# Unraveling the Complexity of Ubiquitin Signaling

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ABSTRACT: Protein ubiquitination, the covalent attachment of ubiquitin to target proteins, has emerged as one of the most prevalent posttranslational modifications (PTMs), regulating nearly every cellular pathway. The diversity of signaling associated with this particular PTM stems from the myriad ways in which a target protein can be modified by ubiquitin, *e.g.*, monoubiquitin, multi-monoubiquitin, and polyubiquitin linkages. In this Review, we focus on developments in both enzymatic and chemical methods that engender ubiquitin with new chemical and physical properties. Moreover, we highlight how these methods have enabled studies directed toward (i) characterizing enzymes responsible for reversing the ubiquitin modification, (ii) understanding the influence of ubiquitin on protein function and crosstalk with other PTMs, and (iii) uncovering the impact of polyubiquitin chain linkage and length on downstream signaling events.
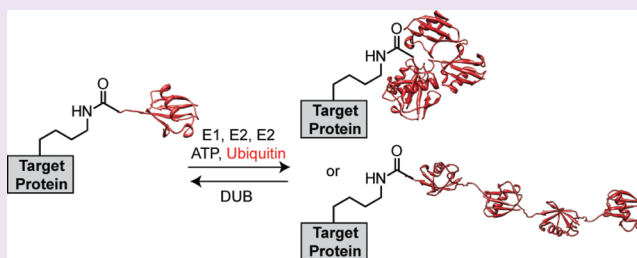
Covalent attachment of ubiquitin (Ub) and ubiquitin-like proteins (Ubls) to the ε-amino group of lysine residues in a target protein, a process termed ubiquitination or ubiquitylation, is one of the most prevalent mechanisms for regulating protein function and stability in eukaryotes.[1,2] Indeed, sequence annotations suggest nearly 5% of the human genome is dedicated to the coupling and removal of Ub/Ubls to and from proteins. Given the central role of the Ub network in cellular physiology, misregulation is often associated with numerous human diseases, including cancer, immune disorders, neurodegenerative diseases, and congestive heart failure.[3-6]

Ubiquitination is unique among the ensemble of posttranslational modifications (PTMs), specifically from the standpoint of signal diversity.[7-9] For example, in contrast to other prevalent PTMs such as phosphorylation, proteins can be modified with Ub on a single lysine residue (monoUb), multiple lysines (multi-monoUb), or a single lysine with a polymeric chain of Ub (polyUb). With regards to polyUb chain formation, Ub possesses seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) and an N-terminal methionine residue (M1), each of which can form isopeptide or peptide linkages with the carboxy terminus of another Ub molecule (Figure 1A). This feature adds significant complexity to intracellular Ub signaling networks as it permits the assembly of chains with many different types of linkages and lengths with the potential to control distinct biological processes.[10]

New experimental approaches have been central to unraveling how Ub signals are formed, recognized, and transduced into various biological responses. In this Review, we describe a combination of enzymatic and chemical methods used to develop molecular probes. We provide a comprehensive overview of enzymatic syntheses of monoUb and polyUb derivatives and compare these to chemoselective reactions used to construct Ub variants unobtainable by other means.

Moreover, we highlight how each method enables the interrogation of Ub signaling, such as the specificity of deubiquitinating enzymes (DUBs), recognition of polyUb chains, and function of ubiquitinated target proteins. Finally, we discuss the utility of each approach in future applications to dissect the importance of polyUb linkage type and chain length.

■ SYNTHESIS OF UBIQUITIN DERIVATIVES: ENZYMATIC CONTROL OVER REGIOSPECIFICITY

Enzymatic transformations provide an efficient and selective means to engender Ub with new chemical and physical properties. In particular, two types of enzymes are used to alter Ub: those that selectively target the C-terminus and others that modify lysines to afford specific homotypic polyUb chains. These approaches have significantly advanced the Ub field by identifying DUBs and discovering tetraUb, with each Ub monomer linked through K48, as the minimal signal for proteasomal substrate targeting.

■ C-TERMINAL UB MODIFICATIONS

Use of Ub-Conjugating E2 Enzymes. An intricate network of enzymes couple Ub to target proteins (Figure 1B). Specifically, three types, *i.e.*, E1, E2, and E3, attach Ub to targets through a sequential enzymatic cascade.[11] E1 initiates the cascade by first catalyzing the adenylation of Ub at the C-terminal glycine residue (Gly76).[12] Following adenylation, a catalytic cysteine of E1 attacks the adenylate affording an E1-*S*-Ub thioester intermediate. The ubiquityl moiety is then shuttled from E1 to an active site cysteine of an E2 through a transthioesterification event affording a Ub-charged E2 (E2-*S*-Ub).[13] To complete the enzymatic sequence, an E3 associates
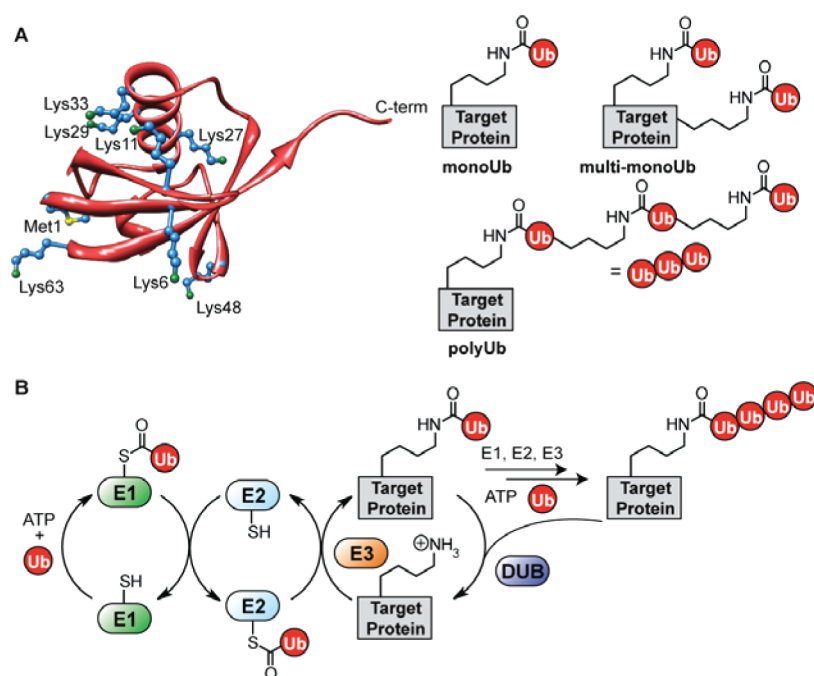
Figure 1. The many facets of protein ubiquitination. (A) The structure of Ub (PDB code 1UBQ[118]) showing all seven lysines (blue, with green nitrogen atom) and the N-terminal methionine (blue, with yellow sulfur atom). Also, each of the different types of Ub modifications are shown: monoUb (where a single lysine of the target is modified), multi-monoUb (where multiple lysines in the target are modified), and polyUb (where one of the eight amino groups in Ub serves as the point of attachment for a growing polymer chain). (B) The enzymatic cascade leading to Ub conjugation and removal: E1 activating, E2 conjugating, E3 ligase, and deubiquitinating (DUB) enzymes.
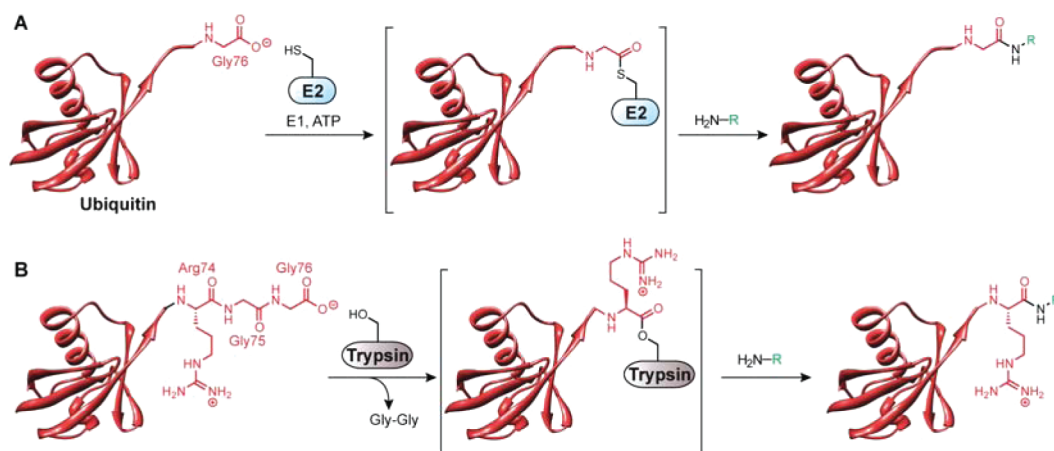

