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Functional and Physical Interaction of Yeast Mgs1 with PCNA: Impact on *RAD6*-Dependent DNA Damage Tolerance

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Proliferating cell nuclear antigen (PCNA), a sliding clamp required for processive DNA synthesis, provides attachment sites for various other proteins that function in DNA replication, DNA repair, cell cycle progression and chromatin assembly. It has been shown that differential posttranslational modifications of PCNA by ubiquitin or SUMO play a pivotal role in controlling the choice of pathway for rescuing stalled replication forks. Here, we explored the roles of Mgs1 and PCNA in replication fork rescue. We provide evidence that Mgs1 physically associates with PCNA and that Mgs1 helps suppress the *RAD6* DNA damage tolerance pathway in the absence of exogenous DNA damage. We also show that PCNA sumoylation inhibits the growth of *mgs1 rad18* double mutants, in which PCNA sumoylation and the Srs2 DNA helicase coordinately prevent *RAD52*-dependent homologous recombination. The proposed roles for Mgs1, Srs2, and modified PCNA during replication arrest highlight the importance of modulating the *RAD6* and *RAD52* pathways to avoid genome instability.

Progression of the replication fork is often impeded by DNA lesions caused by exogenous or endogenous DNA-damaging agents. Replication forks also stall when they encounter tightly bound proteins or aberrant DNA structures (7). Stalled replication forks activate the DNA damage tolerance pathway, which promotes the reinitiation of DNA synthesis with or without removing the replication-blocking lesion.

Genetic studies in *Saccharomyces cerevisiae* indicate that Rad6 and Rad18 play central roles in DNA damage tolerance pathway. This pathway is mediated by the protein products of *RAD6*, *RAD18*, *RAD5*, *MMS2*, and *UBC13*, as well as several other gene products (4, 9, 22). Rad6 is a ubiquitin E2-conjugating enzyme that forms a stable complex with Rad18, an E3 ligase that binds DNA (2, 3). The Ubc13-Mms2 heterodimer is also an E2-conjugating enzyme (14). Rad5 is a DNA-dependent ATPase that functions as an E3 ligase and associates with the Ubc13-Mms2 complex, recruiting this complex to chromatin in response to DNA damage (27). Previous study demonstrated that PCNA is a substrate for *RAD6*-dependent ubiquitination (13). When DNA synthesis on one or both strands is arrested by DNA damage, PCNA is mono-ubiquitinated on lysine 164 (Lys164) by the Rad6-Rad18 complex. Mono-ubiquitinated PCNA may target stalled replication forks to initiate error-prone DNA repair via translesion DNA synthesis, a process that requires low-fidelity polymerases, Polζ or Polη, to synthesize across the damage (10, 15, 25, 29). Alternatively, the Ubc13-Mms2 complex and Rad5 modulate the polyubiquitination of Lys164 through lysine 63-linked ubiquitin chains. When modified in this manner, PCNA promotes error-free DNA repair (13, 25).

PCNA is also sumoylated at Lys164 (and secondarily at Lys127) via a mechanism dependent on a distinct E2-conjugating enzyme, Ubc9, and an E3 ligase, Siz1 (13). Although ubiquitination and sumoylation both occur on Lys164, there is evidence suggesting that the two modifications do not appear to have antagonistic effects and rather that they each have distinct roles. Indeed, PCNA is sumoylated during S phase, suggesting that SUMO modification may play a role in regulating DNA replication in the absence of DNA damage (13, 25). More recently, it has been demonstrated that Srs2 DNA helicase is recruited to the replication fork through sumoylated PCNA, where it facilitates tolerance to replication stalling by preventing unadvantageous recombination (20, 21).

The *S. cerevisiae* Mgs1 (for maintenance of genome stability 1) protein belongs to the AAA^+ class ATPase family and possesses DNA-dependent ATPase and single-stranded DNA annealing activity (11). *MGS1* orthologues are highly conserved from bacteria to human cells (11, 16). *mgs1* mutants are not hypersensitive to UV, methyl methanesulfonate (MMS), or hydroxyurea (HU), but they have a mutator and a hyperrecombination phenotype in the absence of exogenous DNA damage (6, 11). The *mgs1*Δ strain is synthetically lethal with *rad18*, but the lethality of *mgs1*Δ *rad18*Δ is suppressed by overexpression of *RAD52* or mutation in *SRS2* (12). These data suggest that Mgs1 is required to maintain genomic stability during DNA replication and that it may provide a functional link between DNA replication, recombination, and repair.

In the present study, we characterize the in vivo function of Mgs1, focusing on its interaction with PCNA. The results demonstrate that Mgs1 physically associates with PCNA and acts to prevent the *RAD6* DNA damage tolerance pathway in the absence of exogenous DNA damage. We also present evidence suggesting that Srs2 and PCNA sumoylation play a role in preventing *RAD52* recombination pathway, and this activity inhibits the growth of *mgs1 rad18*-deficient cells. Our data suggest that Mgs1-PCNA and PCNA-SUMO-Srs2 play a role in coordinating *RAD6* DNA damage tolerance and *RAD52* recombination pathways when DNA synthesis is arrested.

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype[a]	Source or reference
W1588-4A	W303-1A, *RAD5*	19
W1588-4B	W303-1B, *RAD5*	19
TH301	*MAT*α *mgs1*::*LEU2*	12
TH302	*MAT*α *mgs1*::*HIS3*	12
TH304	*MAT*α *mgs1*::*URA3*	12
C22	*MAT***a** *rad5*::*HIS3*	19
TH203	*MAT***a** *mgs1-18*	12
TH210	*MAT***a** *rad18*::*TRP1*	12
TH211	*MAT***a** *rad51*::*hisG-URA3-hisG*	12
TH221	*MAT***a** *mgs1-18 rad18*::*TRP1*	12
TH223	*MAT***a** *mgs1-18 rad18*::*TRP1 srs2*::*HIS3*	12
TH250	*MAT***a** *srs2*::*HIS3*	12
TH270	*MAT***a** *rev3*::*URA3*	19
TH271	*MAT*α *rad5*::*HIS3 rev3*::*URA3*	This study
TH275	*MAT*α *rad5*::*HIS3 rad30*::*HIS3*	This study
TH280	*MAT***a** *siz1*::*Kan*	This study
TH281	*MAT***a** *siz1*::*Kan rad18*::*TRP1*	This study
TH290	*MAT***a** *pol30 K127R*	This study
TH291	*MAT***a** *pol30 K164R*	This study
TH292	*MAT***a** *pol30 K127R-K164R*	This study
TH293	*MAT***a** *pol30 K127R rad18*::*TRP1*	This study
TH294	*MAT***a** *pol30 K164R rad18*::*TRP1*	This study
TH295	*MAT***a** *pol30 K127R-K164R rad18*::*TRP1*	This study
TH300	*MAT***a** *mgs1-18 siz1*::*Kan*	This study
TH301	*MAT***a** *mgs1-18 rad18*::*TRP1 siz1*::*Kan*	This study
TH310	*MAT***a** *mgs1-18 pol30 K164R*	This study
TH311	*MAT***a** *mgs1-18*, *rad18*::*TRP1*, *pol30 K164R*	This study

[a] All strains are isogenic to W303-1A but *RAD5*$^+$ (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) except for the mutation and mating type described.

