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Synthesis of Branched Triubiquitin Active-Site Directed Probes

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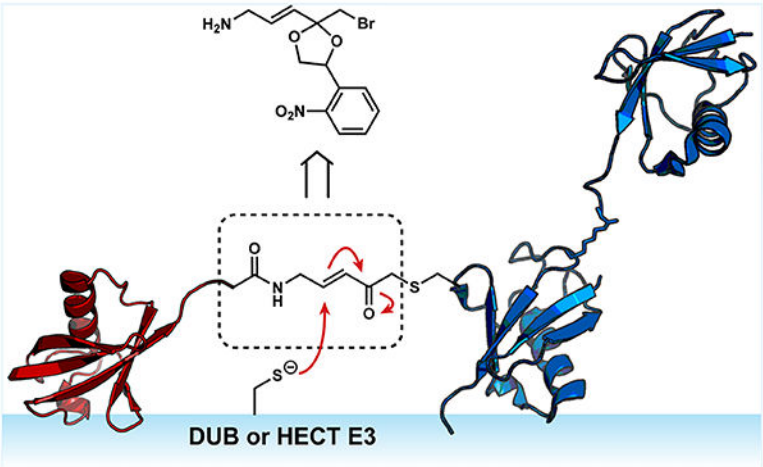
Abstract

Active-site directed probes are powerful tools for studying the ubiquitin conjugation and deconjugation machinery. Branched ubiquitin chains have emerged as important proteasometargeting signals for aggregation-prone proteins and cell cycle regulators. By implementing a new synthetic strategy for the electrophilic warhead, we herein report on the generation and reactivity of a series of branched triubiquitin active-site directed probes. These new tools can be used to dissect the molecular basis of branched chain assembly and disassembly.

Graphical Abstract



Ubiquitin (Ub) chains provide highly nuanced mechanisms of cellular regulation.[1,2] Once a protein is modified with Ub, Ub itself can serve as the substrate to afford polymeric Ub chains. During this elongation process, one of seven amino groups of Ub (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) or its amino terminus (Met1) can be conjugated to

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Supporting Information

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Synthetic protocols, protein expression information, enzyme assays, and characterization of crucial inter-mediates (PDF)

The authors declare no competing financial interest.

the C-terminus of another ubiquitin molecule.[3–5] Moreover, a single Ub moiety can be modified at more than one site, creating a branch point. Thus, in addition to chain length, there is tremendous variability in chain architecture. Effector proteins within the Ub signaling network, i.e., deubiquitinases (DUBs)[6–8] and "reader" proteins containing Ub-binding domains,[9] are capable of exploiting subtle differences in the orientation and spacing of Ub subunits present in different chains to control the net flux through biochemical pathways. With the immense diversity of chains and the number of enzymes involved in Ub signaling, however, there are still many questions that remain unresolved regarding the assembly, recognition, and removal of specific chain types.

Active-site directed probes (also referred to as activity-based probes; ABPs) have been particularly effective as tools for dissecting the Ub conjugation and deconjugation machiner.[10,11] The basic design of an ABP includes a recognition element that directs the probe to its target and an electrophilic warhead that reacts with an enzyme active site to afford a covalent adduct.[12,13] The classic Ub-based ABPs use monoUb as the recognition element.[14–21] These ABPs have been instrumental in the discovery of DUBs[22–27] and elucidating how substrate engagement induces conformational changes in DUBs that are conducive to catalysis.[28–34] More recently, di- and tri-Ub-based probes have been added to toolbox.[35–39] The utility of these probes has been illustrated by biochemical and structural studies that have helped illuminate the molecular details underlying the linkage selectivity of DUBs.[34,39,40]

Despite the impressive advances in Ub ABPs, there is still a need for additional tools. Recent studies have shown that branched Ub chains provide a unique signal for targeting proteins to the proteasome for degradation. During mitosis, for instance, the cullin-RING E3 ligase APC/C (anaphase promoting complex/cyclosome) collaborates with the E2 conjugating enzymes UBE2C and UBE2S to assemble Lys11/Lys48-linked branched chains on several cell cycle regulators.[41] In response to proteotoxic stress, nascent and aggregation-prone proteins are also modified with Lys11/Lys48-linked branched Ub chains to promote their clearance.[42] In this case, branched chains are synthesized, in part, by the HECT E3 ligase UBR5. UBR5 can also introduce Lys48 branch points on pre-existing Lys63 chains to redirect a substrate to the proteasome for degradation.[43] How E3s assemble branched chains is unclear.[44,45] Access to branched chain ABPs would help address this problem. Branched chain ABPs can also be used to understand how this architecture affects the activity of DUBs.[46,47]

