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Synthetic and semi-synthetic strategies to study ubiquitin signaling

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The post-translational modification ubiquitin can be attached to the ε-amino group of lysine residues or to a protein's N-terminus as a mono ubiquitin moiety. Via its seven intrinsic lysine residues and its N-terminus, it can also form ubiquitin chains on substrates in many possible ways. To study ubiquitin signals, many synthetic and semi-synthetic routes have been developed for generation of ubiquitin-derived tools and conjugates. The strength of these methods lies in their ability to introduce chemo-selective ligation handles at sites that currently cannot be enzymatically modified. Here, we review the different synthetic and semi-synthetic methods available for ubiquitin conjugate synthesis and their contribution to how they have helped investigating conformational diversity of diubiquitin signals. Next, we discuss how these methods help understanding the ubiquitin conjugation–deconjugation system by recent advances in ubiquitin ligase probes and diubiquitin-based DUB probes. Lastly, we discuss how these methods help studying post-translational modification of ubiquitin itself.

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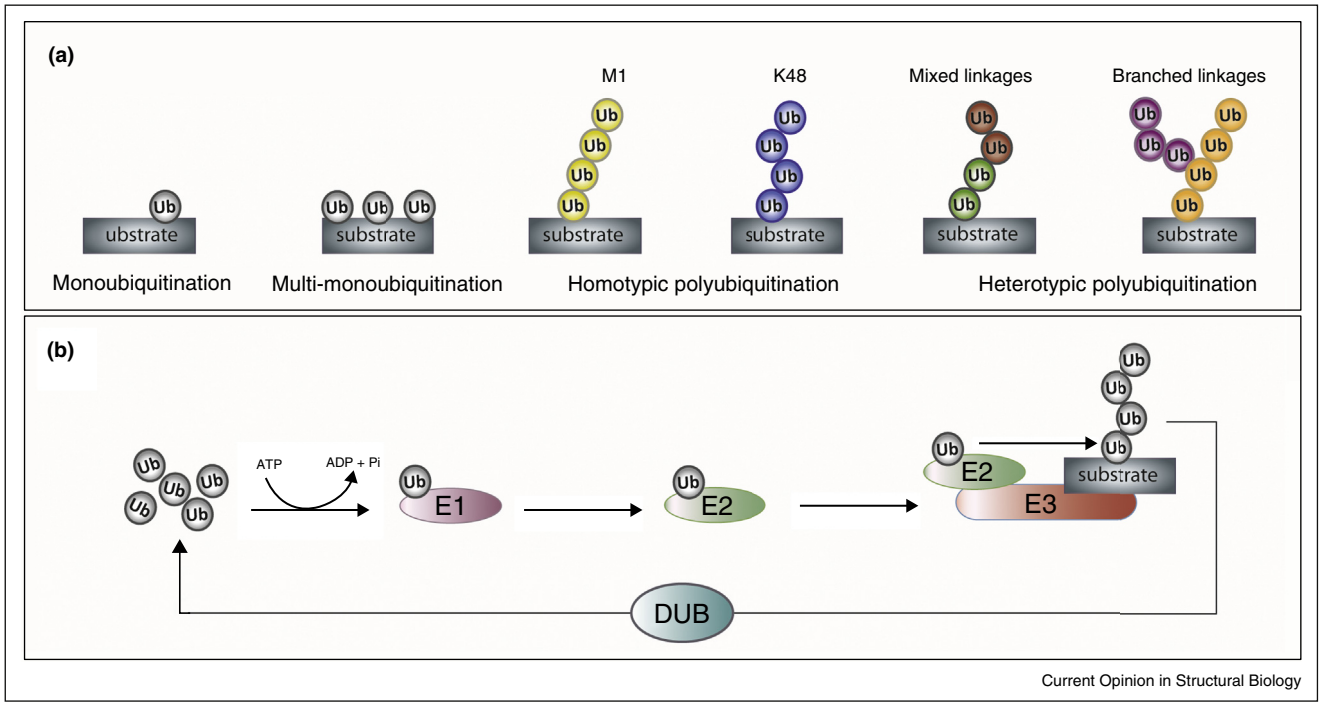
Introduction