Figure 2. Enzymatic modification of the Ub C-terminus. (A) Use of Ub conjugation machinery to selectively modify the C-terminus of Ub Gly76. (B) Trypsin-mediated transpeptidation reactions as a means to modify Ub Arg74.

with a substrate and E2-S-Ub to mediate the delivery of Ub to an ε-amino group of a substrate lysine residue.[2,14]

Early biochemical studies of the Ub pathway indicated the ubiquityl moiety could be directly transferred from E2-S-Ub to amine-based nucleophiles in the absence of an E3.[15] This observation led to the supposition that the reactivity of E2s could be exploited to modify the C-terminus of Ub (Figure 2A). Using this approach, a series of Ub C-terminal adducts bearing either small residues (single amino acids or small amines) or large molecules (proteins such as cytochrome *c* and lysozyme) were synthesized. More recently, several studies have suggested that the molecular basis for the transfer of Ub from E2-S-Ub to amines relies on cooperativity between E2 and the donor Ub moiety in organizing an active site for nucleophilic activation of an amine.[16−19]

The utility of E2-mediated carboxy-terminal modifications is particularly evident when considering the history of DUBs, a class of enzymes whose function is to oppose that of E3 ligases.[20] By 1980 it was clear that the cellular pool of Ub conjugates was dynamic; however, the factors responsible for this activity had not been identified.[21] A likely culprit was an abundant protein in rabbit reticulocytes, now known as Ub C-terminal hydrolase (UCH-L3).[22] Initially, UCH-L3 had been shown to promote the hydrolysis of Ub carboxy-terminal thioesters but its actual function remained unclear.[23] To examine the reactivity and specificity of UCH-L3, a series of E2-derived Ub variants were employed to monitor the rate at which UCH-L3 catalyzed the release of free Ub. The results of these experiments revealed that Ub harboring small C-terminal adducts displayed release rates close to 3 orders of magnitude
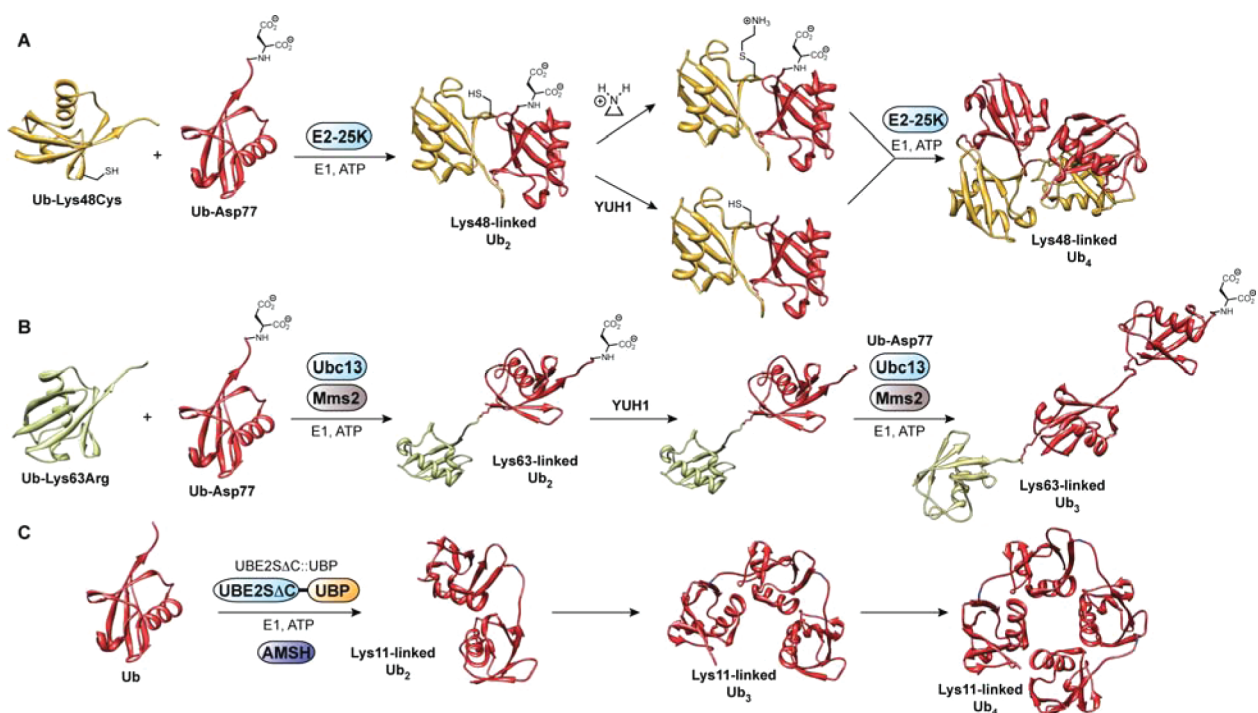
Figure 3. Controlled synthesis of Lys48-, Lys63-, and Lys11-linked polyUb chains. (A) Enzymatic synthesis of Lys48-linked polyUb chains using Lys48-linkage specific E2 conjugating enzyme E2-25K. In the final step leading to Lys48-linked Ub$_4$, two different Lys48-linked Ub dimers are required: one with a C-terminal aspartate cap and thiolysine residue at Lys48, and another with a free C-terminal Gly76 and Lys48 blocked with cysteine. The dimers are coupled through the thiolysine and free C-terminus. The proximal Ub (*i.e.*, the only monomer not tethered to another Ub molecule) has an Asp77 cap, while the distal Ub (*i.e.*, the last Ub in the tetramer) contains a Lys48Cys mutation. YUH1: yeast Ub hydrolase 1. PDB code: 2O6V.[47] (B) Enzymatic synthesis of Lys63-linked polyUb chains using heterodimeric Lys63-linkage specific E2 conjugating system Ubc13/Mms2. The proximal Ub has an Asp77 cap, while the distal Ub (shown in green) contains a Lys63Arg mutation. PDB code: 3HM3.[119] (C) Enzymatic synthesis of Lys11-linked polyUb chains using an engineered Lys11-linkage specific E2 conjugating enzyme UBE2SΔC::UBP. Using UBE2SΔC::UBP alone leads to a mixture of Lys11- and Lys63-linked chains, thus AMSH is added to cleave all Lys63-linkages and obtain a homogeneous product. Dimers, trimers, and tetramers are produced in the same reaction. AMSH: associated molecule with the SH3 domain of STAM. PDB code: 2XEW.[55]

