CHAPTER EIGHT

# Biochemical characterization of SUMO-conjugating enzymes by in vitro sumoylation assays

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## Contents



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## Abstract

The small ubiquitin-related modifier (SUMO) is a protein of ~10 kDa that is covalently conjugated to its substrate proteins in an enzymatic process called sumoylation. This posttranslational modification is an essential regulatory mechanism that plays crucial roles in many cellular pathways. It allows rapid adaptation to environmental changes by switching protein functions due to alternate complex assemblies, changes in intracellular localization, enzymatic activity, or stability. SUMO conjugation is executed by the hierarchical action of E1, E2, and E3 enzymes. Both E2 and E3 enzymes contribute to substrate specificity but with E3 ligases being the more important for this. E1 and E2 activities are essential for all sumoylation reactions but usually—with a few exceptions—modify substrates only inefficiently. Hence, most substrates require the additional action of an E3 ligase or a cofactor. Here, we describe methods to distinguish a bona fide E3 ligase from a cofactor activity by using in vitro sumoylation assays.

## 1. Introduction

Sumoylation is an essential transient posttranslational modification that is predominantly detected in the nucleus and has key functions in many cellular pathways, including transcription, chromatin regulation, DNA replication, DNA damage responses, RNA splicing, cell cycle regulation, protein degradation, and intracellular trafficking (Droescher, Chaugule, & Pichler, 2013; Flotho & Melchior, 2013; Zhao, 2018).

In humans, several different small ubiquitin-related modifier (SUMO) paralogs can be conjugated to cellular proteins. The human genome codes for five SUMO paralogs (SUMO1–5); of these, SUMO1 and the almost identical SUMO2 and SUMO3 are ubiquitously expressed. Sumoylation is executed by an enzymatic triad that covalently attaches SUMO to selected substrates in a hierarchical process. First, the unique heterodimeric SUMO E1 enzyme Aos1/Uba2 activates SUMO by ATP-driven adenylation of the SUMO C-terminus followed by formation of an energy-rich thioester bond between a Uba2 cysteine and the SUMO C-terminus. Next, SUMO is transferred to the unique E2 enzyme Ubc9 again resulting in a thioester linkage (~). Eventually, the SUMO C-terminus is conjugated to a substrate lysine residue, forming an isopeptide bond (*). The final conjugation step usually involves E3 ligases, which stabilize the interaction of the SUMO ~charged E2 enzyme with the substrate. However, exceptional cases allow efficient modification also in the absence of E3 ligases (Pichler, Fatouros, Lee, & Eisenhardt, 2017). Generally, substrates can be modified at single or multiple lysines either with a single SUMO moiety or with poly SUMO chains.

The most abundant sumoylation sites are acceptor lysines embedded in a SUMO consensus motif (SCM) ψKxE (ψ: hydrophobic amino acid with preference for V and I), but non-SCM lysines can also be modified especially upon stress conditions (Hendriks et al., 2017, 2018; Hendriks & Vertegaal, 2016).

The number of known SUMO-ligating enzymes is very limited, with far fewer than the number of corresponding enzymes used for ubiquitin-protein ligation. In mammals, these SUMO pathway enzymes comprise the single E1 and E2 enzymes and a handful E3 ligases that belong to three different classes: 1. the SP-RING family consisting of PIAS1, PIAS2, PIAS3, PIAS4, and MMS21; 2. RanBP2; and 3. the ZNF451-family presented by ZNF451–1, ZNF451–2, ZNF451–3, and the primate-specific KIAA1586 protein (Cappadocia, Pichler, & Lima, 2015; Eisenhardt et al., 2015; Kahyo, Nishida, & Yasuda, 2001; Pichler, Gast, Seeler, Dejean, & Melchior, 2002; Sachdev et al., 2001). This small enzyme number is especially surprising in the light of thousands of SUMO substrates which have been identified in cells (Hendriks et al., 2017, 2018; Hendriks & Vertegaal, 2016).

Several additional proteins have been proposed as SUMO E3 ligases, but so far there are no unambiguous mechanistic data demonstrating their ligase activity. Cellular analyses of SUMO E3 ligase function are often inconclusive in this regard. An increase in cellular substrate sumoylation upon coexpression of a putative E3 protein may not reflect E3 ligase function but could instead result from inhibition of a SUMO protease that normally desumoylates the substrate or, alternatively, a SUMO-targeted ubiquitin ligase that would otherwise promote degradation of the sumoylated protein. In addition, the enzymatic function of an E3 ligase has to be distinguished from a cofactor that can enhance substrate modification in a nonenzymatic manner.

In vitro assays with recombinant proteins represent a pure system that can directly characterize E3 ligase activity. However, one caveat for in vitro studies is the use of excessive enzyme concentrations. High E2 enzyme levels are often sufficient to obtain some substrate sumoylation. Conversely, very high E3 concentrations can deplete SUMO in the reaction (and thereby inhibit substrate sumoylation) because of the efficient automodification activities described for all known SUMO E3 ligases. Therefore, substoichiometric enzyme concentrations relative to the substrate are essential to discriminate between SUMO E3 ligase and cofactor activities.

Detailed biochemical and structural analyses of the three known classes of bona fide SUMO E3 ligases revealed that specific donor-SUMO ($SUMO^D$)

positioning is a hallmark of E3-dependent catalysis along with the ability to bind E2 (Cappadocia et al., 2015; Eisenhardt et al., 2015; Reverter & Lima, 2005; Streich & Lima, 2016; Yunus & Lima, 2009). $SUMO^D$ is the SUMO that forms the thioester linkage with the E2 enzyme and its positioning leads to an optimal orientation, the so-called closed conformation, for nucleophilic attack of the incoming substrate lysine ε-amino group, resulting in efficient isopeptide formation. Thus, $SUMO^D$ positioning and rapid discharge of the $SUMO^D$~E2 are diagnostic features of E3 ligases that can be monitored in in vitro sumoylation reactions.

The interface between the E3 ligase and the E2 enzyme can vary, and ZNF451 and SP-RING ligases stabilize this interaction via noncovalent binding to a scaffold SUMO ($SUMO^B$) on the backside of the E2 (Cappadocia et al., 2015; Eisenhardt et al., 2015; Streich & Lima, 2016). By contrast, RanBP2 does not involve such a scaffold SUMO and interacts directly with the backside of the E2 (Pichler, Knipscheer, Saitoh, Sixma, & Melchior, 2004; Reverter & Lima, 2005). Hence, E2 backside interaction is also important for efficient substrate modification of most but not all E3 ligases and thus can also be addressed by using the in vitro sumoylation assays (see below).