Ubiquitin (Ub) is a 76-amino acid post-translational modifier fundamental to cellular homeostasis. Cellular processes regulated by ubiquitin modification range from classically known Ub-mediated proteasomal degradation to DNA repair, cell division, endoplasmic reticulum-associated degradation (ERAD), mRNA stability and even regulation of the innate immune system [1]. Not surprisingly, deregulation of the ubiquitin system is associated with various serious illnesses such as cancer, immunological disorders and neurodegenerative diseases [2]. The post-translational modifier can be covalently attached to substrate proteins at the ε-amino group of lysine residues or at the protein's N-terminal residue [3,4]. Since ubiquitin harbors seven intrinsic lysine residues, it can also be conjugated to another Ub moiety. In this manner, homotypical ubiquitin chains of a single linkage type consisting of M1, K6, K11, K27, K29, K33, K48 or K63 can be formed, all of which are known to exist *in vivo* [5]. In addition, heterotypical chains of multiple ubiquitin linkage types can be formed, opening up an even more complex layer of post-translational modification (Figure 1a). Conjugation of Ub to a substrate protein is carried out by a cascade of three enzymatic activities: E1 ubiquitin-activating, E2 ubiquitin-conjugating and E3 ubiquitin-ligating activities (Figure 1b). To date, 2 human E1s, about 40 E2s and over 600 E3 enzymes are known. The combination of E2 and E3 enzymes dictates what type of ubiquitin chain is formed and which substrate protein becomes ubiquitinated. In addition, the ubiquitination status of a protein can be regulated by removal or editing of ubiquitin chains, which is carried out by a family of approximately 100 deubiquitinating enzymes (DUBs) [6]. For some of these DUBs, linkage specificity has also been observed. To study the properties of differentially linked ubiquitin chains, traditionally one has relied on generating chains enzymatically by usage of linkage specific E2–E3 pairs. Not all E2-E3 pairs are known however, and some have only been uncovered recently. This resulted in a less extensive knowledge of the 'a-typical' K6-linked, K11-linked, K27-linked, K29-linked and K33-linked ubiquitin chains. Therefore, much effort has been put into making differentially linked ubiquitin derivatives through synthetic and semi-synthetic methods, since these methods allow site specific incorporation of a specific chemoselective ligation handle. In addition, ubiquitin-based DUB probes and fluorescent ubiquitin-based enzyme substrates have seen an enormous boost, producing ubiquitin-based tools in all sorts of different flavors [7]. Two other highly investigated areas of synthetic and semi-synthetic ubiquitin–conjugate research focus on ubiquitinated histones [8,9] and ubiquitinated α-synuclein [10,11]. We will briefly introduce the current synthetic and semi-synthetic methods that can be applied to generate ubiquitin and Ub-based tools and then focus on three other rapidly developing areas. First, we discuss how semi-synthetic diubiquitin synthesis has aided the structural characterization of all differentially linked diubiquitin modules, which further enhanced our understanding of