These findings may provide novel insights into the mechanisms of stalled replication fork rescue.

MATERIALS AND METHODS

Strains and plasmids. All yeast strains used in the present study are listed in Table 1 and are isogenic with the W303 strain, in which the *rad5-G535R* allele of W303 has been replaced by the wild-type *RAD5* gene. YCH292 (*pol30 K127R*), YCH294 (*pol30 K164R*), and YCH298 (*pol30 K127R-K164R*) were kindly provided by Stefan Jentsch. *pol30* mutants were obtained by PCR using the genomic DNA of YCH292, YCH294, and YCH298. These fragments containing the promoter and open-reading frame were cloned into pRS306 or pRS425 for genome replacement or overexpression studies, respectively. A new NdeI site at the ATG initiation codon of *POL30* in pPCNA (pRS425 POL30) was generated to construct SUMO-tagged PCNA by site-directed PCR mutagenesis. A SUMO cassette was constructed by amplifying the coding region of *SMT3* gene by PCR using the genomic DNA as a template and the primers SMT3F (5′-CAAATAA ATACACGACATATGTCGGACTCAGAAG) and SMT3R (5′-TAATACGTA GCCATATGAATCTGTTCTCTGTGAG). PCNA was tagged with the SUMO cassette at their NH_2 terminus, yielding pSUMO-PCNA (pRS425 SUMO-POL30). We also introduced the *pol30*(*K164R*) substitution mutation into plasmid pPCNA and pSUMO-PCNA, giving pPCNA^{K164R} and pSUMO-PCNA^{K164R}. The C-terminal four amino acids of full-length Smt3, including the Gly-Gly found at the C terminus of mature Smt3, were not included in these constructs in order to prevent cleavage of the fusion proteins by C-terminal SUMO hydrolases. The *SIZ1* gene was deleted by use of a PCR-based one-step gene disruption method. All other stains were constructed by standard genetic cross. The DNA sequences of the PCR-amplified fragments were confirmed by sequencing the appropriate regions.

Preparation of yeast cell extracts and Western blot analysis. An exponentially growing culture was blocked with α-factor and released into synthetic complete (SC) medium at 37°C. Protein extracts for Western blots were made by using a trichloroacetic acid precipitation method as described previously (8) but at a small scale (2×10^7 cells). Samples were analyzed by sodium dodecyl sulfate (SDS)–12 or 10% polyacrylamide gel electrophoresis (PAGE) and were detected by Western blot analysis (Amersham Biosciences). Polyclonal rabbit antibodies against yeast PCNA was a gift of A. Sugino. Antibodies to α-tubulin (Sigma) were used.

Protein pull-down assays. Several His-tagged Mgs1 truncation proteins were constructed for the pull-down assay. They were expressed in the *Escherichia coli* BL21(DE3) and purified as previously described (11). PCNA was purified as a glutathione *S*-transferase (GST) fusion protein from *E. coli* using a Bulk-GST purification kit (Amersham Biosciences). Mgs1 (1 μg) was incubated with GST-PCNA (1.5 μg) for 30 min at 25°C in phosphate-buffered saline buffer (200 μl) containing 0.25% Nonidet P-40. Reaction mixtures were centrifuged at 5,000 × *g* for 1 min, and the supernatants were incubated for 2.5 h at 4°C with glutathione-Sepharose beads (3 μl; Amersham Biosciences). For the polyhistidine pull-down assay, GST was removed from the GST-PCNA protein by using a precision protease (Amersham Biosciences). Yeast His-Mgs1 (1.5 μg) or *E. coli* His-MgsA (1.5 μg) was incubated with PCNA (1 μg) for 30 min at 25°C in phosphate-buffered saline buffer (200 μl) containing 0.05% Triton X-100. Reaction mixtures were centrifuged at 5,000 × *g* for 1 min, and the supernatants were incubated for 2.5 h at 4°C with Ni-nitrilotriacetic acid (NTA) beads (3 μl; QIAGEN). The beads were washed three times with reaction buffer and were analyzed by SDS–12% PAGE. Proteins were visualized by silver staining.

Galactose-induced expression of *MGS1*. Cells harboring plasmids with a galactose-inducible wild-type *MGS1* gene (pGal-Mgs1) were grown in liquid SC glucose-Leu (repressed) or SC galactose-Leu (induced). Cells were diluted and spotted onto SC glucose-Leu or SC galactose-Leu plates with or without the indicated concentration of MMS, respectively. For UV sensitivity assays, the plates were irradiated with UV light. The plates were incubated at 30°C for 3 days.

Bacterial two-hybrid assay. PCNA was cloned into pTRG, which contains the N-terminal domain of the α-subunit of RNA polymerase. *MGS1*(1-588), *MGS1* domain I-II(1–386), and *MGS1* domain III(386–588) were cloned into pBT, which contains the DNA-binding domain of λ*c*I. The recombinant plasmids were introduced into the BacterioMatch II reporter strain (Stratagene). To select for interaction of the bait and target hybrid proteins, which would result in the activation of the *HIS3* reporter gene, cells grown on synthetic medium were spotted onto nonselective synthetic medium and onto selective synthetic medium lacking histidine in the presence of 4 mM 3-amino-1,2,4-triazole (3-AT). The plates were incubated at 37°C for 2 days. Chloramphenicol (25 μg/ml), kanamycin (15 μg/ml), and tetracycline (8 μg/ml) were used as needed.

Other methods. Fluorescence-activated cell sorting (FACS) analysis, reversion frequency, and quantitative assay for MMS sensitivity were performed as described previously (12). Unless otherwise indicated, cells were grown at 30°C in YPAD (1% yeast extract, 2% Bacto Peptone, 2% glucose, 0.004% adenine sulfate).

