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Dual roles of the SUMO-interacting motif in the regulation of Srs2 sumoylation

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ABSTRACT

The Srs2 DNA helicase of *Saccharomyces cerevisiae* affects recombination in multiple ways. Srs2 not only inhibits recombination at stalled replication forks but also promotes the synthesis-dependent strand annealing (SDSA) pathway of recombination. Both functions of Srs2 are regulated by sumoylation—sumoylated PCNA recruits Srs2 to the replication fork to disfavor recombination, and sumoylation of Srs2 can be inhibitory to SDSA in certain backgrounds. To understand Srs2 function, we characterize the mechanism of its sumoylation *in vitro* and *in vivo*. Our data show that Srs2 is sumoylated at three lysines, and its sumoylation is facilitated by the Siz SUMO ligases. We also show that Srs2 binds to SUMO via a C-terminal SUMO-interacting motif (SIM). The SIM region is required for Srs2 sumoylation, likely by binding to SUMO-charged Ubc9. Srs2's SIM also cooperates with an adjacent PCNA-specific interaction site in binding to sumoylated PCNA to ensure the specificity of the interaction. These two functions of Srs2's SIM exhibit a competitive relationship: sumoylation of Srs2 decreases the interaction between the SIM and SUMO-PCNA, and the SUMO-PCNA–SIM interaction disfavors Srs2 sumoylation. Our findings suggest a potential mechanism for the equilibrium of sumoylated and PCNA-bound pools of Srs2 in cells.

INTRODUCTION

Homologous recombination (HR) is a major pathway to repair DNA double-strand breaks and single strand gaps, and to facilitate the recovery of stalled or collapsed replication forks (1,2). However, HR can also cause genome rearrangements or impede other DNA repair processes (3). Therefore, multi-layered regulation is critical to harness the benefits of HR and prevent its undesirable outcomes (4). In *Saccharomyces cerevisiae*, one mode of HR regulation utilizes the Srs2 protein, a 3′ to 5′ helicase and ssDNA translocase. It shares sequence homology and functional similarity with the bacterial helicases UvrD, Rep and PcrA, and mammalian Fbh1 and PARI proteins (5–7). A key role of Srs2 is to negatively regulate HR at replication forks and to channel DNA lesions into the post-replicative repair (PRR) pathway mediated by proteins such as Rad6, Rad18 and Rad5. Indeed, mutants of *SRS2* (*s*uppressor of *r*ad *s*ix) were first isolated as suppressors of the DNA damage sensitivity of *rad6* and *rad18* mutants, and this suppression requires HR (8,9). Consistent with this anti-recombinase role, *srs2Δ* confers a hyper-recombination phenotype and results in the accumulation of toxic recombination intermediates (10,11). Biochemical studies later revealed that Srs2 efficiently disrupts Rad51 presynaptic filaments, thus inhibiting an early step of HR (12,13).

The mechanism by which Srs2 is recruited to the replication fork is via its interaction with sumoylated PCNA, the processivity clamp for DNA polymerases (14–16). While the binding between Srs2 and SUMO-PCNA disfavors HR at stalled replication forks, this interaction has been implicated in additional functions such as facilitating replication through trinucleotide repeats (17–20). It is likely that this interaction has even broader effects, because the Srs2–PCNA interaction, but not the Srs2 helicase activity, is required for the toxicity of Srs2 overexpression in 274 deletion mutant backgrounds (21). In contrast with its anti-recombination role, Srs2 can also promote synthesis-dependent strand annealing (SDSA), particularly when the protein is phosphorylated by Cdk1

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(22,23). Interestingly, defects in SDSA caused by non-phosphorylatable Srs2 are alleviated by simultaneously mutating three sumoylation consensus sites, suggesting that sumoylation of Srs2 in this mutant context can be inhibitory to SDSA (23).

Sumoylation entails the covalent attachment of SUMO (Smt3) to target proteins in a three-step mechanism requiring SUMO E1 activating and E2 conjugating enzymes, and often promoted by an E3 ligase. The SUMO E2, Ubc9, can bind directly to the consensus sumoylation sequence ΨKxE/D *in vitro* (24–26). However, this interaction is weak and needs to be stabilized by accessory interactions. Such interactions are often provided by SUMO ligases, though a SUMO-interacting motif (SIM) in the substrate can also promote its interaction with SUMO or SUMO-charged E2 (27–31). Three SUMO E3 ligases, Siz1, Siz2 and Mms21, have been identified in budding yeast (32–34). Although sumoylation has been shown to be critical for DNA replication and repair, the consequences of SUMO attachment to many target proteins are still not known.

As Srs2 sumoylation is strongly induced by DNA damaging agents and negatively affects SDSA in specific situations, it is important to understand how sumoylation of Srs2 impinges on its functions and relates to its interaction with PCNA. Here, we characterize the mechanism of Srs2 sumoylation and highlight the importance of its SIM motif in dictating the balance between unmodified and sumoylated Srs2 in the cell. We show that this motif binds to SUMO-charged Ubc9 to promote the sumoylation of Srs2, but is unable to do so when bound by SUMO-PCNA instead. We also identify a PCNA-specific interaction site that cooperates with the SIM to bind PCNA. These data provide mechanistic insight into Srs2 sumoylation and demonstrate the importance of additional protein-specific interactions in stabilizing the binding between SUMO–SIM interacting partners.

MATERIALS AND METHODS

Yeast strains and plasmids

The *S. cerevisiae* strains used in this study are listed in Supplementary Table S1. The yLK92 strain (*rad18::LEU2 srs2ΔPIM*) was generated by polymerase chain reaction (PCR) using the Srs2Δ1159–1163 mutant, followed by integration of the PCR product into the genome of FF18238 by the PCR-based allele replacement method (35). Correct integration was verified by sequencing. Similarly, the yLK93 (*srs2–3KR*) and yLK94 (*rad18 srs2–3KR*) strains were generated by PCR using the s*rs2-K1081, 1089, 1142R* mutant, followed by integration of the product into the genome of FF1852 and FF18238 respectively.

The $(His)_9$-SRS2::pET11c plasmid has been described elsewhere (36). Plasmids expressing various Srs2 mutants were derived from the original plasmid by site-directed mutagenesis (Stratagene), using primers that are summarized in Supplementary Table S2. To generate the SRS2 (883–1174)::pGEX-6P-1 plasmid, a PCR fragment containing a.a. 883–1174 of Srs2 was cloned into the EcoRI site in pGEX-6P-1. Proteins of the sumoylation pathway were expressed from plasmids AOS1/UBA2::pGEX-4T-1 (37), UBC9::pET21b (38), SMT3::pET-HF (39), SMT3::pGEX-KG (40), UBC9::pGEX-KG, SIZ1 (1–465)::pET21b and SIZ2::pET21b (41), which have been described previously. Plasmids POL30::pPM1088 and POL30-K164R::pPM1088 were used to produce PCNA (42). The yeast two-hybrid plasmids UBC9::pGAD-C1, SMT3::pGAD-C1 (40) and SRS2(783–1174)::GBKT (12) have been described elsewhere. SRS2 (783–1169) in pGBKT7 was generated by insertion of a stop codon using site-directed mutagenesis of SRS2 (783–1174)::pGBKT7. Plasmids SRS2::pBG1805 (43), pCUP1-SRS2::pRS415 and pCUP1-srs2-R1::pRS415 (21) were used for *in vivo* sumoylation studies. Plasmids expressing Srs2 lysine mutants were derived from the SRS2::pBG1805 plasmid by site-directed mutagenesis.

