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# Advancing our Understanding of Ubiquitination Using the Ub-Toolkit

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## Abstract

Post-translational protein modification by ubiquitin (Ub) and Ub-like modifiers is orchestrated by the sequential action of Ub-activating, -conjugating, and -ligating enzymes to regulate a vast array of fundamental biological processes. Unsurprisingly, the dysregulation of the intricate interplay between ubiquitination and deubiquitination gives rise to numerous pathologies, most notably cancer and neurodegenerative diseases. While activity-based probes (ABPs) and assay reagents have been extensively developed and applied for deubiquitinating enzymes, similar tools for the Ub cascade have only recently emerged. Given the recent efforts to develop inhibitors for the Ub system, the urgency for developing ABPs and assay reagents is imminent. In this light, we comprehensively discuss the currently available ABPs with a focus on the newly developed reagents targeting the Ub cascade while illustrating their potential applications.

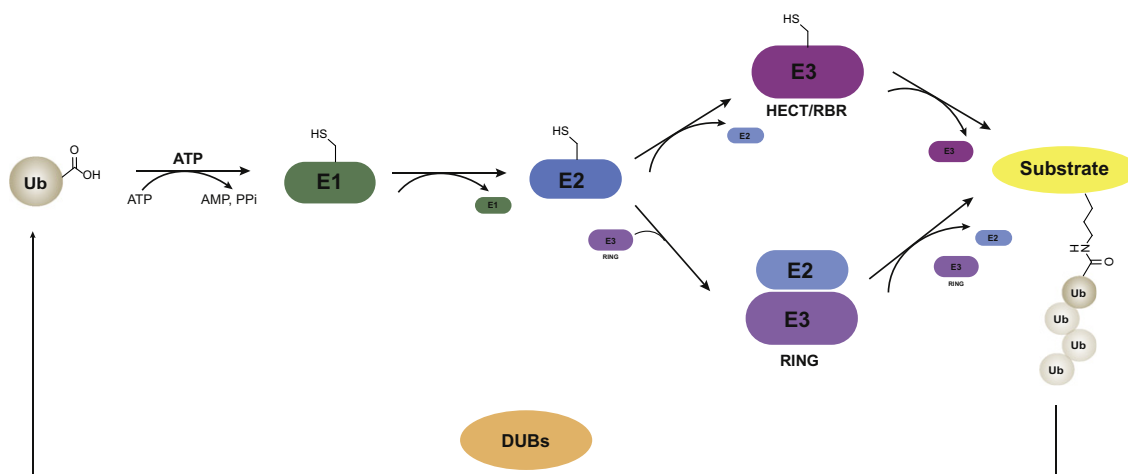
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## Introduction

Ubiquitination, a 76-aa post-translational modification, regulates a plethora of fundamental biological processes [1]. Covalent attachment of ubiquitin (Ub) to its designated substrate proteins occurs by the virtue of an orchestrated action of three specialized enzyme classes—E1, E2, and E3 enzymes. Initiation of ubiquitination commences with the adenylation of the C terminus of Ub at the expense of ATP by forming a high-energy E1-Ub thioester. Subsequently, the activated Ub is transferred by transthioesterification onto the cysteine residue of the E2 enzyme, which permits the Ub transfer on a substrate lysine residue by the means of an E3 ligase (Fig. 1). Ub transfer from the E3 enzyme to the substrate lysine can occur by either directly utilizing the catalytic cysteine residue (HECT/RBR class) or indirectly (RING E3s). Additionally, self-modification of Ub by one of its seven lysine residues to form Ub chains further modulates the biological consequence of substrate ubiquitination, which is counterbalanced by deubiquitinating enzymes (DUBs) of the different classes (UCH, USP, OTU, Josephin, and JAMM/MPN+) and the recently discovered class of MINDY DUBs [1,2] (Fig. 1). Given

the biological importance of Ub, the necessity for a tight regulation of this post-translational modification and maintenance of cellular homeostasis is fulfilled by the orchestrated interplay of two E1 enzymes, 40 E2s, around 600 E3 ligases, and 100 identified DUBs encoded by the human genome [3].