higher than Ub-protein conjugates, indicating UCH-L3 has a strong preference for the former class of substrates.[15] Importantly, these investigations were the first to suggest UCHs play a role in the cotranslational processing of Ub gene products and in recycling Ub from either small molecular weight adducts or Ub-protein conjugates.[20] Furthermore, these experiments established a foundation for the identification and characterization of other DUBs, specifically in the UCH subfamily.[24]

Use of Limited Proteolysis. Protease-mediated transpeptidations are another method for modifying the C-terminus of Ub since the latter is relatively intransigent to proteolytic cleavage by trypsin.[25] In fact, the only site that is efficiently cleaved by trypsin is R74, which leads to the release of the C-terminal GG dipeptide (Figure 2B). Accordingly, proteolysis can be performed in the presence of excess amine-based nucleophile to effectively equip Ub with unique functional groups at the C-terminal position of R74.[26] This approach was pioneered with the Ub C-terminal ethyl ester[27] and subsequently exploited to synthesize an array of Ub-like reagents, including Ub-aldehyde,[28] Ub-nitrile,[29] the fluorogenic Ub-7-amido-4-methyl coumarin (UbAMC) probe,[30] Ub vinyl sulfone (UbVS) derivatives,[31] and nonhydrolyzable Ub-isopeptide isosteres.[32] All of these probes have been utilized to illuminate different aspects of DUBs (some of which have been reviewed in refs 33 and 34). For example, UbAMC has been instrumental in characterizing the biochemical activity of

newly discovered DUBs. That is, by examining a putative DUB against a panel of UblAMC derivatives, the specificity of that particular enzyme can be determined. Another area in which C-terminal probes have been valuable is in the identification of DUBs that are differentially expressed between normal, virus-infected, and tumor-derived human cells.[35]

■ ENZYMATIC SYNTHESIS OF POLYUBIQUITIN CHAINS

Over the past 10 years, a remarkable degree of complexity has been uncovered in the range of Ub signals governing distinct cellular processes.[36] All seven lysine residues of Ub and the amino (N) terminus are used to form chains *in vivo*.[37,38] Currently, the purpose of many of these chain types remains elusive, with the exception of canonical K48- and K63-linked homopolymeric chains. K48-linked chains mediate degradation of a conjugated target protein by shuttling the latter to the proteasome.[39] By contrast, K63-linked chains have non-proteolytic functions, *e.g.*, regulating the target's function during DNA repair and inflammatory signaling pathways.[40] Insight into the physiological roles of these two chain types comes largely from *in vitro* methods that allow synthesis of well-defined polyUb chains. Here, we discuss how these chains are constructed enzymatically and used to characterize their distinct functions. Moreover, we highlight how "atypical" polyUb chains, such as K11-linked homopolymers, can be synthesized in a similar manner using dedicated enzymes.
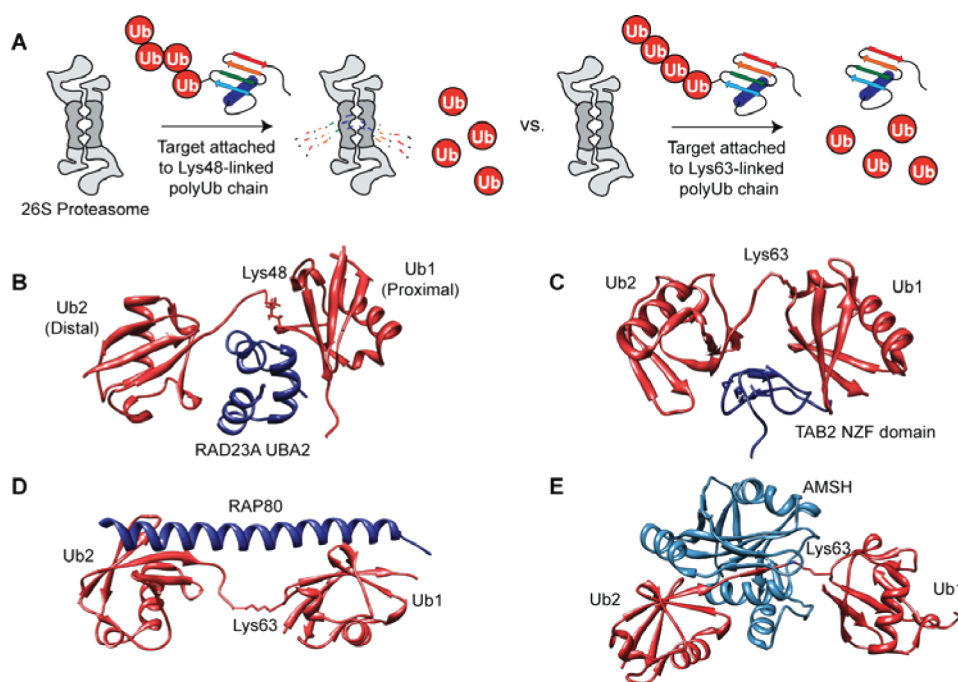
Figure 4. PolyUb signal processing and recognition. (A) Differences between protein degradation through the 26S proteasome when a target is tethered to a Lys48-linked polyUb chain *versus* a Lys63-linked chain. A target tethered to a Lys48-linked chain is translocated into the proteolytic core of the proteasome and rapidly degraded. By contrast, a target conjugated to a Lys63-linked chain binds to the proteasome, but the rate at which deubiquitination occurs exceeds the rate of translocation, ultimately leading to release of the intact target protein and formation of free Ub. (B) Structure of the Ub-associated 2 (UBA2) domain of RAD23A (dark blue) bound to a Lys48-linked Ub dimer (red). PDB code: 1ZO6.[66] UBA2 forms extensive contacts with the linker region and the residues surrounding the isopeptide bond, providing a structural basis for the Lys48-linkage specificity of UBA domains. (C) Structure of the NZF (Npl4 Zinc Finger) domain of TAB2 (dark blue), a subunit of the TAK1 kinase, bound to a Lys63-linked Ub dimer (red). PDB code: 2WWZ.[68] This structure reveals how the linker region is no longer involved in binding and how this particular UBD (Ub binding domain) interacts equally with both monomers. (D) Structure of receptor associated protein 80 (RAP80) tandem Ub interaction motif (tUIM) (dark blue) bound to Lys63-linked Ub dimer (red). PDB code: 3A1Q.[70] (E) Structure of the DUB AMSH (associated molecule with the SH3 domain of STAM) (light blue) bound to Lys63-linked Ub dimer (red). PDB code: 2ZNV.[74]