Expression and purification of recombinant protein

The His-Srs2 protein and its various mutants were expressed and purified as described (44). The GST-Srs2 (883–1174) protein was over-expressed in *E. coli* BL21 DE3 cells. After the cells reached OD_{600} ~ 0.6, the protein expression was induced by adding IPTG to final concentration 1 mM followed by 3 h incubation at 37°C. Cell paste (10 g) was resuspended in 50 ml of cell breakage buffer (50 mM Tris-HCl pH 7.5, 10% sucrose, 10 mM EDTA, 1 mM dithiothreitol, 0.01% Nonidet P-40) containing 150 mM KCl and protease inhibitors. Suspensions were sonicated and cleared by ultracentrifugation. The supernatant was loaded onto 7-ml SP-Sepharose column. The column was developed with 70 ml gradient of 150–700 mM KCl in buffer K (20 mM KH_2PO_4, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Nonidet P-40). Peak fractions were pooled and gently mixed with 1 ml Glutathione-Sepharose 4B beads (GE Healthcare) in buffer K + 500 mM KCl for 45 min at 4°C. The beads were then washed with 20 ml of buffer K + 350 mM KCl. Proteins were eluted with 20 mM reduced glutathione in buffer K + 350 mM KCl. Elution fractions were pooled, 4-fold diluted with buffer K, and loaded onto 0.5 ml MonoS column. The proteins were eluted with 7.5 ml gradient of 150–700 mM KCl in buffer K. The peak fractions were pooled, concentrated, frozen in liquid nitrogen and stored at −80°C.

Expression and purification of proteins required for the sumoylation reaction, including GST-Aos1/Uba2, His-Ubc9, His-Flag-Smt3 (45), GST-Smt3 and untagged Smt3 (40), GST-Ubc9, His-Siz1 (1–465) and His-Siz2 (41) have been characterized previously. In the case of His-Siz2 purification, elution of the protein from Ni-NTA agarose was followed by fractionation in a 1 ml heparin column, using a 10 ml gradient of 100–1000 mM KCl in buffer K.

Untagged PCNA and PCNA-K164R proteins were purified as described (42). All purified proteins were concentrated to 1–5 µg/µl and stored in small aliquots at −80°C. In the following text, the tags of purified proteins are not indicated for simplicity, except in the cases of GST-Smt3, GST-Ubc9 and GST-Srs2 (883–1174), where

the GST tags are specified to differentiate them from their His-tagged analogues.

In vitro sumoylation assay

The Srs2 sumoylation assay was performed in a 10 µl reaction volume containing 0.35 µM Aos1/Uba2 proteins, 1.25 µM Ubc9 protein, 1.6 µM Smt3 protein, 0.75 µM Srs2 protein or its mutants, 100 µM ATP, 150–300 mM KCl and buffer S2 (50 mM HEPES, 10 mM $MgCl_2$). In the indicated cases, 4–300 nM Siz1 (1–465), 12–300 nM Siz2, 0.5–3.2 µM Smt3-PCNA or PCNA-K164R were added to the reaction. Reactions were incubated for 1 h at 4°C, stopped by adding 10 µl of SDS Laemmli buffer (62.5 mM Tris–HCl, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue) and 10 µl of the mixture was analyzed by SDS–PAGE on a 10% gel. Proteins were visualized by staining with Coomassie Blue or by western blotting using anti-Srs2 or anti-Smt3 antibodies.

PCNA sumoylation was carried out in a 150 µl reaction volume containing 0.5 µM Aos1/Uba2 proteins, 5 µM Ubc9 protein, 25 µM untagged Smt3 protein, 2 µM untagged Siz1 (1–465) protein, 20 µM untagged PCNA protein, 1 mM ATP, 250 mM KCl and buffer S2 for 2 h at 30°C. The reaction mixture containing sumoylated PCNA was stored in small aliquots at −80°C.

Pull-down assays

To study Srs2 interaction with Ubc9 and Smt3, purified Srs2 or Srs2ΔSIM (1.25 µM) was incubated with GST-Ubc9 or GST-Smt3 (4 µM) and 10 µl of Glutathione Sepharose 4 Fast Flow (GE Healthcare) in 30 µl of buffer S2 containing 100 mM KCl, for 30 min at RT, with gentle mixing. After incubation, the supernatants were collected and mixed with 30 µl of SDS Laemmli buffer. The beads were washed with 100 µl of the same buffer before being treated with 20 µl of SDS Laemmli buffer to elute the bound proteins. The supernatant, wash and SDS eluate (10 µl each) were analyzed by SDS–PAGE on a 10% gel followed by Coomassie Blue staining. For the pull-down experiments with Srs2 and E3 SUMO ligases, GST-Srs2 (883–1174) (2 µM) was mixed with Siz1 (1–465) (2 µM) or Siz2 (0.6 µM) protein together with 10 µl Glutathione Sepharose 4B (GE Healthcare) in 40 µl of buffer S2 containing 100 mM KCl. Incubation and analysis were performed as described earlier. To study interaction between Srs2 and PCNA, His-tagged Srs2 (1 µM) or its indicated mutant variants were incubated with PCNA (3.5 µM) or Smt3-PCNA (1 µM) and 10 µl of Profinity IMAC Ni-Charged Resin (Bio-Rad) in 40 µl of buffer S2 containing 150 mM KCl. Incubation and analysis were performed as discussed earlier. PCNA and Smt3-PCNA were visualized by western blotting using anti-PCNA antibodies.

Yeast two-hybrid analysis

Yeast two-hybrid analysis was essentially done as described previously (46). The plasmids UBC9::pGAD-C1, SMT3::pGAD-C1, containing *UBC9* and *SMT3* fused to the *GAL4* transcription activation domain, were transformed into the haploid *S. cerevisiae* strain PJ69-4a (MATa). Plasmids SRS2 (783–1174)::GBKT7, SRS2 (783–1169)::pGBKT7, which contained *SRS2* fused to the *GAL4* DNA-binding domain, were introduced into PJ69-4α strain (MATα). Diploid strains were grown to OD_{600} ~1- and 10-fold serially diluted. Activation of the *HIS3* reporter gene was analyzed on medium lacking leucine, tryptophan and histidine. Cells were grown for 3 days at 30°C before analysis.

DNA damage sensitivity assay

Strains FF1852, FF1886, FF18238, D83-5B, yLK92, yLK93 and yLK94 were grown in YPD to OD_{600} ~1- and 10-fold serially diluted. DNA damage sensitivity was assessed on YPD plates, in the absence or presence of 0.00005%, or 0.0005% methyl methane sulfonate (MMS). Pictures of the plates were taken after 2 days of incubation at 30°C.

Detection of Srs2 sumoylation *in vivo*

Protein extracts and immunoprecipitates were prepared essentially as described (47). In brief, cells were disrupted by bead beating under denaturing conditions and diluted protein extracts were immunoprecipitated using either IgG-Sepharose to pull down TAP-tagged Srs2 or Protein G-agarose plus anti-Srs2 antibody to pull down untagged Srs2. Immunoprecipitated proteins were washed and eluted with loading dye before separating by SDS-PAGE and immunoblotting with anti-Smt3 (SUMO) (34), PAP against TAP, or anti-Srs2 antibody (12).

RESULTS

Srs2 is sumoylated at lysines K1081, 1089 and 1142 both *in vitro* and *in vivo*

Previous studies have shown that Srs2 is sumoylated *in vivo* and its sumoylation is stimulated in response to DNA damage [(23) and Supplementary Figure S1]. To define the biochemical requirements for this reaction, we set up an *in vitro* sumoylation assay using purified yeast Aos1, Uba2, Ubc9 and Smt3 proteins. Addition of Srs2 protein to this reaction resulted in its modification by SUMO (Smt3), as confirmed by immunoblotting with both anti-SUMO and anti-Srs2 antibodies (Figure 1A).

