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Strategies to trap enzyme-substrate complexes that mimic Michaelis intermediates during E3-mediated ubiquitin-like protein ligation

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Summary

Most cellular functions rely on pathways that catalyze post-translational modification of cellular proteins by ubiquitin (Ub) and ubiquitin-like (Ubl) proteins. Like other post-translational modifications that require distinct writers, readers and erasers during signaling, Ub/Ubl pathways employ distinct enzymes that catalyze Ub/Ubl attachment, Ub/Ubl recognition and Ub/Ubl removal. Ubl protein conjugation typically relies on parallel but distinct enzymatic cascades catalyzed by an E1 activating enzyme, an E2 carrier protein and an E3 ubiquitin-like protein ligase. One major class of E3, with ca. 600 members, harbor RING or the RING-like SP-RING or Ubox domains. These RING/RING-like domains bind and activate the E2-Ubl thioester by stabilizing a conformation that is optimal for nucleophilic attack by the side chain residue (typically lysine) on the substrate. These RING/RING-like domains typically function together with other domains or protein complexes that often serve to recruit particular substrates. How these RING/RING-like E3 domains function to activate the E2-Ubl thioester while engaged with substrate remains poorly understood. We describe a strategy to generate and purify a unique E2_{Ubc9}-Ubl_{SUMO} thioester mimetic that can be cross-linked to the Substrate_{PCNA} at Lys164, a conjugation site that is only observed in the presence of E3_{Siz1}. We describe two techniques to cross-link the E2_{Ubc9}-Ubl_{SUMO} thioester mimetic active site to the site of modification on PCNA and the subsequent purification of these complexes. Finally, we describe the reconstitution and purification of the E2_{Ubc9}-Ubl_{SUMO}-PCNA complex with the E3_{Siz1} and purification that enabled its crystallization and structure determination. We think this technique can be extended to other E2-Ubl-Substrate/E3 complexes to better probe the function and specificity of RING based E3 Ubl ligases.

Keywords

Ubiquitin; Ubiquitin-like protein; SUMO; Smt3; E2 conjugating enzyme; Ubc9; E3 ligase; Siz/PIAS; PCNA; complex reconstitution; structure

1. Introduction

Post-translational modification of cellular proteins with ubiquitin and ubiquitin-like (Ubl) proteins (such as Nedd8, SUMO (Smt3 in yeast), ISG15, etc.) represent parallel signaling pathways that control and regulate nearly every aspect of cellular homeostasis (1–3). Most

Ubl proteins are expressed as pro-proteins that are proteolytically processed to generate their mature forms, each by a Ubl specific protease, to generate the C-terminal Gly-Gly motif that is subsequently activated for conjugation (4–8). Each member of the canonical Ubl family can be conjugated to cellular targets, typically through the concerted activities of at least three enzymatic activities that are catalyzed by an E1 Ubl activating enzyme, an E2 Ubl carrier protein and an E3 Ubl ligase (9–13). These conjugates can be reversed by proteases similar to those that catalyze Ubl protein maturation, effectively reversing the signal and recycling the Ubl protein, the substrate, or both.

The E1 activating enzyme catalyzes ATP coupled activation of the Ubl C-terminus and E1-thioester bond formation (14–16) and subsequent formation of the E2-Ubl thioester between the E2 active site cysteine and Ubl C-terminus (Fig. 1a)(17, 18). Finally, E3 ligases help catalyze formation of the isopeptide bond between the Ubl and the target substrate (Fig. 1b) (9, 19, 20). A large number of these conjugation events utilize RING type Ubl E3 ligases (21, 22). These ligases harbor RING domains (23, 24) (or the highly related SP-RING (25–29) or Ubox (30) domains) that bind the E2-Ubl thioester complex to promote a closed and activated conformation of the E2-Ubl thioester, amenable to nucleophilic attack (usually by a primary amine, typically a lysine residue) present on the target substrate (31–48). The family of proteins containing RING-like domains is large (~600 members) and diverse. The RING-like domains are found appended to (or complexed with) a diversity of additional domains, many of which recruit the target substrate (22). A major dearth of knowledge in the field of Ubl conjugation is how this diverse repertoire of substrate recruitment domains function in concert with the RING-like domain/E2-Ubl thioester complexes to execute targeted modification of particular substrates.

To better understand how this catalytic machinery functions during conjugation to a specific position on a particular substrate, it is often useful to determine the three-dimensional structure of the reconstituted complex. Historically, reconstituting these complexes in a manner amenable to structural studies proved challenging. First, the E2-Ubl thioester complex is labile in the presence of the E3, which can activate it for non-discriminate discharge to water or buffer components, particularly in the conditions and protein concentrations necessary for structural studies. Other groups developed E2-Ubl thioester mimetics with stabilized covalent linkages between the E2 active site (Cys to Ser or Cys to Lys) and the Ubl C-terminus, making them recalcitrant to discharge of the Ubl. These mimetics have proved useful in obtaining RING/E2-Ubl complex structures (Fig. 1c,d)(33–50). Second, interactions between the E2-Ubl thioester and the E3 and the E2-Ubl/E3 complex and the target substrate are often too transient with rapid association, turnover and dissociation (51). This makes stoichiometric reconstitution and purification challenging. Given these issues, it is perhaps unsurprising that the majority of E2/RING E3 structures resulted from co-crystallization strategies, where crystallization serves to promote protein-protein interactions. Similar co-crystallization strategies have proven useful in structure determination of some substrates in complex with substrate recognition domains. While useful, these strategies have largely been unsuccessful for reconstituting complete E2-Ubl/E3/Substrate complexes. Notably, the Schulman laboratory utilized alternative cross-linking strategies to those discussed here to trap other E2-Ubl/E3/Substrate complexes (52–55).

We pursued numerous unsuccessful attempts to reconstitute a complex between the SUMO specific E3_{Siz1}, the E2_{Ubc9}-SUMO thioester and the Proliferating Cell Nuclear Antigen (PCNA), a well-established target of SUMO modification in yeast(56–58). To overcome this challenge, we adopted a chemical cross-linking strategy to aid in complex reconstitution(59). Other groups have utilized E2 active site Cys to Ser or Lys substitutions that can be used with the E1 activating enzyme to catalyze formation of a stable oxyester or isopeptide linkage, respectively, to the Ubl C-terminus (instead of the native thioester linkage) to create more stable E2-Ubl thioester mimetics (Fig 1c,d). We created an alternative thioester mimetic with the E2_{Ubc9} A129K substitution (adjacent to the active site Cys93) to move the E2-Ubl linkage to an alternative position just adjacent to the active site cysteine (Fig. 1e). This has the advantage of leaving the active site cysteine available for chemical cross-linking to the target substrate. With respect to the substrate, we substituted the E3-dependent site of modification, in this case PCNA Lys164, to cysteine for cross-linking. We hypothesized this covalently linked multi-protein E2-SUMO-PCNA complex would present multiple binding sites to E3_{Siz1}, thus generating a higher affinity, stoichiometric complex with E3_{Siz1}. This turned out to be partially true, however further stabilization and reconstitution of the complex was achieved by fusing a second molecule of SUMO to the C-terminus of the E3 in a location that enabled formation of a stable backside interaction with the E2_{Ubc9}, as observed previously (Fig. 2) (60–62). This strategy resulted in the successful reconstitution of an enzyme-substrate complex that crystallized readily.

Presented here are two chemical cross-linking strategies, both of which employ straightforward and technically accessible methods that utilize standard protein purification equipment and commercially available reagents. The first technique utilizes bismaleimidoethane (BMOE) that creates a cross-link that is stable to reducing agents post reaction between the respective cysteine residues. Utilizing BMOE, we reconstituted the E2-SUMO/E3/Substrate complex and solved the crystal structure. The structure revealed that the BMOE linkage was suboptimal, perhaps due to its bulky maleimide ring structures and linker that creates a suboptimal distance between the E2 and substrate after cross-linking (Fig. 3). This led us to employ 1,2-ethanedithiol (EDT) as an alternative cysteine-to-cysteine cross-linker, creating an atomic bridge with the same number of atoms as the predicted tetrahedral intermediate (Fig. 1b and Fig. 4). Although less stable to reducing agents, in comparison to a BMOE cross-link, EDT is a better mimic of conformation and distance for isopeptide bond formation despite having unique dihedral angles at the two disulfide bonds. The EDT strategy also led to successful enzyme-substrate complex reconstitution, crystallization and structure determination.

The strategy of E2-Ubl thioester mimetic formation, cross-linking of the E2 active site to the site of modification on the substrate and reconstitution with the E3 may prove useful in reconstituting other E2-Ubl/E3/Substrate complexes for structural study (see Note 1). Elucidating structural details for other such complexes will shed light on the manner in which the E2-Ubl/RING association works in concert with the immense variety and diversity of substrate recruitment domains/substrate interactions to affect specific conjugation across the variety of extant RING E3 ligases.