RESULTS

Synthetic lethality of *mgs1 rad18* double mutant is dependent on sumoylation of the PCNA lysine 164. Previous studies and results presented here showed that *mgs1* deletion is synthetically lethal in combination with *rad6* or *rad18* mutations but confers a less severe growth defect in combination with *rad5*Δ (Fig. 1A, B, and C) (12). One possible explanation for this result is that error-prone translesion DNA synthesis occurs in *rad5* but not in *rad18* mutants, because mono-ubiquitination of PCNA is dependent on Rad18 but not on Rad5. Here, a genetic approach was used to examine why the phenotype of *rad5 mgs1* mutants is less severe than that of *rad18 mgs1* mutants. Although the phenotype of *mgs1*Δ was not exacerbated by mutations in the translesion polymerases *REV3* (polζ) or *RAD30* (polη), the deletion of *RAD30* slightly reduced the growth rate of *mgs1*Δ *rad5*Δ cells (Fig. 1C). We determined growth rates of these mutants and appropriate controls in liquid YPAD medium at 30°C. The wild-type strain and *mgs1*, *rev3*, and *rad30* mutants had similar doubling times (~93 min), whereas a *rad5* mutation caused a slight growth delay (105 min). The doubling times of the *mgs1 rad5* and *mgs1 rad5 rad30* mutants were approximately 138 and 155 min, respectively. Moreover, *mgs1 rad5 rev3* triple mutants showed severe growth

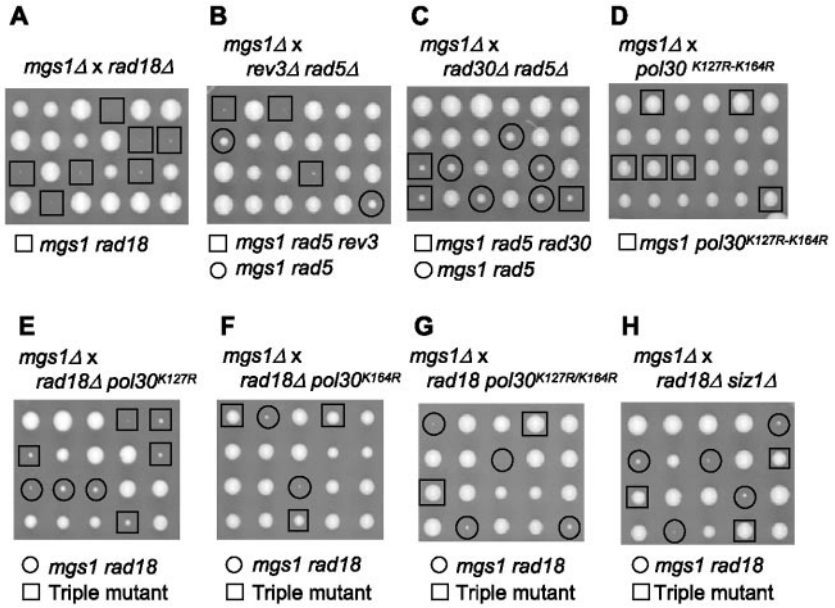

FIG. 1. Loss of SUMO modification of PCNA at K164 suppresses the growth defect of *mgs1*Δ *rad18*Δ cells. Tetrads from indicated heterozygous diploids were dissected and grown on YPAD at 30°C for 3 days. (A) Squares indicate *mgs1 rad18* mutants. (B and C) Circles indicate *mgs1 rad5* mutants; squares indicate *mgs1 rad5 rev3* and *mgs1 rad5 rad30* triple mutants. (D) Squares indicate *mgs1 pol30*(*K127R*/*K164R*) mutants. (E, F, G, and H) *mgs1 rad18* double mutants are indicated by circles. Squares indicate *mgs1 rad18 pol30*(*K127R*), *mgs1 rad18 pol30*(*K164R*), *mgs1 rad18 pol30*(*K127R*/*K164R*), *mgs1 rad18 siz1* triple mutants.

defects, as did the *mgs1 rad18* double mutant strain (Fig. 1A and B). We could not determine the doubling times of *mgs1 rad5 rev3* and *mgs1 rad18* mutants because they grew very poorly and were unstable, spontaneously producing fast-growing colonies. These results indicate that the ability of *rad5*Δ *mgs1*Δ cells to grow is dependent on translesion DNA synthesis.

Previous studies showed that PCNA is ubiquitinated at Lys164 and can also be sumoylated at Lys164 and Lys127 (13). Therefore, experiments were performed to determine the effect of PCNA modification on the viability of *mgs1*Δ cells by using the PCNA mutant *pol30*(*K127R*/*K164R*), which lacks lysine residues critical for ubiquitination and sumoylation. Interestingly, the *mgs1*Δ *pol30*(*K127R*/*K164R*) double mutant grew at the same rate as wild-type cells, despite the absence of *RAD6*-dependent ubiquitination of PCNA (Fig. 1D). Whereas ubiquitin conjugation is absent in the *rad6*, *rad18*, or *pol30* (*K127R*/*K164R*) mutants, SUMO conjugation can still occur in the *rad6* or *rad18* mutant but not in the *pol30*(*K127R*/*K164R*) mutant (13, 20), suggesting the possibility that SUMO conjugation at Lys164 and/or Lys127 interferes with the growth of the *mgs1 rad18* double mutant. This possibility was tested by examining the phenotype of *mgs1*Δ *rad18*Δ cells with or without *pol30*(*K127R*), *pol30*(*K164R*), or *pol30*(*K127R*/*K164R*). The results showed that *pol30*(*K127R*) had no effect on the viability of *mgs1*Δ *rad18*Δ cells (Fig. 1E) but that *pol30*(*K164R*) or *pol30*(*K127R*/*K164R*) clearly suppressed the lethal phenotype (Fig. 1F and G). In addition, the lethality of *mgs1*Δ *rad18*Δ was also suppressed by mutation of *SIZ1*, an E3 ligase required for sumoylation of PCNA at Lys164 (Fig. 1H). Thus, these results indicate that the severe growth defect of *mgs1*Δ *rad18*Δ is related to the sumoylation of PCNA at Lys164.

SUMO modification of PCNA in *mgs1 rad18* cells. To examine the effect of PCNA sumoylation in *mgs1 rad18*-deficient cells, we used *mgs1-18 rad18* mutants, which are temperature sensitive for growth at 37°C (12). *siz1*Δ or *pol30*(*K164R*) suppressed the growth defect of *mgs1-18 rad18*Δ cells at the restrictive temperature (Fig. 2A and B). These data confirm that the loss of SUMO modification of PCNA at Lys164 suppresses the growth defect in *mgs1*Δ *rad18*Δ cells.

PCNA sumoylation was monitored by using a mobility shift assay under denaturing conditions. Slow-migrating bands, representing covalently modified PCNA, were observed in wild-type, *mgs1*Δ, *rad18*Δ, and *pol30*(*K127R*) strains, as reported previously (13). These species were not observed in *siz1*Δ, *pol30*(*K164R*), or *pol30*(*K127R*/*K164R*) mutants (Fig. 2C), indicating that these specific slow-migrating species represent PCNA sumoylation at Lys164.

