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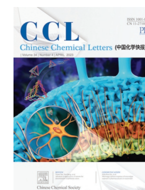

# Chemical tools for E3 ubiquitin ligase study

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E3 ubiquitin ligases catalyze the final step of ubiquitylation, a crucial post-translational modification involved in almost every process in eukaryotic cells. E3 ubiquitin ligases are key regulators of cellular events, and the investigation into their functions and functioning mechanisms are research areas with great importance. Synthetic or semi-synthetic tools have greatly facilitated the research about the enzyme activity, distribution in different physiological events, and catalytic mechanism of E3 ubiquitin ligase. In this review, we summarize the development of chemical tools for E3 ubiquitin ligases with an emphasis on the synthetic routes. We show the utility of these chemical tools by briefly discussing their applications in biological research.

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## 1. Introduction

Ubiquitylation is a post-translational modification of proteins regulating most physiological activities that covalently links the target protein with the C terminus of a 76 amino acids (aa) protein called ubiquitin (Ub) through the free amino groups from N terminus or side chains of Lys [1,2]. Canonical ubiquitylation is an enzymatic cascade catalyzed by ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3), during which E3 ligase plays important roles in recognizing substrates and coordinating with E2 to catalyze the final step of substrate ubiquitylation (Fig. 1) [3].

Human genome encodes more than 600 E3s, and they can be classified into three families according to their catalytic mechanism: RING (Really Interesting New Gene), HECT (Homologous to E6AP C-terminus), and RBR (RING-between-RING) [4]. RING E3s catalyze the nucleophilic attack of substrate amino group at E2~Ub thioester bond, mediating the direct transfer of ubiquitin from E2 to substrate [5]. HECT and RBR E3s, however, form thioester linkage between their catalytic cysteine and ubiquitin, which will be attacked by substrate amino group later, resulting in the conjugation of ubiquitin to the substrate (Fig. 1) [6,7]. E3s are involved in substantially every biological process, and their dysfunction is closely related to diseases like cancer, neurodegenerative diseases, and pathogen infection (Fig. 2) [8–12]. Despite their significance, E3s remain inadequately investigated mainly due to the dynamic and transient nature of E3-catalyzed ubiquitylation [13].

Chemical tools can capture the dynamic enzymatic intermediate of E3 ligase during ubiquitylation, and they can be used to profile E3s and to investigate the molecular mechanism of E3-catalyzed ubiquitylation, which will deepen our understanding of these vital enzymes [14–17].

## 2. Rationale and synthetic routes of different chemical tools

The difference in catalytic nature of E3 ligases from different families requires the customized design of chemical tools targeting a specific class of E3s. For HECT/RBR family E3s, activity-based probes (ABPs) are used to covalently capture the catalytic cysteine of E3s for either E3 identification, characterization of regulatory mechanism, or structural studies. These ABPs generally contain two parts: a reactive group (*e.g.*, electrophilic group) for covalent capture of E3s and an E3-interacting module (*e.g.*, ubiquitin) for specific recognition of the corresponding E3s. In some cases, they can also have a reporter group (*e.g.*, HA-tag) for enrichment, visualization, or purification.

For RING family E3s catalyzing ubiquitylation without a catalytic cysteine, strategies like photo-crosslinking probes and stable construct for structural studies are needed (Fig. 3).

Regardless of the E3 targeting strategies, E3 chemical tools can be obtained by total synthesis or semi-synthesis. For total synthesis, E3 chemical tool segments are synthesized by modern solid-

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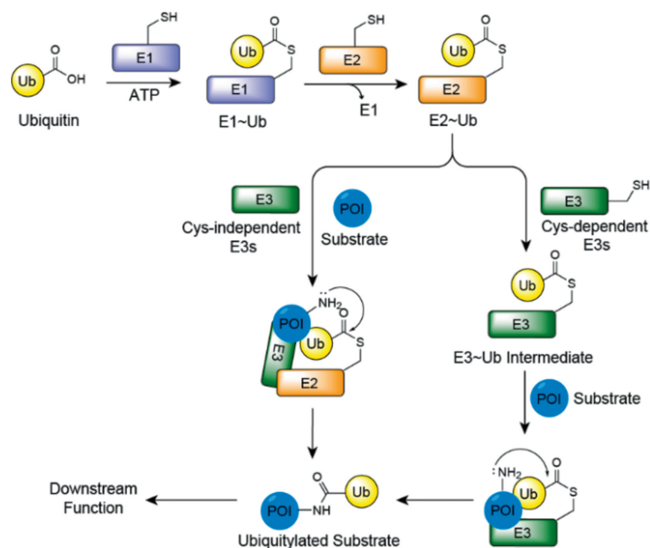


**Fig. 1.** Ubiquitylation mechanism of Cys-dependent and independent E3 ubiquitin ligase.

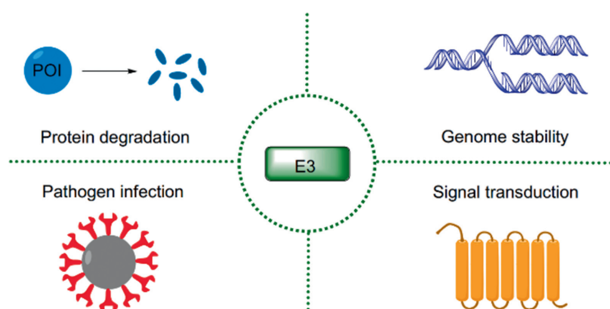


**Fig. 2.** Biological significance of E3 ubiquitin ligase.

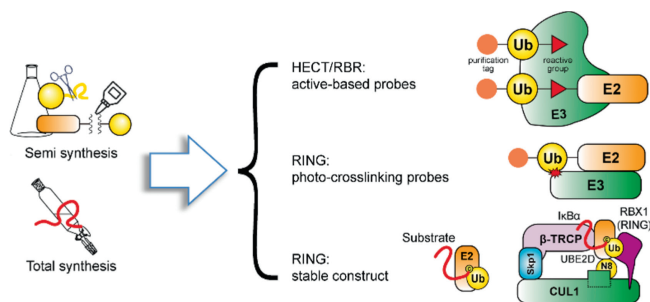


**Fig. 3.** Design and synthetic routes of E3 chemical tools.

phase peptide synthesis [18,19] and ligated by methods including native chemical ligation (NCL), serine and threonine ligation (STL), or $\alpha$-Ketoacid–Hydroxylamine (KAHA) ligation [20–26]. While total synthesis allows the structural tuning and atomic tailoring of the E3 chemical tools, semi-synthesis enables the expedient, modular, and large-scale preparation of them. It also allows the E2 component to be readily altered which might be necessary for labelling of certain E3s. Biologically expressed E3-interacting modules can be conjugated to reactive groups or other chemically synthesized parts bio-orthogonally [27–29]. Semi-synthesis provides a technically less challenging way to prepare E3 chemical tools while allowing the fine-tuning of the chemical structure of chemically synthesized parts.

In the following sections, we will introduce the design, synthesis, and applications of chemical tools including probes targeting catalytic cysteine (activity-based probes for HECT/RBR-type E3s), photo-crosslinking probes, and stable construct for structural studies (for RING-type E3s), with a focus on the synthetic routes to prepare those tools.