Substrate sumoylation can be visualized by SDS polyacrylamide gel electrophoresis (PAGE) due to the stability of the isopeptide bond in SDS and DTT. SUMO attachment results in a ~15–20 kDa size shift in substrate mobility as measured by SDS-PAGE (the predicted ~10 kDa SUMO migrates more slowly in gels because of its flexible N-terminus). Thus, SUMO–protein conjugates can be distinguished from noncovalent SUMO interactions such as those that occur through a SUMO interaction motif (SIM) or with the backside of the E2 (Pichler et al., 2017). Also thioester bonds, such as the linkage in $SUMO^D$~E2, can be visualized by SDS-PAGE, but in contrast to isopeptide bonds, this linkage is sensitive to reducing agents and should not exceed a final concentration of 0.1 m*M* DTT.

In this methods paper, we aim to summarize how to use the power of in vitro sumoylation assays to characterize the activities of SUMO-conjugating enzymes and to distinguish E3 regulators or cofactors from true SUMO E3 ligases without laborious structural analyses. Structural study can nevertheless validate the inference of a protein being a bona fide SUMO E3 ligase.

## 2. Substoichiometric substrate modification

E3 ligases catalyze the rapid transfer of the $SUMO^D$ from the E2 enzyme to the substrate. By definition, enzymes are recycled in the reaction,

allowing multiple rounds of substrate modification by a single enzyme. Thus, enzymes function at substoichiometric amounts relative to the substrate (low enzyme to substrate ratio) in a concentration- and time-dependent manner.

To monitor sumoylation in vitro we use purified recombinant enzymes following the protocols described in detail by the Melchior lab (Werner, Moutty, Moller, & Melchior, 2009). Ideally, all assay components are purified from *Escherichia coli* as bacteria lack a SUMO system, thus avoiding copurification of SUMO E3 ligases or proteases which at undetectable concentrations could affect the outcome of the in vitro reaction. Any known and putative SUMO substrate can be tested in this system if can be purified. All known SUMO E3 ligases show some substrate specificity in vitro, but they usually have broad substrate spectra; this is especially when working with a truncated E3 ligase (Cox et al., 2015; Koidl et al., 2016; Pichler et al., 2004). Here, we use GST-Sp100 as model substrate as it is a promiscuous substrate that can be sumoylated by all known classes of SUMO E3 ligases as well as by the sumoylated E2 (S*E2) without an E3 (Knipscheer et al., 2008; Koidl et al., 2016; Pichler et al., 2002, 2004; Sternsdorf, Jensen, & Will, 1997). For in vitro sumoylation reactions, all components can simply be added together in a test tube with ATP and $Mg^{2+}$ and incubated for up to 30 min in a multiturnover reaction. Detection of sumoylation is performed by immunoblotting using substrate-specific antibodies. We use anti-GST antibodies that detect the N-terminally fused GST-tag of Sp100, and thus we can rule out that sumoylation interferes with detection.

Because the E2 enzyme directly recognizes a SCM, it can modify substrates in vitro at high enzyme concentrations as we illustrate in Fig. 1A. Hence, it is important to use a low E2 concentration in E3-dependent reactions that either show no or only marginal substrate modification by the E2 alone. At high enzyme concentrations, the mammalian E2 itself gets sumoylated at Lys 14 (S*E2). We have shown that this particular modification stabilizes the E2 interaction with GST-Sp100 due to a SIM in the substrate that is in close proximity to the SCM. The E2*S adduct therefore also enhances GST-Sp100 modification to a degree comparable to some E3 ligases (Knipscheer et al., 2008) (Fig. 1A). However, E3 ligases are usually more potent and promote higher sumoylation rates when at low (substoichiometric) levels; this can be seen both by varying E3 concentrations (Fig. 1B) and by following sumoylation over time (Fig. 1C).

