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Peeling Away the Layers of Ubiquitin Signaling Complexities with Synthetic Ubiquitin-Protein Conjugates

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Abstract

Covalent attachment of ubiquitin, a process termed ubiquitination, affects the location, function, and stability of modified proteins. Significant advances have been made in building synthetic ubiquitin-protein conjugates that can be used to investigate how ubiquitin regulates diverse biological processes. Herein we describe recent advances and discuss how chemical methods have been implemented to address the molecular underpinnings of ubiquitin-dependent cellular signaling.

Over the last 40 years, it has become increasingly evident that ubiquitin (Ub) plays a critical role in nearly every biochemical pathway in eukaryotes [1]. Ub is one of the most highly conserved proteins in eukaryotic organisms, with only three mutations between the yeast and human homologs. The basic enzymatic logic by which Ub is covalently tethered to lysine side chains of substrate proteins is also conserved [2,3] (Figure 1A). Approximately 3% of the human genome is dedicated to Ub conjugation [4]. Consistent with the predicted abundance of Ub conjugation machinery, proteomics studies have recently uncovered ~19,000 modification sites in ~5,000 human proteins [5•]. Further underscoring the physiological importance of Ub modifications, defects in Ub conjugation and deconjugation are known to have severe deleterious consequences [6].

A longstanding mystery in the field of Ub research is how cells distinguish between different Ub-modified substrates [7,8]. Ub is a small protein with only 76 amino acids in its sequence, yet the surface area (~3800 Å²) is still much larger than those of other prevalent lysine modifications (e.g., acetylation). Consequently, mono-ubiquitinated substrate proteins gain several new structural features, which can be further expanded if the substrates are modified with multiple monoUb molecules or Ub chains. In Ub chains, the seven lysines of Ub (K6, K11, K27, K29, K33, K48, and K63) along with the N-terminal methionine (M1) can all serve as points of conjugation to the C-terminus of another Ub molecule (Figure 1B). The resulting Ub chains can vary in length, linkage, and topology (i.e., chains can be linear or branched) (Figure 1C). Many Ub receptors — including deubiquitinases that reverse the

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ubiquitination process — can recognize distinct chain types, suggesting that Ub chain topology is a critical determinant of Ub-mediated cellular signaling [9].

In this review, we focus on how chemical methods have facilitated efforts to understand the functions of Ub modifications. With the exception of M1-linked chains, Ub is connected to substrate protein lysines through an isopeptide bond, which cannot be made recombinantly with proteinogenic amino acids. Instead, enzymatic or chemical methods are required to forge an isopeptide linkage. Enzymatic methods are powerful in their own right, but they offer limited control over which substrate lysine can be modified [10-15]. Chemical approaches, on the other hand, are relatively tunable and high yielding. A number of chemical strategies have been developed over the last few years to generate either native or non-native isopeptide linkages for biochemical or biophysical studies [16,17]. Here, we provide a brief overview of existing chemical methods before discussing how they can be used to address important biological problems.

Synthesis of Defined Ub-Substrate Conjugates — An Overview

There are several state of the art methods to generate native and non-native isopeptide bonds. Chief among those that afford native isopeptide linkages are native chemical ligation (NCL) and expressed chemical ligation (EPL). Both NCL and EPL utilize temporary auxiliaries to facilitate chemical ligation with a Ub C-terminal thioester (Figure 2A) [16-18]. The auxiliaries are typically removed under reducing conditions to furnish a native linkage. Depending on the size of the polypeptide substrate, the auxiliary can be introduced using solid-phase peptide synthesis [19••] or the nonsense-suppression strategy involving an orthogonal transfer RNA synthetase and tRNA_{CUA} pair (where CUA is the anticodon of the tRNA) [20]. Nonsense-suppression technology can also be used to incorporate orthogonal amine-protecting groups for site-specific isopeptide bond formation (Figure 2B) [21,22•].

For the construction of non-native linkages, the latent reactivity of cysteine can be exploited. The general strategy is to convert the side chain thiol into a thiolate anion or thiyl radical. Availing the reactivity of these intermediates has led to the synthesis of Ub-protein conjugates and Ub chains connected by disulfide [23•-25] or thioether linkages [26•,27] (Figure 2C). From a practical standpoint, the chemistry is straightforward; coupling reactions are efficient and minimal synthetic manipulations are required.