Our approach focused on a two-step sequence starting from two different Ub variants. The idea was to site-specifically couple a set of di-Ubs to a Ub C-terminally modified with an α-bromo-vinylketone (BVK) moiety to afford tri-Ub derivatives containing a native isopeptide bond on one side of the branch point and a Michael acceptor on the other (Scheme 1). Linkage-specific enzymes provide a powerful set of tools to assemble dimers with amino acid substitutions in specific subunits.[48] With Cys introduced at specific sites in lieu of Lys, we can couple dimers to Ub-BVK using standard Cys thiolate substitution chemistry.

To synthesize the Ub-BVK derivative we turned to previous work[37] in which a latent form of BVK was coupled to Ub-MESNA prior to deprotection under acidic conditions (4 mM p-TsOH, 54% (v/v) TFA). In our hands, however, we observed extensive hydrolysis during the

deprotection of the ethylene glycol ketal, resulting in the formation of $Ub_{1–75}$, thereby precluding the generation of sufficient quantities of the tri-Ub probe. To overcome these challenges, we sought to devise a slightly different synthetic route to Ub-BVK.

Our synthesis commenced with a cross-metathesis reaction between N-allylphthalimide (2) and methyl vinyl ketone using the Hoveyda–Grubbs second-generation catalyst and Lewis acid Cy_2BCl (Figure 1).[49] This reaction reproducibly furnished 3 in approximately 40% yield. After α-bromination, we subjected ketone 4 to ketalization conditions with 1-(2-nitrophenyl)-1,2-ethanediol (5). We reasoned the photolability of the *o*-nitrophenyl group would enable deprotection under milder conditions relative to those used for the ethylene glycolbased ketal. With the photolabile ketal in place, the amino group was unveiled using methyl hydrazine to give 6 and coupled to $Ub_{1–75}$-MESNa (7) to obtain 8. We found that the aminolysis reaction occurs readily with only 30-fold excess of amine 6 relative to $Ub_{1–75}$-MESNa, minimizing the loss of material.

Irradiation of the resulting photolabile Ub-BVK derivative (8) was examined at different pHs. Reactions were monitored by MALDI-TOF mass spectrometry (MS) (Figure S3). Under all conditions, we detected significant amounts of Ub-BVK (9). Although hydrolysis still occurs, the amount of $Ub_{1–75}$ is still markedly less than what we observed with ethylene glycol as a protecting group. Moreover, compared to the deprotection of the ethylene glycol ketal, which affords at least four products with Ub-BVK as a minor species, removal of the photolabile ketal resulted in Ub-BVK as the major product.

With Ub-BVK in hand, we sought to assemble branched tri-Ub probes. We started with di-Ub variants containing K6C, K48C, or K63C substitutions in the proximal subunit (**10a–d**). Upon mixing Ub-BVK with the Cys-containing dimers, we observed robust trimer formation (Figure 2). MS analysis confirmed the generation of each probe (Figures 2 and S6).

Next, we investigated the reactivity of the branched tri-Ub probes toward cysteine-dependent DUBs. OTUB1 is Lys48-specific DUB[50] in the ovarian tumor (OTU) subfamily and is capable of rapidly cleaving the Lys48 linkage within branched trimers. In accord, we found that OTUB1*–a more active version with the E2 UBE2D2 genetically fused to the N-terminus[51]–reacts with the branched probes **11a** and **11b** (Figure 3A). The catalytic Cys residue is critical for covalent complex formation, as higher molecular weight bands are not observed with the inactive C91A variant (Figure 3A). OTUB1* also displays selectivity for the electrophilic warhead at position-48. When other non-Lys48 residues are replaced with the warhead (e.g., Lys6 in **11c** or Lys63 in **11d***), complex formation is not observed (Figures 3A and S7). In addition, the time course assay shows the kinetics of covalent complex formation are on par with the overall cleavage rate (Figure 3B).

