Site-specific inhibition of the small ubiquitin-like modifier (SUMO)-conjugating enzyme Ubc9 selectively impairs SUMO chain formation

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Posttranslational modifications by small ubiquitin-like modifiers (SUMOs) regulate many cellular processes, including genome integrity, gene expression, and ribosome biogenesis. The E2-conjugating enzyme Ubc9 catalyzes the conjugation of SUMOs to ε-amino groups of lysine residues in target proteins. Attachment of SUMO moieties to internal lysines in Ubc9 itself can further lead to the formation of polymeric SUMO chains. Mono- and poly-SUMOylations of target proteins provide docking sites for distinct adapter and effector proteins important for regulating discrete SUMO-regulated pathways. However, molecular tools to dissect pathways depending on either mono- or poly-SUMOylation are largely missing. Using a protein-engineering approach, we generated high-affinity SUMO2 variants by phage display that bind the back side binding site of Ubc9 and function as SUMO-based Ubc9 inhibitors (SUBINs). Importantly, we found that distinct SUBINs primarily inhibit poly-SUMO chain formation, whereas mono-SUMOylation was not impaired. Proof-of-principle experiments demonstrated that in a cellular context, SUBINs largely prevent heat shock-triggered poly-SUMOylation. Moreover, SUBINs abrogated arsenic-induced degradation of promyelocytic leukemia protein. We propose that the availability of the new chain-selective SUMO inhibitors reported here will enable a thorough investigation of poly-SUMO-mediated cellular processes, such as DNA damage responses and cell cycle progression.

Post-translational modifications by the small ubiquitin-like modifiers (SUMOs) regulate a plethora of cellular processes, including genome integrity, gene expression, and ribosome biogenesis (1–3). Unlike ubiquitination in vertebrates that is mediated by a complex machinery composed of two E1 activating enzymes, roughly 36 E2 enzymes and >600 E3 enzymes, SUMOylation is mediated by only one heterodimeric E1 enzyme (SAE1/SAE2), a single E2 enzyme (Ubc9), and only a limited number of E3 ligases. Complexity in SUMOylation processes is introduced through the conjugation of at least three formally different proteins to substrates: SUMO1 and the highly similar SUMO2 and -3. After posttranslational processing by SUMO-specific proteases (SENPs), the mature forms of SUMO2 and -3 share a 97% identity, whereas SUMO1 shares only 47% identity to SUMO2. The current view is that SUMO2 and -3 can be assembled into poly-SUMO chains, whereas SUMO1 predominantly serves in mono-SUMOylation of substrates or acts as chain terminator. However, MS-based proteomics provide evidence for the existence of SUMO1 chains as well as mixed SUMO1-SUMO2/3 chains (4, 5).

SUMO-chain formation is typically triggered by heat or osmotic stress that leads as a cellular response mechanism to a rewiring of binding partners that harbor specific SUMO interaction motifs. So far only a few processes have been defined as being dependent on poly-SUMOylation. The best-studied pathway is the SUMO-primed ubiquitination of cellular proteins by the evolutionary conserved family of SUMO-targeted E3 ubiquitin ligases (STUBLs) (6). In mammals, the RING-type ligases RNF4 and RNF111 belong to the STUBL family and specifically bind to polymeric SUMO2/3 chains via tandem repeats of SUMO-interaction motifs (SIMs). Thus, poly-SUMOylated substrates provide a docking site for the recruitment of STUBLs, which subsequently label the proteins by conjugating ubiquitin to target sites. The best-characterized STUBL target is promyelocytic leukemia protein (PML), which subsequently undergoes osmotic stress that leads as a cellular response mechanism to a rewiring of binding partners that harbor specific SUMO interaction motifs. So far only a few processes have been defined as being dependent on poly-SUMOylation. The best-studied pathway is the SUMO-primed ubiquitination of cellular proteins by the evolutionary conserved family of SUMO-targeted E3 ubiquitin ligases (STUBLs) (6). In mammals, the RING-type ligases RNF4 and RNF111 belong to the STUBL family and specifically bind to polymeric SUMO2/3 chains via tandem repeats of SUMO-interaction motifs (SIMs). Thus, poly-SUMOylated substrates provide a docking site for the recruitment of STUBLs, which subsequently label the proteins by conjugating ubiquitin to target sites. The best-characterized STUBL target is promyelocytic leukemia protein (PML), which subsequently undergoes

The abbreviations used are: SUMO, small ubiquitin-like modifier; PML, promyelocytic leukemia; S2vs, S2 variant; TEV, tobacco etch virus; ITC, isothermal titration calorimetry; HSQC, heteronuclear single quantum correlation; CSP, chemical shift perturbation; RFP, red fluorescent protein; SIM, SUMO-interaction motif; SUBE, poly-SUMO-specific affinity traps.

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elocytic leukemia (PML) protein, which is ubiquitinated and degraded by either RNF4 or RNF111 in a poly-SUMOylation-dependent manner (7–9). Importantly, poly-SUMOylation of PML and the leukemogenic fusion protein PML-RARα is specifically induced by arsenic trioxide providing a molecular explanation for the therapeutic effect of this drug in PML-RARα-positive acute promyelocytic leukemia. In addition to PML turnover, the StUBL pathway is also instrumental for the control of transcriptional processes and proper DNA damage response (10). In double-strand break repair RNF4 controls the dynamics of DNA repair foci. One critical target in this process is mediator of DNA damage checkpoint 1 (MDC1), which is ubiquitinated by RNF4 in a poly-SUMO-dependent manner (11, 12). Although StUBL-mediated ubiquitination is well established, the functional significance of other proteins that bind poly-SUMO chains has remained largely unclear (13).

