

Carpe Diubiquitin**

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Eukaryotic cells are made more diverse and complex by an intricate ensemble of post-translational modifications to their proteins. Of these, ubiquitination is perhaps the most fateful. Ubiquitin (Ub) is a small, robust, highly conserved protein (Figure 1). The covalent attachment of ubiquitin marks cellular proteins for degradation by the proteasome, and can elicit other consequences as well. Defects in ubiquitin demarcation correlate with multiple diseases, including cancers, inflammatory diseases, and a variety of neurodegenerative disorders.

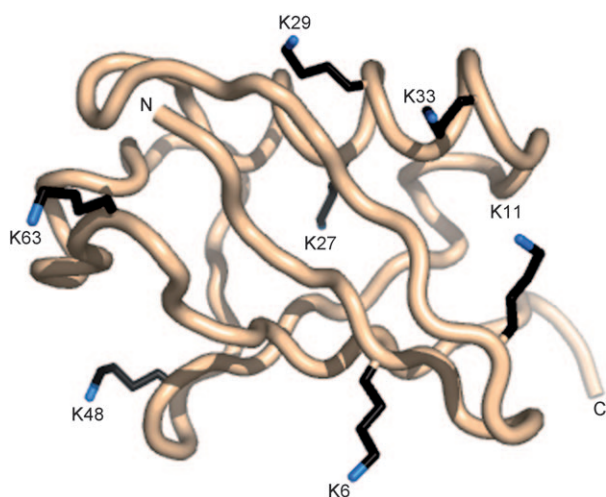


Figure 1. Three-dimensional structure of ubiquitin, which contains 76 amino acid residues, with its seven lysine residues indicated explicitly (black; blue = NH₂ groups).

Ubiquitination is forged through an isopeptide bond—a nonstandard amide linkage between the ϵ -nitrogen of a lysine residue on a target “acceptor” protein and the C-terminal carboxy group of ubiquitin “donor”. A lysine residue on the appended ubiquitin can be decorated subsequently with another ubiquitin, and so on. Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48, and K63; Figure 1). Each possible isopeptide bond exists in living cells, and particular linkages stimulate disparate responses.

The production of proteins containing non-canonical linkages, such as isopeptide bonds, is an ongoing challenge in chemical biology. Recently, three independent groups^[1] have announced the construction of diubiquitin, including the total synthesis of all possible diubiquitin chains.^[1a] This accomplishment is a milestone in chemical biology, enabling the first explorations of the structure and function of diubiquitin regioisomers.

The isopeptide bond is not accessible with the standard techniques of recombinant DNA. Isopeptide bond-forming enzymes can, however, provide access. In pioneering work, the Pickart group developed a chemoenzymatic route to di- and polyubiquitin (Table 1).^[2] This work led to the discovery of K48-linked chains containing at least four ubiquitins as a signal for proteasomal degradation.

Methods of modern protein chemistry are expanding the horizons of isopeptide bond formation. The initial products were precise conjugates with a single ubiquitin. In 2007, the Muir group used a photolabile auxiliary group to effect the semisynthesis of a histone with a ubiquitin pendant at a specific site,^[3] a modification that can have marked effects on transcription. In addition to being a harbinger, the Muir approach is noteworthy in being traceless—no residual atoms or groups remain in the synthetic target. Other chemical means to decorate targets with a single ubiquitin moiety have availed a pyrrolysine analogue that enables native chemical ligation,^[4] disulfide bond formation,^[5] or an isopeptide-like bond with an oxime.^[6]

More recent work has expanded to the synthesis of diubiquitin. Using thioether formation as a mechanism for concatenating synthetic peptides, the Przybylski group completed a total synthesis of K63-linked diubiquitin.^[7] In their product, thioethers replace several native peptide bonds.

The new work by the groups of Liu and Liu,^[1c] Chin and Komander,^[1b] and Brik^[1a] is most exciting. Their distinct, complementary approaches have led to the traceless total synthesis of designated diubiquitin chains in quantities useful for structure–function analyses.

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Table 1: Diubiquitins from biosynthesis, semisynthesis, and synthesis.