SUMO-PCNA conjugates were quantified in *mgs1-18 rad18*Δ cells at the permissive (26°C) and restrictive (37°C) temperatures. For this experiment, *mgs1-18 rad18*Δ cells were synchronized at G_1 with α-factor and released into S phase at 37°C. FACS analysis shows that the cells arrested with a 2C DNA content at 37°C (Fig. 2D). In contrast, the presence of *siz1*Δ or *pol30*(*K164R*) in the *mgs1-18 rad18*Δ background suppressed cell cycle arrest (Fig. 2D). SUMO-PCNA conjugates at Lys164 were detected in wild-type or *mgs1-18 rad18*Δ cells after release from α-factor but not in G_1-arrested cells (Fig. 2E). This result was consistent with the previously reported results that SUMO modification of PCNA is cell cycle regulated and occurs primarily during S phase (13). As shown in Fig. 2E, SUMO-PCNA conjugates were not detected in *mgs1-18 rad18*Δ *siz1*Δ or *mgs1-18 rad18*Δ *pol30*(*K164R*) cells. On the other hand, SUMO-PCNA conjugates were detected at

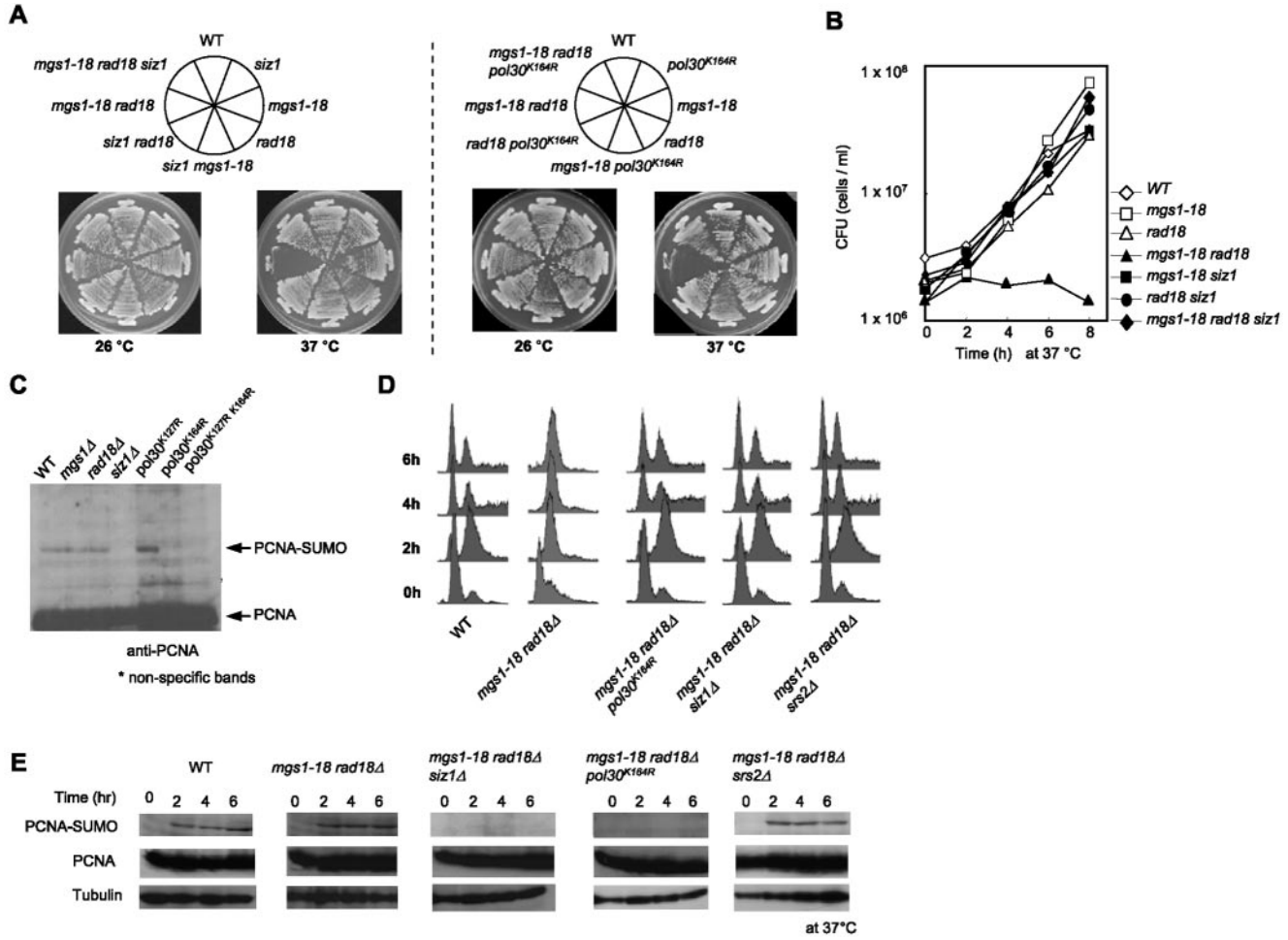

FIG. 2. Suppression of temperature sensitivity in *mgs1-18 rad18*Δ cells. (A) The *siz1* or *pol30*(*K164R*) mutations suppress the temperature sensitivity for growth of the *mgs1-18 rad18*Δ cells. Cells were streaked onto YPAD plates and incubated at 26°C (left) or 37°C (right) for 3 days. (B) Growth of the mutant cells after temperature shift to 37°C. Cells were grown in liquid YPAD to early logarithmic phase at 26°C and then shifted to 37°C. Cells were taken at the indicated time points and plated onto YPAD plates. CFU were counted after 3 days of incubation at 26°C. (C) Detection of SUMO modification of PCNA at K164. Yeast cell extracts derived from the indicated cells were separated by SDS-PAGE, and the Western blots were probed with anti-PCNA antibody. The positions of the PCNA-SUMO conjugates and PCNA are indicated on the left. ✻, nonspecific band. (D) FACS analysis of the DNA content of synchronized cells. Cells grown to early log phase at 26°C were arrested in G_1 with α-factor for 2 h. The cells were then released into SC medium at 37°C. Aliquots were taken at the indicated time points. DNA content was measured by FACS. (E) PCNA-SUMO modification in the *mgs1-18 rad18*Δ strain at the restrictive temperature. Synchronized cells were released at 37°C and PCNA-SUMO conjugates were detected by Western blotting as described for panel C. α-Tubulin was used as a loading control.

a similar level in wild-type and *mgs1-18 rad18*Δ cells at the restrictive temperature (Fig. 2E). These results suggest that the loss of SUMO-PCNA conjugations at Lys164 correlates with cell cycle progression and that the growth inhibition of *mgs1*Δ *rad18*Δ cells caused by SUMO-PCNA conjugates is not due to an increased abundance of SUMO-PCNA conjugates.

Srs2 and PCNA sumoylation act in a coordinated manner to inhibit the homologous recombination pathway. We previously showed that deletion of *SRS2* suppresses the synthetic lethality of a *mgs1*Δ *rad18*Δ strain by a mechanism that requires a functional *RAD52* recombination pathway (12). Moreover, it was recently demonstrated that Srs2 DNA helicase is recruited to the replication fork through sumoylated PCNA (20, 21). Therefore, we examined whether a deficiency in Srs2 affects PCNA sumoylation in a *mgs1-18 rad18*Δ strain. While deletion of *SRS2* in the *mgs1-18 rad18*Δ background suppressed cell cycle arrest at 37°C, wild-type levels of sumoylated-PCNA species were observed in *mgs1-18 rad18*Δ *srs2*Δ cells (Fig. 2D and E). These results indicate that deletion of *SRS2* abrogates the negative effect of PCNA sumoylation on the growth of *mgs1-18 rad18*Δ cells without affecting the level of PCNA sumoylation.