The sole E2-conjugating enzyme Ubc9 is the linchpin of the SUMO system that functionally connects the E1 activating enzyme with substrate SUMOylation. In general, Ubc9 conjugates SUMO to target proteins at the surface-exposed e-NH₂ group of a lysine residue that is frequently but not exclusively embedded in a consensus motif ψKXE(D/E) (ψ indicates a hydrophobic amino acid, whereas X indicates any amino acid) (14). SUMOylation does not strictly depend on E3 ligases, but E3 enzymes facilitate the conjugation by inducing a conformation of Ubc9—SUMO that is primed for transfer of the donor SUMO (15). Importantly, Ubc9 not only interacts covalently with the donor SUMO via a thioester bond but can also bind a second SUMO molecule by non-covalent interactions on the opposite surface or back side located on the N-terminal α-helix of Ubc9 (16, 17). Structural and biochemical analysis of the Ubc9 back side binding site revealed that mutations in the N-terminal helix alter SUMO back side binding and also modulate chain formation activity and thioester formation (16, 17). Additionally, back side binding is critical for poly-SUMO chain formation catalyzed by tandem SIM-containing SUMO-E3 ligases (18, 19). Based on these insights, we hypothesized that high affinity inhibitors that disrupt the binding of SUMO to the back side of Ubc9 could be used to block SUMOylation processes. Therefore, we developed SUMO2 variants (SUBINs (SUMO-based Ubc9 inhibitors)) that selectively bind the back side of Ubc9 with high affinity and compete with SUMO2 WT. SUBINs have been developed using a previously established phage display approach that yielded highly selective inhibitors and modulators of enzymes in the ubiquitination pathway (20–27). SUBINs inhibit poly-SUMOylation in vitro, limit cellular SUMO chain formation in response to heat stress, and prevent arsenic-induced degradation of PML.

**Results**

**Library design and selection of high affinity Ubc9-binding S2 variants (S2vs)**

We based our library on SUMO2/3 because they are the most abundant SUMO family members (28). We displayed an N-terminally truncated SUMO2 (herein referred to S2.WT) that lacks the first 14 unstructured residues (ΔN-14) including the primary site of SUMO chain formation (14). It was previously shown that the deletion of the unstructured N-terminal residues does not affect overall stability of SUMO2 (29). S2vs derived from such a library that additionally lack the C-terminal di-Gly motif (ΔGG) cannot be activated by the E1 complex or become a part of SUMO2/3 chains when expressed in cells. Residues for randomization were chosen based on structural analysis of SUMO family members in complex with Ubc9 and SENPs (16, 17, 30). Interface residues of SUMO2 buried in complexes with Ubc9 or de-conjugating enzymes are located on the β1 and β2 (region 1), β3 (region 2), and the C-terminal β4 (region 3) strand extending into the C-terminal di-Gly motif. We soft-randomized a total of 25 residues that are surface-exposed, and their side chains are part of the interface (Fig. 1A and supplemental Fig. 1). Soft-randomization allows for a sufficient diversity across the binding interface and ensures that the overall structure of SUMO2 is maintained (20). In total, the library contains $1.5 \times 10^{10}$ independent S2vs that were displayed as N-terminal fusion with the minor coat protein (pIII) on the surface of filamentous phage. After 5 rounds of phage display selection, we identified 45 unique S2vs selectively binding to Ubc9 compared with S2.WT (supplemental Fig. 1). Sequence analysis showed that mutations are distributed over the complete interface. Nevertheless, the residues Ala23, Gln25, and Thr83 of S2.WT show preferred mutations to Gln, Glu, and Leu or Gln, respectively, indicating that these residues establish critical contacts to Ubc9. Additionally, the prevalence of these mutations implies that the selected variants bind a similar epitope in Ubc9. Initial specificity tests against Ubc9 and SENPs as controls showed that the majority of the selected variants are highly specific and exhibit in comparison to S2.WT enhanced binding to Ubc9 (Fig. 1B and supplemental Fig. 1). In a next step we estimated the IC₅₀ whereas the Ubc9-specific variants were still displayed on phage by competing in solution with increasing amounts of Ubc9 (supplemental Fig. 1). The binding of most variants could be reduced by >50% with as little as 25 nM Ubc9 as competitor, indicating that the selected variants have an IC₅₀ value better than 25 nM when displayed on phage.

For further characterization, we chose three variants that showed high affinity and specificity toward Ubc9 (Fig. 1C). For *in vitro* characterization, we cloned the S2.WT and the variant E2.15 without the C-terminal di-Gly motif, whereas the variant E2.34 was cloned including the mutation G93T at the C terminus. The proteins were purified as N-terminal fusions with a hexahistidine tag followed by a tobacco etch virus (TEV) cleavage site, and we tested their binding to Ubc9 by isothermal titration calorimetry (ITC) (Fig. 1D). In initial experiments and in agreement with previous reports (17), we found that at 25 °C S2.WT ΔGG interacted with Ubc9 only with low enthalpy change, making a reliable fit of the binding constants difficult. Consequently, all ITC experiments were carried out at 37 °C to allow sufficient enthalpy difference between the unbound and bound state for the S2.WT ΔGG and S2vs binding to Ubc9. The S2vs E2.15 ΔGG and E2.34 have $K_d$ values of 58 nM and 16 nM, respectively (Table 1). In contrast to previously reported $K_d$ values between 0.08 and 0.15 μM for SUMO2/Ubc9 interaction (17, 18), we measured a $K_d$ of 2.44 μM for S2.WT ΔGG (Table 1). The most likely explanations for the differences in $K_d$ for the WT interaction are that we used an N-terminal trunc-
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Figure 1. S2 library design and binding of high affinity S2vs to Ubc9. A, phage-displayed S2 library design mapped onto the SUMO2 structure (PDB code 2ckh). SUMO2 (gray) and randomized residues of three regions (yellow, region 1; purple, region 2; teal, region 3) are marked as colored spheres. B, S2vs bind selectively to Ubc9. Binding of phage displaying S2vs measured by phage ELISA to immobilized proteins Ubc9, SENP1, SENP2, SENP6 and SENP7. Color intensity corresponds to absorption at 450 nm from A$_{450}$ = 0.75 (maximum) to 0.0 (minimum). C, sequence of S2.WT and selected S2vs binding to Ubc9 (S2v.E2.15, S2v.E2.20, and S2v.E2.34). Only positions within the randomized regions that were mutated in the selected variants are shown; conserved residues compared to S2.WT or S2vs were titrated to 20 μM Ubc9. Affinity of the S2v.E2.34 was measured by competing for the binding site with S2.WT