We have previously shown that Srs2 sumoylation is abolished when lysines K1081, 1089 and 1142 are mutated to arginines (23). To determine the individual contributions of these lysines to Srs2 sumoylation, we constructed and purified Srs2 mutants lacking one or two of these SUMO-conjugation sites. These mutants were sumoylated to a level comparable with wild-type Srs2 (Figure 1B). Only mutation of all three lysines abolished the sumoylation *in vitro* (Figure 1B, lanes 15 and 16). To examine the sumoylation status of the mutated Srs2 proteins *in vivo*, we tested wild-type and lysine mutant Srs2 expressed from centromeric plasmids under the control of the GAL1 promoter. Consistent with our *in vitro* data, only mutation of all three lysine residues

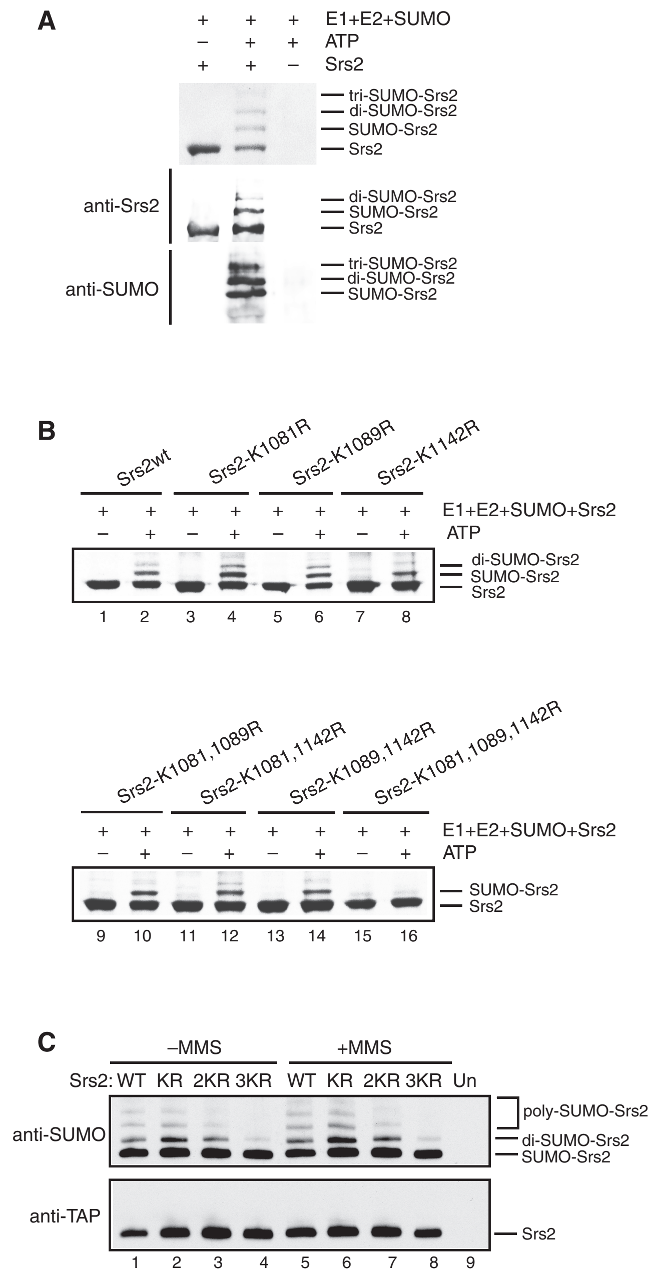

Figure 1. Srs2 is sumoylated at lysines K1081, 1089 and 1142. (**A**) Sumoylation of Srs2 *in vitro*. Sumoylation assay was performed using recombinant Aos1/Uba2 (0.35 µM), Ubc9 (1.25 µM) and SUMO (1.6 µM) in the presence or absence of ATP (100 µM) or Srs2 (0.75 µM) as indicated. Reactions were carried out in buffer S2 containing 300 mM KCl, incubated for 1 h at 4°C, stopped by adding SDS Laemmli buffer and analyzed by 10% SDS–PAGE, followed by staining with Coomassie Blue (top). Sumoylated Srs2 protein was confirmed by western blotting using anti-Srs2 (middle) or anti-SUMO (bottom) antibodies. Markers on the side of the gel indicate positions of unmodified, mono-, di- and tri-sumoylated Srs2. (**B**) Sumoylation of Srs2 lysine mutants. *In vitro* sumoylation assay of Srs2 or its various mutants was performed as in (A) in buffer containing 150 mM KCl. Only the relevant portions of the Coomassie-stained gels are shown for clarity. (**C**) Sumoylation of Srs2 lysine mutants *in vivo*. Yeast strains expressing TAP-tagged Srs2 or Srs2 mutants (Srs2-K1142R, Srs2-K1081, 1142R, Srs2-K1081, 1089, 1142R) from the *GAL1* promoter were grown in the absence or presence of 0.3% MMS for 2 h, followed by immunoprecipitation and western blotting for SUMO (above) and TAP (below). Strain containing untagged Srs2 is included as negative control.

(K1081R, K1089R, K1142R) decreased Srs2 sumoylation to almost undetectable level (Figure 1C). These data demonstrate that all three lysine residues—K1081, 1089 and 1142, represent major sumoylation sites both *in vitro* and *in vivo*.

Srs2 sumoylation is stimulated by Siz1 and Siz2 SUMO E3s

To define the enzymatic requirements for Srs2 sumoylation, we assessed the role of the E3 SUMO ligases. Though sumoylation of Srs2 can occur in the absence of a SUMO ligase *in vitro* (Figure 1), it is promoted by the ligases Siz1 or Siz2 (Figure 2A). Moreover, Siz1 and Siz2 target the same three lysines of Srs2 described for E3-independent sumoylation (Supplementary Figure S2). We also tested the requirement for the ligases *in vivo* by analyzing the sumoylation of endogenous Srs2 protein in *siz1*Δ *siz2*Δ cells. Correlating with our *in vitro* findings, the level of Srs2 sumoylation in these mutant cells was decreased (Figure 2B). To address the mechanism of E3 action, we performed pull-down experiments using purified GST-Srs2, Siz1 and Siz2 proteins. When applied to GTH-beads, GST-Srs2 was able to retain both Siz1 and Siz2, indicating a direct interaction between the Siz proteins and Srs2 (Supplementary Figure S3). In summary, Srs2 sumoylation is stimulated by the action of Siz1 and Siz2 ligases, but they are not indispensable for Srs2 sumoylation.

SUMO E3-independent sumoylation of Srs2 requires its SIM

Because Srs2 sumoylation can occur even in the absence of the Siz ligases, we wished to understand the mechanism of Siz-independent sumoylation by investigating Srs2 interactions with SUMO and Ubc9, which have been reported previously using yeast two-hybrid analysis (14). In pull-down experiments, GST-SUMO-, but not GST-Ubc9-, bound beads were able to retain Srs2 (Figure 3A, lane 2; Figure 3B, lane 3), suggesting that Srs2 interacts with Ubc9 via SUMO. Indeed, SUMO-modified Ubc9 gained the ability to interact with Srs2 (Figure 3C). The interaction between Srs2 and SUMO has been suggested to be mediated by a SIM motif comprising the last five amino acids of the protein. Supporting this notion, deletion of this motif abolished the Srs2–SUMO and Srs2–SUMO-Ubc9 interactions (Figure 3A, lane 6; Figure 3C, lane 6). Interestingly, the SIM domain was also required for Srs2 interaction with SUMO and Ubc9 in the yeast two-hybrid system (Figure 3D). To investigate the importance of the SIM-mediated Srs2-SUMO interaction for Srs2 sumoylation, we purified Srs2 protein lacking the SIM motif. Sumoylation of Srs2ΔSIM was abolished *in vitro* (Figure 3E). Consistent with this result, sumoylation of Srs2ΔSIM was also dramatically decreased *in vivo* (Figure 3F). Taken together, these data show that the SIM motif is required for Srs2 sumoylation.

