# Activation of the Slx5–Slx8 Ubiquitin Ligase by Poly-small Ubiquitin-like Modifier Conjugates*[S]

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Protein sumoylation is a regulated process that is important for the health of human and yeast cells. In budding yeast, a subset of sumoylated proteins is targeted for ubiquitination by a conserved heterodimeric ubiquitin (Ub) ligase, Slx5–Slx8, which is needed to suppress the accumulation of high molecular weight small ubiquitin-like modifier (SUMO) conjugates. Structure-function analysis indicates that the Slx5–Slx8 complex contains multiple SUMO-binding domains that are collectively required for *in vivo* function. To determine the specificity of Slx5–Slx8, we assayed its Ub ligase activity using sumoylated Siz2 as an *in vitro* substrate. In contrast to unsumoylated or multisumoylated Siz2, substrates containing poly-SUMO conjugates were efficiently ubiquitinated by Slx5–Slx8. Although Siz2 itself was ubiquitinated, the bulk of the Ub was conjugated to SUMO residues. Slx5–Slx8 primarily mono-ubiquitinated the N-terminal SUMO moiety of the chain. These data indicate that the Slx5–Slx8 Ub ligase is stimulated by poly-SUMO conjugates and that it can ubiquitinate a poly-SUMO chain.

Sumoylation regulates a diverse set of cellular processes and is essential for viability in budding yeast (1). Sumoylation resembles ubiquitination in that the C terminus of SUMO[2] is conjugated to lysine residues of target proteins. In yeast, this occurs through the sequential activity of an activating (E1) enzyme (Aos1/Uba2), a conjugating (E2) enzyme (Ubc9), and one of several SUMO ligases (E3) (*e.g.* Mms21 and Siz2) (2–4). Although a large class of Ub E3 ligases are RING-domain proteins (5), SUMO E3 ligases often contain a variant domain known as SP-RING (3, 6). Sumoylation can be reversed by the Ulp1 and Ulp2 isopeptidases, which catalyze the cleavage of SUMO polypeptides from target proteins (7, 8).

In yeast, recombinational DNA repair depends on sumoylation. It has been known for some time that DNA damage tolerance is compromised in yeast strains with defects in Ubc9, Mms21, Ulp1, or Ulp2 (7, 9–11). More recently, cells lacking the Srs2 anti-recombinase have been shown to require the Ulp1 isopeptidase for viability (12), and cells defective in *UBC9* and *MMS21* accumulate Rad51-dependent cruciform structures during DNA replication (13). In the best characterized cases, SUMO has been shown to link Srs2 to proliferating cell nuclear antigen (14, 15) and to be conjugated to Rad52 (16, 17).

Sumoylation is distinct from ubiquitination in that target proteins are typically modified by single SUMO moieties that result in mono- or multisumoylated products as opposed to targets bearing poly-SUMO chains (1). Nonetheless, poly-SUMO conjugates are commonly observed in *in vitro* sumoylation reactions (3, 18, 19), and they accumulate in yeast cells deficient in the Ulp2 SUMO protease or Ub-mediated proteolysis (20, 21). The polymerization of SUMO in yeast (called Smt3) occurs preferentially through its three N-terminal lysine residues (lysines 11, 15, and 19) that are found in a domain of 20 amino acids (aa) unique to SUMO (18–20). The function of such poly-sumoylation is unknown, although in the case of *ulp2Δ* cells, it appears to be toxic (20).

*SLX5* and *SLX8* are required for the viability of yeast cells lacking the Sgs1 DNA helicase (22). These genes encode a heterodimeric Ub ligase that links sumoylation to recombinational DNA repair (21, 23–25). On their own, *slx5Δ* and *slx8Δ* null mutants display similar phenotypes, including slow growth, sensitivity to hydroxyurea (HU), and increased rates of gross chromosomal rearrangements and mitotic recombination (22, 26, 27). Like mutants deficient in sumoylation, *slx5Δ* and *slx8Δ* mutants display a clonal lethality that is dependent on the 2µ circle (9, 28, 29), and this can be suppressed by eliminating genes in the *RAD51*-independent recombination pathway (26). The *SLX5* and *SLX8* genes were also isolated in a screen for suppressors of a *mot1–301* allele, along with mutations in most of the enzymes involved in the sumoylation pathway (30). Importantly, there is an accumulation of sumoylated proteins in *slx5Δ* and *slx8Δ* cells that correspond to high molecular weight SUMO conjugates (21, 24, 30).

The Slx5–Slx8 complex interacts directly with SUMO via SUMO interacting motifs in each subunit (21, 24, 28, 31). The complex appears to be functionally conserved in *Schizosaccharomyces pombe*, where the related RING-finger proteins Rfp1 and Rfp2 also interact with SUMO and Slx8 to suppress the build-up of sumoylated proteins (21, 24, 32–34). The idea that the Ub ligase activity of the complex is directed toward sumoylated targets is supported by the finding that substrates containing a SUMO sequence are preferentially ubiquitinated (24, 32, 33).

[2] The abbreviations used are: SUMO, small ubiquitin-like modifier; HU, hydroxyurea; SIM, SUMO interacting motif; GST, glutathione S-transferase; wt, wild type; Ulp1$_{UD}$, Ulp1 Ulp domain; SD, synthetic complete medium; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; aa, amino acid(s); aR , protein in which all lysine residues are changed to arginine; Ub,ubiquitin.

In this study we investigated the specificity of the Slx5–Slx8 Ub ligase toward sumoylated substrates. We found that its activity on free SUMO or a multisumoylated test substrate was low, whereas ubiquitination of a poly-sumoylated substrate was high. Characterization of this reaction revealed that Slx5–Slx8 primarily mono-ubiquitinated the N-terminal end of the SUMO chain. Although there is no known physiological role for poly-sumoylation, we found increased levels of these conjugates in cells lacking the Sgs1 DNA helicase. Thus, it appears that one essential function of the Slx5–Slx8 Ub ligase is to ubiquitinate poly-sumoylated proteins that arise in *sgs1Δ* cells.

## EXPERIMENTAL PROCEDURES

*Yeast Strains and Plasmids*—Standard methods and media were used for the propagation, transformation, and culturing of *Saccharomyces cerevisiae* (35). Strain JD194 (MATα *ura3Δ5 his3–11,15 leu2–3,112 pre1–1 pre2–2*) was kindly provided by Dr. Kiran Madura. Additional genotypes and plasmid construction details are available on request.

*Expression and Purification of Recombinant Proteins*—GST- and His$_6$-tagged proteins were produced in *Escherichia coli* BL21-RIL cells (Stratagene) using the T7 expression system of Studier (36). His$_6$-tagged proteins were expressed and purified as described previously (37). GST-tagged proteins were expressed similarly but purified following lysis in Buffer A (25 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 0.01% Nonidet P-40, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 200 mM NaCl and protease inhibitors. The extract was applied to a 1-ml GST-TRAP column (GE Healthcare), washed with 20 ml of Buffer A containing 200 mM NaCl, and eluted with the same buffer containing 10 mM glutathione. Peak protein fractions were dialyzed in Buffer A containing 100 mM NaCl. Additional versions of GST–Smt3 and GST-Ub fusion proteins were constructed with protease 3C and cAMPdPK consensus sites downstream of the GST domain. Following treatment with cAMPdPK and γ-[$^{32}$P]ATP, proteins were re-bound to glutathione beads and washed free of ATP prior to eluting the radiolabeled protein via protease 3C cleavage. Expression and purification of Ulp1$_{UD}$ (38) has been described (28).

*SUMO Binding Assay*—Physical interactions between His$_6$/FLAG-tagged Smt3 (HF-Smt3) (2) and GST-Slx5 or GST-Slx8 were detected following incubation on ice for 1 h in a final volume of 0.1 ml using Buffer A with 50 mM NaCl as the incubation buffer. This reaction was then diluted with 0.3 ml of incubation buffer and mixed with 20 μl of glutathione beads (40 μl of 50% slurry) for 1 h at 4 °C. The beads were recovered by low speed spin and washed three times with incubation buffer. Bound proteins were eluted with 25 μl of SDS sample buffer and detected by immunoblotting as described before (37).

*In Vitro Sumoylation Assay*—The standard sumoylation reaction was performed in the presence of 20 mM HEPES (pH 7.5), 5 mM MgCl$_2$, 2 mM ATP, 5 μM ZnSO$_4$, and 0.1 mM dithiothreitol. Unless otherwise stated, reactions were incubated at 30 °C for 60 min and contained 2 nM Aos1/Uba2, 30 nM Ubc9, 100 nM Siz2-V5, and 2 μM HF-Smt3-G98 in a total volume of 20 μl. Immunoblotting was carried out essentially as described (37) except that proteins were transferred to polyvinylidene difluoride or a 0.22-μm nitrocellulose membrane. Where indi-

cated, mature HF-Smt3-G98 was substituted with HF-Smt3-G97 or HF-Smt3-G98A mutants, whereas latter experiments employed Smt3 and variants that were simply His$_6$-tagged.