Figure 1



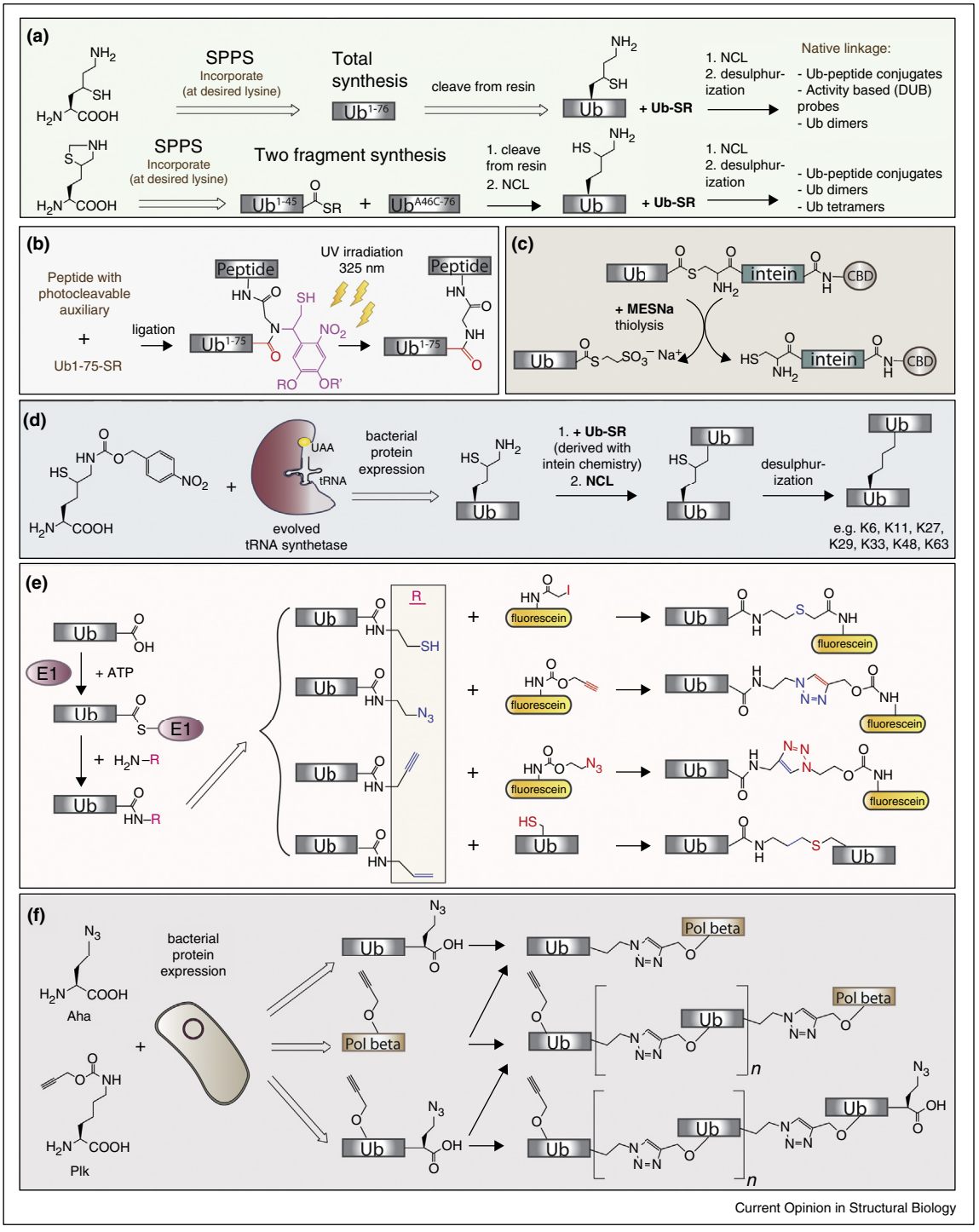
The ubiquitin system. **(a)** Types of ubiquitin conjugation: ubiquitin (UB) can be conjugated as a monomer on one site, or on multiple sites of the substrate protein (multi-monoubiquitination). It can also form homotypical ubiquitin polymers through its N-terminus (M1-linked) or either one of its seven lysine residues (e.g. K48-linked). Mixing of different linkage types gives rise to heterotypic polyubiquitin chains. **(b)** General overview of ubiquitin conjugation and deubiquitination by E1, E2, E3 enzymes and deubiquitinating enzymes (DUBs). Ubiquitin is activated by an E1 enzyme, transferred to a specific E2 enzyme and conjugated to a substrate protein with the help of an E3 ligase.

how different linkages can confer specific signals. Next, we focus on recent advances in diubiquitin-based DUB probes and the development of ubiquitin ligase probes that open up new interesting areas of ubiquitin structural biology. Finally, we discuss advances in making synthetic and semi-synthetic ubiquitin molecules containing other post-translational modifications such as phosphorylation and acetylation.

Chemical strategies

Native chemical ligation (NCL) has been an extremely useful tool to make ubiquitin, ubiquitin–peptide conjugates, ubiquitin dimers and ubiquitin tetramers, as reviewed recently by Pham *et al.* [11]. Chemical synthesis of ubiquitinated peptides was first established by Muir and co-workers and utilizes a ligation auxiliary where the auxiliary group is removed under photolytic conditions; yielding a natively linked Ub-peptide conjugate [12]. Today, most reported methods rely on the incorporation of a γ-thiolysine or δ-thiolysine moiety at a designated lysine residue to allow NCL with a thioester moiety. The thiol containing ubiquitin module can be synthesized with total, linear synthesis [13], or from two fragments [14]. During the total, linear fluorenylmethyloxycarbonyl (Fmoc)-based solid phase peptide synthesis (SPPS) approach, the growing peptide chain is stabilized by the incorporation of special building blocks that prevent the formation of aggregates as the ubiquitin chain grows. In the two segment approach, an N-terminal Ub(1–45)-SR fragment is synthesized and ligated to a synthetic C-terminal Ub[(46–76)-A46C] fragment. In the latter fragment, alanine 46 is replaced by N-methylcysteine to allow NCL with the first fragment and is afterwards converted into the native alanine residue through a desulphurization step. To make ubiquitin dimers and other conjugates, a thioester needs to be introduced at the ubiquitin C-terminus for NCL with the Ub thiolysine-containing module. The thioester functionality can be incorporated by E1-mediated enzymatic conversion with sodium 2-mercaptoethane sulfonate (MESNa) [13] or during Fmoc-based SPPS [15]. Next, NCL can be performed and a subsequent desulphurization step results in a ubiquitin conjugate that bears the native isopeptide linkage. The above described methods are schematically represented in Figure 2a,b. In addition, other strategies yielding a non-native isopeptide linkage have been reported and include oxime-based ligation for non-hydrolysable ubiquitin–conjugate synthesis [16] and thioether based ligation to prepare diubiquitin [17], branched triubiquitin [18] and polyubiquitin [19] modules that retain

