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Stimulation of in-vitro sumoylation by Slx5-Slx8: evidence for a functional interaction with the SUMO pathway

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Abstract

The yeast genes *SLX5* and *SLX8* were identified based on their requirement for viability in the absence of the Sgs1 DNA helicase. Loss of these genes results in genome instability, nibbled colonies, and other phenotypes associated with defects in sumoylation. The Slx5 and Slx8 proteins form a stable complex and each subunit contains a single RING-finger domain at its C-terminus. To determine the physiological function of the Slx5-8 complex, we explored its interaction with the SUMO pathway. Curing 2μ circle from the mutants suppressed their nibbled colony phenotype and partially improved their growth rate, but did not affect their sensitivity to hydroxyurea. The increase in sumoylation observed in *slx5*Δ and *slx8*Δ mutants was found to be dependent on the Siz1 SUMO ligase. Physical interactions between the Slx5-8 complex and both Ubc9 and Smt3 were identified and characterized. Using in vitro reactions, we show that Slx5, Slx8, or the Slx5-8 complex stimulates the formation of SUMO chains and the sumoylation of a test substrate. Interestingly, a functional RING-finger domain is not required for this stimulation in vitro. These biochemical data demonstrate for the first time that the Slx5-8 complex is capable of interacting directly with the SUMO pathway.

Keywords

genome stability; recombination; SUMO; Smt3; Sgs1 DNA helicase

1. Introduction

Ubiquitin (Ub) and ubiquitin-like (Ubl) variants modify proteins by forming an isopeptide bond between their C terminus and a lysine side-chain present in a target protein. One of the best studied Ubls is SUMO (Small Ubiquitin-related MOdifier; aka Ubl1, hSmt3, and sentrin) [1]. Unlike Ub's role in directing protein degradation, the biological functions of SUMO modification are diverse and include promoting protein-protein interactions [2,3], nuclear import [4], and protein stabilization [5].

Sumoylation has been effectively studied in budding yeast where the process is known to be essential for viability. As in other species, modification by the yeast SUMO (Smt3) proceeds through a three-step process analogous to Ub conjugation. These steps require an ATP-dependent E1 activating enzyme (Aos1/Uba2), an E2 conjugating enzyme (Ubc9), and one of several E3 ligases that confer substrate specificity [1]. Sumoylation normally takes place at lysine residues that fall within the consensus sequence ΨKXE/D, where Ψ is a hydrophobic residue. Unlike Ub, typical SUMO modifications are believed to be monomeric although

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SUMO chains can form in vitro and in vivo [1,6]. Sumoylation is also reversible due to the activity of the SUMO-specific isopeptidases Ulp1 and Ulp2/Smt4 [6–9].

In yeast there are four known SUMO E3 ligases. The homologous Siz1/Ull1 and Siz2/Nfi1 proteins were the first SUMO E3 ligases to be identified [10]. These proteins share a RING-finger related sequence motif, the SP-RING domain, with the PIAS1 (Protein Inhibitor of Activated Signal transducer and activator of transcription) protein of human cells [11,12]. The Siz1 and Siz2 ligases are active on septins and may have partially overlapping specificities [10,13,14]. Together, Siz1 and Siz2 account for 90% of the total sumoylation in yeast, and cells lacking both *SIZ1* and *SIZ2* are viable but slow growing [9,10]. The *SIZ*-independent sumoylation may be partly due to a third ligase, Mms21, which is essential for viability and also contains an SP-RING domain [15,16]. Mms21 is conserved in humans [17] and co-purifies with the essential Smc5-Smc6 that is required for recombination-mediated DNA repair [16, 18]. Zip3 is a meiotic SUMO E3 ligase with an SPRING domain that plays a role in the formation of the synaptonemal complex [19]. In addition to the SP-RING proteins, human cells contain at least two SUMO E3 ligases, RanBP2 and PC2, that lack any form of RING domain [20,21].

Yeast strains with defects in a variety of sumoylation components (i.e., Ulp1, Ulp2, Siz1 Siz2, or Ubc9) sometimes display an irregular colony phenotype that was first described as “nibbled” in *S. carlsbergensis* [22,23]. Rather than being perfectly circular, these colonies have uneven edges due to an unstable population of enlarged slow-growing cells that exhibit clonal lethality [7,24]. The phenotypes of these cells are exacerbated at low temperature and include a delay in the G2/M phase of the cell cycle [22,23,25]. It has recently been shown that the nibbled phenotype of sumoylation mutants occurs due to over-replication of the 2μ circle which is normally an innocuous plasmid found in most laboratory strains of *Saccharomyces* [24–26].

Accumulating evidence points to a role of the *SLX5* and *SLX8* genes in regulating sumoylation. These genes were first isolated in an *SGS1* synthetic-lethal screen and, on their own, null mutations in *SLX5* and *SLX8* produce nearly identical phenotypes [27]. The *slx5*Δ and *slx8*Δ mutants display slow growth, a reduced plating efficiency compared to wild type (wt) cells, and a nibbled colony phenotype [27]. Like *sgs1*Δ and *top3*Δ mutants, *slx5*Δ or *slx8*Δ cells are sensitive to the DNA synthesis inhibitor hydroxyurea (HU) and homozygous mutant cells display a reduced sporulation frequency compared to wt [27]. Consistent with a role in controlling genome stability, these mutants display a large increase in Gross Chromosomal Rearrangements (GCRs) [28]. The Slx5 and Slx8 proteins associate in yeast extracts and can be purified as a recombinant complex [27,29]. Yeast two-hybrid (Y2H) studies have detected interactions between Slx5 and multiple components of the sumoylation pathway [30,31]. More recently, *slx5*Δ-*slx8*Δ mutants have been shown to accumulate highly-sumoylated proteins and to have a variety of genetic interactions with genes that control sumoylation [32].

Both Slx5 and Slx8 contain predicted RING-finger motifs of the C3HC4 type. Although most RING-finger proteins are ubiquitin E3 ligases, we tested the possibility that the nibbled colony morphology and other defects observed in these mutants reflected a direct biochemical role in stimulating Ubc9-dependent sumoylation. We found that the Slx5 and Slx8 proteins, as well as the Slx5-Slx8 complex, could stimulate SUMO conjugation activity in vitro. This activity did not require the RING-finger of Slx5 and was resistant to mutations in critical RING-domain residues in Slx8. Our results indicate that the Slx5-Slx8 complex directly interacts with sumoylation proteins in-vitro and suggests that it may have a more complex role regulating sumoylation in-vivo.

2. Materials and methods

2.1 Yeast Strains and Plasmids

The yeast strains used in this study are listed in Table 1. Gene deletions remove the entire open reading frames. Yeast two-hybrid vectors and strains were obtained from Clontech. Cells were cured of 2μ circle using plasmid pBIS-KFLP, which expresses a mutant FLP recombinase [33]. The loss of 2μ circle was confirmed by PCR. His6-tagged UD domain of Ulp1 ($Ulp1_{UD}$) [34] was expressed from plasmid pTI7269 which consists of a PCR fragment encoding residues 347–621 of Ulp1 ligated into the NdeI and BamHI sites of pET28a. The yeast expression plasmids pTI7271 and pTI7272 contain the open reading frames of $Ulp1_{UD}$ and Ulp2, respectively, downstream of the *GPD1* promoter in vectors pRS424 and pRS425 [35].

2.2 Expression and purification of recombinant proteins

Recombinant proteins were produced using the T7 expression system of Studier [36]. Slx5, Slx8, and Slx5-8 complex were purified as N-terminal His6-tagged fusion proteins, as described [29]. The same procedure was used to purify the following point mutants as single subunits: Slx5-7 (C556S), Slx5-8 (C556S, H558A, C561S), Slx8-2 (C221S), Slx8-3 (C221S, H223A, C226S). The sumoylation enzymes His6-Aos1/Uba2, His6-Ubc9, and HF-Smt3 were purified from plasmids generously provided by Erica Johnson using published methods [37, 38].

2.3 In vitro Sumoylation assay

Sumoylation reactions were 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 DTT. Unless otherwise indicated, the standard reaction was incubated at 30°C for 90 min and contained 3.3 ng E1, 20 ng E2, 10 – 300 ng Slx5, Slx8, or Slx5-8 complex, 150 ng HF-Smt3, and 100 ng substrate in a total volume of 20 μl. Reductive methylation of HF-Smt3 was carried out as described [39]. Reactions were terminated by the addition of Laemmli buffer, boiled and subjected to SDS-PAGE and immunoblot analysis with the appropriate antibodies. Antibody detection was performed using a chemiluminescent substrate for HRP (Pierce) and an LAS-3000 chemiluminescence camera (Fujifilm). Reaction products were quantitated using ImageGauge software (Fujifilm).