2. Materials

2.1 Cloning, Protein Expression and Purification.

Stock solutions should be prepared in water unless otherwise noted and 0.2 μ M filtered.

1. 18.2 M Ω -cm ultrapure water for all solutions.
2. Plasmids: pET15b, pET21a, pET28b, pSMT3 and TOPO-SMT3 (see Note 2).
3. Oligonucleotide primers.
4. Thermostable proofreading fusion polymerase (Herculase II, Agilent).
5. dNTP mixture.
6. Thermocycler.
7. Luria-Bertani (LB) medium: 10 g tryptone, 10 g NaCl, 5 g yeast extract, 1.5 g TRIS-HCl in 1 L water, autoclaved.
8. Luria-Bertani (LB) agar medium: 10 g tryptone, 10 g NaCl, 5 g yeast extract, 1.5 g TRIS-HCl, 15 g agar in 1 L water, autoclaved and poured in plates.
9. Super Broth (SB) medium: 32 g tryptone, 20 g yeast extract, 5 g NaCl, 9.87 g potassium phosphate dibasic in 1 L water, autoclaved.
10. Antibiotics: 100 mg/ml Ampicillin in water; 50 mg/ml Kanamycin in water; 30 mg/ml Chloramphenicol in ethanol.
11. Bacterial strains: *Escherichia coli* (*E. coli*) Top10 (Thermo Fisher) or *E. coli* (Lucigen) (for cloning), *E. coli* BL21 (DE3) RIL Codon Plus (Agilent) (for expression).
12. Shaking incubators capable of a temperature range of 18–37 °C.
13. 2 L baffled shaking flasks.
14. 1 M Isopropyl β -D-thiogalactopyranoside (IPTG) in water.
15. Bovine thrombin (Sigma): 1 U/ μ L resuspended in 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl (pH 7.4) (PBS). Aliquot, flash freeze and store at –20 °C.
16. Ulp1 protease catalytic domain (amino acids 403–621)(63): 3 mg/ml in 20 mM TRIS-HCl (pH 8.0), 350 mM NaCl, 1 mM β -mercaptoethanol (BME), 10% glycerol (see Note 3).
17. HIS-GST-Ulp1 protease catalytic domain (amino acids 403–621): 4.8 mg/ml in 20 mM TRIS-HCl (pH 8.0), 350 mM NaCl, 1 mM BME (see Note 3).
18. 1 M N-Ethylmaleimide (NEM) in ethanol stock solution, diluted to 20 mM in water.
19. 1 M TRIS-HCl (pH 8.0 at 4 °C).
20. 14.3 M BME.

21. 100 mM phenylmethanesulfonyl fluoride (PMSF) in ethanol.
22. 1 M MgCl_2 .
23. 2 M CaCl_2 .
24. 4 M imidazole.
25. 10 % IGEPAL CA-630.
26. 5 M NaCl.
27. 10 mg/ml lysozyme in 20 mM TRIS-HCl (pH 8.0), 50 mM NaCl. Aliquot, flash freeze and store at -20°C .
28. 10 mg/ml DNase I in 20 mM TRIS-HCl (pH 8.0), 50 mM NaCl. Aliquot, flash freeze and store at -20°C .
29. Suspension buffer: 50 mM TRIS-HCl (pH 8.0), 20 % sucrose.
30. Lysis buffer A: 50 mM TRIS-HCl (pH 8.0), 20% sucrose, 20 $\mu\text{g/ml}$ lysozyme, 5 mM BME, 2.5 mM MgCl_2 , 0.5 mM CaCl_2 , 20 mM imidazole, 0.05% IGEPAL CA-630, 100 $\mu\text{g/ml}$ DNase I, 350 mM NaCl (see Note 4).
31. Lysis buffer B: 50 mM TRIS-HCl (pH 8.0), 20% sucrose, 20 $\mu\text{g/ml}$ lysozyme, 5 mM BME, 1 mM PMSF, 2.5 mM MgCl_2 , 0.5 mM CaCl_2 , 20 mM imidazole, 0.05% IGEPAL CA-630, 100 $\mu\text{g/ml}$ DNase I, 350 mM NaCl.
32. Lysis buffer C: 50 mM TRIS-HCl (pH 8.0), 20% sucrose, 20 $\mu\text{g/ml}$ lysozyme, 5 mM BME, 1 mM PMSF, 2.5 mM MgCl_2 , 0.5 mM CaCl_2 , 0.05% IGEPAL CA-630, 100 $\mu\text{g/ml}$ DNase I, 350 mM NaCl.
33. Lysis buffer D: 50 mM TRIS-HCl (pH 8.0), 20% sucrose, 20 $\mu\text{g/ml}$ lysozyme, 5 mM BME, 2.5 mM MgCl_2 , 0.5 mM CaCl_2 , 0.05% IGEPAL CA-630, 100 $\mu\text{g/ml}$ DNase I, 150 mM NaCl.
34. Branson Ultrasonics Digital Sonifier with 1/2-inch horn, or equivalent.
35. Ni-NTA Superflow agarose resin (Qiagen).
36. Buffer A: 20 mM TRIS-HCl (pH 8.0), 350 mM NaCl, 20 mM imidazole, 1 mM BME.
37. Buffer B: 20 mM TRIS-HCl (pH 8.0), 350 mM NaCl, 250 mM imidazole, 1 mM BME.
38. Buffer C: 20 mM TRIS-HCl (pH 8.0), 350 mM NaCl, 1 mM BME.
39. Buffer D: 20 mM TRIS-HCl (pH 8.0), 100 mM NaCl, 1 mM BME.
40. Buffer E: 20 mM TRIS-HCl (pH 8.0), 1 M NaCl, 1 mM BME.
41. Buffer F: 20 mM TRIS-HCl (pH 8.0), 50 mM NaCl, 1 mM BME.
42. Buffer G: 20 mM TRIS-HCl (pH 8.0), 200 mM NaCl, 1 mM BME.

43. AKTA-FPLC (GE Healthcare) or an equivalent workstation equipped with gel filtration columns (HiLoad 26/60 Superdex 75 and Superdex 200) and ion exchange columns (MonoQ 10/100 GL and Mono S 10/100 GL) (*see* Note 5).
44. Amicon Ultra centrifugal filter devices (Millipore) with 3K, 10K and 30K molecular weight cutoffs.
45. Bradford reagent.
46. NanoDrop 2000 Spectrophotometer (Thermo Scientific) or equivalent.
47. Glutathione Sepharose 4B (GE Healthcare).
48. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE):
 - a. NuPAGE system (Invitrogen) for SDS-PAGE utilizing either MES or MOPS running buffer.
 - b. 12% and 4–12% gradient polyacrylamide BIS-TRIS gels (Invitrogen).
49. Ammonium sulfate, (NH₄)₂SO₄ (powder).
50. 2K and 8K molecular weight cutoff dialysis tubing.

2.2 E2-SUMO Formation Reaction.

1. 1 M BIS-TRIS Propane (pH 9.5).
2. 1 M MgCl₂.
3. 5 M NaCl.
4. 10 % Triton X-100.
5. 1 M DTT.
6. 100 mM ATP in 10 mM HEPES (pH 7.5).

2.3 E2-SUMO-PCNA Cross-linking Reaction.

1. 1 M TCEP in 10 mM HEPES (pH 7.5).
2. PD-10 (GE Healthcare) and/or Zeba (Thermo Fisher) desalting columns, or equivalent.
3. Cross-linking buffer: 20 mM HEPES, 200 mM NaCl, 5 mM EDTA.
4. Bismaleimidoethane (BMOE, Pierce) solution: 100 mM BMOE in dimethyl sulfoxide (DMSO).
5. Aldrithiol-2 (AT2) solution: 300 mM AT2 in DMSO.
6. 1,2-ethanedithiol (EDT) solution: 300 mM EDT in DMSO.
7. Buffer H: 20 mM TRIS-HCl (pH 8.0), 350 mM NaCl.
8. Buffer I: 20 mM TRIS-HCl (pH 8.0), 200 mM NaCl.
9. Buffer J: 20 mM TRIS-HCl (pH 8.0), 1000 mM NaCl.