## 3. Probes targeting catalytic cysteine

### 3.1. Mono-Ub activity-based probes

Mono-Ub activity-based probes (mono-Ub ABPs) are ubiquitin variants that contain electrophilic active groups at the carboxyl end of ubiquitin. Mono-Ub ABPs, initially developed for studying deubiquitinating enzymes (DUBs) [30–32], were later repurposed for the study of E3 ligase since they can also covalently crosslink to E3 ligase and therefore could be used in proteomic screening and structural mechanism study for HECT/RBR family E3s [33]. Mono-Ub ABPs with different electrophilic groups have been developed, including Ub-Dha, Ub-VS, and HA-Ub probes.

A representative mono-Ub ABP is the Ub-VS probe, which utilizes its vinyl sulfone group (VS) as the reaction warhead to crosslink the active Cys of E3s [34]. Ub-VS ABP can be synthesized using Ub(1-75)-MesNa [35] (obtained by the intein method) and H-Gly-VS (Fig. 4A). Similar to the Ub-VS probe, Ploegh *et al.* developed a series of mono-Ub ABPs that have HA tag on their N-terminal for pull-down assay in cell lysates [36]. These probes include HA-Ub-Lac ($\beta$-lactone), HA-Ub-OEtVS (vinylethoxysulfone), and HA-UbTF$_3$BOK (2,6-trifluoromethyl-benzyloxymethylketone), and they can be synthesized with Ub(1-75)-MesNa and corresponding warhead molecules (Fig. 4A). These probes, without the need of E2 enzyme, can covalently capture E3 since ubiquitin itself could form a number of productive interactions with the E3 [37].

Ub-Dha probe is another type of mono-Ub ABP which exerts cross-linking activity by introducing dehydroalanine (Dha) at the C terminal end of Ub. Ub-Dha probe labels E3 ligase in an E1-E2-E3 enzymatic cascade manner. It is converted *in situ* to an E2~Ub conjugate first and then forming the final E3~Ub-Dha product. Therefore, Ub-Dha probes could also be defined as E2-Ub probes technically but since the formation of E2~Ub conjugate is *in situ*, we define them as mono-Ub ABPs here. These probes could be used for enzymatic activity detection and proteomic screening [38]. Ub-Dha can be prepared by total synthesis. First, Ub(1-75) was obtained by Fmoc solid-phase peptide synthesis (SPPS), and then H-Cys(Bn)-OMe was attached to the carboxyl end of the protected Ub(1-75) to obtain Ub(1–75)-Cys(Bn)-OMe. After deprotection by trifluoroacetic acid (TFA), Ub(1–75)-Cys(Bn)-OMe is converted into Ub-Dha-OMe by oxidation and elimination using 2-[(aminooxy)sulfonyl]-1,3,5-trimethylbenzene (MSH), and Ub-Dha probe is obtained from Ub-Dha-OMe by hydrolysis [39,40]. Another way for Ub-Dha probe preparation is the semi-synthetic route [41]. 2,5-Dibromohexanediamide is used to directly convert the C-terminal cysteine of recombinantly expressed UbG76C into the Dha group (Fig. 4B). Ub-Dha probe can, demonstrated by the high similarity between the crystal structure of UBE3D3~UbDha and UBE2D3~Ub [38], crosslink to enzymes without affecting their conformation, making it a practical tool to investigate the enzymes involved in ubiquitylation process. However, since Ub-Dha probe undergoes sequential transthiolation reactions before labelling the E3 ligase, the Dha warhead could also irreversibly trap E1 and E2 enzymes to form E1-Ub or E2-Ub conjugate, making it inefficient to be used as a functional E3 probe.

Mono-Ub ABPs are widely used in E3 proteomic screenings. Ovaa *et al.* attached biotin to the Ub-Dha probe for proteomic screening of ubiquitin-related enzymes in HeLa uterine tumor cells and MelJuSo melanoma cells [38]. Mass spectrometry analysis showed that in the presence of ATP, the Ub-Dha probe identified two E1 enzymes (UBE1 and UBA6) and multiple downstream E2

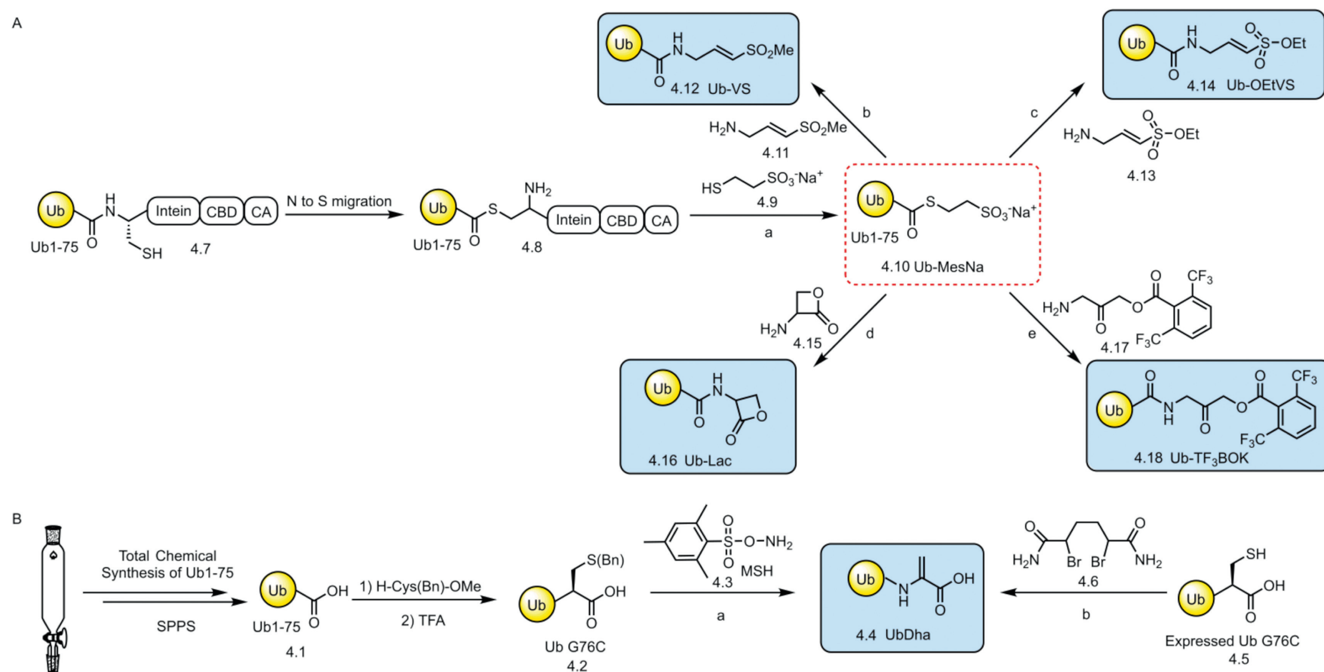


**Fig. 4.** Synthetic routes for mono-Ub ABPs. (A) Preparation of mono-Ub ABPs with different warheads by Ub-MesNa intermediate. (B) Preparation of Ub-Dha ABP through desulfurization.

enzymes such as UBE2S and UBE2K. In addition, they also detected E2/E3 hybrid enzymes like UBE2O, BIRC6, and HECT-type E3 ligases. It is worth noting that the E3 enzymes UBE3A and HECTD1 were detected in both two cell lines, while the TRIP12 E3 enzyme was only detected in the MelJuSo cell line, indicating that E3 enzyme expression levels are different in different cells. In the absence of ATP, Ub-Dha was unable to capture the above-mentioned ubiquitin-related enzymes. Therefore, background noises in proteomic results can be reduced by eliminating ATP, which could benefit research in complex biological environments like stimulation or starvation. Similarly, the Ub-VS probe has been used for the activity screening of E3 enzymes like Parkin [42].