Building polyUb Chains. Due to the recalcitrant nature of isopeptide bonds, enzymatic construction remains one of the most effective methods currently available to synthesize well-defined polyUb chains. However, specific requirements must be met in order to obtain chains of specific linkages in quantities suitable for functional studies: (1) the enzyme must exhibit linkage specificity and (2) the enzyme must be active with free Ub. Currently, three E2 conjugating enzymes satisfy these criteria: K48-specific E2-25K (also known as UbcH1 or UBE2K),[41] K63-specific Ubc13/Mms2,[42,43] and K11-specific UBE2S.[44]

To control K48-linked chain synthesis using E2-25K, two Ub variants were required: one bearing a K48C mutation and another harboring a C-terminal aspartate cap (Ub-D77).[45,46] Conjugation of these two monomers is performed with E2-25K, E1, and ATP (Figure 3A). It is important to note that if the desired end product is a K48-linked diUb, Ub K48C is replaced with Ub K48R to prevent elongation at the distal end. However, if longer chains are required, Ub K48C serves as the distal monomer and additional steps must be conducted to extend the chain. Namely, the C-terminal D77 cap is displaced using a UCH enzyme, often yeast Ub hydrolase 1 (YUH1), and the C48 residue is site-specifically alkylated with ethyleneimine affording an S-aminoethylcysteine lysine surrogate. Subsequently, ligation of the deprotected dimers with E2-25K furnishes K48-linked Ub tetramers. Importantly, structural analysis shows that Ub dimers linked through S-amino-ethylcysteine resemble native dimers.[47] At this time, the molecular basis for K48 selectivity is unclear, but the C-terminal Ub-associated (UBA) domain of E2-25K, which binds free Ub, is thought to play a role.[48]

An analogous approach has been used to assemble K63-linked polyUb chains, with a few critical differences (Figure 3B). For instance, E2-25K was replaced with a heterodimeric complex consisting of Ubc13 and Mms2, an E2 enzyme and an E2 variant (UEV) lacking the active site cysteine normally present in an E2. Notably, structural details have suggested Mms2 plays a role in properly arranging K63 of the acceptor Ub in the active site of Ubc13.[49] The inefficiency with which Ubc13/Mms2 utilizes tagged Ub substrates, including Ub containing the β-thiolysine at position 63, necessitates a synthetic strategy using native K63 residues as the nucleophile in chain extension.[50] Thus, instead of the K48-linked chain-building strategy, K63-linked chains are generated by successive addition of Ub monomers.

To avoid complications in chain assembly caused by unnatural isopeptide linkages, a hybrid approach was recently developed using the genetically encoded lysine analogue Nε-(*tert*-butyloxycarbonyl)-L-lysine, also referred to as H-Lys-(Boc)-OH.[51,52] In this case, a pyrrolysyl (Pyl)-tRNA-synthetase (PylRS)/tRNA^{Pyl} pair from the methanogen *Methanosarcina barkeri* facilitates incorporation of non-proteinogenic pyrroly-sine analogue H-Lys(Boc)-OH into the genetic code of *E. coli*. Ub is then produced with a single Lys(Boc), site-specifically encoded by a TAG stop codon through a process referred to as amber suppression (for a review of this topic, see ref 53). Thus,

in conjunction with E2-25K or Ubc13/Mms2, controlled syntheses of well-defined polyUb chains with natural linkages can be achieved following chain extension of Boc-deprotected Ub dimers.

Once considered an atypical linkage, K11-linked polyUb chains have recently made a mark as an abundant form of polyUb *in vivo*, accounting for approximately 30% of all linkages in yeast and 2% in mammalian cells.[54] To understand the function of these chains, the E2 UBE2S, which is selective for K11, was engineered to synthesize free chains.[55] Critical to the success of this approach was the replacement of the lysine-rich C-terminal tail of UBE2S, a target for *in cis* autoubiquitination, with the Ub-binding zinc finger UBP domain of USP5/IsoT. This domain binds Ub with nanomolar affinity and, importantly, does not block the K11 side chain from serving as the nucleophile during chain extension. Using the UBE2S variant (UBE2SΔC::UBP) in combination with K63-specific DUB AMSH to prevent the formation of mixed linkage chains, K11-linked polymers of defined length can be assembled with high efficiency (Figure 3C).

In contrast to methods designed to produce chains of controlled length through multistep processes, linkage-specific E2 conjugating enzymes have also been used to produce multimilligram quantities of pure wild type polymers after a single elongation step.[56] Given the simplicity of this approach and the ability to readily incorporate fluorescent probes, this strategy may prove the most effective for studying the molecular details of these polymers.

Understanding the Function of polyUb Chains. Enzymatic access to K48- and K63-linked polyUb chains has proven vital to understanding how the 26S proteasome processes substrates bearing polyUb chains. Using a model substrate for the 26S proteasome (dihydrofolate reductase (DHFR) fused to structurally defined K48-linked polyUb chains), several key aspects of proteasomal targeting have been unveiled.[57] For instance, tetraUb was identified as the minimal signal for proteasomal targeting, with markedly increased degradation rates observed for substrates carrying longer polyUb chains. The molecular basis of this phenomenon is that by increasing chain length the affinity to the proteasome increases, which confers a greater commitment to degradation with substrates carrying long polyUb chains (>4 monomers). Subsequent to this report, K48- and K63-linked chains of the same length were shown to possess similar affinities to the proteasome despite significant differences in the structures of the two chains:[58] K48-linked chains are compact with significant contacts between monomeric units,[47,59] whereas K63-linked chains form more extended structures with minimal intersubunit contacts.[60,61] Why, then, are substrates tethered to K48-linked polyUb chains degraded while those fused to K63-linked chains are not? The answer to this question may rest in the kinetic partitioning between deubiquitination and unfolding of the proteasome-bound substrate.[62] In the case of K63-linked polyUb chains, the rate of deubiquitination by proteasome-associated DUBs is faster than the rate of unfolding, resulting in release of the intact substrate from the proteasome prior to degradation. By contrast, the residence time for a substrate bearing a K48-linked polyUb chain is sufficient for both unfolding and proteolytic cleavage (Figure 4A). As a result, substrates bearing K48-linked chains are preferentially degraded by the 26S proteasome despite both types of chains displaying the ability to interact with the proteasome.