Since both DUBs and E2 conjugating enzymes and E3 ligases together coordinate ubiquitination governing the cellular outcome, their aberrant activity unsurprisingly leads to the dysregulation of cellular homeostasis, promoting the pathogenesis of numerous diseases, most notably cancer and neurodegenerative disorders [4]. So far, there are only a handful of drugs targeting the Ub system that have been FDA-approved or have even entered clinical trials. The considerable success of proteasome inhibitors such as bortezomib and carfilzomib has sparked interest to develop more small-molecule inhibitors targeting other components of the Ub system [5]. Due to their participation in a vast array of biological roles, specific inhibitor development for the Ub conjugation or deconjugation has proven to be challenging. E1 enzymes activate Ub in an ATP-dependent manner; inhibition at the apex of the Ub/Ubl cascades controls the ubiquitination of



**Fig. 1.** Schematic overview of the ubiquitination cascade. Activation of ubiquitin (Ub) as a thioester is catalyzed under ATP consumption by the Ub-activating enzyme E1 and subsequent transfer to the Ub-conjugating enzyme (E2). Finally, the activated Ub is covalently attached to the substrate lysine residue with the direct (HECT/RBR class) or indirect (RING class) participation of an E3 ligase. DUBs cleave or trim ubiquitination and poly-ubiquitination to reverse or modulate the biological outcome.

downstream targets. To date, two E1 inhibitors having reached clinical evaluation are the NAE1 inhibitor pevonedistat (MLN4924) for the treatment of acute myeloid leukemia and myelodysplastic syndromes and, more recently, a UBE1 inhibitor MNL7243 [6–8]. Similarly, only a few small molecules targeting Ub E3 ligases have progressed to clinical evaluation. For example, the immunomodulatory imide drugs thalidomide and its derivatives, which were first introduced as sedatives, have been demonstrated to modulate the cullin-RING E3 ligase cereblon and are now approved for use in hematological malignancies [5,9]. Additionally, several different compound classes exhibiting inhibitory properties toward the E3 ligase MDM2 have entered clinical testing [10]. Identifying and understanding the enzymatic activities and substrate specificity of the Ub- and Ub-like (Ubl) modifier-activating, -conjugating, -ligating, and -deconjugating enzymes are critical for the development of pharmacological inhibitors. Although reversed proteolysis and intein chemistry were originally used to generate Ub-based reagents [11], the introduction of effective synthetic procedures greatly expanded the Ub toolkit. While DUBs have been identified and extensively characterized utilizing these reagents, increasing efforts have recently been made toward developing activity-based probes (ABPs) to study the conjugating and ligating enzymes of the Ub cascade. ABPs consist of three main elements: a reactive group, a recognition element, and a retrieval or detection tag [12]. In contrast to assay reagents, ABPs react covalently with the active site nucleophile of specific enzymes, allowing the identification, isolation, and characterization of active enzymes [13] (Fig. 2b). In this review, the advances of Ub- and Ubl-based reagents and probes will be discussed while highlighting the advances of Ub ABPs targeting Ub ligases and

how these ABPs can potentially provide insights on these molecular processes.

## Synthetic and Semi-Synthetic Methods to Generate Ub ABPs

### Semi-synthetic approaches

Given the importance of understanding Ub conjugation and deconjugation for pharmacological intervention, both semi-synthetic and synthetic approaches to develop these reagents have been undertaken. One of the first approaches to develop Ub-based assay reagents Ub-AMC used transpeptidation to modify Ub [14,15]. However, one of the most powerful semi-synthetic approaches to generate Ub-based active site probes has been intein-based chemistry [16]. This methodology relies on protein *trans*-splicing, which through a series of acyl shifts forms a thioester that can react with thiol or amine nucleophiles [17,18]. In the recent years, advances in the genetic incorporation of unnatural amino acids using genetic code expansion permitted the incorporation of unnatural amino acids facilitating the production of Ub-based reagents [19–21]. Another semi-synthetic approach to generate fluorogenic Ub-based DUB substrates and di-Ub mimics exploits the E1-enzyme-mediated C-terminal amidation of Ub [22].

