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Chemical strategies to understand the language of ubiquitin signaling

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Abstract

Ubiquitin (Ub) is a small 76 amino acid long protein that is highly conserved in all eukaryotes studied to date. In humans, more than 600 ligases are involved in the reversible modification of specific lysine side-chain ϵ -amines in substrate proteins by conjugation with the C-terminal carboxylate of Ub. Initially monoubiquitylated proteins can undergo repetitive ubiquitylation starting from one of seven lysine residues or the α -amine in the first Ub to generate a variety of polyUb chains with different topologies and functions. The most well known role for protein ubiquitylation is in targeting substrates for proteolytic destruction by 26S proteasomes. However, a growing body of evidence indicates that both mono- and polyubiquitylation play proteasome-independent roles in modulating the structure, function, and localization of protein substrates. Understanding the complexity of Ub-mediated functions in our cells is a major challenge for modern biology. In addition to well-established *in vivo* genetic methods, biochemical and biophysical investigations of ubiquitylated proteins *in vitro* can shed light on the direct mechanistic roles for Ub in different contexts. Such studies have traditionally been limited by the ability to obtain sufficient quantities of homogeneously ubiquitylated proteins with precisely defined linkages. This review focuses on recent advances in both synthetic and recombinant protein-based methods that have yielded access to homogeneously site-specifically ubiquitylated proteins. Mechanistic studies of the roles for protein ubiquitylation and of the enzymes involved in protein deubiquitylation that are enabled by these chemical tools are highlighted.

Introduction

The reversible post-translational modification (PTM) of proteins adds diversity to the limited set of functional groups encoded by their genes, and thereby allows these proteins to participate in multiple temporally regulated processes.¹ While all twenty common amino acids may undergo PTM, the nucleophilic side-chain ϵ -amine of the amino acid Lys is noteworthy due to its modification by small chemical groups such as methyl and acetyl groups, as well as by entire proteins such as ubiquitin (Ub) and the family of Ub-like proteins (Ubls). Ub is a small 76 amino acid long protein that is highly conserved in plants and eukaryotes but not found in prokaryotes.² Early evidence for the multiple important roles for protein modification by Ub (termed ubiquitylation or ubiquitination) in mammals came from its discovery in two very different contexts. Goldknopf et al. first identified the nucleosomal protein A24 to be a ubiquitylated form of the histone protein H2A.^{3–5} From the

reduced amounts of A24 found in transcriptionally active nuclei of regenerating or thioacetamide-treated rat liver cells, H2A ubiquitylation was associated with the modulation of gene activity.³ In another series of elegant experiments, Hershko and coworkers identified and characterized Ub as the ATP-dependent proteolysis factor 1 that was covalently conjugated to protein Lys side-chains and necessary for their degradation in rabbit reticulocytes.^{6–8} In comparison with PTMs such as acetylation, and methylation, modification by Ub presents a larger and more chemically varied surface, and is therefore well-suited to diverse functions such as modulating the conformation, function, localization, and intracellular half-lives of its targets.

The majority of our knowledge regarding the roles for protein ubiquitylation comes from genetic experiments *in vivo* that involve the mutagenesis of specific Lys sites of ubiquitylation in substrates/Ub and the knockdown/deletion of specific enzymes that are thought to be involved in the ubiquitylation cascade.⁹ An additional and powerful approach that yields insight on the direct mechanistic roles for Ub involves subjecting ubiquitylated proteins to biophysical and biochemical investigations *in vitro* under chemically defined conditions. However, until recently, such a reductionist approach was largely limited by the inability to access homogeneously ubiquitylated substrates in sufficient quantities from either natural sources or by ubiquitylation with purified Ub ligases. The recent explosion of chemical methodologies to obtain ubiquitylated proteins holds much promise in overcoming these challenges and deepening our understanding of the mechanistic consequences of protein ubiquitylation.^{10,11} This review will briefly discuss synthetic and semisynthetic approaches to obtain ubiquitylated proteins and highlight the structural and biochemical insights gained from these efforts.

The protein ubiquitylation cascade

Protein ubiquitylation involves a series of chemical reactions mediated by the family of E1–E3 Ub ligases.¹² There are two distinct E1 ligases in the human proteome that use ATP to activate Ub at its C-terminus, first as a Ub-adenylate then, following nucleophilic attack by an active site Cys thiol, as a C-terminal thioester that remains attached to the enzyme. Following this, a family of E2 (~40 in humans) and E3 ligases (over 600 in humans) catalyze the condensation of the Ub thioester with lysine side-chain ϵ -amines in substrates to link the two by an isopeptide bond.¹³ Ubiquitin itself is a target for ubiquitylation and monoubiquitylated proteins can undergo further rounds of ubiquitylation starting from one of seven lysine residues in the first Ub to generate polyUb chains with different topologies. For example, the NF- κ B pathway uses at least two types of Ub linkages, Lys63 and N-terminally linked polyUb, to activate gene expression.¹⁴ A family of over 100 deubiquitylating enzymes (DUBs) use either an active site cysteine or water activated by bound Zn^{2+} to hydrolyze the isopeptide linkage between Ub and its substrates, thus conferring reversibility to ubiquitylation and removing Ub in response to cellular cues.¹⁵ Although the length of polyUb chains found attached to substrates *in vivo* shows significant variations, effective degradation by the 26S proteasome typically requires a chain of at least 4 Ub molecules to be present on substrates (Figure 1).^{16,17} On the other hand, monoubiquitylation is primarily involved in numerous non-proteolytic processes such as receptor endocytosis, gene regulation by histone modifications, DNA double-strand break

repair, and the modulation of protein aggregation associated with neurodegenerative disorders.^{18,19} In rare instances, monoubiquitylation may also target a protein for proteolysis.²⁰ The absence of a known consensus sequence for ubiquitylation and the relatively few Ub ligases for which substrates have been identified largely limit the ability to generate site-specifically mono- and polyubiquitylated proteins in enzymatic assays.