*In Vitro Ubiquitination Assay*—Ubiquitination was performed using the same buffer conditions as the sumoylation assay. Reactions were incubated at 30 °C for 15 min and contained 2 nM Uba1, 30 nM Ubc5, 10 nM unlabeled ubiquitin, and 350,000 cpm $^{32}$P-Ub in a total volume of 30 μl. The products of this reaction were resolved by SDS-PAGE (typically 15% acrylamide). The gel was then fixed and visualized on a PhosphorImager (Amersham Biosciences).

## RESULTS

*Multiple SUMO Binding Domains Are Required for Slx5–Slx8 Function*—To locate potential SUMO-binding domains within the two subunits of the Ub ligase, we fused portions of the N and C termini of Slx5 or Slx8 to the C terminus of GST. Purified recombinant proteins were then assayed for their ability to bind SUMO following incubation with glutathione beads. In contrast to GST alone, GST-Slx5 bound strongly to SUMO (Fig. 1A). Two small N-terminal truncations (ΔN100 and ΔN200) showed diminishing affinity to SUMO, whereas deletion of 300 aa or more eliminated this activity. Analysis of C-terminal deletions revealed a strong SUMO-binding domain within the first 100 aa of Slx5, although additional mapping identified a second larger domain between aa 101 and 300 (Fig. 1B). Thus, the first 300 aa of Slx5 contains at least two SUMO-binding domains. When the N-terminal deletions were examined for their ability to complement *sgs1Δ* synthetic lethality, a correlation was found between loss of SUMO binding and loss of complementation (Fig. 1A). And consistent with its role in promoting Ub ligase activity (23), the RING domain of Slx5 was required to complement *sgs1Δ* synthetic lethality. However, deletion of a single N-terminal SUMO-binding domain was tolerated in *sgs1Δ* cells. A similar analysis revealed a SUMO binding activity in Slx8 that was localized to the C-terminal 111 aa (Fig. 1C). As with Slx5, removal of the one SUMO-binding domain in Slx8 was tolerated in *sgs1Δ* cells. Consistent with the mapping of multiple SUMO interacting motifs within these subunits (21, 24), we conclude that there are at least three SUMO-binding domains within the Slx5–Slx8 Ub ligase.

*The Severity of slx5 and slx8 Phenotypes Correlates with in Vivo Sumoylation Levels*—To correlate the sumoylation defects of *slx5* and *slx8* mutants with their other phenotypes, we characterized a collection of point and truncation alleles with respect to their effect on *in vitro* Ub ligase activity, accumulation of hyper-sumoylated proteins, HU sensitivity, and *sgs1Δ* synthetic lethality. Among point mutation alleles that map to the RING domains of Slx5 and Slx8, four were shown to eliminate Ub ligase activity *in vitro* (*slx5-6*, *slx5-8*, *slx8-1*, and *slx8-3*) and those displayed lethality in the *sgs1Δ* background (Fig. 2A) (23, 37)). Those retaining Ub ligase activity (*slx5-5*, *slx5-7*, and *slx8-2*) displayed intermediate complementation in the *sgs1Δ* background (Fig. 2A) (37). To assay hyper-sumoylation, a denatured extract from each strain was immunoblotted with α-Smt3 antibody. All mutations that were synthetically lethal with *sgs1Δ* showed extreme hyper-sumoylation, whereas the intermediate alleles displayed partially elevated sumoylation levels
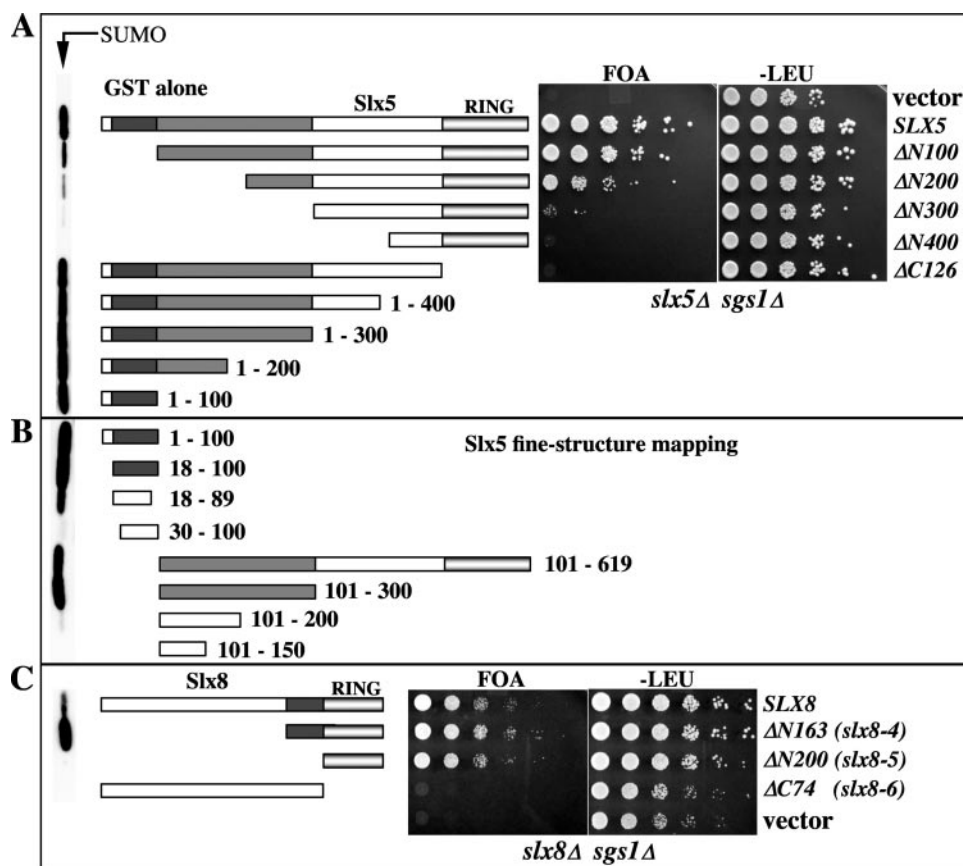
FIGURE 1. **Identification of SUMO-binding domains in Slx5 and Slx8.** *A, right panel,* the *SLX5* ORF or fragments corresponding to the indicated N- and C-terminal truncations were cloned downstream of the *SLX5* promoter in vector pRS415 (*LEU2/CEN/ARS*). These constructs were then transformed into strain JMY1464 [(*sgs1Δ slx5Δ* plus pJM500 (*SGS1/URA3/ADE3*)]. Transformants were resuspended at a concentration of $A_{600}$ = 3.0, and following 10-fold serial dilution, ~5 $\mu$l was pinned onto solid synthetic complete media (SD) lacking leucine (-LEU) or containing 5-fluoro-orotic acid to select against pJM500. Plates were photographed following 3 days of growth at 30 °C. *Left panel,* the indicated proteins were fused to the C terminus of GST and purified from bacteria. GST fusions or GST alone (1 $\mu$g) were then incubated on ice with 1 $\mu$g of HF-Smt3, and bound proteins were detected by glutathione bead pulldown assay and immunoblotting with anti-FLAG antibody. *B,* fine structure mapping of the N-terminal SUMO-binding domains of Slx5 was carried out as in *A. Shading* is used in the schematic to indicate the minimal SUMO-binding domains. *C,* the *SLX8* open reading frame or fragments corresponding to the indicated truncations were assayed as in *A* except that strain VCY1524 [(*sgs1Δ slx8Δ* plus pJM500 (*SGS1/URA3/ADE3*)] was used as host.

(Fig. 2B). In these and subsequent experiments the 5% stacking gel was retained on the blot, because it has been shown that highly poly-sumoylated proteins are retained in the stacking gel (20). Interestingly, removing one SUMO-binding domain (*slx5-ΔN100*, *-ΔN200*, and *slx8-ΔN200*) resulted in intermediate levels of hyper-sumoylation, whereas deleting an additional domain (*slx5-ΔN300 and slx5-ΔN400*) or a RING domain (*slx5-ΔC126* and *slx8-ΔC74*) produced a null phenotype. Consistently, strains with high sumoylation levels grew poorly on HU (Fig. 2C) and required *SGS1* to survive (Fig. 2B). Strains with slightly elevated sumoylation levels grew like wild type on HU and survived loss of *SGS1*. We conclude that the *in vivo* sumoylation levels and HU sensitivities of *slx5* and *slx8* alleles correlate well with their *sgs1Δ* synthetic-lethal phenotypes.