2.4 Immunological techniques

Purified recombinant Slx5, Slx8, and HF-Smt3 were used as antigens to raise rabbit antisera (Covance, Denver PA). Physical interactions between Slx5, Slx8, or the Slx5-Slx8 complex and various GST-fusion proteins were detected following incubation on ice for one hour in a final volume of 0.1 ml using Buffer A (25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.01% [vol/vol] NP-40, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM DTT) containing 50 mM NaCl as the incubation buffer. This reaction was then diluted with 0.3 ml incubation buffer and mixed with 20 μl glutathione beads for one hour 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 SDS sample buffer and detected by immunoblotting as described [40]. Antibodies to Cdc11 were obtained from Santa Cruz Biotechnology. Antibodies to PCNA were generously provided by Zhiguo Zhang.

2.5 Analysis of sumoylated yeast proteins

Unless otherwise noted, crude yeast extracts were made using the TCA lysis method. Briefly, cells were harvested, washed consecutively with water and 20% TCA, and resuspended in 25 – 200 μl of 20% TCA. An equal volume of glass beads was added and cells were lysed by vortexing with at 4°C by hand or in a FastPrep shaker (Savant). Extracts were removed and

microcentrifuged at 3000 RPM for 15 min at 4°C. Protein precipitates were then resuspended in 2X SDS-loading buffer, heated for 5 min and subjected to SDS-PAGE. To purified His6-tagged proteins, extracts were prepared in guanidine as described [13]. Cleared extracts were made 10 mM in imidazole and continuously applied to a 1 ml Ni His-Trap column (GE Healthcare) at room temperature overnight. The column was then washed with 20 volumes guanidine buffer, 10 volumes N buffer (25 mM Tris·HCl [pH 8.0], 10% glycerol, 500 mM NaCl, 0.01% NP40, 0.1 mM PMSF, and 1mM dithiothreitol) containing 10 mM imidazole, and the protein was eluted in N buffer containing 0.25M imidazole. To cleave sumoylated proteins an aliquot of the eluate was diluted with an equal volume of Buffer A and incubated with 250 ng $Ulp1_{UD}$ for 60 min at 30°C.

3. Results

3.1 Sumoylation defects in slx5Δ and slx8Δ mutants

The nibbled colony morphology of *slx5*Δ and *slx8*Δ mutants is reminiscent of that seen in SUMO mutants where it is known to be caused by overreplication of the 2μ circle plasmid (2μ) [24–27]. To determine whether 2μ was responsible for this phenotype in *slx5*Δ and *slx8*Δ mutants, we cured the cells of the plasmid by expressing a step-arrest mutant of the Flp recombinase [33]. Wild type (wt) cells formed large circular colonies whose size and morphology were unaffected by curing 2μ circle (Fig. 1A). In contrast, the *slx5*Δ *cir^o* and *slx8*Δ *cir^o* colonies no longer displayed nibbled edges and were uniformly round. Curing 2μ circle also improved the growth rates of these mutants (Fig. 1B). However, the doubling times of *slx5*Δ *cir^o* and *slx8*Δ *cir^o* cells remained 30 – 40% greater than that of wt cells. In addition, *slx5*Δ *cir^o* and *slx8*Δ *cir^o* strains remained as sensitive to HU as the uncured strains (Fig. 1B). We conclude that the nibbled phenotype of *slx5*Δ and *slx8*Δ cells is dependent on 2μ circle and that these cells have a second slow-growth defect that is independent of 2μ circle.

3.2 Genetic interactions between SLX5-8 and components of the sumoylation pathway

To explore their role in the sumoylation pathway, we searched for genetic interactions between *slx5*Δ or *slx8*Δ and the known sumoylation enzymes. Following crosses to a strain carrying a hypomorphic temperature-sensitive allele of *UBC9* (*ubc9-1*) [41], we found that both *slx5*Δ *ubc9-1* and *slx8*Δ *ubc9-1* double mutants were inviable (Fig. 2A and Table 2). This result is consistent with a previously reported interaction with a different allele of *UBC9* [32]. The synthetic lethality observed in these strains is likely to be due to defects in sumoylation since *ubc9-1 slx5*Δ lethality could be suppressed by eliminating the non-essential *ULP2/SMT4* SUMO iso-peptidase (Table 2). In addition, we were unable to isolate double mutants between *slx5*Δ or *slx8*Δ and a hypomorphic mutation in the essential *ULP1* iso-peptidase (Table 2), and this lethality again was suppressed by eliminating *ULP2*. The fact that Ubc9 and Ulp1 catalyze very different reactions in the sumoylation process suggests that *SLX5* and *SLX8* become essential when the balance of sumoylation:desumoylation is altered. Consistent with this idea, overexpression of Ulp2 or the Ulp1 UD domain [34] was lethal in the *slx5*Δ strain (Table 2). The mechanism of suppression by *ulp2*Δ was not further explored, however it has previously been suggested that yeast cells may require a balance between Smt3-conjugating and de-conjugating activities, and that there is a feedback mechanism that limits Smt3 conjugation when Smt3 cleavage rates are severely impaired [7].

A synthetic growth defect between either *slx5*Δ or *slx8*Δ and *siz2* has previously been observed [32]. This defect was further exacerbated in *siz1 siz2 slx5* or *siz1 siz2 slx8* triple mutants (Table 2). On their own, *siz1 siz2* double mutants displayed a synthetic growth defect [10,42] with a doubling time (DT) of 123 min, and loss of *SLX5* or *SLX8*Δ in this background exacerbated this defect (DT = 200 min). The fact that all of these proteins contain RING- or SP-RING motifs suggested that Siz1 and/or Siz2 may functionally overlap with, or regulate the levels of

Slx5 and Slx8. However, no consistent change in their abundance was dected by immunoblotting extracts of *siz1 siz2* cells (data not shown).

We considered the possibility that the growth defect resulting from the loss of SUMO E3 ligases in *slx5*Δ or *slx8*Δ cells might be due to global changes in the levels of their sumoylated proteins. To test this idea, we prepared cell extracts and immunoblotted them with antibodies against yeast SUMO. As previously observed, wild type cells displayed a range of sumoylated proteins while *siz*Δ*1, siz2*Δ, and *siz*Δ*1 siz2*Δ double mutants showed a significant reduction in their abundance [10] (Fig. 2B). In contrast, *slx5*Δ and *slx8*Δ mutants displayed an increase in the abundance of sumoylated species, particularly in the high molecular-weight region of the gel [32] (Fig. 2B). This increase was dependent on *SIZ1*, which is known to be responsible for the majority of the sumoylation in yeast. The loss of *SIZ2* did not have a significant effect on the levels of sumoylated proteins in *slx5*Δ or *slx8*Δ cells. The sumoylation levels in the triple-mutant background (e.g., *siz1*Δ *siz2*Δ *slx5*Δ*)* were very low, although they were elevated slightly relative to *siz1*Δ *siz2*Δ cells. Given that the elevated sumoylation levels of *slx5-8*Δ mutants were suppressed in this background, we tested whether lowering of sumoylation levels could suppress the synthetic lethality of *slx5*Δ *sgs1*Δ cells. However, *sgs1*Δ *slx5*Δ *siz1*Δ *siz2*Δ cells remained inviable as did *sgs1*Δ *slx5*Δ cells overexpressing $ULP1_{UD}$ and/or *ULP2* (Table 2). Thus, it is unlikely that the elevation of global sumoylation levels is responsible for *sgs1*Δ *slx5*Δ synthetic lethality. In summary, *SLX5-SLX8* has a complex relationship to the sumoylation pathway in which loss of either gene results in increased levels of *SIZ1*-dependent sumoylated proteins, in addition to synthetic sickness with mutations that are expected to either diminish (e.g, *ubc9-1, siz2*Δ) or enhance (e.g., $ulp1^{ts}$) sumoylation levels.

3.3 Physical interactions between Slx5-8 and sumoylation proteins

We next searched for physical interactions between Slx5 or Slx8 and components of the sumoylation pathway using the yeast two-hybrid (Y2H) assay. We obtained positive evidence for interactions between Slx5 (as bait) and both Smt3 and Ubc9, but not a variety of negative-control preys (Fig. 3A). This assay also detected the known interaction between Slx5 and Slx8, as well as an Slx5 self-interaction. In a second series of experiments, we found evidence that Slx8 interacted with Ubc9, Smt3, and Slx5 (Fig. 3B). All of these interactions were confirmed by reciprocal Y2H experiments (data not shown). The Y2H interactions between Slx5 and both Slx8 and Smt3 were previously observed in a large-scale study of yeast protein-protein interactions [30]. In order to test whether these proteins interact biochemically, the Slx5, Slx8, and Slx5-Slx8 complex were expressed in bacteria and purified [29]. The Slx5 or Slx8 subunits were then incubated together with GST-Ubc9 or GST-Smt3 fusion proteins. As expected for a direct physical interaction, Slx5 bound to glutatione-beads in the presence of either fusion protein, but not GST alone (Fig. 3C). Slx8 also showed specific binding to Ubc9, although binding to Smt3 was no greater than background. Consistent with these data, both subunits of the Slx5-Slx8 complex were found to co-precipitate with the Ubc9 or Smt3 fusion proteins. Taken together, these data indicate that Slx5-Slx8 interacts both genetically and physically with components of the SUMO pathway. These results led us to test whether Slx5-Slx8 was capable of directly regulating sumoylation *in vitro*.