Cysteine chemistry also enables site-specific conjugation of Ub oligomers and monoUb to substrate proteins through nonhydrolyzable linkages. This strategy has been successfully employed with coupling reagents such as dichloroacetone [28], bromoacetamide [29], and maleimide [29] (Figure 2D). The resulting conjugates are stable in the presence of deubiquitinases and can be used to selectively probe binding interactions.

Another useful strategy to link mono-Ub and Ub chains to substrate proteins is the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction (Figure 2E). The azide and alkyne can be incorporated into both Ub and the substrate protein using nonsense suppression [30-32•]. Alternatively, EPL can be employed to install the alkyne at the C-terminus of Ub [33]. Either approach furnishes a Ub-conjugate containing a stable triazole linkage.

There is data showing that, depending on the interacting partner, modifications in the vicinity of isopeptide amide can adversely affect binding and bond scission [34]. Since non-native linkages introduce perturbations, it is important to evaluate whether the linkage of interest is a good surrogate of the native isopeptide bond. There are two factors to consider when comparing non-native and native Ub conjugates: the thermodynamics and kinetics of interactions with Ub-binding domains and deubiquitinases. Few studies have addressed these quantitative aspects.

Conformational dynamics of Ub conjugates contribute to the specificity of Ub receptors, and are therefore fundamental to the thermodynamics and kinetics of binding and catalysis. Consequently, there have been a couple reports examining whether artificial linkages affect structure. Computational methods can be used to compare the native and non-native linkages. Calculations using Rosetta, for example, revealed that a disulfide-linked Ub conjugate samples the same conformational space as the native conjugate [35]. On the other hand, molecular dynamics simulations of Ub dimers bearing a triazole linker showed the artificial linker renders the conjugate less flexible compared to the native dimer [36]. These results suggest that the disulfide linkage might not affect the on/off rates of binding interactions while the triazole linker could introduce some deviation. Nevertheless, linkage-specific antibodies can correctly identify Ub chains bearing non-native linkages [27,32]. Additionally, hydrolysis experiments with select deubiquitinases showed similar processing of the disulfide, thioether, and native linkages [23•, 24•, 26•]. To further solidify the utility of non-native linkages for studying ubiquitin-mediated signaling, future studies should employ quantitative analyses to fully assess the extent to which these linkages mimic the native isopeptide bond.

Chemical methods provide a powerful arsenal to tackle mechanistic questions related to Ub functions. In the following sections, we seek to highlight what can be learned from using designer ubiquitin-protein conjugates in biochemical and structural studies.

How Do Receptors Specifically Recognize Ub-Modified Substrates?

It is estimated that the human genome encodes ~250 Ub-binding domains (UBDs) [9]. The large number of receptors poses the question of how UBDs specifically recognize Ub-modified substrates. Since most UBDs bind mono- or diUb with relatively low affinity (μM range), additional layers of structural complexity must be required to specifically direct Ub-dependent processes (Figure 3) [37,38]. Understanding the relationship between Ub structure and signaling outcomes requires access to an array of well-defined ubiquitinated substrates.

One of the best-understood examples of how UBD-bearing proteins select their ligands involves proliferating cell nuclear antigen (PCNA). PCNA is a cofactor of DNA polymerases that recruits proteins to the DNA replication fork. Different Ub modifications of PCNA at an evolutionarily conserved lysine (K164) activate distinct post-replication DNA repair pathways [39]. For example, monoUb-PCNA promotes error-prone DNA translesion synthesis (TLS), while PCNA conjugated to K63-linked Ub chains signals for an error-free lesion bypass mechanism. Initial biochemical studies showed that monoUb-

PCNA, generated by enzymatic methods, activates TLS polymerases, such as Pol η , through a bipartite interaction: the PCNA-interacting protein (PIP) motif and the Ub-binding zinc finger (UBZ) domain of Pol η simultaneously engage monoUb-PCNA [40-43]. However, enzyme synthesis alone could not generate sufficient quantities of monoUb-PCNA conjugates for detailed structural analyses. To overcome this limitation, disulfide exchange and CuAAC chemistry was applied to site-selectively construct large quantities of monoUb-PCNA [23,31]. Small angle X-ray scattering analysis combined with computational modeling revealed distinct binding interfaces between Ub and PCNA that are critical for coordinating polymerase activity [44,45].