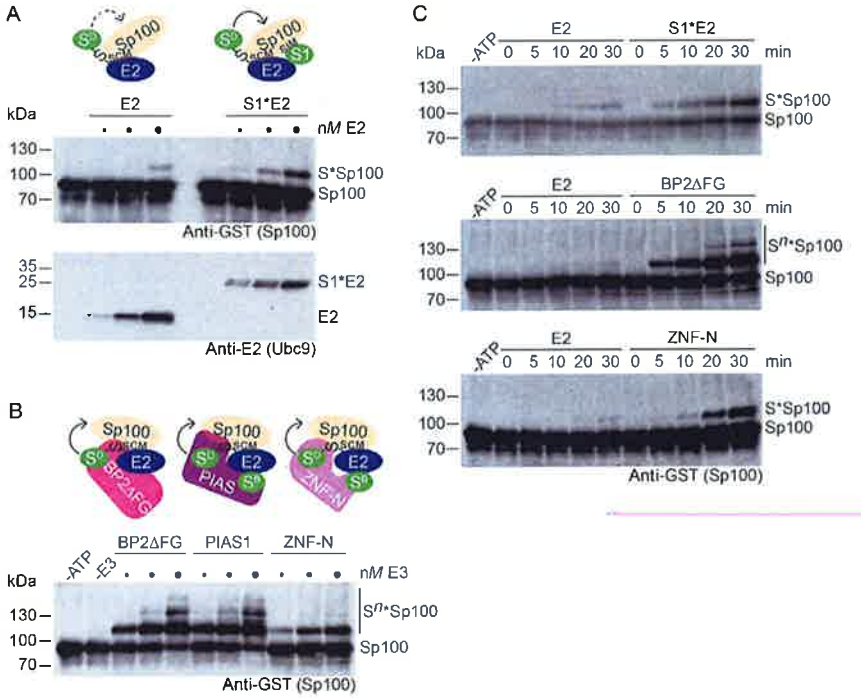


**Fig. 1** Substrate sumoylation at substoichiometric enzyme concentrations. (A) Schematic presentation of substrate–E2 interactions and immunoblots of E2-dependent sumoylation reactions. Diagrams depict E2 or S1*E2 charged with a donor-SUMO ($S^D$) interacting with the substrate Sp100. The E2 catalytic cleft directly binds the substrate via a SUMO consensus motif (SCM). S1*E2 additionally interacts with Sp100 via a SIM in close distance to the SCM that stabilizes the interaction. In vitro sumoylation assays with increasing concentrations (0, 10, 50, or 250 n*M*) of E2 or S1*E2 were incubated with 200 n*M* GST-Sp100, 60 n*M* E1, 2 µ*M* SUMO2, and 5 m*M* ATP in 20 µL reactions for 30 min at 30°C. Samples were stopped with SDS sample buffer and analyzed by immunoblotting using anti-GST (*upper panel*) or anti-E2 (anti-Ubc9) antibodies (*lower panel*). (B) Schematic presentation of substrate–E2–E3 interactions and immunoblot of E3-dependent sumoylation reactions. Diagrams show the different E3s RanBP2ΔFG, PIAS1, or ZNF-N interacting with a $SUMO^D$-charged E2 ($S^D$~E2) and the substrate Sp100. In vitro sumoylation assays with increasing concentrations of the individual E3s (0.2, 1, and 5 n*M* BP2ΔFG or 0.6, 3, and 15 n*M* PIAS1 or 11.25, 45, and 180 n*M* ZNF-N) were incubated with 200 n*M* GST-Sp100, 60 n*M* E1, 50 n*M* E2, 2 µ*M* SUMO2, and 5 m*M* ATP in 20 µL reactions for 30 min at 30°C. Samples were stopped with SDS sample buffer and analyzed by immunoblotting using anti-GST antibodies. (C) Time course of E2- or E3-dependent sumoylation reactions. 250 n*M* of E2 or S1*E2 in E3-independent reactions, or 50 n*M* E2 combined with E3 (5 n*M* BP2ΔFG or 180 n*M* of ZNF-N) were incubated with 200 n*M* GST-Sp100, 60 n*M* E1, 2 µ*M* SUMO2, and 5 m*M* ATP for 0, 5, 10, 20, or 30 min in 20 µL reactions at 30°C. Samples were stopped with SDS sample buffer and analyzed by immunoblotting using anti-GST antibodies. Conclusion: E3 ligases and S1*E2 accelerate Sp100 sumoylation at substoichiomentric enzyme concentrations.

## 2.1 Equipment

- Heat block
- Standard SDS-PAGE equipment
- 7% and 15% SDS polyacrylamide gels
- Immunoblotting and detection system

## 2.2 Buffers and reagents

- Sumoylation assay buffer (SAB): 20 m*M* HEPES pH 7.3, 110 m*M* KOAc, 2 m*M* $Mg(OAc)_2$, 1 m*M* DTT, 0.2 mg/mL ovalbumin, 0.05% Tween 20 (v/v)
- Recombinant enzymes and substrate: E1: His-Aos1/His-Uba2; E2s: E2 wt or S*E2; E3s: His-MBP-PIAS1, RanBP2 ΔFG, His-MBP-ZNF-N; SUMO2; substrate: GST-Sp100. Proteins are expressed recombinantly and purified as described (Eisenhardt et al., 2015; Knipscheer et al., 2008; Pichler et al., 2002; Werner et al., 2009). All recombinant proteins should be snap frozen in small single-use aliquots at the highest possible concentrations and stored at −80°C.
- ATP: 100 m*M* in 20 m*M* HEPES pH 7.3, 100 m*M* $Mg(OAc)_2$
- SDS sample buffer (3×): 150 m*M* Tris–HCl (pH 6.8), 6% SDS, 30% glycerol, 0.3% bromophenol blue, 100 m*M* DTT
- Antibodies: mouse anti-GST (from Dr. Egon Ogris, MFPL Vienna) and rabbit anti-E2 primary antibodies (anti-Ubc9 (Eisenhardt et al., 2015)), corresponding secondary antibodies (here: antimouse and antirabbit HRP, respectively from Jackson ImmunoResearch Laboratories, Inc.)

## 2.3 Procedure

1. Set up in vitro sumoylation reactions in a final volume of 20 μL by mixing together 60 n*M* E1, different concentrations of E2 enzymes: E2 wt or S*E2 (10, 50, 250 n*M*), 200 n*M* of the substrate (GST-Sp100), and 2 μ*M* of SUMO2 in E3-independent reactions or 50 n*M* E2 and different concentrations of E3s (0.2, 1 and 5 n*M* for RanBP2ΔFG, 0.6, 5 and 15 n*M* for PIAS1, and 11.25, 45 and 180 n*M* for ZNF-N). Add ATP to a final concentration of 5 m*M* to start the reaction.
2. Incubate at 30°C for 30 min.
3. Stop the reaction by adding 10 μL 3× SDS sample buffer and heat denature for 5 min at 95°C.
4. Run 13 μL of the sample on a 7% SDS gel.

5. Transfer onto nitrocellulose membranes by standard semidry or wet protein transfer.
6. Detect the substrate and the SUMO2 modification by immunoblotting using substrate-specific antibodies.
7. To ensure comparable concentrations of E2 and S*E2, run 13 µL of the sample on a 15% SDS gel. Transfer onto nitrocellulose membrane by standard semidry or wet protein transfer and probe with E2-specific (anti-Ubc9) antibodies.

### 2.4 Notes

1. Sumoylation reactions should always be set up on ice.
2. Use SAB for all predilutions.
3. Titrate the enzymes using serial dilutions.
4. Include a "minus ATP" and "minus E3" control in all assays.
5. Prepare a master mix for all components in common to minimize pipetting errors and split them into individual tubes. Usually E1, SUMO, and the substrate are added as part of the master mix in E3-independent reactions. In E3-dependent reactions the E2 is also added to the master mix.
6. Sumoylation is detected as an apparent size shift of ~15–20 kDa for each SUMO added.
7. Use of anti-SUMO2 antibodies is not recommended to detect substrate sumoylation due to additional sumoylation events happening in the reaction, e.g., E1 sumoylation, E2 sumoylation, SUMO chain formation, and automodification of E3s.
8. Be aware that substrate specific antibodies may not recognize the sumoylated substrate if SUMO attachment interferes with epitope accessibility.

## 3. E3-mediated E2 discharge

E3 ligases simultaneously interact with the substrate and the $SUMO^D$ charged E2 enzyme to catalyze the discharge of the thioester-bound $SUMO^D$ from the E2 to the substrate. E3 interaction with $SUMO^D$ via a SIM results in a closed conformation which is highly reactive and leads to rapid discharge of the thioester bond as it was shown for all three classes of bona fide SUMO E3 ligases (Cappadocia et al., 2015; Eisenhardt et al., 2015; Reverter & Lima, 2005; Streich & Lima, 2016). By using E1 and E2 enzymes together with ATP, $Mg^{2+}$, and SUMO, a $SUMO^D$~E2

thioester is formed. To monitor the discharge of $SUMO^D$ from the E2, ATP needs to be hydrolyzed by apyrase (an ATP diphosphohydrolase) to prevent recharging of the E2. As this assay allows only a single round of E2 discharge (called a "single-turnover" assay), it requires much higher enzyme concentrations compared to the "multiturnover" reactions described in Fig. 1 that cycle through multiple rounds of charging and discharging. The major challenge of single-turnover reactions is the ability of the E2 to also be discharged in the absence of an E3, and it discharges within minutes at 30°C. This is likely due to the high E2 enzyme concentration that allows E3-independent modification of the E1, the E2, SUMO, and the substrate. Hence, E3-dependent discharge has to be executed at high E3 concentrations and in a short time frame to visualize $SUMO^D$~E2 discharge and substrate modification (Fig. 2).