Access to well-defined polyUb chains has also been instrumental in understanding how binding partners other than the proteasome recognize Ub signals. Current estimates predict the human genome encodes approximately 200 Ub binding domain (UBD)-containing proteins.[63] UBDs are effector molecules that transduce specific Ub signals to downstream cellular events. A particular family of UBDs that has received a significant amount of attention is the class of Ub-associated (UBA) domain-containing proteins, whose function is usually ascribed to proteosomal targeting and regulation. In a set of groundbreaking studies, UBAs were found to preferentially bind K48-linked chains over K63-linked chains, providing the first sign that UBDs exhibit linkage selective recognition.[64] However, when UBAs work in concert (*i.e.*, upon homodimerization or heterodimerization) their intrinsic linkage selectivity can be overcome. This results in the ability of other linkages, *e.g.*, K63, to effectively compete with K48-linkages for binding.[65] From a structural standpoint, monomeric UBAs favor K48-polyUb because the latter presents an ideal compact epitope to which a contiguous surface can bind.[66] That is, UBAs have a tendency to directly interact with the isopeptide linkage as well as distinct surfaces on each monomer (Figure 4B).

UBDs selective for K63- and M1-linkages also interact with multiple Ub moieties simultaneously, but the actual isopeptide linker plays less of a role in comparison to UBAs. For instance, the Npl4 zinc-finger (NZF) domains in TAB2 and TAB3, both of which activate the NF-κB pathway, selectively recognize K63-linked polyUb chains by serving as a molecular ruler that measures the length between the two hydrophobic patches in a Ub dimer and interacts with both monomers equally (Figure 4C).[67,68] This strategy is also observed for the NF-κB essential modulator (NEMO), which interacts with linear chains through the UBAN domain (<u>U</u>b <u>b</u>inding in <u>A</u>20-binding inhibitor of <u>N</u>F-κB proteins), and the K63 specific tandem UIMs (<u>U</u>b <u>i</u>nteraction <u>m</u>otifs) in receptor associated protein 80 (RAP80) (Figure 4D), a critical responder to DNA damage.[69−71] In each of these examples, the isopeptide linkage does not participate in the interaction and, instead, likely facilitates proper orientation of the two Ub surfaces.

DUBs are another class of UBDs that exhibit linkage selectivity. For instance, both USP14, the DUB associated with the 26S proteasome, and OTUB1 show specificity for K48-linked chains.[72,73] By contrast, several DUBs selectively cleave K63-linked chains, including CYLD, TRABID, OTUD5, AMSH, CSN5, BRCC36, POH1, and MYSM1.[20] Structural analyses suggest that linkage specificity is achieved by interactions that encompass a specific region of Ub surrounding the Lys residue forming the isopeptide bond. This is best illustrated by the structure obtained for a catalytically inactive variant of AMSH bound to K63-linked Ub dimer (Figure 4E). Here, AMSH binds the surface surrounding K63 of the proximal Ub (*i.e.*, the Ub with a free C-terminus), providing a molecular basis of K63-linkage specificity.[74]

Collectively, these studies have dramatically expanded our knowledge of how different Ub signals are recognized and processed. To further illuminate the dynamics of the Ub network, future work must focus on atypical polyUb chains of defined length. Although significant advances have been made along these lines, *e.g.*, in identifying the K11 linkage-specific DUB Cezanne and developing K11 linkage-specific antibodies used to dissect the role of these chains in mitosis,[55,75] further developments must rely on the arduous task of identifying
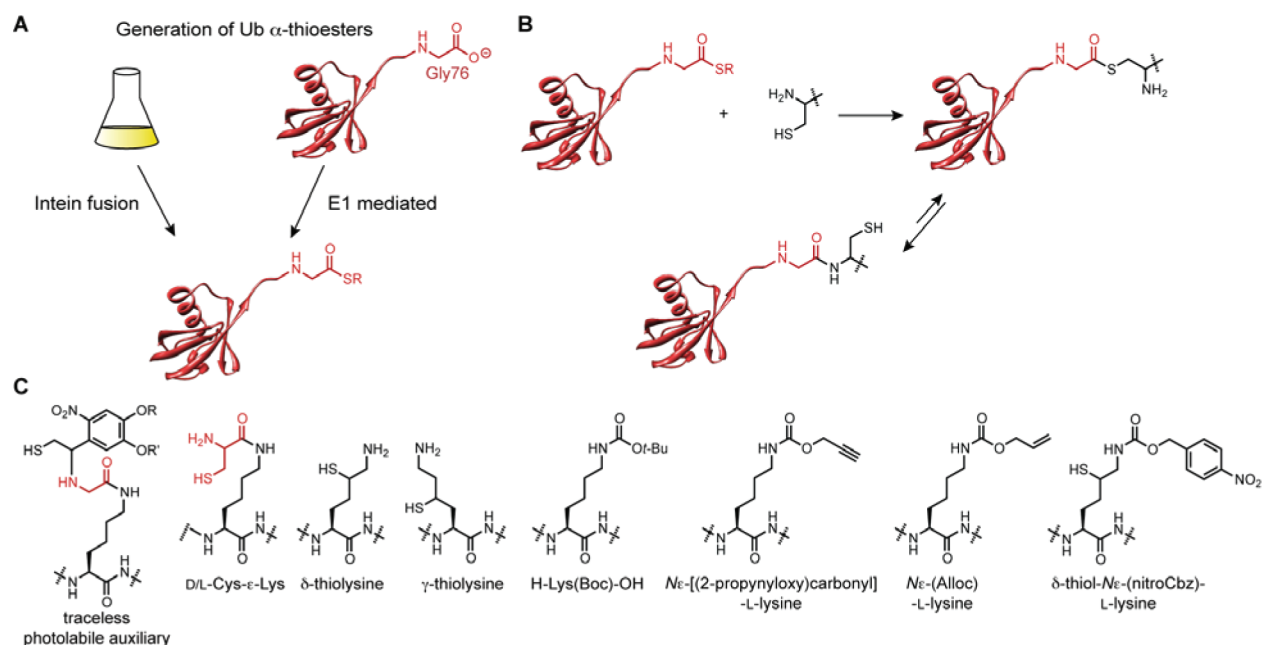
Figure 5. Components of EPL. (A) Two different methods are available for generating recombinant Ub α-thioesters. The first method is based on the production of Ub-intein fusion proteins as thiolysis of the fusion protein leads to the desired α-thioester. The second method exploits the reactivity of E1 Ub-activating enzymes. This reaction proceeds through an E1-S-Ub acyl enzyme intermediate that is captured by a small molecule bearing a free thiol to afford Ub(1−76) α-thioester. (B) The mechanism of EPL with 1,2-aminothiols. The reaction commences with a transthioesterification followed by a rapid S-to-N acyl shift to afford a native amide bond. (C) Structures of various non-proteinogenic amino acids used to construct isopeptide linkages with Ub α-thioesters. Both H-Lys(Boc)-OH and $N\varepsilon$-[(2-propynyloxy)carbonyl]-L-lysine have not been used to forge an isopeptide linkage with Ub α-thioester; rather, other Ub C-terminal variants are required.[102,104]

enzymes that synthesize chains of specific linkage. Thus, chemical approaches to synthesizing polyUb chains of defined linkage and length will likely have an immediate impact on uncovering salient features of atypical polyUb chains.

SYNTHESIS OF UB DERIVATIVES: CHEMICAL CONTROL OVER REGIOSPECIFICITY

Recent advances in bioconjugate chemistry have led to the discovery of numerous routes to regiospecifically conjugate Ub to target proteins. In this section, we highlight these breakthroughs while showcasing how each method has been employed to illuminate details associated with Ub biology (for other general reviews, see refs 76 and 77).