Chemical synthesis of ubiquitylated peptides and polyUbs

Access to fully synthetic Ub and ubiquitylated substrates greatly simplifies the incorporation of a variety of spectroscopic probes to study Ub folding, stability and function. With this goal in mind, Ramage and co-workers reported the first successful total synthesis of Ub, in ~4% overall yield, by utilizing a fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS) approach.²¹ Somewhat surprisingly, the authors observed a single additional tryptic fragment from the C-terminus of synthetic Ub than from recombinant Ub, which suggested possible differences in the tertiary structures of these two forms. However, later studies demonstrated the identical recognition of both synthetic and recombinant Ub by anti-Ub antibodies,²² indistinguishable X-ray crystal structures,²³ and the ability of Ub ligases to accept synthetic Ub as a substrate.²⁴ These pioneering studies established total synthesis as a means to obtain biologically functional Ub. The ability to easily append residues to the C-terminus of fully synthetic Ub was employed to profile differences in the abilities of various DUBs to cleave ubiquitin from linear peptide fusions and from the ϵ -amine of Lys.²⁵ This revealed a degree of substrate promiscuity in the mixture of DUBs associated with 26S proteasomes, which is undoubtedly essential for the proteasome's ability to degrade a variety of ubiquitylated targets. More recently, Ovaa and coworkers reported significantly improved yields (~14% after refolding) in the linear synthesis of Ub by the incorporation of structure-disrupting pseudoproline and dimethoxybenzyl dipeptide building blocks.²⁶ The strategic placement of these molecules in the growing peptide chain prevented its aggregation and premature folding and facilitated otherwise difficult coupling steps. The synthesis was readily adaptable for the conjugation of fluorophores at both the N- and C-terminus of Ub, and the latter was shown to be a substrate for the DUBs, HAUSP/USP7 and UCH-L3.

Shortly after the first reports of the total synthesis of Ub, Kent and co-workers reported a major advance in protein chemistry, namely the technique known as Native Chemical Ligation (NCL).²⁷ NCL overcomes the size limitations of linear peptide synthesis by ligating two unprotected peptide fragments by a native amide bond. The minimal requirements for successful NCL are the presence of a C-terminal α -thioester in one peptide fragment and an N-terminal Cys in the other. Following an initial trans-thioesterification reaction that links the two fragments, an amide bond is formed by an S-to-N acyl transfer step proceeding through a favorable 5-membered ring intermediate (Figure 2). Although Ub does not have any Cys residues, the facile conversion of Cys to Ala by free-radical²⁸ or Raney-Nickel-mediated desulfurization permits temporary Ala-to-Cys substitutions in Ub for NCL.²⁹ This permitted Brik and co-workers to apply NCL to the synthesis of Ub directly from the ϵ -amine of internal lysines in short resin-bound peptides by employing an orthogonal 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) protecting group to temporarily protect the Lys side-chain. Following the initial assembly of the short

main chain, the Lys was deprotected and residues 46–76 of Ub were sequentially coupled to the ϵ -amine with an Ala46Cys mutation. The Ub(1-45) fragment was synthesized as a C-terminal thioester and ligated to the isopeptide-linked Ub(46-76) peptide (Figure 3A). By incorporating a Cys protected as a thiazolidine residue (Thz) at the N-terminus of ubiquitylated peptides, the authors were able to unmask the Cys and perform a second NCL to yield full-length substrates, such as the monoubiquitylated histone H2B, uH2B. In order to overcome the synthetic limitations inherent in relying on naturally occurring Ala residues as sites for NCL in ubiquitin, Brik and co-workers developed a 5-mercaptolysine derivative (also referred to as δ -mercaptolysine) that could be inserted at any Lys position in synthetic Ub in order to conjugate a full-length ubiquitin- α -thioester with the ϵ -amine (Figure 3B).³⁰ The ability to desulfurize 5-mercaptolysine under similar conditions as for Cys rendered the ligation traceless and greatly increased its appeal for structural and biochemical studies of ubiquitylated proteins. The versatility of 5-mercaptolysine was demonstrated in the tour de force synthesis of seven isomeric diUbs generated by the site-specific attachment of one Ub at one of seven lysines at positions 6, 11, 27, 29, 33, 48 and 63 in a second Ub.³¹

Different topologies that arise from different sites of isopeptide linkage within polyUbs add to the vast complexity of ubiquitin signaling in cells. For example, Lys48-linked polyUb primarily serves as a signal for proteasomal degradation,³² while Lys63-linked polyUb chains are involved in NF κ B activation,^{33,34} post-replication DNA repair,^{35,36} and endosomal transportation.³⁷ Atypical polyUb chains, such as those linked at Lys11 can also function as degradation signals.³⁸ Lys6-linked polyUb is associated with the DNA damage response by the breast cancer associated protein BRCA1, although its precise role in this process awaits elucidation.³⁹ Since the E2/E3 ligases for generating all 7 isomeric Ub-linkages are not known, this precludes the generation of isomeric polyUbs by enzymatic means for biochemical studies of their function and for the identification of DUBs involved in their disassembly. Therefore, synthetic access to the diUbs permitted an investigation of the Ub-linkage tolerance of the human isopeptidase T (IsoT) and UCH-L3 enzymes. While UCH-L3 failed to hydrolyze any of the diUbs, which is consistent with its proposed role as a Ub-scavenging hydrolase that removes Ub primarily from small molecule adducts,⁴⁰ IsoT showed remarkable promiscuity and failed to hydrolyze only the Lys27 linked diUb at an enzyme-to-protein ratio of 1:100. However, at the lower enzyme-to-substrate ratio of 1:1000, IsoT showed a clear preference for Lys48 linked diUb, which is consistent with its role in hydrolyzing the isopeptide linkage and releasing free Ub from unanchored Lys48-linked ubiquitin chains *in vivo*. In another study from the same group, it was observed that UCH-L3 could indeed deubiquitylate a short peptide (FKT) ubiquitylated at the ϵ -amine by Lys48- and Lys63-linked polyUb chains of differing length. However, the efficiency of deubiquitylation by UCH-L3 decreased with increasing chain-length and upon changing the Ub site of linkage from Lys48 to Lys63.⁴¹ Thus, the total synthesis of polyUb chains with different sites of linkage holds great promise as probes to identify DUBs specific for each linkage-type and for the characterization of different topologies by NMR/X-ray crystallography. However, the demanding multi-step synthesis of the 5-mercaptolysine derivative, the multiple protecting group manipulations required to ensure the correct sequence of peptide fragment ligations, and the requirement for global desulfurization at the

end of the multi-step polyUb assembly challenge the broad application of entirely synthetic approaches for studies of ubiquitin-mediated signaling.