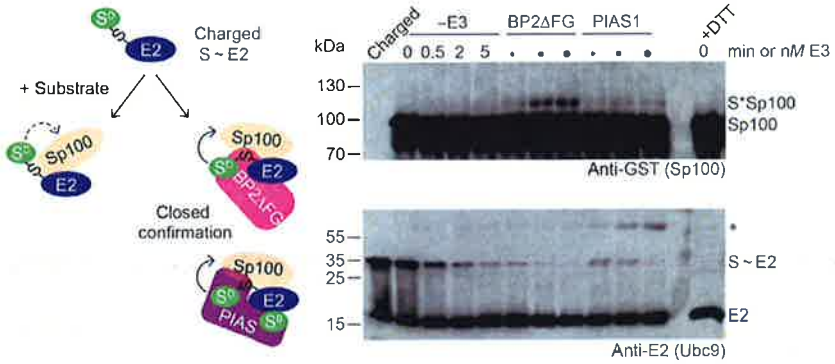


**Fig. 2** E2- and E3-dependent $SUMO^D$~Ubc9 discharge reactions. Schematic presentation of the donor $SUMO^D$ charged E2 enzyme ($S^D$~E2) and its discharge to the substrate (Sp100) in the absence and presence of E3 ligases. Charging reaction was with 700 n*M* E2 incubated with 350 n*M* E1, 1400 n*M* SUMO2, and 5 m*M* ATP in 10 µL per reaction (mastermix) for 5 min at 30°C. Charging was stopped by addition of 0.5 U of Apyrase on ice. $S^D$~E2 discharge (final concentration 350 n*M*, 175 n*M* E1, 700 n*M* SUMO2) was induced by addition of 100 n*M* GST-Sp100 without or with increasing concentration of the indicated E3 ligase (1.1, 3.3, and 10 n*M* of BP2ΔFG or 5.6, 16.7, and 50 n*M* of PIAS1) at a final volume of 20 µL. E2-dependent discharge was for 0, 0.5, 2, or 5 min, and E3-dependent reaction for 5 min at 30°C. Reactions were stopped with UREA buffer. To distinguish between thioester and isopeptide bond modified E2, one sample (discharge 0 min) was treated with DTT to reduce thioester bonds. Samples for E2 detection were separated on nonreducing 15% SDS-PAGE, and substrate modification was on reducing 7% SDS-PAGE. Detection was with anti-GST or anti-E2 (Ubc9) antibodies. *Uncharacterized band detected in PIAS1 containing reactions. Conclusion: In the presence of E3 ligases, both $SUMO^D$~E2 discharge and transfer to the substrate are accelerated.

## 3.1 Equipment

- As in Section 2.1

## 3.2 Buffers and reagents

- 5× Discharge assay buffer (DAB): 100 m*M* Tris–HCl pH 7.5, 250 m*M* NaCl, 50 m*M* $MgCl_2$, 0.25% Tween 20, 1 mg/mL ovalbumin
- Recombinant enzymes and substrate: E1: His-Aos1/His-Uba2; E2: E2 wt; SUMO2; E3s: His-MBP-PIAS1, RanBP2ΔFG; substrate: GST-Sp100 (Eisenhardt et al., 2015; Pichler et al., 2002; Werner et al., 2009)
- ATP: 100 m*M* in 20 m*M* HEPES pH 7.3, 100 m*M* $Mg(OAc)_2$
- Apyrase (e.g., from Sigma): 1 U/μL in $H_2O$
- DTT: 1 *M* in $H_2O$
- UREA nonreducing sample buffer (3×): 150 m*M* Tris pH 6.8, 30% glycerol (v/v), 6 *M* urea, 3% SDS, 0.3% Bromophenol blue
- Antibodies: as in Section 2.2

## 3.3 Procedure

1. Set up in vitro charging reactions as master mix in a volume of 10 μL/reaction containing 350 n*M* E1, 700 n*M* E2, and 1400 n*M* of SUMO2 in 1× DAB. Add ATP to a final concentration of 5 m*M* to start the reaction. Incubate at 30°C for 5 min.
2. Stop charging reaction by adding 0.5 U of apyrase (0,5 μL/reaction).
3. Incubate on ice for 5 min.
4. Prepare in advance tubes on ice for each reaction with 100 n*M* of GST-Sp100. Add E3s or buffer (for E3-independent reactions) in a concentration-dependent manner (1.1, 3.3, or 10 n*M* of RanBP2ΔFG or 5.6, 16.7, and 50 n*M* of PIAS1) and adjust to 9.5 μL with 1× DAB. All concentrations are calculated for a final volume of 20 μL.
5. Add 10.5 μL of the charging reaction and discharge for 0–5 min at 30°C.
6. Stop reaction by addition of 10 μL of 3× UREA buffer and immediate heat denature for 5 min at 95°C.
7. Run 13 μL of each sample on a nonreducing 15% SDS gel for E2 detection.
8. Add 1 μL of 1 *M* DTT to all remaining samples.
9. Run 14 μL of the sample on a 7% gel for GST-Sp100 detection.
10. Transfer proteins from both gels to nitrocellulose membranes by standard semidry or wet protein transfer.

11. Detect $SUMO^D$~E2 and the (modified) substrate by immunoblotting using anti-E2 (Ubc9) and antisubstrate (anti-GST) antibodies, respectively.

### 3.4 Notes

1. $SUMO^D$~E2 charging reactions should always be set up on ice.
2. Be aware that thioesters are DTT sensitive and most recombinant proteins are purified and stored in buffers containing DTT. Thioesterification reactions should not exceed a final concentration of 0.1 m*M* DTT. However, a minimal amount of DTT is required as otherwise the catalytic cysteines of the E1 and E2 can form disulfide bonds, rendering them inactive.
3. All protein dilutions should be done in DAB.
4. Prepare a master mix for the charging reaction.
5. Substrate can be added to the master mix after the apyrase treatment and before splitting into the individual reactions.
6. Take one sample right after charging and substrate addition to analyze the efficiency of the charging reaction (time 0 for the discharge).
7. Instead of apyrase treatment, EDTA, which chelates $Mg^{2+}$, can also be used. It is important that the charging is stopped completely and no recharging of the E2 occurs. Usually $SUMO^D$~E2 dissociates within 5–10 min at 30°C. If recharging is still ongoing, $SUMO^D$~E2 appears more "stable" and can be detected for longer.
8. To monitor dissociation of $SUMO^D$~E2, we perform E3-independent discharge in a time-dependent manner.
9. To distinguish $SUMO^D$~E2 from S*E2 we analyze one sample (time 0 for the discharge) and add 1 µL of 1 *M* DTT to reduce thioester bonds without affecting isopeptide bonds. For loading this sample on the gel at least one lane has to be kept as a spacer as DTT can diffuse into neighboring lanes.