NATIVE CHEMICAL LIGATION (NCL)

Semisynthetic Approaches to Ub-Protein Conjugates. Expressed protein ligation (EPL) is the most common method to construct Ub-protein conjugates (Figure 5A). In general, EPL involves a chemoselective native chemical ligation (NCL) between a recombinant protein harboring a C-terminal thioester (α-thioester) and a synthetic peptide with a free thiol adjacent to a primary or secondary amine.[78] α-Thioesters are typically generated upon thiolysis of an intein fusion protein, while the peptide component bearing a non-proteinogenic amino acid specifically is obtained through solid-phase peptide synthesis (SPPS). With these building blocks, EPL commences with transthioesterification between the thiol of a modified amino acid and α-thioester, followed by a spontaneous intramolecular S-to-N acyl transfer resulting in a native amide bond (Figure 5B).[79]

As an alternative to intein-derived Ub α-thioesters, the intrinsic reactivity of the E1 Ub-activating enzyme can be exploited to generate similar products, namely Ub(1-76) α-

thioester (Figure 5A).[80] Recall that E1 activates Ub as an adenylate, which is then attacked by a cysteine residue to afford an E1-S-Ub thioester intermediate. Since E1 does not possess the basic residues necessary for deprotonating amine-based nucleophiles, the E1-S-Ub intermediate readily reacts with nucleophilic thiols to afford a Ub(1-76) α-thioester.[81] This method has proven extremely effective, as yields approaching 80% have been observed for the 2-mercaptoethanesulfonate (MESNa) thioester.[82] An additional benefit to using the E1 approach is the ability to label a single monomer in a polyUb chain with a chemical tag.

With regards to the peptide component of EPL, several strategies have been developed to promote ligations with Ub α-thioesters. A common approach is to use the canonical cysteine-mediate ligation,[80,83,84] but several recent studies have focused on the use of non-proteinogenic amino acids (Figure 5C). The distinction between these two is that the cysteine-mediated ligation affords a peptide linkage while non-proteinogenic amino acids promote isopeptide bond formation. Due to limitations in space our discussion is centered on the latter.

A $N\varepsilon$-(Gly)-L-lysine variant decorated with a photolabile auxiliary group was the first non-proteinogenic amino acid used to forge an isopeptide tether between Ub and the synthetic peptide.[85] After difficulties were encountered in scaling up this protocol, an alternative method was pursued involving the classic cysteine-mediated ligation strategy with $N\varepsilon$-(L-Cys)-L-Lys.[86] Unlike the strategy based on photolabile auxiliary groups, the cysteine-mediated approach, albeit more effective in terms of yield, is no longer traceless as subsequent desulfurization reactions are required to remove the residual thiols. Moreover, the use of $N\varepsilon$-(L-Cys)-L-Lys ultimately results in the replacement of Ub Gly76 with alanine, which could be

problematic if one is to use this method to study the activity of DUBs; Ala76-ε-Lys is more resistant to cleavage than the native Gly76-ε-Lys. To overcome some of these limitations, the Brik and Liu laboratories independently reported on the utility of thiolysines.[87−89] Both γ-thiolysine and δ-thiolysine benefit from the same principles that promote NCL with cysteine residues, although δ-thiolysine is thought to stimulate faster ligations relative to γ-thiolysine due to differences in rates associated with five-membered *versus* six-membered ring formation.

Functional Studies of Ub Using Semisynthesis. Chromatin biology was the first area targeted by Ub semisynthesis (for more detailed reviews of this topic, see refs 90 and 91). Ub plays a critical role in orchestrating gene transcription, replication, DNA repair and recombination by spatially and temporally regulating interactions at the interface of chromatin.[92] Covalent attachment of Ub to the core components of chromatin, histones H2A and H2B, is one of the most common modifications, often regulating additional modifications such as histone methylation. This level of interplay between ubiquitylation and methylation is referred to as "crosstalk". Until recently, the molecular details associated with crosstalk were unclear because of the difficulty associated with enzymatically generating well-defined histone modifications. To address this issue, Muir and co-workers elegantly employed protein semisynthesis to construct Ub-modified H2B (uH2B).[86,93] Semisynthetic uH2B was then incorporated into nucleosomes, the fundamental unit of chromatin, to obtain chemically defined probes for biochemical studies with the methyltransferases responsible for H3 K4 and K79 methylation (hSet1L and hDot1L). The results of these experiments revealed that (i) methylation was dependent on the presence of uH2B and (ii) a noncanonical surface of Ub (*i.e.*, a surface not composed of the hydrophobic patch consisting of L8, I44, and V70) associates with the methyltransferases. One of the most surprising findings from these studies, however, was that Ub does not function by enhancing binding of the methyltransferases to nucleosomes but rather by stimulating a catalytically competent conformation of the enzyme through allosteric regulation.

Thiolysine-based semisynthetic approaches have also proven effective in understanding how Ub alters the function of a protein. In particular, δ-thiolysine was used to investigate the ubiquitination of α-synuclein K6 (Ub-α-synuclein).[94] α-Synuclein is a presynaptic protein implicated in the etiology of several neurodegenerative diseases including Parkinson's disease (PD) and is modified in myriad ways, *e.g.*, through phosphorylation, glycosylation, truncation, and/or ubiquitination. Each of these modifications is associated with the pathogenesis of PD and commonly found in α-synuclein aggregates (also referred to as fibrils). Consequently, there is significant interest in understanding how fibrils are formed. In this context, recent biophysical studies using semisynthetic Ub-α-synuclein demonstrated that, contrary to some studies,[95] ubiquitination of K6 does not promote aggregation relative to the unmodified form. These results suggest Ub stabilizes the monomeric form of α-synuclein, which would be consistent with previous observations indicating the majority of ubiquitinated species are found in soluble form and not in fibrils.[96] Future work with defined mixtures of unmodified and modified α-synuclein (both monoUb and multi-monoUb) may shed more light on how Ub alters the morphology of fibrils.

Efforts to understand how K33-linked polyUb chains serve in a non-proteolytic capacity have also relied on the use of thiolysine-based semisynthesis.[97] In this example, ^{15}N-isotopic labeling of the distal Ub unit (obtained as a recombinant protein and activated by E1 as α-thioester) enabled spectroscopic interrogation of the conformation and ligand binding properties of K33-linked Ub dimer; δ-thiolysine was installed into the proximal Ub monomer through total synthesis. These studies revealed two critical differences between K33- and K48-linkages, the canonical signal for protein degradation. First, unlike K48-linked Ub dimers where both subunits form strong interdomain contacts through the hydrophobic patch of Ub (L8, I44, V70), the interface between Ub monomers in the K33-linked dimer is transient due to the engagement of only one hydrophobic patch. Second, K33-linked Ub dimer interactions with a proteasomal chaperone (the human homologue of protein Rad23, hHR23a) indicated that binding affinity is reduced by an order of magnitude relative to K48-linked Ub dimers. This result is consistent with studies on the ubiquitination of T-cell receptors,[98] which suggest K33-linkages do not promote protein degradation through the 26S proteasome.