The SIM motif could contribute to Srs2 sumoylation by interacting either with SUMO-charged Ubc9 or sumoylated Ubc9. SUMO can be covalently linked to

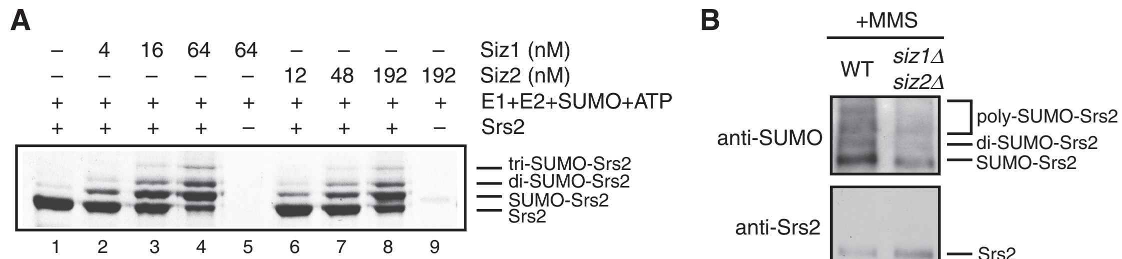

Figure 2. Srs2 sumoylation is stimulated by the SUMO E3 ligases Siz1 and Siz2. (**A**) Standard *in vitro* sumoylation assay was performed using Aos1/Uba2 (0.35 µM), Ubc9 (0.3 µM), SUMO (1.6 µM), Srs2 (0.75 µM) and ATP (100 µM) in the absence or presence of increasing amounts of Siz1 (1–465) or Siz2 as indicated. Reactions containing 300 mM KCl were incubated for 1 h at 4°C and analyzed by 10% SDS–PAGE, followed by Coomassie Blue staining. (**B**) *In vivo* requirement for SUMO E3 ligase. Endogenous Srs2 from MMS-treated wild-type or *siz1*Δ *siz2*Δ cells was immunoprecipitated using anti-Srs2 antibody and western blotted for SUMO (above) and Srs2 (below).

Ubc9 by a thioester linkage through cysteine C93 of Ubc9 (SUMO-charged Ubc9), or by isopeptide linkages on lysines K157 or K153 via auto-sumoylation (30,37,48). We found that Ubc9-K153, 157R did not affect Srs2 sumoylation *in vitro*, suggesting that auto-sumoylation of Ubc9 is not required for Srs2 sumoylation (Supplementary Figure S4). In summary, the SIM motif likely promotes Srs2 sumoylation by interacting with SUMO-charged Ubc9.

Srs2 sumoylation is inhibited by SUMO-PCNA *in vitro*

The anti-recombinogenic function of Srs2 at stalled forks is mediated by its binding to sumoylated PCNA. As this interaction also requires the Srs2 SIM domain, we examined the effect of PCNA and SUMO-PCNA on Srs2 sumoylation. To this end, SUMO-PCNA or its non-sumoylatable mutant (PCNA-K164R) was added to the Srs2 sumoylation reaction. Inclusion of SUMO-PCNA resulted in a strong decrease in Srs2 sumoylation (Figure 4A, lanes 2–4), while unmodified PCNA had a very slight effect only when present in excess of Srs2 (Figure 4A, lanes 8–10). The inhibitory effect of SUMO-PCNA on Srs2 sumoylation may be explained by the competition with SUMO-charged Ubc9 for SIM binding. To test this hypothesis, we asked if sumoylated PCNA could outcompete SUMO in Srs2 binding. We found that addition of sub-equimolar amounts of SUMO-PCNA completely disrupted the interaction between Srs2 and GST-SUMO (Figure 4B), supporting the notion that SUMO-PCNA inhibits Srs2 sumoylation by competing with SUMO-charged Ubc9 for SIM binding. We also addressed this question *in vivo* by analyzing the sumoylation status of endogenous Srs2 protein in a non-sumoylatable PCNA mutant (*pol30-K127, 164R*). Although MMS-induced poly-sumoylated Srs2 levels were higher in *pol30-K127, 164R* than in wild-type cells, mono-sumoylated levels were lower (Figure 4C). Since Srs2 is not properly localized at replication forks in *pol30-K127, 164R* cells and the PRR repair pathway is blocked, the *in vivo* sumoylation result may reflect the combined effect of these factors, as well as the direct inhibitory effect of SUMO-PCNA.

As Srs2 sumoylation is induced by DNA damage, we tested potential mechanisms by which the inhibitory effect of sumoylated PCNA can be overcome in this situation. We first pre-incubated Srs2 with an excess of sumoylated PCNA to form a complex, and then analyzed the complex in the sumoylation reaction. We found that increasing amounts of SUMO and Siz1 can counteract the inhibitory effect of SUMO-PCNA on Srs2 sumoylation (Figure 4D). This suggests that a local increase in SUMO enzyme concentrations after DNA damage could overcome the inhibitory effect of sumoylated PCNA and result in Srs2 sumoylation.

Srs2 sumoylation inhibits its interaction with SUMO-PCNA and SUMO *in vitro*

As Srs2 is recruited to stalled replication forks via interaction with sumoylated PCNA, we asked whether Srs2 sumoylation affects this interaction. To ensure that the same quantities of unmodified and modified Srs2 protein were used for the experiment, we performed two identical sumoylation reactions, one in the presence of ATP, the other in its absence. Following the reaction, we tested both unmodified and sumoylated Srs2 for their ability to bind SUMO-PCNA. Interestingly, sumoylation of Srs2 decreased its affinity for SUMO-PCNA (Figure 5A). To examine whether this effect is PCNA specific, we next tested the effect of Srs2 sumoylation on its interaction with SUMO alone. We observed that Srs2 sumoylation also inhibits its interaction with SUMO (Figure 5B). These results suggest that Srs2 sumoylation could reduce the availability of its SIM motif for interaction with sumoylated proteins in general. It has been shown that lack of the interaction between Srs2 and SUMO-PCNA can suppress the MMS sensitivity of *rad18Δ* cells (14,15). Therefore, we tested whether defective Srs2 sumoylation has an opposite effect. Unlike *srs2Δ, srs2–3KR* did not affect the *rad18Δ* MMS sensitivity (Supplementary Figure S5), suggesting that Srs2 sumo-deficient protein is sufficient for the function *in vivo*.

Srs2 interacts with SUMO-PCNA via two sites

Our results showed that sumoylated PCNA binds to Srs2 with higher affinity than SUMO alone (Figure 4A and B), consistent with previous results. This indicates the existence of a second, PCNA-specific interaction site. The observation that Srs2 also directly interacts with unmodified