Table 1
Thermodynamic parameters of the Ubc9 S2.WTΔGG and S2vs interactions derived from ITC experiments at 37 °C

| Ubc9          | ΔH (kcal/mol) | ΔS (kcal/mol·K) | ΔG (kcal/mol) | K_d (μM) | N   |
|---------------|---------------|-----------------|---------------|----------|-----|
| +S2.WTΔGG     | -2.80 ± 0.03  | -5.15           | -7.95         | 2.44     | 1.01 ± 0.01 |
| +S2v.E2.15ΔGG | -8.49 ± 0.04  | -1.78           | -10.27        | 0.058    | 1.02 ± 0.01 |
| +S2.WTΔGG/S2v.E2.34 | -11.33 ± 0.05 | +0.27           | -11.06        | 6.29 ± 0.23 × 10^7 | 0.016 |

S2v binding overlaps with SUMO2 WT binding to Ubc9

To understand where the variants bind Ubc9, we recorded HSQC spectra of 15N-labeled Ubc9 in the absence and presence of ligands and analyzed the chemical shift perturbation of contact residues (Fig. 2A). Residues were assigned based on earlier published HSQC spectra of Ubc9 (32). The chemical shift perturbation (CSP) analysis shows that the same resonances undergo changes upon the addition of ligand, confirming overlapping binding sites for the S2vs and S2.WTΔGG. Differences in the directions and chemical shift perturbation values are most likely due to variations in the chemical environment of the Ubc9 residues contacting mutated S2vs residues. Additionally, the interaction for S2.WTΔGG is in the intermediate exchange on the NMR time scale, which is consistent with an affinity in the μM range corroborating the results from ITC measurements (supplemental Fig. 2). The chemical shifts observed in the titration with the high affinity variants reflect an interaction in the slow exchange mode, which is also consistent with the low μM K_d measured by ITC.

Mapping residues with a CSP larger than 1 S.D. from the mean CSP onto the surface of Ubc9 indicates that the binding site of the variants is predominantly located at the C-terminal end of the first α-helix and a loop connecting to the first β-strand of Ubc9 (Fig. 2B). This binding site corresponds to the
earlier determined back side binding site of Ubc9 (supplemental Fig. 3) (16, 17). To better understand which residues are required for binding, we introduced Ala mutations of the surface-exposed residues Lys14, Lys18, Asp19, Phe22, Lys49, and Gln111 in the Ubc9 epitope and simultaneously tested the binding of the variant E2.34 and E2.15. Although Lys49 in Ubc9 did not undergo significant chemical shift perturbation, it was included in this analysis to test if the Glu25 in the variant E2.34 establishes polar contacts to this residue. Our data showed that the binding of the S2vs to Ubc9 with single mutations at the positions Lys14, Lys18, and Phe22 is reduced, whereas it was completely abolished when Ubc9 harbored simultaneous alanine mutations at all three residues (Fig. 2C). Conversely, when testing the binding to Ubc9 with K49A or Q111A mutations, no reduction in binding of the variants could be observed, indicating that these residues were not in direct contact with this residue. Our data showed that the binding of the S2vs to Ubc9 with single mutations at the positions Lys14, Lys18, and Phe22 is reduced, whereas it was completely abolished when Ubc9 harbored simultaneous alanine mutations at all three residues (Fig. 2C). Conversely, when testing the binding to Ubc9 with K49A or Q111A mutations, no reduction in binding of the variants could be observed, indicating that these residues were not in direct contact with this residue.

In parallel, we tested back-to-WT mutations at the paratope position 83 in the variant E2.15 and at the paratope positions 25, 83, and the C-terminal residues 89, 90, and 93 in the variant E2.34 to probe their respective contribution to the improved binding to Ubc9 (Fig. 2C). Interestingly, the binding of the variants depended almost exclusively on the enriched mutation Gln83, whereas the back mutation of the equally well-conserved mutation at position 25 or the C-terminal residues did not contribute to the binding to Ubc9. Nevertheless, the mutation at position 25 may have accessory roles as shown by the reduced binding to Ubc9 when mutated at Lys18 or Phe22. Additionally, the C-terminal mutations in E2.34 may have inhibitory effects in vitro and in cells. Importantly, the S2.WT had no detectable binding in this assay indicating that the micromolar affinity measured in our ITC experiments was above the detection threshold. Taken together, these data demonstrate that the variants compete with S2.WT binding and recognize an epitope centered on the residues Lys18 and Phe22 located on the first α-helix of SUMO2. Nevertheless, the additional mutations in E2.34 are most likely beneficial in improving specificity and strengthening existing contacts.