Total Synthesis of Ub-Protein Conjugates. Total chemical synthesis of Ub derivatives has emerged as a powerful strategy to generate Ub-target conjugates as evidenced by the recent synthesis of the 304-residue K48-linked tetraUb.[99] Building on previous efforts in synthesizing all seven isopeptide-linked Ub dimers,[100] the Brik group reported a linear synthesis of tetraUb in which each monomer is incorporated consecutively. Key to success of their approach was the development and implementation of a chemical synthesis of Ub α-thioester using a C-terminal *N*-methylcysteine residue.[101] This allows for the incorporation of δ-thiolysine into the same Ub sequence without self-ligating, thus enabling chain elongation to proceed. Three distinct monomers, each constructed from two peptide fragments using NCL, were prepared, two of which contained both the thioester and orthogonally protected δ-thiolysine. After each round of ligation a desulfurization step led to the removal of the thiols and formation of native isopeptide bonds. Ultimately, K48-linked tetraUb was produced in a 5% overall yield starting from a single Ub monomer. As mentioned by the authors, further improvements could be made by exploiting recent developments in SPPS.[82] Upon implementing these approaches, this methodology may lend itself to the construction other types of homopolymers (*e.g.*, K6, K27, K29, and K33) as well as linear heteropolymers (monomers linked through different lysine residues), both of which are unattainable by enzymatic means in a well-defined manner.

Incorporation of Unnatural Amino Acids. Undoubtedly, the most streamlined method to synthesizing Ub-protein conjugates is the regiospecific ligation of recombinant Ub α-thioester to another recombinant protein. This strategy avoids the laborious synthetic steps required for semisynthesis and total synthesis and takes advantage of the ease with which recombinant proteins can often be obtained. To this end, the PylRS/tRNA[Pyl] pair from *M. barkeri* has been used to insert pyrrolysine analogues into specific sites of Ub and target proteins. Several different pyrrolysine analogues have successfully been incorporated into recombinant Ub, namely, H-Lys(Boc)-OH,[102] D/L-Cys-ε-Lys,[103] *N*ε-[(2-propynyloxy)-carbonyl]-L-lysine,[104] *N*ε-(Alloc)-L-lysine,[105] and a latent form of δ-thiolysine referred to as δ-thiol-*N*ε-(*p*-nitrocarbobenzyloxy)-L-lysine or δ-thiol-*N*ε-(nitroCbz)-L-lysine[106] (Figure 5C). Of these, δ-thiol-*N*ε-(nitroCbz)-L-lysine may prove the most
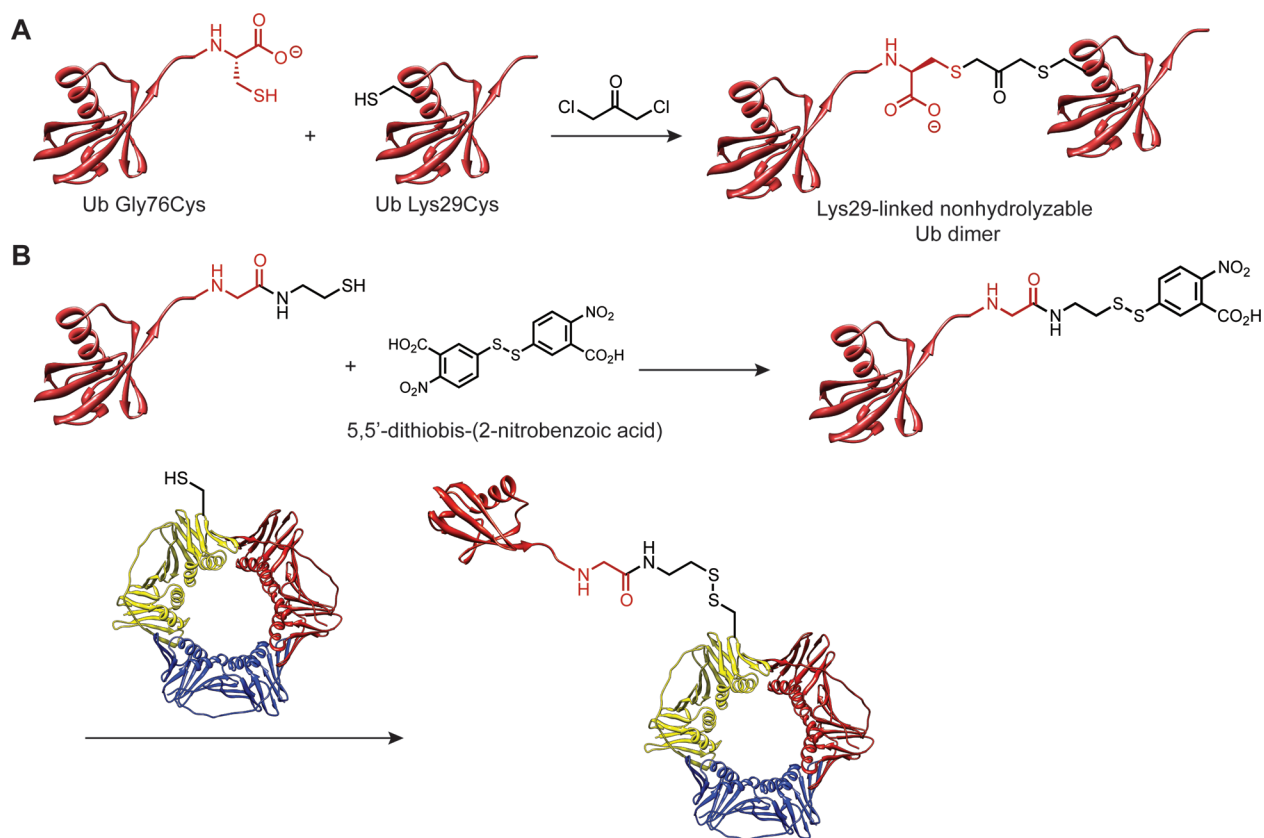
Figure 6. Synthesis of Ub-protein conjugates using methods other than NCL. (A) Synthesis of nonhydrolyzable Ub dimers by constructing a thioether bridge between monomers. (B) Conjugation of Ub to target protein using directed disulfide exchange. The target protein shown here is proliferating cell nuclear antigen (PCNA).[111] PCNA PDB code: 1VYM.[120]

versatile as it allows for a traceless synthesis of Ub dimers as well as Ub-Ubl heterodimers with user-defined control over the linkage site. Another interesting feature of δ-thiol-Nε-(nitroCbz)-L-lysine is that the nitroCbz is removed *in situ*, enabling direct isolation of Ub bearing δ-thiolysine.