*Sumoylated Siz2 Is Ubiquitinated by Slx5–Slx8*—To examine the specificity of the Slx5–Slx8 ubiquitin ligase toward sumoylated proteins, we took advantage of the fact that the SUMO E3 ligase Siz2, which is known to be sumoylated *in vivo* (39), exhibits auto-sumoylation *in vitro*. Sumoylated Siz2 was then used as

a substrate for *in vitro* ubiquitination. To validate this system, sumoylation reactions were carried out with epitope-tagged Siz2 (Siz2-V5) and a variety of mutant SUMO substrates. These products were analyzed on duplicate immunoblots that were probed with $\alpha$-V5 or $\alpha$-Smt3 (Figs. 3, *A* and *B*). Using mature HF-Smt3-G98 as substrate (2), we observed two major species of Siz2 products (Fig. 3*A, lane 1*). One product migrated as a doublet at 125–150 kDa and was judged to be conjugated to one or more SUMO groups. The upper band is most likely multisumoylated, because it co-migrated with the single product obtained with Smt3-aR (Fig. 3*A, lane 4*). All nine lysine residues have been mutated to arginine in Smt3-aR, so it is unable to form SUMO chains (20). The second product was a highly polysumoylated form of Siz2, which displayed a characteristic mobility just entering the stacking gel (Fig. 3, *A* and *B, lane 1*). As expected, Smt3-G97 (lacking the essential C-terminal glycine) gave no sumoylated product, because it cannot form covalent attachments to target proteins (Fig. 3, *A* and *B, lane 2*), and Smt3-G98A produced both multi- and poly-sumoylated Siz2 in addition to short multimers of Smt3. Finally, synthesis of these products required functional Smt3, E1, E2 and Siz2 itself (Fig. 3, *A* and *B, lanes 5– 8*). We conclude that Siz2 has multiple sites for Smt3 attachment, and that it can catalyze the formation of poly-SUMO chains.

To detect Ub E3 ligase activity, Siz2 sumoylation products were incubated with Uba1, Ubc5, ATP, and the Slx5–Slx8 dimer as described (23), together with radiolabeled ubiquitin ($^{32}$P-Ub) as substrate. Following a 15-min incubation, the products were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3*C*, samples containing sumoylated Siz2 produced a labeled band near the well of the gel (lanes 1–4). In addition to this band was a background smear of auto-ubiquitinated products such as poly-Ub chains ($Ub_n$) in the resolving gel. The major signal that was trapped in the stacking gel was dependent on Ub E1, Ub E2, and Slx5–Slx8 (lanes 5–7). Importantly, the level of signal was responsive to a titration of Slx5–Slx8 as expected for an Ub E3 ligase activity (Fig. 3*C, lanes 7–14*). Moreover, products were obtained using Slx5–Slx8 at concentrations as low as 30 pM, which indicates a significant affinity relative to the levels (~3 nM) needed for robust auto-ubiquitination (23).
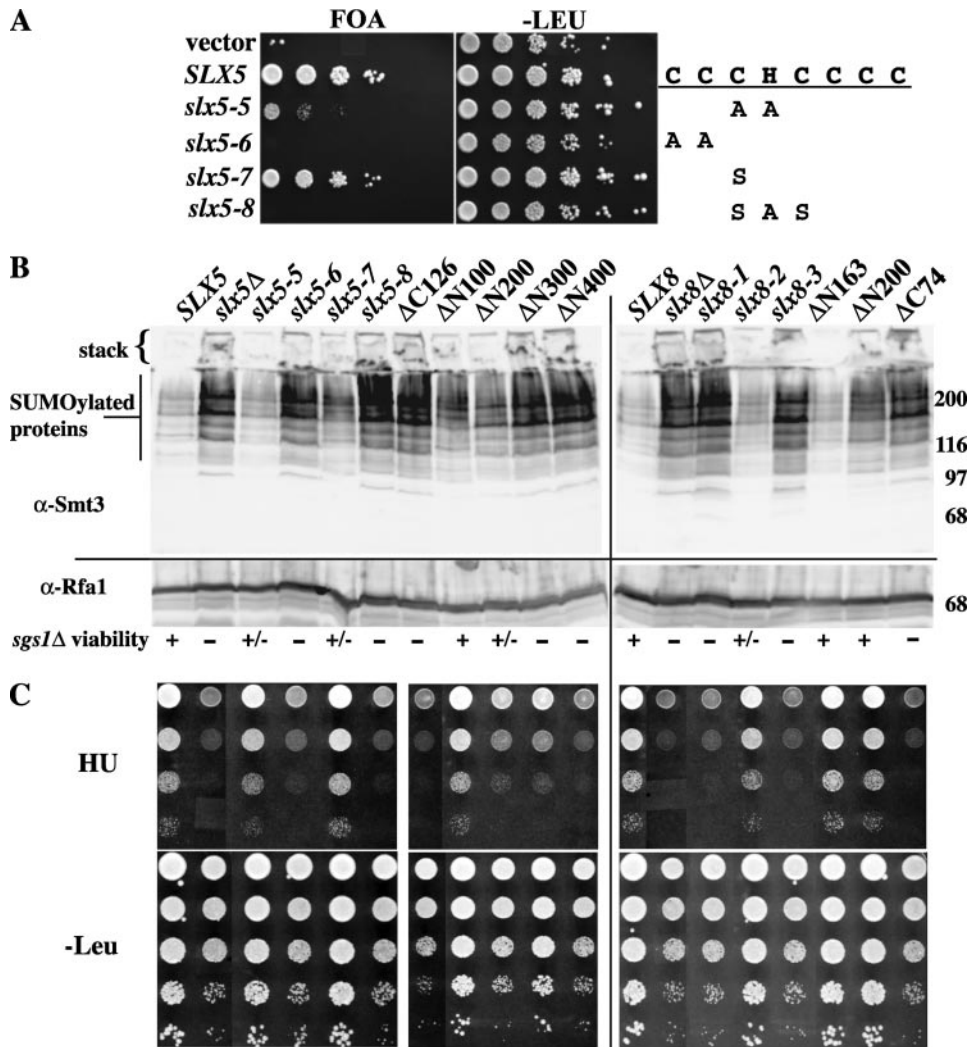
FIGURE 2. **Synthetic-lethal phenotype of *SLX5* and *SLX8* mutations correlates with the accumulation of hyper-sumoylated proteins.** *A*, alleles of *SLX5* containing the indicated RING-finger mutations were tested for complementation of *sgs1Δ slx5Δ synthetic* lethality as in Fig. 1. *B*, the indicated *SLX5* or *SLX8* alleles were integrated into strain JMY1699 (*slx5Δ*) or SIY778 (*slx8Δ*), and *N*-ethylmaleimide extracts were analyzed for Smt3-protein conjugates by 8% SDS-PAGE and immunoblotting using antibodies against Smt3 (*upper panel*) or Rfa1 (*lower panel*). A *bracket* marks the stacking gel. Also shown is the alleles' ability to complement the relevant *slxΔ sgs1Δ* synthetic lethality. To determine *sgs1Δ* viability, strains JMY1464 or VCY1525 [(*sgs1Δ slx8Δ* plus pJM500 (*SGS1/URA3/ADE3*)] were transformed with *LEU2/CEN/ARS* plasmids carrying the indicated *SLX5* or *SLX8* alleles and evaluated for growth on plates containing 5-fluoro-orotic acid. Symbols: +, good growth; +/−, slow growth; −, no growth. *C*, strains JMY1699 or SIY778 were transformed with *LEU2/CEN/ARS* plasmids carrying the indicated alleles, were assayed for growth as in Fig. 1*A* but in the presence or absence of 0.1 M hydroxyurea. The plates were photographed after 3 days of growth at 30 °C.

*Poly-SUMO Chains Are a Preferred Substrate of Slx5–Slx8*— Because the ubiquitinated products shown in Fig. 3*C* could only just enter the stacking gel, it was not possible to determine the structure of these molecules. We therefore considered the following possibilities: (*a*) the signal in the well could be due to the attachment of Ub chains onto mono- or multisumoylated Siz2 or (*b*) the signal could be due to one or more Ub residues ligated to poly-sumoylated Siz2 (Siz2-(Smt3)$_n$). These possibilities were distinguished by repeating the assay using radiolabeled Ub mutants in which some or all of its conjugable lysines had been mutated to arginine. As shown in Fig. 4*A* (*lanes 1–4*), the ubiquitination signal was unchanged regardless of whether the $^{32}$P-Ub was wt, contained Lys-48 as its only lysine (K48O), contained Lys-63 only (K63O), or contained no lysines (aR). The

fact that the product obtained with $^{32}$P-Ub-aR, which is unable to form chains, is indistinguishable from wt $^{32}$P-Ub suggests that the substrate is not mono- or multisumoylated Siz2, which, if mono-ubiquitinated, should migrate into the resolving gel (Fig. 3, *A* and *B*). Ubiquitination was also strictly dependent on the synthesis of poly-SUMO chains, because neither wild-type nor mutant $^{32}$P-Ubs can be ligated onto Siz2 following sumoylation with Smt3-aR (Fig. 4*A*, *lanes 5–9*). We conclude that poly-sumoylated Siz2 is a preferred substrate of Slx5–Slx8.