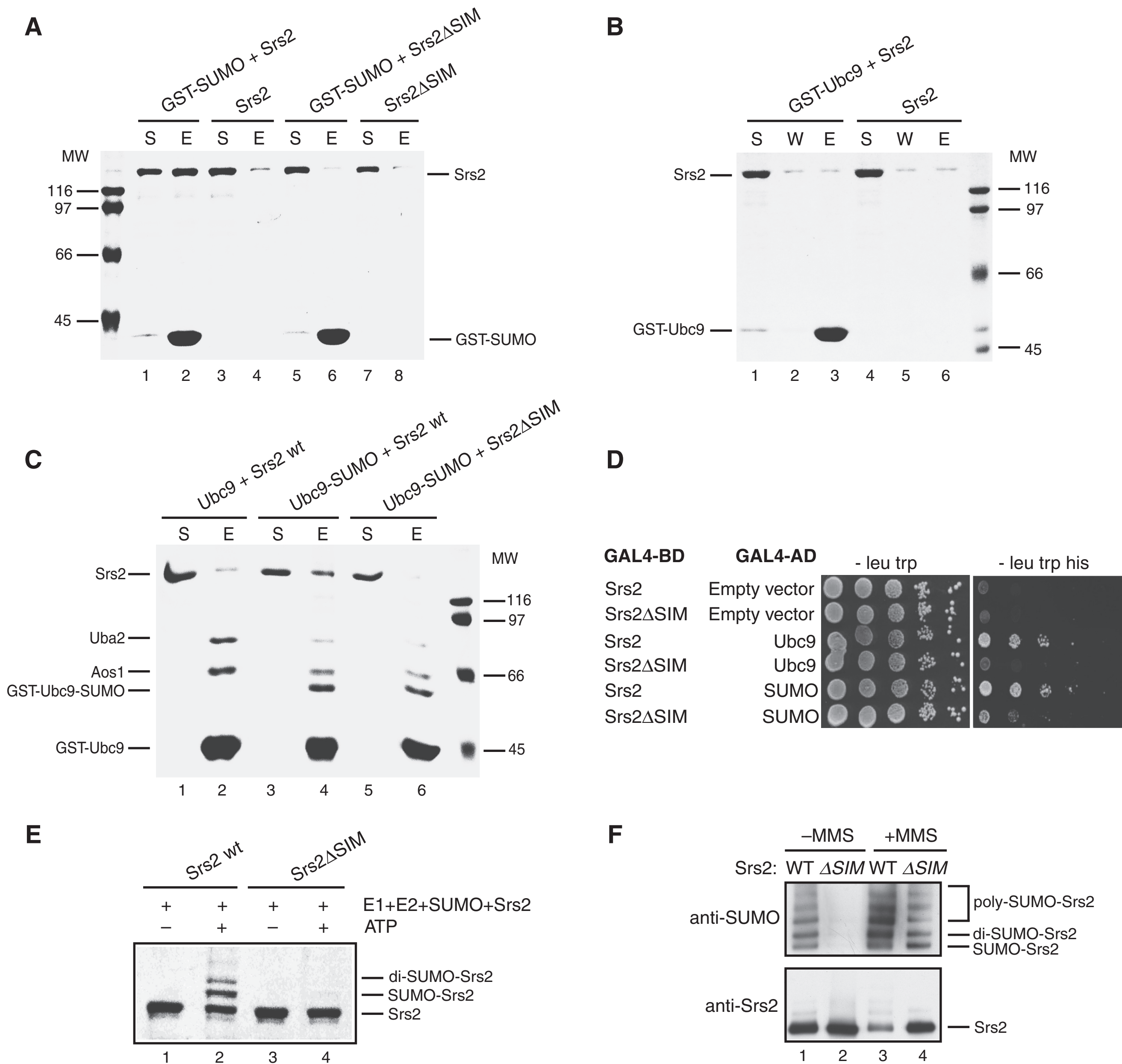

Figure 3. The interaction between the Srs2 SIM motif and SUMO is necessary for Srs2 sumoylation. (**A**) SUMO interacts with the Srs2 SIM motif. Purified GST-SUMO (2 µM, lanes 1, 2 and 5, 6) was incubated with Srs2 (0.6 µM, lanes 1–4) or its mutant lacking the SIM motif—Srs2ΔSIM (0.6 µM, lanes 5–8) and GTH-Sepharose in buffer S2 containing 100 mM KCl for 30 min at RT. The beads were washed and treated with SDS Laemmli buffer to elute the bound proteins. The supernatant (S) containing unbound Srs2 protein, and the SDS eluate (E) (10 µl each) were analyzed by 10% SDS–PAGE followed by staining with Coomassie Blue. Reactions containing only GTH-Sepharose and Srs2 (lanes 3 and 4) or Srs2ΔSIM (lanes 7 and 8) were included as negative control. Numbers on the left side of the gel indicate molecular weights (in kDa) of protein standards. (**B**) Ubc9 does not interact with Srs2. Interaction between purified GST-Ubc9 (4 µM, lanes 1–3) and Srs2 (1.25 µM) was analyzed as in (A). (**C**) SUMO modification of Ubc9 triggers its interaction with Srs2. GST-Ubc9 (4 µM, lanes 1–2) or GST-Ubc9-SUMO (4 µM, lanes 3–6), prepared by sumoylation reaction in the absence or presence of ATP, was mixed with Srs2 (1.25 µM, lanes 1–4) or Srs2ΔSIM (1.25 µM, lanes 5–6) and analyzed as in (A), except β-mercaptoethanol was excluded from the Laemmli buffer to prevent denaturation of SUMO-charged Ubc9. (**D**) Yeast two-hybrid interaction of Ubc9 and SUMO with Srs2 is mediated by its SIM motif. Strain PJ69-4 containing *UBC9* or *SUMO* fused to the GAL4 transcription activation domain and *SRS2 (aa 783–1174)* or *SRS2ΔSIM (a.a. 783–1169)* fused to the GAL4 DNA-binding domain, were spotted as 10-fold serial dilutions on medium lacking leucine and tryptophan or leucine, tryptophan and histidine. The empty vector (pGADT7) was included as negative control. (**E**) Srs2 SIM motif is necessary for Srs2 sumoylation *in vitro*. The standard *in vitro* sumoylation reaction was done with wild-type Srs2 (lanes 1 and 2) or Srs2ΔSIM (lanes 3 and 4) in buffer S2 containing 100 mM KCl. (**F**) *In vivo* sumoylation of Srs2 requires its SIM motif. Yeast cells, expressing His-tagged wild-type Srs2 or Srs2ΔSIM mutant under the copper-responsive *CUP1* promoter, were grown in the absence or presence of 0.3% MMS and immunoprecipitated using anti-Srs2 antibody. Western blotting was performed as in Figure 2B.