We also examined the reactivity of OTUD1 toward the branched probes. In vitro, it has been shown that OTUD1 displays moderate selectivity toward Lys63-linked chains.[52] We found that OTUD1 cleaves the native Lys63 linkage in probes **11b** and **11b***, leaving behind a di-Ub ABP with a warhead at position-48 (Figure 3C). By contrast, when the warhead is at position-63 (**11d***) a covalent adduct is readily observed. Together, these results demonstrate

that certain DUBs can selectively react with branched ABPs, similar to what has been observed with di-Ub probes.

Lastly, we investigated the reactivity of branched probes toward E3 ligases bearing active-site Cys residues. Two HECT E3 ligases were chosen: UBE3C and AREL1. The purpose here was not to assess whether the E3s display selectivity toward certain probes since these enzymes synthesize heterotypic Ub chains containing a mixture of linkages.[53–55] Instead, we wanted to measure the efficiency of labeling for future structural studies aiming to identify cryptic Ub binding sites that are important for chain assembly. With both UBE3C and ARE1L, we detected the formation of a covalent adduct between probes **11a**, **11b**, and **11d***, and the catalytic domains of UBE3C and AREL1 only when the catalytic Cys is present (Figures 4A,B and S8). In general, the labeling efficiency is higher with UBE3C compared to AREL1 (Figure S9). Interestingly, there does appear to be some selectivity. Probe **11c** containing the warhead at position-6 and an isopeptide bond at position-63 does not react with either UBE3C or ARE1L even though it does label nonselective DUBs, e.g., USP7, to some extent (Figure S10). This observation may reflect the linkage preferences of UBE3C and ARE1L considering Lys6 and Lys63 are not produced by these enzymes to a significant degree.[53–55] These results demonstrate that, in addition to DUBs, the branched probes can be used to generate complexes with HECT ligases.

In conclusion, we report on the synthesis and reactivity of a new set of Ub ABPs based on branched trimers. To generate these probes, we devised an alternative synthetic approach to the Michael acceptor linking one arm of the branched trimer to the proximal subunit. The advances made include a facile cross-metathesis reaction and photodeprotection of a ketal. We found that like other Ub ABPs the branched probes readily react with E3 ligases and DUBs containing active-site Cys residues. These probes will be valuable tools for uncovering cryptic Ub binding sites on E3s and DUBs that lead to linkage selectivity in both chain assembly and disassembly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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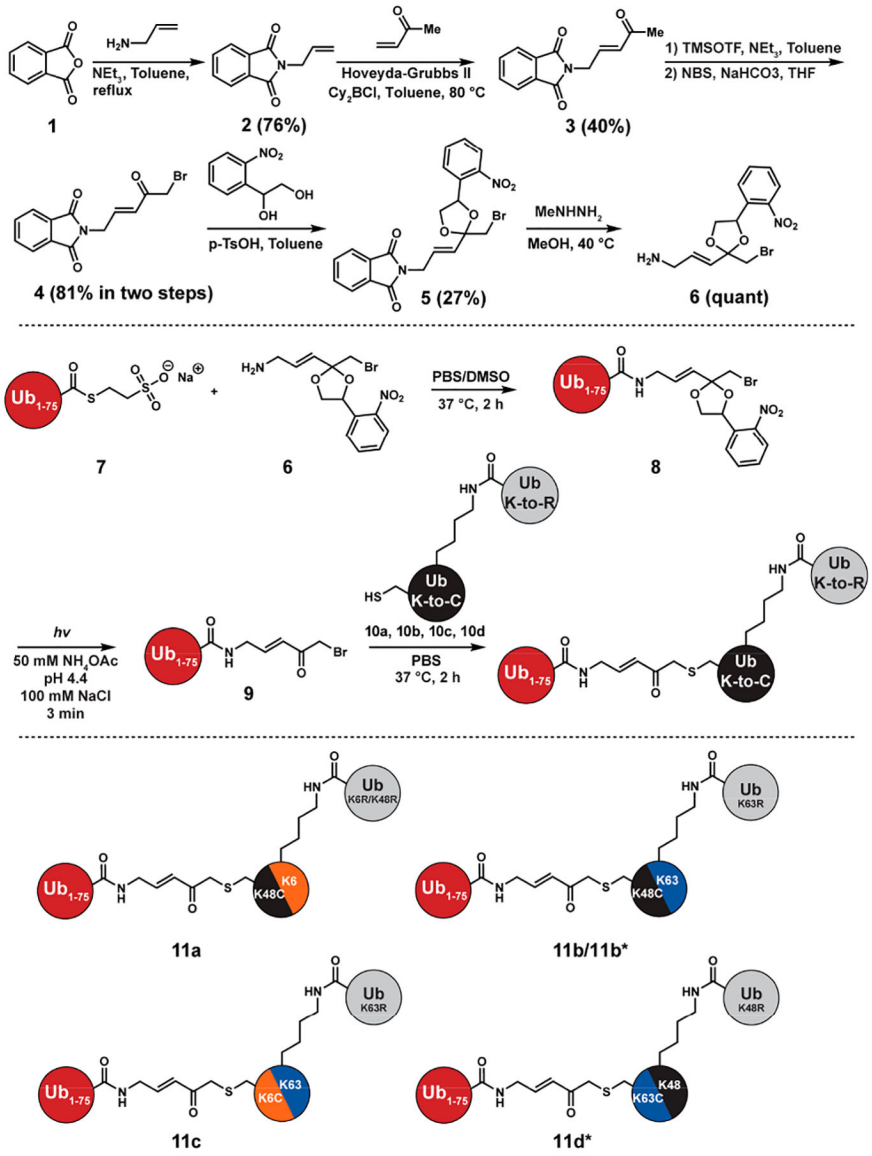