Semisynthesis of ubiquitylated proteins

The application of molecular biological methods to generate the Ub- α -thioester used in NCL can greatly reduce the number of synthetic steps required to access ubiquitylated proteins and hence expand the scope of studies of protein ubiquitylation. Muir et al. first demonstrated the utility of intein-mediated chemistry to generate protein- α -thioesters for NCL in a process they termed expressed protein ligation, or EPL.⁴² Inteins are proteins involved in a process called protein splicing, wherein a protein undergoes an intramolecular rearrangement that results in the extrusion of an internal protein sequence (intein) and the joining of its N- and C-terminal flanking sequences (exteins) by a native amide bond.⁴³ A mutant intein that is defective in splicing but capable of forming the intermediate thioester at its N-terminus may be linearly fused to the C-terminus of ubiquitin. Following intein-mediated rearrangement, the thioester-linked ubiquitin may be intercepted with thiol-bearing small molecules, such as 2-mercaptoethanesulfonic acid, in order to release the Ub- α -thioester in solution (Figure 4).

Ploegh and co-workers first reported the use of a recombinant Ub(1-76)- α -thioester to generate mechanism-based probes of DUB activity.⁴⁴ These probes were used in cell lysates to profile the reactivity of different DUBs and led to the identification of several new DUBs in mouse thymoma-derived cells. Inspired by these early studies, Muir and coworkers devised a synthetic strategy that used a Ub(1-75)- α -thioester to produce ubiquitylated peptides.⁴⁵ Key to their synthetic strategy was the use of a photolytically removable ligation auxiliary⁴⁶ attached to the specific Lys target side-chain in the form of *N*-substituted Gly76 in Ub (Figure 5A). This permitted the site-specific ligation of a Ub(1-75)- α -thioester, which terminates in Gly75, to Gly76 attached to the target. The sterically undemanding ligation between two glycines led to reasonable yields of the ubiquitylated peptide product despite the relatively slow kinetics of acyl transfer onto the sterically hindered secondary amine of Gly76. Following ligation, the auxiliary was cleanly removed by photolysis at 365 nm to yield a native secondary amide bond between Gly75-Gly76. By employing a Thz residue at the N-terminus of the ubiquitylated peptide, which presented an N-terminal Cys for a second EPL after treatment with methoxylamine, McGinty et al. extended the use of the ligation auxiliary to achieve full-length uH2B (Figure 5A).⁴⁷ This landmark synthesis permitted the first biochemical studies of the relationship between uH2B and dimethylation at Lys79 (H3 K79me₂) in histone H3 and established a nucleosome-dependent role for ubiquitin in stimulating the methyltransferase, hDot1L. An interesting observation from these studies was that although levels of H3 K79me₂ far exceed the levels of uH2B *in vivo*, the latter is strictly required for efficient methylation of H3 by hDot1L, and the stimulatory effect of Ub is restricted to methylation of the nucleosome to which it is directly attached. This suggests that histone ubiquitylation may be more prevalent but also more dynamic at different stages of the cell cycle than is evident from snapshots at specific time-points. Another possibility that remains to be established is that factors independent of uH2B may contribute to the degree of H3 K79me₂ in cells.⁴⁸

Disulfide-directed protein ubiquitylation

Although the Muir,^{45,47} Brik^{30,49} and Liu^{50,51} research groups have illustrated the high utility of ligation auxiliaries for NCL and EPL approaches to obtain ubiquitylated proteins, these approaches are somewhat limited by the challenging multi-step synthesis of the auxiliaries themselves. With this in mind, Muir and co-workers reported a simplified strategy to rapidly access monoubiquitylated histones that may, in principle, be applied to access most ubiquitylated proteins. Taking advantage of the absence of Cys in both H2B and Ub, and the fact that the only Cys in mononucleosomes present in H3 can be mutated to Ala without significant changes in nucleosome function, the authors reported a disulfide-directed strategy for histone ubiquitylation.⁵² In this study, an expressed Ub-intein fusion protein was reacted with cysteamine, which resulted in Ub with a C-terminal aminoethanethiol group (Ub-SH, Figure 5B). A single thiol was introduced at the desired site of ubiquitylation in H2B with the Lys120Cys mutation, and activated by the formation of a reactive asymmetric disulfide with 2-thio(5-nitropyridine).⁵³ Reaction between Ub-SH and the activated H2B K120C mutant under denaturing conditions readily generated a disulfide-linked analogue of uH2B (uH2B_{ss}). Surprisingly, after its incorporation in mononucleosomes, uH2B_{ss} stimulated methyltransferase activity of the enzyme hDot1L to a similar level as wild-type uH2B. The good yields, easy scalability, and functional equivalency of the disulfide-linkage allowed the authors to interrogate the efficiency of hDot1L-mediated methylation in response to the precise position of Ub on the nucleosome. This revealed a significant degree of plasticity in hDot1L function *in vitro* and suggested that multiple sites of ubiquitylation in H2B may direct methylation at H3 K79. In support of this hypothesis, Dou and co-workers recently demonstrated the *in vitro* methylation of H3 K79 by hDot1L in response to ubiquitylation at H2B K34.⁵⁴ However, not all reported sites of ubiquitylation on the nucleosome may stimulate hDot1L activity.⁵⁵ The disulfide-directed approach was also used by Fierz et al. to probe the direct effect of uH2B on chromatin structure in a series of biophysical experiments with chromatin-like 12-mer arrays of ubiquitylated nucleosomes.⁵⁶ Consistent with the association of uH2B with transcriptionally active regions of chromatin,⁵⁷ the authors demonstrated that ubiquitylation is inhibitory for both the intra- and inter-array compaction events that are implicated in transcriptional silencing by heterochromatin formation. Although uH2B and H4 K16 acetylation were shown to have similar inhibitory effects toward the intra-array compaction of nucleosomal arrays, the effects were not additive and the effect of acetylation dominated that of ubiquitylation. However, in assays of inter-array association between strands the two modifications were shown to have an additive effect and to greatly inhibit higher order structure formation when present together.

In an independent study, Zhuang and co-workers also developed a disulfide-directed monoubiquitylation strategy that made use of a reactive asymmetric disulfide of Ub-SH with 5-thio(2-nitrobenzoic acid).⁵⁸ This was applied to study the mechanistic role for ubiquitylation of the homotrimeric proliferating cell nuclear antigen (PCNA) in translesion DNA synthesis. Unlike histones, each PCNA monomer has four Cys residues that were mutated to Ser and an additional Cys was introduced at the desired Lys164 site of ubiquitylation. The ubiquitylated form of PCNA was found to be stable in the presence of 0.5 mM of the reducing agents dithiothreitol or glutathione, and also in yeast lysates for up

to 3 h. This suggests that for some proteins the disulfide-linked ubiquitylated analogue may be suitable for biochemical experiments in complex protein mixtures such as cellular lysates. Although Lys164 is the primary site of ubiquitylation *in vivo*,⁵⁹ it was observed that ubiquitylation at several other positions in PCNA (Lys27, Lys 107 and Arg44) had similar effects on the exchange of the replicative DNA Pold for the translesion synthesis polymerase Pol η . It is possible that the flexible and unstructured C-terminus of Ub allows sufficient repositioning in order to recruit proteins/enzymes to identical regions in its protein substrates even when Ub is attached to different lysines. However, the precise mechanisms underpinning the plasticity observed in these systems awaits further investigation.