3.4 Slx5 and Slx8 proteins stimulate SUMO conjugation activity in vitro

The Slx5 and Slx8 subunits were incubated with purified E1 (Aos1-Uba2), E2 (Ubc9), and FLAG-tagged SUMO (HF-Smt3) together with ATP and test substrates as targets for sumoylation. Immunoblotting with anti-FLAG antibodies was then used to reveal the presence of HF-Smt3-conjugated proteins. Although initial attempts failed to detect activity on a variety of standard substrates (e.g., beta-galactosidase, calf histones, bovine serum albumin, human topoisomerase II), SUMO chains were reproducibly generated in an Slx5- and Slx8-dependent manner. This activity has previously been observed using several SUMO E3 ligases [15,19,

43]. As shown in Fig. 4A, the synthesis of SUMO chains was dependent on the E1 and E2 and was strongly stimulated by either Slx5 or Slx8, although Slx5 was routinely more active in this assay. Testing of additional proteins revealed that Slx8, but not Slx5, promoted the sumoylation of the 69 kd subunit of yeast RPA (Fig 4B and data not shown). Another substrate was identified in the process of assaying Slx5 on a variety of general transcription factors. As shown in Figure 4C, Slx5 strongly sumoylated Ydr1/Ncb2 (a subunit of the NC2 transcriptional regulator). Because of its small size, Ydr1 was chosen as a substrate to further characterize this activity.

Ydr1 is composed of 146 amino acid residues with a single consensus sumoylation site at K141 (140-VKSE-143). We assayed the sumoylation activity of Slx5 using Ydr1 and a mutant, Ydr1-K141R, as substrates. In the absence of Slx5, there was a low level of SUMO chain formation (Fig. 5A; lanes 1–2), but Ydr1 was not efficiently sumoylated (Fig. 5B, lanes 1–2). As before, Slx5 strongly stimulated the formation of SUMO chains in the complete reaction (Fig. 5A). Immunoblotting with anti-Ydr1 antibody (Fig. 5B) revealed that Slx5 promoted the conjugation of SUMO onto Ydr1 (Ydr-S1) and produced at least three higher-molecular weight forms. As expected for a single sumoylation site, none of these modifications were observed using Ydr1-K141R (Fig. 5B, lane 4). Moreover, the abundance of SUMO chains could be inhibited through the use of methylated SUMO (Me-SUMO) (Fig. 5B, lanes 5–6). This inhibition indicates that the higher molecular weight forms arise from SUMO self-conjugation and that a chain of at least four residues could be attached to Ydr1. As expected for a stimulatory activity, all conjugation in the reaction was dependent on the presence of the E1 and E2 (data not shown). The specificity of this reaction was confirmed by demonstrating that conjugation of Me-SUMO to Ydr1 could be stimulated by Slx5 and Slx8, but not BSA (Fig. 5C). The findings that Slx5 and Slx8 interact with Ubc9 and SUMO, they stimulate SUMO conjugation to Ydr1 specifically, and they do so in an E1- and E2-dependent manner, suggested that the complex could also stimulate this activity.

In order to directly compare the stimulatory activities of these proteins, we assayed titrations of Slx5, Slx8, and the Slx5-Slx8 complex on Ydr1 using Me-SUMO. As shown in Figure 6A, substantial and similar levels of the primary product (Ydr1-S1) were obtained using 60 ng of either monomer (Slx5 or Slx8), or 120 ng of the dimeric complex (Slx5-Slx8). These quantities of the E3s also produced similar levels of a secondary product (Ydr1-S2) which probably represents a dimeric chain arising from incomplete methylation of the SUMO. We conclude that, under these conditions, the three forms of the proteins have roughly similar activities toward Ydr1. We next tested whether the activities differed using unmethylated SUMO. As shown in Figure 6B, titration of Slx5 produced singly-modified Ydr1 in addition to substantial levels of Ydr1-containing SUMO chains. In contrast, only the highest levels of Slx8 produced Ydr1 containing SUMO chains. Both Slx5-Slx8 dimer and a reconstitution of Slx5 plus Slx8 produced chains that resembled the activity of Slx5 alone. Thus, while Slx5 and Slx8 produce similar levels of mono-sumoylated Ydr1, the Slx5 protein is more active in promoting the formation of SUMO chains on Ydr1. To test whether SUMO chain formation was specific for the Ydr1 target protein, the same blot was probed with α-FLAG to detect SUMO conjugation. As shown in Figure 6C, the conjugation of free SUMO by Slx5 is also greater than that of Slx8. It should also be noted that Slx8 did not inhibit chain formation by Slx5 (Slx5+Slx8), and that comparable amounts of Slx5-Slx8 complex produced chains that were sufficiently long as to be retained at the top of the resolving gel. Taken together, we conclude: (1) that Slx5 and Slx8 have similar substrate specificity, (2) that Slx5 is more active than Slx8 in promoting SUMO chain formation, (3) that stimulation of chain formation by the Slx5-Slx8 complex is slightly greater than Slx5 alone, and (4) this enhanced activity cannot be reconstituted from the individual subunits.

3.5 Role of the RING finger in in-vitro sumoylation

Most SUMO E3 ligases are characterized by an SP-RING domain that is essential for SUMO ligase activity [10,14,42,44,45]. To test the role of the RING finger in sumoylation activity, we expressed and purified some mutant proteins lacking this domain for in vitro assays. Surprisingly, deletion of the RING-finger domain from Slx5 (Slx5-ΔC126) did not affect its ability to stimulate the conjugation of SUMO chains (Fig. 7A). Chain formation also did not require the N-terminal 200 amino acids of Slx5, although removal of an additional 200 aa (Slx5-ΔN400) completely eliminated the activity. In contrast, removal of the RING domain of Slx8 (Slx8-ΔC74) eliminated its ability to stimulate SUMO conjugation (Fig. 7B). And surprisingly, the 74 amino acid RING domain alone (Slx8-ΔN200) was capable of stimulating the reaction (Fig. 7B).

To determine whether this stimulation relied on the conserved activity of the RING domain, as opposed to a novel activity, we assayed proteins bearing mutations in conserved RING domain residues that are known to be important for function. To quantify the results, we assayed their ability to conjugate a single Me-SUMO onto Ydr1 as a function of time. After 90 min of incubation, sumoylation of Ydr1 was stimulated about 9-fold by wild type Slx5 and Slx8, compared to the activity of the E1 and E2 alone (Fig. 7C and D). Consistent with the RING-independent activity observed above, the Slx5-7 and Slx5-8 point-mutant proteins displayed approximately wild-type levels of activity (Fig. 7C). In the case of Slx8, both point-mutant proteins displayed similar intermediate levels of activity (Fig. 7D). Importantly, it has previously been shown that one of these alleles is active in-vivo (*slx8-2*) while the other is null (*slx8-3*) [29]. Therefore, the lack of correlation between in-vitro and in-vivo activities suggests that the in-vitro sumoylation by Slx8 is a secondary activity of this domain. Taken together, we conclude that the stimulation of sumoylation by both Slx5 and Slx8 does not depend on the conserved function of the RING-domain.

3.6 Regulation of specific sumoylation targets in-vivo

In addition to investigating changes in global sumoylation levels, we were interested in learning how the loss of *SLX5* affected the sumoylation of specific target proteins. We therefore examined two well-characterized sumoylated proteins: PCNA [46] and Cdc11 [10]. Denaturing extracts were first prepared from wt and *slx5*Δ strains carrying His-tagged PCNA. Following chromatography over a Ni resin, the partially purified fraction was resolved by SDS-PAGE and immunoblotted with antibody against PCNA. Wild-type extracts revealed two higher molecular weight forms of PCNA (Mr ~ 48 and 60 kD) that were judged to represent sumoylated species based on their sensitivity to Ulp1 treatment (Fig. 8A, lanes 1 and 2). Probing these samples with anti-Smt3 antibody revealed that multiple sumoylated proteins co-purified with PCNA, perhaps non-specifically. The effectiveness of the Ulp1 treatment was confirmed on this blot although (Fig. 8A). We conclude that the indicated bands represent PCNA containing one and two SUMO moieties, respectively. Although both forms were present in the *slx5*Δ mutant, their abundance was reduced about 50% compared to wt (Fig. 8A, compare lanes 1 and 3). This result was surprising as it contrasts with the elevated levels of total sumoylated proteins found in this mutant.