It is worth mentioning that although mono-Ub chemical probes can crosslink to E3s in an activity-based manner, the enzymatic nature of E3-catalyed ubiquitylation requiring the formation of protein-protein complex together with E2 enzymes and/or substrates makes mono-Ub probes alone unsuitable for studying many E3-catalyzed processes like transthiolation. Therefore, probes containing E2s or substrates as E3-interacting modules have been developed and will be discussed in the following sections.

### *3.2. E2-Ub activity-based probes*

The first step of HECT/RBR catalyzed ubiquitylation is the transfer of ubiquitin from E2s to E3s by transthiolation reaction. E3s recognize corresponding Ub-charged E2s by non-covalent interactions to form the E3-E2-Ub complex, preparing the catalytic cysteine of E3s to attack E2~Ub thioester bonds. Therefore, E2-Ub activity probes with electrophilic active groups near the E2~Ub thioester bonds could covalently capture the E3s to form a stable E3-E2-Ub complex for either E2-E3 pairing study or transthiolation mechanism investigation [43].

In 2016, Virdee *et al.* reported an E2-Ub-activated vinylsulfide (AVS) activity-based probe [44]. In order to make the probe better resemble the native structure of the E2-Ub intermediate, they reduced the number of amino acids at the C-terminus of ubiquitin to accommodate the active electrophilic group (Fig. 5A). The authors first expressed Ub(1-73) intein and converted it to a thioester by thiolysis reaction. They then introduced the azide group to Ub(1-73) thioester by aminolysis reaction and linked TDAE (tosyl-substituted doubly activated enes) to the probe through cycloaddition to obtain Ub-TDAE. Incubation of Ub-TDAE with E2 mutant that contain only the catalytic cysteine led to the formation of the E2-Ub-AVS probe (Fig. 5A).

Apart from E2-Ub-AVS, E2-Ub-Dha, another type of E2-based probe with different chemical structures and good synthetic accessibility, became a valuable supplement to E2-Ub chemical probes. Li *et al.* reported an E2-Ub-Dha activity-based probe through a semi-synthetic method in 2019 [45]. The authors selected the UBE2D2 (UbcH5b) mutant with all non-catalytic Cys mutated to Ser as the E2 enzyme in the probe, and then converted the active Cys to Dha, yielding the E2-Dha protein. They then introduced compound 5.15 to react with the Dha group and then removed the protecting group to obtain the E2 intermediate, which reacted with ubiquitin through native chemical ligation (NCL). The exposed sulfhydryl group was eliminated to obtain the final E2-Ub-Dha probe (Fig. 5B). Another E2-Ub-Dha active-based probe called E2-Dap-Ub-Dha was reported by Liu *et al.* recently [17]. Through chemical total synthesis, they prepared an E2-Ub probe that could possibly mimic the native intermediate better when crosslinked to E3s because of the exactly same atom number between the $\alpha$-carbon of the active site amino acid on E2 enzyme and the C-terminus of ubiquitin. The authors used solid-phase peptide synthesis to synthesize three fragments of UbcH7, the selected E2 enzyme, in which the active cysteine was replaced by Acm-protected cysteine-linked 2,3-diaminopropionic acid (Dap). Then the authors ligated the three fragments by native chemical ligation, removed the two sulfhydryl groups and the Acm protecting group, and then connected it with Ub(1-75)-MesNa to obtain the intermediate E2-Dap-Ub. Finally, the intermediate was reacted with 5.13 to obtain the E2-Dap-Ub-Dha probe (Fig. 5C).

E2-Ub activity probes have demonstrated potentials to be used in E3 profiling and transthiolation mechanism study. For example, the E2-Ub-AVS probe was used to directly monitor the activity of Parkin, an RBR-typed E3 enzyme whose mutation will lead to Parkinson's disease (PD), in different cell extracts [44]. Biochemical