**S2v.E2.34 limits SUMO chain formation by Ubc9 in vitro**

We next aimed to determine whether SUBINs affect the SUMO conjugation process. In addition to the variants E2.15ΔGG and E2.34, we included the variant E2.20ΔGG (Fig. 1B) in the experiments because we were interested in how a single mutation of threonine to leucine at position 83 of S2.WTΔGG affects the activity of Ubc9. To this end we performed in vitro S2.WT Ubc9 thioster formation and SUMO2 conjugation assays in a purified biochemical system composed of recombinantly expressed E1, Ubc9, and S2.WT or wild-type
SUMO2 as substrate. First, we asked if the formation of the S2.WT—Ubc9 thioester conjugate is influenced in the presence of the high-affinity SUBINs that block the back side of Ubc9 (Fig. 3, A and B). We used the S2.WT construct for thioester formation because it can be activated by E1 and E2 due to the presence of the di-Gly motive but cannot form poly-SUMO chains, as it still lacks the primary site for poly-SUMOylation (N-14). To investigate concentration-dependent effects, experiments were performed at 1:1, 1:4, and 1:6 ratios of Ubc9 against SUBINs or the corresponding S2.WTΔGG, respectively (Fig. 3, A and B). Reaction products were separated by SDS-PAGE, and band intensities were quantified. In control reactions, a S2.WT—Ubc9 thioester conjugate migrating at 35 kDa was detected on SDS-PAGE in the absence of DTT upon anti-Ubc9 immunoblotting. The addition of the N-terminally truncated, conjugation-deficient S2.WTΔGG to the reaction at a 1:1 concentration slightly reduced the charging of Ubc9 with S2.WT compared with the corresponding positive control. Importantly, however, the addition of the high-affinity Ubc9-binding variants did not further reduce thioester formation. Increasing concentration of either S2.WTΔGG or SUBINs led to a more significant decrease in E2-charging, but again the differences between SUBINs and S2.WTΔGG at the same concentration were statistically not significant (Fig. 3B). Notably, we also observed that upon reduction of the thioester bond by DTT, a Ubc9-S2.WT conjugate was still detectable. This DTT-resistant conjugate likely represents a lysine-linked SUMO2-Ubc9 conjugate that has been described previously (33). It is worth noting that the formation of this conjugate was also not affected by SUBINs.

With respect to the proposed involvement of SUMO backside binding on Ubc9 in SUMO chain formation, we asked whether this process is affected by SUBINs. In the absence of other target proteins, SUMO2 is converted in a reconstituted in vitro system in the presence of ATP mainly to polymeric lysine-linked SUMO2-chains. Accordingly, after separation
of reaction products of an in vitro SUMOylation assay by SDS-PAGE and immunoblotting with an anti-SUMO2/3 antibody, di-SUMO2 (~35 kDa), tri-SUMO2 (~53 kDa), and higher order SUMO2 conjugates are detected in the presence of ATP, whereas only monomeric SUMO2 is found in the control sample lacking ATP (Fig. 3C). To avoid a rate-limiting E1–E2 trans-thiolation reaction, we performed the poly-SUMOylation assay in excess of E1 to Ubc9. This yields higher order Uba2-SUMO2 conjugates that have been described before (17). The addition of S2.WTΔGG, S2v.E2.15ΔGG, and S2v.E2.20ΔGG only moderately impairs the formation of poly-SUMO2 species relative to the positive control. Importantly, the variant E2.34 with the highest affinity and mutated di-Gly motif (G93T) shows a strong inhibition of poly-SUMO chains that contain more than two SUMO2 moieties. These data are consistent with the notion that charged Ubc9 can at least transfer one SUMO2 moiety to acceptor sites independent of back-side binding. Nevertheless, blocking the back side of Ubc9 by E2.34 impairs elongation of di-SUMO2 moieties by additional SUMO2 units. The variants E2.15ΔGG and E2.20ΔGG do not lead to a reduction of poly-SUMO chains to the same extent as E2.34, indicating that high affinity and a mutated di-Gly motif (G93T) is required to compete with the binding of poly-SUMO chains and free SUMO2 substrate as well as E1 binding.

To further examine the inhibitory potential of the variants on SUMO conjugation, we performed an in vitro conjugation assay using p53 (generated by in vitro transcription/translation using a rabbit reticulocyte system) as a bona fide substrate. In this in vitro assay, p53 became poly-SUMOylated resulting in distinct bands at ~70 kDa, 87 kDa, and 104 kDa corresponding to the attachment of one, two, or three SUMO2 moieties, respectively (Fig. 3D). As additional control, we used a modified SUMO2 that can still be conjugated but lacks the major SUMO conjugation sites at the N-terminus and, therefore, cannot efficiently form poly-SUMO chains. Similar to the results in the substrate-independent in vitro assay experiment, the transfer of one SUMO2 unit to the substrate is not impaired in the presence of the S2.WTΔGG and variants when compared with the control reactions. However, the variant E2.34 strongly impairs the formation of di- and tri-SUMOylated p53 species without affecting mono-sumoylation. In summary, our data show that E2.34 inhibits the assembly of poly-SUMO chains by blocking the back side of Ubc9.

**S2vs bind Ubc9 in cells**

We next proceeded to characterize the S2vs in a cellular setting and first monitored the association of the variants with endogenous Ubc9 in a co-immunoprecipitation experiment (Fig. 4A). To this end, RFP-tagged S2.WTΔGG and the respective variants E2.15ΔGG, E2.20ΔGG, and E2.34 were transiently expressed in HeLa cells and after cell lysis proteins were captured with anti-RFP beads. Precipitates were separated by SDS-PAGE and probed for the presence of endogenous Ubc9 by immunoblotting with an anti-Ubc9 antibody. Indicative of its relatively weak affinity, S2.WTΔGG retained only a minor amount of Ubc9. However, in agreement with the in vitro binding studies, the S2vs co-precipitate a significant fraction of Ubc9. Next, we asked if the variants do specifically associate with Ubc9 in cells. Therefore, we analyzed by mass spectrometry anti-FLAG immunoprecipitates from HEK293T cells expressing FLAG-tagged S2.WTΔGG or the S2vs (Fig. 4B, supplemental Table 1). The variants selectively bound Ubc9 and led in the case of the variant E2.34 to a >70-fold enrichment of Ubc9-derived peptides over S2.WTΔGG background. The variants E2.15ΔGG and E2.20ΔGG resulted in a lower enrichment of Ubc9 peptides of around 25 and 7 times, respectively. In summary, these results indicate that the variant E2.34 is highly specific to Ubc9 in cells and that the selectivity of the variants correlates with affinity and number of mutations accumulated in the SUMO2 scaffold.