2.4 Final Reconstitution with the E3.

1. Buffer K: 20 mM TRIS-HCl (pH 8.0), 50 mM NaCl, 5 mM EDTA.

3. Methods

3.1 Protein Expression and Purification (described previously (29, 32, 58, 64–66)).

3.1.1 Cloning and Purification of N18Smt3.

1. Residues 19–98 of yeast Smt3, representing mature C-terminally processed Smt3 lacking the disordered N-terminal tail, are amplified by PCR from yeast genomic DNA and subsequently cloned into the 5' NdeI and 3' XhoI sites of pET28b. The expressed protein will have an N-terminal, thrombin cleavable, His₆ fusion tag.
2. Transform the plasmid into *E. coli* BL21 (DE3) RIL Codon Plus cells.
3. Inoculate 50 ml of LB or SB with the Codon Plus cells harboring the plasmid and let grow at 37 °C overnight for ca. 16 hours with shaking. Inoculate 4 × 1 L of Superbroth in 2 L baffled shaking flasks, from the overnight culture, to a final optical density at 600 nm (OD₆₀₀)=0.1 and let grow at 37 °C. When the culture OD₆₀₀=1.0–2.0, induce with 1 mM IPTG and let grow 3–4 hours at 30 °C.
4. Collect cells by centrifugation (4500 × g). Suspend cell pellets in suspension buffer (2 ml of buffer per g of wet cell mass). Flash freeze in liquid nitrogen and store at –80 °C for future use.
5. Rapidly thaw suspended cells in warm water. On ice, adjust composition of the cell solution to that of Lysis Buffer A by adding each of the components from stock solutions, with rapid mixing (assuming a volume increase of ca. 1.2-fold).
6. On ice/water slurry with rapid stirring (ideally in a cold room), sonicate at 60% output for ca. 1 minute for every 100 ml of lysate with 5 seconds on, 8 seconds off to disrupt cells.
7. Pellet cell debris for 30 minutes at 40,000 × g at 4 °C and collect the cleared supernatant (see Note 6).
8. Apply the supernatant to 10 ml of NiNTA resin in a chromatography column, equilibrated with Buffer A. Wash column with 10 column volumes of Buffer A followed by elution with Buffer B. Locate the peak of the eluate with Bradford reagent and/or analyze by SDS-PAGE.
9. Pool the eluate peak and incubate with 100 units of thrombin and dialyze overnight against 2 L Buffer C in 2K molecular weight cutoff dialysis tubing at 4 °C. Confirm cleavage of the His₆ fusion tag by SDS-PAGE.
10. Resolve the dialysate over Superdex 75 gel filtration column equilibrated with Buffer C. Analyze the fractions by SDS-PAGE.
11. Collect the peak fractions and concentrate to ca. 30–45 mg/ml. Measure the OD₂₈₀ and determine the final protein concentration with the extinction coefficient 1490 M^{–1} cm^{–1} or 1 OD₂₈₀ = 6.22 mg/ml. Extinction coefficient

determined by Protean software within Lasergene 8 suite (DNASTAR). Aliquot, flash freeze and store at -80°C for later use.

3.1.2 Cloning and Purification of the Yeast SUMO/Smt3 E1 Activating Enzyme.

1. The yeast SUMO E1 activating enzyme is a heterodimer complex of Aos1 and Uba2. The yeast genes for full-length Aos1 and C-terminally truncated Uba2 (C-term, amino acids 1–554) are amplified by PCR from yeast genomic DNA.
2. Yeast Aos1 is cloned into pET15b using the 5' NcoI and 3' BamHI sites and Uba2 C-term is ligated into pET28b using the 5' NdeI and 3' XhoI sites. Uba2 C-term is expressed with an N-terminal, thrombin cleavable, His₆ fusion tag.
3. Transform both plasmids, together, into *E. coli* BL21 (DE3) RIL Codon Plus cells for coexpression.
4. The Codon Plus cells with the plasmids are inoculated into 200 ml of LB or SB and let grow at 37°C overnight for ca. 16 hours with shaking. Inoculate $12 \times 1\text{ L}$ of Superbroth in 2 L baffled shaking flasks, from the overnight culture, to a final $\text{OD}_{600}=0.1$ and let grow at 37°C . When the culture reaches $\text{OD}_{600}=1.0\text{--}2.0$, cold shock in ice water for 30 minutes, induce with 2% ethanol and 1 mM IPTG. The temperature is shifted to 18°C and cells are induced overnight for ca. 16 hours.
5. Cells are harvested and the pellets are processed for lysis by sonication as described in Sect. 3.1.1, with the exception of using Lysis Buffer B composition.
6. Apply the supernatant to 10 ml of NiNTA resin in a chromatography column, equilibrated with Buffer A. Wash column with 10 column volumes of Buffer A followed by elution with Buffer B. Locate the peak of the eluate with Bradford reagent and/or analyze by SDS-PAGE.
7. Pool the eluate peak and incubate with 100 units of thrombin and dialyze overnight against 2 L Buffer C in 8K molecular weight cutoff dialysis tubing at 4°C . Confirm cleavage of the His₆ fusion tag by SDS-PAGE.
8. Resolve the dialysate over Superdex 200 gel filtration column equilibrated with Buffer C. Analyze the fractions by SDS-PAGE.
9. Pool peak fractions containing the Aos1/Uba2 C-term heterodimer complex. Dilute with 20 mM TRIS-HCl (pH 8.0), 1 mM BME to final $[\text{NaCl}]=100\text{ mM}$ or desalt to Buffer D.
10. Load this mixture onto anion-exchange resin (Mono-Q). The protein is eluted with a linear gradient from Buffer D to 35% Buffer E over 20 column volumes. The Aos1/Uba2 C-term complex elutes between ca. 200–300 mM NaCl.
11. Collect the peak fractions and concentrate to ca. 10 mg/ml. Measure the OD_{280} and determine the final protein concentration with the extinction coefficient $65,710\text{ M}^{-1}\text{ cm}^{-1}$ or $1\text{ OD}_{280}=1.55\text{ mg/ml}$. Aliquot, flash freeze and store at -80°C for later use.

3.1.3 Cloning and Purification of the Yeast E2 Ubc9^{C5S/A129K/K153R} Mutant.

1. The SUMO specific yeast E2, Ubc9, is amplified by PCR from yeast genomic DNA and subsequently cloned into the 5' NdeI and 3' HindIII sites of pET28b. The expressed protein will have a N-terminal, thrombin cleavable, His₆ fusion tag.
2. Three successive site directed mutagenesis reactions generate the K153R mutation, the A129K/K153R mutation combination and the C5S/A129K/K153R mutation combination yielding the pET28b-E2_{Ubc9}^{C5S/A129K/K153R} construct (see Note 7).
3. The plasmid is transformed into *E. coli* BL21 (DE3) RIL Codon Plus cells.
4. Inoculate 50 mL of LB or SB with the Codon Plus cells harboring the plasmid and let grow at 37 °C overnight for ca. 16 hours with shaking. Inoculate 4 × 1 L of Superbroth in 2 L baffled shaking flasks from the overnight culture to a final OD₆₀₀=0.1 and let grow at 37 °C. When the culture reaches OD₆₀₀=1.0–2.0, induce with 1 mM IPTG and let grow 3–4 hours at 30 °C.
5. Cells are harvested and the pellets are processed for lysis by sonication as described in Sect. 3.1.1, again using Lysis Buffer A composition.
6. Apply the supernatant to 10 ml of NiNTA resin in a chromatography column, equilibrated with Buffer A. Wash column with 10 column volumes of Buffer A followed by elution with Buffer B. Locate the peak of the eluate with Bradford reagent and/or analyze by SDS-PAGE.
7. Pool the eluate peak and incubate with 100 units of thrombin and dialyze overnight against 2 L Buffer C in 8K molecular weight cutoff dialysis tubing at 4 °C. Confirm cleavage of the His₆ fusion tag by SDS-PAGE.
8. Resolve the dialysate over Superdex 75 gel filtration column equilibrated with Buffer C. Analyze the fractions by SDS-PAGE.
9. Typically, the protein is sufficiently pure at this point, however, if further purity is required, pool peak fractions containing Ubc9^{C5S/A129K/K153R}. Dilute with 20 mM TRIS-HCl (pH 8.0), 1 mM BME to final [NaCl]= 100 mM or desalt to Buffer D.
10. Load this mixture onto cation-exchange resin (Mono-S). The protein is eluted with a linear gradient from Buffer D to 35% Buffer E over 20 column volumes. The Ubc9^{C5S/A129K/K153R} protein elutes at approximately 150–230 mM NaCl.
11. Collect the peak fractions and concentrate to between 15–45 mg/ml. Measure the OD₂₈₀ and determine the final protein concentration with the extinction coefficient 39,545 M⁻¹ cm⁻¹ or 1 OD₂₈₀ = 0.45 mg/ml. Aliquot, flash freeze and store at –80 °C for later use.