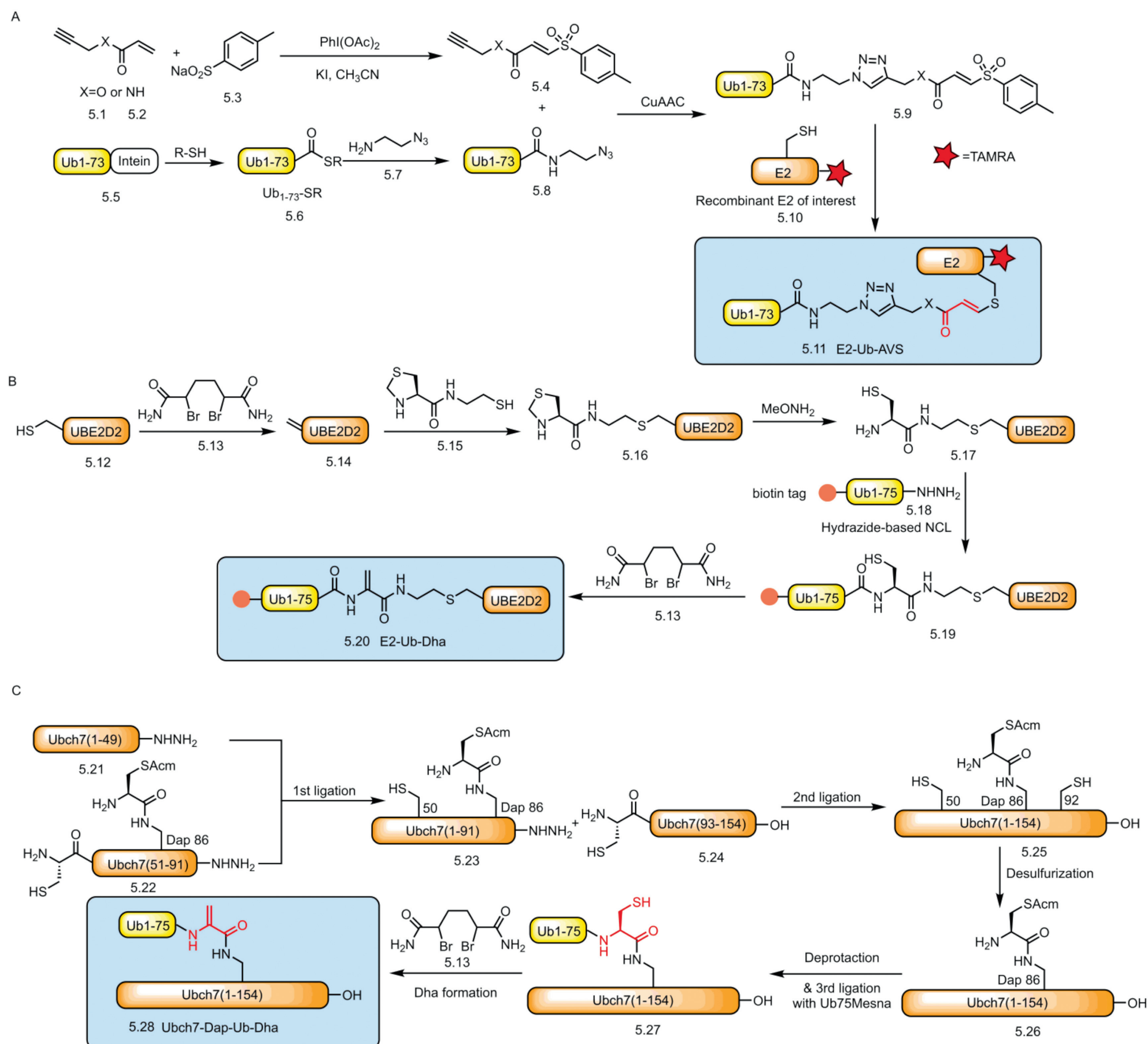

**Fig. 5.** Synthetic routes for E2-Ub probes. (A) Preparation of E2-Ub-AVS probes using bio-orthogonal reactions. (B) Preparation of E2-Ub-Dha probes by ligating expressed E2 and Ub using hydrazide-based NCL. (C) Preparation of E2-Dap-Ub-Dha probes by total chemical synthesis.

experiments showed that the phosphorylation of S65 of ubiquitin and S65 of Parkin is essential for Parkin to exert trans-thioester activity, and that phosphorylation will lead to an open conformation of Parkin's self-inhibitory structural region known as the REP element. Studies on the activity of Parkin in SH-SY5Y cells showed that, in response to mitochondria depolarization, >75% of intracellular Parkin will be phosphorylated and activated by PINK1. The probe was also used in transthiolation activity screening of E3 ligases in cell extracts. Biotin-E2-Ub-AVS probe was incubated with SH-SY5Y neuroblastoma cell extract and after streptavidin enrichment and digestion with trypsin, the sample was sent for mass spectrometry analysis. The results showed that the probe not only detected more than 50 currently known RBR and HECT E3 ligases but also unexpectedly found 33 RING E3 ligases, in which MYCBP2 was confirmed to have a unique transthioester activity that was believed to be possessed only by HECT or RBR-type E3s [16].

For transthiolation mechanism investigation, E2-Ub-AVS and E2-Ub-Dha probes were used to obtain a stable E3-E2-Ub complex by providing additional covalent linkages for structural analysis tools. For instance, Virdee *et al.* obtained the catalytic intermediate of MYCBP2 and RNF213 when transferring ubiquitin from E2s to E3s, enabling the further structural mechanism study on how MYCBP2 employs two different active Cys to ubiquitylate substrate threonine and how RNF213 ubiquitylate substrate in a Cys-dependent and ATP-dependent manner [46,47]. Similar strategies were also employed by Schulman *et al.* (study of the transthiolation process between UBE2L3 and ARIH1) [48] and Liu *et al.* (study of the transthiolation process between UbcH7 and Nedd4/Parkin) [17], further demonstrating the utility of E2-Ub activity-based probes in both activity-based E3 profiling and transthiolation mechanism study.

### 3.3. Substrate-Ub activity-based probes

After the transfer of ubiquitin from E2s to HECT/RBR E3s, the E3~Ub complex will engage with substrates to position the sub-

strate lysine for the attack of the E3~Ub thioester bond to achieve the transfer of ubiquitin from E3s to substrates, the last step of HECT/RBR-catalyzed ubiquitylation. Therefore, substrate-Ub containing an electrophilic group near the iso-peptide bonds could serve as chemical probes to covalently capture corresponding E3s. Reported substrate-Ub activity-based probes are relatively rare, possibly because of the less stable nature of the E3-substrate-Ub complex and the greater diversity of substrates.

A good example of substrate-Ub activity-based probes is the TS2 ABP used in the study of SCF-ARIH1-mediated ubiquitylation by the Schulman lab. Ub(1-75)MesNa was ligated to a substrate peptide with a Cys on the N-terminal by native chemical ligation to form substrate-Ub. After the conversion of cysteine to dehydroalanine by 2,5-dibromohexanediamide, the substrate-Ub probe bearing a double bond as the electrophilic group was prepared. The probe was used to reconstitute the SCF-ARIH1-substrate complex in the ubiquitin transfer intermediate state and the structure of this intermediate was solved using cryo-EM. Together with the structure of the transthiolation intermediate mentioned in 3.2, they were able to show how these two E3 ligases form "super-assembly" and coordinate with each other to ubiquitylate a wider range of substrates than alone [48].

While similar strategies could be applied to other HECT/RBR family E3s to study the ubiquitylation priming and elongation process of a specific substrate in the future [49], selecting a representative substrate of great synthetic accessibility could be challenging. Nevertheless, we envision that the development of substrate-Ub activity-based probes will facilitate the mechanism investigation of many HECT/RBR family E3s in the future.

## 4. Photo-crosslinking probes

RING-type E3s are Cys-independent enzymes catalyzing the direct transfer of ubiquitin from E2s to the substrates, and therefore they cannot be covalently captured by the activity-based probes targeting catalytic cysteine. Therefore, photo-crosslinking becomes a feasible strategy to covalently crosslink to the RING-type E3s using photo-crosslinking functional groups. These photo-crosslinking probes could be used in proteomic studies since the covalent capture of E3s could enable future enrichment and mass spectrometry analysis.

Photo-crosslinking groups can be introduced into chemical probes to yield highly reactive free radical species under light conditions for crosslinking to nearby residues in E3s. It is worth noting that the incorporation site of the photo-crosslinking group is crucial to the efficiency of crosslinking even though these probes crosslink E3s in an activity-independent manner.