Pratt and co-workers recently demonstrated the excellent scope of disulfide-directed ubiquitylation for *in vitro* studies of the aggregation of α -synuclein (α -syn).¹⁹ This 140 amino acid protein is the principal component of intracellular protein aggregates called Lewy bodies and Lewy neurites that are hallmarks of Parkinson's disease.^{60–62} α -Syn is known to undergo ubiquitylation at nine distinct Lys sites, however the roles for ubiquitylation at these sites are not well understood.^{63,64,65} In order to directly ascertain the role of Ub, the authors generated multi-milligram quantities of all 9 ubiquitylated variants and performed Thioflavin T fluorescence assays and transmission electron microscopy imaging to measure the degree of fibril formation by ubiquitylated α -syn. Interestingly, it was observed that the effect of Ub on fibril formation varied with its location on α -syn. Thus, ubiquitylation at Lys10 and Lys23 led to changes in the kinetics of fibril formation from wild-type α -syn but eventually formed similar amounts of fibrils. On the other hand, ubiquitylation at Lys6, Lys12, and Lys21 led to reduced aggregation. Indeed, a similar inhibitory effect on aggregation by ubiquitylation at Lys6 was also reported by Laushel and co-workers.⁶⁶ Finally, the attachment of Ub to Lys32, Lys34, Lys43, and Lys96 showed significantly less fibril formation than unmodified α -syn. Thus the precise effect of Ub on fibril formation is strongly dependent on the Lys site of modification. The stretch of residues from Lys32 to Lys96 in α -syn occurs in the region that comprises the fiber core and the role of Ub at these sites may be to act as steric bulk that prevents inter-strand contact. However, additional studies are needed to confirm this and other possible roles of Ub, particularly when it is attached near the N- or C-terminus of α -syn.

Thioether-directed protein ubiquitylation

Although disulfide-directed ubiquitylation is readily achieved with recombinant proteins and requires minimal chemical manipulation, the final product is susceptible to deubiquitylation by reduction. This typically limits its application to *in vitro* studies in reductant free buffers with a few exceptions, such as seen for PCNA. With this limitation in mind, Strieter and co-workers recently reported an elegant strategy to construct stable thioether-linked polyUb chains using radical-mediated thiol-ene coupling (TEC) chemistry.⁶⁷

The addition of thiyl radicals to alkenes⁶⁸ has recently gained popularity as a bioconjugation technique and is classified as a click-reaction, due to its high efficiency, bioorthogonality, and the stability of the final product.⁶⁹ Following the photochemical or thermal induction of a thiyl radical, TEC proceeds by addition of the radical across an alkene to yield the anti-Markovnikov thioether product.^{70,71} Instead of an intein-based approach to generate a Ub- α -

thioester for the introduction of the alkene group, the authors employed a Ub-DUB acyl-enzyme intermediate thioester (Figure 6). Thus, Ub was expressed with a C-terminal Asp77 extension and subjected to the yeast Ub hydrolase 1 (YUH1) which forms a Ub₁₋₇₆-S-UCH acyl-enzyme intermediate in the process of removing Asp77.⁷² This intermediate was readily intercepted with allylamine to generate the desired Ub-allylamine (Ub-AA). TEC was then used to attempt linkage of Ub-AA to all seven Lys-to-Cys single mutants of Ub by irradiation at 365 nm in the presence of the free-radical photoinitiator lithium acyl phosphinate (LAP) (Figure 6). This yielded site-specifically linked diUbs joined at Lys6, Lys11, Lys48, and Lys63 in good quantities, but not all linkages were accessible to the same extent and the Lys27Cys mutant was altogether refractory to TEC. Since wild-type Ub lacks Cys this is an excellent method to generate Ub oligomers. However, its application for the ubiquitylation of target proteins that have more than one Cys residue may require additional mutagenesis to prevent unwanted coupling. The resulting N^ε-Gly-L-homothiaLys isopeptide linkage is one atom longer than the native N^ε-Gly-L-Lys isopeptide linkage (Figure 6). The thioether-linked diUbs were tested with both promiscuous and linkage-specific DUBs and were hydrolyzed at similar rates as native Lys48 and Lys63-linked diUbs. This validated the thioether-linkage as a reasonable mimic of the native isopeptide linkage. The thioether strategy was also used to generate regioisomeric branched triUbs in order to study the effect of ubiquitylation at Lys6/11/63 on hydrolysis of the isopeptide bond at Lys48. Unlike linear polyUbs, branched polyUbs have two or more Ubs attached to the same molecule of Ub.^{73,74} Three branched tri-Ubs were made by TEC between Ub double mutants (Lys6Cys, Lys48Cys; Lys11Cys, Lys48Cys; and Lys48Cys, Lys63Cys) and Ub-AA. Not surprisingly, the non-specific DUB IsoT was capable of hydrolyzing all three tri-Ubs. Interestingly, however, ubiquitylation at Lys6, but not at Lys11/63, prevented hydrolysis of the Lys48-linked ubiquitin by the linkage-specific ovarian tumor domain (OTU) DUB, OTU-A20. The authors suggested that ubiquitylation at Lys6 may be a mechanism by which Lys48-linked polyubiquitylated proteins escape proteasomal degradation and instead aggregate in cells. It is noteworthy that Lys6-linked polyUb chains themselves are deubiquitylated by 26S proteasomes *in vitro*⁷⁵ and further studies are needed to test the degree and proximity of the site of Lys6 ubiquitylation that may be required to inhibit degradation of Lys48-linked chains.