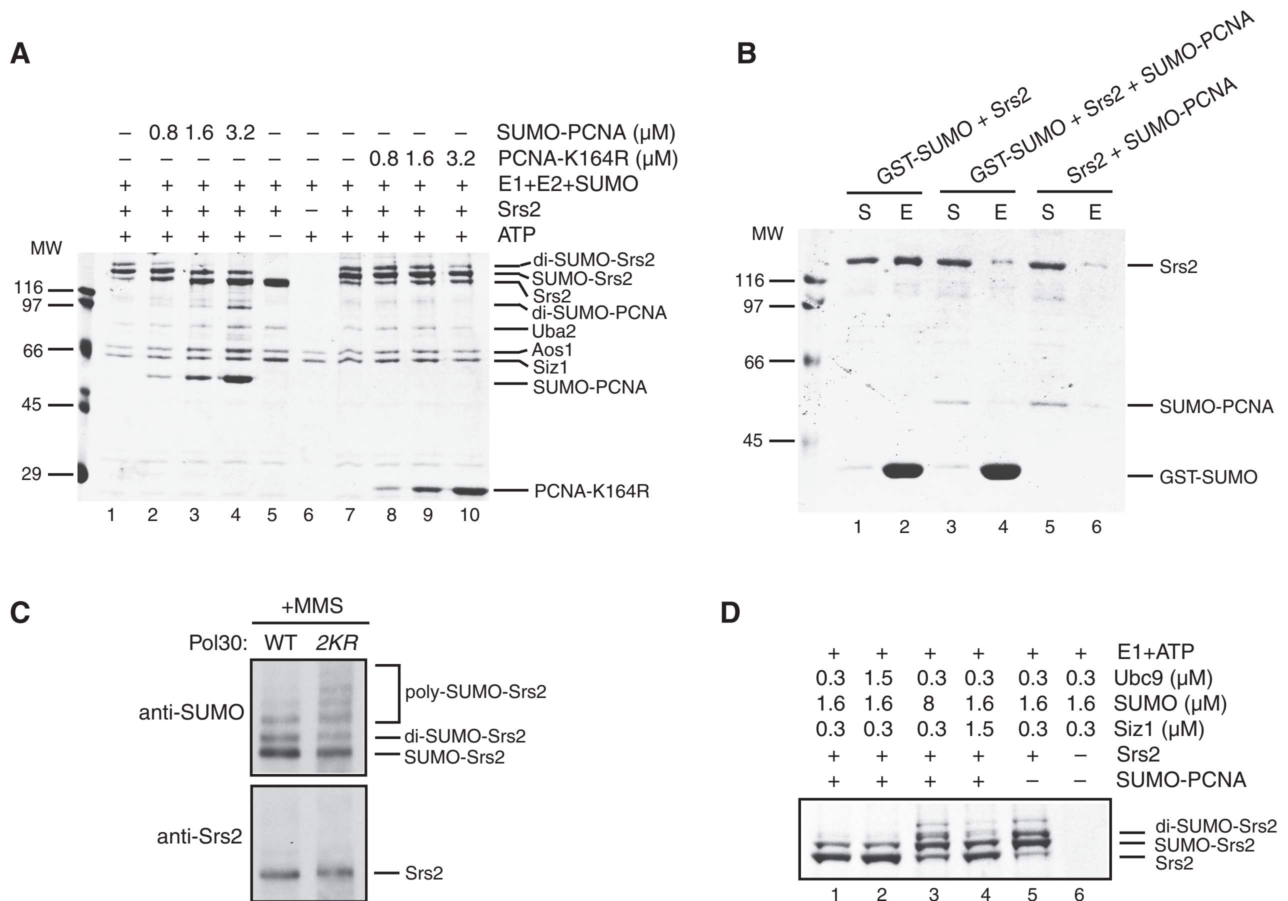

Figure 4. SUMO-PCNA inhibits Srs2 sumoylation by binding to the SIM of Srs2. (**A**) SUMO-PCNA inhibits Srs2 sumoylation *in vitro*. *In vitro* sumoylation assay was performed using Aos1/Uba2 (0.35 μM), Ubc9 (1.25 μM), SUMO (1.6 μM), Siz1 (1–465) (0.4 μM), Srs2 (0.75 μM) and ATP (100 μM) in the absence or presence of increasing amounts of SUMO-PCNA (0.8, 1.6, 3.2 μM, lanes 2–4) or PCNA-K164R (lanes 8–10). The reactions were stopped, resolved on 10% SDS-PAGE gel and stained with Coomassie Blue. (**B**) SUMO-PCNA outcompetes SUMO in Srs2 binding. Pull-down experiments using purified GST-SUMO (2 μM, lanes 1–4) and Srs2 (0.6 μM) in the absence (lanes 1 and 2) or presence of SUMO-PCNA (0.6 μM, lanes 3–6) were performed as in Figure 3. (**C**) Lack of PCNA sumoylation alters the Srs2 sumoylation profile *in vivo*. Endogenous Srs2 from wild-type or *pol30-K127, 164R* yeast strains treated with 0.3% MMS was analyzed as in Figure 2B. (**D**) The inhibitory effect of SUMO-PCNA on Srs2 sumoylation can be overcome by increasing amounts of SUMO and Siz1. Srs2 (0.75 μM) was pre-incubated with SUMO-PCNA (1.5 μM, lanes 1–4) for 15 min at RT, after which the sumoylation assay was performed using Aos1/Uba2 (0.35 μM), indicated amounts of Ubc9, SUMO and Siz1(1–465) in buffer containing 100 μM ATP and 300 mM KCl. Reactions were stopped and analyzed by 10% SDS-PAGE, followed by Coomassie Blue staining.

PCNA [Figure 6B, lane 2, (15)] motivated us to map the Srs2 domain responsible for this interaction. As deletion of the last 22 amino acids of Srs2 (Srs2 1–1152) abolished the PCNA interaction (Supplementary Figure S6, lane 4), we constructed and purified Srs2 mutant proteins lacking the last 5, 11 and 16 amino acids (Figure 6A), respectively. We observed that deletion of the last 5 or 11 amino acids had no effect on PCNA interaction; however, deletion of the last 16 amino acids (Srs2 1–1158) disrupted the interaction, suggesting that amino acids 1159–1163 likely constitute a PCNA binding site (Figure 6B). Indeed, deletion of these five amino acids abolished Srs2-PCNA binding (Figure 6B, lane 10, summarized in Figure 6C). We designate this site PCNA-interacting motif (PIM). This region contains three consecutive lysine residues (K1160, 1161 and 1162), mutation of which significantly decreased PCNA binding, indicating that these lysines are critical for PCNA interaction (Supplementary Figure S6, lane 8).

Next, we tested the aforementioned Srs2 mutants for interaction with sumoylated PCNA. We found that ΔSIM or ΔPIM each decreased SUMO-PCNA binding, and deletion of both abolished the interaction completely (Figure 6D, summarized in Figure 6E). This is in good correlation with our results that SUMO or PCNA alone is able to interact with Srs2 *in vitro* (Figures 3A and 6B). To understand how the PIM affects Srs2 sumoylation, we asked whether sumoylated PCNA is still able to inhibit Srs2 sumoylation in the absence of this motif. Notably, though sumoylated PCNA efficiently inhibited sumoylation of wild-type Srs2, it was unable to inhibit that of the mutant (Figure 6F, lanes 8–10). This observation further supports our conclusion that SUMO-PCNA outcompetes SUMO-Ubc9 in Srs2 binding due to a second, PCNA-specific interaction site. We also examined the importance of the Srs2 PIM motif *in vivo* by assessing its ability to suppress the sensitivity of *rad18Δ*

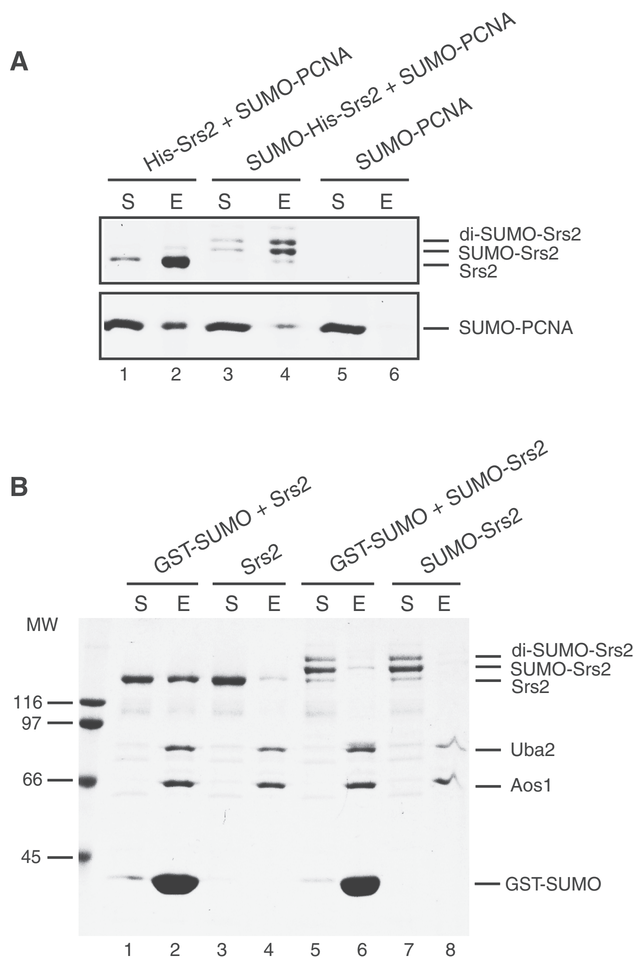

Figure 5. Sumoylation of Srs2 inhibits its interaction with SUMO-PCNA and SUMO *in vitro*. (**A**) Sumoylation of Srs2 inhibits its interaction with SUMO-PCNA. His-tagged Srs2 (1.5 µM, lanes 1 and 2) or SUMO-Srs2 (1.5 µM, lanes 3 and 4), prepared by sumoylation reaction using untagged SUMO and Siz1 proteins, was mixed with SUMO-PCNA (1.5 µM) and Ni-charged resin. The beads were washed and treated with SDS Laemmli buffer to elute the bound proteins. The supernatant (S) containing unbound Srs2 protein and the SDS eluate (E) (10 µl each) were analyzed by 10% SDS–PAGE followed by Coomassie Blue staining. Sumoylation reaction in the absence of Srs2 was mixed with Ni-charged resin as a negative control (lanes 5 and 6). (**B**) Sumoylation of Srs2 inhibits its interaction with SUMO. Srs2 (1.5 µM, lanes 1–4) or SUMO-Srs2 (1.5 µM, lanes 5–8), prepared by sumoylation reaction in the absence or presence of ATP, was mixed with GST-SUMO (1.5 µM, lanes 1, 2, 5 and 6) or without it (lanes 3, 4, 7 and 8) and pulled-down on GTH-Sepharose beads. The analysis was performed as in (A).

cells to the alkylating agent MMS. Interestingly, *srs2ΔPIM* was able to suppress the sensitivity, similar to *srs2Δ* and *srs2ΔSIM* (Figure 6G), indicating that the PIM mediates PCNA-interaction *in vivo*. These data show that SUMO-PCNA interacts with Srs2 through two interaction sites, one SUMO-specific and the other PCNA-specific, and both are necessary for efficient function *in vivo*.