### Synthetic methods

Although efforts to synthesize Ub have been pioneered by Briand *et al.* and Ramage *et al.* in the late 1980s [23,24], only the introduction of an

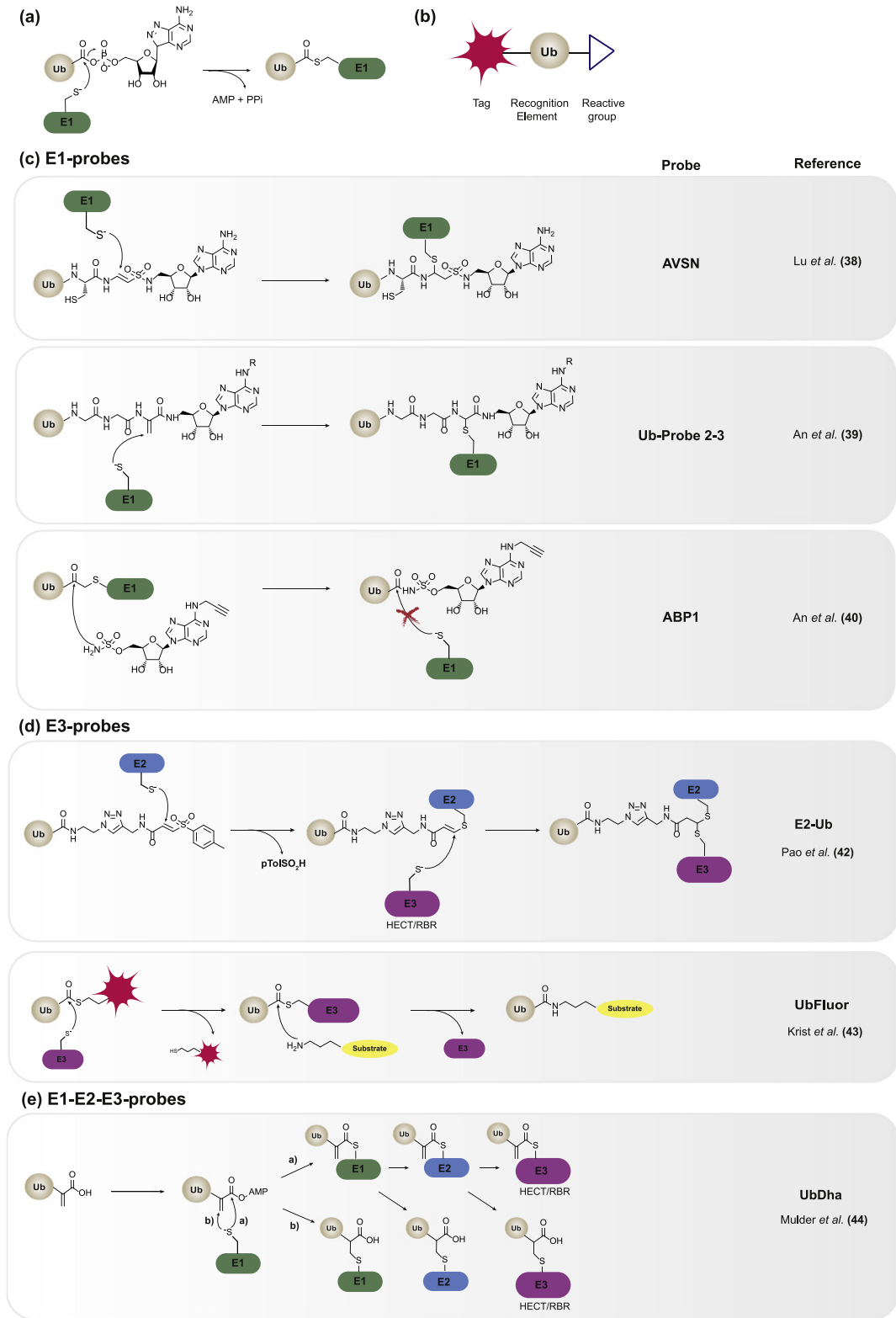


Fig. 2 (legend on next page)