We then examined the sumoylation of Cdc11 using crude extracts obtained from exponentially growing cells that contained His6-modified Smt3 (HF-Smt3) as their only source of SUMO. As shown in Figure 8B, wt cells contained a slower migrating form of Cdc11 (Mr ~ 83 kD) that was elevated in mutant cells. Unfortunately, non-specific binding of the antibody obscured other potentially sumoylated Cdc11 proteins in these extracts. To improve the sensitivity of this assay we took advantage of the His6-tagged Smt3 in this strain. Thus, we repeated the immunoblot using fractions that had been enriched in HF-Smt3-modified proteins by Ni chromatography. As shown in Figure 8C, a band corresponding to mono-sumoylated Cdc11

was detected in fractions from wt cells. Interestingly, the intensity of this band, and one corresponding to di-sumoylated Cdc11, increased in the *slx5Δ* mutant. Taking these results together, we conclude that loss of *SLX5* results in complex changes in the pattern of protein sumoylation in the cell. Although bulk protein sumoylation increases in the mutant, there are cases in which the abundance of sumoylated forms is reduced.

4. Discussion

One of the goals of this study was to gain insight into the molecular function of Slx5-Slx8 which is a complex needed for viability in the absence of *SGS1-TOP3*. Our results indicate that the complex interacts directly and functionally with Smt3. Interestingly, it was previously shown that *SLX5* and *SLX8* are required to suppress gross chromosomal rearrangements [28]. This phenotype suggests that *slx5*Δ and *slx*8Δ mutants may exhibit increased recombination which is conisistent with the need for *SGS1-TOP3*. One explanation for the various phenotypes of *slx5*Δ and *slx8*Δ mutants is that they arise indirectly from the deregulation of sumoylation. It is known, for example, that an allele of *ULP1* can produce a hyper-recombination phenotype that is lethal in the absence of *SRS2* or homologous recombination [47]. Similarly, *ulp2*Δ mutants are sensitive to HU and to a variety of DNA damage agents [7]. Thus, the increase in global sumoylation levels seen in the *slx5*Δ and *slx8*Δ mutants may mimic the increase seen in *ulp1ts* or *ulp2*Δ cells which could lead indirectly to the observed increase in genome instability.

Other results, however, suggest that *SLX5* and *SLX8* act more directly to control genome stability. First, *slx5*Δ and *slx8*Δ phenotypes are distinct from other sumoylation mutants. Although there is some overlap in drug sensitivities (e.g., HU sensitivity), *slx5*Δ and *slx8*Δ cells are not sensitive to MMS, UV or benomyl (J.R.M. and S.J.B., unpublished results), like *ulp2*Δ mutants [7]. Second, the genetic interaction between *SGS1* and *SLX5* or *SLX8* is very specific. No other SUMO E3 ligases or *ulp* mutants were identified in our screen or in two other genome-wide screens for synthetic interactors with *SGS1* [48,49]. Indeed, *sgs1*Δ *siz1*Δ *siz2*Δ cells are viable, as are *sgs1*Δ *ulp2*Δ cells (J.R.M. and S.J.B., unpublished results). And although certain genetic interactions between *slx5*Δ or *slx8*Δ and either *ubc9-1* or *ulp1ts* can be suppressed by *ulp2*Δ (Table 2), we have been unable to suppress *sgs1*Δ *slx5*Δ lethality with various combinations of *ulp2*Δ, *siz1*Δ, and/or *siz2*Δ. Thus, the synthetic lethality is unlikely to be due simply to elevated global levels of sumoylation.

In contrast, the suppression observed in *ulp1ts slx5*Δ *ulp2*Δ cells may be related to the fact that these two isopeptidase mutations (*ulp1ts ulp2*Δ*)* display reciprocal suppression and reduced levels of Smt3-protein conjugates compared to that seen in the single mutants [7]. These and other results previously suggested the existence of a feedback mechanism whereby severely reduced de-conjugation activity inhibits SUMO conjugation [7]. For example, it has been proposed that sumoylation of the E2 or an unknown E3 might inhibit conjugation activity. Some of the *slx5-8*Δ defects we observed (e.g., increased global sumoylation levels) might be explained if Slx5-8 is involved in this feedback mechanism. In this model, Slx5-8 complex might regulate Siz1 via sumoylation. This might inhibit its activity in wt cells and, by comparison, lead to the excess sumoylation observed in *slx5*Δ strains. We have not yet tested whether Siz1 is sumoylated by Slx5-8, so it remains a possibility that the increase in sumoylation levels observed in *slx5*Δ and *slx8*Δ mutants is due to unleashing of the Siz1 ligase. Left unexplained by this model is why the *siz2 slx5*Δ and *siz1 siz2 slx5*Δ mutants display synthetic sickness. One possibility, suggested by the ability of Slx5 and Slx8 to promote sumoylation in vitro, is that these three proteins promote the sumoylation of a common target.

A second goal of this work was to test the possibility of a direct link between the Slx5-Slx8 complex and the sumoylation pathway. Such a connection could provide a mechanism by which

Slx5-8 controls genome stability. The ability to stimulate SUMO chain formation in vitro supports the idea that Slx5-8 interacts directly with this pathway, however we have not yet identified any in vivo substrates whose sumoylation is directly stimulated by Slx5-8. Preliminary experiments have failed to reveal Ydr1 sumoylation in-vivo, and Slx5-8 localizes to the nucleus [29] where it is unlikely to interact directly with Cdc11. Identifying in vivo substrates for Slx5-8 is made more difficult by the unusual deregulation of sumoylation in the mutants which leads to both increases and decreases in sumoylation depending on the substrate. If some of these effects arise due to compensatory mechanisms, it will be very difficult to identify authentic substrates. Moreover, substrates relevant to genome stability may be modified only under specific conditions, such as in the presence of DNA damage or replication arrest.

The stimulation of sumoylation by Slx5 and Slx8 raises the question as to the function of their RING-finger domains. The RING-fingers may mediate subunit dimerization, promote the interaction with other proteins, or contribute to an additional activity. The C3HC4-type RING-finger domains found in these proteins are typical of Ub ligases. The fact that these domains are dispensible for sumoylation activity in vitro raises the possibility that Slx5-8 might serve dual roles in promoting both Ub and SUMO conjugation. Precedent for such a dual-activity protein is provided by the conserved RING-finger protein, Topors. Topors binds DNA topoisomerase I [50] and p53 [51,52] and it displays both Ub E3 ligase [53,54] and SUMO ligase activity [55]. Topors Ub ligase activity is dependent on its RING-finger domain, while its SUMO ligase activity is not [55]. Slx5-Slx8 may represent another example of such a dual-function ligase. It will be interesting to test this hypothesis by searching for interactions between Slx5-Slx8 and the known Ub conjugating enzymes.

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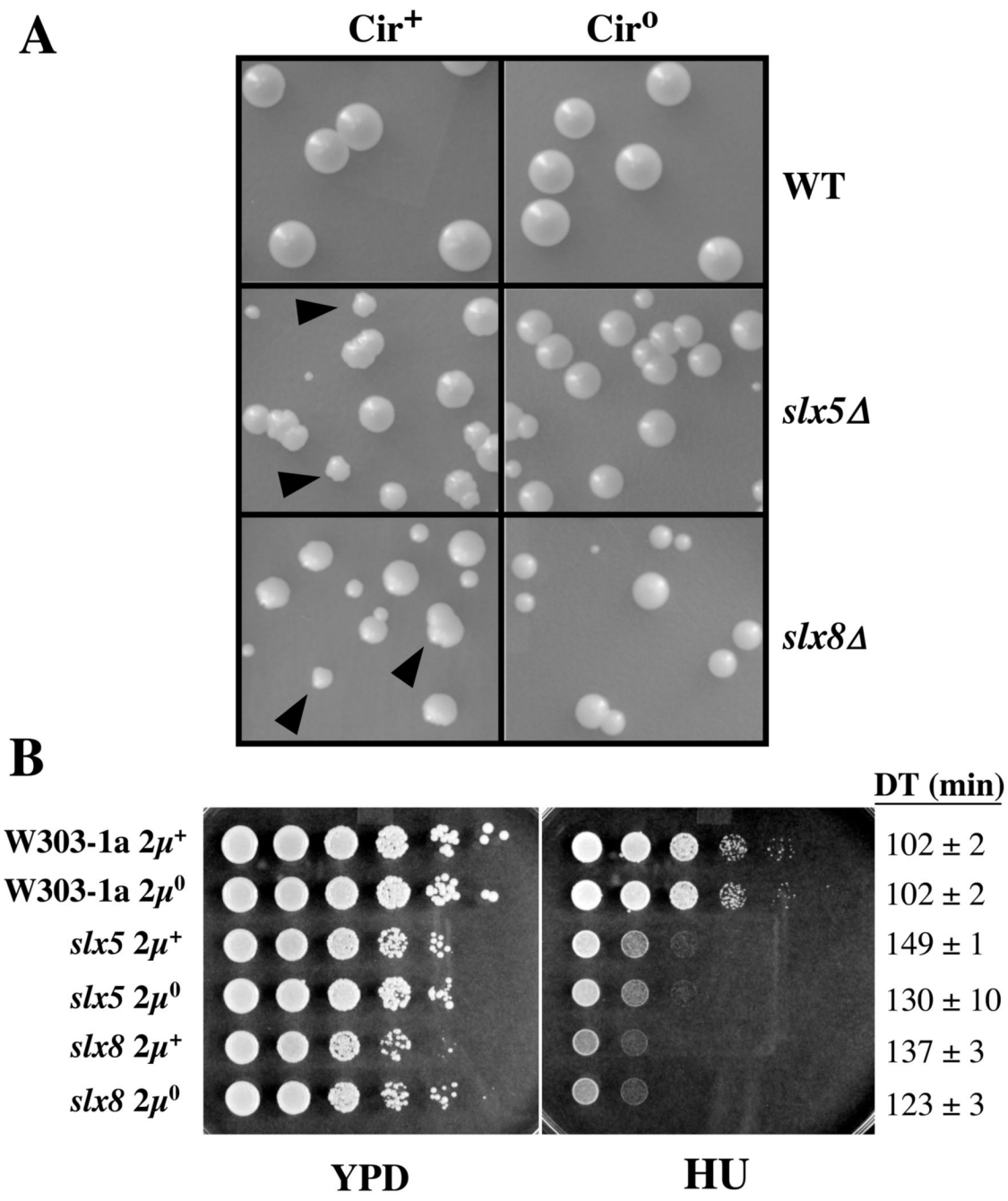

Figure 1.