The breast cancer type 1 susceptibility (BRCA1)-BRCA1 associated RING domain protein 1 (BARD1) complex is a heterodimeric ubiquitin ligase complex that assembles Lys6-linked polyUb on itself^{76,77} and is localized to DNA double strand break regions in response to phosphorylation of the histone variant H2A.X.⁷⁸ Studies to understand the precise role of Lys6-linked polyUb in the DNA damage response pathway and to investigate the proposed helical structure of these chains⁷⁹ would benefit from the ability to access polyUb of precisely defined linkage and length. A straightforward extension of the TEC strategy to generate oligomeric Ubs was recently reported by Trang et al.⁸⁰ Dual functionalization of a Ub molecule with a C-terminal allylamine and internal Lys-to-Cys mutation enabled TEC to occur at both the thiol group and at the C-terminus (Figure 6). This provided easy access to chromatographically separable chains of up to seven Ubs. In order to probe the effect of linkage type on the rate of Ub hydrolysis by 26S proteasome associated DUBs, tetra-Ubs linked at Lys48, Lys63 and Lys6 were assayed with purified human PA700, the 19S

proteasomal regulatory particle that has deubiquitylating activity.^{81,82} Lys63-linked chains were processed faster than either Lys48 or Lys6-linked tetra-Ub, which were processed at similar rates. This led the authors to suggest that Lys6-linked polyUb may suffice to target proteins for degradation by 26S proteasome, which is consistent with a previous study that reported Lys6Ala and Lys6Trp mutations in Ub inhibited ATP-dependent degradation of substrates in reticulocyte lysates.⁸³ However, further studies with bona-fide polyUb substrates are required to test this hypothesis.

While thioether-directed ubiquitylation affords a simple and rapid means to obtain ubiquitylated proteins, the success of this strategy for all substrates cannot be predicted at this early stage in its development. Although issues arising from steric inaccessibility of the reactive thiol (as was proposed for Ub Lys27Cys) may potentially be resolved by the inclusion of denaturing reagents during TEC, additional factors such as the precise sequence context of the thiol may influence reactivity of the thiyl radical and dictate reaction outcomes.

Additional strategies for protein ubiquitylation

Most synthetic and semisynthetic approaches to study protein ubiquitylation aim to generate the native isopeptide linkage or close structural mimics thereof. However, structurally divergent linkages have also yielded important insight on specific protein binders of atypical ubiquitin linkages and have the added advantage of being stable to hydrolysis by DUBs. An early example of a non-hydrolyzable linker was generated by first introducing a Gly76Cys mutation in one Ub monomer and a Lys-to-Cys mutation at positions 11, 29, 48, or 63 in another Ub monomer.⁸⁴ The two Cys thiols were then reacted with 1,3-dichloroacetone to form a non-hydrolyzable mimic of the Ub-Ub isopeptide bond. This method was effectively utilized to generate chains of tetra-Ub immobilized on a resin, which was used to identify the protein Ufd3 as a Lys29-linked diUb-binding protein.⁸⁵ Other methods to link Ub to proteins include the azide-alkyne click reaction^{86,87} and oxime formation.⁸⁸ Surprisingly, these poor structural mimics of the isopeptide linkage have also proven to be useful in identifying various diUb-binding proteins and DUBs, which indicates the wide variety of Ub surfaces that are recognized by proteins/enzymes associated with Ub-signaling.

Conclusions and Outlook

The protein Ub plays a key role in eukaryotes by its involvement in a myriad of functions that range from mediating proteasomal degradation of oxidatively damaged and terminally misfolded proteins to changing the structure of chromatin and triggering receptor endocytosis.⁸⁹ By using the seven ϵ -amines and one α -amine in Ub as a starting point for building Ub chains of varying linkage and length, a diversity of topologies are generated by the large family of Ub ligases. Not surprisingly, these different polymers interact with different binding partners and linkage-specific DUBs, resulting in rich functional diversity. However, the precise functions of modification by Ub clearly appear to be substrate dependent, akin to words in a language whose precise meanings are best inferred from context. Even the simplest form of monoubiquitylation may serve as a signal for receptor internalization,⁹⁰ enzyme activation,⁴⁷ and protein degradation.²⁰ Similar to what is already

known regarding histone PTMs,⁹¹ protein ubiquitylation also appears to form a complex signaling language.⁹ Increasingly sophisticated synthetic and molecular biological toolkits have permitted access to a growing number of homogeneously and site-specifically ubiquitylated proteins and regioisomeric oligomeric Ub chains. Biochemical and biophysical studies with these molecules have shed light on some of the many roles for protein monoubiquitylation, and revealed both similarities and differences in signaling mediated by Lys48/63-linked and atypically linked polyUbs. Amber codon suppression techniques that employ orthogonal pairs of aminoacyl tRNA synthetases/tRNA to incorporate ligation auxiliaries, such as 5-mercaptolysine,⁹² for EPL and azides or alkynes for click chemistry⁹³ will no doubt expand the scope of protein ubiquitylation to include substrates that are not amenable to Cys mutagenesis or to refolding from the denatured state.

However, despite the rapid progress in chemical ubiquitylation strategies, it is clear that no single technique may address the many challenges posed by the complexity of ubiquitin signaling in our cells. Challenges for the future of chemical ubiquitylation include the design of ligation auxiliaries that are easily synthesized in high overall yields, are removable under conditions that do not affect other essential protein sulfhydryl groups, and that yield the native isopeptide linkage. Another significant challenge for current strategies is posed by the discovery of mixed-linkage, or heterotypic, Ub chains such as those containing Lys6/11, Lys11/63, Lys27/29, and Lys29/33 linkages.^{74,94} Thioether-directed ubiquitylation holds particular promise for the construction of such complex linkages but steric factors may be limiting for some linkages. Beyond this, the discovery of heterologous Ub chains that play critical physiological roles - such as mixed chains of Ub and the small ubiquitin-like modifier (SUMO) that prevent the accumulation of leukemogenic fusions of the Promyelocytic Leukemia protein and retinoic acid receptor - will benefit from the application of synthetic strategies for mechanistic studies.⁹⁵ Finally, the continued improvement in mass spectrometric^{96,97} and biochemical tools that permit the determination of the precise sites of Ub attachment in substrates and specific linkages in polyUb chains^{98,99} will undoubtedly continue to inspire the development of new chemical techniques to understand the language of ubiquitin signaling.