Figure 2



Overview of described synthetic methods that yield a native isopeptide bond (**a** and **b**) and described semi-synthetic ligation methods (**c**–**f**). (a) Native chemical ligation using γ-thiolysine (top) or δ-mercaptolysine (bottom) auxiliaries. (b) Photo-crosslinking ligation using a photolytically (UV 325 nm) removable auxiliary. (c) MESNa mediated thiolysis of a ubiquitin–intein construct generates a ubiquitin thioester which can be used for ligation purposes. (d) Semi-synthetic incorporation of δ-thiol-L-lysine with genetic code expansion by addition of δ-thiol-Nε-(p-nitrocarbobenzyloxy)lysine to bacterial cell cultures. (e) Semi-synthetic functionalization of the ubiquitin C-terminus with different moieties utilizing the E1-enzyme. Thioether-based linkages and triazole-based linkages can also be made using synthetic methods. (f) Semi-synthetic incorporation of Aha and Plk allows click chemistry to make several ubiquitin–peptide conjugates and ubiquitin polymers. NCL, native chemical ligation; MESNa, 2-mercaptoethane sulfonate; CBD, chitin binding domain; Aha, azidohomoalanine, Plk, propargyl-derivatized lysine.

a sulfur atom in the forged isopeptide bond. It is clear that synthetic protein chemistry has much to offer to the biological community, but one must keep in mind that the chemistry is not always straightforward. In addition, protein folding must always be checked, although this is less of an issue with the very stable ubiquitin protein.

Semi-synthetic strategies

Next to synthetic strategies, protein semi-synthesis has also become a very convenient strategy for the production of large peptides and small proteins since it was first introduced. Using expressed protein as the starting material, a number of strategies have been invented to equip the protein with chemical reactive groups, labels or fluorogenic dyes. For instance, the use of inteins has become a useful tool for the formation of protein thioesters through MESNa-mediated thiolysis (Figure 2c) [20]. Expansion of the genetic code with unnatural amino acids (UAAs) has further aided the field of protein semi-synthesis [21]. Briefly, an UAA can be incorporated by a modified transfer RNA (tRNA) at the site of a rarely used codon, such as the amber stop codon in *E. coli*. To this end, the active site of the complementary aminoacyl tRNA synthetase (aaRS) needs to be mutated to allow selective recognition of the UAA, which will then be transferred to the modified tRNA and eventually incorporated into the newly translated protein. While genetic code expansion-based methods are clearly useful, most do require certain expertise that can only be found in specialized labs and often require specific *E. coli* strains and tRNA pairs. Below, we will discuss genetic code expansion strategies employed on ubiquitin, while a more extensive overview of incorporation of chemoselective unnatural amino acids in general is discussed by Lang *et al.* [22].

The GOPAL approach (genetically encoded orthogonal protection and activated ligation) uses genetic code expansion for site specific incorporation of tert-butyloxycarbonyl (Boc)-protected lysine in ubiquitin with a specific *Mb*PylRS/*Mb*PylRS$_{CUA}$ pair. Initially, two bioorthogonal protection groups were used that allowed selective deprotection of the desired lysine residue on the proximal ubiquitin module [23]. Later, a specific tRNA pair was found that allows site-specific incorporation of δ-thio-L-lysine and δ-hydroxy-L-lysine without the Boc protecting group, circumventing the need of the previously reported extensive protection and deprotection strategy (Figure 2d) [24].

Another semi-synthetic strategy for making diubiquitin mimics was reported in 2014 and utilizes E1 enzyme to equip the ubiquitin C-terminus with several reactive groups through an amidation reaction (Figure 2e). The advantage of this method is that it does not require extensive knowledge of peptide chemistry nor of genetic code expansion. In this case, allylamine was used to equip the Ub C-terminus with an alkene. Expressed ubiquitin lysine to cysteine mutants at the desired lysine conjugation site were converted into ubiquitin dimers by UV irradiation [25•]. Through this procedure, a C-terminal alkyne moiety was also introduced, which in turn can be used as a reactive group to form non-hydrolysable ubiquitin conjugates with Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC, or 'click chemistry').

