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Smc5/6 Mediated Sumoylation of the Sgs1-Top3-Rmi1 Complex Promotes Removal of Recombination Intermediates

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SUMMARY

Timely removal of DNA recombination intermediates is critical for genome stability. The DNA helicase-topoisomerase complex, Sgs1-Top3-Rmi1 (STR), is the major pathway for processing these intermediates to generate conservative products. However, the mechanisms that promote STR-mediated functions remain to be defined. Here we show that Sgs1 binds to poly-SUMO chains and associates with the Smc5/6 SUMO E3 complex in yeast. Moreover, these interactions contribute to the sumoylation of Sgs1, Top3, and Rmi1 upon the generation of recombination structures. We show that reduced STR sumoylation leads to accumulation of recombination structures, and impaired growth in conditions when these structures arise frequently, highlighting the importance of STR sumoylation. Mechanistically, sumoylation promotes STR inter-subunit

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SUPPLEMENTAL INFORMATION

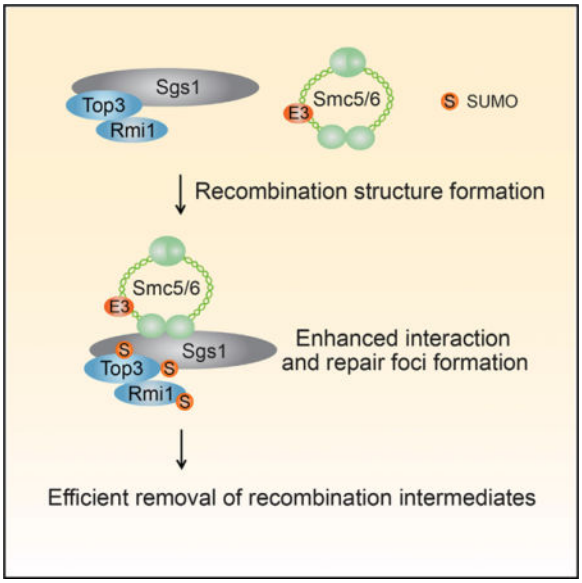
Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.06.015.

AUTHOR CONTRIBUTIONS

J.N.B., K.C., and X.Z. conceived experiments, performed and analyzed most of the in vivo tests, and wrote the paper; B.S. and D.B. conducted 2D gel experiments; N.P.T. and G.W.B. performed and analyzed STR foci studies in wild-type and *ubc9-10* cells; X.X. and P.S. tested in vitro Sgs1-SUMO chain interaction; M.A., J.M., L.W., and B.W. provided reagents and additional data.

interactions and accumulation at DNA repair centers. These findings expand the roles of sumoylation and Smc5/6 in genome maintenance by demonstrating that they foster STR functions in the removal of recombination intermediates.

Graphical Abstract



INTRODUCTION

During DNA replication, strand breaks or gaps resulting from template lesions can be processed by recombinational repair. In this context, homologous pairing and DNA synthesis produce joint molecules (JMs), such as Holliday junctions and D-loop structures, which are subsequently processed via mechanistically distinct pathways to yield different DNA products (reviewed in Symington et al., 2014). Both the formation and removal of JMs are important for proper DNA repair. Defects in the latter can be more deleterious in some situations, as alternative DNA repair processes could compensate for the complete lack of recombinational repair in dealing with certain DNA lesions but could not remove the JMs formed as recombination intermediates. If JMs persist, they can block chromosome segregation, leading to genetic alterations or cell death (reviewed in Sarbajna and West, 2014).

Consistent with the importance of timely JM removal, cells possess a group of enzymes to process and eliminate JMs. A key member is a conserved DNA helicase-topoisomerase complex, composed of the Sgs1 helicase and the Top3-Rmi1 sub-complex (together referred to as STR) in yeast and the BLM helicase and TopoIIIa-RMI1-RMI2 (referred to as BTR) in humans. The DNA branch-migrating activity of Sgs1/BLM and the strand cleavage/passing activity of Top3/TopoIIIα collaborate to disentangle double Holliday junctions in a process termed dissolution, yielding non-crossover products (reviewed in Bizard and Hickson, 2014). In parallel to STR/BTR, several conserved structure-specific nucleases, such as Mus81-Mms4, Slx1-Slx4, and Yen1 in yeast and their orthologs in mammals, can directly

cleave JMs in a process called resolution, generating both non-crossover and crossover products (reviewed in Sarbajna and West, 2014).