## 4. E3–E2 backside interaction

The E2 possesses an important regulatory interface which is termed its backside as it is opposite to the catalytic cleft that bears the active-site cysteine forming the thioester with $SUMO^D$. This backside site interacts noncovalently with a scaffold $SUMO^B$ and was originally shown to be important for E2-mediated SUMO chain formation in vitro (Capili & Lima, 2007; Knipscheer, van Dijk, Olsen, Mann, & Sixma, 2007). Moreover, it partially overlaps with the E2–E1 interface (Duda et al., 2007) and is required for

direct or indirect E2–E3 interactions. SUMO$^{B}$ is essential for the E3 activity of ZNF451 family members (Cappadocia et al., 2015; Eisenhardt et al., 2015) and strongly enhances the activity of Siz/Pias family members (Mascle et al., 2013; Streich & Lima, 2016), whereas RanBP2 directly interacts with the backside of the E2 independent of a SUMO (Pichler et al., 2004; Reverter & Lima, 2005).

The requirement of the E2-SUMO$^{B}$ interaction for E3 activity can be tested in vitro by employing a SUMO or E2 mutant which are specifically impaired in this interaction: SUMO2 D62R abolishes, and E2 F22A weakens this particular interaction (Capili & Lima, 2007; Knipscheer et al., 2007). Here we show RanBP2 as an example of an E3 that displays no major defects with either mutant (Fig. 3A, upper panel) as SUMO$^{B}$ is dispensable for its catalytic activity. However, E2 F22A has mild effects on RanBP2's activity as E2-Phe22 contributes to the larger RanBP2–E2 interface (Pichler et al., 2004; Reverter & Lima, 2005). By contrast, PIAS1 clearly requires the SUMO$^{B}$-E2 interaction for efficient Sp100 sumoylation (Fig. 3A, lower panel) as does ZNF451 (Eisenhardt et al., 2015; Koidl et al., 2016). At low PIAS1 concentrations, SUMO2 D62R results in minimal GST-Sp100 modification. The E2 F22A mutation only partially interrupts the E2-SUMO$^{B}$ interaction and hence causes a more mild reduction in PIAS1-dependent substrate sumoylation. Of note, Sp100 modification in the absence of an E3 (E2 or S*E2 dependent) remains unaffected (Fig. 3B). In vitro sumoylation assays used here are similar to substrate modification described in Section 2 and also set up in a multiturnover reaction.

## 4.1 Equipment

- As in Section 2.1

## 4.2 Buffers and reagents

- As in Section 2.2
- E2 variants: E2 wt or S*E2 or E2 F22A and SUMO: SUMO2 wt or SUMO2 D62R (Eisenhardt et al., 2015)

## 4.3 Procedure

1. Set up in vitro sumoylation reactions in a total volume of 20 µL as in Section 2.3.

## 4.4 Notes

1. As in Section 2.4

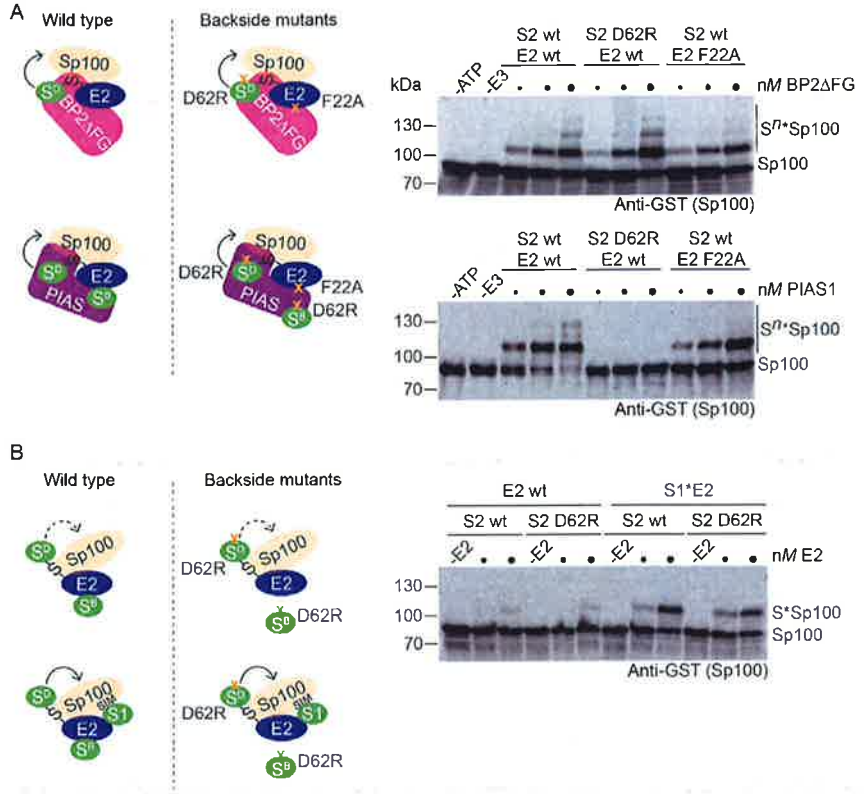


**Fig. 3** Requirements of scaffold SUMO$^B$-E2 backside interactions for E2 and E3 activities. (A) Schematic presentation of S$^D$~E2–scaffold SUMO$^B$ (S$^B$)–E3–substrate interactions and consequences of mutations in SUMO (S2 D62R) and the E2 (E2 F22A) that impair the E2–S$^B$ interaction. RanBP2 interacts directly with the backside of the E2, while PIAS1 bridges this interaction via S$^B$. In vitro sumoylation reactions with increasing E3 concentrations (0.2, 1, and 5 n*M* of BP2ΔFG or 0.6, 3, and 15 n*M* of PIAS1) incubated with 200 n*M* GST-Sp100, 60 n*M* E1, 50 n*M* E2 or E2 F22A, 2 µ*M* SUMO2 or 2 µ*M* SUMO2 D62R, and 5 m*M* ATP in 20 µL reactions for 30 min at 30°C. Samples were stopped with SDS sample buffer and analyzed by immunoblotting using anti-GST antibodies. (B) Schematic presentation of S$^D$~E2 or S$^D$~E2*S1–S$^B$–substrate interactions and consequences of mutations in SUMO (S2 D62R) and the E2 (E2 F22A) that impair E2-S$^B$ interaction. In vitro sumoylation reaction with increasing concentration (0, 50, or 250 n*M*) of the E2 or the S1*E2 incubated with 200 n*M* GST-Sp100, 60 n*M* E1, 2 µ*M* SUMO2 or 2 µ*M* SUMO2 D62R, and 5 m*M* ATP in 20 µL reactions for 30 min at 30°C. Reactions were stopped with SDS sample buffer and analyzed by immunoblotting using anti-GST antibodies. Conclusion: Backside SUMO$^B$–E2 interaction is important for PIAS1-dependent substrate modification, but dispensable for BP2ΔFG-, E2-, and S1*E2-dependent reactions. Of note, the mild impairment of BP2ΔFG in the presence of E2 F22A mutant is due to the direct interaction with this residue as part of the E3–E2 binding interface.