3.1.4 Cloning and Purification of the Yeast E3 Siz1_(167–449)^{C361D}- N18Smt3 Fusion Protein.

1. The SUMO specific yeast E3, Siz1, catalytic fragment (residues 167–449) is amplified by PCR from yeast genomic DNA and subsequently cloned into the 5' BamHI and 3' HindIII sites of pSmt3, creating pSmt3-Siz1_(167–449). Subsequently, N18Smt3 is amplified from the plasmid in Sect. 3.1.1 and ligated into the 5' HindIII and 3' XhoI of the pSmt3-Siz1_(167–449) plasmid, creating pSmt3-Siz1_(167–449)- N18Smt3. The expressed Siz1_(167–449)- N18Smt3 fusion protein will have an N-terminal, Ulp1 cleavable, His₆-Smt3 fusion tag.
2. The Siz1 C361D mutation is introduced into pSmt3-Siz1_(167–449)- N18Smt3 by site directed mutagenesis. Cysteine 361 occurs within the SP-RING domain but is not involved in Zn²⁺ coordination (29) and may be prone to oxidation and Siz1 inactivation. In *Candida albicans* and *Caenorhabditis elegans* the homologous residue is an aspartate, thus our choice for mutation.
3. The plasmid is transformed into *E. coli* BL21 (DE3) RIL Codon Plus cells.
4. Inoculate 200 mL of LB or SB with the Codon Plus cells harboring the plasmid and let grow at 37 °C overnight for ca. 16 hours with shaking. Inoculate 8 × 1 L of Superbroth in 2 L baffled shaking flasks from the overnight culture to a final OD₆₀₀=0.1 and let grow at 37 °C. When the culture reaches OD₆₀₀=1.0–2.0, cold shock in ice water for 30 minutes, then induce with 2% ethanol and 1 mM IPTG and let grow ca. 16 hours at 18 °C.
5. Cells are harvested and the pellets are processed for lysis by sonication as described in Sect. 3.1.1, with the exception of using Lysis Buffer C composition.
6. Apply the supernatant to 10 ml of NiNTA resin in a chromatography column, equilibrated with Buffer A. Wash column with 10 column volumes of Buffer A followed by elution with Buffer B. Locate the peak of the eluate with Bradford reagent and/or analyze by SDS-PAGE.
7. Pool the eluate peak and incubate with 1:10,000 His₆-Ulp1 or His₆-GST-Ulp1 (mg Ulp:mg Smt3-Siz1- N18Smt3)(see Note 3) and dialyze overnight against 2 L Buffer C in 8K molecular weight cutoff dialysis tubing at 4 °C. Confirm cleavage of the His₆-Smt3 fusion tag by SDS-PAGE.
8. If using His₆-GST-Ulp1, pass dialysate cleavage product over 3 ml glutathione beads equilibrated in Buffer C collecting the unadsorbed protein flow-through.
9. Resolve the flow-through over Superdex 200 gel filtration column equilibrated with Buffer C. Analyze the fractions by SDS-PAGE.
10. Pool peak fractions and dilute with 20 mM TRIS-HCl (pH 8.0), 1 mM BME to final [NaCl]= 100 mM or desalt to Buffer D.
11. Load this mixture onto anion-exchange resin (Mono-Q). The protein is eluted with a linear gradient from Buffer D to 35% Buffer E over 20 column volumes.

The Siz1_(167–449)^{C361D}- N18Smt3 protein elutes between ca. 220–280 mM NaCl.

12. Collect the peak fractions and concentrate to ca. 10–12 mg/ml. Measure the OD₂₈₀ and determine the final protein concentration with the extinction coefficient 33,890 M⁻¹ cm⁻¹ or 1 OD₂₈₀ = 1.23 mg/ml. Aliquot, flash freeze and store at –80 °C for later use.

3.1.5 Cloning and Purification of Yeast PCNA_{K127G/K164C} and PCNA_{K77D/C81E/R110D/K127G/K164C}

1. The current chemical cross-linking protocol was performed with both trimeric (PCNA_{K127G/K164C}) and monomeric (PCNA_{K77D/C81E/R110D/K127G/K164C}). Both monomeric and trimeric versions were reconstituted into final E2-SUMO/E3/ Substrate complexes and crystallized, however the crystals for trimeric did not diffract to sufficient resolution to yield a structure.
2. Full-length PCNA is amplified by PCR from yeast genomic DNA and subsequently cloned into the 5' NdeI and 3' XhoI sites of pET21a in order to express a tag-less protein with native termini.
3. Serial site directed mutagenesis generates the K127G mutation and then the K127G/K164C mutation combination to utilize a thiol-based cross-linking strategy at the E3-dependent site of modification (K164). Subsequent mutagenesis created the K77D/C81E/R110D/K127G/K164C combination of mutations to create monomeric PCNA with the same ability to chemically cross-link.
4. The plasmids are transformed, separately, into *E. coli* BL21 (DE3) RIL Codon Plus cells.
5. Inoculate 50 mL of LB or SB with the Codon Plus cells harboring the plasmid and let grow at 37 °C overnight for ca. 16 hours with shaking. Inoculate 4 × 1 L of Superbroth in 2 L baffled shaking flasks from the overnight culture to a final OD₆₀₀=0.1 and let grow at 37 °C. When the culture reaches OD₆₀₀=1.0–2.0, induce with 1 mM IPTG and let grow ca. 16 hours at 18 °C.
6. Cells are harvested and the pellets are processed for lysis by sonication as described in Sect. 3.1.1, with the exception of utilizing Lysis Buffer D composition.
7. Collect the supernatant and, with rapid stirring at 4 °C, slowly add dry (NH₄)₂SO₄ to 40% (for trimeric PCNA_{K127G/K164C}) or 50% (for monomeric PCNA_{K77D/C81E/R110D/K127G/K164C}). Let this mix at 4 °C for 4 hr to overnight. Pellet the precipitate at 40,000 × g for 30 min at 4 °C. Collect the supernatant and, with rapid stirring at 4 °C, slowly add dry (NH₄)₂SO₄ to 70%. Let mix 4 hr at 4 °C. Pellet the precipitate at 40,000 × g for 30 min at 4 °C. Dissolve the pellet in 4 ml 20 mM TRIS-HCl (pH 8.0), 1 mM BME. Dialyze the dissolved pellet(s) against 2 L Buffer C for 4 hr to overnight at 4 °C in 8K MWCO dialysis tubing.

8. Resolve the dialysate over Superdex 200 gel filtration column equilibrated with Buffer C (may require two runs). Analyze the fractions by SDS-PAGE.
9. Pool peak fractions and dilute with 20 mM TRIS-HCl (pH 8.0), 1 mM BME to final [NaCl]= 200 mM or desalt to Buffer G.
10. Load one fourth of this mixture (to avoid overloading) onto anion-exchange resin (Mono-Q). The protein is eluted with a linear gradient from Buffer G to 30% Buffer E over 10 column volumes. The trimeric PCNA_{K127G/K164C} protein elutes between ca. 330–440 mM NaCl and monomeric PCNA_{K77D/C81E/R110D/K127G/K164C} elutes between ca. 200–380 mM NaCl. Repeat for three more column runs. Analyze the fractions by SDS-PAGE.
11. Collect the peak fractions and concentrate PCNA_{K127G/K164C} to ca. 70 mg/ml and measure the OD₂₈₀ and determine the final protein concentration with the extinction coefficient 6,585 M⁻¹ cm⁻¹ or 1 OD₂₈₀ = 4.38 mg/ml. Concentrate monomeric PCNA_{K77D/C81E/R110D/K127G/K164C} to 40–100 mg/ml and measure the OD₂₈₀ and determine the final protein concentration with the extinction coefficient 6,460 M⁻¹ cm⁻¹ or 1 OD₂₈₀ = 4.46 mg/ml. Aliquot, flash freeze and store at –80 °C for later use.

3.2 E2-SUMO Formation Reaction

1. The E2-SUMO thioester mimetic is formed in a reaction containing the E1_{Aos1/Uba2}, E2_{Ubc9}, and Ubl_{N18SUMO}, followed by purification of the mimetic from the E1, the unreacted E2 and SUMO and in some instances any E2–2(SUMO) or E2-(SUMO)₂ side products (see Note 8). Our early explorations with the formation of the E2-SUMO mimetic, prior to employing the chemical cross-linking strategy, were performed with E2_{Ubc9}^{A129K/K153R} (Fig. 5). The E2_{Ubc9}^{C5S/A129K/K153R} mutant, necessary for specificity of cross-linking, behaves similarly in these types of reactions. In our explorations, we found that the presence of the E3_{Siz1} increased the rate of E2-SUMO formation, in the case of the E2_{Ubc9}^{A129K/K153R} mutant but not the E2_{Ubc9}^{C93K} mutant. We interpret this result to indicate that, in the case of the E2_{Ubc9}^{C5S/A129K/K153R} mutant, SUMO is transferred from the E1 onto the E2 active site cysteine to form an E2-SUMO thioester. This thioester is then attacked by the lysine installed at position 129 to form the isopeptide linkage adjacent to the active site cysteine. These data suggest binding of the E3 to the charged E2 stimulates conjugation to the installed lysine residue by orienting SUMO in the closed and most productive conformation for nucleophilic attack. Our final preparative protocol did not employ E3 to form the E2-SUMO mimetic as we opted to bypass the requirement of the E3 by performing the reactions at higher pH to ensure lysine deprotonation with fewer protein components.
2. Set up a reaction with 50 mM BIS-TRIS Propane (pH 9.5), 50 mM NaCl, 10 mM MgCl₂, 0.1% Tween-20, 1 mM DTT, 0.5 μM E1_{Aos1/Uba2}, 100 μM E2_{Ubc9}^{C5S/A129K/K153R}, and 200 μM N18Smt3. Save aliquot for analysis by

SDS-PAGE. Initiate the reaction with the addition of 2 mM buffered ATP. Let reaction incubate 1 hr at 30 °C (Fig. 5) (*see* Note 9).