To further examine the relationship between PCNA sumoylation and Srs2 in *mgs1 rad18*-deficient cells, we fused SUMO to the N terminus of PCNA to generate a constitutively sumoylated form at their N terminus. *mgs1-18 rad18*Δ *siz1*Δ, *mgs1-18 rad18*Δ *pol30*(*K164R*), and *mgs1-18 rad18*Δ *srs2*Δ cells were transformed with the SUMO-PCNA fusion construct. Expression of the SUMO-PCNA fusion proteins inhibited the growth of *mgs1-18 rad18*Δ *siz1*Δ cells at 37°C, whereas expression of wild-type PCNA did not (Fig. 3A). However, expression either of wild-type PCNA or of the SUMO-PCNA fusion protein inhibited the growth of *mgs1-18 rad18*Δ *pol30*(*K164R*) cells at

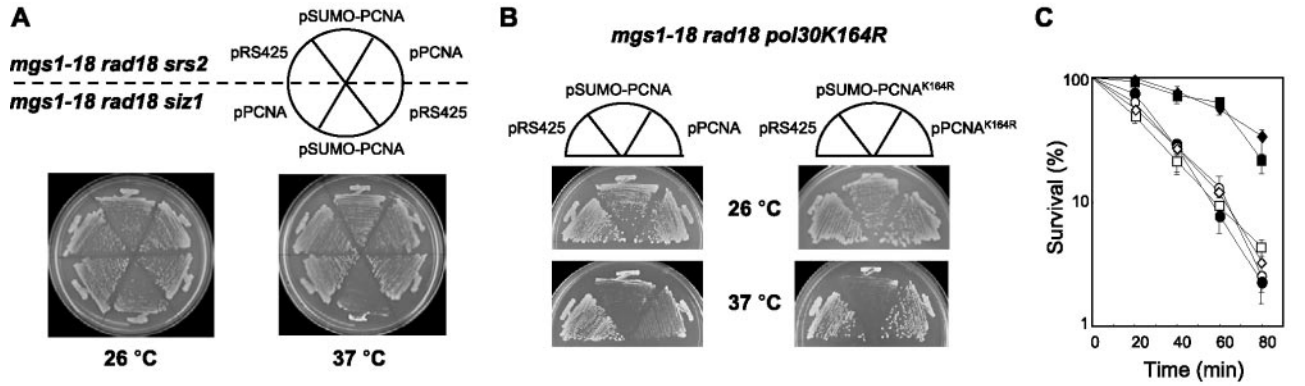

FIG. 3. Epistatic relationship between SUMO-PCNA and Srs2. (A) *mgs1-18 rad18*Δ *siz1*Δ or *mgs1-18 rad18*Δ *srs2*Δ cells were transformed with pRS425 (vector), pPCNA, and pSUMO-PCNA. Cells were streaked onto SC-Leu plates and incubated at 26°C (left) or 37°C (right) for 3 days. (B) *mgs1-18 rad18*Δ *pol30K164R* cells that carried the indicated *POL30* or SUMO-fused *POL30* alleles on a plasmid were streaked onto SC-Leu plates and incubated at 26°C (top panel) or 37°C (bottom panel) for 3 days. (C) MMS sensitivity of wild-type and *srs2*Δ cells expressing SUMO-PCNA fusion protein. For quantitative assay, cells were incubated in SC-Leu containing 0.1% MMS at 30°C. At the indicated times, samples were withdrawn and plated on medium lacking MMS. Error bars indicate the standard deviations of independent experiments. Symbols: ◆, WT/vector; ■, WT/pPCNA; ●, WT/pSUMO-PCNA; ◇, *srs2*Δ/vector; □, *srs2*Δ/pPCNA; ○, *srs2*Δ/pSUMO-PCNA. WT, wild type.

37°C, as indicated by their reduced ability to grow at 37°C (Fig. 3B). This could have been due to the fact that excess wild-type PCNA can act as a dosage suppressor of ubiquitin and SUMO modification defects of *pol30*(*K164R*) mutants (13). Therefore, $PCNA^{K164R}$ and SUMO-$PCNA^{K164R}$ constructs were transformed into *mgs1-18 rad18 pol30*(*K164R*) cells to exclude the possibility of indirect effects associated with overexpression of wild-type PCNA. As shown in Fig. 3B, expression of the SUMO-$PCNA^{K164R}$ fusion proteins inhibited the growth of *mgs1-18 rad18*Δ *pol30*(*K164R*) cells at 37°C, whereas the expression of $PCNA^{K164R}$ did not. In addition, SUMO-PCNA and SUMO-$PCNA^{K164R}$ fusion proteins did not inhibit the growth of *mgs1-18*, *rad18*, *siz1* and *pol30*(*K164R*) single mutants at 37°C (data not shown). Thus, covalent attachment to PCNA of SUMO at a different position seems to have the same effect as sumoylation of the Lys164 residue. Importantly, SUMO-PCNA fusion proteins failed to inhibit the growth of *mgs1-18 rad18*Δ *srs2*Δ cells (Fig. 3A). Taken together, these results suggest that the growth inhibition conferred by SUMO-PCNA conjugates in *mgs1 rad18*-deficient cells depends on the Srs2 function.

We also found that expression of the SUMO-PCNA fusion protein in wild-type cells increased their sensitivity to MMS, suggesting that constitutive expression of this protein impairs DNA damage repair (Fig. 3C). Notably, expression of the SUMO-PCNA fusion protein did not enhance the MMS sensitivity of the *srs2* mutant (Fig. 3C), suggesting that the inhibitory effects on DNA damage repair caused by the SUMO-PCNA fusion protein depends on Srs2. Indeed, we found that cells with a high level of expression of the *SRS2* gene, which was cloned into a pRS425 plasmid under its native promoter, became more sensitive to MMS (data not shown). Thus, our data support a direct role for SUMO modification as a regulatory switch that controls Srs2 functions.