**SUBIN E2.34 inhibits poly-SUMOylation in cells**

Next, we set out to test whether SUBINs, in particular the E2.34 variant, can be used as a tool to specifically interfere with the formation of SUMO chains and SUMO-regulated pathways in a cellular context. We first monitored whether the expression of E2.34 alters SUMO chain formation upon induction of heat stress. HeLa cells were transfected with control vectors or vectors encoding N-terminally truncated wild-type S2.WTΔGG
or SUBIN E2.34. Cells were either left untreated or incubated for 1 h at 43 °C, and cell lysates were prepared. Using validated poly-SUMO-specific affinity traps (SUBE), poly-SUMO conjugates were captured from these lysates (34), separated by SDS-PAGE, and immunoblotted with an anti-SUMO2/3 antibody. In control cells, a smear of anti-SUMO2/3-reactive poly-SUMO conjugates migrating above 150 kDa could be observed (Fig. 5A). As expected, the extent of poly-SUMOylation is significantly enhanced upon heat stress. More importantly, in the presence of SUBIN E2.34 but not the corresponding wild-type SUMO control, poly-SUMOylated species are drastically diminished, further supporting our conclusion that E2.34 limits SUMO chain formation (Fig. 5A).

A well-established SUMO-mediated pathway, which particularly relies on the formation of SUMO2 chains, is the arsenic-induced degradation of PML. As outlined in the Introduction, arsenic triggers the formation of SUMO chains on PML that are recognized by the SUMO-targeted ubiquitin-ligase RNF4 mediating UPS-dependent degradation of PML. To investigate whether SUBINs interfere with this process, HeLa cells were transiently transfected with SUBINs (E2.15ΔGG, E2.20ΔGG, and E2.34) or the respective wild-type S2.WTΔGG. Two days after transfection, cells were treated with arsenic for 6 h, and PML levels were monitored by immunoblotting with an anti-PML antibody (Fig. 5B). In cells expressing S2.WTΔGG, an anti-PML-reactive band migrating at 110 kDa was specifically lost upon the addition of arsenic. By siRNA experiments, we validated that this band indeed corresponds to PML (supplemental Fig. 4). Expression of E2.15ΔGG and E2.20ΔGG slightly reduces the arsenic-induced degradation of the 110-kDa PML species relative to S2.WTΔGG control. However, E2.34, which has the highest binding affinity toward Ubc9, almost completely abolished PML degradation.

To further validate that E2.34 affects PML degradation by directly impairing its poly-SUMOylation, we used a cell line stably overexpressing the HA-tagged isoform III of PML. Short-term treatment with arsenic (2 h) triggers poly-SUMOylation of HA-PML without reduction of PML levels in this experimental system due to overexpression of HA-PML (Fig. 5C). Anti-HA immunoblotting reveals four major

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Figure 5. SUBIN E2.34 inhibited heat shock-induced poly-SUMOylation and PML poly-SUMOylation in cells. A, HeLa cells were transfected with the indicated RFP-tagged constructs. Before lysis, cells were heat shocked for 1 h at 43 °C. Pulldown of poly-SUMO conjugates with GST or SUBE were analyzed by immunoblotting using the indicated antibodies. B, HeLa cells were transfected with the indicated RFP-tagged constructs. Cells treated with AsO$_3$/H$_2$O for 6 h and untreated controls were analyzed by immunoblotting using the indicated antibodies. C, HeLa cells stably expressing HA-PML III were transfected with the indicated RFP-tagged constructs. Cells treated with AsO$_3$/H$_2$O for 2 h, and untreated controls were analyzed by immunoblotting using the indicated antibodies.
bands in untreated cells expressing S2.WTΔGG. The anti-HA reactive band at 100 kDa represents unmodified PML, whereas the 120 kDa, 135 kDa, and 180 kDa species correspond to SUMO conjugates, with one, two, or three SUMO moieties attached to distinct lysine residues of PML (35). Treatment with arsenic triggered the appearance of higher molecular weight anti-HA-reactive bands migrating above 180 kDa, which represent PML species that are modified by SUMO chains or mixed Ub/SUMO chains (8, 9). Expression of which indicate that SUBINs and in particular the E2.34 variant inhibit arsenic-induced PML degradation, most likely by preferentially interfering with SUMO chain formation on PML. Altogether, the data suggest that the E2.34 variant is a valuable tool to study poly-SUMO controlled cellular processes.