The ability of Siz2-(Smt3)$_n$ to serve as a preferred ubiquitination substrate leaves open the question of whether Ub is ligated directly to the target protein (Siz2) or to the SUMO chain itself. To answer this question, ubiquitination reactions were first performed on Siz2-(Smt3)$_n$ in the presence of wt $^{32}$P-Ub. Subsequently, the products were treated with the Ulp1$_{UD}$ SUMO protease, which cleaves Smt3 from modified proteins and proteolyzes Smt3 chains into monomers. As shown in Fig. 4*B*, titration of Ulp1$_{UD}$ resulted in the disappearance of the Siz2-(Smt3)$_n$-Ub* signal from the stacking gel, and the appearance of a major radiolabeled product at 27 kDa (Fig. 4*B*, *lanes 5–7*). The identification of this band as Smt3-Ub* is based on its predicted molecular weight (HF-Smt3 + $^{32}$P-Ub = 20 + 7 kDa) as well as the following control. We attempted to ubiquitinate monomeric SUMO by incubating Uba1,

Ubc5, ATP, and $^{32}$P-Ub together with free HF-Smt3 and Slx5–Slx8. Monomeric HF-Smt3 was a poor substrate in this reaction; however, some ubiquitinated forms of HF-Smt3 were observed, including diminishing amounts of di- and tri-Ub conjugates (supplemental Fig. S1). The primary mono-ubiquitinated product, Smt3-Ub*, co-migrated with the Ulp1$_{UD}$-proteolytic product at 27 kDa (Fig. 4*B*, *lane 8*), which migrates as a doublet. The source of this doublet is unknown; however, we suspect that proteolysis may be imprecise or that the two bands represent isomers in which Ub is conjugated to different lysine residues of Smt3.

The relatively small amounts of di-ubiquitinated Smt3 (Fig. 4*B*, *lanes 5–7*) indicate that this E3 ligase primarily mono-ubiquitinates its substrates. This is consistent with two test proteins
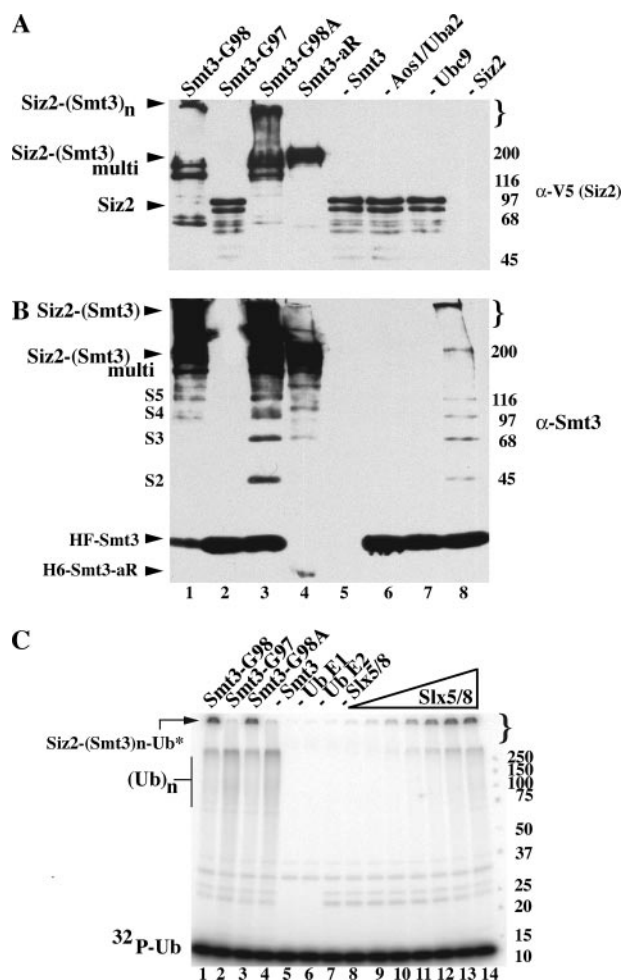
FIGURE 3. **Poly-sumoylated Siz2 is preferentially ubiquitinated by Slx5–Slx8.** *A*, standard sumoylation assays were performed in the presence of the indicated Smt3 variants or in the absence of the indicated component. Siz2-V5 products were analyzed by SDS-PAGE and immunoblotting with antibodies against the V5 epitope. *B*, duplicate sumoylation reactions were analyzed by SDS-PAGE and immunoblotting with antibodies against Smt3. Oligomeric chains of Smt3 are indicated by S2-S5. Note that all Smt3 proteins were N-terminally tagged with $His_6$-FLAG, except for Smt3-aR, which was tagged with $His_6$ only. *C*, sumoylation reactions were first performed in the presence of wt HF-Smt3 (*lanes 1* and *5–14*), the indicated HF-Smt3 mutants (*lanes 2* and *3*), or no Smt3 (*lane 4*). The reaction products were then subjected to a standard ubiquitination assay in the presence of $^{32}$P-Ub and either 10 nM Slx5–Slx8 (*lanes 1–6*), or 0, 10 pM, 30 pM, 100 pM, 300 pM, 1 nM, 3 nM, or 10 nM Slx5–Slx8 (*lanes 7–14*). The Ub E1 or E2 was omitted where indicated (*lanes 5* and *6*).



FIGURE 4. **Poly-SUMO chains are targeted for ubiquitination.** *A*, Siz2 sumoylation reactions were first performed in the presence of wt HF-Smt3 (+), no Smt3 (−), or H6-Smt3-aR (*aR*). The products were subsequently ubiquitinated in the presence of $^{32}$P-Ub and 10 nM Slx5–Slx8 prior to analysis by SDS-PAGE and autoradiography. Ub was either wt or contained the indicated mutations. *B*, standard sumoylation reactions were performed in the presence of 4 μM HF-Smt3, and the products were subjected to ubiquitination with wt $^{32}$P-Ub as above. The products of this reaction were then subjected to a 1-h incubation with recombinant $Ulp1_{UD}$ at the following concentrations: 0, 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, and 1 nM (*lanes 1–7*, respectively). As a marker for HF-Smt3-Ub*, free HF-Smt3 was subjected to a standard ubiquitination reaction in the presence 100 nM Slx5–Slx8 (*lane 8*). Bands were identified based on the relative molecular weights of HF-Smt3 (20 kDa) and Ub (7 kDa). The bands migrating at ~107 and 73 kDa represent mono-ubiquitinated Slx5 and Slx8, as indicated. The molecular weight markers are identical to those in Fig. 3C.

used previously (23) and the prominent Slx5–Slx8 auto-ubiquitination products visible in Fig. 4. The $Ulp1_{UD}$ titration resulted in a transient product at 47 kDa (Fig. 4B, *lane 4*) consistent with the expected $(Smt3)_2$-Ub* intermediate. Larger intermediate products, such as $(Smt3)_3$-Ub* were probably not observed due to rapid proteolysis of the SUMO chain, however we cannot rule out the possibility that Smt3-Ub is a preferred substrate of $Ulp1_{UD}$. In addition, we did not detect a 54-kDa band (representing $(Smt3-Ub*)_2$) that would be expected from a SUMO chain saturated with mono-Ub residues. Along with the release of the major 27-kDa band was a band at 112 kDa (Fig. 4B, *lanes 5–7*). This band is consistent with mono-ubiquitinated Siz2-V5 (*i.e.* 105 plus 7 kDa), which indicates that Slx5–Slx8 also disp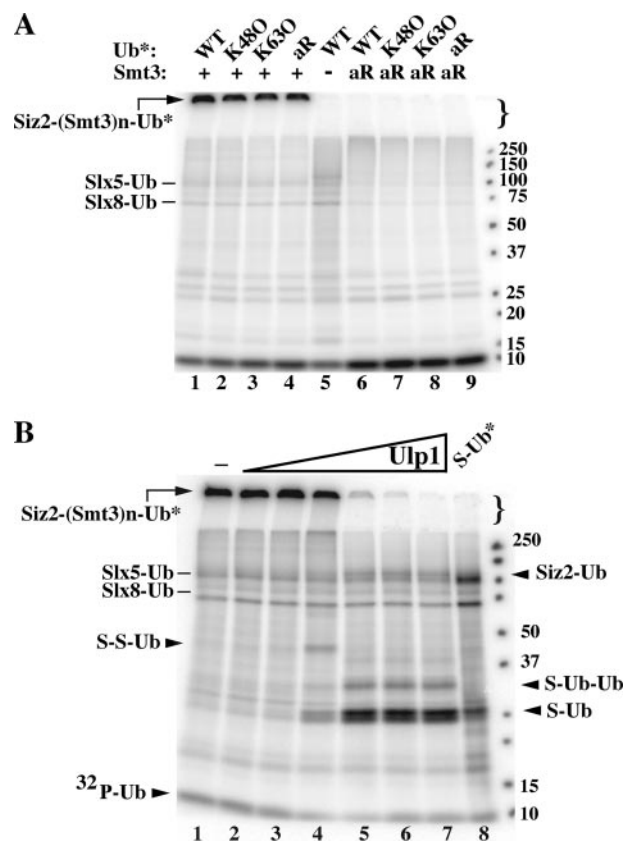lays activity against the target protein. Taken together, we conclude that Slx5–Slx8 is activated by poly-SUMO chains and that it mono-ubiquitinates both Siz2 and SUMO.