Physical interaction between Mgs1 and PCNA. While the experiments described above indicate functional interactions among Mgs1, Rad18 and PCNA, it is unclear whether Mgs1 physically interacts with PCNA. Because Mgs1 has similarity to replication factor C (RFC), which plays roles in loading PCNA onto DNA, it seemed possible that Mgs1 forms an RFC-like complex and interacts with PCNA. However, Aroya and Kupiec showed that Mgs1 did not coprecipitate with any of the small RFC subunits (1). Moreover, a study of the human *MGS1* orthologue WRNIP1 demonstrated that WRNIP1 protein predominantly forms a homo-octamer and does not associate with RFC2-5 subunits of RFC (26). Indeed, Mgs1 protein possesses DNA-dependent ATPase activity by itself (11). Thus, Mgs1 is functionally distinguishable from other RFC-like components such as Rad24, Ctf18, and Elg1. Therefore, we examined whether Mgs1 interacts directly with PCNA. This was tested by examining whether purified GST-PCNA and Mgs1 coprecipitated in vitro (Fig. 4A). The results showed that Mgs1 coprecipitated with GST-PCNA in the presence of 0.25% NP-40 (Fig. 4B, lane 2). A strong Mgs1-PCNA interaction domain was mapped to a segment containing domain I-II of Mgs1 (Fig. 4B, lane 6), and a weaker Mgs1-PCNA interaction domain was mapped to domains II and II-III (Fig. 4B, lanes 8 and 10). A complex between Mgs1-domain I and PCNA was detected in the presence of 0.02% NP-40 but not at higher detergent concentrations (Fig. 4B, lane 16). Domain III of Mgs1 did not interact with PCNA under any of these conditions. A reciprocal pull-down experiment demonstrated the presence of PCNA in the fractions coprecipitated with His-Mgs1 but not with His-MgsA (MgsA is an *E. coli* orthologue of yeast Mgs1) (Fig. 4C). An interaction between PCNA and either full-length Mgs1 or Mgs1 domain I-II was also seen in the two-hybrid assay, although the interaction between PCNA and Mgs1 domain I-II were reduced in this assay (Fig. 4D). In addition, Mgs1 domain III did not interact with PCNA (Fig. 4D), which is consistent with the pull-down assays. These results suggest that domain II of the Mgs1 protein interacts directly with PCNA and that domain I may stabilize this interaction, possibly through the weak interaction with PCNA.

High levels of Mgs1 inhibits the RAD6-dependent DNA damage tolerance. We previously showed that overexpression of *MGS1* confer sensitivity to MMS and HU, but not to UV

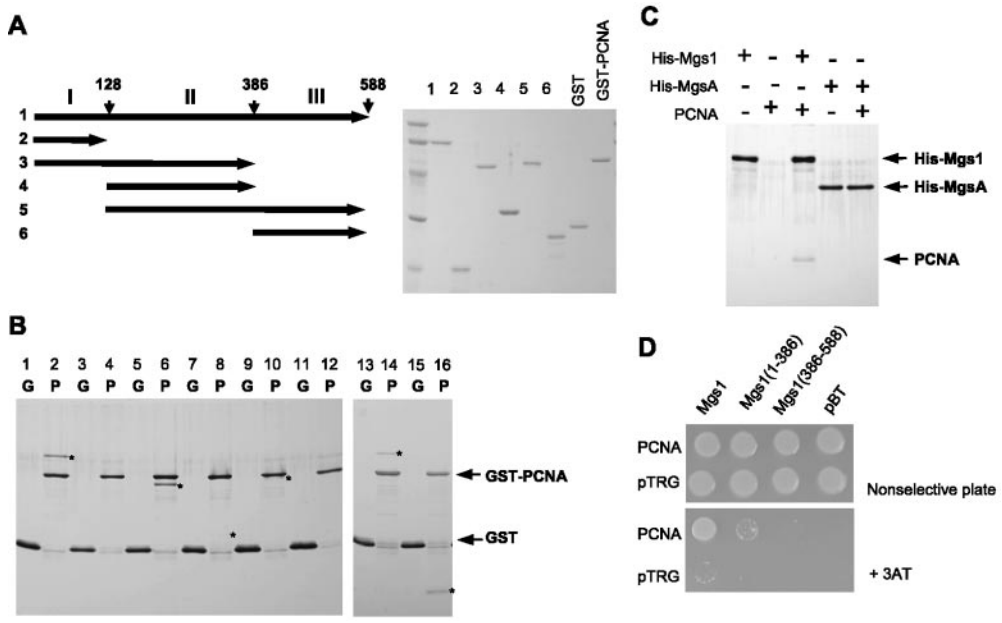

FIG. 4. Mgs1 physically interacts with PCNA. (A) Mgs1 truncated proteins are indicated schematically on the left. Purified proteins were detected by Coomassie blue staining and are shown on the right. Lanes 1 to 6 of the gel correspond to constructs 1 to 6 shown on the left. (B) Interaction of PCNA with Mgs1. Proteins were incubated with GST (lanes G) or GST-PCNA (lanes P) for 30 min at 15°C in the presence of 0.25% NP-40 (lanes 1 to 12) or 0.02% NP-40 (lanes 13 to 16). The complexes were analyzed by SDS-PAGE and detected by silver staining. Lanes 1 and 2, wild-type Mgs1; lanes 3 and 4, Mgs1(1–128); lanes 5 and 6, Mgs1(1–386); lanes 7 and 8, Mgs1(128–386); lanes 9 and 10, Mgs1(128–588); lanes 11 and 12, Mgs (386–588); lanes 13 and 14, wild-type Mgs1; lanes 15 and 16, Mgs1(1–128). (C) His-Mgs1 or His-MgsA protein was incubated with PCNA. Protein complexes were precipitated by using Ni-NTA beads. The complexes were analyzed by SDS-PAGE and detected by silver staining. (D) Full-length and truncated versions of Mgs1 were tested for two-hybrid interaction with PCNA. Positive interactions were detected by growth on synthetic medium lacking histidine in the presence of 3-AT.

(11). However, we found that cells overexpressing Mgs1 in the presence of galactose prior to MMS and HU exposure and UV irradiation were not only sensitive to MMS and HU but also sensitive to UV (Fig. 5). In our previous study, cells grown on glucose were UV irradiated, after which the cells were grown onto galactose-containing medium to induce *MGS1* transcription (11). To examine whether the higher sensitivity of cells overexpressing *MGS1* is due to an altered *RAD6* pathway func-