## 5. Donor SUMO positioning

Initially, E3 ligases were thought to interact simultaneously with the charged E2 enzyme and the substrate to bring them in close distance for an efficient $SUMO^D$ transfer. As this definition may better describe a cofactor than an enzymatic activity, the question was raised as to whether E3 ligases also involve a catalytic component. The first evidence in this direction came from the crystallographic analysis of RanBP2 interacting with a donor $SUMO^D$-charged E2 mimic; this revealed that the E3 ligase also binds the modifier $SUMO^D$ (Reverter & Lima, 2005). Subsequent biochemical analysis demonstrated that this SIM-dependent interaction is indeed essential for RanBP2's catalytic activity (Reverter & Lima, 2005). Meanwhile, all three classes of SUMO E3 ligases were shown to depend on this feature that is also called the "closed conformation" as it positions the $SUMO^D$ modifier optimally for the nucleophilic attack of the incoming substrate lysine (Cappadocia et al., 2015; Eisenhardt et al., 2015; Reverter & Lima, 2005; Streich & Lima, 2016; Yunus & Lima, 2009).

Based on the SUMO–SIM interaction involved in $SUMO^D$ positioning, a SUMO2ΔSBD (SIM-binding domain; Q30A, F31A, I33A) mutant can be investigated that disrupts this important binding interface (Eisenhardt et al., 2015; Meulmeester, Kunze, Hsiao, Urlaub, & Melchior, 2008). In Fig. 4A, multiturnover assays are presented for RanBP2 and PIAS1 that both show clearly that substrate sumoylation depends on interactions of SUMO with the SIM of the E3. However, such assays are only conclusive for E3 ligases that do not require the $SUMO^B$-E2 backside interaction, which is true for RanBP2 (Fig. 3). E3 ligases such as ZNF451 or Siz/PIAS family members have additional SBD(SUMO)–SIM(E3) interactions involving the scaffold $SUMO^B$ (see Figs. 3 and 4); analysis of these E3s demands single-turnover assays to clearly distinguish between SIM-dependent $SUMO^D$ positioning and scaffold $SUMO^B$ binding. For such assays, the E2 is charged with SUMO2 wt or the SUMO2ΔSBD mutant, and discharge reactions are performed in the presence of SUMO2 wt added along with the substrate and the E3 (Fig. 4B) to allow E3 scaffold $SUMO^B$ binding. E3-independent sumoylation of Sp100 by the E2 or S*E2 is independent of both $SUMO^D$ positioning (Fig. 4C) and scaffold $SUMO^B$ binding (Fig. 3B).

Taken together, these assays demonstrate that $SUMO^D$ positioning is essential for all E3 ligases but dispensable for E2 and S*E2 sumoylation

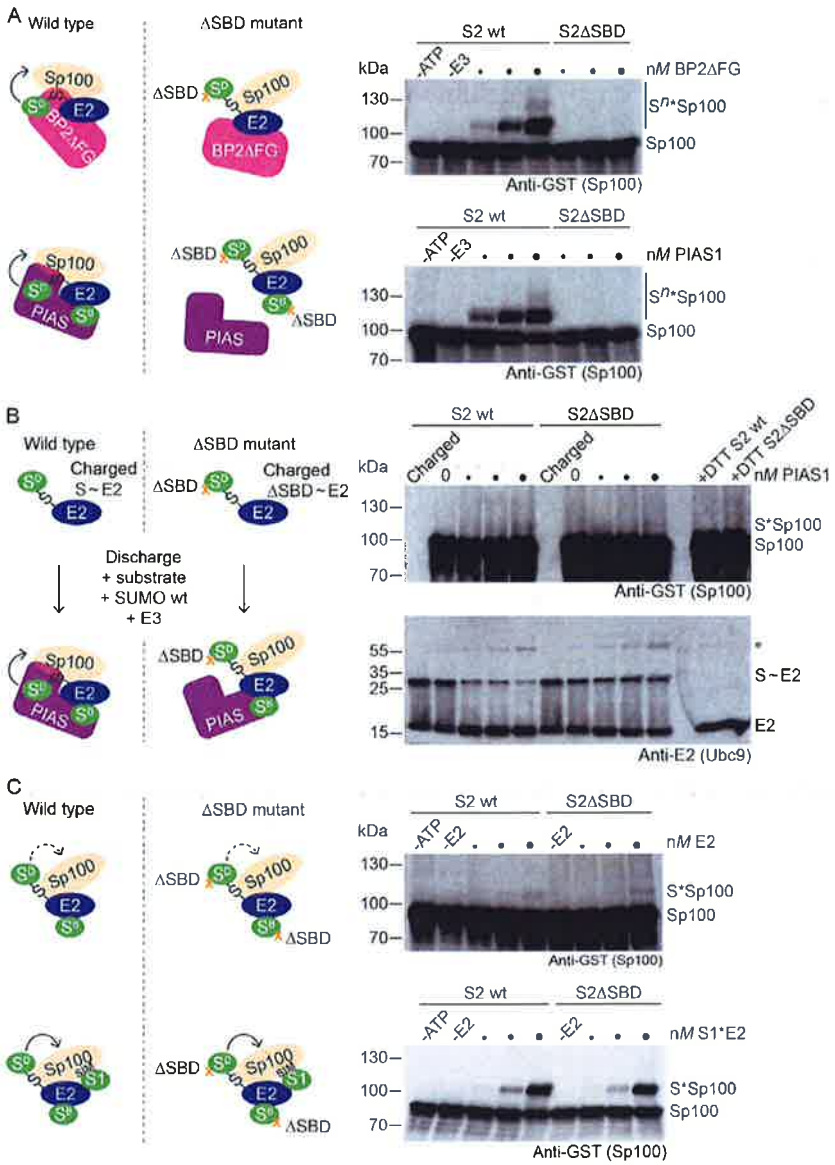


**Fig. 4** Requirements of donor SUMO positioning for E2 and E3 activities. (A) Schematic presentation of E3-dependent donor SUMO ($S^D$)-positioning and consequences of a mutant, SUMO2ΔSBD, impaired in this interaction. While BP2ΔFG involves SUMO–SIM interactions only in $S^D$ positioning, PIAS1 also depends on this interface for scaffold $S^B$ binding. Hence, multiturnover assays using SUMO2ΔSBD are only conclusive for enzymes independent of scaffold $S^B$ binding. In vitro sumoylation assays with increasing E3 concentrations (0.2, 1, and 5 n*M* of BP2ΔFG or 0.6, 3, and 15 n*M* of PIAS1) incubated