*Slx5–Slx8 Ubiquitination Requires the N-terminal Lysines of Smt3*—The above experiment raised the question of where on the SUMO chain the Ub was conjugated. We addressed this question by first determining which of the nine lysines of Smt3 were required for efficient ubiquitination by Slx5–Slx8. Johnson and colleagues have previously shown that Smt3 preferentially polymerizes through its three N-terminal lysine residues (lysines 11, 15, and 19), although a SUMO E3 ligase is capable of promoting chain formation through alternative lysine residues when the primary sites have been mutated to arginine (20). We therefore prepared sumoylated Siz2 using an array of Smt3 mutants in which individual lysines, or groups of lysines, had been mutated to arginine. A portion of each reaction (one-third) was analyzed by immunoblotting with either anti-V5 (Fig. 5A) or anti-Smt3 (Fig. 5B). The accumulation of high
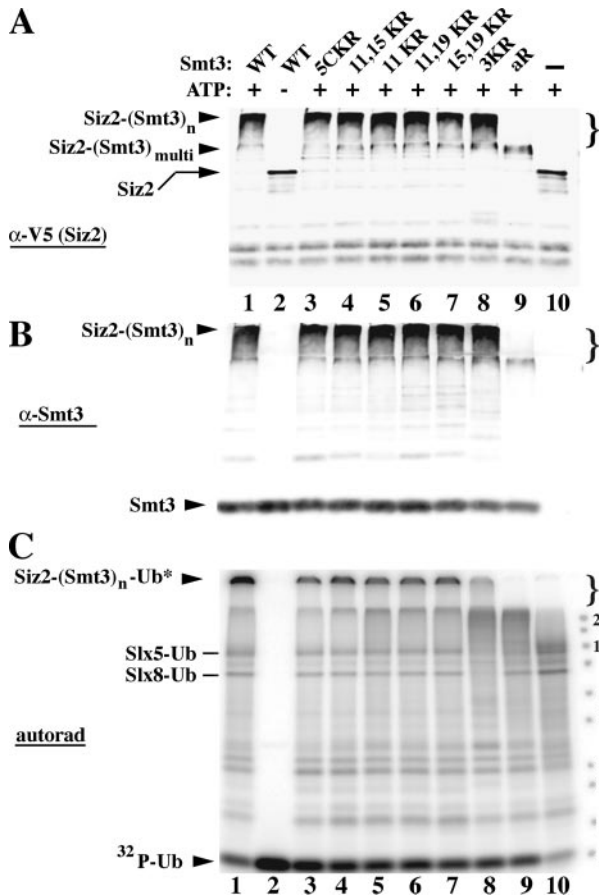
## A



## B

## C

FIGURE 5. **The N-terminal lysines of Smt3 are important for ubiquitination by Slx5–Slx8.** *A*, standard sumoylation reactions were performed except that the duration of the incubation was 45 min and included His$_6$-Smt3 (*WT*), the indicated His$_6$-Smt3 mutant proteins, or no Smt3 (*lane 10*). In *lane 2*, ATP was excluded from the sumoylation reaction. One-third of each reaction was analyzed by SDS-PAGE and immunoblotting with anti-V5. Unmodified Siz2 is indicated with an *arrow*. *B*, one-third of each of the above reactions was immunoblotted with antibodies against Smt3. *C*, one-third of each of the above reactions was subjected to ubiquitination in the presence of 30 nM Slx5–Slx8 and standard levels of Uba1 and Ubc5.

## A



## B

## C

FIGURE 6. **Characterization of alternative poly-SUMO chains ubiquitinated by Slx5–Slx8.** *A*, consecutive sumoylation-ubiquitination reactions were performed exactly as in Fig. 3*C* except that the following Smt3 derivatives were used: wt HF-Smt3 (*lane 1*), HF-Smt3-G97 (*lane 2*), HF-Smt3-G98A (*lane 3*), no Smt3 (*lane 4*), or His$_6$-Smt3–3KR (*lanes 6–15*). *B*, consecutive sumoylation-ubiquitination reactions were performed exactly as above except that His$_6$-Smt3-aR was used as substrate in *lanes 6–15*. *C*, consecutive sumoylation-ubiquitination reactions were untreated or cleaved with 10 nM Ulp1$_{UD}$ as in Fig. 4*B*, except that the initial sumoylation reactions were performed in the presence of the following Smt3 proteins: His$_6$-Smt3 (*WT*), His$_6$-Smt3–3KR (*3KR*), His$_6$-Smt3-CT5KR (*CT5KR*), His$_6$-Smt3-aR (*aR*), or no Smt3 (*None*).

molecular weight signal in these blots indicates that polysumoylation of Siz2 occurs using any of the mutant Smt3 proteins, except for Smt3-aR (Fig. 5, *A* and *B*, *lanes 9*). The remaining portion of the sumoylated reaction product was ubiquitinated by Slx5–Slx8 using $^{32}$P-Ub (Fig. 5*C*). Surprisingly, most of these poly-sumoylated proteins were efficiently ubiquitinated, including Siz2-(Smt3-CT5KR)$_n$, which lacks the five C-terminal lysine residues of Smt3. Smt3s lacking one or two of the N-terminal lysine residues were also ubiquitinated following polymerization (Fig. 5*C*, *lanes 4–7*). However, chains of Smt3 lacking all three N-terminal lysines (Smt3–3KR) were altered in their ubiquitination (Fig. 5*C*, *lane 8*). Although immunoblotting showed that bulk Siz2-(Smt3–3KR)$_n$ product appeared to just enter the stacking gel like chains prepared with wt Smt3 (Fig. 5, *A* and *B*, compare *lanes 1* and *8*), there was less ubiquitinated product at the well, more signal spread throughout the stacking gel, and more intense background signal in the 150- to 250-kDa range of the resolving gel. This increased signal in the resolving gel was also observed with Smt3-aR and, as shown below, appears to be due to ongoing sumoylation of auto-ubiquitinated Slx5 and Slx8 subunits. The enhanced
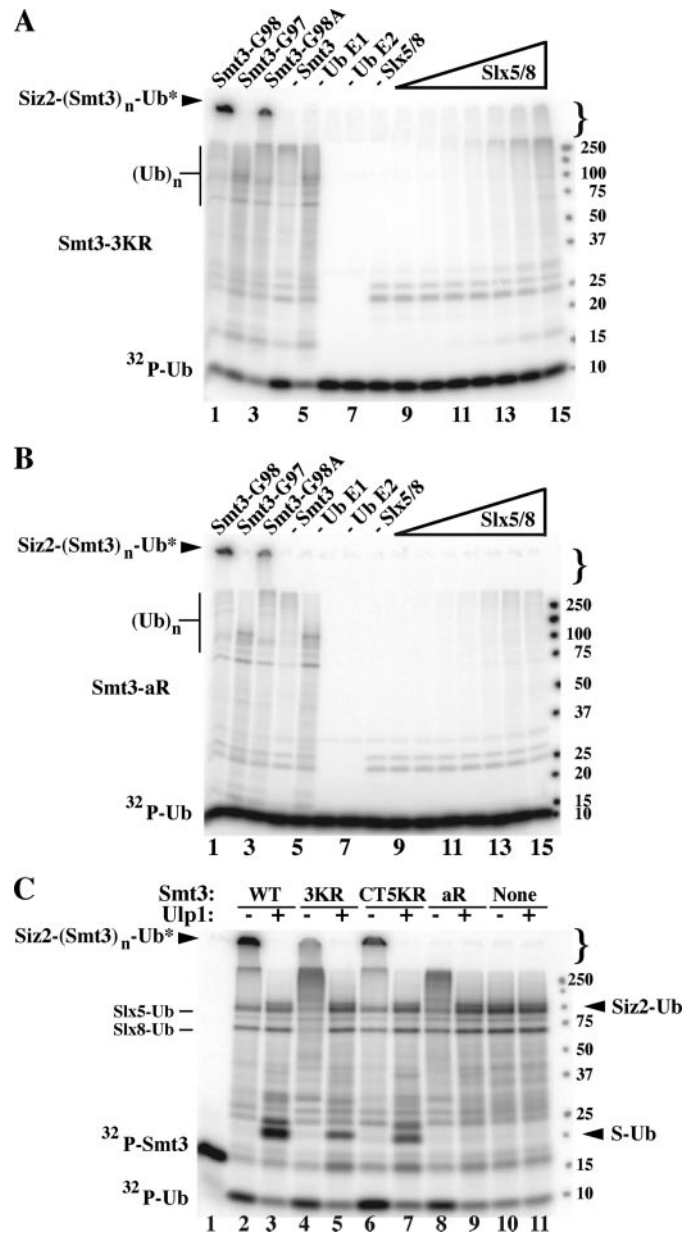
sumoylation of reaction components has previously been noted in cases where poly-sumoylation is inhibited using mutant SUMOs (20).