Two-micron circle is responsible for the nibbled colony phenotype of *slx5*Δ and *slx8*Δ strains. (A) WT, *slx5*Δ, and *slx8*Δ strains were cured of 2μ circle and the indicated cultures were spread on YPD plates. Colonies were photographed following 3 days growth at 30°C. Filled arrowheads indicate nibbled colonies. (B) The indicated strains were resuspended at an OD = 3, serially diluted in 10-fold steps, and approximately 5 μl spotted on solid yeast extract-peptone-dextrose (YPD) media in the absence or presence of 0.1 M hydroxyurea (HU). The plates were photographed following 2 (YPD) or 4 (HU) days growth at 30°C. The growth rates of these strains were determined in liquid YPD at 30°C. These doubling times (DT) are presented at right.

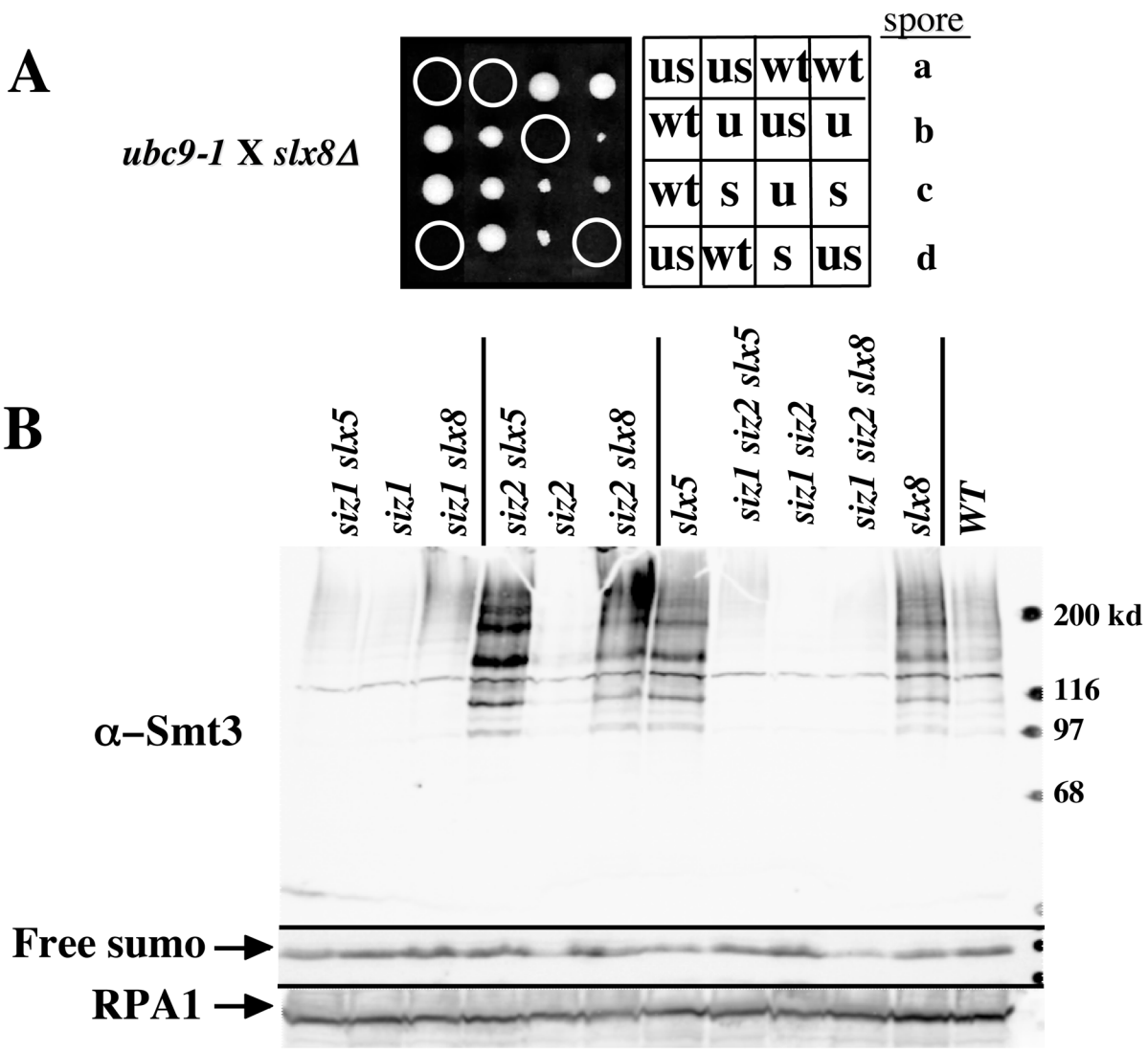

Figure 2.

SLX5 and *SLX8* show genetic interactions with known sumoylation components. (A) Four tetrads from the cross YO174 (*ubc9-1*) X JMY1604 (*slx8*Δ) were dissected vertically onto a YPD plate, allowed to germinate for 4 days at 25°C, and photographed. Genotypes of the spore clones were determined and are indicated as follows: WT, wild type; *u, ubc9-1; s, slx8*Δ; *us, ubc9-1 slx8*Δ. Note the nibbled colony morphology of *slx8*Δ and *ubc9-1* single mutants. (B) Extracts from the indicated yeast mutants were prepared by the NaOH method [56] and analyzed for Smt3-protein conjugates by 10% SDS-PAGE and immunoblotting using antibodies against Smt3. Identical samples were run on 17% SDS-PAGE, blotted, and probed for free Smt3 or RPA1 as internal loading controls.

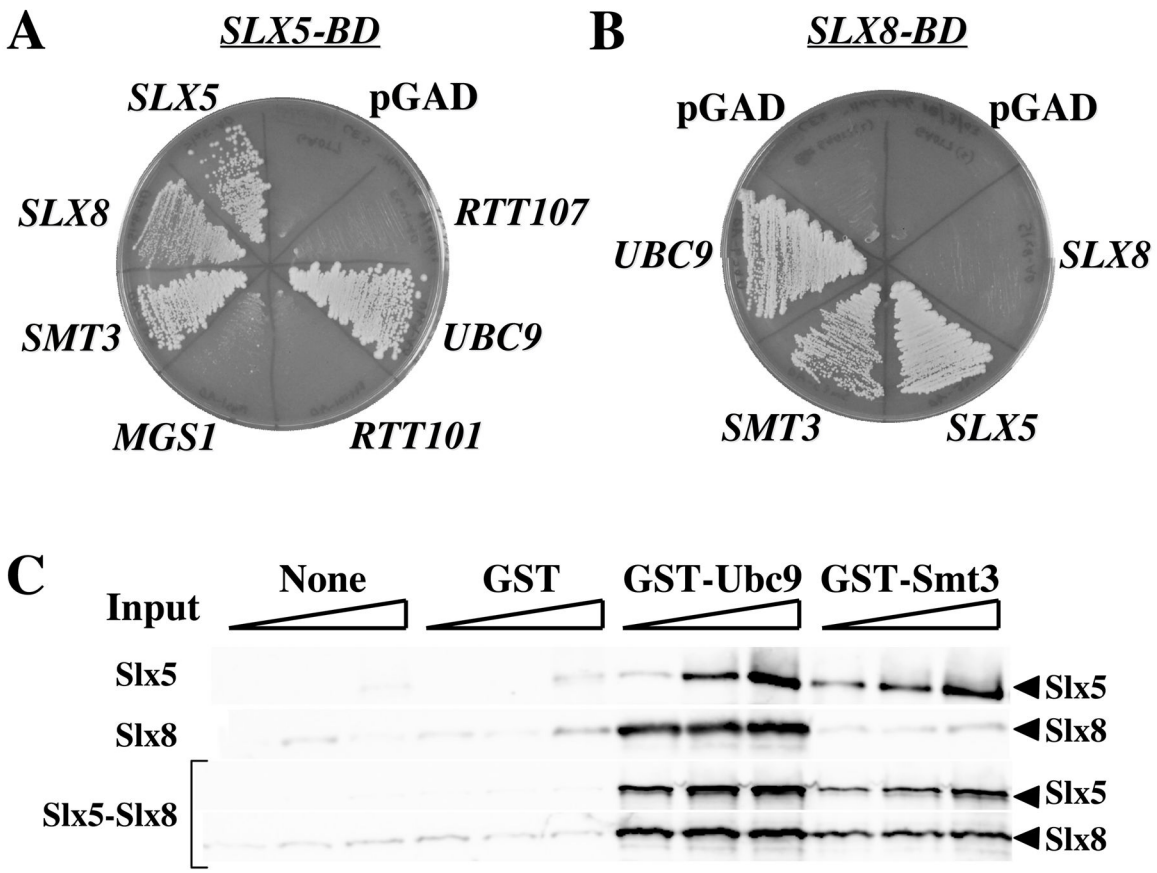

Figure 3.