Discussion

Protein engineering of intracellular modulators based on ubiquitin and ubiquitin-like proteins has recently been shown to be an effective strategy to study distinct functional epitopes in ubiquitin-specific proteases (USPs), HECT-E3 ligases, F-box proteins, and linear ubiquitin binding motifs (20–22, 27). Here, we have expanded this approach to SUMO-dependent processes and developed site-specific inhibitors of the sole SUMO-specific E2-conjugating enzyme Ubc9 by engineering amino acid substitutions in SUMO2 that enhance affinity and specificity. ITC measurements and NMR studies showed that the inhibitors block selectively the back side binding site of Ubc9 with an up to ×150 stronger affinity than S2.WTΔGG. Unlike the high entropic contribution in S2.WTΔGG binding, the increase in affinity of the S2 variants is enthalpy-driven. Importantly, intracellular experiments and in vitro SUMOylation assays show that the variant E2.34 with the highest affinity and mutated di-Gly motif has the strongest inhibitory potential and blocks the formation of poly-SUMO chains. The decreased charging of Ubc9, which we observed in the thioester formation assay, is most likely due to a partial overlap of the SUMO back side and the E1 binding site, which has been shown, at least for the yeast Uba2<sup>Uld</sup>, to be centered also on the first α-helix of Ubc9 (36). Consequently, the reduction of E2-charging independent of the binding affinity of the SUMO variants suggests a weak interaction between the heterodimeric E1 with Ubc9 that is already outcompeted by the 2.4 μM interaction of S2.WTΔGG. A recurring property of the variant E2.34 is that the transfer of a second SUMO moiety relative to the substrate is impaired, suggesting that the coordination of the growing poly-SUMO chain by the back side binding site is required for chain elongation by Ubc9. Recently, a similar finding has been reported that describes the back side binding site of Ubc9 as being required for chain elongation by E3 ligases (18, 19). Additionally, mutations that disrupt back side binding of Ubc9 to SUMO make the enzyme incapable of forming poly-SUMO chains in vitro (16, 17). Aside from the molecular aspects of Ubc9 inhibition, RNA interference-mediated Ubc9 knockdown has been used to study SUMOylation processes in cell based cancer models (37, 38). However, Ubc9 knockdown is not able to discriminate between different processes that depend exclusively on mono- or poly-SUMOylation. Our results show that the SUBIN E2.34 causes an overall reduction of poly-SUMOylation in cells, which suggests that reversible inhibition of the back side of Ubc9 is a valuable approach to investigate the role of poly-SUMO chains in a proteome wide scale. Thus, site-specific inhibitors that act reversibly on Ubc9 and selectively block poly-SUMOylation are important tools to study the role of poly-SUMOylation in DNA damage response and cell cycle progression.

Experimental procedures

Construction of a phage-displayed SUMO2 library

SUMO2 (ΔN-14) wild-type (S2.WT) library was constructed using previously described methods (39). In brief, S2.WT DNA was cloned into the phagemid pNE (20), and the library was constructed using site-directed mutagenesis by simultaneously targeting three regions of the S2.WT gene with degenerate oligonucleotides (Fig. 1A) (39, 40). Mutations were introduced using a soft randomization approach, where the nucleotide ratio at degenerate positions was adjusted to 70% of the WT nucleotide and 10% of each of the other nucleotides (41). The degenerate oligonucleotides used to generate the S2 library are summarized in supplemental Table 2. The resulting library was used to electroporate Escherichia coli SS320 cells, resulting in 1.5 × 10<sup>10</sup> independent S2vs (39).

Selection of S2 variants specifically binding to Ubc9

Phage display of individual S2 variants were harvested by precipitation with PEG/NaCl (20% PEG-8000 (w/v), 2.5 M NaCl) and resuspended in PBT buffer (1× PBS, 1% BSA, 0.1% Tween 20). Immobilization of the target protein and subsequent binding selections were done using established methods (39). In short, 4 wells of a 96-well Maxisorp microtiter plate (NUNC) were immobilized with 100 μl of 2 μM GST-fused Ubc9 in PBS overnight at 4 °C. After blocking with PBT buffer for 1 h, the phage library pool was added to each well and incubated for 1 h at room temperature. The plate was washed 8 times with cold PT buffer (1× PBS, 0.1% Tween 20), and bound phage were eluted with 0.1 M HCl and immediately neutralized with 1.0 M Tris/HCl, pH 8.0. Eluted phage was directly used to infect exponentially growing E. coli XL1-blue supplemented with helper phage M13K07 (New England BioLabs) and incubated overnight at 37 °C. In each successive selection round, the selection stringency was increased by two additional washing steps. After five rounds of enrichment, individual S2 variants with improved binding properties toward Ubc9 were identified by clonal phage ELISA as described (39) and sequenced (supplemental Fig. 1).

Binding specificity of selected S2 variants

The specificity of all 45 selected S2vs and S2.WT was tested in a phage ELISA format (42) using GST-tagged SENP1, SENP2, SENP6, SENP7, GST alone, and BSA as controls and GST-tagged Ubc9 as a positive control. Phage-displaying individual S2 variants were prepared from single colonies of bacte-
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ria harboring phagemids encoding S2vs using established methods (20).

**Competitive phage ELISA**

The IC_{50} value of phage displayed S2vs was estimated in a competitive phage ELISA (39). Culture supernatant containing phage that display an individual S2v was diluted 1:3 in PBS and incubated with buffer control or increasing concentrations of Ubc9 at final concentrations of 25 nM, 100 nM, 250 nM, and 500 nM in solution for 30 min at 25 °C. The preincubated phage solution was transferred to immobilized GST-tagged Ubc9 (0.5 μM in PBS) and incubated for 30 min at 25 °C. After washing with PT buffer, bound phage was detected by the addition of anti-M13 antibody (GE Healthcare) fused to horseradish peroxidase and developed with tetramethylbenzidine (TMB) substrate (BD Biosciences). Binding signals were normalized to phage signals incubated without Ubc9. The IC_{50} values correspond to the concentration of Ubc9 that blocks 50% of Ubc9-phage binding to immobilized Ubc9.