PCNA modified with K63-linked Ub chains has been shown to inhibit the activity of Pol η and other low-fidelity TLS polymerases while recruiting proteins for error-free repair. To interrogate the effect of polyubiquitination on PCNA function, disulfide exchange chemistry was used to build polyubiquitinated PCNA with discreet Ub chain lengths [46••]. PolyUb-PCNA with Ub chains longer than dimers were observed to inhibit TLS by trapping Pol η in an unproductive state and preventing association with Pol δ , another polymerase required for replication and repair. Synthetic polyubiquitinated PCNA will be important for defining structural aspects of Pol η inhibition and interrogating how effectors such as ZRANB3 (zinc finger, RAN-binding domain containing 3) specifically interact with PCNA-tethered K63-linked chains [47-49].

How Do Ub Modifications Impact the Function of Protein Substrates?

A large proportion of proteins from all eukaryotic proteomes are modified with Ub [5], and there is significant interest in understanding the biochemical and biophysical consequences of Ub modifications on protein function. Advances in chemical ubiquitination have enabled a growing number of efforts to address these questions. We highlight a couple recent case studies below. With approximately 30% of human tumors containing an activating mutation, the GTPase Ras is one of the most prevalent oncogenes found in human cancers [50]. Ras is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs facilitate the exchange of GDP with GTP and activate Ras, whereas GAPs potentiate GTP hydrolysis and deactivate Ras. Monoubiquitination of K-Ras has been shown to promote tumorigenesis [51]; however, the mechanism by which monoUb leads to activation of Ras has not been fully resolved. A series of biochemical and structural studies, in which cysteine chemistry was used to ubiquitinate Ras, showed that monoubiquitination disrupts regulation of Ras by GAPs in a site-specific manner by reducing the binding affinity between the two proteins [35••]. The decrease in binding affinity explains in part why loss of monoubiquitination impairs oncogenic Ras-mediated tumorigenesis.

The Wnt/ β -catenin signaling pathway controls many aspects of cellular fate during animal development. When extracellular Wnt ligands bind their cognate receptor, a signaling cascade is triggered in which the protein Dishevelled is recruited to the membrane and polymerized in a head-to-tail manner through its DIX domain, establishing a signaling platform from which many downstream processes emanate [52]. Studies have shown that the DIX domain is ubiquitinated [53-55], and that this modification attenuates head-to-tail polymerization. However, the exact mechanism by which ubiquitination affects DIX

polymerization has remained elusive. To address this problem, chemical ubiquitination using nonsense suppression technology was employed to generate site-specifically modified variants of the DIX domain [56•]. The differential effects of K54- and K58-linked Ub on DIX polymerization are striking; DIX54-Ub inhibits the head-to-tail interaction between DIX monomers, while DIX58-Ub remains polymerization-competent. Structural studies demonstrated that ubiquitination of K54 would result in a steric clash with the interacting DIX monomer. These results provide a molecular-level view of how Ub can affect protein function—and ultimately the entire Wnt/ β -catenin signaling pathway—by regulating protein self-association.

How Do Ub Modifications Affect the Stability of Proteins?

Eukaryotes use three major cellular degradative pathways: the endosomal/lysosomal pathway, the proteasome system, and autophagy [57]. Ub modification is a critical component of all three, raising the question: How are substrates targeted to a particular pathway? For proteasomal degradation, the current paradigm is that a substrate must be modified with a K48-linked Ub chain containing at least four subunits. For the endocytic and autophagic pathways, substrates must be marked with monoUb or a K63-linked chain.