Method	Donor Ub	Acceptor Ub	Product	Isopeptide linkage(s)	Notes
In vivo				K(all), N terminus	Free diubiquitin is known only as a degradative byproduct
Brik ^[1a]				K(all)	Used to determine the substrate specificity of isopeptidase T
Liu & Liu ^[1c]				K48	Mercaptolysine residue promotes both peptide and isopeptide bond formation
Chin ^[1b]				K6, K29	Enabled structure determination of K6-linked diubiquitin, and characterization of isopeptidases
Pickart ^[2]				K48, K63	Requires an E2 enzyme and C-terminal hydrolase
Przybylski ^[7]				K63	First total chemical synthesis of a diubiquitin motif

The groups of Liu and Liu uses a combination of solid-phase peptide synthesis and native chemical ligation (NCL) to obtain K48-linked diubiquitin.^[1c] Their key reagent is a lysine analogue containing a photolabile protecting group on the ϵ -nitrogen and a thiol group on the γ -carbon. The thiol promotes native chemical ligation (to form a peptide bond with the α -nitrogen) as part of the assembly of the acceptor ubiquitin. A second NCL reaction completes the synthesis of this ubiquitin, and the protecting group is removed by photolysis. The γ -thiol then promotes a subsequent NCL, this time attaching a full-length ubiquitin thioester (obtained by using an intein) to form the desired isopeptide bond. Desulfurization excises the exogenous sulfur.

The Chin and Komander groups also uses a ubiquitin thioester as the source for the donor ubiquitin, but their method of assembly is otherwise divergent.^[1b] By using stop-codon suppression to incorporate a single N^{ϵ} -Boc-protected lysine residue followed by global benzyloxycarbonyl (Cbz) protection, they ultimately produce ubiquitin containing a single unprotected amino group. Its condensation with a ubiquitin containing a C-terminal N -hydroxysuccinimide ester generates the isopeptide bond. Global deprotection affords “native” diubiquitin. This method led to the crystal structure of K6-linked diubiquitin, as well as substrates for assays of deubiquitinases—enzymes that catalyze the hydrolysis of isopeptide bonds.

Brik and co-workers eschewed biosynthesis, opting instead for the total chemical synthesis of all seven diubiquitins.^[1a] Their strategy is congruous with the synthesis of Liu

and Liu, with subtle but noteworthy differences. Their key reagent was also a mercaptolysine, though with the δ -carbon bearing the thiol. All ubiquitin monomers were assembled from two peptide precursors by using an A46C variant for NCL. The δ -mercaptolysine was protected as a thiazolidine during solid-phase peptide synthesis, and deprotected immediately prior to isopeptide bond formation. The thioester was generated from an appended C-terminal N -methylcysteine amide residue with clever methodology developed in the Brik laboratory. Reacting the donor thioester with each of the seven δ -mercaptolysine-containing ubiquitin acceptors afforded the entirety of ubiquitin–ubiquitin isopeptide bonds. Desulfurization made for a traceless synthesis.

After completing these seven total syntheses, Brik and co-workers subjected their diubiquitin substrates to novel degradation assays with human isopeptidase T. This enzyme disassembles polyubiquitin chains, preventing them from inhibiting the proteasome and enabling the liberated ubiquitins to be reused by the cell. Although this enzyme was most active towards the K48 isopeptide, nearly equivalent activity was observed for the K63 linkage. This finding was surprising, given that the signals elicited by these two linkages are different and that the K11 linkage (which is as abundant in yeast as the K48 linkage and could also be a signal for proteasomal degradation) does not appear to be a substrate.^[8]

These efforts are true milestones. The physiologically prevalent but synthetically recalcitrant isopeptide bonds are the essence of posttranslational modification by ubiquitin (and ubiquitin-like) proteins. Chemical biologists are now on

the verge of teasing out the panoply of signals contained within the “ubiquitin code”. It is noteworthy, however, that the current state of the art is limited to targets containing a single isopeptide linkage, either between ubiquitin and a target protein or between two ubiquitins. Hence, we look forward to work that expands these recent initiatives to the tetraubiquitins, which are the true beacons for protein turnover.

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