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References

1. Walsh, CT. Posttranslational modification of proteins: expanding nature's inventory. Roberts and Company Publishers; Greenwood Village: 2005.
2. Schlesinger DH, Goldstein G. *Nature*. 1975; 255:423–424. [PubMed: 1128706]
3. Goldknopf IL, Taylor CW, Baum RM, Yeoman LC, Olson MO, Prestayko AW, Busch H. *J Biol Chem*. 1975; 250:7182–7187. [PubMed: 1165239]
4. Olson MO, Goldknopf IL, Guetzow KA, James GT, Hawkins TC, Mays-Rothberg CJ, Busch H. *J Biol Chem*. 1976; 251:5901–5903. [PubMed: 972147]
5. Goldknopf IL, Busch H. *Proc Natl Acad Sci USA*. 1977; 74:864–868. [PubMed: 265581]
6. Ciechanover A, Hod Y, Hershko A. *Biochem Biophys Res Comm*. 1978; 81:1100–1105. [PubMed: 666810]

7. Hershko A, Ciechanover A, Rose IA. *Proc Natl Acad Sci USA*. 1979; 76:3107–3110. [PubMed: 290989]
8. Ciechanover A, Elias S, Heller H, Ferber S, Hershko A. *J Biol Chem*. 1980; 255:7525–7528. [PubMed: 6249802]
9. Komander D, Rape M. *Annu Rev Biochem*. 2012; 81:203–229. [PubMed: 22524316]
10. Strieter ER, Korasick DA. *ACS Chem Biol*. 2012; 7:52–63. [PubMed: 22196026]
11. Spasser L, Brik A. *Angew Chem Int Ed Eng*. 2012; 51:6840–6862.
12. Hershko A, Ciechanover A. *Ann Rev Biochem*. 1998; 67:425–479. [PubMed: 9759494]
13. Metzger MB, Hristova VA, Weissman AM. *J Cell Sci*. 2012; 125:531–537. [PubMed: 22389392]
14. Skaug B, Jiang X, Chen ZJ. *Annu Rev Biochem*. 2009; 78:769–796. [PubMed: 19489733]
15. Reyes-Turcu FE, Ventii KH, Wilkinson KD. *Annu Rev Biochem*. 2009; 78:363–397. [PubMed: 19489724]
16. Pickart CM, Faseb J. 1997; 11:1055–1066. [PubMed: 9367341]
17. Finley D. *Annu Rev Biochem*. 2009; 78:477–513. [PubMed: 19489727]
18. Hicke L. *Nat Rev Mol Cell Biol*. 2001; 2:195–201. [PubMed: 11265249]
19. Meier F, Abeywardana T, Dhall A, Marotta NP, Varkey J, Langen R, Chatterjee C, Pratt MR. *J Am Chem Soc*. 2012; 134:5468–5471. [PubMed: 22404520]
20. Boutet SC, Disatnik MH, Chan LS, Iori K, Rando TA. *Cell*. 2007; 130:349–362. [PubMed: 17662948]
21. Ramage R, Green J, Ogunjobi OM. *Tet Lett*. 1989; 30:2149–2152.
22. Ramage R, Green J, Muir TW, Ogunjobi OM, Love S, Shaw K. *Biochem J*. 1994; 299(Pt 1):151–158. [PubMed: 8166633]
23. Alexeev D, Bury SM, Turner MA, Ogunjobi OM, Muir TW, Ramage R, Sawyer L. *Biochem J*. 1994; 299(Pt 1):159–163. [PubMed: 8166634]
24. Love SG, Muir TW, Ramage R, Shaw KT, Alexeev D, Sawyer L, Kelly SM, Price NC, Arnold JE, Mee MP, Mayer RJ. *Biochem J*. 1997; 323(Pt 3):727–734. [PubMed: 9169606]
25. Layfield R, Franklin K, Landon M, Walker G, Wang P, Ramage R, Brown A, Love S, Urquhart K, Muir T, Baker R, Mayer RJ. *Anal Biochem*. 1999; 274:40–49. [PubMed: 10527495]
26. El Oualid F, Merckx R, Ekkebus R, Hameed DS, Smit JJ, de Jong A, Hilkmann H, Sixma TK, Ovaa H. *Angew Chem Int Ed Eng*. 2010; 49:10149–10153.
27. Dawson PE, Muir TW, Clark-Lewis I, Kent SBH. *Science*. 1994; 266:776–778. [PubMed: 7973629]
28. Wan Q, Danishefsky SJ. *Angew Chem Int Ed Eng*. 2007; 46:9248–9252.
29. Yan LZ, Dawson PE. *J Am Chem Soc*. 2001; 123:526–533. [PubMed: 11456564]
30. Kumar KSA, Haj-Yahya M, Olschewski D, Lashuel HA, Brik A. *Angew Chem Int Ed Eng*. 2009; 48:8090–8094.
31. Kumar KSA, Spasser L, Erlich LA, Bavikar SN, Brik A. *Angew Chem Int Ed Eng*. 2010; 49:9126–9131.
32. Chau V, Tobias JW, Bachmair A, Marriott D, Ecker DJ, Gonda DK, Varshavsky A. *Science*. 1989; 243:1576–1583. [PubMed: 2538923]
33. Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ. *Mol Cell*. 2006; 22:245–257. [PubMed: 16603398]
34. Wu CJ, Conze DB, Li T, Srinivasula SM, Ashwell JD. *Nat Cell Biol*. 2006; 8:398–406. [PubMed: 16547522]
35. Hofmann RM, Pickart CM. *Cell*. 1999; 96:645–653. [PubMed: 10089880]
36. Ulrich HD, Jentsch S, Embo J. 2000; 19:3388–3397. [PubMed: 10880451]
37. Piper RC, Lehner PJ. *Trends Cell Biol*. 2011; 21:647–655. [PubMed: 21955996]
38. Baboshina OV, Haas AL. *J Biol Chem*. 1996; 271:2823–2831. [PubMed: 8576261]
39. Sobhian B, Shao G, Lilli DR, Culhane AC, Moreau LA, Xia B, Livingston DM, Greenberg RA. *Science*. 2007; 316:1198–1202. [PubMed: 17525341]
40. Larsen CN, Krantz BA, Wilkinson KD. *Biochemistry*. 1998; 37:3358–3368. [PubMed: 9521656]