The group of Marx has also developed a strategy to achieve non-hydrolysable ubiquitin–conjugate synthesis through bacterial expression and click chemistry (Figure 2f) [26••]. For click chemistry, both an azide and an alkyne are needed. The methionine analog azidohomoalanine (Aha) was incorporated on the ubiquitin C-terminus as the azide containing unnatural amino acid. The alkyne functionality was introduced at one of the desired lysine conjugation positions by using propargyl-derivatized pyrrolysine (Plk) and the PylRS/tRNA^{Pyl} pair of *Methanosarcina barkeri* [27]. After introduction of both azide and alkyne functionalities, non-hydrolysable ubiquitin conjugates can be formed by CuAAC. Such non-hydrolysable ubiquitin-based tools can be utilized to address the selective nature of ubiquitin binding domains towards a certain diubiquitin molecule [28], to study the impact of a monoubiquitination event such as on Polβ [27] and PCNA [29], or to study the effect of non-hydrolysable polyubiquitin chains such as K11 on cell cycle related events [30].

Using synthetic and semi-synthetic tools to study ubiquitin chain structure and biochemistry

Over the recent years, much effort has gone into structural and biochemical characterization of di-ubiquitin and polyubiquitin molecules representing all eight different homogenously linked ubiquitin types. In these efforts, people have been hampered by the lack of specific E2 and E3 enzymes to generate the so-called atypical (K6, K11, K27, K29, K33) chains. The K6 diubiquitin structure was first solved by using the semi-synthetic GOPAL approach [23]. Only recently an enzymatic approach for making K6-polyubiquitin chains was reported [31]. K11 diubiquitin structure elucidation was achieved by two groups independently in 2010 [32,33] after a K11-specific ligase was identified in 2009 [34]. Recently, K29-linked and K33-linked diubiquitins have been structurally characterized after a combinatorial ligation–deubiquitination approach was developed to generate such linkages enzymatically [35,36]. Currently, only K27-linked ubiquitin cannot be prepared fully enzymatically, although interestingly a recent study reports K27 polyubiquitination by the E3 ligase RNF168 [37]. Nevertheless, semi-synthetic and synthetic strategies have been an indispensable tool for studying the structural and biophysical properties of atypical diubiquitins.

Two recent papers describe NMR structural conformation of all atypical diubiquitins in solution. With the exception of K11-linked diubiquitin, all were prepared by a variant of the semi-synthetic GOPAL method. Based on SANS (small-angle neutron scattering), 15N relaxation and RDC (residual dipolar coupling) NMR data, Castañeda *et al.* found that the available diubiquitin crystal structure data poorly overlaps with their in-solution NMR data, which suggests a great flexibility in conformation of Ub chains [38••]. Multiple crystal conformations for the same diubiquitin molecules have indeed been reported, which is consistent with this notion. The same approach was next used to further characterize the atypical K27 diubiquitin linkage in particular [39•]. Interestingly, K27 diubiquitin was processed very inefficiently by the DUBs tested, including more promiscuous ones. It must be noted however that in other studies, DUBs have been described that do cleave K27-linked diubiquitin quite similarly to other diubiquitin molecules, albeit not specifically [40,41]. The study by Castañeda *et al.* provides the first structural analysis of K27 diubiquitin: it shows that the K27 residue is almost completely buried in the diubiquitin structure and that it has very low solvent accessibility. In addition, the C-terminal Gly75–76 region of the distal Ub moiety is more ordered compared to the other known diubiquitin structures. This structural aspect might explain why K27 diubiquitin is processed less efficiently by some DUBs.

Using ubiquitin-based activity probes for structural and biochemical characterization of the ubiquitin conjugation–deconjugation machinery

Ubiquitin-based probes have been used extensively in the past to investigate the mechanism of DUB activation and to trap deubiquitinating enzymes in their active state. Numerous structures were solved with ubiquitin aldehyde (Ubal), the vinyl methyl ester (VME) warhead or the propargyl (PA) warhead (Table 1). While activity-based probes have been recently reviewed [7], we would like to highlight a few recently emerged probes which open up new interesting areas of ubiquitin biology.

The introduction of diubiquitin probes with a warhead in-between the distal and proximal ubiquitin module has allowed a more selective investigation of diubiquitin recognition by DUBs [42–45]. These covalent probes certainly allow more detailed structural investigation of diubiquitin-specific DUB recognition, but do not allow investigation of potential additional binding sites. A recent report was made on DUB probes that do allow investigation of supplementary binding sites, such as the so-called DUB S2 site, by utilizing click chemistry and incorporation of a C-terminal propargyl warhead [46••]. Generated fluorogenic substrates allowed for detailed studies of enzymatic turnover and in a more recent report [47], one of these probes was used to structurally characterize the K48 polyubiquitin cleaving mechanism of the SARS DUB PLpro.

