Angew. Chem.* **2009**, *121*, 7108–7133.
[4] a) C. P. Hackenberger, D. Schwarzer, *Angew. Chem. Int. Ed.* **2008**, *47*, 10030–10074; *Angew. Chem.* **2008**, *120*, 10182–10228; b) L. R. Malins, R. J. Payne, *Curr. Opin. Chem. Biol.* **2014**, *18*, 70–78; c) C. D. Spicer, B. G. Davis, *Nat. Commun.* **2014**, *5*, 4740; d) B. L. Nilsson, M. B. Soellner, R. T. Raines, *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 91–118.
[5] a) L. Wang, P. G. Schultz, *Angew. Chem. Int. Ed.* **2005**, *44*, 34–66; *Angew. Chem.* **2005**, *117*, 34–68; b) S. Milles, S. Tyagi, N. Banterle, C. Koehler, V. VanDelinder, T. Plass, A. P. Neal, E. A. Lemke, *J. Am. Chem. Soc.* **2012**, *134*, 5187–5195.
[6] a) G. Volkmann, H. D. Mootz, *Cell. Mol. Life Sci.* **2013**, *70*, 1185–1206; b) A. Reif, S. Siebenhaar, A. Troster, M. Schmalzlein, C. Lechner, P. Velisetty, K. Gottwald, C. Pohner, I. Boos, V. Schubert, S. Rose-John, C. Unverzagt, *Angew. Chem. Int. Ed.* **2014**, *53*, 12125–12131; *Angew. Chem.* **2014**, *126*, 12321–12327.
[7] S. Liebscher, M. Schopfel, T. Aumuller, A. Sharkhuukhen, A. Pech, E. Hoss, C. Parthier, G. Jahreis, M. T. Stubbs, F. Bordusa, *Angew. Chem. Int. Ed.* **2014**, *53*, 3024–3028; *Angew. Chem.* **2014**, *126*, 3068–3072.
[8] H. Mao, S. A. Hart, A. Schink, B. A. Pollok, *J. Am. Chem. Soc.* **2004**, *126*, 2670–2671.
[9] J. Yin, P. D. Straight, S. M. McLoughlin, Z. Zhou, A. J. Lin, D. E. Golan, N. L. Kelleher, R. Kolter, C. T. Walsh, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 15815–15820.
[10] I. Chen, M. Howarth, W. Lin, A. Y. Ting, *Nat. Methods* **2005**, *2*, 99–104.
[11] M. Fernández-Suárez, H. Baruah, L. Martínez-Hernández, K. T. Xie, J. M. Baskin, C. R. Bertozzi, A. Y. Ting, *Nat. Biotechnol.* **2007**, *25*, 1483–1487.
[12] K. Heller, P. Ochtrop, M. F. Albers, F. B. Zauner, A. Itzen, C. Hedberg, *Angew. Chem. Int. Ed.* **2015**, *54*, 10327–10330; *Angew. Chem.* **2015**, *127*, 10467–10471.
[13] P. Wu, W. Shui, B. L. Carlson, N. Hu, D. Rabuka, J. Lee, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 3000–3005.
[14] M. Rashidian, J. K. Dozier, M. D. Distefano, *Bioconjugate Chem.* **2013**, *24*, 1277–1294.
[15] M. W. Popp, J. M. Antos, G. M. Grotenbreg, E. Spooner, H. L. Ploegh, *Nat. Chem. Biol.* **2007**, *3*, 707–708.
[16] a) M. Rudiger, J. Wehland, K. Weber, *FEBS J.* **1994**, *220*, 309–320; b) A. Szyk, A. M. Deaconescu, G. Piszczek, A. Roll-Mecak, *Nat. Struct. Mol. Biol.* **2011**, *18*, 1250–1258.
[17] A. Banerjee, T. D. Panosian, K. Mukherjee, R. Ravindra, S. Gal, D. L. Sackett, S. Bane, *ACS Chem. Biol.* **2010**, *5*, 777–785.

  

[18] C. Hamers-Casterman, T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E. B. Songa, N. Bendahman, R. Hamers, *Nature* **1993**, *363*, 446 – 448.
[19] a) U. Rothbauer, K. Zolghadr, S. Tillib, D. Nowak, L. Schermelleh, A. Gahl, N. Backmann, K. Conrath, S. Muyldermans, M. C. Cardoso, H. Leonhardt, *Nat. Methods* **2006**, *3*, 887 – 889; b) L. Trinkle-Mulcahy, S. Boulon, Y. W. Lam, R. Urcia, F. M. Boisvert, F. Vandermoere, N. A. Morrice, S. Swift, U. Rothbauer, H. Leonhardt, A. Lamond, *J. Cell Biol.* **2008**, *183*, 223 – 239.
[20] a) J. Guizetti, L. Schermelleh, J. Mantler, S. Maar, I. Poser, H. Leonhardt, T. Muller-Reichert, D. W. Gerlich, *Science* **2011**, *331*, 1616 – 1620; b) J. Ries, C. Kaplan, E. Platonova, H. Eghlidi, H. Ewers, *Nat. Methods* **2012**, *9*, 582 – 584.
[21] A. Kirchhofer, J. Helma, K. Schmidthals, C. Frauer, S. Cui, A. Karcher, M. Pellis, S. Muyldermans, C. S. Casas-Delucchi, M. C. Cardoso, H. Leonhardt, K. P. Hopfner, U. Rothbauer, *Nat. Struct. Mol. Biol.* **2010**, *17*, 133 – 138.
[22] N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046 – 15047.
[23] E. Saxon, C. R. Bertozzi, *Science* **2000**, *287*, 2007 – 2010.
[24] a) N. Nischan, A. Chakrabarti, R. A. Serwa, P. H. Bovee-Geurts, R. Brock, C. P. Hackenberger, *Angew. Chem. Int. Ed.* **2013**, *52*, 11920 – 11924; *Angew. Chem.* **2013**, *125*, 12138 – 12142; b) R. Serwa, P. Majkut, B. Horstmann, J. M. Swiecicki, M. Gerrits, E. Krause, C. P. R. Hackenberger, *Chem. Sci.* **2010**, *1*, 596 – 602; c) R. Serwa, I. Wilkening, G. Del Signore, M. Muhlberg, I. Claussnitzer, C. Weise, M. Gerrits, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2009**, *48*, 8234 – 8239; *Angew. Chem.* **2009**, *121*, 8382 – 8387.
[25] S. Zink, L. Grosse, A. Freikamp, S. Banfer, F. Muksch, R. Jacob, *J. Cell Sci.* **2012**, *125*, 5998 – 6008.

Received: June 14, 2015
Revised: August 17, 2015
Published online: September 25, 2015

  