Challenging the dogma of proteasomal targeting is the finding that monoubiquitination of α -synuclein is sufficient for degradation [58•]. α -Synuclein is a small protein (140 residues) implicated in several neurodegenerative diseases, including Parkinson's disease. Failure to maintain appropriate levels of α -synuclein can lead to aggregation and progression of neurodegeneration. By stimulating proteasomal degradation, monoubiquitination is thought to play a major role in controlling the levels of α -synuclein [59]. To determine whether protein turnover is dependent on the site of modification, a disulfide exchange approach was used to synthesize α -synuclein bearing monoUb at each of the nine different lysine side chains [60•]. Incubating these conjugates with purified proteasomes led to the observation that Ub attachment near the N-terminus stimulates degradation, while modifications near the middle of the α -synuclein sequence do not trigger turnover. A similar finding was recently reported with the substrate protein cyclin B [61•]. In this case, a single Ub modification is not sufficient for degradation, but proximity of the first ubiquitinated lysine to the N-terminus is associated with faster proteasomal degradation. Chemical strategies for generating defined ubiquitinated substrates are critical for identifying how each modification site affects protein degradation.

Chemical methods to generate polyUb-protein conjugates also afford the opportunity to interrogate the effect of Ub linkage type on protein stability. K48-linked Ub chains may not be the only mark for proteasomal degradation, as K11-linked chains have also been shown to act as targeting signals in the proteasome pathway during mitosis [62]. To test whether other Ub chains can target substrates to the proteasome, CuAAC chemistry was used to construct K11-, K27-, and K29-linked chains [32•]. Since CuAAC furnishes a nonhydrolyzable linkage, any Ub chain that is recognized by the proteasome should remain bound and inhibit the degradation of the native substrate cyclin B. Consistent with the role of K11-linked chains in mitosis, nonhydrolyzable K11-linked chains were found to completely abolish cyclin B degradation. By contrast, monoUb and Ub chains bearing

nonhydrolyzable K27 and K29 linkages did not interfere with cyclin B turnover, suggesting the latter chain types are unable to support proteasomal degradation of substrate proteins. Nonhydrolyzable Ub chains can thus be used in cell free systems to study how distinct Ub chain types regulate protein stability.

What are the Substrates of Deubiquitinases?

Deubiquitinases antagonize the action of Ub ligases by catalyzing the removal of Ub from substrate proteins and dismantling Ub chains [63]. The human genome encodes ~90 DUBs, subdivided into five distinct subfamilies: four subfamilies are cysteine proteases (e.g., the Ub-specific proteases or USPs, the Ub C-terminal hydrolases or UCHs, the ovarian tumor proteases or OTUs, and Josephins) and one is composed of zinc-dependent metalloproteases (the JAMM subfamily) [64]. The sheer number and diversity of these enzymes suggests they must carry out specific functions. However, it remains challenging to elucidate the biochemical function of these enzymes. Approximately one quarter of the human protein-coding genes are modified with Ub, and there is tremendous variation in the types Ub chains that are produced. Thus matching a particular deubiquitinase to its physiological substrates is akin to finding a needle in a haystack. Even when the substrates of a particular deubiquitinase are known, it is difficult to define the molecular mechanisms underlying selective substrate recognition and hydrolysis.

Synthetic Ub-protein conjugates and unanchored/substrate-free Ub chains can help shed light on deubiquitinase functions. Recent work on the human OTU deubiquitinases nicely illustrates this point. In a screen with all eight types of native Ub dimers synthesized via semi-synthetic approaches, ten out of the fourteen catalytically active OTUs displayed selectivity towards one or two linkages [65••]. Biochemical and structural studies with synthetic dimers and Ub-peptide conjugates showed that linkage selectivity is achieved by different mechanisms. In cases where UBDs are embedded within the polypeptide sequences of OTUs, these domains can both restrict and broaden linkage selectivity through interactions with individual Ub subunits. The subsites (termed S1' sites) within the catalytic domain of OTUs can also confer preference for specific Ub linkages. Both UBDs and S1' sites serve to position the proximal Ub moiety and orient the isopeptide bond in the active site. Identifying UBDs and S1' sites is critical for verifying whether OTUs act as linkage selective enzymes in cells. Indeed, with structural information obtained for the OTU family member TRABID, K29/K33-linked Ub chains were found to be the preferred substrates both *in vitro* and *in vivo* [66•].