3. Filter the reaction and resolve over Superdex 75 gel filtration column equilibrated with Buffer C. Analyze the fractions by SDS-PAGE.
4. Collect the peak fractions and concentrate to ca. 30–40 mg/ml. Measure the OD₂₈₀ and determine the final protein concentration with the extinction coefficient 41,035 M⁻¹ cm⁻¹ or 1 OD₂₈₀ = 0.66 mg/ml. Aliquot, flash freeze and store at –80 °C for later use.

3.3 E2-SUMO-PCNA Cross-linking Reaction

3.3.1 Cross-linking with BMOE

1. BMOE is a robust and highly reactive reagent to generate stable cross-links between two cysteine residues (Fig. 3).
2. Rapidly thaw the E2-Smt3 thioester mimetic and PCNA proteins to be cross-linked. Add TCEP to 1 mM and incubate 15 min at 22 °C. Desalt to cross-linking buffer (*see* Note 10).
3. To 800 µL of 493 µM and desalted E2_{Ubc9}^{C5S/A129K/K153R}-N18Smt3, add 12 µL of 100 mM BMOE (ca. 3-fold excess BMOE over E2-SUMO mimetic) and incubate 3 min at 22 °C. Desalt to cross-linking buffer to remove excess BMOE.
4. Mix the desalted E2-Smt3-BMOE with the 480 µL of 3,290 µM desalted PCNA_{K77D/C81E/R110D/K127G/K164C} (ca. 4-fold excess PCNA) (*see* Note 11) and let react 15 min at 22 °C (Fig. 6).
5. Quench with 1 µL BME.
6. Resolve the quenched reaction over Superdex 200 gel filtration column equilibrated with Buffer C. Analyze the fractions by SDS-PAGE.
7. Pool peak fractions and dilute with 20 mM TRIS-HCl (pH 8.0), 1 mM BME to final [NaCl] = 200 mM or desalt to Buffer G.
8. Load this mixture onto anion-exchange resin (Mono-Q). The protein is eluted with a linear gradient from Buffer G to 30% Buffer E over 10 column volumes. The Ubc9-Smt3-BMOE-PCNA complex elutes approximately at the [NaCl] that the source PCNA species eluted originally. Analyze the fractions by SDS-PAGE.
9. Collect the peak fractions and measure the OD₂₈₀ and determine the final protein concentration with the extinction coefficient 81,510 M⁻¹ cm⁻¹ or 1 OD₂₈₀ = 1.20 mg/ml (for E2-SUMO-BMOE-PCNA_{K77D/C81E/R110D/K127G/K164C}) or with the extinction coefficient 81,635 M⁻¹ cm⁻¹ or 1 OD₂₈₀ = 1.20 mg/ml (for E2-SUMO-BMOE-PCNA_{K127G/K164C}). Proceed to final reconstitution with E3 or concentrate to at least 10 mg/ml, aliquot, flash freeze and store at –80 °C for later use.

3.3.2 Cross-linking with EDT

1. While BMOE is a robust cross-linker, with two bulky maleimide rings and long distance between reactive centers, it is a poor mimetic of the distance and geometry expected for the tetrahedral intermediate (Fig. 1b and Fig. 3). Cross-linking *via* EDT provides a bridge between the E2 active site cysteine and the Lys to Cys mutation on the target substrate that has the same number of atoms as the predicted tetrahedral intermediate (Fig. 4). Thus, EDT is a better structural mimetic of the reaction intermediate, despite the two disulfide bonds that impart unique dihedral angles.
2. Flash thaw the E2-Smt3 thioester mimetic and PCNA proteins to be cross-linked. Add TCEP to 1 mM and incubate 15 min at 22 °C. Desalt to cross-linking buffer (*see* Note 12).
3. To 400 µL of 1,238 µM and desalted E2_{Ubc9}^{C5S/A129K/K153R}- N18Smt3, add 4 µL of 300 mM AT2 and incubate 15 min at 22 °C. The AT2 forms a disulfide adduct to the active site cysteine. Desalt to cross-linking buffer to remove excess AT2 (Fig. 4).
4. Add 5 µL of 300 mM EDT to the desalted E2-Smt3-AT2 and incubate 15 min at 22 °C. The AT2 acts as a good leaving group and helps catalyze the formation of the E2-Smt3-EDT disulfide adduct on the active site cysteine. Desalt to cross-linking buffer to remove excess EDT (Fig. 4).
5. Add 5 µL of 300 mM AT2 to the desalted E2-Smt3-EDT and incubate 15 min at 22 °C. This second round of AT2 forms a disulfide adduct to the distal end of the EDT molecules attached to the E2 active site cysteines (Fig. 4). This second AT2 will act as a leaving group to catalyze the disulfide bond formation between the distal end of the EDT and the PCNA cysteine. Desalt to cross-linking buffer.
6. Mix the desalted E2-Smt3-EDT-AT2 with the 825 µL of 1,575 µM and desalted PCNA_{K77D/C81E/R110D/K127G/K164C} (ca. 3-fold excess PCNA) and let react 20 min at 22 °C (Fig. 7).
7. Resolve the cross-linked reaction over Superdex 200 gel filtration column equilibrated with Buffer H. Analyze the fractions by SDS-PAGE.
8. Pool peak fractions and dilute with 20 mM TRIS-HCl (pH 8.0), 1 mM BME to final [NaCl]= 200 mM or desalt to Buffer I.
9. Load this mixture onto anion-exchange resin (Mono-Q). The protein is eluted with a linear gradient from Buffer I to 30% Buffer J over 10 column volumes. The Ubc9-Smt3-EDT-PCNA complex elutes approximately at the [NaCl] that the source PCNA species eluted originally. Analyze the fractions by SDS-PAGE.
10. Collect the peak fractions and measure the OD₂₈₀ and determine the final protein concentration as in Sect. 3.3.1. Because of the potential instability of the EDT linkage, proceed to final reconstitution with E3.

3.4 Reconstitution with E3

1. For the final reconstitution, mix the E2_{Ubc9}-Ubl_{Smt3}-PCNA cross-linked complex with the E3_{Siz1}-N18Smt3 in a 1:1 stoichiometry. Dialyze to Buffer F (if using BMOE) or Buffer K (if using EDT) (*see* Note 13).
2. Resolve the reconstituted complex over Superdex 200 gel filtration column equilibrated with Buffer F or K, depending on BMOE *versus* EDT as the cross-linker, respectively. Analyze the fractions by SDS-PAGE (Fig. 8).
3. Collect the peak fractions and concentrate to 10–15 mg/ml. Proceed to downstream structure determination technique of choice or concentrate to at least 10 mg/ml, aliquot, flash freeze and store at –80 °C for later use.

4. Notes

1. The generality of this strategy will depend primarily on the particular E2 and target substrate and the number and surface accessible cysteine residues to which cross-linking is not desired. In the case of the E2, it may be necessary to mutate any cysteine, other than the active site, to a compatible residue (perhaps serine, threonine, alanine or an alternative side chain based on sequence analysis and alignment with orthologous proteins). In the case of the target substrate, whether any surface exposed cysteine residues need to be removed by mutation should be determined empirically with pilot cross-linking assays. For instance, Cys22, Cys30 and Cys62 remained in our engineered PCNA K164C monomeric substrate, while Cys22, Cys30, Cys62 and Cys81 remained in the trimeric version. While not employed here, it is possible that the E3 could provide specificity during the cross-linking reaction.
2. The pSMT3 vectors derive from pET-28b and include an N-terminal His₆ fusion to *S. cerevisiae* SUMO/Smt3(63). pSMT3 enables cloning of the protein of interest (POI) in the MCS, *via* the BamHI and XhoI sites, in frame with the N-terminal His₆-Smt3 to generate the His₆-Smt3-Gly-Gly-Ser-POI fusion polypeptide. The Ulp1 protease cleaves the His₆-Smt3 from the POI, C-terminal to the di-Gly motif, liberating the POI with an N-terminal serine residue artifact. The TOPO-SMT3 vector is modified pSMT3, with custom TOPO adaptation (Invitrogen) for downstream flap ligation. The N-terminal Smt3 fusion can enhance expression, solubility and yield for challenging proteins.
3. This protocol describes formation of a Smt3/SUMO conjugate. Even trace amounts of contaminating Ulp1 can quickly reverse the conjugation reaction. A pilot cleavage reaction should be performed to identify the minimal amount of Ulp1 needed to cleave the protein of interest in the time frame necessary. Additionally, using His₆-GST-Ulp1 (purified *via* the His₆ tag) allows for cleavage of the His₆-Smt3 fusion tag and the ability to subsequently deplete the Ulp1 with immobilized glutathione resin. Additionally, trace Ulp1 can deposit on columns, become solubilized and can contaminate protein preparations previously free of Ulp1. To prevent this, any remaining Ulp1 can be inactivated

by applying 10 ml of 20 mM NEM to columns prior to equilibration and use. NEM can be inactivated with excess BME.