DISCUSSION

Modification by SUMO has emerged as an important regulator of DNA repair processes (49–51). Increasing number of proteins involved in HR have been shown to undergo sumoylation, and more prominently so after DNA damage, suggesting a direct role for sumoylation in the DNA damage response (49,52,53). A better understanding of the function of sumoylation in HR regulation requires detailed study of the effects of sumoylation on each substrate. Here, we report the biochemical study of sumoylation of Srs2, an important, multi-functional helicase involved in the regulation of DNA repair on several levels.

Mechanism of Srs2 sumoylation

Both *in vitro* and *in vivo* results suggest that the three consensus sumoylation sites in the C-terminus of Srs2 (K1081, 1089, 1142) are targeted for modification, because mutating these lysines resulted in the disappearance of two sumoylated Srs2 forms and a strong reduction in the intensity of the lowest SUMO-Srs2 band (Figure 1). The presence of trace amounts of the lowest SUMO-Srs2 band suggests that one more lysine could be targeted for modification. The appearance of slow-migrating sumoylated forms of Srs2 (Figures 1C, 3F and 4C) probably reflects the attachment of more than one SUMO peptide to the lysine residue. We have addressed the requirement of two SUMO E3 ligases, Siz1 and Siz2, for Srs2 sumoylation (Figure 2). E3 SUMO ligases are thought to act as adaptors that enhance the interaction between Ubc9 and substrates, and the SP-RING domains of Siz1 and Siz2 bind directly to Ubc9 (40,41,54–56). Here, we show that Siz proteins bind directly to the substrate Srs2 (Supplementary Figure S3), thus providing evidence for a direct physical interaction between E3s and substrate and supporting their proposed role as adaptors. Our *in vitro* and *in vivo* results show that Siz1 and Siz2 function redundantly in sumoylating Srs2, consistent with and extending previous findings (23,57). However, our data also reveal Siz-independent sumoylation of Srs2, which we show is mediated directly by Ubc9 (Figure 1, summarized in Figure 7).

Although Srs2 does not bind Ubc9, it binds to SUMO via its SIM domain (Figure 3). The failure to detect SUMO-Srs2 binding in a previous report is possibly due to the low affinity of Srs2 for SUMO (15). Our pull-down experiments showed that when Ubc9 is attached to SUMO, it gains the ability to interact with Srs2 via the SIM. Indeed, sumoylation of the Srs2ΔSIM mutant is compromised both *in vitro* and *in vivo*. Our work in yeast is consistent with findings of SIM-dependent sumoylation of mammalian proteins and suggests that this is a conserved mechanism of sumoylation (27–31).

Srs2 interacts with sumoylated PCNA and this interaction is required for its localization to the replication fork (14,15). As the SIM of Srs2 is necessary for its interaction with SUMO-PCNA (16) as well as its sumoylation, we tested the effect of SUMO-PCNA on Srs2 sumoylation. We found that sumoylated PCNA inhibits Srs2 sumoylation *in vitro* by outcompeting SUMO-Ubc9 binding (Figure 4). We speculate that SUMO-PCNA blocks Srs2 sumoylation under normal conditions. However, DNA damage may lead to an increase in the

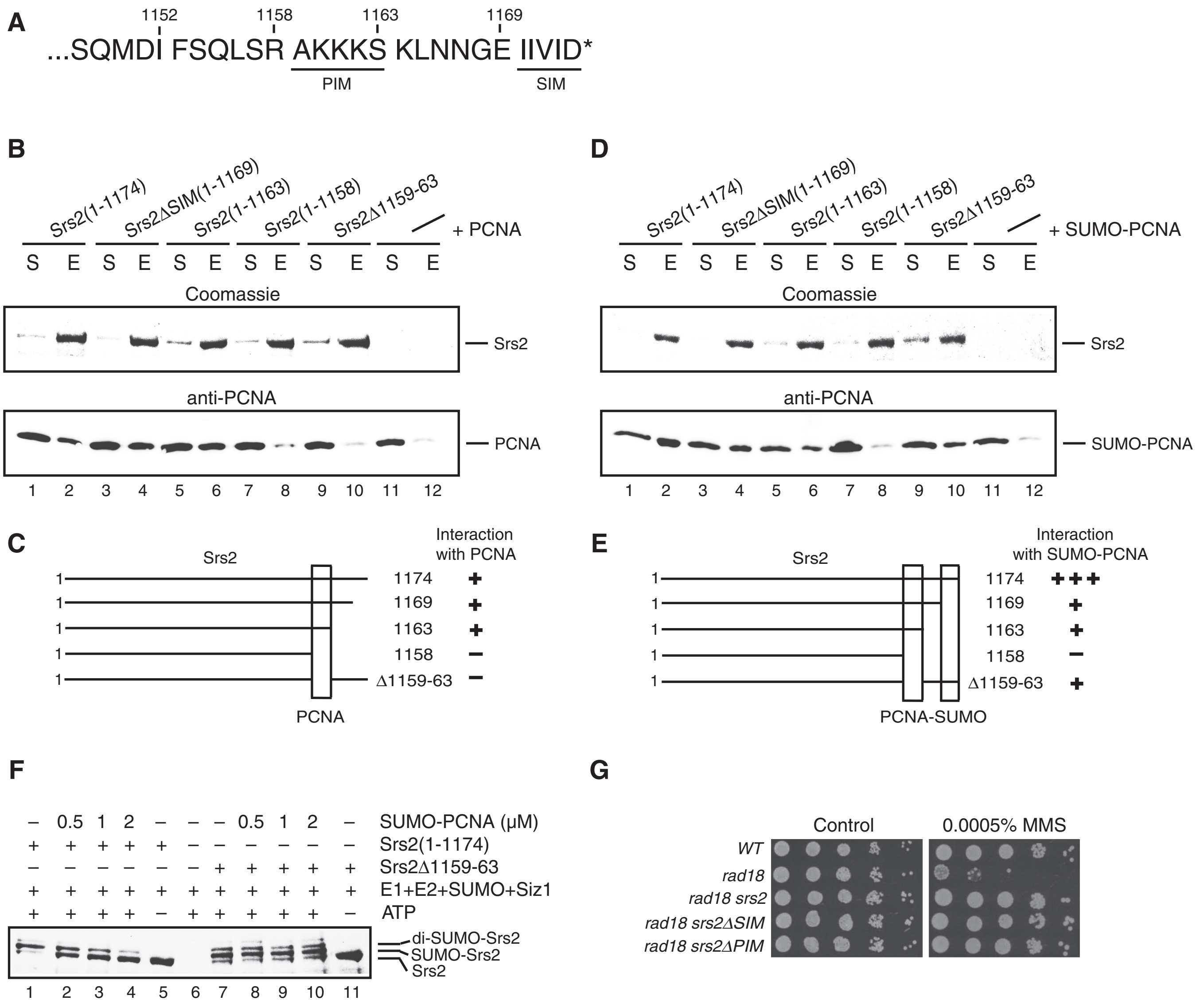

Figure 6. Srs2 contains a PCNA-specific interaction motif. (**A**) Amino acid sequence of the C-terminus of Srs2 (a.a. 1148–1174). (**B**) PIM is localized between amino acids 1159 and 1163 of Srs2. His-tagged Srs2 (1 μM, lanes 1 and 2) or the indicated deletion mutants were incubated with PCNA (3.5 μM) and captured on Ni-charged resin. The beads were washed and treated with 20 μl of SDS Laemmli buffer. The supernatant (S) and the SDS eluate (E) were analyzed by 10% SDS–PAGE followed by staining with Coomassie Blue or by western blotting using anti-PCNA antibodies. Reaction containing only PCNA with Ni-charged resin was included as negative control (lanes 11 and 12). Results of the interaction mapping are summarized in (**C**). (**D**) Srs2 interacts with SUMO-PCNA via two interaction sites. Interaction between Srs2 and its mutants with SUMO-PCNA was analyzed as in (B). Results are summarized in (**E**). (**F**) SUMO-PCNA does not inhibit sumoylation of Srs2ΔPIM. Effect of SUMO-PCNA on sumoylation of Srs2 (lanes 2–4) and Srs2ΔPIM (lanes 8–10) was assessed as in Figure 4A. Reactions were stopped, analyzed on 10% SDS-PAGE gel and stained with Coomassie Blue. (**G**) *Srs2ΔPIM* suppresses the MMS sensitivity of *rad18Δ* cells. Indicated strains were spotted as 10-fold serial dilutions on YPD plates in the absence or presence of 0.0005% MMS.

local concentration of sumoylation enzymes, which could overcome the inhibitory effect of sumoylated PCNA, a situation we observed *in vitro* (Figure 4D).