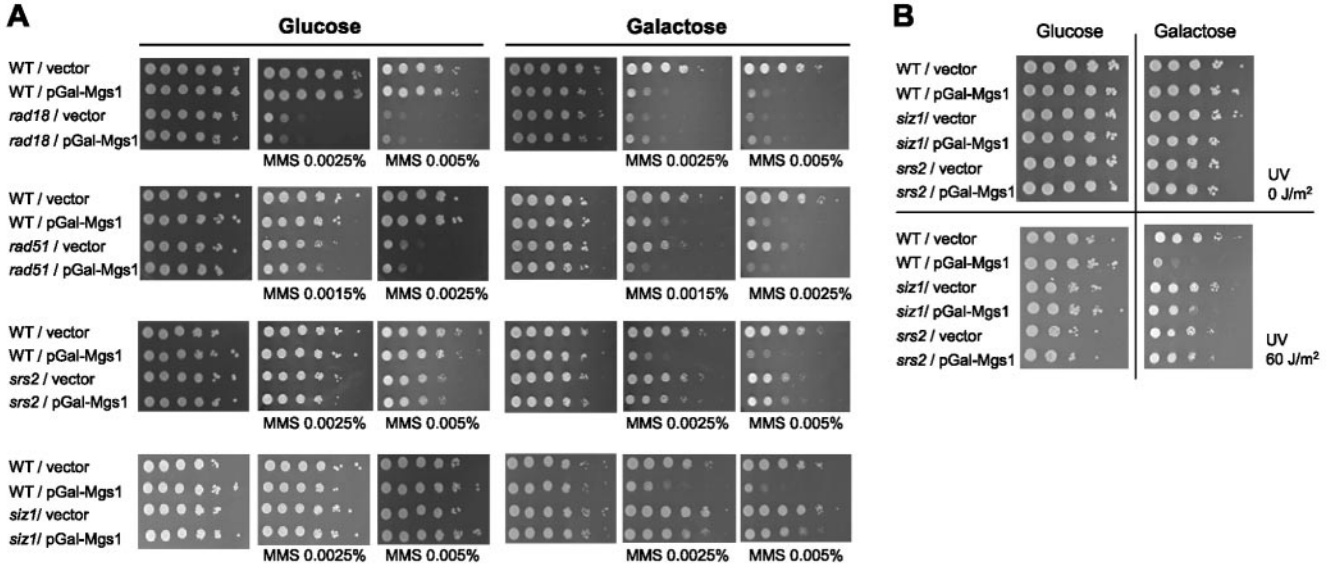

FIG. 5. Overexpression of *MGS1* inhibits Rad18 function. (A) Wild-type and *rad18*Δ, *rad51*Δ, *srs2*Δ, and *siz1*Δ mutant cells harboring empty vector (vector) or plasmids with a galactose-inducible wild-type *MGS1* gene (pGal-Mgs1) were grown in liquid SC glucose-Leu or SC galactose-Leu, respectively. Cells were diluted and spotted onto SC glucose-Leu or SC galactose-Leu plates with the indicated concentrations of MMS. The plates were incubated at 30°C for 3 days. (B) Cells harboring plasmid were grown and spotted onto plates as described for panel A. DNA damage was induced by UV.

TABLE 2. Spontaneous and DNA damage-induced reversion frequency at the *trp1-1* allele

Strain	Trp^+ reversion frequency/10^7 viable cells (fold increase) with[a]:			
	0.03% MMS[b]		UV	
			10 J/m^2	40 J/m^2
Wild type	2.1 (1)	65.4 (1)	13.0 (1)	57.8 (1)
mgs1 mutant	14.4 (6.9)	46.5 (0.7)	27.7 (2.1)	45.1 (0.8)
rev3 mutant	0.26 (0.1)	0.18 (0.003)	0.23 (0.02)	ND
mgs1 rev3 mutant	0.34 (0.2)	0.29 (0.004)	0.47 (0.04)	ND

[a] Relative to the wild-type strain. The results represent the average of three independent measurements. ND, not determined.
[b] Cells were treated with 0.03% MMS for 2 h.

tion under conditions of DNA damage or stalled DNA replication forks, we measured the MMS sensitivity of wild-type and *rad18*Δ cells expressing *MGS1* from the inducible *GAL1* promoter. The results showed that overexpression of *MGS1* enhanced MMS-sensitivity in wild-type and *rad51*Δ cells but not in *rad18*Δ cells (Fig. 5A). Notably, the MMS sensitivity caused by overexpression of *MGS1* was suppressed by *srs2* or *siz1* mutation (Fig. 5A), which is consistent with the fact that deletion of *SRS2* or *SIZ1* suppresses the repair deficiency of *rad18*Δ. Similarly, overexpression of Mgs1 enhanced UV sensitivity in wild-type cells (Fig. 5B), which was less sensitive than *rad18*Δ cells, but not in *rad18*Δ cells (data not shown). Deletion of *SRS2* or *SIZ1* could also suppress the UV sensitivity caused by overexpression of *MGS1* (Fig. 5B). These data do not result from variations in the expression level of *MGS1* because the expression level of Mgs1 protein was similar in all strains (data not shown). Therefore, these results suggest that overexpression of *MGS1* inhibits the *RAD6* DNA damage tolerance.

Mgs1 suppresses *RAD6*-dependent DNA damage tolerance in the absence of exogenous DNA damage. If Mgs1 suppresses the *RAD6*-dependent DNA damage tolerance, *mgs1* mutations would activate the *RAD6* pathway and therefore be expected to cause a *RAD6*-dependent increase of mutation frequency. To test this possibility, we examined the mutation frequency in an *mgs1* mutant in the presence or absence of exogenous DNA damage. *mgs1*-deficient cells showed increased spontaneous mutation frequencies at the *trp1-1* allele compared to the wild type, and this increase was completely dependent on the *REV3*-encoded error-prone DNA polymerase ζ (Table 2). Interestingly, when cells were treated with MMS or UV, *mgs1*Δ cells had a DNA damage-induced mutation frequency similar to that for the wild type (Table 2), suggesting that *mgs1* mutation reduced the DNA damage induced mutagenesis only partially. These differences suggest that Mgs1 helps suppress the *RAD6*-dependent damage tolerance in the absence of induced DNA damage rather than in its presence.

DISCUSSION

PCNA not only serves as a polymerase processivity factor but also provides attachment sites for various other proteins that function in DNA replication, DNA repair, cell cycle progression, and chromatin assembly. Thus, PCNA functions as a molecular switch to coordinate DNA replication-associated events. In the present study, we analyzed the interaction between Mgs1 and PCNA and its impact on *RAD6*-dependent DNA damage tolerance. Previous studies and the present study showed that *mgs1* is synthetically lethal in combination with *rad6* or *rad18*, and *mgs1 rad5* double mutants show a slow-growth phenotype. Furthermore, *mgs1 rad5 rev3* triple mutants showed severe growth defects, as did *mgs1 rad18* mutants (Fig. 1A), suggesting that in the *mgs1*Δ strain, the absence of the Rad5-dependent error-free repair pathway results in the partial channeling of blocking lesions into an alternative Rev3-dependent error-prone pathway. This hypothesis is supported by the fact that *mgs1 rad5* cells have a higher mutation frequency than *mgs1* or *rad5* cells (12). In addition, the *mgs1* mutation causes a mutator phenotype that is dependent on Rev3, showing that when Mgs1 is absent, the load on translesion DNA synthesis is increased. These results suggest an important role for the Mgs1 protein in protecting the genome from spontaneous DNA damage. Therefore, *mgs1* mutation might spontaneously generate replication-blocking lesions during S phase in the absence of exogenous DNA damage, at least some of which could be bypassed by the *REV3*-encoded translesion DNA polymerase ζ.