Slx5 and Slx8 show physical interactions with known sumoylation components. (A) Yeast Two-Hybrid interactions with *SLX5*. *SLX5* was subcloned into a binding-domain vector (pGAD) and transformed into strain AH109 along with an activating domain plasmid containing the indicated gene. Transformants were streaked onto selective media lacking histidine and adenine as selection for the two reporter genes in this strain. (B) Yeast Two-Hybrid interactions with *SLX8* in the binding-domain vector were assayed as in (A). (C) Increasing amounts (12, 48, or 120 pmol) of Slx5 (top panel), Slx8 (second panel), or the Slx5-Slx8 complex (lower panel) were incubated on ice with either no protein, or 24 pmol of GST, GST-Ubc9, or GST-Smt3 as indicated. Bound proteins were detected following glutathione bead pull-down and immunoblotting with Slx5 or Slx8 antisera.

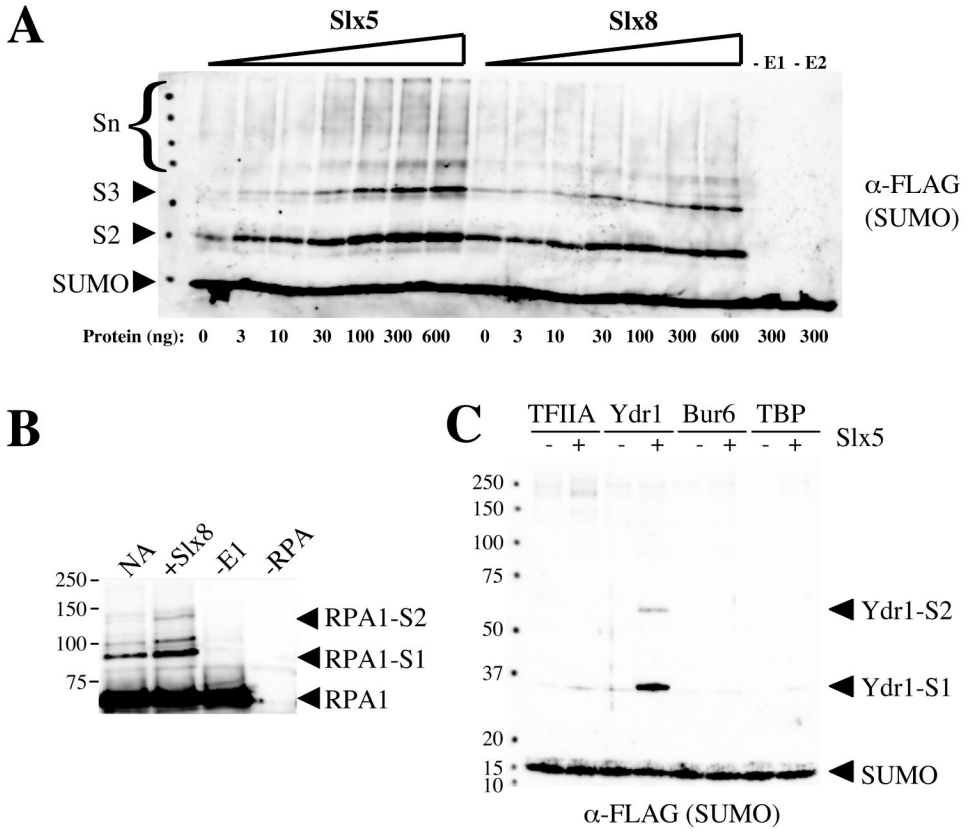

Figure 4.

Purified Slx5 and Slx8 stimulate SUMO conjugation in vitro. (A) Standard sumoylation reactions were carried out as described in the Materials and Methods, but included the indicated amounts of Slx5 or Slx8, in addition to the following levels of yeast Aos1/Uba2 (E1, 3.3 ng in lanes 1-14 and 16), Ubc9 (E2, 8.3 ng in lanes 1-15), and HF-Smt3 (SUMO, 150 ng). Following incubation, the reaction products were analyzed by SDS-PAGE and immunoblotting using antibodies against the FLAG epitope to detect SUMO. The positions of monomeric (SUMO) and polymerized SUMO chains (di = S2; tri = S3; multiple = Sn) are indicated. (B) Sumoylation reactions were carried out under standard conditions but contained RPA (50 ng) as substrate and methylated HF-Smt3 (150 ng) in place of HF-Smt3 to limit SUMO chain formation. Reactions contained either no addition (NA), Slx8 (225 ng), no Aos1-Uba2 (-E1), or no RPA (−RPA). Following incubation, the reaction products were analyzed by SDS-PAGE and immunoblotting using antibody against yeast RPA1. Arrowheads indicate the positions of RPA1 and its sumoylated products. (C) Sumoylation reactions were carried out under standard conditions, but contained methylated HF-Smt3 (150 ng) in addition to the following yeast transcription factors as substrates: TFIIA (100 ng, lanes 1 and 2), Ydr1 (100 ng, lanes 3 and 4), Bur6 (100 ng, lanes 5 and 6), or TBP (100 ng, lanes 7 and 8). Reactions were performed in the presence (+) or absence (−) of Slx5 (300 ng). Following incubation the reactions were analyzed as in (A) to detect SUMO. The positions of SUMO and sumoylated Ydr1 products are indicated.

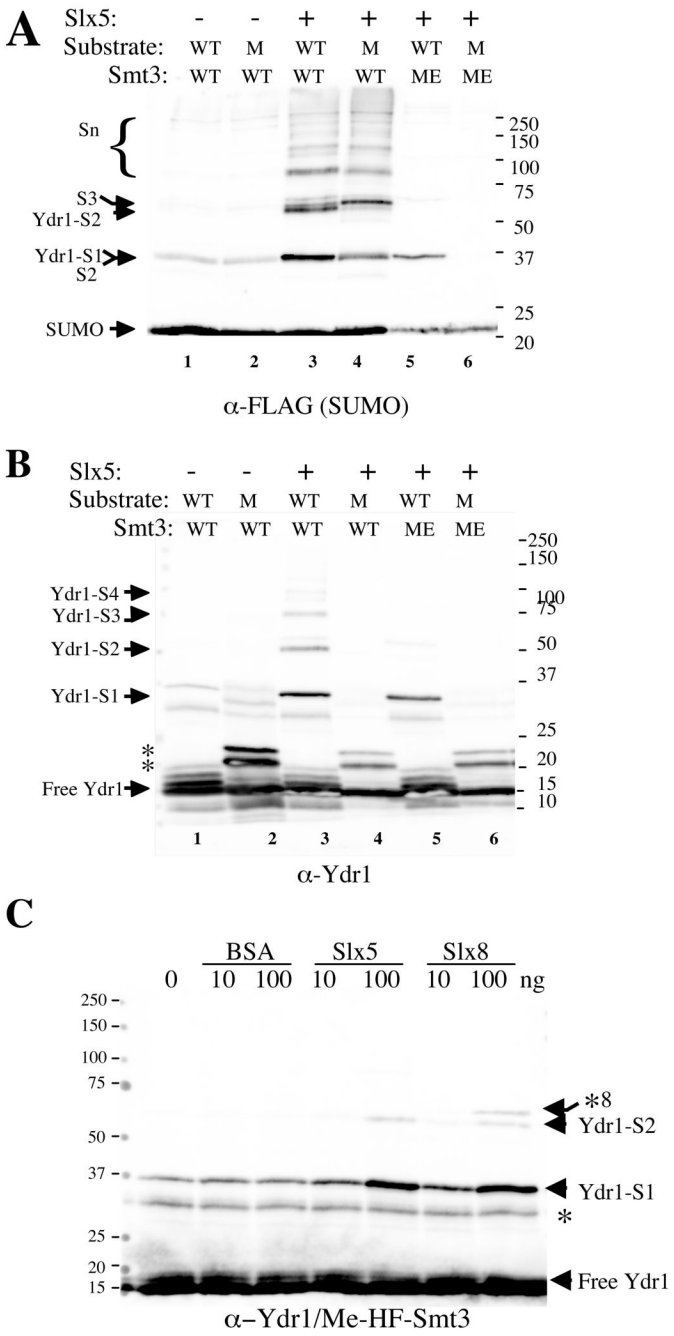

Figure 5.