efficient linear Fmoc-based solid-phase peptide synthesis of Ub and the native chemical ligation strategy unlocked the potential of these ABPs through the site-specific installation of a wide variety of reactive groups, unnatural amino acids, fluorescent labels, or pull-down handles [25]. Undoubtedly, one of the most important methods to generate Ub chains or ubiquitinated proteins and peptides is native chemical ligation—a method that relies on the incorporation of  $\gamma$ - or  $\delta$ -thiolysine moieties at defined lysine residues to yield the Ub–isopeptide linkage [25–27].

### The Ub Toolkit—An Assortment Ranging from DUB Reagents to Di-Ub Probes

The development of a variety of Ub-based probes targeting the cysteine protease DUB classes has facilitated their discovery, extensive mechanistic and biochemical investigation, and structural elucidation. Activity-based probes equipped with the classical Ub aldehyde, the vinyl methyl ester, the vinyl sulfone, and the propargyl reactive groups enabled the crystallization and solution of numerous DUB structures [16,28–30]. With the recent addition of di-Ub probes featuring a warhead between the distal and the proximal Ub module to covalently trap enzymes, the aspect DUB linkage specificity could be addressed in structural and biochemical studies [31–36]. Although these di-Ub probes have led to the characterization of DUBs [32], they do not allow the study of the DUB S1 and S2 binding site participation in Ub chain processing. To study this aspect, Flierman *et al.* reported non-hydrolysable di-Ub probes that have been generated by click chemistry and feature a C-terminal propargyl warhead [37]. Despite the broad assortment of ABPs and fluorogenic assay reagents available for DUBs, corresponding reagents to assess Ub conjugation and ligation have been lacking due to the complexity and dynamics involved.

### Beyond Deubiquitinating Enzymes—Targeting the Ub Cascade

Although a number of ABPs have been developed for DUBs, the advancement of probes to monitor the

E1-E2-E3 enzyme cascade has been lagging behind due to challenges of targeting a sequential enzymatic cascade rather than a single enzyme. Since ABPs mechanistically utilize an active-site nucleophile, the current probe developments for E3 ligases target only the HECT and RBR E3-enzymes. Early attempts described by Lu *et al.* toward developing a Ub-based probe for the E1 enzyme involved a 5'-sulfonyl-adenosine modification of the C terminus of Ub or a Ubl modifier [38] (Fig. 2c). This semi-synthetic approach, which permitted the introduction of an electrophilic trap through the covalent crosslinking of the E1 enzyme to the Ub/Ubl probe, allowed the mechanistic study of these enzymes, that is, insights into E1-catalyzed adenylation and thioesterification. To generate Ub/Ubl-AMP probes more efficiently, An *et al.* later devised an easier protocol based on the approach by Lu *et al.* using native-chemical ligation followed by the conversion of a cysteine residue to dehydroalanine (Dha). This allows a covalent bond formation with the active-site cysteine of the E1 enzyme, thereby trapping the “tetrahedral E1-Ubl-AMP intermediate” [39] (Fig. 2c). This methodology utilizes Ub/Ubls prepared by intein-based methods, retaining the “native” sequence and permitting E1-mediated chemoenzymatic synthesis, while in contrast, the approach by Lu *et al.* requires changes in the Ub/Ubl sequence [38]. In contrast to previous Ub/Ubl activity probes, these Ub/Ubl-AMP probes (Ub-Probe 2 and 3) are equipped with a tag for the installation of a pull-down handle or a fluorescent tag to facilitate detection (Fig. 2c). Other advancements by An and Statsyuk employed a mechanism-based approach using an AMP-derived compound (ABP1), which reacts with the E1-Ub/Ubl thioester intermediate due to its structural resemblance to AMP, forming the covalent Ub/Ubl-ABP1 conjugate structurally reminiscent of the Ub-AMP adenylylate [40] (Fig. 2c). One major advantage of this ABP is its cell permeability, which renders it amenable to profiling Ub/Ubl E1 enzyme inhibitors in living cells. Due to cross-reactivity with DUBs such as IsoT (USP5), utility of the Dha Ub-Probes 2 and 3 are restricted to monitoring E1 enzyme activity *in vitro* [39,40]. A major limitation of these first-generation ABPs due to their mimicry of the Ub/Ubl-adenylylate intermediate is that they are restricted to the E1 but cannot be transferred to the downstream E2 and HECT- and RBR-E3 enzymes. In order to overcome this obstacle, Pao *et al.* developed a more advanced activity probe by generating an azide-functionalized