Future Applications for Synthetic Ub-Protein Conjugates

Synthetic Ub-protein conjugates have proven to be invaluable tools for systematically interrogating the diverse functions of Ub modifications. Heroic efforts have been made toward site-specifically attaching Ub chains to substrate proteins *via* total protein synthesis or disulfide chemistry. However, total-synthesis based strategies are limited by technical challenges, and disulfide chemistry restricts downstream applications to non-reducing conditions. New and innovative methods are needed to site-selectively and efficiently attach

any Ub chain—with different linkages, lengths and topologies—to target proteins ranging in size and complexity.

The development of robust and general methods to build Ub chains and Ub conjugates will propel efforts to decipher Ub-mediated processes. A particularly exciting and uncharted area of research with defined Ub modifications is with the ATP-dependent chaperone valosin-containing protein (VCP)/p97 and its many interacting partners. With its ability to unfold ubiquitinated substrate proteins and edit Ub modifications, p97 adds an additional level of control to the cellular degradative systems [67]. It is still unclear how different Ub modifications, in particular Ub chains, are processed by p97 to regulate Ub-dependent processes. One strategy to investigate p97 function is to use well-defined ubiquitinated substrate proteins for binding and activity-based experiments.

Deciphering the specificity and enzymatic logic of deubiquitinases will require moving beyond Ub dimers as test substrates. The UCH deubiquitinases play important roles in a number of cellular processes ranging from metabolism to DNA damage repair, yet their biochemical functions remain poorly defined. Members of the UCH family are capable of removing N-terminal Ub from substrate proteins [68]; however, the physiological significance of these results awaits further testing. Recent advances in the synthesis of activity-based Ub probes could facilitate these efforts [69]. The USPs, which constitute the largest family of deubiquitinases, were originally thought to display little selectivity towards Ub chains of different linkages. Emerging data on USP30, however, argues that some USPs could be selective for certain chain types if the chain is long enough [70]. Having access to a diverse array of Ub chains varying in both length and topology will be important for understanding the selectivities of USPs and UCHs. It will also be imperative to test the full panel of deubiquitinases against Ub-protein conjugates of interest, as the DUBs Cezanne and CYLD were reported to efficiently cleave Ub from the DIX domain of Dishevelled [56].

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Highlights

- Chemical methods enable facile production of a diverse array of ubiquitin-protein conjugates
- Defined ubiquitin conjugates can be used to characterize selective interactions with receptors; intermolecular protein-protein interactions; and fundamental aspects of ubiquitin-mediated protein degradation.

Figure 1.

Protein ubiquitination. A. Covalent attachment of Ub to substrate proteins through a process referred to as protein ubiquitination. Ub modifications can be removed by deubiquitinases (DUBs) and these enzymes can also dismantle Ub chains. B. Chains can be constructed using any of the seven lysines of Ub (K6, K11, K27, K29, K33, K48, and K63) along with the N-terminal methionine. C. Chains varying in length, linkage and topology are formed on substrates. The numbers denote which lysine is used to link each of the Ub subunits.

Figure 2.

Chemical methods for synthesizing well-defined Ub-protein conjugates. A. NCL and EPL approach using auxiliary groups that are removed under reducing conditions. B. Nonsense suppression technology can be used to incorporate unnatural amino acid derivatives that facilitate coupling with activated Ub molecules. C. Cysteine chemistry can be used to forge linkages that are cleaved by deubiquitinases. D. Cysteine chemistry can also be used to build nonhydrolyzable linkages. E. Strategies for using Cu-catalyzed azide-alkyne cycloaddition (CuAAC) chemistry to link Ub to substrate proteins.

A Discriminating between monoubiquitinated substrates **B** Discriminating between polyubiquitinated substrates **Figure 3.**

Cooperative, low-affinity interactions between Ub receptors and their binding partners can impart specificity in Ub-dependent processes. Substrates carrying distinct monoUb (A) and Ub chain (B) modifications can bind specific Ub receptors through substrate-binding sites and Ub-binding sites.