4. PMSF is only used for E1 and E3 lysis where there is an observed benefit. Imidazole is omitted from the E3 lysis buffer to minimize the exposure of the RING domain to imidazole where it might compete for Zn^{2+} coordination.
5. All buffers and solutions for use during FPLC are prepared with ultrapure water, 0.2 μM filtered and degassed for at least 1 hour to maximize reproducibility and column integrity.
6. All steps of protein purification were performed at 4 °C or on ice. Each sample is passed through a 0.2 μM filter prior to application on chromatography columns. All proteins are concentrated (ideally to at least 10 mg/ml or greater), aliquoted and flash frozen prior to storage at -80 °C.
7. The C5S mutation in Ubc9 results in a single cysteine in the E2, the active site cysteine, which is critical for specific cross-linking in later steps. The K153R mutation is introduced to prevent a known E2 SUMO conjugate from forming (67). The A129K mutation is introduced to install a lysine residue proximal to the active site cysteine in a position that enables nucleophilic attack of the E2-SUMO thioester and formation of a stable isopeptide linkage. This position for the lysine works well for E2_{Ubc9} and E2_{Rad6}; however, other E2s may differ in their active site configurations. The extension of this technique to more divergent E2s may require exploration of alternative positions for installation of a suitable lysine.
8. Separation of the E2_{Ubc9}-Ubl from unreacted E2_{Ubc9} or any E2_{Ubc9}-2(Ubl) or E2_{Ubc9}-(Ubl)₂ represents one of the more challenging aspects of this strategy. While E2_{Ubc9} binds to Mono-S resin, full resolution of E2_{Ubc9} from E2_{Ubc9}-Smt3 is not readily achieved. Full-length Smt3 binds to Mono-Q resin which enables resolution of free E2_{Ubc9} from E2_{Ubc9}-Smt3. Additionally, E2_{Ubc9} with two full-length Smt3 proteins (i.e. E2_{Ubc9}-2(Smt3) or E2_{Ubc9}-(Smt3)₂) elutes from the MonoQ at higher [NaCl] than E2_{Ubc9} with a single Smt3 (E2_{Ubc9}-Smt3). However, this purification advantage is lost with the N18Smt3 construct since lowering the [NaCl] sufficiently to bind the Mono-Q causes the E2_{Ubc9}-N18Smt3 complex to precipitate. This advantage is also not observed for the few ubiquitin E2s we have tested. In these instances, the E2, E2-Ubl, E2-2(Ubl) and E2-(Ubl)₂ species do not fully resolve from one another on gel filtration or ion exchange. In these cases, it is paramount to optimize the reaction conditions to maximize yield of desired product and minimize components that are challenging to remove (*see* Note 9). In cases such as these, two additional strategies may be employed. First, leaving the N-terminal His₆ tag on the Ubl can allow for immobilization of the Ubl and E2-Ubl mimetic on NiNTA resin while washing away unreacted E2 (35). Second, a His₆-Smt3-Ubl (or perhaps His₆-GST-Ubl, His₆-MBP-Ubl, etc.) construct could be used to form the E2-His₆-Smt3-Ubl mimetic. This strategy provides the advantage of a sufficiently large difference in the Stokes radius for E2-2(Ubl) or E2-(Ubl)₂ to better

separate them from E2-Ubl by gel filtration. The His₆-Smt3 (or His₆-GST, His₆-MBP, etc.) can be subsequently removed with Ulp1 (or Thrombin, TEV, etc.).

9. When extending this technique to other E2-Ubl mimetics, it will be advantageous to screen a range of E1, E2 and Ubl concentrations in addition to a range of reaction times and pH to identify conditions that maximize E2-Ubl conjugate yield while minimizing formation of undesirable E2-2(Ubl) or E2-(Ubl)₂ species.
10. An alternate technique would be to purify the E2-Ubl thioester mimetic and substrate to be cross-linked in the presence of TCEP instead of BME. TCEP is compatible with BMOE cross-linking, however, in the present protocol, we removed it by desalting at the same time as removing BME. Because BME will react and interfere with BMOE based cross-linking, it is crucial to remove it from all proteins prior to cross-linking. Once proteins are cross-linked with BMOE they are stable and no longer susceptible to reducing agents.
11. For monomeric PCNA, it was desirable to attempt to react all of the E2-Ubl-BMOE species and maximize yield, since the monomeric unmodified PCNA could be easily separated from the E2-Ubl-BMOE-PCNA species. In the case of trimeric PCNA, the reverse was true and the incubation was maintained at a 1.2 to 1.5-fold excess of E2-Ubl-BMOE over PCNA in an attempt to modify all three Cys164 positions around the PCNA ring to achieve near homogeneity and to then purify away any unreacted E2-Ubl-BMOE by gel filtration.
12. The EDT cross-link is susceptible to reducing agents. It is critical to remove all BME or other reducing agents from the input proteins, the columns and FPLC system prior to purification of the EDT cross-linked proteins. For this reason, it may be advantageous to use the more robust BMOE technique to optimize reconstitution conditions and structure determination before attempting EDT preparation and reconstitution for structural analysis.
13. The stoichiometry of this complex is due in part to the E3-SUMO fusion, where the SUMO binds the backside of E2_{Ubc9} in addition to the other E2/E3 and E3/PCNA binding interactions. Because the backside affinities between E2s and Ubls differ, it is possible that other complexes may not reconstitute with similar stoichiometry to enable purification by gel filtration. In such cases, structural analysis may require a co-crystallization strategy.

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5. References

- (1). Kerscher O, Felberbaum R, Hochstrasser M (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* 22:159–180 [PubMed: 16753028]

- (2). Hochstrasser M (2009) Origin and function of ubiquitin-like proteins. *Nature* 458:422–429 [PubMed: 19325621]
- (3). Gareau JR, Lima CD (2010) The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat Rev Mol Cell Biol* 11:861–71 [PubMed: 21102611]
- (4). Rose IA, Warms JV (1983) An enzyme with ubiquitin carboxy-terminal esterase activity from reticulocytes. *Biochemistry* 22:4234–4237 [PubMed: 6313036]
- (5). Pickart CM, Rose IA (1985) Ubiquitin carboxyl-terminal hydrolase acts on ubiquitin carboxyl-terminal amides. *J Biol Chem* 260:7903–10 [PubMed: 2989266]
- (6). Mayer AN, Wilkinson KD (1989) Detection, resolution, and nomenclature of multiple ubiquitin carboxyl-terminal esterases from bovine calf thymus. *Biochemistry* 28:166–72 [PubMed: 2539853]
- (7). Amerik AY, Hochstrasser M (2004) Mechanism and function of deubiquitinating enzymes. *Biochim Biophys Acta* 1695:189–207 [PubMed: 15571815]
- (8). Mevissen TET, Komander D (2017) Mechanisms of Deubiquitinase Specificity and Regulation. *Annu Rev Biochem* 86:159–192 [PubMed: 28498721]
- (9). Hershko A, Ciechanover A, Heller H, Haas AL, Rose IA (1980) Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. *Proc Natl Acad Sci U S A* 77:1783–6 [PubMed: 6990414]
- (10). Schulman BA, Harper JW (2009) Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. *Nat Rev Mol Cellular Biol* 10:319–331 [PubMed: 19352404]
- (11). Streich FC, Jr, Lima CD (2014) Structural and functional insights to ubiquitin-like protein conjugation. *Annu Rev Biophys* 43:357–79 [PubMed: 24773014]
- (12). Streich FC, Haas AL (2010) Activation of Ubiquitin and Ubiquitin-Like Proteins, in: Groettrup M (Ed.), *Conjugation and Deconjugation of Ubiquitin Family Modifiers*, Landes Bioscience and Springer, Austin and New York, pp. 1–16.
- (13). Cappadocia L, Lima CD (2017) Ubiquitin-like Protein Conjugation: Structures, Chemistry, and Mechanism. *Chem Rev Article ASAP*
- (14). Haas AL, Rose IA (1982) The mechanism of ubiquitin activating enzyme. A kinetic and equilibrium analysis. *J Biol Chem* 257:10329–37 [PubMed: 6286650]
- (15). Haas AL, Warms JV, Hershko A, Rose IA (1982) Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation. *J Biol Chem* 257:2543–8 [PubMed: 6277905]
- (16). Haas AL, Warms JV, Rose IA (1983) Ubiquitin adenylate: structure and role in ubiquitin activation. *Biochemistry* 22:4388–4394 [PubMed: 6313038]
- (17). Pickart CM, Rose IA (1985) Functional-Heterogeneity of Ubiquitin Carrier Proteins. *J Biol Chem* 260:1573–1581 [PubMed: 2981864]
- (18). Haas AL, Bright PM (1988) The resolution and characterization of putative ubiquitin carrier protein isozymes from rabbit reticulocytes. *J Biol Chem* 263:13258–67 [PubMed: 3417657]
- (19). Hershko A, Heller H, Elias S, Ciechanover A (1983) Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J Biol Chem* 258:8206–14 [PubMed: 6305978]
- (20). Hershko A, Heller H, Eytan E, Reiss Y (1986) The protein substrate binding site of the ubiquitin-protein ligase system. *J Biol Chem* 261:11992–9 [PubMed: 3017957]
- (21). Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM (1999) RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc Natl Acad Sci U S A* 96:11364–9 [PubMed: 10500182]
- (22). Deshaies RJ, Joazeiro CA (2009) RING domain E3 ubiquitin ligases. *Annu Rev Biochem* 78:399–434 [PubMed: 19489725]
- (23). Freemont PS, Hanson IM, Trowsdale J (1991) A novel cysteine-rich sequence motif. *Cell* 64:483–484 [PubMed: 1991318]
- (24). Barlow PN, Luisi B, Milner A, Elliott M, Everett R (1994) Structure of the C3HC4 domain by 1H-nuclear magnetic resonance spectroscopy. A new structural class of zinc-finger. *J Mol Biol* 237:201–11 [PubMed: 8126734]