Figure 1.

Synthesis of Ub-BVK and tri-Ub ABPs. Di-Ub variants **10a** and **10b** contain a K48C substitution in the proximal Ub subunit and a native isopeptide bond at position-6 or −63, respectively. **10c** contains a K6C substitution in the proximal and a native isopeptide bond at position-63 (Lys63-linked). **10d** is a Lys48-linked dimer with a K63C substitution in the proximal subunit. **11b*** is a branched tri-Ub probe where the distal Ub attached to position-48 is N-terminally biotinylated. **11d*** is a branched tri-Ub probe where the distal Ub attached to position-63 is N-terminally biotinylated.

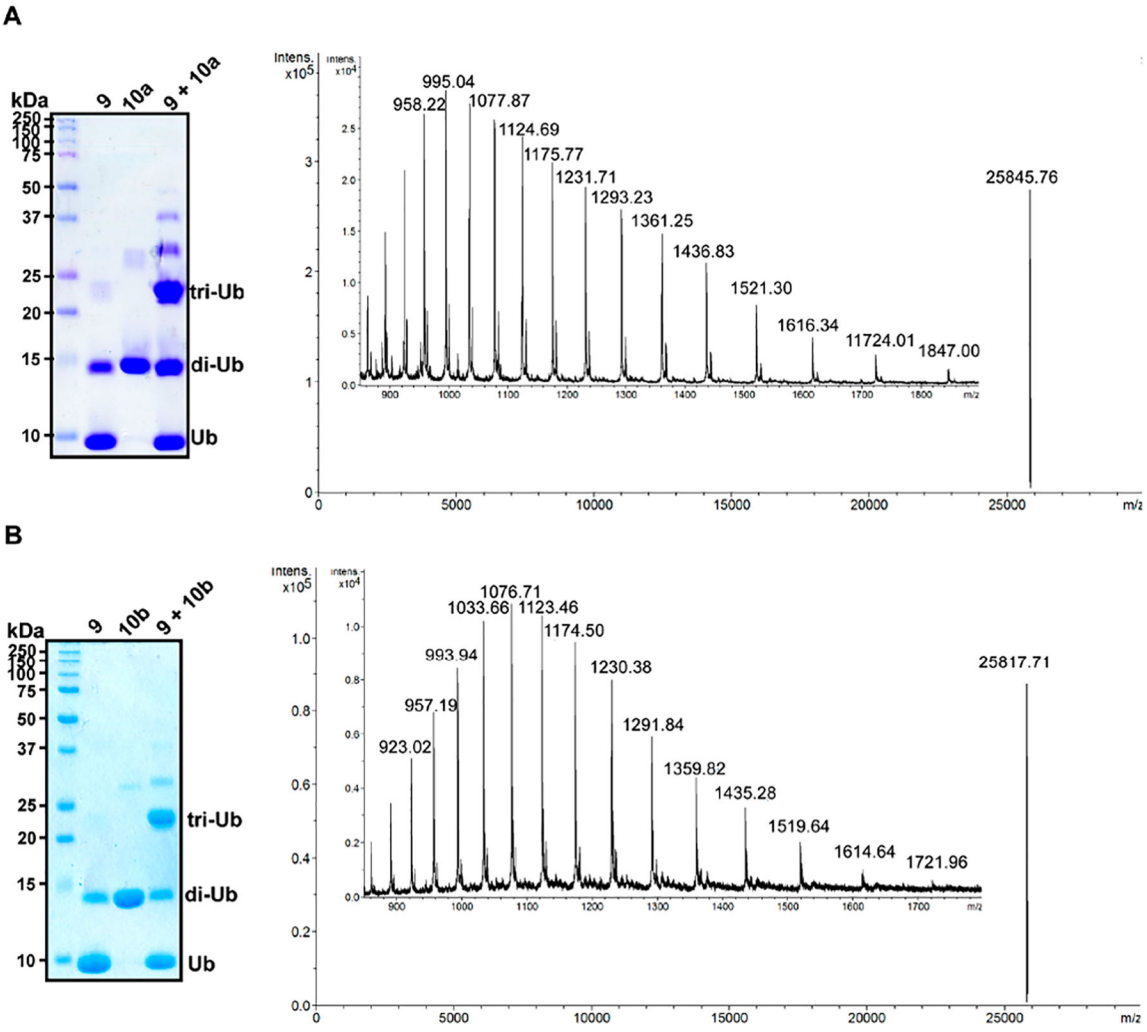


Figure 2.