Slx5-dependent sumoylation of Ydr1 in vitro. (A) Standard sumoylation reactions were carried out as described in the Materials and Methods, but where indicated contained Slx5 (300 ng), either Ydr1 (WT, 100 ng) or Ydr1-K141R (M, 100 ng) as substrate, and either HF-Smt3 (WT, 150 ng) or Me-HF-Smt3 (ME, 150 ng). Following incubation, the reactions were analyzed by SDS-PAGE and immunoblotting using antibody against FLAG to detect SUMO (A) or against Ydr1 (B). The identity of the reaction products are indicated. Asterisks in (B) indicate cross-reacting proteins present in the Ydr1-K141R preparation. (C) The specificity of SUMO ligase activity was tested using standard reaction conditions and included Ydr1 (100 ng), methylated HF-Smt3 (150 ng), and the indicated amount of BSA, Slx5, or Slx8. Following incubation, the

reactions were analyzed by SDS-PAGE and immunoblotting with antibodies against Ydr1. Arrowheads indicate the positions of sumoylated products and asterisks indicate cross-reacting bands. The cross-reacting band denoted by *8 is His6-Slx8.

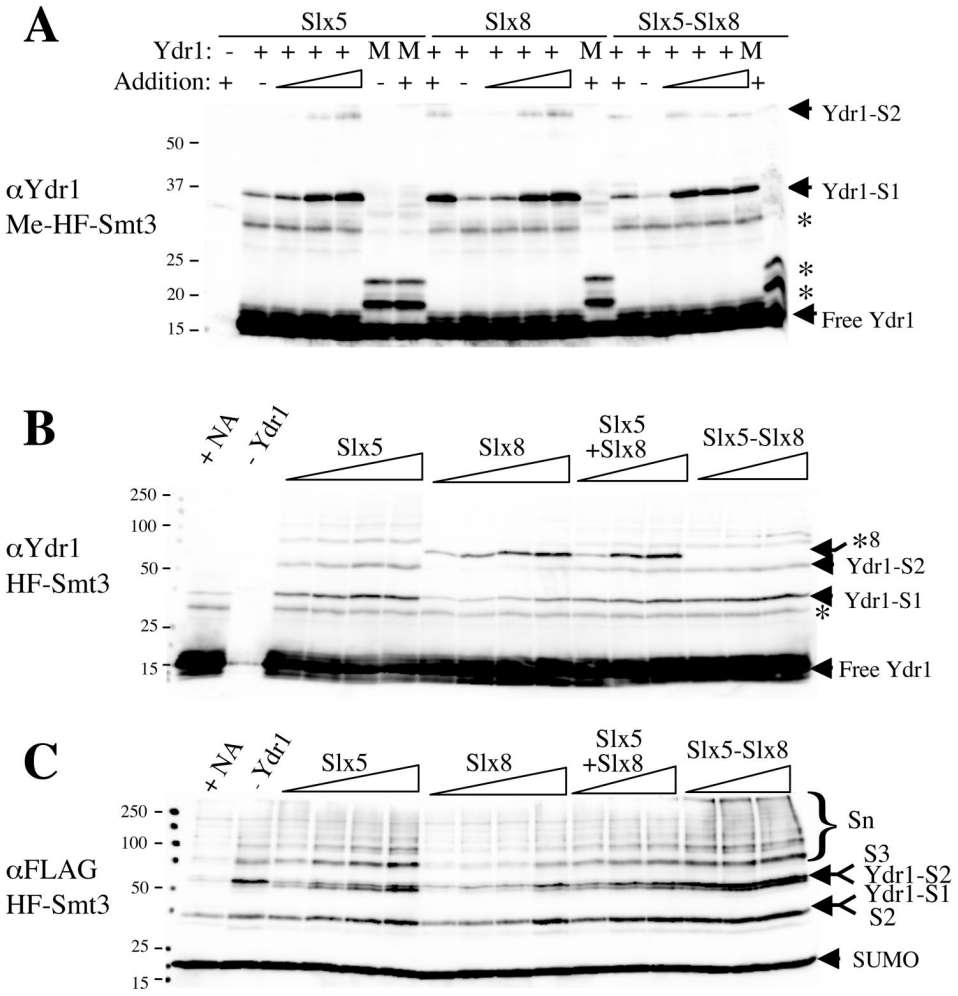

Figure 6.

Stimulation of SUMO conjugation to Ydr1 by Slx5, Slx8, and the Slx5-Slx8 complex. (A) The SUMO ligase activities of the monomeric and heterodimeric forms of Slx5-Slx8 were compared using standard reactions conditions and either Ydr1 (+, 50 ng) or Ydr1-K141R (M, 50 ng), as indicated. Reactions contained methylated HF-Smt3 (150 ng) and various amounts of either Slx5 (225, 0, 10, 60, 225, 225 ng), Slx8 (225, 0, 10, 60, 225, 225 ng), or Slx5-Slx8 complex (450, 0, 20, 120, 450, 450 ng), as indicated. (B) and (C) Additive effects of Slx5 and Slx8 were tested in reactions containing Ydr1 (100 ng), HF-Smt3 (150 ng), and either Slx5 (0, 180, 30, 60, 120, 180 ng in lanes 1–6), Slx8 (30, 60, 120, 180 ng in lanes 7–10); Slx5 plus Slx8 (constant 30 ng Slx5 plus 30, 90, 150 ng Slx8 in lanes 11–13), or the Slx5-Slx8 complex (60, 120, and 180 ng in lanes 14–16). Incubation and analysis was performed as in Figure 5 using antibodies against Ydr1 (A) and (B) or FLAG (C).

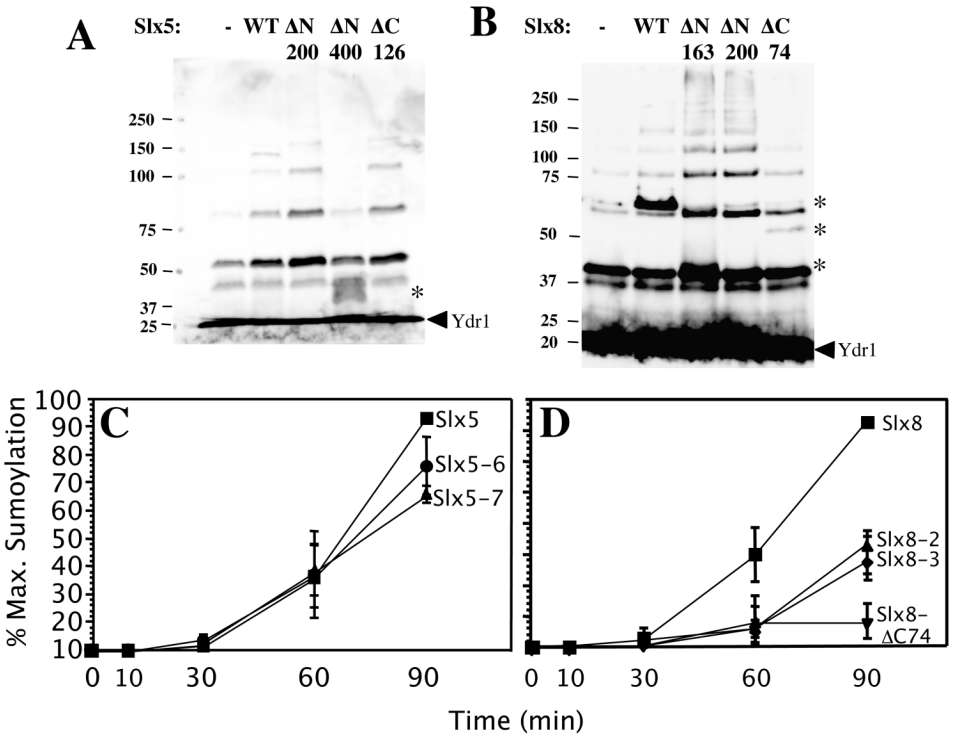

Figure 7.