Genetic studies show that *RAD6*-dependent DNA damage tolerance pathway is essential in *mgs1*-deficient cells even in the absence of exogenous DNA damage and that the lethality of *mgs1*Δ *rad18*Δ cells can be suppressed by inhibiting sumoylation of Lys164 of PCNA. These results suggest that the lethality of *mgs1*Δ *rad18*Δ cells is related to the sumoylation of PCNA at Lys164, and not to the absence of the *RAD18*-controlled PCNA ubiqutination pathways. Furthermore, evidence is presented here that SUMO-PCNA conjugates do not accumulate in *mgs1-18 rad18*Δ cells, even at restrictive temperature, suggesting that SUMO and ubiquitin do not compete for Lys164. It should be noted that bands representing PCNA K127-SUMO conjugates were not detected in the present study. This may be due to a strain or antibody difference. However, our genetic study showed that mutations in PCNA/K164 and PCNA/K127-K164, but not PCNA/K127, could suppress the lethality of *mgs1*Δ *rad18*Δ cells, suggesting that the inhibitory effect of K127 sumoylation is likely to be minimal.

We have also taken several approaches to elucidate the role of PCNA sumoylation. We fused SUMO to the N terminus of PCNA, a strategy that has been used to examine the effect of sumoylation of other target proteins (23, 24). We found that the growth of *mgs1-18 rad18*Δ *siz1*Δ and *mgs1-18 rad18*Δ *pol30*(*K164R*) cells was inhibited at 37°C by SUMO-PCNA and SUMO-PCNA^{K164R} fusion proteins, respectively. Notably, the growth of *mgs1-18 rad18*Δ *srs2*Δ cells was not inhibited by SUMO-PCNA fusion proteins. In support of this, Lys164 of PCNA was sumoylated in *mgs1-18 rad18*Δ *srs2*Δ cells to the same extent as in wild-type cells, although the *srs2* mutation suppressed the growth defect of *mgs1-18 rad18*Δ cells. These findings suggest that the Srs2 helicase, in conjunction with sumoylation of PCNA at Lys164, inhibits the growth of *mgs1*Δ cells when the *RAD6*-dependent DNA damage tolerance pathway is inactivated.

Previously, our studies showed that the lethality of *mgs1*Δ *rad18*Δ cells is suppressed by *srs2*Δ in a *RAD52*-dependent manner (12). As shown for *srs2* mutants, *mgs1-18 rad18*Δ *siz1*Δ and *mgs1-18 rad18*Δ *pol30*(*K164R*) cells displayed a synthetic lethal phenotype when combined with *rad51*Δ at restrictive

temperature (data not shown). Thus, suppression of the *mgs1 rad18* lethality, either by inhibiting PCNA sumoylation or by deleting *SRS2*, requires a functional *RAD52* pathway, indicating that Srs2 and PCNA sumoylation act on the same pathway to prevent homologous recombination. Accordingly, Srs2 was recently shown to block the *RAD52*-dependent homologous recombination by actively disrupting the Rad51-nucleoprotein filament (18, 28). Furthermore, recent studies showed that Srs2 preferentially associates with sumoylated PCNA (20, 21). Therefore, our results, taken together with those of previous studies, support the conclusion that sumoylated PCNA facilitates the recruitment of the Srs2 helicase to replication forks and thus helps it to prevent the *RAD52*-dependent recombination during S phase, which might contribute to determine the mode of action (*RAD6* or *RAD52* pathway) to be taken when the progression of DNA replication forks is impeded. Thus, distinct PCNA modifications alter the function of PCNA and serve as a regulatory switch in the DNA damage tolerance pathway. One possible explanation for these results is that fork rescue is facilitated by PCNA trimers carrying sumoylation and ubiquitination of Lys164, as suggested previously (10, 20). SUMO and ubiquitin could coexist either in one PCNA trimer or in two different PCNA trimers present at the same stalled replication fork, but it is not yet clear if the former or latter (or both) occur at stalled replication forks in vivo.

We showed here that Mgs1 bound to PCNA and that high levels of *MGS1* suppress the *RAD6*-dependent DNA damage tolerance pathway, resulting in hypersensitivity to MMS, HU, and UV. Importantly, deletion of *SIZ1* suppressed the MMS sensitivity caused by Mgs1 overexpression, suggesting that PCNA sumoylation still occurs in Mgs1-overexpressing cells. Thus, high levels of *MGS1* might lead to sequestration of PCNA and specifically interfere with the PCNA ubiquitination. Alternatively, the switch of polymerase is impaired by the PCNA-bound Mgs1 even if PCNA is ubiquitinated. These findings are consistent with previous observations where MMS-induced mutagenesis, which depends on *REV3*-edcoded DNA polymerase ζ, is abolished upon Mgs1 overexpression (5). While overexpression of *MGS1* provides important clue for understanding its biological role, it might alter cellular function such as the spatiotemporal regulation of Mgs1. Indeed, deletion of the *MGS1* gene does not confer hypersensitivity to MMS, HU, and UV. Furthermore, we showed that *mgs1*-deficient cells exhibited elevated spontaneous mutation frequencies, which were completely abolished by the *rev3* mutation, but had minor effect on MMS- or UV-induced mutation frequencies. Thus, the mutator phenotype of *mgs1*Δ cells is restricted to spontaneous events. It therefore seems that the interaction between Mgs1 and PCNA helps prevent the attendant risk of increased genome instability caused by inappropriate *RAD6* DNA damage tolerance in the absence of induced DNA damage rather than in its presence. This may account for why high levels of *MGS1* sensitizes cells to DNA-damaging agents.

The Mgs1 family is highly conserved from bacteria to humans. Since the ubiquitin modification system is unique to eukaryotes, Mgs1 seems to have another function besides its role in preventing the *RAD6* pathway. We previously showed that *mgs1*Δ could suppress the temperature sensitivity of DNA polymerase δ (Polδ) mutants, but the ability of *mgs1* mutation to suppress the growth defect of Polδ mutants was associated with synergistic increase of the mutation frequency (12), suggesting that deficiency of Mgs1 allows replication fork progression in Polδ mutants at the expense of replication fidelity. Furthermore, other studies have suggested that Mgs1 contributes to faithful replication fork movement by activating Fen1 activity, although direct interaction between them is not observed (17). Thus, it is likely that Mgs1 not only modulates the *RAD6* pathway but also plays a role in preventing replication problems and the generation of DNA damage, both of which might be closely related through interaction with PCNA.

The proposed roles for Mgs1, Srs2, and modified PCNA during replication arrest highlight the importance of modulating the *RAD6* and *RAD52* pathways to avoid genome instability. Although the precise molecular events modulating the two pathways remain to be fully elucidated, the genetic and physical interactions strongly suggest that the PCNA-SUMO-Srs2 interactions are important for blocking the *RAD52*-dependent recombination, and Mgs1-PCNA interaction is at least involved in the regulation of the *RAD6* DNA damage tolerance when the DNA synthesis is arrested by natural impediments, such as DNA secondary structures and DNA-protein complexes. Further biochemical characterization of these proteins should provide significant insights into the spatiotemporal regulation of the *RAD6* DNA damage tolerance and *RAD52* recombination pathways at stalled replication forks.

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