Representative SDS–PAGE and MS analysis of tri-Ub ABPs. (A) Coomassie-stained SDS–PAGE gel showing the formation of probe **11a** using Ub-BVK and di-Ub **10a**. ESI-MS analysis of purified **11a** (observed deconvoluted m/z ratio for $[M + H]^+$ is 25845.8; calculated m/z is 25846.6). The di-Ub species present along with Ub-BVK 9 is formed by self-condensation of Ub-MESNa 7 while concentrating the sample. (B) SDS–PAGE gel showing the formation of **11b** starting from Ub-BVK and di-Ub **10b**. ESI-MS analysis of purified **11b** (observed deconvoluted m/z ratio for $[M + H]^+$ is 25817.7; calculated m/z is 25817.1).

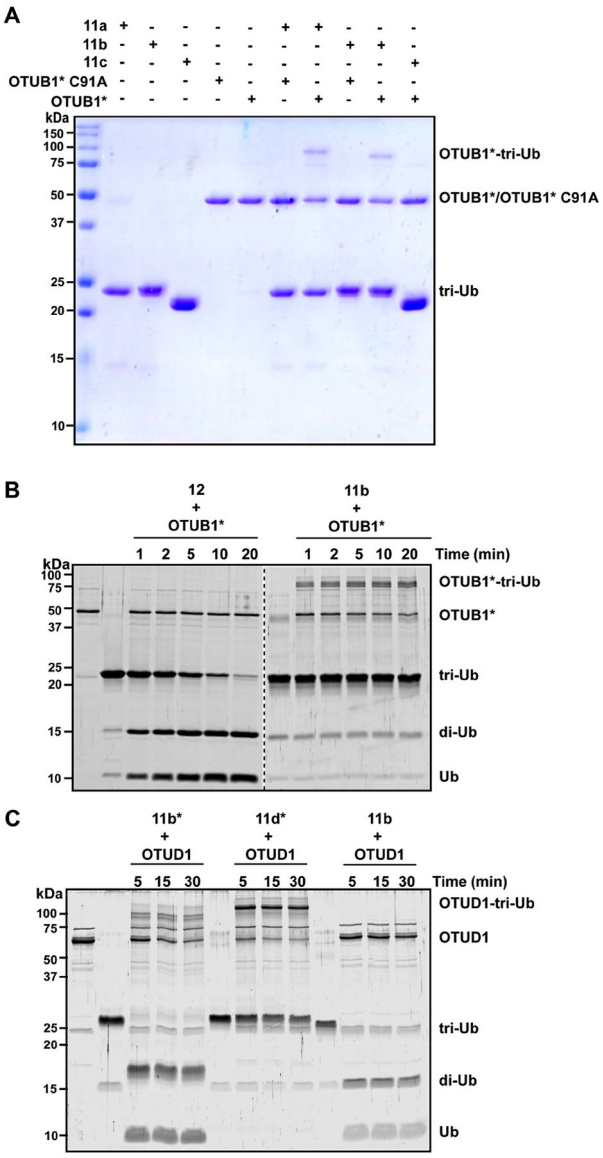


Figure 3.