While there is a plethora of DUB probes available, until recently the E1–E2–E3 machinery has been falling behind in this respect. An AMP-based E1 probe has been described recently and could be of use for studying E1 enzymes for other ubiquitin-like proteins as well [63•]. In addition, a probe that reacts with purified E2 and E3 ligases has been reported and was used to study activation of the Parkin E3 ligase in detail [64••]. Lastly, a recent report was made on a Ub-dehydroalanine-based ligase probe, which can be passed on in the ligation cascade to E1, E2 and E3 ligases of the HECT/RBR type [65••].

Using synthetic and semi-synthetic tools to study post-translational modifications on ubiquitin

An exciting emerging area currently under investigation is the post-translational modification of ubiquitin itself. In 2010, a report was made about deamidation of Gln40 on ubiquitin by the bacterial effector protein CHBP, which effectively impaired ubiquitin chain synthesis [66]. Recently, it was found that ubiquitin can be acetylated on Lys6, Lys48 and Lys63 by mass spectrometric efforts [67]. The effects of acetylation were studied by incorporation of N-acetyllysine on positions 6 and 48 of ubiquitin through genetic code expansion. Interestingly, acetylation at Lys6 and Lys48 on mono ubiquitin prevented ubiquitin chain elongation by several E2 enzymes *in vitro*. Histone H2B was identified as a substrate for acetylated mono Ub, further suggesting a chain terminating effect of the acetylation event. In addition to the three acetylation sites, Ohtake et al. also detected phosphorylation on Thr14 and Ser65 in conjugated ubiquitin species. Ub has eight potential phosphorylation sites: Thr7, Thr12, Thr14, Ser20, Ser57, Tyr59, Ser65 and Thr66. A general overview of post translational modifications on Ub is presented in Figure 3a.

The best studied example of ubiquitin phosphorylation is Ser65 phosphorylated Ub. Phosphorylation on Ser65 is mediated by PTEN-induced kinase 1 (PINK1) and activates the RING-in-between-RING (RBR) E3 ligase Parkin. This in turn leads to clearance of damaged mitochondria in a process called 'mitophagy' [68]. Mutations in both Parkin and PINK1 have been implicated in the early onset of autosomal-recessive Parkinson's disease and players in this pathway are therefore heavily investigated for therapeutic purposes [69]. Although Ser65 phosphorylated Ub is easily made enzymatically using recombinant PINK1, this is restricted to this residue only. Not surprisingly, several efforts have been put into making phosphorylated ubiquitin available through synthetic and semi-synthetic approaches that allow investigation of other potential phosphorylation sites. Phosphorylated

Table 1

Overview of DUB structures with Ub-based activity-based probes and key findings summarized. Ubal, ubiquitin aldehyde; Ub-VME, ubiquitin vinyl methyl ester; Ub-PA, ubiquitin propargyl