*(Continued)*

reactions. Thus, we propose $SUMO^D$ positioning as a key criterion to describe the enzymatic function of SUMO E3 ligases that distinguishes them from other enhancing activities like cofactors or the S*E2.

## 5.1 Equipment

- As in Section 2.1

## 5.2 Buffers and reagents

- As in Sections 2.2 and 3.2
- SUMO: SUMO2 wt and SUMO2ΔSBD (Q30A, F31A, I33A) (Eisenhardt et al., 2015)

## 5.3 Procedure

1. Set up in vitro multiturnover sumoylation assays as in Section 2.3 but with 2 μ*M* of SUMO2 or SUMO2ΔSBD.
2. Single-turnover assays as in Section 3.3 but with 1100 n*M* of SUMO2 wt or SUMO2ΔSBD (instead of 1400 n*M* SUMO) for charging. For the discharge reaction, add 350 n*M* SUMO2 wt together with the substrate and the E3 ligase to each reaction tube.

**Fig. 4—Cont'd** with 200 n*M* GST-Sp100, 60 n*M* E1, 50 n*M* E2, 2 μ*M* SUMO2 orSUMO2 ΔSBD, and 5 m*M* ATP in 20 μL reactions for 30 min at 30°C. Samples were stopped with SDS sample buffer and analyzed by immunoblotting using anti-GST antibodies. (B) Single turnover assay to analyze scaffold SUMO ($S^B$)-dependent E3 ligases (like PIAS1) for donor SUMO positioning. Schematic presentation of the $S^D$~charged E2 enzyme interacting with indicated E3 ligases and consequences of an impaired $S^D$-E3 interaction. Charging reaction was with 700 n*M* E2 incubated with 350 n*M* E1, 1100 n*M* SUMO2 or SUMO2ΔSBD, and 5 m*M* ATP in 10 μL per reaction (mastermix) for 5 min at 30°C. Charging was stopped by addition of 0.5 U of Apyrase on ice. $S^D$~E2 discharge (final concentration 350 n*M*, 175 n*M* E1, 700 n*M* SUMO2 or SUMO2ΔSBD) was induced by addition of 100 n*M* GST-Sp100 with increasing concentration of PIAS1 (5.6, 16.7, and 50 n*M*) and additional 350 n*M* SUMO2 wt to provide $SUMO^B$ in a final volume of 20 μL. Discharge was for 5 min at 30°C. Reactions were stopped with UREA buffer and analyzed as in Fig. 2. *Uncharacterized band detected in PIAS1-containing reactions. (C) Schematic presentation of the E2 and S1*E2 that are independent in donor $S^D$-positioning for substrate modification. In vitro sumoylation assay with increasing E2 or S1*E2 concentrations (0, 10, 50, or 250 n*M*) incubated with 200 n*M* GST-Sp100, 60 n*M* E1, 2 μ*M* SUMO2 or SUMO2ΔSBD, and 5 m*M* ATP in 20 μL reactions for 30 min at 30°C. Reactions were stopped with SDS sample buffer and analyzed by immunoblotting using anti-GST antibodies. Conclusion: Donor SUMO positioning is essential for E3-dependent substrate modification but dispensable for E2-dependent reactions. Thus, donor SUMO positioning is a hallmark of SUMO E3 ligases.

## 5.4 Notes

1. As in Sections 2.4 and 3.4.
2. Less SUMO2 or SUMO2ΔSBD is used in charging reaction compared to Section 3.3 as the E2 backside should not be saturated to allow binding of a $SUMO^{B}$ (SUMO wt) in the discharge reaction.

# 6. Summary and conclusions

In vitro sumoylation assays offer powerful tools to study sumoylation. They can be used to address several aspects, such as identification and analysis of SUMO substrates, mapping their specific sumoylation sites or determining their specific conjugating and deconjugating enzymes. In addition, these assays allow the functional characterization of the enzymes themselves, like which surfaces are important for certain functions, including E1–E2, E2–substrate, E3–substrate, and E2–E3 interactions. They further permit conclusions about the stoichiometric ratio between the substrate and the enzymes required for efficient substrate modification. A stoichiometric modifier/substrate ratio rather describes a cofactor, while potent activity at substoichiometric ratios points to what we define as E3 ligases. However, there can be some ambiguity in such an assignment as we demonstrate for S*E2. S*E2 definitely shows enhanced catalytic activation compared to the unmodified E2 and is activated in a substrate-specific manner. However, the enhancing role of the additional SUMO is better described as a cofactor function that stabilizes the interaction of the substrate with the E2, and no additional catalytic activity appears to be involved (Knipscheer et al., 2008). Thus, it is important to also investigate other characteristics of SUMO E3 ligases, like the dependence of a scaffold $SUMO^{B}$ binding to the backside of the E2 that is important for all known SUMO E3 ligases except for RanBP2. Ultimately, the most conclusive assay to demonstrate SUMO E3 ligase activity is by demonstrating dependence of donor $SUMO^{D}$ positioning for the discharge of the E2 and the $SUMO^{D}$ transfer to the substrate.

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## Author contributions

N.E. and A.P. designed the experiments, N.E., D.I., and E.N. performed the in vitro sumoylation assays, N.E. and A.P. prepared the figures and wrote the manuscript. All authors commented on the manuscript.

## References

Capili, A. D., & Lima, C. D. (2007). Structure and analysis of a complex between SUMO and Ubc9 illustrates features of a conserved E2-Ubl interaction. *Journal of Molecular Biology*, *369*(3), 608–618. https://doi.org/10.1016/j.jmb.2007.04.006.

Cappadocia, L., Pichler, A., & Lima, C. D. (2015). Structural basis for catalytic activation by the human ZNF451 SUMO E3 ligase. *Nature Structural & Molecular Biology*, *22*(12), 968–975. https://doi.org/10.1038/nsmb.3116.