Labeling DUBs with branched tri-Ub probes. (A) Coomassie-stained SDS–PAGE gel showing the reaction between tri-Ub probes **11a–c** and either active or inactive (C91A) OTUB1*. Probes were incubated with OTUB1* for 5 min at room temperature.(B) SYPRO Ruby-stained SDS–PAGE gel showing the cleavage of native K48/K63 tri-Ub (**12**) by OTUB1* over time at 37 °C (left side of the gel). Right side shows the labeling of OTUB1* with the **11b** probe at 37 °C. (C) SYPRO Ruby-stained SDS–PAGE gel showing the labeling of OTUD1 with different tri-Ub probes. Reactions were performed at 37 °C.

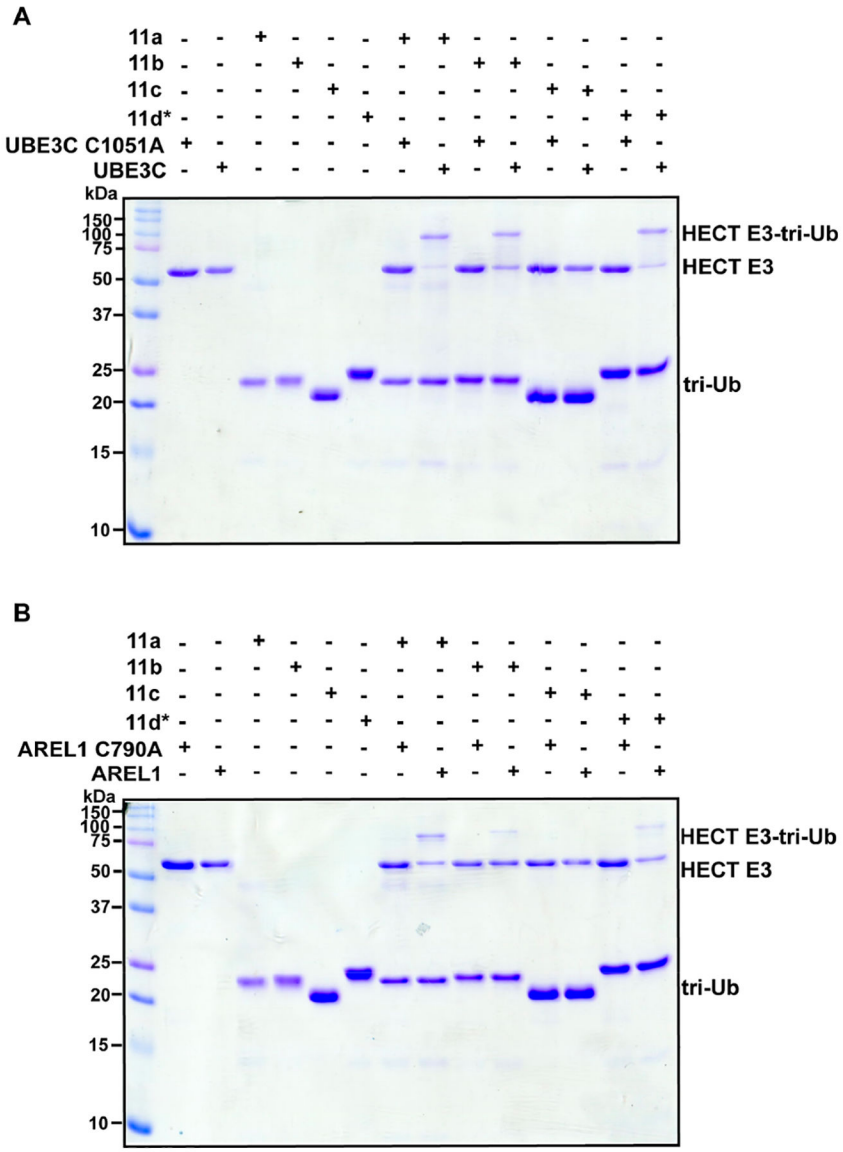
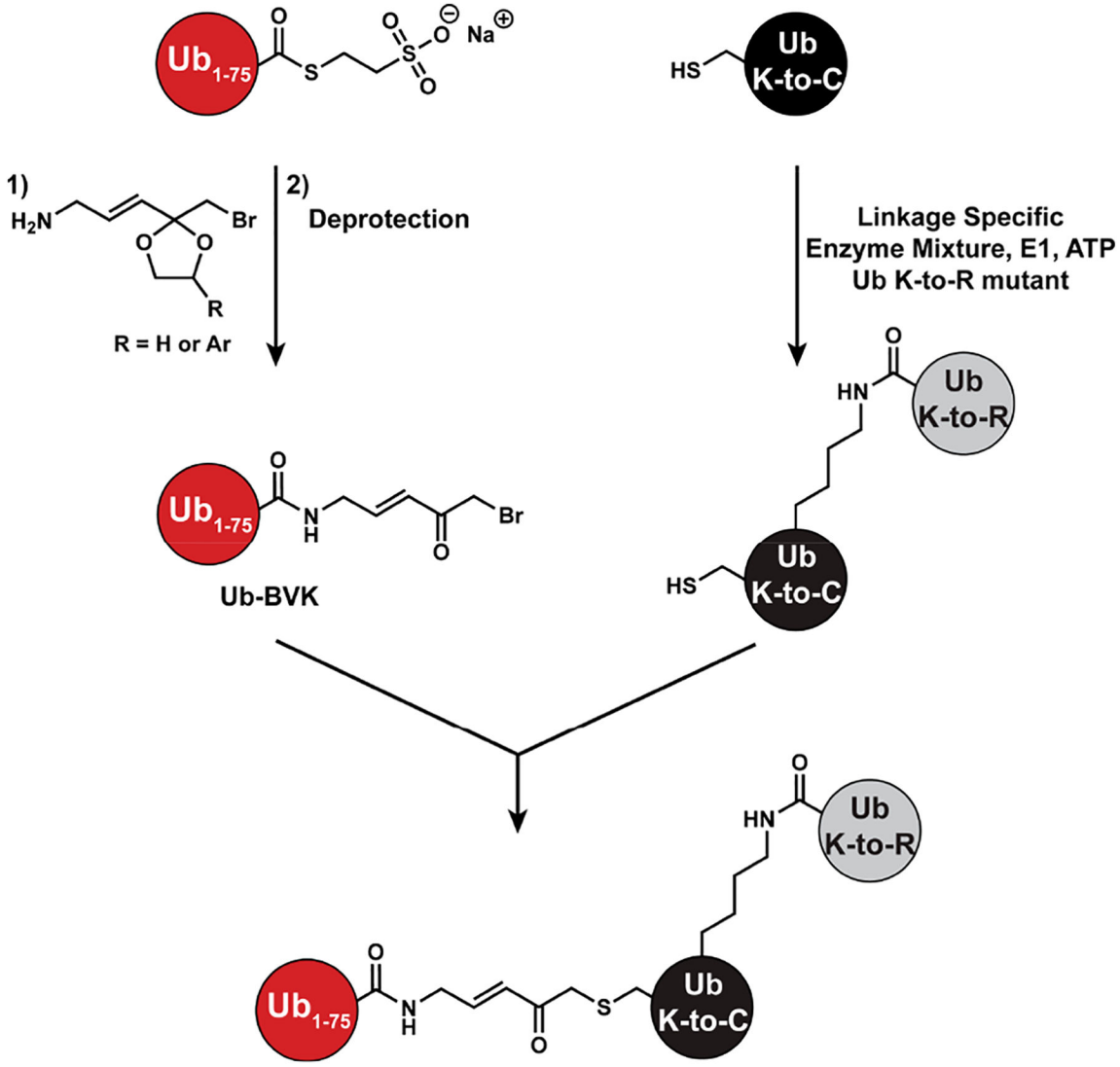


Figure 4.

Labeling of HECT E3 ligases with branched tri-Ub probes. Coomassie-stained SDS–PAGE gel showing the formation of a covalent complex between the branched ABPs **11a**, **11b**, and **11d*** and active, but not inactive, UBE3C (A) and AREL1 (B).



Scheme 1.
Strategy for Generating Branched Tri-Ub ABPs