41. Bavikar SN, Spasser L, Haj-Yahya M, Karthikeyan SV, Moyal T, Kumar KSA, Brik A. *Angew Chem Int Ed Eng.* 2012; 124:782–787.
42. Muir TW, Sondhi D, Cole PA. *Proc Natl Acad Sci USA.* 1998; 95:6705–6710. [PubMed: 9618476]
43. Perler FB, Adam E. *Curr Opin Biotechnol.* 2000; 11:377–383. [PubMed: 10975457]
44. Borodovsky A, Ovaa H, Kolli N, Gan-Erdene T, Wilkinson KD, Ploegh HL, Kessler BM. *Chem Biol.* 2002; 9:1149–1159. [PubMed: 12401499]
45. Chatterjee C, McGinty RK, Pellois J-P, Muir TW. *Angew Chem Int Ed Eng.* 2007; 46:2814–2818.
46. Marini C, Offer J, Longhi R, Dawson P. *Bioorg Med Chem.* 2004; 12:2749–2757. [PubMed: 15110856]
47. McGinty RK, Kim J, Chatterjee C, Roeder RG, Muir TW. *Nature.* 2008; 453:812–816. [PubMed: 18449190]
48. Nakanishi S, Lee JS, Gardner KE, Gardner JM, Takahashi Y-h, Chandrasekharan MB, Sun Z-W, Osley MA, Strahl BD, Jaspersen SL, Shilatifard A. *J Cell Biol.* 2009; 186:371–377. [PubMed: 19667127]
49. Kumar KSA, Bavikar SN, Spasser L, Moyal T, Ohayon S, Brik A. *Angew Chem Int Ed Eng.* 2011; 50:6137–6141.
50. Yang R, Pasunooti KK, Li F, Liu XW, Liu CF. *Chem Commun (Camb).* 2010; 46:7199–7201. [PubMed: 20737108]
51. Yang R, Pasunooti KK, Li F, Liu XW, Liu CF. *J Am Chem Soc.* 2009; 131:13592–13593. [PubMed: 19728708]
52. Chatterjee C, McGinty RK, Fierz B, Muir TW. *Nat Chem Biol.* 2010; 6:267–269. [PubMed: 20208522]
53. Rabanal F, DeGrado W, Dutton P. *Tet Lett.* 1996; 37:1347–1350.
54. Wu L, Zee BM, Wang Y, Garcia BA, Dou Y. *Mol Cell.* 2011; 43:132–144. [PubMed: 21726816]
55. Geng F, Tansey WP. *Mol Biol Cell.* 2008; 19:3616–3624. [PubMed: 18562693]
56. Fierz B, Chatterjee C, McGinty RK, Bar-Dagan M, Raleigh DP, Muir TW. *Nat Chem Biol.* 2011; 7:113–119. [PubMed: 21196936]
57. Nickel BE, Allis CD, Davie JR. *Biochemistry.* 1989; 28:958–963. [PubMed: 2713375]
58. Chen J, Ai Y, Wang J, Haracska L, Zhuang Z. *Nat Chem Biol.* 2010; 6:270–272. [PubMed: 20208521]
59. Hoege C, Pfander B, Moldovan GL, Pyrowolakis G, Jentsch S. *Nature.* 2002; 419:135–141. [PubMed: 12226657]
60. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. *Nature.* 1997; 388:839–840. [PubMed: 9278044]
61. Braak H, Sandmann-Keil D, Gai WP, Braak E. *Neurosci Lett.* 1999; 265:67–69. [PubMed: 10327208]
62. Recchia A, Debetto P, Negro A, Guidolin D, Skaper SD, Giusti P, FASEB J. 2004; 18:617–626. [PubMed: 15054084]
63. Hasegawa M, Fujiwara H, Nonaka T, Wakabayashi K, Takahashi H, Lee VMY, Trojanowski JQ, Mann D, Iwatsubo T. *J Biol Chem.* 2002; 277:49071–49076. [PubMed: 12377775]
64. Rott R, Szargel R, Haskin J, Shani V, Shainskaya A, Manov I, Liani E, Avraham E, Engelender S. *J Biol Chem.* 2008; 283:3316–3328. [PubMed: 18070888]
65. Engelender S. *Autophagy.* 2008; 4:372–374. [PubMed: 18216494]
66. Hejjoui M, Haj-Yahya M, Kumar KSA, Brik A, Lashuel HA. *Angew Chem Int Ed Eng.* 2011; 50:405–409.
67. Valkevich EM, Guenette RG, Sanchez NA, Chen Y-C, Ge Y, Strieter ER. *J Am Chem Soc.* 2012; 134:6916–6919. [PubMed: 22497214]
68. Posner T. *Berichte der Deutschen Chemischen Gesellschaft.* 1905; 38:646–657.
69. Dondoni A. *Angew Chem Int Ed Eng.* 2008; 47:8995–8997.
70. Mayo FR, Walling C. *Chem Rev.* 1940; 27:351–412.
71. Griesbau K. *Angew Chem Int Edit.* 1970; 9:273–287.
72. Johnston SC, Riddle SM, Cohen RE, Hill CP, Embo J. 1999; 18:3877–3887. [PubMed: 10406793]