- (25). Johnson ES, Gupta AA (2001) An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell* 106:735–44 [PubMed: 11572779]
- (26). Kahyo T, Nishida T, Yasuda H (2001) Involvement of PIAS1 in the sumoylation of tumor suppressor p53. *Mol Cell* 8:713–8 [PubMed: 11583632]
- (27). Takahashi Y, Toh-e A, Kikuchi Y (2001) A novel factor required for the SUMO1/Smt3 conjugation of yeast septins. *Gene* 275:223–31 [PubMed: 11587849]
- (28). Hochstrasser M (2001) SP-RING for SUMO: new functions bloom for a ubiquitin-like protein. *Cell* 107:5–8 [PubMed: 11595179]
- (29). Yunus AA, Lima CD (2009) Structure of the Siz/PIAS SUMO E3 ligase Siz1 and determinants required for SUMO modification of PCNA. *Mol Cell* 35:669–82 [PubMed: 19748360]
- (30). Aravind L, Koonin EV (2000) The U box is a modified RING finger - a common domain in ubiquitination. *Curr Biol* 10:R132–134 [PubMed: 10704423]
- (31). Hamilton KS, Ellison MJ, Barber KR, Williams RS, Huzil JT, McKenna S, Ptak C, Glover M, Shaw GS (2001) Structure of a conjugating enzyme-ubiquitin thiolester intermediate reveals a novel role for the ubiquitin tail. *Structure* 9:897–904 [PubMed: 11591345]
- (32). Reverter D, Lima CD (2005) Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. *Nature* 435:687–92 [PubMed: 15931224]
- (33). Dou H, Buetow L, Sibbet GJ, Cameron K, Huang DT (2012) BIRC7-E2 ubiquitin conjugate structure reveals the mechanism of ubiquitin transfer by a RING dimer. *Nat Struct Mol Biol* 19:876–83 [PubMed: 22902369]
- (34). Page RC, Pruneda JN, Amick J, Klevit RE, Misra S (2012) Structural insights into the conformation and oligomerization of E2~ubiquitin conjugates. *Biochemistry* 51:4175–87 [PubMed: 22551455]
- (35). Plechanovová A, Jaffray EG, Tatham MH, Naismith JH, Hay RT (2012) Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis. *Nature* 489:115–20 [PubMed: 22842904]
- (36). Pruneda JN, Littlefield PJ, Soss SE, Nordquist KA, Chazin WJ, Brzovic PS, Klevit RE (2012) Structure of an E3:E2~Ub complex reveals an allosteric mechanism shared among RING/U-box ligases. *Mol Cell* 47:933–42 [PubMed: 22885007]
- (37). Dou H, Buetow L, Sibbet GJ, Cameron K, Huang DT (2013) Essentiality of a non-RING element in priming donor ubiquitin for catalysis by a monomeric E3. *Nat Struct Mol Biol* 20:982–6 [PubMed: 23851457]
- (38). Branigan E, Plechanovova A, Jaffray EG, Naismith JH, Hay RT (2015) Structural basis for the RING-catalyzed synthesis of K63-linked ubiquitin chains. *Nat Struct Mol Biol* 22:597–602 [PubMed: 26148049]
- (39). Hodge CD, Ismail IH, Edwards RA, Hura GL, Xiao AT, Tainer JA, Hendzel MJ, Glover JN (2016) RNF8 E3 ubiquitin ligase stimulates Ubc13 E2 conjugating activity that is essential for DNA double strand break signaling and BRCA1 tumor suppressor recruitment. *J Biol Chem* 291:9396–410 [PubMed: 26903517]
- (40). Koliopoulos MG, Esposito D, Christodoulou E, Taylor IA, Rittinger K (2016) Functional role of TRIM E3 ligase oligomerization and regulation of catalytic activity. *EMBO J* 35:1204–18 [PubMed: 27154206]
- (41). Lechtenberg BC, Rajput A, Sanishvili R, Dobaczewska MK, Ware CF, Mace PD, Riedl SJ (2016) Structure of a HOIP/E2~ubiquitin complex reveals RBR E3 ligase mechanism and regulation. *Nature* 529:546–50 [PubMed: 26789245]
- (42). Sanchez JG, Chiang JJ, Sparrer KM, Alam SL, Chi M, Roganowicz MD, Sankaran B, Gack MU, Pornillos O (2016) Mechanism of TRIM25 Catalytic Activation in the Antiviral RIG-I Pathway. *Cell Rep* 16:1315–25 [PubMed: 27425606]
- (43). Wagner JM, Roganowicz MD, Skorupka K, Alam SL, Christensen D, Doss G, Wan Y, Frank GA, Ganser-Pornillos BK, Sundquist WI, Pornillos O (2016) Mechanism of B-box 2 domain-mediated higher-order assembly of the retroviral restriction factor TRIM5alpha. *eLife* 5:e16309 [PubMed: 27253059]
- (44). Wright JD, Mace PD, Day CL (2016) Secondary ubiquitin-RING docking enhances Arkadia and Ark2C E3 ligase activity. *Nat Struct Mol Biol* 23:45–52 [PubMed: 26656854]