**Phage ELISA for binding site evaluations**

Alanine mutants of GST-tagged Ubc9 (K14A, K18A, D19A, F22A, K49A, Q111A) were generated by site-directed mutagenesis using QuikChange (Agilent) following the user manual. Point mutations in S2.E2.15 and E2.34 were introduced using site-directed mutagenesis (41). Purified alanine mutants of Ubc9 (0.5 μM in PBS) were immobilized in a 384 Maxisorp microtiter plate (NUNC), and S2vs mutants were prepared from single colonies of bacteria transformed with phagemids encoding S2vs using established methods (43). Binding was tested using phage ELISA format (42).

**Cloning of S2 variants**

DNA fragments encoding the variants E2.15, E2.20, and the S2.WT without the C-terminal di-Gly motif (S2.WTGG) were replaced by a TEV cleavage site by site-directed mutagenesis using specific primers adding attB1 and attB2 sites at the 5’ and 3’ ends compatible for Gateway cloning (Invitrogen). E2.34 was cloned with the mutated di-Gly motif (G93T). For thioester formation assay, S2.WT bound to glutathione-Sepharose 4 Fast Flow beads (GE Healthcare) was cleaved on-column by the addition of 0.33 mg/ml purified GST-tagged PreScission Protease and incubated for 30 min, and subsequently precipitated by centrifugation at 10,000 × g for 30 min, and subsequently the supernatant was purified using nickel-nitriolitri-acetic acid chromatography (Qiagen) for His-tagged proteins or glutathione Sepharose 4 Fast Flow beads (GE Healthcare) for GST-tagged proteins at 4 °C following the manufacturer’s instructions. Eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Fractions with purified protein were combined and dialyzed into 150 mM NaCl, 50 mM Tris/HCl, pH 7.5, and 1 mM DTT. Protein concentrations were determined by measuring the absorption at 280 nm (NanoDrop). For NMR and ITC experiments, the hexahistidine tag of S2 variants and S2.WTGG was removed by TEV cleavage as described (45). Uniformly ^15N-labeled Ubc9 was expressed as a polyhistidine-tagged ubiquitin fusion protein (46) in T7 Express competent E. coli (New England BioLabs). Cells were grown in M9 medium supplemented with [^15N]-NH₄Cl (1 g/liter) and induced at 18 °C for 20 h with 0.2 mM isopropyl 1-thio-β-d-galactopyranoside. Purification included lysis, immobilized metal affinity chromatography (IMAC), tag removal by TEV protease, and a second IMAC step followed by gel filtration. For *in vitro* thioester formation and SUMOylation assays, the heterodimeric E1 was purified from bacteria expressing His-tagged Uba2 together with GST-tagged Aos1 as described (47). For thioester formation assay, the GST tag of Ubc9 was removed by PreScission protease cleavage. In brief, GST-fused Ubc9 bound to glutathione-Sepharose 4 Fast Flow beads (GE Healthcare) was cleaved on-column by the addition of 0.33 mg/ml purified GST-tagged PreScission Protease and incubated over night at 4 °C. Eluates were collected and analyzed by SDS-PAGE.

**Isothermal titration calorimetry**

ITC measurements were performed at 37 °C in 25 mM HEPES/NaOH, pH 7.0, and 150 mM NaCl using a VP-ITC microcalorimeter (MicroCal). S2v.E2.34 (150 μM) and S2v.E2.15ΔGG (150 μM) were titrated into 10 μM Ubc9; S2.WTΔGG (550 μM) was titrated into 40 μM Ubc9 (12.5 μl injections). ITC data were analyzed using ITC-Origin 7.0 software with a one-site binding model. For precise determination of the thermodynamic parameters of the E2.34 binding, a displacement assay was used; Ubc9 (20 μM) was saturated with S2.WTΔGG (160 μM) and titrated with E2.34 (300 μM). The resulting curve was analyzed with a competitive binding model (31) using the thermodynamic parameters previously obtained for the Ubc9:S2.WTΔGG interaction.

**NMR spectroscopy**

NMR experiments of apoUbc9 and Ubc9 in complex with ligand were performed in 25 mM HEPES/NaOH, pH 7.0, and 150 mM NaCl supplemented with 5% D₂O for field frequency lock. Spectra were acquired on a 600 MHz Bruker Avance spec-
trometer equipped with a 5-mm triple-resonance cryogenic z axis gradient probe. Data were processed using TopSpin 3.0 software (Bruker) and analyzed with the NMR assignment and integration software Sparky (UCSF). Titration of 15N-labeled Ubc9 (0.2 mM) with S2.WT ΔAGG or S2vs was monitored by a series of 1H,15N HSQC spectra until saturation was reached. Spectra were acquired at Ubc9:S2.WT ΔAGG or Ubc9:S2vs molar ratios of 0, 1:8, 1:4, 1:2, 3:4, 1, 3:2, 2, and 4. Backbone resonance assignment of human Ubc9 was adopted from Liu et al. (32), and CSP were calculated using $\Delta \delta = (\delta_{\text{sat}} - \delta_{\text{free}})^2 \times 0.17 + (\delta_{\text{sat}} - \delta_{\text{free}})^2 / 2$.

**In vitro E2 thioester formation assay**

Ubc9 and S2.WT ΔAGG was performed in a total volume of 20 μl in buffer containing 20 mM Tris/HCl, pH 8.0, 100 mM NaCl, and 5 mM MgCl₂. 190 nM Ubc9 (previously cleaved with PreScission protease) was incubated with 1140 nM, 760 nM, or 190 nM of the indicated S2vs or S2.WT ΔAGG for 30 min at room temperature. 200 nM E1 and 3 μM S2.WT were added, and the reaction was started by the addition of 4 μM ATP. Reactions were performed at 30°C for 10 min and stopped by the addition of 3× SDS Laemmli buffer with or without reducing agent (200 mM DTT) and incubated for 5 min. Samples were run on SDS-PAGE, immunoblotted, and analyzed with anti-Ubc9 antibody (Ubc9 clone 50; BD Biosciences).