73. Ben-Saadon R, Zaaroor D, Ziv T, Ciechanover A. *Mol Cell*. 2006; 24:701–711. [PubMed: 17157253]
74. Kim HT, Kim KP, Lledias F, Kisselev AF, Scaglione MK, Skowrya D, Gygi SP, Goldberg AL. *J Biol Chem*. 2007; 282:17375–17386. [PubMed: 17426036]
75. Nishikawa H, Ooka S, Sato K, Arima K, Okamoto J, Klevit RE, Fukuda M, Ohta T. *J Biol Chem*. 2004; 279:3916–3924. [PubMed: 14638690]
76. Chen A, Kleiman FE, Manley JL, Ouchi T, Pan ZQ. *J Biol Chem*. 2002; 277:22085–22092. [PubMed: 11927591]
77. Wu-Baer F, Lagrazon K, Yuan W, Baer R. *J Biol Chem*. 2003; 278:34743–34746. [PubMed: 12890688]
78. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. *Curr Biol*. 2000; 10:886–895. [PubMed: 10959836]
79. Virdee S, Ye Y, Nguyen DP, Komander D, Chin JW. *Nat Chem Biol*. 2010; 6:750–757. [PubMed: 20802491]
80. Trang VH, Valkevich EM, Minami S, Chen Y-C, Ge Y, Strieter ER. *Angew Chem Int Ed Eng*. 2012; 51:13085–13088.
81. Guterman A, Glickman MH. *J Biol Chem*. 2004; 279:1729–1738. [PubMed: 14581483]
82. Koulich E, Li X, DeMartino GN. *Mol Biol Cell*. 2008; 19:1072–1082. [PubMed: 18162577]
83. Shang F, Deng G, Liu Q, Guo W, Haas AL, Crosas B, Finley D, Taylor A. *J Biol Chem*. 2005; 280:20365–20374. [PubMed: 15790562]
84. Yin L, Krantz B, Russell NS, Deshpande S, Wilkinson KD. *Biochemistry*. 2000; 39:10001–10010. [PubMed: 10933821]
85. Russell NS, Wilkinson KD. *Biochemistry*. 2004; 43:4844–4854. [PubMed: 15096053]
86. Weikart ND, Mootz HD. *ChemBioChem*. 2010; 11:774–777. [PubMed: 20209558]
87. Weikart ND, Sommer S, Mootz HD. *Chem Commun (Camb)*. 2012; 48:296–298. [PubMed: 22095407]
88. Shanmugham A, Fish A, Luna-Vargas MPA, Faesen AC, El Oualid F, Sixma TK, Ovaa H. *J Am Chem Soc*. 2010; 132:8834–8835. [PubMed: 20540574]
89. Varshavsky A. *Annu Rev Biochem*. 2012; 81:167–176. [PubMed: 22663079]
90. Terrell J, Shih S, Dunn R, Hicke L. *Mol Cell*. 1998; 1:193–202. [PubMed: 9659916]
91. Dhall A, Chatterjee C. *ACS Chem Biol*. 2011; 6:987–999. [PubMed: 21827195]
92. Virdee S, Kapadnis PB, Elliott T, Lang K, Madrzak J, Nguyen DP, Riechmann L, Chin JW. *J Am Chem Soc*. 2011; 133:10708–10711. [PubMed: 21710965]
93. Eger S, Scheffner M, Marx A, Rubini M. *J Am Chem Soc*. 2010; 132:16337–16339. [PubMed: 21033666]
94. Goto E, Yamanaka Y, Ishikawa A, Aoki-Kawasumi M, Mito-Yoshida M, Ohmura-Hoshino M, Matsuki Y, Kajikawa M, Hirano H, Ishido S. *J Biol Chem*. 2010; 285:35311–35319. [PubMed: 20833710]
95. Tatham MH, Geoffroy MC, Shen L, Plechanovova A, Hattersley N, Jaffray EG, Palvimo JJ, Hay RT. *Nat Cell Biol*. 2008; 10:538–546. [PubMed: 18408734]
96. Peng JM, Schwartz D, Elias JE, Thoreen CC, Cheng DM, Marsischky G, Roelofs J, Finley D, Gygi SP. *Nat Biotechnol*. 2003; 21:921–926. [PubMed: 12872131]
97. Xu P, Peng JM. *Anal Chem*. 2008; 80:3438–3444. [PubMed: 18351785]
98. Newton K, Matsumoto ML, Wertz IE, Kirkpatrick DS, Lill JR, Tan J, Dugger D, Gordon N, Sidhu SS, Fellouse FA, Komuves L, French DM, Ferrando RE, Lam C, Compaan D, Yu C, Bosanac I, Hymowitz SG, Kelley RF, Dixit VM. *Cell*. 2008; 134:668–678. [PubMed: 18724939]
99. Sims JJ, Scavone F, Cooper EM, Kane LA, Youle RJ, Boeke JD, Cohen RE. *Nat Methods*. 2012; 9:303–309. [PubMed: 22306808]

Figure 1. The protein ubiquitylation cascade

Ub is activated as a C-terminal thioester by an E1 activating enzyme and then transferred to an E2 ligase. The E2 ligase transfers Ub to substrates, often in conjunction with an E3 ligase that imparts substrate specificity. Monoubiquitylated substrates may undergo multiple rounds of ubiquitylation at one of seven Lys side-chains to form regioisomeric chains of polyUb. Such Ub chains play a role in targeting substrates to proteasomes, or in other signaling pathways. DUBs= deubiquitylating enzymes. Ub, PDB code 1UBQ. Substrate, PDB code 1NW3.

Figure 2. The principle of native chemical ligation (NCL)

Peptide 1, which contains a C-terminal α -thioester undergoes nucleophilic attack by the Cys side-chain thiol at the N-terminus of Peptide 2. The initial thioester-linked product undergoes rapid intramolecular rearrangement through a favorable 5-membered ring to yield the ligated product with a native amide bond joining the two peptide fragments.

Figure 3. Chemical synthesis of ubiquitylated peptides and proteins

(A) An orthogonally protected Lys residue in H2B(118-125) is selectively deprotected and the Ub(46-76)A46C fragment is synthesized starting from the ϵ -amine. Cleavage of the branched peptide from the resin is followed by NCL with a synthetic Ub(1-45)- α -thioester to generate full-length Ub(1-76)A46C. Deprotection of the N-terminal thiazolidine permits a second NCL with H2B(1-116)- α -thioester. Finally, desulfurization of the ligation product yields the wild-type ubiquitylated protein. (B) A thiazolidine-protected form of 5-mercaptolysine may be introduced in either the thioester-containing or the N-terminal Cys bearing fragment of Ub. The order of two NCLs - main-chain or side-chain first - depends upon the placement of the ligation auxiliary as shown. Finally, global desulfurization yields the wild-type ubiquitylated protein. ivDde= 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl, SPPS= solid-phase peptide synthesis, Boc= *tert*-butoxycarbonyl, Fmoc= fluorenylmethoxycarbonyl.

Figure 4. Intein-mediated C-terminal thioester formation

Ub fused to a mutant intein undergo N-to-S acyl transfer onto the side-chain of the N-terminal Cys. The thioester-linked Ub- α -thioester intermediate is intercepted with 2-mercaptoethanesulfonic acid, or any nucleophilic water-soluble thiol, to yield the corresponding Ub- α -thioester. Ub, PDB code 1UBQ. GyrA intein, PDB code 1AM2.

Figure 5. Semisynthetic protein ubiquitylation

(A) Photolytically removable auxiliary-mediated peptide ubiquitylation. (B) Disulfide-directed protein ubiquitylation. $\text{R}' = -\text{CH}_3$, $\text{R}'' = -\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{N}(\text{CH}_3)\text{H}$.

Figure 6. Thioether-directed ubiquitylation

The deubiquitylase enzyme YUH1 forms a stable acyl-enzyme intermediate that undergoes aminolysis with allylamine (AA) to generate the mutant Ub(K48C)-AA protein. TEC in the presence of a light-activated free radical initiator results in formation of diUb. The initial diUb undergoes multiple rounds of TEC to produce polyUb chains of varying length. LAP= lithium phenyl-2,4,6-trimethylbenzoylphosphinate. Ub, PDB code 1UBQ.