Functional Studies Based on Unnatural Amino Acid Incorporation. Methods utilizing PylRS/tRNA[Pyl] pairs to obtain native Ub dimers have exposed important details related to atypical linkages.[102] For instance, structural analysis of K6-linked diUb showed a compact conformation distinct from previously characterized K11-, K48-, and K63-linked diUb derivatives. This observation suggests K6-linked polyUb chains may bind novel UBDs and mediate distinct biological processes, which would be congruent with the prevalence of this linkage in DNA repair pathways.[107] Having dimers linked through atypical lysine residues also provides an opportunity to profile the selectivity and specificity of DUBs. For example, biochemical studies with 10% of the known human DUBs led to the identification of TRABID, an ovarian tumor (OTU)-domain containing DUB, which cleaves K29-linked diUb 40-fold more efficiently than K63-linked diUb. Importantly, these synthetic strategies provide a means of investigating the biological effects of distinct Ub and Ubl chain topologies *in vitro*, as well as developing other biochemical tools for *in vivo* studies, *e.g.*, linkage-specific antibodies.

■ ALTERNATIVES TO NCL

Thioether Ligations. As highlighted above, the objective behind synthesizing all possible isopeptide Ub linkages is to enable studies designed to identify and characterize linkage-selective binding partners. The source of these binding partners is often an extract from a lysed population of cells. However, cellular extracts present a difficult environment in which to study polyUb linkage-selective interactions due to the vast number of DUBs that reduce the half-life of the specific polyUb chain under investigation. To overcome this problem, Wilkinson and co-workers pioneered the development of nonhydrolyzable linkages between Ub molecules based on thioether bonds.[108] Their strategy was to fashion a thioether tether between recombinant Ub monomers by exploiting the absence of native cysteines. Two different Ub monomers are required for this site-specific ligation: one where G76 is mutated to cysteine and another where a specified lysine is mutated to cysteine (Figure 6A). Subsequent fusion of these two monomers through a thioether bridge results in a linkage that differs slightly from that of the native isopeptide bond in length (one carbon longer) and polarity (there is an acetone spacer along with a free carboxylate). Nevertheless, the ability of these dimers to serve as inhibitors of DUBs and Ub conjugation machinery is testament of their ability to target enzymes that process polyUb chains. Further extension of this methodology toward the synthesis of nonhydrolzable tetraUb chains immobilized on resin led to the development of novel tools for identifying binding partners in cell lysates.[109] Indeed, a key finding from these experiments was the identification of Ufd3 (an important player in sorting membrane proteins into multivesicular bodies) as a novel interacting partner of what is ostensibly referred to as a nonhydrolyzable variant of K29-linked tetraUb.

Directed Disulfide Bond Formation. Isopeptide-like linkages between recombinant proteins can also be constructed through a selective exchange of disulfide bonds.[110,111] In this approach, the addition of cysteamine to a Ub-intein fusion affords Ub with a C-terminal aminoethanethiol linker. Combining this Ub derivative with 5,5′-dithiobis(2-nitrobenzoic acid) and a target containing a single cysteine residue results in the formation of a site-specific disulfide tether between the two proteins (Figure 6B). It is important to note that despite the disulfide linkage being ~2.4 Å longer than the native isopeptide bond, the function of the Ub-protein conjugate is not altered. The advantages of this approach relative to other methods are that (i) chemical synthesis is no longer required and (ii) the conjugation site can be genetically encoded with standard techniques of recombinant DNA similar to the thioether strategy.

Functional Studies with Directed Disulfide Bond Formation. The utility of the disulfide-directed methodology is highlighted by the ability to rapidly construct uH2Bss variants and glean information on the impact Ub has on nucleosome structure and function.[110,112] For instance, by applying a positional scanning approach, whereby the Ub attachment is varied on the nucleosome surface, two key observations were made. First, the Ub attachment point is not important for stimulating hDot1L activity since methylation of H3 K79 occurred when Ub was attached to other positions on H2B as well as on H2A. Second, mononucleosomes harboring regioisomers of uH2Bss exhibited different structural characteristics, thus presaging a role for Ub in regulating chromatin structure. Indeed, experiments with nucleosome arrays led to the proposal that uH2B prevents folding of the entire chromatin fiber, thereby promoting the formation of a structure more conducive to active transcription.

Another example where disulfide bond formation has illuminated biochemical details is with proliferating cell nuclear antigen (PCNA).[111] PCNA is a homotrimeric protein involved in processive DNA synthesis. When PCNA is ubiquitinated following DNA damage, the DNA polymerase Polη is recruited to the site of damage to promote translesion DNA synthesis (TLS). To investigate how PCNA-Ub specifically stimulates TLS, a chemical approach using the disulfide-directed methodology was employed. In this case, several PCNA mutants (K107C, K127C, and K164C) were coupled to Ub and another Ubl, SUMO (<u>s</u>mall <u>u</u>biquitin-like <u>mo</u>difier), both harboring a C-terminal aminoethanethiol linker to afford PCNA-Ubl conjugates at different positions. Using these probes, it was revealed that SUMO has little effect on the degree to which Polη suppresses Polδ-PCNA-catalyzed DNA synthesis. By contrast, ubiquitination of PCNA rapidly promotes the exchange between Polδ and Polη. An additional finding indicated that the position of Ub attachment on PCNA does not influence the inhibition of DNA synthesis through Polη-PCNA-Ub complex formation, suggesting a great deal of conformational flexibility in the latter ternary complex. These results, along with the uH2B studies described above, illustrate the power of the disulfide-directed approach in establishing structure—activity relationships.

SUMMARY AND OUTLOOK

Although Ub-based probes have played a central role in elucidating the basic principles of Ub signaling, there remains much to be discovered with regards to the function of diverse Ub signals in specific biological processes. In this Review, we have discussed methods, both enzymatic and non-enzymatic, that lead to the generation of specific Ub modifications. The resulting Ub-based probes have been used to (i) identify and characterize DUB activity, (ii) understand the influence of Ub on protein function and crosstalk with other PTMs, and (iii) help comprehend the impact of polyUb chain linkage and length on downstream signaling events. Despite these achievements, we are currently left with a vast number of uncharacterized polyUb signals, DUBs and UBDs, many of which play important roles in human disease.[36]

Recent developments in synthesizing specific isopeptide bonds between Ub molecules and other Ubls offer tremendous potential in unraveling many aspects of Ub signaling. The DNA damage response (DDR) is one pathway in particular where access to a diverse array of tools could help dissect the role of different polyUb chains (reviewed in ref 113). For example, upon induction of DNA double-strand breaks, chromatin is modified with K63-linked polyUb chains, which recruit additional E3 ligases including BRCA1. Since BRCA1 has been shown to assemble atypical K6-linked polyUb chains,[107] the question is: What role(s) might these chains serve during DDR? Is it possible they act as an additional platform on which other proteins are mobilized to the response, as a switch to facilitate the exchange between repair mechanisms, or both? Another area of interest is in understanding how different linkage types promote degradation through the 26S proteasome. Quantitative proteomics experiments with yeast suggest the kinetics of chain disassembly *via* the proteasome correlate with the extent to which different chains accumulate during proteasomal inhibition.[37] While this is consistent with K48 linkages functioning as the major degradative signal due to their overall abundance, the identification of linkage-selective chaperones responsible for delivering substrates to the proteasome implies there are additional factors that could regulate the rates of protein degradation.[114,115] Finally, the emergence of mixed Ub-Ubl species, particularly Ub-SUMO and Ub-NEDD8, as distinct cellular signals presents an opportunity for non-enzymatic approaches to the construction of these molecules to play an important role in understanding their function.[116,117]

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