**In vitro SUMOylation assays**

S2vs, S2.WT ΔAGG, and SUMO2 full length were used for in vitro SUMOylation. His-tagged Ubc9 (265 nM) and the indicated S2vs (1.5 μM) was preincubated in in vitro SUMOylation buffer (50 mM Tris/HCl, pH 7.5, 2 mM ATP (freshly added)) at room temperature for 30 min. Subsequently, E1 (3.6 μM), SUMO2 (2.65 μM), and where indicated 3 μl of p53 (Tx) were added, and in vitro SUMOylation was carried out at 30°C for 30 min or for 60 min if p53 was added. The total reaction volume was 30 μl. The reaction was stopped by adding 6× SDS Laemmli buffer and subsequent incubation for 5 min at 95°C. After SDS-PAGE and immunoblotting, products were detected using the following antibodies: anti-SUMO2/3 (clone 1E7, MBL) and anti-p53 (Pab1801 and DO1, Santa Cruz). In vitro translated p53 was prepared using the TnT® Quick Coupled Transcription/Translation System (Promega).

**Cell culture**

HEK293T (ATCC), HeLa (ATCC), and HeLa HA-PML III stable cells were grown under standard conditions in DMEM high glucose, 10% FCS, and 100 units/ml penicillin and 100 units/ml streptomycin. Where indicated, cells were treated with 1 μM As₂O₃ for 2 h or 6 h to induce PML body formation and poly-SUMOylation.

**Immunoprecipitation of RFP fusion proteins**

RFP-tagged SUMO2 variants were transiently transfected in HeLa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 1 μg of DNA per 2 × 10⁶/ml HeLa cells and 2 μl transfection reagent were used. Cells were lysed 48 h after transfection, and RFP-tagged proteins were immunoprecipitated from cell lysate using RFP-Trap®_MA kit (Chromotek) following the protocol from Chromotek for RFP-Trap®_MA co-immunoprecipitation. Bound RFP immune complexes were eluted with Laemmli buffer and analyzed by immunoblotting using antibodies against endogenous Ubc9 (Ubc9 clone 50, BD Biosciences) and against overexpressed RFP-tagged S2vs (Clontech).

**Pulldown of poly-SUMOylated conjugates**

Using Lipofectamine 2000 (Invitrogen), RFP-tagged SUMO2 variants were transiently transfected in HeLa cells. Cells were cultured for 48 h followed by 1 h of heat shock at 43°C to induce poly-SUMOylation. Lysis of cells and pulldown experiments were performed as described previously (34) using SUBEs as SUMO affinity traps. For each condition we used 1 mg of total cell lysate and incubated with 50 μl of GST-agarose beads containing either 50 μg of GST or SUBE. Elution was performed with 50 μl of 2× Laemmli buffer, and analysis was done by immunoblotting with antibodies against RFP-tagged S2vs (Clontech) and SUMO2/3 (clone 1E7, MBL).

**siRNA transfection**

For siRNA-mediated knockdown, 1 × 10⁵/ml HeLa cells were transfected with the indicated siRNAs (30 pmol) and 2 μl of transfection reagent using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s instructions. The following siRNA sequences (sense) were used for depletion experiments: control, CGUACGCAGAAUACUUCGAdTdT; PML_1, GGAGAAAGAUCAGCUGAdTdT; PML_2, CGAUCGUCCGAGAUCdTdT; PML_3, GGGCCUAGG-UGCAGACACdTdT.

**Mass spectrometry**

For mass spectrometry analysis, 4 × 150 cm² dishes/variant (S2.E2.15ΔGG, E2.20ΔGG, E2.34, and S2.WT ΔAGG) were used and plated with 2 × 10⁶/ml HEK293T cells. Using the CaCl₂ transfection method, pcDNA3.1-NT-FLAG constructs were transiently transfected 24 h after plating. In brief, for each dish 12 μg of plasmid DNA was mixed with 150 μl of CaCl₂ (2 M), 1 ml of H₂O, and 1.2 ml of 2× HBS-buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 7.04). After 30 min of incubation at room temperature, the mixture was added dropwise to the cells. Cells were scraped into PBS 48 h post-transfection, pooled, and washed twice in PBS. After centrifugation, the cell pellets were resuspended in lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, and protease inhibitor mixture, Roche Applied Science) and incubated for 30 min at 4°C with end-over-end rotation. The lysates were centrifuged, and supernatants were transferred to pre-equilibrated FLAG-M2-agarose beads (Sigma) and incubated overnight at 4°C. Beads were washed 5 times with 1 ml of lysis buffer and 5 times with PBS. Elution was performed 3 times by incubating the beads with 50 μl of FLAG peptide (0.1 μg/μl). Eluates were combined, and further steps of sample preparation for trypsin digest and mass spectrometry were done as described before (48). Samples were analyzed in technical duplicates on a LTQ Velos (Thermo Scientific). Data analysis was done using the software CompPASS (49).
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Author contributions—S. W., A. G., S. M., and A. E. designed the study. S. W. and A. G. performed the in vitro and in vivo experiments and analyzed the data. M. S. R. provided the SUMO affinity traps. A. E. designed the SUMO2 libraries. A. K. performed the ITC and NMR experiments. A. S. performed the library construction and selections. C. B. and S. W. performed the mass spectrometry measurements, and C. B., S. W., and A. E. analyzed the mass spectrometry data. V. V. R., V. D., and A. K. analyzed the NMR and ITC data. S. W., A. G., S. M., and A. E. wrote the paper.

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