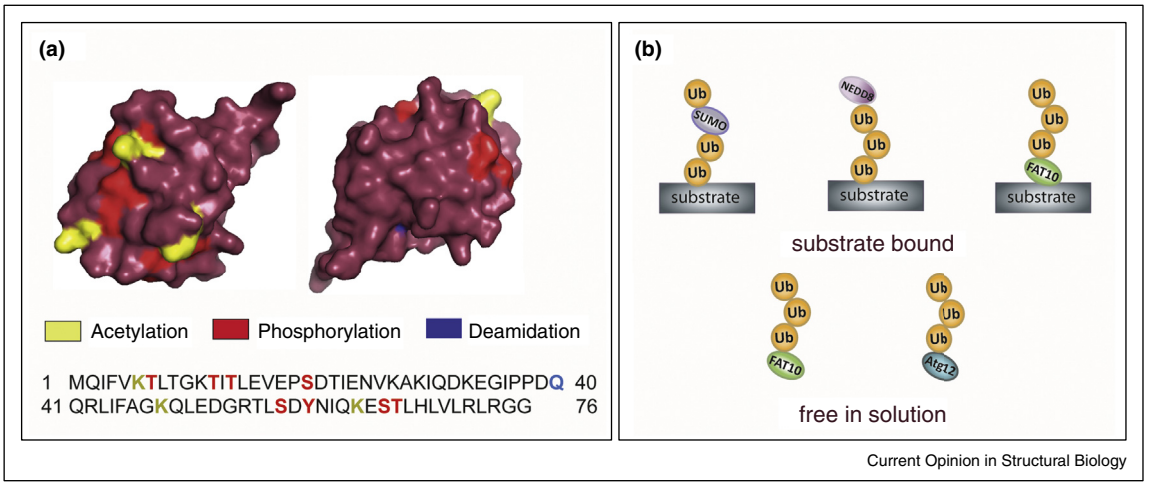
Probe	Ref.	DUB	PDB code	Key findings
Ubal	[48]	Otubain-1	4LDT	Binding of free ubiquitin to Otubain-1 (OTUB1) triggers conformational changes in the OTU domain of OTUB1 and allosterically increases the affinity for Ubc13∼Ub.
	[49]	SAGA DUB module	3MHS	Integrity of the SAGA module (Ubp8, Sgf11, Sus1 and Sgr73) is essential for proper Ubp8 catalytic activity. Although the Ubp8 active site is well ordered, binding of Ubal shows important structural rearrangements in the vicinity of the active site. The intact SAGA module stabilizes Ubp8 so it is catalytically competent and able to bind Ub.
	[50]	SARS-CoV PLpro	4MM3	Structure elucidation allowed characterization of the binding surface while subsequent mutational analysis and structural modeling suggest the existence of a second binding site to provide K48 and ISG15 specificity of PLpro.
	[51]	USP14 (catalytic domain)	1XD3	In the apo USP14 structure, the active site is well formed *before* substrate binding. Ubiquitin binding is blocked by two nearby surface loops (BL1 and BL2). Ub binding translocates the two loops to allow access of its C-terminus to the USP14 active site.
	[52]	USP7/HAUSP (catalytic domain)	1NBF	In the apo USP7 structure, the catalytic histidine is nearly 10 Å away from the catalytic cysteine. The C-terminal segment of Ubal induces backbone conformational changes in the vicinity of the active site, leading to alignment of the catalytic triad.
	[53]	Yuh1	1CMX	Ubal binding is coupled to ordering of a 21-residue loop which blocks the active-site cleft in the absence of substrate.
Ub-VME	[54]	Legionella DUB module	5CRA	The $SDeA_{DUB}$ modules engage Gln40 of Ub instead of the common Ile44 hydrophobic patch. The sparse use of molecular contacts likely allows dual specificity of the DUB module towards Ub and NEDD8.
	[55]	$M48^{USP}$	2J7Q	An acidic cleft on $M48^{USP}$ accommodates the complementary basic C-terminal Ub stretch. Specificity is additionally mediated by a β-hairpin to recognize Ub over other UbL's such as ISG15.
	[56]	UCHL1	3KW5, 3IFW (S18Y), 3KVF (I93M)	Binding of the N-terminal ubiquitin β-hairpin results in placement of the UCHL1 active site histidine in the correct location, which in the apo structure is located 7.7 Å from the catalytic cysteine.
	[57]	hUCHL3	1XD3	UCH-L3 features a crossover loop that is disordered in the substrate-free structure and ordered above the active-site cleft when Ub is bound.
	[58]	*Pf*UCHL3	2WDT	The *Plasmodium falciparum (Pf)* UCHL3 structure provides a basis for dual recognition of both NEDD8 and Ub, but also shows distinct differences in the Ub binding site compared to human UCHL3.
	[59]	USP37	4I6N, 4IG7	The UCH37-like domain (ULD) contacts Ub and stabilizes an unusual salt bridge between Lys48 and Glu51 of Ub. This results in an inhibited enzyme state in the proteasome free form.
Ub-PA	[60]	UCH-L5	4UEL (RPN13)	UCH-L5 can be activated by RPN13 and deactivated by INO80G. Ub binding to RPN13 is identical to the canonical ubiquitin-binding mode found in all UCH family members. Ub binds via its C-terminal tail close to the UCH-L5 active site and via its core relatively far from the active site in a series of three specific exosites that lead to several structural rearrangements.
	[61]	vOTU	3ZNH	Structure elucidation of viral (v)OTU with Ub-propargyl closely resembles previous vOTU-Ub complexes and shows that the Ub-propargyl group forms a vinyl thioether linkage in complex.
Ub-Br_2	[62]	Otubain-2	4FJV	Ub binding is different from vOTU due to two extra β-strands in vOTU. Upon Ub binding, the OTUB2 catalytic residues show only subtle movements within the catalytic center. On either side of bound Ub however, large conformational changes occur in OTUB2. The Ub C-terminus is extended to reach the OTUB2 catalytic core and forms the main points of contact. N-terminal domain swapping reveals how OTUB1 can cleave different Ub-linkages than OTUB2.

ubiquitin variants other than Ser65 are indeed currently commercially available.