**Fig. 2.** Overview of currently developed activity-based probes (ABPs) for ubiquitin (Ub) E1, E2, and E3 enzymes. (a) Mechanism of E1-catalyzed Ub thioester formation. Initially, the Ub adenylylate undergoes a nucleophilic attack by the active-site cysteine of the E1 enzyme, releasing the AMP to yield the thioester. (b) Basic modular structure of an ABP and (c) ABPs targeting E1-activating enzyme only. (d) Modular E2-Ub probe targeting specific HECT/RBR E3 ligases and fluorescent Ub thioester (UbFluor) reagent monitoring transthiolation activity. (e) Cascading E1-E2-E3 mechanism-based probe that is transferred and simultaneously labels the activating, conjugating, and ligating enzymes but not the substrates.

Ub that was conjugated to an alkyne-modified tosyl-substituted doubly activated ene (TDAE) by click chemistry to yield the E2-Ub using an approach based on E2-TDAE [41,42]. This modified Ub was reacted with a respective E2 enzyme forming a stable E2-Ub conjugate that was subsequently used to recruit the RBR-E3 ligase Parkin and thus monitor the transthiolation activity of an E3 ligase (Fig. 2d). Using this approach, the transthiolation activity of this ligase dependent on the phosphorylation status of both the ligase and Ub was profiled [42]. Despite the emergence of ABPs to interrogate the Ub-conjugation cascade, reagents to monitor efficiently the transthiolation of HECT- and RBR-E3 enzymes in high-throughput settings are urgently required. To address this need, Park *et al.* demonstrated that a mimimalistic ABP mimicking a Ub thioester in the form of UbMes (mercaptoethanesulfonate) can react with catalytically active E3 enzymes in the absence of ATP and E1 and E2 enzymes in their "Bypassing System" [43]. In a follow-up study, Krist *et al.* generated a fluorescent Ub thioester that permits a direct readout of both transthiolation and ligation activities of HECT E3 ligases [44] (Fig. 2d). Since monitoring the transthiolation activity of E2/E3 enzymes requires both the activation and transfer of Ub/Ubl, Mulder *et al.* addressed this challenge by developing an ABP that participates in the sequential transthiolation reactions and has the option to covalently react with the active-site cysteine residues of the E1, E2, or HECT/RBR-E3 enzyme [45] (Fig. 2e). Replacement of Gly76 at the C terminus of Ub with Dha moiety allows it to react with active-site cysteine residues in an ATP-dependent manner reminiscent of the native transthiolation or to alternatively form the covalent thioether adduct (Fig. 2). The ability of UbDha to participate in the cascade reaction and to react covalently with the active-site cysteine residue of the E1, E2, and HECT enzymes was confirmed by *in vitro* experiments and proteomics studies. These features make this the first ligase ABP amenable not only to biochemistry and structural studies but also especially to proteomics and in-cell enzymology of the entire Ub cascade.

### Expanding the Scope of E1-E2-E3 ABPs

Despite all the recent advances in the development of ligase activity probes, their applicability for drug discovery still remains to be demonstrated. E3 ligases, which are beginning to emerge as drug targets especially in oncology, are commonly tested for inhibition by assaying the ubiquitination of a substrate, which is usually overexpressed together with a ligase [5]. Although this approach has led to the discovery of several inhibitors, its largest caveat is that the substrates need to be known. Additionally, overexpression may lead to biological alterations that may not