Virdee *et al.* developed an E2-Ub photo-crosslinking probe containing the photo-crosslinking non-natural amino acid Bpa (*p*-benzoyl-L-phenylalanine) for capturing RING-type E3s in 2020 [50]. In order to determine the appropriate photo-crosslinking sites, they selected some amino acids in ubiquitin that are adjacent to E3 based on the E3-E2-Ub crystal structure and carried out optimization through *in vitro* crosslinking activity test. To facilitate the enrichment and analysis for proteomic research, a His-tag was also introduced at the N-terminus of ubiquitin. This ubiquitin variant His-Ub-Bpa can be expressed through unnatural amino acid incorporation, and then recognized and catalyzed by E1 to form iso-peptide bonds with E2 UBE2D3 whose catalytic Cys had been mutated into Lys to obtain the E2-Ub photo-crosslinking probe (Fig. 6A). Although the probe does not target a specific active residue on E3 ligase, it is activity-based because it has been shown to undergo activity-dependent labelling of RING E3s. Using this probe as a tool to monitor the enzymatic activity, they showed that the activity of c-Cbl, a RING-type E3, depends on the co-expression with

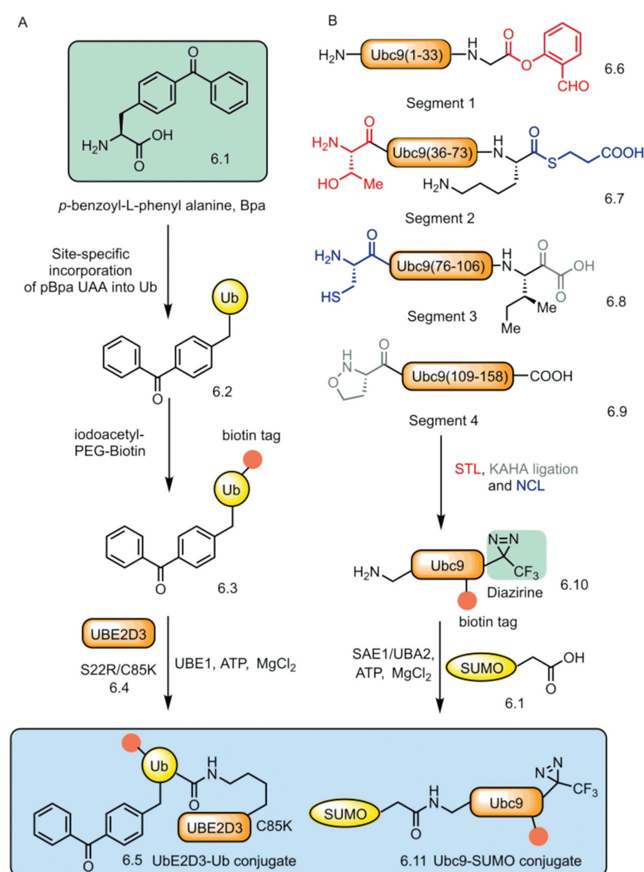


**Fig. 6.** Synthetic routes for photo-crosslinking probes. (A) Preparation of Bpa-based probes. (B) Preparation of diazirine-based probes.

c-Src kinase, phosphorylation of c-Cbl Y371, and interaction between c-Cbl RING domain and E2 enzyme. Similar to previous findings [51,52], c-Cbl phosphorylation was found to enhance its own activity. This example highlights the promising application of the photo-crosslinking strategy in RING-type E3 enzyme activity study.

Another representative photo-crosslinking probe was developed by Bode *et al.* for the study of E3 ligases of a small ubiquitin-like modifier, named SUMO [53]. They prepared the E2-SUMO photo-crosslinking probe by *in vitro* SUMOylation, in which the E2 enzyme (Ubc9) part was prepared by total synthesis. First, four fragments of Ubc9 were obtained by solid-phase peptide synthesis, and then fragments 1 and 2 were ligated by the serine/threonine ligation method (STL), while fragments 3 and 4 were connected by KAHA ligation. Finally, the full-length protein is obtained by native chemical ligation. The authors mutated the catalytic Cys93 to 2,3-diaminopropionic acid (Dap) on Ubc9 to avoid hyperSUMOylation of the substrates, and they chose diazirine as the photoactive group. The E2-SUMO photo-crosslinking probe was prepared by connecting the modified Ubc9 with SUMO (Fig. 6B). The probe was used to crosslink RanBP2, the E3 ligase for SUMO with the help of substrate Sp100 to form SUMO-Ubc9-RanBP2 ternary complex in both reconstituted biochemical assays and cell lysates, proving the potential for trapping new E3 ligases of this chemical probe and broadening the application of E2-Ub probe to ubiquitin-liker modifiers like SUMO. However, the fact that no evidence shows that the probe could label E3 ligase in an activity-dependent manner makes it questionable whether this probe could be used in E3 activity monitoring.

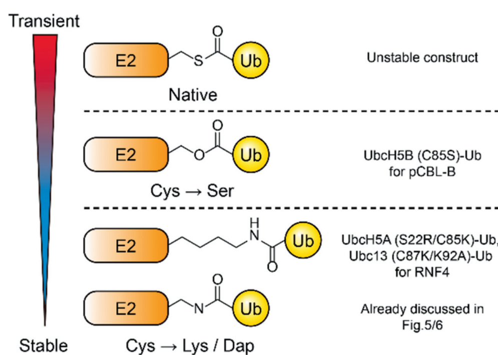


**Fig. 7.** Chemical nature of native E2∼Ub and stable E2-Ub constructs.

## 5. Stable constructs for structural studies

For structural studies of E3-catalyzed ubiquitylation, capturing the dynamic catalytic intermediate is challenging for visualizing the ubiquitylation reaction in act. Constructing the stable mimic for the native, highly transient intermediate has provided a practical approach to "chemically stabilize" the catalytic intermediate for structural analysis using biophysical methods like X-ray crystallography, cryogenic electron microscopy (cryo-EM), and so on. In the following section, we will discuss the design and synthetic approaches for constructing these stable mimic for structural studies.

### *5.1. E2-Ub stable construct*

As we have mentioned above, RING-type E3s catalyze the direct transfer of ubiquitin from E2s to substrates. To achieve this, RING-type E3s need first engage with E2∼Ub to position the thioester bond for later nucleophilic attack by amino groups of substrate lysine (called the activation of E2∼Ub). Therefore, the structure of the E3-E2-Ub ternary complex is needed to help us understand the molecular details of this process. However, the native E2∼Ub is an unstable construct in the presence of E3s [54], making it difficult to obtain this complex for structural analysis. E2-Ub chemically stabled mimics through amide bond or oxyester bond linkage have been developed to tackle this issue (Fig. 7).

In 2012, Hay *et al.* reported an amide bond-linked E2-Ub construct UbcH5A-Ub for the mechanism study of ubiquitin transferring from E2 to the substrate of a RING-type E3 enzyme RNF4 [54]. The construct was prepared using chemoenzymatic methods. UbcH5A mutant (S22R/C85K) was incubated with ubiquitin and E1 enzyme UBE1 with the presence of ATP, $MgCl_2$, and TCEP to allow E1 to transfer ubiquitin to E2 to form UbcH5A-Ub stable conjugate. Similarly, Ubc13 (C87K/K92A)-Ub stable conjugate was constructed to study the mechanism of K63-linked ubiquitin chain formation [55]. Oxyester bond linkage, although less stable than amide bond linkage from chemical perspectives, was also proven to be effective in stabilizing E2-Ub, as illustrated by the UbcH5B (C85S)-Ub stable conjugate [56].

With these stable E2-Ub conjugates in hand, many E3-E2-Ub ternary complexes were obtained and their structures were solved using X-ray crystallography. These structures have provided direct and key evidence for the proposed catalytic mechanism of RING-type E3s: they bind directly to both E2s and ubiquitin, and by immobilizing the donor ubiquitin through several key interaction interfaces, RING-type E3s help orientate the E2∼Ub thioester bond to be positioned in an optimal arrangement for the incoming attack by the amino group from substrate lysine. This lay the foundation for the investigation into the ubiquitin transfer step catalyzed by RING-type E3s, which requires the use of E2-Ub-substrate stable constructs to obtain the catalytic intermediate.