Chemical synthesis of phosphorylated ubiquitin

Last year, the total chemical synthesis of Ser65 phosphorylated mono and Lys63-linked diubiquitin was reported [70•]. Lys63-linked diubiquitin was prepared with a phospho Ser65 at the distal, proximal or both moieties of the diubiquitin module. Characterization of biological consequences of enzymatically prepared Ser65 phospho-Ub was reported that year and showed that the majority of tested DUBs were impaired in hydrolyzing phosphorylated

Figure 3



Crosstalk between ubiquitin and other post-translational modifications. **(a)** Reported post-translational modifications on ubiquitin shown on the Ub surface structure and the amino acid sequence of Ub. **(b)** Representation of *in vivo* found Ub/UbL heterologous chains.

ubiquitin chains [71]. An additional layer of DUB regulation was implied by using synthetic Lys63-linked distal, proximal and dual phospho-Ser65 diubiquitin, which showed that diminished DUB activity could be affected by position of the phosphorylation site at either the proximal or distal ubiquitin [70•]. Biological relevance of this observation is currently unknown.

Semi-synthesis of phosphorylated ubiquitin

A recently published semi-synthetic strategy to obtain phosphorylated ubiquitin involves native chemical ligation of an expressed N-terminal ubiquitin–intein fusion fragment and a synthetic C-terminal ubiquitin fragment [72•]. This allows introduction of a phosphorylated residue at 4 out the 8 known phosphorylation sites on ubiquitin in the C-terminal Ub(46–76) fragment.

Crosstalk with other post-translational modifications

Many reports have already been made implying crosstalk between ubiquitin and ubiquitin-like (UbL) proteins: a class of proteins that share high structural similarity and a common β-grasp fold with Ub. Best studied is the crosstalk between ubiquitin and SUMO [73], but ubiquitinated-NEDD8 chains have also been reported [74], as well as the existence of ubiquitinated FAT10 [75], ISGylated ubiquitin [76] and ubiquitinated Atg12 [77]. The protein (semi)-synthesis community is moving rapidly to catch up with this phenomenon and semi-synthetic strategies for obtaining ubiquitinated SUMO-2 [24] and ubiquitinated Rub1, the yeast NEDD8 homolog, have already been reported [78•]. Synthetic strategies for obtaining full length UbL proteins such as Nedd8 [61], SUMO-1 [79,80•] and Ufm1 [81] are also starting to emerge. It is not hard to imagine the existence of other PTMs on ubiquitin such as methylation or glycosylation, both of which could be incorporated through (semi-)synthetic approaches. Plenty of opportunities are available in this area and we expect that future research will provide many more interesting insights into this exciting new field.

Future directions for the field

Although much has been accomplished already, there are many more future challenges in the ubiquitin and ubiquitin-like field for (semi)-synthetic scientists. The synthesis of lysine-specific, homotypical polyubiquitin chains is such an example; as is the (semi-)synthesis of heterotypical ubiquitin chains and mixed Ub/UbL chains. With regard to probes, much more can be achieved by extending the scope towards ubiquitin-like proteins, which in general is a much less characterized class of proteins. Many opportunities also still lie in the (semi-)synthesis of these UbL proteins, which would allow incorporation of fluorogenic dyes, warheads, ligation sites or other useful chemical handles. Finally, the seemingly limitless emerging post-translational crosstalk between protein modifiers reveals a whole new layer of interesting protein regulation. Here, (semi)-synthetic biology would certainly add great value to help understanding the expanded ubiquitin code. Not only for ubiquitin itself, but also for ubiquitin-like proteins and possibly even their decoding sequences: (ubiquitin) binding domains.

Summary

The post-translational modifier ubiquitin is an essential constituent of the cell and participates in many cellular processes. Synthetic and semi-synthetic strategies to obtain ubiquitin and ubiquitin-based derivatives have provided indispensable tools to study ubiquitin biochemistry. Structural NMR-based characterization of semi-synthetic

atypical diubiquitins has revealed great flexibility of the ubiquitin signal and helped further understanding of how different Ub linkages can confer specific signals. Ubiquitin-based probes have helped understanding DUB activation mechanisms and the coming of new diubiquitin-based DUB probes will shed more insight into polyubiquitin recognition. Ub-based ligase probes are starting to emerge and will provide more mechanistic investigation of the ubiquitin ligation process. Additionally, techniques are starting to emerge to study an even more intricate layer of the ubiquitin signal: post-translational modification of ubiquitin itself. Deamidation, acetylation and phosphorylation of ubiquitin have been reported and in addition, crosstalk between ubiquitin-like proteins and Ub is known to exist *in vivo*. We expect that the use of synthetic and semi-synthetic strategies will be very useful to study this exciting new field of ubiquitin regulation as they allow control over the exact site of the modification.

Conflict of interest

H.O. is founder and shareholder of the company UbiQ that markets reagents in the ubiquitin field. H.O. is part of the DUB Alliance that includes Cancer Research Technology and FORMA Therapeutics

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