reflect the endogenous situation, not to mention that this methodology is labor intensive. Utilizing ABPs capable of monitoring the activity of the entire cascade would simplify assays for drug screening by enabling high-throughput screens and providing information on the E2 and E3 enzyme activity. However, this necessitates the generation of specific ABPs and assay reagents for probing E3 ligase functions, for example, by combining phage display with synthetic strategies. In a recent study, Zhang *et al.* demonstrate the generation of specific Ub variants reactive toward specific HECT E3 ligases, enabling the discovery of novel biological functions for these enzymes [46]. As the current ligase ABPs either are limited to a modular approach (E2-Ub conjugates) or broadly target only HECT- and RBR-type E3 ligases, specificity of these tools could be introduced by combining this strategy with synthetic efforts to yield a powerful platform for unraveling the E3 ligase biology and for drug discovery. While modular approaches, such as the Ub-TDAE probe, have been applied to biochemically assess the effect of mutations of Parkin, UbDha is the first ABP facilitating the chemoproteomic study of the Ub cascade in the context of diverse perturbations (i.e., silencing, bacterial and viral infections, inhibitors, etc.). This opens up the possibility of exploring new avenues of studying HECT/RBR E3-ligase activity in a complex biological context, for example, the effect of mutations in a specific HECT/RBR E3 ligase activity [42,45].

### Concluding Remarks

In comparison to phosphorylation, the temporal, spatial, and substrate context of ubiquitination dictating the biological outcome is extremely intricate, presenting challenges in the development of specific ABPs and warranting careful evaluation of drug intervention. For instance, direct inhibition of Ub E3 enzyme activity could abrogate auto-ubiquitination, which regulates both enzyme and substrate stability [5] and thus directs that inhibition could lead to substrate accumulation that adversely affects cellular homeostasis [47]. Given these ramifications, can a single pharmacological inhibitor for such a complex post-translational modification be the answer? To facilitate the discovery of effective yet tailored pharmacological intervention strategies, we must further explore the dynamic interplay and crosstalk in the ubiquitination system by exploiting the available tools for assaying these enzyme activities and by obtaining structural insights and proteomic data. Given that ubiquitination relies on the highly orchestrated action of all enzymes in the Ub cascade, both conjugation and deconjugation aspects must be regarded in evaluating potential pharmacological intervention strategies to effectively counterbalance aberrant ubiquitination. However, design of such pharmacological perturbations requires the accessibility of novel advanced tools to elucidate and exploit the

dynamic nature of the Ub system *in vitro* and even more importantly *in vivo*. In the light of this increasing demand for effective inhibitors for the Ub conjugation cascade, we anticipate that novel tools to study E1, E2, and E3 enzymes will emerge in the coming years. Additionally, application of existing tools reviewed here for addressing biochemical, structural questions, and chemoproteomics will shed light into the complexity of the Ub system and potentially advance drug discovery.

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E2 and E3 enzymes;  
ligases

#### Abbreviations used:

Ub, ubiquitin; DUB, deubiquitinating enzyme; Ubl, ubiquitin-like; ABP, activity-based probe; Dha, dehydroalanine; TDAE, tosyl-substituted doubly activated ene.