To confirm the above result, Siz2-(Smt3–3KR)$_n$ was ubiquitinated with a titration of Slx5–Slx8 (Fig. 6*A*). Compared with wt Siz2-(Smt3)$_n$ (Fig. 3*C*), Siz2-(Smt3–3KR)$_n$ failed to generate a discrete band near the well, even at the highest level of Ub ligase, and it produced background signal in the resolving gel

that was similar to that obtained with Siz2-Smt3-aR (Fig. 6*B*). Thus, the same lysine residues that are primarily used for SUMO polymerization (lysines 11, 15, and 19) are important for ubiquitination by Slx5–Slx8. A variety of these labeled ubiquitinated reaction products were then subjected to proteolysis by Ulp1$_{UD}$ (Fig. 6*C*). Treatment of Siz2-Smt3–3KR and Siz2-Smt3-CT5KR ubiquitination products with Ulp1$_{UD}$ reduced the material present in the stack and the higher molecular weight region of the resolving gel. It also generated bands corresponding to Smt3-Ub* (23 kDa; Fig. 6*C*, *lanes 5* and *7*), although these were less intense than that obtained with wt Smt3. In all cases, including Smt3-aR, Ulp1$_{UD}$ treatment also released Siz2-Ub* (112 kDa). However, the level of Siz2-Ub* that was released was no greater than that obtained in the absence of sumoylation (Fig. 6*C*, *lanes 10* and *11*). Also, the enhanced sumoylation of Slx5–Slx8 subunits with Smt3 mutants that fail to polymerize well (Smt3–3KR and Smt3-aR), did not stimulate their auto-ubiquitination (Fig. 6*C*, *lanes 5* and *7*). Thus, Slx5–Slx8-dependent ubiquitination preferentially targets poly-SUMO chains and is sensitive to the loss of the three N-terminal lysines of SUMO. Because of their role in SUMO conjugation, these residues are expected to be most readily available on the terminal SUMO moiety of the poly-SUMO chain.

*The Terminal SUMO Moiety Is Targeted for Ubiquitination by Slx5–Slx8*—If lysine residues 11, 15, or 19 of the terminal SUMO moiety are targeted for ubiquitination by Slx5–Slx8, then ubiquitination should interfere with further SUMO chain elongation. To test this idea, the Siz2-(Smt3)$_n$ chains were purified away from any free Smt3 used in its preparation. We reasoned that the very large molecular weight of the Siz2-(Smt3)$_n$ product may allow it to be purified by ultracentrifugation. To validate this method, we prepared poly-sumoylated Siz2 using $^{32}$P-Smt3 and subjected the products to centrifugation at 113,000 × *g*. The pellet was then washed, repelleted, and resuspended before loading equivalent samples from each step on an SDS gel for analysis by autoradiography. As shown in Fig. 7*A*, the Siz2-(Smt3)$_n$ product was successfully purified away from the $^{32}$P-Smt3. We then prepared unlabeled poly-sumoylated Siz2 using cold Smt3 and subjected it to the same purification scheme. The product was then ubiquitinated with increasing concentrations of Slx5–Slx8 using either $^{32}$P-Ub (as a control) or cold Ub. The products of the cold ubiquitination were then subjected to a new round of sumoylation using $^{32}$P-Smt3-aR. Consistent with previous results, purified Siz2-(Smt3)$_n$ was efficiently labeled with $^{32}$P-Ub as the concentration of Slx5–Slx8 was increased in the control reactions (Fig. 7*B*, *lanes 2–6* and supplemental Fig. S2*A*) while unmodified Siz2 was weakly labeled (Fig. 7*B*, *lane 1*). In the absence of ubiquitination, Siz2-(Smt3)$_n$ was capable of being labeled with $^{32}$P-Smt3-aR (Fig. 7*B*, *lane 9*). However, increasing ubiquitination of the purified Siz2-(Smt3)$_n$ led to the inhibition of further SUMO chain elongation using $^{32}$P-Smt3-aR (Fig. 7*B*, *lanes 10–13*). We conclude that ubiquitination of poly-SUMO chains inhibits further growth of the SUMO chain.

The above experiment suffers from the possibility that ubiquitination inhibits Siz2 activity. More importantly, the experiment does not rule out the possibility that Ub is also added to
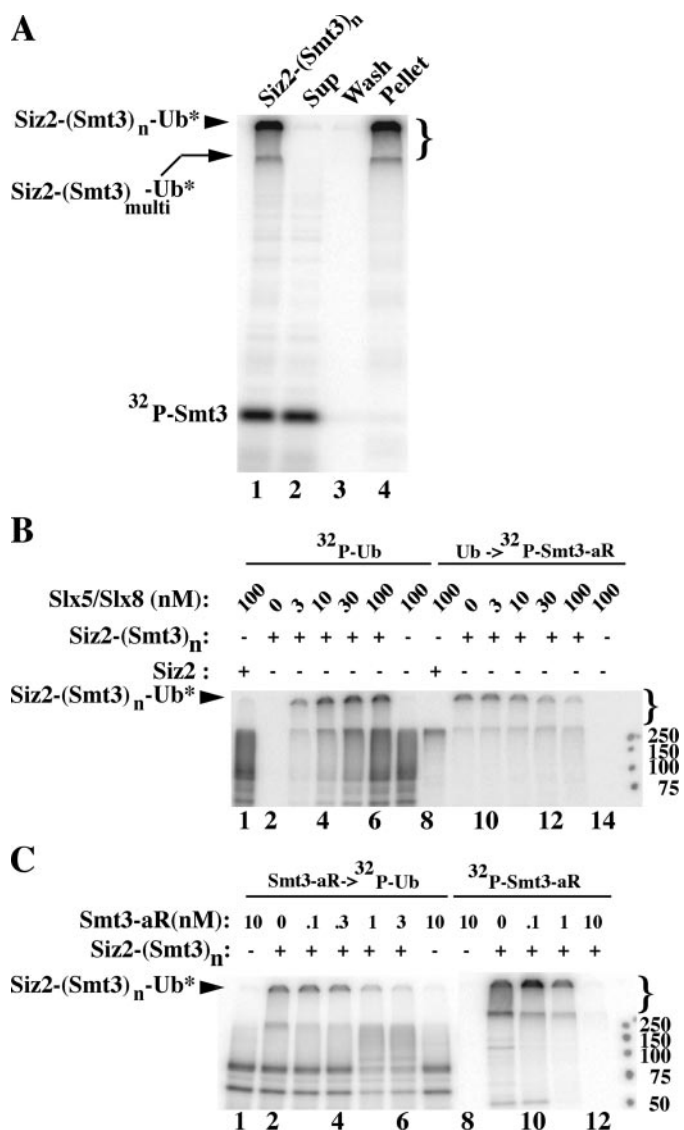