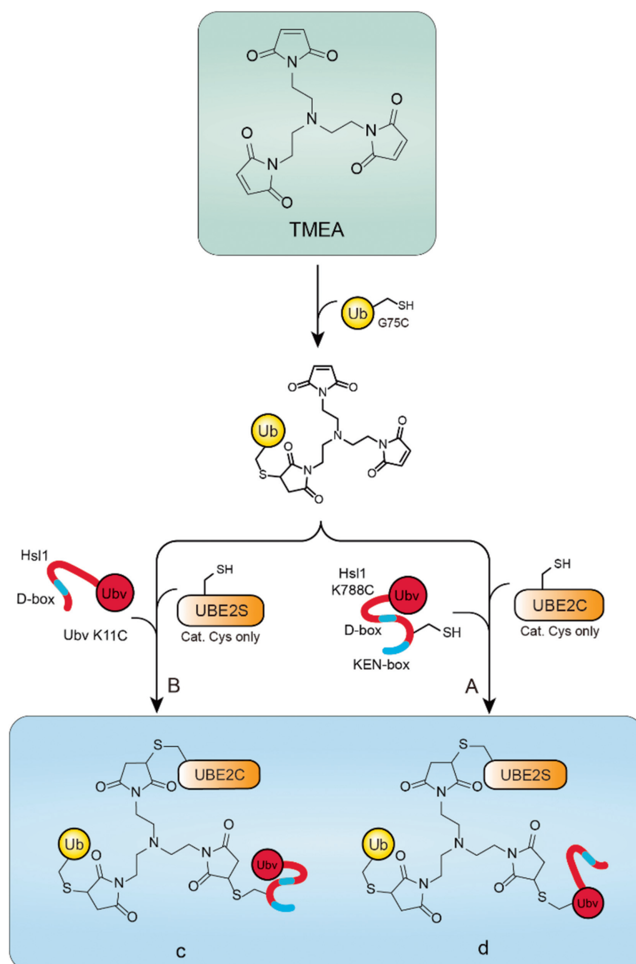


**Fig. 8.** Construction of E2-Ub-substrate stable construct by trifunctional molecule-assisted chemical trapping strategies.

### *5.2. E2-Ub-substrate stable construct*

In order to further elucidate the molecular mechanism of how E3 ligase transfers Ub to the substrate in coordination with E2, capturing the intermediate state of the E3-E2∼Ub-substrate complex for structural biology studies is needed. The highly unstable chemical nature of the high-energy tetrahedral structure formed by amino group attacking makes it challenging to directly capture the E3-E2∼Ub-substrate complex for structural study. To solve this problem, covalently linked E2-Ub-substrate stable constructs were developed to form a relatively stable E3-E2-Ub-substrate complex for mimicking the native intermediate state during ubiquitin transfer.

Using trifunctional molecules to connect ubiquitin with E2 and substrate is one way to construct the intermediate mimics. Three-headed crosslinker TMEA (tris(2-maleimidoethyl)amine) was used to investigate the role of RING-type E3 APC/CCDH1 complex in catalyzing multi-monoubiquitylation (construct c, Fig. 8) and K11 polyubiquitylation (construct d, Fig. 8) of substrate Hsl1 [57]. All three arms of TMEA are sulfydryl-reactive maleimide groups, which are crosslinked to $Ub^{G75C}$, E2 enzyme UBE2C/UBE2S (all Cys were mutated except the catalytic site), and substrate (ubiquitinated Hsl1 fragment variant) successively. The two constructs were assembled with APC/CCDH1 complex respectively and subse-

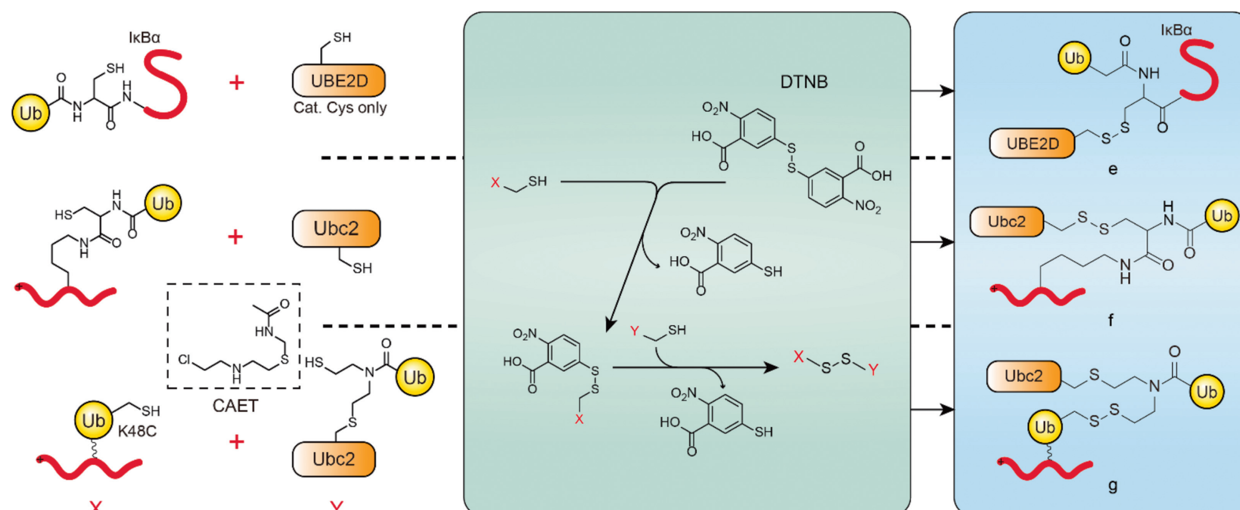


**Fig. 9.** Construction of E2-Ub-substrate stable construct by disulfide-bond linking strategies.

quently used for structural analysis to explore the similarities and differences between multi-monoubiquitylation and polyubiquitylation processes catalyzed by the same E3 APC/C$^{CDH1}$.

Another strategy to construct stable intermediate mimics is disulfide bond cross-linking, using molecules like DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) and AT2 (Aldrithiol-2) to form disulfide bond linkages between key sites. In 2020, Schulman *et al.* used this method to construct an E2-Ub-substrate ternary construct (construct e, Fig. 9) [58] and explored the mechanism of ubiquitylation through E2 UBE2D and RING E3 ligase complex CRL1. The construct adopts a disulfide bond to connect E2 (all Cys were mutated except the catalytic site) to a ubiquitinated substrate *IκBα* degron peptide. The ubiquitinated substrate peptide was synthesized by NCL reaction between Ub(1-75)-MesNa and *IκBα* degron peptide with Cys at the N-terminal obtained by SPPS, and then the only reactive Cys in UBE2D was activated by DTNB [59] and crosslinked to the exposed sulfhydryl group in the ubiquitinated substrate (Fig. 9, upper panel). UBE2D-Ub-*IκBα* degron construct e was used to assemble a complex with CRL1 as the specimen for single-particle cryo-EM, and the structure revealed the molecular mechanism of the initiation of the substrate ubiquitylation process catalyzed by E2-CRL complex and activated by NEDD8 covalent modification.