RING-finger function of Slx5 and Slx8 is dispensible for in vitro sumoylation. (A) Slx5 (WT) or Slx5 protein lacking the N-terminal 200 aa (ΔN200), N-terminal 400 aa (ΔN400), or C-terminal 126 aa (ΔC126) was purified from *E. coli*, and 100 ng was assayed for the ability to stimulate SUMO chain formation using 10% SDS-PAGE. The RING domain is comprised of the C-terminal 126 residues. (B) Slx8 (WT) or Slx8 protein lacking the N-terminal 163 aa (ΔN163), the N-terminal 200 aa (ΔN200), or the C-terminal 74 aa (ΔC74) was titrated into in vitro sumoylation assays and analyzed as in (A) except for the use of 12.5% SDS-PAGE. The RING domain is comprised of the C-terminal 74 residues. (C) and (D) Time course of *in vitro* Ydr1 sumoylation reactions. Standard sumoylation reactions were carried out as described in the Materials and Methods, but contained 10 ng of the indicated Slx5 or Slx8 protein in addition to Ydr1 (50 ng) and methylated HF-Smt3p (150 ng). Following incubation at 30°C for 0, 10, 30, 60, or 90 min, the reactions were terminated, analyzed by SDS-PAGE, and immunoblotted using anti-Ydr1 antibody. The level of singly-modified Ydr1 was determined by densitometry and is presented as the percent of maximal sumoylation as a function of time. Point mutations are as follows: Slx5-7 (C556S), Slx5-8 (C556S, H558A, C561S), Slx8-2 (C221S), Slx8-3 (C221S, H223A, C226S).

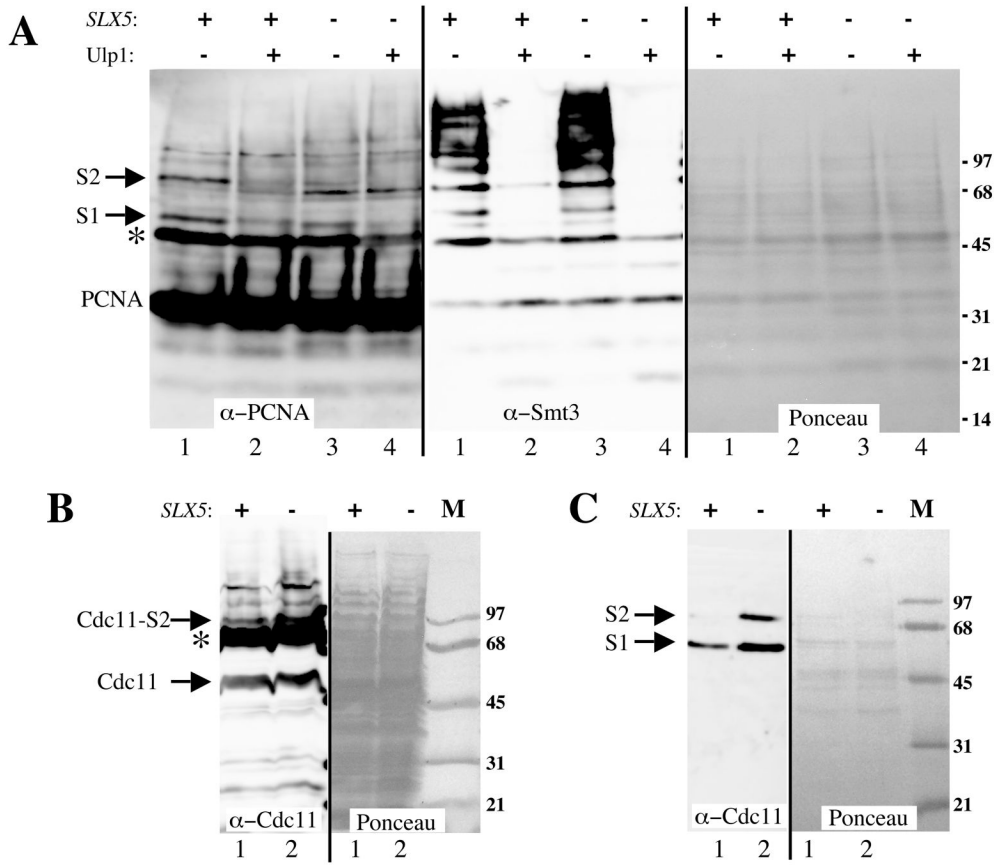

Figure 8.

Sumoylation of PCNA and Cdc11 is altered in *slx5Δ* mutants. Guanidine extracts from wt or *slx5Δ* strains that express His7-tagged PCNA were partially purified on a Ni resin and treated with or without Ulp1 protease prior to immunoblotting with antibodies against PCNA or Smt3. (B) TCA extracts from wt or *slx5Δ* strains that express HF-Smt3 as their only source of SUMO were immunoblotted with antibody against Cdc11. (C) Guanidine extracts from the strains in (B) were partially purified on a Ni resin and immunoblotted with antibodies against Cdc11. Ponceau S-stained membranes are shown as gel-loading controls. S1, S2: the relevant protein conjugated to 1 or 2 Smt3 moieties, respectively. Asterisks indicate non-specific cross-reacting bands. Strains: NJY2504, NJY2505, NJY2510, NJY2543.

Table 1

Yeast strains used in this study

Strain	Genotype	Reference or Source
W303-1a	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 rad5-535*	Thomas and Rothstein
JMY1699	*MAT***α** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 rad5-535 slx5-11::HPH*	This study
JMY1604	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 rad5-535 slx8-10::KAN::loxP*	This study
SIY778	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 rad5-535 slx8-10::KAN::loxP*	Mullen *et al*., 2001
EJY326	*MAT***a** *ura3-52 his3-200 leu2-3,112 trp1-63 lys2-801 CAN1 siz1::LEU2 siz2::TRP1*	Johnson and Gupta, 2001
JMY1831	*MAT***α** *ade2-1 his3 leu2-3,112 trp1 siz1::LEU2 siz2::TRP1 slx5-11::HPH lys2-801 can1-100*	This study
NJY1855	*MAT***a** *ura3 his3 leu2-3,112 trp1 lys2-801 siz1::LEU2 siz2Δ::TRP1 slx5-11::HPH*	This study
JMY1799	*MAT***α** *ura3 his3 leu2-3,112 trp1 lys2-801 can1-100 siz1Δ::LEU2 siz2Δ::TRP1 slx8-10::KAN::loxP*	This study
MYH1614	*MAT***a** *his3-Δ200 leu2-3,112::LEU2::ulp1-333 ura3-52 lys2-801 trp1-1 ulp2-1::HIS3 ulp1-Δ1::his3::URA3*	Li and Hochstrasser, 2000
JMY1743	W303-1a *slx5-11::HPH*	This study
JMY1464	*MAT***a** *ade2 ade3::hisG ura3 his3-11,15 leu2 trp1-1 lys2 slx5-10::TRP1 sgs1-20::HGR can1-100* + pJM500	Mullen *et al*., 2001
VCY1525	*MAT***a** *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 lys2 sgs1-20::HPH slx8-10::KAN* + pJM500	This study
Y0174	*MAT***α** *his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52 ubc9Δ::TRP1 LEU2::ubc9-1 ts*	Mao *et al*., 2000
AH109	*MAT***a** *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1uas-HIS3 MEL1 GAL2uas-ADE2 URA3::MEL1uas-lacZ*	Clontech
NJY2504	*MAT***α** *ade2-1 ADE3 ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 can1-100 His7pol30::HIS3 RAD5*	This study
NJY2505	*MAT***α** *ade2-1 ADE3 ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 can1-100 slx5Δ::NAT His7pol30::HIS3 RAD5*	This study
NJY2510	*MAT***α** *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 can1-100 smt3Δ::loxP RAD5 + pNJ7264 (HF-Smt3/LEU2)*	This study
NJY2543	*MAT***α** *ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 LYS2 smt3Δ::KAN::loxP slx5Δ::NAT RAD5 + pNJ7264 (HF-SMT3/LEU2)*	This study

Table 2

SLX5-SLX8 Genetic Interactions [a]

Genotype	Growth
ubc9^{ts} *slx5Δ*	−
ubc9^{ts} *slx5Δ ulp2Δ*	+
ubc9^{ts} *slx8Δ*	−
ubc9^{ts} *slx8Δ ulp2Δ*	ND
ulp1^{ts} *slx5Δ*	−
ulp1^{ts} *slx5Δ ulp2Δ*	+
ulp1^{ts} *slx8Δ*	−
ulp1^{ts} *slx8Δ ulp2Δ*	+
ulp2Δ slx5Δ	+
ulp2Δ slx8Δ	+
siz1Δ slx5Δ	+
siz1Δ slx8Δ	+
siz2Δ slx5Δ	sick
siz2Δ slx8Δ	sick
siz1Δ siz2Δ	sick
siz1Δ siz2Δ slx5Δ	very sick
siz1Δ siz2Δ slx8Δ	very sick
siz1Δ siz2Δ slx5Δ sgs1Δ	−
ULP1$_{UD}$ OE *SLX5*	+
ULP2 OE *SLX5*	+
ULP1$_{UD}$ OE *slx5Δ*	−
ULP2 OE *slx5Δ*	−
ULP1$_{UD}$ OE *sgs1Δ slx5Δ*	−
ULP2 OE *sgs1Δ slx5Δ*	−
ULP2+ULP1$_{UD}$ OE *sgs1Δ slx5Δ*	−

[a] +, viable; −, inviable; ND, not done