- (45). Dove KK, Olszewski JL, Martino L, Duda DM, Wu XS, Miller DJ, Reiter KH, Rittinger K, Schulman BA, Klevit RE (2017) Structural Studies of HHARI/UbcH7 approximately Ub Reveal Unique E2 approximately Ub Conformational Restriction by RBR RING1. *Structure* 25:890–900.e5 [PubMed: 28552575]
- (46). Yuan L, Lv Z, Atkison JH, Olsen SK (2017) Structural insights into the mechanism and E2 specificity of the RBR E3 ubiquitin ligase HHARI. *Nat Commun* 8:211 [PubMed: 28790309]
- (47). Dawidziak DM, Sanchez JG, Wagner JM, Ganser-Pornillos BK, Pornillos O (2017) Structure and catalytic activation of the TRIM23 RING E3 ubiquitin ligase. *Proteins* 85:1957–61 [PubMed: 28681414]
- (48). Nomura K, Klejnot M, Kowalczyk D, Hock AK, Sibbet GJ, Vousden KH, Huang DT (2017) Structural analysis of MDM2 RING separates degradation from regulation of p53 transcription activity. *Nat Struct Mol Biol* 24:578–587 [PubMed: 28553961]
- (49). Eddins MJ, Carlile CM, Gomez KM, Pickart CM, Wolberger C (2006) Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation. *Nat Struct Mol Biol* 13:915–20 [PubMed: 16980971]
- (50). Kamadurai HB, Souphron J, Scott DC, Duda DM, Miller DJ, Stringer D, Piper RC, Schulman BA (2009) Insights into ubiquitin transfer cascades from a structure of a UbcH5B~ubiquitin-HECT(NEDD4L) complex. *Mol Cell* 36:1095–1102 [PubMed: 20064473]
- (51). Kleiger G, Saha A, Lewis S, Kuhlman B, Deshaies RJ (2009) Rapid E2-E3 assembly and disassembly enable processive ubiquitylation of cullin-RING ubiquitin ligase substrates. *Cell* 139:957–68 [PubMed: 19945379]
- (52). Kamadurai HB, Qiu Y, Deng A, Harrison JS, Macdonald C, Actis M, Rodrigues P, Miller DJ, Souphron J, Lewis SM, Kurinov I, Fujii N, Hammel M, Piper R, Kuhlman B, Schulman BA (2013) Mechanism of ubiquitin ligation and lysine prioritization by a HECT E3. *eLife* 2:e00828 [PubMed: 23936628]
- (53). Brown NG, VanderLinden R, Watson ER, Qiao R, Grace CR, Yamaguchi M, Weissmann F, Frye JJ, Dube P, Ei Cho S, Actis ML, Rodrigues P, Fujii N, Peters JM, Stark H, Schulman BA (2015) RING E3 mechanism for ubiquitin ligation to a disordered substrate visualized for human anaphase-promoting complex. *Proc Natl Acad Sci U S A* 112:5272–9 [PubMed: 25825779]
- (54). Brown NG, VanderLinden R, Watson ER, Weissmann F, Ordureau A, Wu KP, Zhang W, Yu S, Mercredi PY, Harrison JS, Davidson IF, Qiao R, Lu Y, Dube P, Brunner MR, Grace CR, Miller DJ, Haselbach D, Jarvis MA, Yamaguchi M, Yanishevski D, Petzold G, Sidhu SS, Kuhlman B, Kirschner MW, Harper JW, Peters JM, Stark H, Schulman BA (2016) Dual RING E3 Architectures Regulate Multiubiquitination and Ubiquitin Chain Elongation by APC/C. *Cell* 165:1440–53 [PubMed: 27259151]
- (55). Qiao R, Weissmann F, Yamaguchi M, Brown NG, VanderLinden R, Imre R, Jarvis MA, Brunner MR, Davidson IF, Litos G, Haselbach D, Mechtler K, Stark H, Schulman BA, Peters JM (2016) Mechanism of APC/CCDC20 activation by mitotic phosphorylation. *Proc Natl Acad Sci U S A* 113:E2570–8 [PubMed: 27114510]
- (56). Pfander B, Moldovan GL, Sacher M, Hoege C, Jentsch S (2005) SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. *Nature* 436:428–33 [PubMed: 15931174]
- (57). Papouli E, Chen S, Davies AA, Huttner D, Krejci L, Sung P, Ulrich HD (2005) Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p. *Mol Cell* 19:123–33 [PubMed: 15989970]
- (58). Armstrong AA, Mohideen F, Lima CD (2012) Recognition of SUMO-modified PCNA requires tandem receptor motifs in Srs2. *Nature* 483:59–63 [PubMed: 22382979]
- (59). Streich FC, Jr., Lima CD (2016) Capturing a substrate in an activated RING E3/E2-SUMO complex. *Nature* 536:304–8 [PubMed: 27509863]
- (60). Capili AD, Lima CD (2007) Structure and analysis of a complex between SUMO and Ubc9 illustrates features of a conserved E2-Ubl interaction. *J Mol Biol* 369:608–18 [PubMed: 17466333]
- (61). Duda DM, van Waardenburg RC, Borg LA, McGarity S, Nourse A, Waddell MB, Bjornsti MA, Schulman BA (2007) Structure of a SUMO-binding-motif mimic bound to Smt3p-Ubc9p:

- conservation of a non-covalent ubiquitin-like protein-E2 complex as a platform for selective interactions within a SUMO pathway. *J Mol Biol* 369:619–30 [PubMed: 17475278]
- (62). Knipscheer P, van Dijk WJ, Olsen JV, Mann M, Sixma TK (2007) Noncovalent interaction between Ubc9 and SUMO promotes SUMO chain formation. *EMBO J* 26:2797–807 [PubMed: 17491593]
- (63). Mossesso E, Lima CD (2000) Ulp1-SUMO crystal structure and genetic analysis reveal conserved interactions and a regulatory element essential for cell growth in yeast. *Mol Cell* 5:865–76 [PubMed: 10882122]
- (64). Bernier-Villamor V, Sampson DA, Matunis MJ, Lima CD (2002) Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. *Cell* 108:345–56 [PubMed: 11853669]
- (65). Lois LM, Lima CD (2005) Structures of the SUMO E1 provide mechanistic insights into SUMO activation and E2 recruitment to E1. *EMBO J* 24:439–51 [PubMed: 15660128]
- (66). Yunus AA, Lima CD (2009) Purification of SUMO conjugating enzymes and kinetic analysis of substrate conjugation. *Methods Mol Biol* 497:167–86 [PubMed: 19107417]
- (67). Knipscheer P, Flotho A, Klug H, Olsen JV, van Dijk WJ, Fish A, Johnson ES, Mann M, Sixma TK, Pichler A (2008) Ubc9 sumoylation regulates SUMO target discrimination. *Mol Cell* 31:371–82 [PubMed: 18691969]

Fig. 1.

Chemical diagrams for Ubl transfer from the E2-Ubl thioester complex to the target substrate and chemical diagrams of thioester mimetics used in structural studies. **(a)** Chemical diagram of a lysine residue undergoing nucleophilic attack of a native E2-Ubl thioester. **(b)** Chemical diagram of the predicted tetrahedral intermediate transition state formed after nucleophilic attack on the way to isopeptide bond formation. Chemical diagram of an E2-Ubl thioester mimetic formed using **(c)** an E2 catalytic Cys to Ser mutant, which forms an oxyester linkage or **(d)** an E2 catalytic Cys to Lys mutant, which forms an isopeptide linkage. **(e)** Chemical diagram of the thioester mimetic utilized in the current protocol. Alanine 129, neighboring the catalytic cysteine, is mutated to Lys. The E1 charges the E2 catalytic Cys, followed by nucleophilic attack by the installed Lys129 to form and E2-Ubl thioester mimetic with a stable isopeptide linkage and an intact active site cysteine residue available for chemical cross-linking.

Fig. 2. E2_{Ubc9}-UblSUMO/E3_{Siz1}/SubstratePCNA complex reconstitution. **(a)** UV trace of Superose 6 analytical gel filtration runs to investigate complex formation between individual components. An E3-SUMO fusion along with chemical cross-linking of the E2 active site to the site of modification on PCNA were both required for stoichiometric complex reconstitution, depicted in the cartoon complex. **(b)** SDS-PAGE analysis of the eluted fractions from the indicated gel filtration runs, Coomassie stained.

Fig. 3.
Chemical diagram of BMOE based cross-linking of the E2 and target substrate PCNA.
BMOE, bismaleimidoethane.

Fig. 4. Chemical diagram of the progressive steps to cross-link the E2 and target substrate with EDT. The number of atoms present in the EDT bridging cross-link equals that in the predicted tetrahedral intermediate (Fig. 1b). AT2, Aldrithiol-2; EDT, 1,2-ethanedithiol.

Fig. 5.

E2_{Ub9}-SUMO thioester mimetic formation and purification. **(a)** SDS-PAGE analysis of a time course for reactions forming the E2_{Ub9}^{C93K}-SUMO *versus* E2_{Ub9}^{A129K}-SUMO mimetics in the presence and absence of the E3, Coomassie stained. Time points are 0, 2, 5, 20, and 60 minutes. The presence of the E3 speeds the reaction in the case of A129K, but not C93K. This unexpected observation suggests E3 binding activates the E2-SUMO thioester for nucleophilic attack by the lysine exogenously installed proximal to its own active site cysteine. This observation supports the model that the E1 catalyzes transfer of the SUMO to Cys93 and not directly to the installed Lys129. **(b)** SDS-PAGE analysis of the purification of the E2_{Ub9}^{C93K}-SUMO thioester mimetic, Coomassie stained. Gel filtration can isolate the E2_{Ub9}-SUMO mimetic from the E1, free E2 and free SUMO. The larger than expected shift in stokes radius and trailing peak results, in part, from the non-covalent

E2_{Ubc9}/SUMO backside interaction creating a daisy chain effect. (c) The purification of the E2_{Ubc9}^{A129K}-Ubl_{SUMO} thioester mimetic proceeds similar to the C93K mutant.

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Fig. 6. BMOE cross-linking reaction and complex purification. **(a)** SDS-PAGE analysis of reactions exploring optimal BMOE concentrations, time of reaction and ratio of PCNA to E2-SUMO-BMOE for maximizing yield of final product, Coomassie stained. **(b)** SDS-PAGE analysis of the purification of the E2-SUMO-BMOE-PCNA cross-linked complex, Coomassie stained.

Fig. 7. EDT cross-linking reaction and complex purification. **(a)** SDS-PAGE analysis of reactions exploring the optimal PCNA ratio to E2-SUMO-EDT-AT2 for maximizing yield of final product, Coomassie stained. **(b)** SDS-PAGE analysis of the gel filtration purification of the E2-SUMO-EDT-PCNA complex, Coomassie stained.

Fig. 8. Final reconstitution of the E2-SUMO-PCNA cross-linked complex with the E3-SUMO fusion. SDS-PAGE analysis of the gel filtration purification of the final reconstituted complex between (a) the E2-SUMO-BMOE-PCNA complex or (b) the E2-SUMO-EDT-PCNA complex with E3-SUMO fusion, Coomassie stained.