FIGURE 7. **Ubiquitination of poly-SUMO chains blocks further sumoylation.** *A*, radiolabeled Siz2-(Smt3*)$_n$ was formed in a 45-min sumoylation reaction containing 1 μM His$_6$-Smt3 and $^{32}$P-Smt3. The products were centrifuged at 113,000 × *g*, the pellet was washed with reaction buffer lacking ATP, pelleted again, and resuspended in 20 mM HEPES (pH 7.5), 300 mM NaCl, 0.02% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. The indicated fractions from this regimen were analyzed by SDS-PAGE and autoradiography. Each *lane* represents one-tenth of the original reaction. *B*, unlabeled Siz2-(Smt3)$_n$ was prepared by centrifugation as above from a scaled-up reaction. Portions of the purified material were then subjected to 30-min ubiquitination reactions containing 10 nM Uba1, 100 nM Ubc5, 50 nM unlabeled Ub, and 350,000 cpm $^{32}$P-Ub in the presence of the indicated concentrations of Slx5–Slx8 to monitor ubiquitination (*lanes 1–8*). Alternatively, portions of Siz2-(Smt3)$_n$ were first subjected to a 30-min ubiquitination reaction containing 10 nM Uba1, 100 nM Ubc5, and 2 μM Ub in the presence of the indicated concentrations of Slx5–Slx8. These products were then subjected to a 30-min sumoylation reaction containing 2 nM Aos1/Uba2, 30 nM Ubc9, 1 μM Smt3-aR, and 350,000 cpm $^{32}$P-Smt3-aR (*lanes 9–14*). Following this incubation, the reaction products were analyzed by SDS-PAGE and autoradiography. In *lanes 1* and *8* unmodified Siz2 was used in place of Siz2-(Smt3)$_n$. *C*, purified Siz2-(Smt3)$_n$ or mock (−) was sumoylated for 30 min with the indicated concentration of His$_6$-Smt3-aR in the presence of 10 nM Aos1/Uba2 and 100 nM Ubc9. The products were then ubiquitinated for 20 min with 250,000 cpm $^{32}$P-Ub and 5 nM Uba1, 50 nM Ubc4, 50 nM Slx5–Slx8, plus 20 nM unlabeled Ub (*lanes 1–7*). As control, the sumoylation step was monitored by incubating Siz2-(Smt3)$_n$ or mock (−) under the same conditions but with 250,000 cpm $^{32}$P-Smt3-aR and the indicated concentrations of unlabeled His$_6$-Smt3-aR (*lanes 8–12*).

internal sites within the SUMO chain. To test this, we performed the reverse experiment: purified Siz2-(Smt3)$_n$ was first incubated under sumoylation conditions with increasing concentrations of unlabeled Smt3-aR, and the products were then ubiquitinated with $^{32}$P-Ub. As shown in Fig. 7C (*lanes 1–7*), increasing concentrations of Smt3-aR specifically inhibited ubiquitination of Siz2(Smt3)$_n$: background signal resulting from auto-ubiquitination of Slx5–Slx8 and other factors was unaffected by Smt3-aR (Fig. 7C and supplemental Fig. S2B). In control reactions Siz2-(Smt3)$_n$ efficiently incorporated $^{32}$P-Smt3-aR (*lanes 8–12*). Thus, ubiquitination of poly-SUMO chains was dependent on a SUMO chain whose terminal SUMO moiety could be modified.

*Highly Sumoylated Proteins Accumulate in Specific Strain Backgrounds*—The requirement for the Slx5–Slx8 Ub ligase in *sgs1Δ* strains may be related to the requirement for Ulp1 in *srs2Δ* strains (12). For example, both factors may act to suppress the accumulation of sumoylated proteins. To test whether these DNA repair mutants displayed altered patterns of sumoylated proteins, total denatured protein was isolated from several strains (*sgs1Δ*, *srs2Δ*, and *slx5Δ*) and analyzed by immunoblot. As shown in Fig. 8A, *sgs1Δ* and *srs2Δ* cells contained an excess of hyper-sumoylated proteins that was similar to that found in *slx5Δ* strains. In contrast, the post-replication repair mutant *rad6Δ* did not accumulate these proteins (data not shown). The abundance of hyper-sumoylated proteins in these two recombinational repair mutants suggests that Slx5–Slx8 or Ulp1, respectively, are needed to suppress the accumulation of toxic levels of these modified proteins.

To test the idea that Ub-mediated proteolysis controls the abundance of sumoylated proteins, total denatured protein was isolated from a temperature-sensitive 20 S proteasome mutant strain (*pre1-1 pre2-2*) and immunoblotted with antibody against Smt3 (Fig. 8C). At the permissive temperature, the *pre1 pre2* mutant had wt levels of sumoylated proteins (Fig. 8C, *lanes 4* and *7*), but after increasing time at the semi-permissive temperature (30 °C) or the non-permissive temperature (37 °C), there was an increased abundance of sumoylated proteins in the 100- to 250-kDa range similar to that seen in the *slx5Δ* and *slx8Δ* strains (Fig. 8C, *lanes 6, 8,* and *9*). As observed using other conditions to limit Ub-mediated proteolysis (21), evidence of long poly-SUMO chains was visible in the stacking gel following 4 h at 37 °C. Thus, the hyper-sumoylation observed in *slx5Δ* and *slx8Δ* cells may result from the failure of poly-sumoylated proteins to be degraded by the proteasome.

## DISCUSSION

The main finding of this study is that the Slx5–Slx8 Ub ligase is activated by poly-SUMO conjugates. Activation by this substrate is consistent with the preferential interaction between Slx5–Slx8 and poly-SUMO conjugates (21), and with the presence of multiple SUMO-binding domains in Slx5–Slx8. We suggest that these multiple SUMO-binding domains are needed to interact with polymerized SUMO moieties. Indeed, the SUMO-binding domain located in the N terminus of Slx5 and the one in Slx8 have been shown to contain well conserved amino acid sequence motifs that match the SUMO interacting motif consensus (21, 24). We have previously reported that Slx5
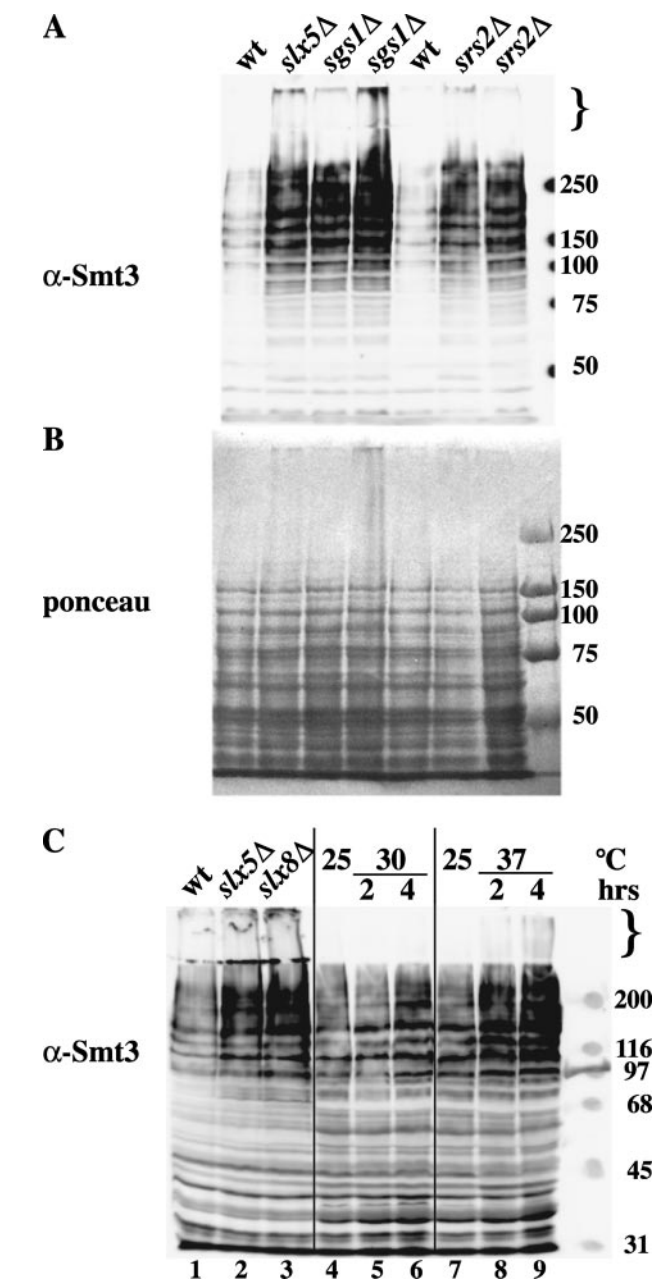