## References

- [1] D. Komander, M. Rape, The ubiquitin code, *Annu. Rev. Biochem.* 81 (2012) 203–229.
- [2] S.A. Abdul Rehman, et al., MINDY-1 is a member of an evolutionarily conserved and structurally distinct new family of deubiquitinating enzymes, *Mol. Cell* 63 (2016) 146–155.
- [3] S.M. Nijman, et al., A genomic and functional inventory of deubiquitinating enzymes, *Cell* 123 (2005) 773–786.
- [4] D. Popovic, D. Vucic, I. Dikic, Ubiquitination in disease pathogenesis and treatment, *Nat. Med.* 20 (2014) 1242–1253.
- [5] X. Huang, V.M. Dixit, Drugging the undruggables: exploring the ubiquitin system for drug development, *Cell Res.* 26 (2016) 484–498.
- [6] T.A. Soucy, et al., An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer, *Nature* 458 (2009) 732–736.
- [7] J. Sarantopoulos, et al., Phase I study of the investigational NEDD8-activating enzyme inhibitor pevonedistat (TAK-924/MLN4924) in patients with advanced solid tumors, *Clin. Cancer Res.* 22 (2016) 847–857.
- [8] Inc., MP, A phase I, open-label, multicenter, dose escalation study to access the safety and tolerability of MLN7243, an inhibitor of ubiquitin-activating enzyme (UAE), In Adult Patients with Advanced Solid Tumors. *ClinicalTrials.gov* [Internet]. Bethesda (MD): National Library of Medicine (US). 2017-(cited 2017 Feb27).
- [9] T. Ito, et al., Identification of a primary target of thalidomide teratogenicity, *Science* 327 (2010) 1345–1350.
- [10] Y. Zhao, et al., Small-molecule inhibitors of the MDM2-p53 protein-protein interaction (MDM2 inhibitors) in clinical trials for cancer treatment, *J. Med. Chem.* 58 (2015) 1038–1052.
- [11] R. Ekkebus, et al., Catching a DUB in the act: novel ubiquitin-based active site directed probes, *Curr. Opin. Chem. Biol.* 23 (2014) 63–70.
- [12] B.F. Cravatt, A.T. Wright, J.W. Kozarich, Activity-based protein profiling: from enzyme chemistry to proteomic chemistry, *Annu. Rev. Biochem.* 77 (2008) 383–414.
- [13] L.E. Sanman, M. Bogoy, Activity-based profiling of proteases, *Annu. Rev. Biochem.* 83 (2014) 249–273.
- [14] C.N. Larsen, B.A. Krantz, K.D. Wilkinson, Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases, *Biochemistry* 37 (1998) 3358–3368.
- [15] L.C. Dang, F.D. Melandri, R.L. Stein, Kinetic and mechanistic studies on the hydrolysis of ubiquitin C-terminal 7-amido-4-methylcoumarin by deubiquitinating enzymes, *Biochemistry* 37 (1998) 1868–1879.
- [16] A. Borodovsky, et al., A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14, *EMBO J.* 20 (2001) 5187–5196.
- [17] H.D. Mootz, Split inteins as versatile tools for protein semisynthesis, *Chembiochem* 10 (2009) 2579–2589.
- [18] P.E. Dawson, et al., Synthesis of proteins by native chemical ligation, *Science* 266 (1994) 776–779.
- [19] S. Virdee, et al., Engineered diubiquitin synthesis reveals Lys29-isopeptide specificity of an OTU deubiquitinase, *Nat. Chem. Biol.* 6 (2010) 750–757.
- [20] S. Virdee, et al., Traceless and site-specific ubiquitination of recombinant proteins, *J. Am. Chem. Soc.* 133 (2011) 10,708–10,711.
- [21] N.D. Weikart, H.D. Mootz, Generation of site-specific and enzymatically stable conjugates of recombinant proteins with ubiquitin-like modifiers by the Cu(I)-catalyzed azide-alkyne cycloaddition, *Chembiochem* 11 (2010) 774–777.
- [22] X.A. Wang, et al., E1-catalyzed ubiquitin C-terminal amidation for the facile synthesis of deubiquitinase substrates, *Chembiochem* 15 (2014) 37–41.
- [23] R. Ramage, et al., Synthetic, structural and biological studies of the ubiquitin system: the total chemical synthesis of ubiquitin, *Biochem. J.* 299 (1994) 151–158.
- [24] J.P. Briand, et al., Total chemical synthesis of ubiquitin using BOP reagent: biochemical and immunochemical properties of the purified synthetic product, *Pept. Res.* 2 (1989) 381–388.
- [25] F. El Oualid, et al., Chemical synthesis of ubiquitin, ubiquitin-based probes, and diubiquitin, *Angew. Chem. Int. Ed.* 49 (2010) 10,149–10,153.
- [26] K.S. Kumar, et al., Total chemical synthesis of di-ubiquitin chains, *Angew. Chem. Int. Ed. Engl.* 49 (2010) 9126–9131.
- [27] K.S. Ajish Kumar, et al., Highly efficient and chemoselective peptide ubiquitylation, *Angew. Chem. Int. Ed. Engl.* 48 (2009) 8090–8094.
- [28] G.B. van Tilburg, A.F. Elhebieshy, H. Ovaa, Synthetic and semi-synthetic strategies to study ubiquitin signaling, *Curr. Opin. Struct. Biol.* 38 (2016) 92–101.
- [29] R. Ekkebus, et al., On terminal alkynes that can react with active-site cysteine nucleophiles in proteases, *J. Am. Chem. Soc.* 135 (2013) 2867–2870.
- [30] A. de Jong, et al., Ubiquitin-based probes prepared by total synthesis to profile the activity of deubiquitinating enzymes, *Chembiochem* 13 (2012) 2251–2258.
- [31] M.P.C. Mulder, et al., A native chemical ligation handle that enables the synthesis of advanced activity-based probes: diubiquitin as a case study, *Chembiochem* 15 (2014) 946–949.