Additionally, Zhao and Liu synthesized the Ubc2~Ub-substrate constructs f and g (Fig. 9, middle and lower panel) by disulfide bonding method, and constructed the reaction intermediate mimics revealing how Ubc2 and yeast RING E3 Ubr1 transfer the first Ub and second Ub to the substrate, respectively [60]. The synthetic pathway of construct f for the initiation state of ubiquitylation is similar to that of Schulman's group as described above. For construct g mimicking the elongation process of polyubiquitylation, the ubiquitinated substrate peptide was obtained through SPPS and NCL, and the Lys48 of ubiquitin was mutated to Cys. The E2 Ubc2 (all Cys were mutated except the catalytic site), was firstly modified by a bifunctional small molecule CAET through the reactive sulfydryl group. CAET can not only mediate the NCL reaction to donor ubiquitin but also connect the C48 sulfydryl group on the ubiquitinated substrate through the disulfide bond after the removal of the Acm protecting group by PdCl2. Using these two constructs, they reported the first full-length structure of yeast Ubr1 and investigated the mechanism of how Ubr1 recognizes the N-degron and E2~Ub to perform K48-linked ubiquitylation initiation and elongation.

Compared with tri-functional linking, the advantage of disulfide bond connection is that it does not need to introduce long linker and cross-linking groups with large steric hindrances such as maleimide into the construct. This makes the connection distance closer to the distance between complex key sites in native state, and the assembled complex is more dynamically stable and close to the native conformation [61]. However, sensitivity to reducing environment and potential disulfide bond exchange reactions still partly limit the application of this strategy.

## 6. Summary and perspective

We reviewed above the design and synthesis of E3 chemical tools and provided examples of their applications in proteomic screening and structural mechanism investigation. The use of E3 chemical tools can deepen our understanding of these important enzymes, which are crucial players in ubiquitin and ubiquitin-like systems [62]. However, considering the large amount of E3s in the natural world, there is still a lot of E3s whose functions, potential regulation pathways and unique catalytic mechanisms remain to be discovered. These pose new challenges for the development and application of E3 chemical tools.

At present, the variety of E3 chemical tools is limited, and therefore developing new synthetic methods for more efficient tool preparation and designing new E3 tools to enrich the "toolbox" for E3 ligase will provide more opportunities for ubiquitin-related research. Most substrates in substrate-Ub constructs are short peptides or truncated proteins, which may behave differently from full-length protein substrates in the complex structure. Besides, Schulman and Ovaa *et al.* discovered that the distance between the polypeptide backbone and primary amine has a strong influence on chain formation for diverse polyubiquitylating enzymes [63]. This indicates that when capturing E3 ligase covalently by chemical tools, space factors should be taken into consideration to prevent that the chemical tool cannot crosslink to the E3s or the crosslinking product cannot imitate the true intermediate.

Since most E3 chemical tools developed are unable to cross through the plasma membrane, they can only be used for E3 ligase screening in cell lysis, which will greatly limit their application in intact cells. Introducing membrane penetrating peptides to the probes provides a possible way for tackling this issue. For example, Zhuang *et al.* reported a warhead-modified photocaged cell-permeable ubiquitin probe that can identify DUBs in intact cells

after being activated by UV irradiation *in situ* [64]. Li *et al.* reported a backbone modification strategy which enabled the preparation of cell-permeable photocaged ubiquitin probes with warheads difficult to block and activate, and these probes were used for time-resolved profiling of DUBs in cells under oxidative response [65]. These examples highlight the promising potentials of using cell-permeable probes to study the ubiquitylation system in intact cells. It becomes natural therefore that cell-permeable E3 chemical probes can be designed and applied to intact cells for E3-related research, which will help monitor the highly dynamic activity of E3 ligase. Since the ubiquitin system components are highly spatial and temporal heterogeneous in cells [66], developing such tools to study the distribution, abundance, and activity of ubiquitin modifiers and erasers are of broad prospective.

The development of E3 chemical tools will be helpful to resolve the unknown catalytic mechanisms of many important E3 ligases. For instance, E6AP is a HECT-type E3 ligase closely related to cancer. Huang *et al.* revealed the structure of E6AP and UbcH7 complex [67], but how E6AP catalyzes the conjugation of ubiquitin and substrate remained unclear so far. Our understanding of the ubiquitin transfer mechanism of this important E3 ligase can be deepened if a stable E6AP-E2-substrate complex can be obtained using E3 chemical tools for structure determination. Also, the mechanism of many RING-type E3s-catalyzed ubiquitin transferring to the substrate remains unknown, and developing E3 chemical tools will be helpful for us to solve it. In addition, apart from monoubiquitin, ubiquitin chains with different topology and therefore different physiological functions exist on the substrates, and how E3s achieve chain-type specificity calls for the development and application of new E3 chemical tools.

We anticipate more E3 tools to be developed and applied to the living system to gain us deeper and more accurate knowledge of ubiquitin systems, and therefore provide insights for the understanding and interference of ubiquitin systems for diagnostic and therapeutic purposes.

Chemical tools have been widely used to study biomacromolecules. This review summarized recent progress in designing and synthesizing chemical tools targeting E3 ubiquitin ligase.

## Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

## References

[1] D. Komander, M. Rape, Annu. Rev. Biochem. 81 (2012) 203–229.
[2] A. Hershko, A. Ciechanover, Annu. Rev. Biochem. 67 (1998) 425–479.
[3] L. Buetow, D.T. Huang, Nat. Rev. Mol. Cell Biol. 17 (2016) 626–642.
[4] C.E. Berndsen, C. Wolberger, Nat. Rev. Mol. Cell Biol. 21 (2014) 301–307.
[5] R.J. Deshaies, C.A. Joazeiro, Annu. Rev. Biochem. 78 (2009) 399–434.
[6] D. Rotin, S. Kumar, Nat. Rev. Mol. Cell Biol. 10 (2009) 398–409.
[7] H. Walden, K. Rittinger, Nat. Struct. Mol. Biol. 25 (2018) 440–445.
[8] H.D. Ulrich, H. Walden, Nat. Rev. Mol. Cell Biol. 11 (2010) 479–489.
[9] K.S. McNaught, C.W. Olanow, B. Halliwell, O. Isacson, P. Jenner, Nat. Rev. Neurosci. 2 (2001) 589–594.
[10] B. Dale, M. Cheng, K.S. Park, et al., Nat. Rev. Cancer 21 (2021) 638–654.
[11] Z.J. Chen, Nat. Cell Biol. 7 (2005) 758–765.
[12] H. Ashida, M. Kim, C. Sasakawa, Nat. Rev. Microbiol. 12 (2014) 399–413.
[13] A. Craney, M. Rape, Curr. Opin. Cell Biol. 25 (2013) 704–710.
[14] D.S. Hewings, J.A. Flygare, M. Bogyo, I.E. Wertz, FEBS J. 284 (2017) 1555–1576.
[15] K.F. Witting, M. Mulder, H. Ovaa, J. Mol. Biol. 429 (2017) 3388–3394.
[16] K.C. Pao, N.T. Wood, A. Knebel, et al., Nature 556 (2018) 381–385.
[17] L.J. Liang, G.C. Chu, Q. Qu, et al., Angew. Chem. Int. Ed. 60 (2021) 2–9.
[18] L.A. Carpino, J. Am. Chem. Soc. 92 (1970) 5748.
[19] C.D. Chang, J. Meienhofer, Int. J. Pept. Protein Res. 11 (1978) 246–249.
[20] P.E. Dawson, T.W. Muir, I. Clark-Lewis, S.B.H. Kent, Science 266 (1994) 776.
[21] Q. Wan, S.J. Danishefsky, Angew. Chem. Int. Ed. 46 (2007) 9248–9252.
[22] G.M. Fang, Y.M. Li, F. Shen, et al., Angew. Chem. Int. Ed. 50 (2011) 7645–7649.
[23] G.M. Fang, J.X. Wang, L. Liu, Angew. Chem. Int. Ed. 51 (2012) 10347–10350.
[24] S. Tang, Y.Y. Si, Z.P. Wang, et al., Angew. Chem. Int. Ed. 54 (2015) 5713–5717.
[25] Y. Zhang, C. Xu, H.Y. Lam, C.L. Lee, X. Li, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 6657–6662.
[26] J.W. Bode, R.M. Fox, K.D. Baucom, Angew. Chem. Int. Ed. 45 (2006) 1248–1252.
[27] J. Shi, T.W. Muir, J. Am. Chem. Soc. 127 (2005) 6198–6206.
[28] Y.M. Li, Y.T. Li, M. Pan, et al., Angew. Chem. Int. Ed. 53 (2014) 2198–2202.
[29] G.C. Chu, M. Pan, J. Li, et al., J. Am. Chem. Soc. 141 (2019) 3654–3663.
[30] Y. Gao, A. Koppen, M. Rakhshandehroo, et al., Nat. Commun. 4 (2013) 2656.
[31] E. Kummari, N. Alugubelly, C.Y. Hsu, et al., Plos One 10 (2015) e0135531.
[32] X. Sui, Y. Wang, Y.X. Du, et al., Chem. Sci. 11 (2020) 12633–12646.
[33] A.M. Sadaghiani, S.H. Verhelst, M. Bogyo, Curr. Opin. Chem. Biol. 11 (2007) 20–28.
[34] A. Borodovsky, H. Ovaa, W.J. Meester, et al., Chembiochem 6 (2005) 287–291.
[35] K.D. Wilkinson, T. Gan-Erdene, N. Kolli, Methods Enzymol. 399 (2005) 37–51.
[36] K.R. Love, R.K. Pandya, E. Spooner, H.L. Ploegh, ACS Chem. Biol. 4 (2009) 275–287.
[37] H.B. Kamadurai, J. Souphron, D.C. Scott, et al., Mol. Cell 36 (2009) 1095–1102.
[38] M.P. Mulder, K. Witting, I. Berlin, et al., Nat. Chem. Biol. 12 (2016) 523–530.
[39] F. El Oualid, R. Merkx, R. Ekkebus, et al., Angew. Chem. Int. Ed. 49 (2010) 10149–10153.
[40] G.J. Bernardes, J.M. Chalker, J.C. Errey, B.G. Davis, J. Am. Chem. Soc. 130 (2008) 5052–5053.
[41] J.M. Chalker, S.B. Gunnoo, O. Boutureira, et al., Chem. Sci. 2 (2011) 1666.
[42] C. Gladkova, S.L. Maslen, J.M. Skehel, D. Komander, Nature 559 (2018) 410–414.
[43] D.L. Vaux, J. Silke, Nat. Rev. Mol. Cell Biol. 6 (2005) 287–297.
[44] K.C. Pao, M. Stanley, C. Han, et al., Nat. Chem. Biol. 12 (2016) 324–331.
[45] L. Xu, J. Fan, Y. Wang, et al., Chem. Commun. 55 (2019) 7109–7112.
[46] J. Ahel, A. Fletcher, D.B. Grabarczyk, et al., bioRxiv (2021), doi:10.1101/2021.05.10.443411.
[47] P.D. Mabbitt, A. Loreto, M.A. Déry, et al., Nat. Chem. Biol. 16 (2020) 1227–1236.
[48] D. Horn-Ghetko, D.T. Krist, J.R. Prabu, et al., Nature 590 (2021) 671–676.
[49] H.B. Kamadurai, Y. Qiu, A. Deng, et al., eLife 2 (2013) e00828.
[50] S. Mathur, A.J. Fletcher, E. Branigan, R.T. Hay, S. Virdee, Cell Chem. Biol. 27 (2020) 74–82.
[51] L. Buetow, G. Tria, S.F. Ahmed, et al., BMC Biol. 14 (2016) 76.
[52] H. Dou, L. Buetow, G.J. Sibbet, K. Cameron, D.T. Huang, Nat. Struct. Mol. Biol. 20 (2013) 982–986.
[53] Y. Zhang, T. Hirota, K. Kuwata, et al., J. Am. Chem. Soc. 141 (2019) 14742–14751.
[54] A. Plechanovová, E.G. Jaffray, M.H. Tatham, J.H. Naismith, R.T. Hay, Nature 489 (2012) 115–120.
[55] E. Branigan, A. Plechanovová, E.G. Jaffray, J.H. Naismith, R.T. Hay, Nat. Struct. Mol. Biol. 22 (2015) 597–602.
[56] H. Dou, L. Buetow, G.J. Sibbet, K. Cameron, D.T. Huang, Nat. Struct. Mol. Biol. 19 (2012) 876–883.
[57] N.G. Brown, R. VanderLinden, E.R. Watson, et al., Cell 165 (2016) 1440–1453.
[58] K. Baek, D.T. Krist, J.R. Prabu, et al., Nature 578 (2020) 461–466.
[59] G.L. Ellman, Arch. Biochem. Biophys. 82 (1959) 70–77.
[60] M. Pan, Q. Zheng, T. Wang, et al., Nature 600 (2021) 334–338.
[61] F.C.Jr. Streich, C.D. Lima, Methods Mol. Biol. 1844 (2018) 169–196.
[62] L. Cappadocia, C.D. Lima, Chem. Rev. 118 (2018) 889–918.
[63] J. Liwocha, D.T. Krist, G.J. van der Heden van Noort, et al., Nat. Chem. Biol. 17 (2021) 272–279.
[64] W. Gui, S. Shen, Z. Zhuang, J. Am. Chem. Soc. 142 (2020) 19493–19501.
[65] Y. Wang, J. Chen, X. Hua, et al., Angew. Chem. Int. Ed. 61 (2022) e202203792.
[66] C. Grabbe, K. Husnjak, I. Dikic, Nat. Rev. Mol. Cell Biol. 12 (2011) 295–307.
[67] L. Huang, E. Kinnucan, G. Wang, et al., Science 286 (1999) 1321–1326.