Cox, E., Uzoma, I., Guzzo, C., Jeong, J. S., Matunis, M., Blackshaw, S., et al. (2015). Identification of SUMO E3 ligase-specific substrates using the HuProt human proteome microarray. *Methods in Molecular Biology*, *1295*, 455–463. https://doi.org/10.1007/978-1-4939-2550-6_32.

Droescher, M., Chaugule, V. K., & Pichler, A. (2013). SUMO rules: Regulatory concepts and their implication in neurologic functions. *Neuromolecular Medicine*, *15*(4), 639–660. https://doi.org/10.1007/s12017-013-8258-6.

Duda, D. M., van Waardenburg, R. C., Borg, L. A., McGarity, S., Nourse, A., Waddell, M. B., et al. (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. *Journal of Molecular Biology*, *369*(3), 619–630. https://doi.org/10.1016/j.jmb.2007.04.007.

Eisenhardt, N., Chaugule, V. K., Koidl, S., Droescher, M., Dogan, E., Rettich, J., et al. (2015). A new vertebrate SUMO enzyme family reveals insights into SUMO-chain assembly. *Nature Structural & Molecular Biology*, *22*(12), 959–967. https://doi.org/10.1038/nsmb.3114.

Flotho, A., & Melchior, F. (2013). Sumoylation: A regulatory protein modification in health and disease. *Annual Review of Biochemistry*, *82*, 357–385. https://doi.org/10.1146/annurev-biochem-061909-093311.

Hendriks, I. A., Lyon, D., Su, D., Skotte, N. H., Daniel, J. A., Jensen, L. J., et al. (2018). Site-specific characterization of endogenous SUMOylation across species and organs. *Nature Communications*, *9*(1), 2456. https://doi.org/10.1038/s41467-018-04957-4.

Hendriks, I. A., Lyon, D., Young, C., Jensen, L. J., Vertegaal, A. C., & Nielsen, M. L. (2017). Site-specific mapping of the human SUMO proteome reveals co-modification with phosphorylation. *Nature Structural & Molecular Biology*, *24*(3), 325–336. https://doi.org/10.1038/nsmb.3366.

Hendriks, I. A., & Vertegaal, A. C. (2016). A comprehensive compilation of SUMO proteomics. *Nature Reviews. Molecular Cell Biology*, *17*(9), 581–595. https://doi.org/10.1038/nrm.2016.81.

Kahyo, T., Nishida, T., & Yasuda, H. (2001). Involvement of PIAS1 in the sumoylation of tumor suppressor p53. *Molecular Cell*, *8*(3), 713–718. https://doi.org/10.1016/S1097-2765(01)00349-5.

Knipscheer, P., Flotho, A., Klug, H., Olsen, J. V., van Dijk, W. J., Fish, A., et al. (2008). Ubc9 sumoylation regulates SUMO target discrimination. *Molecular Cell*, *31*(3), 371–382. https://doi.org/10.1016/j.molcel.2008.05.022.

Knipscheer, P., van Dijk, W. J., Olsen, J. V., Mann, M., & Sixma, T. K. (2007). Noncovalent interaction between Ubc9 and SUMO promotes SUMO chain formation. *The EMBO Journal*, *26*(11), 2797–2807. https://doi.org/10.1038/sj.emboj.7601711.

Koidl, S., Eisenhardt, N., Fatouros, C., Droescher, M., Chaugule, V. K., & Pichler, A. (2016). The SUMO2/3 specific E3 ligase ZNF451-1 regulates PML stability. *The International Journal of Biochemistry & Cell Biology*, *79*, 478–487. https://doi.org/10.1016/j.biocel.2016.06.011.

Mascle, X. H., Lussier-Price, M., Cappadocia, L., Estephan, P., Raiola, L., Omichinski, J. G., et al. (2013). Identification of a non-covalent ternary complex formed by PIAS1, SUMO1, and UBC9 proteins involved in transcriptional regulation. *The Journal of Biological Chemistry*, *288*(51), 36312–36327. https://doi.org/10.1074/jbc.M113.486845. [pii].

Meulmeester, E., Kunze, M., Hsiao, H. H., Urlaub, H., & Melchior, F. (2008). Mechanism and consequences for paralog-specific sumoylation of ubiquitin-specific protease 25. *Molecular Cell*, *30*(5), 610–619. https://doi.org/10.1016/j.molcel.2008.03.021.

Pichler, A., Fatouros, C., Lee, H., & Eisenhardt, N. (2017). SUMO conjugation—A mechanistic view. *Biomolecular Concepts*, *8*(1), 13–36. https://doi.org/10.1515/bmc-2016-0030.

Pichler, A., Gast, A., Seeler, J. S., Dejean, A., & Melchior, F. (2002). The nucleoporin RanBP2 has SUMO1 E3 ligase activity. *Cell*, *108*(1), 109–120. https://doi.org/10.1016/S0092-8674(01)00633-X.

Pichler, A., Knipscheer, P., Saitoh, H., Sixma, T. K., & Melchior, F. (2004). The RanBP2 SUMO E3 ligase is neither HECT- nor RING-type. *Nature Structural & Molecular Biology*, *11*(10), 984–991. https://doi.org/10.1038/nsmb834.

Reverter, D., & Lima, C. D. (2005). Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. *Nature*, *435*(7042), 687–692. https://doi.org/10.1038/nature03588.

Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., & Grosschedl, R. (2001). PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. *Genes & Development*, *15*(23), 3088–3103.

Sternsdorf, T., Jensen, K., & Will, H. (1997). Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1. *The Journal of Cell Biology*, *139*(7), 1621–1634.

Streich, F. C., Jr., & Lima, C. D. (2016). Capturing a substrate in an activated RING E3/E2-SUMO complex. *Nature*, *536*, 304–308. https://doi.org/10.1038/nature19071.

Werner, A., Moutty, M. C., Moller, U., & Melchior, F. (2009). Performing in vitro sumoylation reactions using recombinant enzymes. *Methods in Molecular Biology*, *497*, 187–199. https://doi.org/10.1007/978-1-59745-566-4_12.

Yunus, A. A., & Lima, C. D. (2009). Structure of the Siz/PIAS SUMO E3 ligase Siz1 and determinants required for SUMO modification of PCNA. *Molecular Cell*, *35*(5), 669–682. https://doi.org/10.1016/j.molcel.2009.07.013.

Zhao, X. (2018). SUMO-mediated regulation of nuclear functions and signaling processes. *Molecular Cell*, *71*(3), 409–418. https://doi.org/10.1016/j.molcel.2018.07.027.