FIGURE 8. **Poly-sumoylated proteins accumulate in the absence of *SGS1, SRS2*, or proteasome function.** *A*, total yeast extracts were prepared by the trichloroacetic acid method and resolved by 10% SDS-PAGE prior to immunoblotting and staining with antibodies against Smt3. The strains are JMY2472 (*WT*), NJY2402 (*slx5Δ*), NJY506 (*slx5Δ*), JMY1460 (*sgs1Δ*) JMY2472 (*WT*), JMY286 (*srs2Δ*), and NJY379 (*srs2Δ*). *B*, shown is the blot in *A* stained with Ponceau Red to reveal protein loading. *C*, extracts from the following strains were prepared and immunoblotted with antibodies against Smt3 as above: W303–1a (*WT*), SIY778 (*slx8Δ*), and JMY1699 (*slx5Δ*) grown at 30 °C. Strain JDY194 (*pre1-1 pre2-2*) was grown at 25 °C (*lanes 4* and *7*), at 30 °C for 2 or 4 h (*lanes 5* and *6*), or at 37 °C for 2 or 4 h (*lanes 8* and *9*).

and Slx8 can promote SUMO chain formation in the presence of Aos1/Uba2 and Ubc9, but their RING domains were not required for this activity (28). The identification of multiple SUMO-binding domains may explain this *in vitro* result. For example, the Slx5–Slx8 heterodimer, or Slx5 alone, may bind multiple SUMO moieties in a way that would increase their local concentration and their conjugation efficiency by Ubc9. Further, the multiple SUMO-binding domains of Slx5 may confine adjacent

## Activation of the Slx5–Slx8 Ub Ligase

SUMO polypeptides to the same orientation found in a SUMO chain. Consistent with this idea, Slx5 is significantly more active in promoting high molecular weight SUMO chains than is Slx8 (28).

Siz2 was chosen as a test substrate because it is known to be sumoylated *in vivo* (39) and because auto-sumoylated Siz2 was expected to carry an assortment of SUMO modifications. Based on the fact that most *in vivo* sumoylation is monomeric, we expected that a single SUMO polypeptide would be sufficient to bind the ligase to the target so that it could be ubiquitinated directly. Instead, we found that the Ub ligase not only prefers the poly-sumoylated form of Siz2, but that it modifies its N-terminal SUMO moiety. This conclusion is supported by the lack of ubiquitination of Siz2 that has been modified by Smt3-aR, which is a good model of a mono-sumoylated substrate. An alternative explanation for this result is that Smt3-aR is not recognized efficiently by Slx5–Slx8 or that Siz2 is a poor receptor for Ub. However, Smt3-aR can partially complement the *smt3Δ* null mutation *in vivo* (20), and Siz2 must contain at least one site for ubiquitination, because it was ubiquitinated independent of its sumoylation status. Although *in vivo* evidence for mixed SUMO-Ub chains has not yet been obtained, it may not be unexpected given that the spectrum of Ub modifications is extremely diverse, including Ub chains with mixed linkages (40).

Several results are consistent with the idea that the Slx5–Slx8 Ub ligase specifically targets three N-terminal lysine residues of SUMO. First, the inhibition of ubiquitination by capping SUMO chains with Smt3-aR demonstrates that there is little or no internal ubiquitination of the SUMO chain. For ubiquitination to occur at an internal SUMO moiety, a poly-SUMO chain with Lys-15 linkages, for example, would have to be ubiquitinated as close as four residues away from the inter-SUMO linkage (at Lys-11 or Lys-19), which is likely to be sterically excluded. Second, if a SUMO chain were saturated with Ub at these sites, then we should have observed a unique intermediate following Ulp1 cleavage, $(Smt3-Ub)_2$, which we have never seen. Lastly, SUMO chain elongation is inhibited by ubiquitination, suggesting that Ub is competing for these N-terminal residues.

The recent discovery of similar SUMO-targeted Ub ligases in fission yeast suggests that mechanisms to eliminate sumoylated proteins are highly conserved and functionally important (21, 32, 33). In the case of Slx5–Slx8, it remains to be determined whether its targets are limited to poly-SUMO conjugates. Moreover, it will be interesting to determine whether these conjugates are biologically active or just toxic artifacts. In this regard we find it interesting that yeast Smt3 can tolerate the mutation of many of its lysine residues to arginine, individually and collectively, but mutating all lysines (Smt3-aR) confers a sickness to otherwise wt cells (20). Although it is reasonable to suspect that Smt3-aR is structurally defective due to its many mutations, it remains possible that some of its defect is due to an inability to form poly-SUMO chains. Thus, it will be interesting to determine whether the accumulation of hyper-sumoylated proteins that we observed in the absence of *SGS1* or *SRS2* represents a functional response to their DNA repair defects. And regardless of whether these hyper-sumoylated proteins are functionally important or simply toxic intermediates, a major
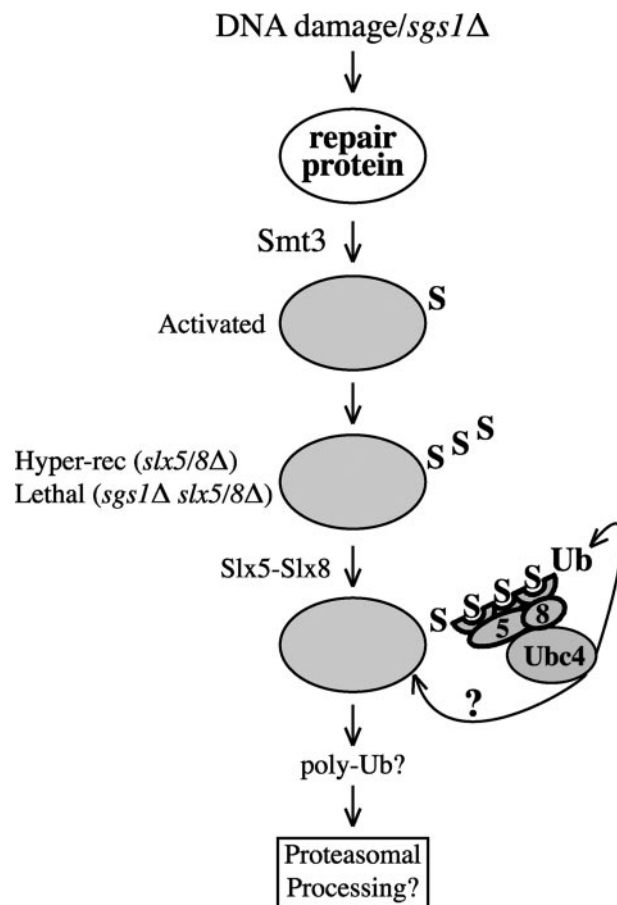


FIGURE 9. **Model for the generation of poly-sumoylated proteins and their Ub-dependent proteasomal degradation.** In response to replicative DNA damage, a DNA repair protein is activated by mono-sumoylation (*S*). This damage is expected to be exacerbated in the absence of the Sgs1 DNA helicase. Poly-SUMO chain formation may alter the activity of the repair protein or act as a first step in its destruction. The Slx5–Slx8 Ub ligase, together with Ubc4 or Ubc5, modifies the SUMO chain at its growing end to limit its length or to direct it to the proteasome following Ub chain elongation via an unknown mechanism.

unanswered question is why Slx5–Slx8 is specifically needed in the absence of *SGS1*. Unlike Slx5–Slx8, which represents a module in the DNA integrity network (41), loss of other SUMO regulators such as Ulp2, Siz1, or Siz2 does not result in lethality in the absence of *SGS1*.[3] A potential explanation for this specificity is that Slx5–Slx8 ubiquitinates poly-SUMO chains that have a particular length or type of linkage. Alternatively, Slx5–Slx8 may show specificity due to its localization to DNA replication foci (26).

Presented in Fig. 9 is a working model to accommodate the above findings, including the accumulation of poly-sumoylated proteins and their destruction by Slx5–Slx8. In this model replicative DNA damage, which is exacerbated in the absence of the Sgs1 DNA helicase, leads to the "activation" of one or more recombinational repair proteins by sumoylation. Although poly-sumoylation is not a major feature of SUMO function (20) it may be a transient step following recombinational repair, perhaps to eliminate or further modify the repair protein. In support of this possibility we have found that *smt3-aR* cells are

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sensitive to DNA damage by methyl methane sulfonate.[4] In the absence of the Slx5–Slx8 Ub ligase, the poly-sumoylated protein remains active in stimulating recombination. In wt cells, we propose that the Slx5–Slx8 Ub ligase promotes Ubc4/5-dependent mono-ubiquitination of lysines 11, 15, or 19 on the terminal SUMO residue. Future studies will be needed to address the question of whether extension of the Ub chain is necessary for proteasomal targeting and whether the redundant Uls1 E3 ligase plays a role in that reaction (21). However, until a biologically relevant substrate for the Slx5–Slx8 Ub ligase is identified, we cannot rule out the possibility that the ligase ubiquitinates its sumoylated substrate directly.

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