Consequences of Srs2 sumoylation

We show that sumoylation of Srs2 leads to decreased affinity for sumoylated PCNA and SUMO *in vitro* (Figure 5). We envision two possible biological roles for Srs2 sumoylation. First, Srs2 sumoylation may sequester its SIM by intra-, or intermolecular SIM–SUMO interactions that lead to self-association or dimerization of sumoylated Srs2 molecules. Intra-molecular SIM–SUMO binding has previously been reported for thymine DNA glycosylase, where sumoylation triggers a conformational change mediated by an internal SIM–SUMO interaction (58,59). Similar scenarios have also been described for ubiquitination (60). Thus, sumoylation of Srs2 may release it from complexes with sumoylated proteins or prevent such interactions by engaging its SIM in intra- or intermolecular SUMO–SIM binding. Second, sumoylation of Srs2 may stimulate its interactions

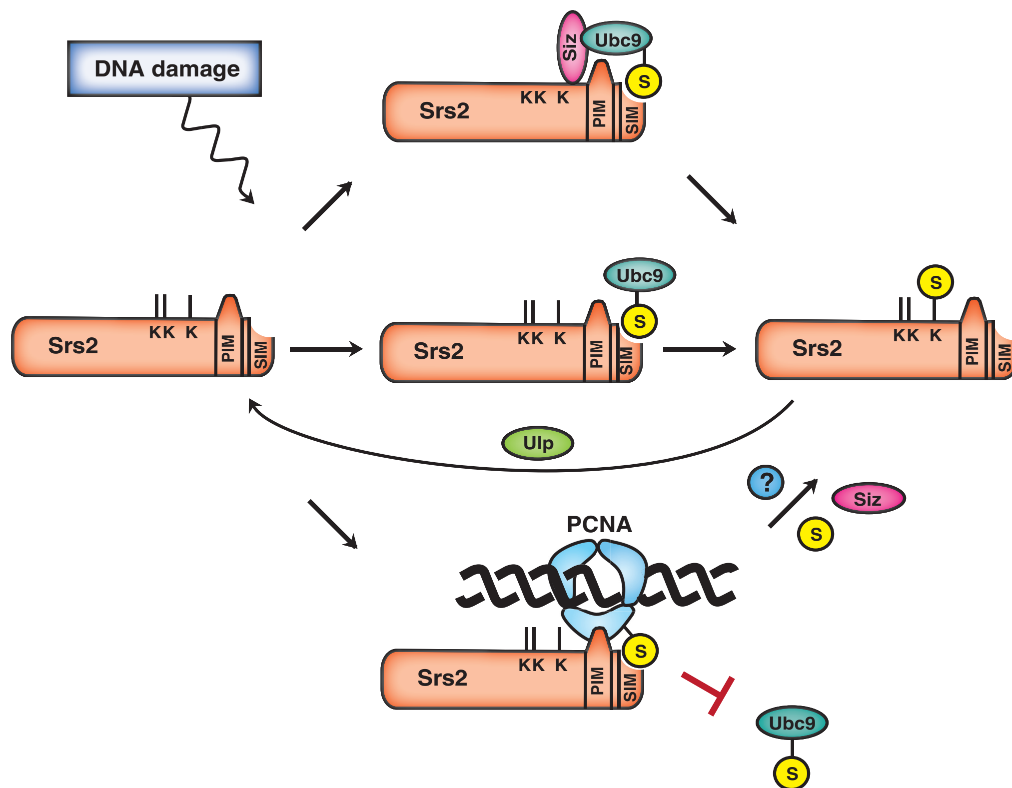

Figure 7. Model depicting the described features of Srs2 sumoylation. Srs2 sumoylation is induced by DNA damage and requires interaction between Srs2's SIM motif and SUMO. Sumoylation can proceed with or without the stimulatory effect of the Siz proteins. Srs2 can be recruited to the replication fork by sumoylated PCNA, with which it interacts via two sites, one SUMO-specific and the other PCNA-specific. SUMO-PCNA inhibits Srs2 sumoylation; however, this inhibitory effect might be overcome by local increase in SUMO enzyme concentrations.

with other SIM-containing proteins. Srs2 sumoylation appears to occur in response to DNA damage; therefore, it may play a role in the assembly of protein complexes designated for DNA repair, as has been suggested earlier (50). Such complexes may be stabilized by multiple SUMO–SIM interactions, in a manner similar to the formation of PML-nuclear bodies (61). Consistent with this idea, replication-coupled Srs2 foci are not detectable in an Srs2 mutant that lacks the SIM (16), illustrating the importance for damage-induced foci formation.

Srs2–PCNA interaction

We show that Srs2 interacts with sumoylated PCNA via two interaction sites, one SUMO-specific and the other PCNA-specific (Figure 6). Either site is able to mediate interaction that can be observed *in vitro*. However, the suppression of *rad18Δ* DNA damage sensitivity in the *srs2ΔSIM* and *srs2ΔPIM* mutants suggests that neither of these two interaction sites is sufficient to mediate efficient interaction in the cell. This is consistent with the finding that Srs2ΔSIM is sufficient to disrupt the interaction with SUMO-PCNA *in vivo* (16).

During peer review of this work, a structural study of the SUMO-PCNA—Srs2 complex was published (62). Consistent with our interpretation, Armstrong *et al.* also identified a PCNA-specific binding site within Srs2 and showed that both this site and the SIM motif are required for efficient interaction with SUMO-PCNA. However, the PCNA-interacting sites reported by us and Armstrong *et al.* differ slightly. The Srs2 PIP-like motif described by Armstrong *et al.* comprises amino acids 1148–1161, with I1152-L1156 being the most important. Our data suggest that the basic amino acids (lysines 1160, 1161 and 1162) are pivotal for this interaction, as their deletion suppresses the MMS sensitivity of *rad18Δ* to the same extent as *srs2Δ*.

Many proteins have been identified as targets for sumoylation, and many proteins contain SIMs. This observation raises an important question—how is the specificity between SUMO-modified proteins and their SIM-containing interactors achieved? Association between SUMO and a SIM motif is relatively weak (63,64), and to play a meaningful biological role, it needs to be stabilized by other interactions. These may be provided by yet another binding site between the sumoylation substrate and its SIM-containing partner. More importantly, it would also confer substrate specificity for SUMO–SIM interactions. Though this mode of interaction has been suggested previously (50,56,65), we provide direct evidence for this model by identifying a second, PCNA-specific binding site on Srs2 that is essential for efficient interaction between sumoylated PCNA and Srs2 *in vivo*. Identification and study of additional SUMO–SIM interacting partners will be needed to discover whether this is a common feature of SIM specificity.

Sumoylation of PCNA in S phase is conserved among eukaryotic species (7, 66–68). Although Srs2 homologs in higher eukaryotic cells have not been identified, the human protein PARI was recently shown to bind to PCNA analogous to Srs2 [i.e. via both PIM and SIM, (6)]. The consequences of the PARI-PCNA and Srs2-PCNA interactions are also similar, as PARI, like Srs2, inhibits HR and is required for genomic stability. However, it is not clear whether the mechanisms of HR inhibition conferred by these interactions are the same, because only Srs2 possesses ATPase activity. Future work will be needed to test how these mechanisms can contribute to HR regulation during replication in yeast and humans.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2, Supplementary Figures 1–6 and Supplementary References [69,70].

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