- [32] T.E. Mevissen, et al., Molecular basis of Lys11-polyubiquitin specificity in the deubiquitinase Cezanne, *Nature* 538 (2016) 402–405.
- [33] N. Haj-Yahya, et al., Dehydroalanine-based diubiquitin activity probes, *Org. Lett.* 16 (2014) 540–543.
- [34] G. Li, et al., Activity-based diubiquitin probes for elucidating the linkage specificity of deubiquitinating enzymes, *Chem. Commun. (Camb.)* 50 (2014) 216–218.
- [35] J.F. McGouran, et al., Deubiquitinating enzyme specificity for ubiquitin chain topology profiled by di-ubiquitin activity probes, *Chem. Biol.* 20 (2013) 1447–1455.
- [36] A. Iphofer, et al., Profiling ubiquitin linkage specificities of deubiquitinating enzymes with branched ubiquitin isopeptide probes, *Chembiochem* 13 (2012) 1416–1420.
- [37] D. Flierman, et al., Non-hydrolyzable diubiquitin probes reveal linkage-specific reactivity of deubiquitylating enzymes mediated by S2 pockets, *Cell Chem. Biol.* 23 (2016) 472–482.
- [38] X. Lu, et al., Designed semisynthetic protein inhibitors of Ub/Ubl E1 activating enzymes, *J. Am. Chem. Soc.* 132 (2010) 1748–1749.
- [39] H. An, A.V. Statsyuk, Facile synthesis of covalent probes to capture enzymatic intermediates during E1 enzyme catalysis, *Chem. Commun. (Camb.)* 52 (2016) 2477–2480.
- [40] H. An, A.V. Statsyuk, Development of activity-based probes for ubiquitin and ubiquitin-like protein signaling pathways, *J. Am. Chem. Soc.* 135 (2013) 16,948–16,962.
- [41] M. Stanley, et al., Orthogonal thiol functionalization at a single atomic center for profiling transthioylation activity of E1 activating enzymes, *ACS Chem. Biol.* 10 (2015) 1542–1554.
- [42] K.C. Pao, et al., Probes of ubiquitin E3 ligases enable systematic dissection of parkin activation, *Nat. Chem. Biol.* 12 (2016) 324–331.
- [43] S.D. Park, T. Krist, A.V. Statsyuk, Protein ubiquitination and formation of polyubiquitin chains without ATP, E1 and E2 enzymes, *Chem. Sci.* 6 (2015) 1770–1779.
- [44] D.T. Krist, et al., UbFluor: a mechanism-based probe for HECT E3 ligases, *Chem. Sci.* 7 (2016) 5587–5595.
- [45] M.P.C. Mulder, et al., A cascading activity-based probe sequentially targets E1-E2-E3 ubiquitin enzymes, *Nat. Chem. Biol.* 12 (2016) 523–530.
- [46] W. Zhang, et al., System-wide modulation of HECT E3 ligases with selective ubiquitin variant probes, *Mol. Cell* 62 (2016) 121–136.
- [47] V. Landre, et al., Screening for E3-ubiquitin ligase inhibitors: challenges and opportunities, *Oncotarget* 5 (2014) 7988–8013.