Recent studies have shown that JM nucleases are tightly regulated by phosphorylation. For example, phosphorylation of Mus81-Mms4 by Cdc5 and CDK kinases activates the nuclease specifically during mitosis, whereas dephosphorylation of Yen1 at anaphase is critical for its function (Blanco et al., 2014; Eissler et al., 2014; Gallo-Fernández et al., 2012; Matos et al., 2011; Szakal and Branzei, 2013). Compared with nuclease-based JM resolution, JM dissolution is important especially during DNA replication and is preferred during mitotic growth because it exclusively generates the more conservative DNA products (reviewed in Bizard and Hickson, 2014). Thus far, the molecular mechanisms that promote JM dissolution have remained elusive.

Two candidates that could promote JM dissolution have been suggested by genetic data. Previous studies have noted partial phenotypic overlap between mutants of STR and mutants of sumoylation and the conserved Smc5/6 complex. Specifically, yeast mutants of SUMO (small ubiquitin-like modifier) E2 (Ubc9) or the SUMO E3 subunit of the Smc5/6 complex (Mms21) accumulate JMs, like the sgs1-null mutant (Branzei et al., 2006; Liberi et al., 2005). In addition, mammalian cells deficient for Ubc9 or Mms21 homologs are prone to chromosomal nondisjunction, similarly to BLM mutant cells (Jacome et al., 2015; Nacerddine et al., 2005; Payne et al., 2014). However, other findings indicate that STR/BTR and Smc5/6 mutants sensitize each other during growth or under genotoxic stress, thus precluding a simple interpretation of their relationship (Chen et al., 2009; Jacome et al., 2015). While not directly demonstrated, such a complex relationship can conceivably stem from distinct functions assigned to STR and Mms21, such as a role in DNA end resection by STR (Mimitou and Symington, 2008; Zhu et al., 2008b) and sister chromatid cohesion by Mms21 (Almedawar et al., 2012; McAleenan et al., 2012). Thus far, the multi-functional natures of these factors have made it difficult to discern whether Smc5/6 and sumoylation can affect JM metabolism via direct regulation of STR or via other means, such as affecting cohesion. In addition, it remains to be elucidated whether SUMO and Smc5/6 influence JM metabolism through shared or independent mechanisms.

Addressing the aforementioned issues will provide mechanistic insights into the regulation of JM dissolution, which is indispensable for genomic integrity, and clarify the roles of SUMO and Smc5/6 in this process. Here, we use a combination of approaches to reveal a molecular connection among STR, SUMO, and Smc5/6. We show that Sgs1 physically associates with SUMO and Smc5/6 and that these interactions aid STR sumoylation. Importantly, disrupting these interactions or reducing Sgs1 sumoylation leads to increased JM levels and downregulation of STR subunit foci in response to DNA damage. These findings shed light on how Smc5/6 and SUMO work together to directly promote STR function in JM dissolution.

RESULTS

Association of Sgs1 with Smc5 Increases upon Replication Stress

To understand the mechanistic roles of Smc5/6 in JM removal, we probed for its physical interactions with STR using the yeast two-hybrid (Y2H) assay. Pairwise tests among all subunits of the two complexes revealed an association between Sgs1 and Smc5 (Figure 1A; Figure S1A). This association was confirmed by co-immunoprecipitation and was more noticeable when cells were arrested in S phase than when they were in G1 or G2/M (Figures 1B, S1B, and S1C). The Sgs1-Smc5 interaction was also detected upon methyl methanesulfonate (MMS) treatment, which induces DNA replication stress and increases JM levels (Figures 1C and S1D) (Branzei et al., 2006; Liberi et al., 2005). These results suggest that Sgs1 and Smc5 associate more prominently during replication.

A SIM Cluster within Sgs1 Contributes to Its Interaction with SUMO and Smc5

We asked whether the observed Sgs1-Smc5 association is mediated by SUMO, based on the following rationale. First, Sgs1 interacts with conjugatable SUMO in Y2H assays (Böhm et al., 2015; Branzei et al., 2006). Second, SUMO moieties often promote protein-protein interactions (Jentsch and Psakhye, 2013). Third, several subunits of the Smc5/6 complex are sumoylated, likely at multiple sites (Bermúdez-López et al., 2015; Zhao and Blobel, 2005). Fourth, genetic data implicate both SUMO and Smc5/6 in JM metabolism as described earlier. These observations suggest a possible dependence of the Sgs1-Smc5 interaction on SUMO. Based on this reasoning, one would predict mutations of the SUMO-interacting motifs (SIMs) in Sgs1 to compromise its interactions with both SUMO and Smc5.

SIMs harbor two to four hydrophobic residues and one acidic residue in the core sequence, as well as several acidic residues in the flanking region (Hannich et al., 2005; Hecker et al., 2006; Zhu et al., 2008a). As the SIM-SUMO interaction has a low affinity, SIMs often cluster to achieve enhanced association with SUMO (Song et al., 2004; Zhu et al., 2008a). Sgs1 contains four putative SIMs clustered in a previously noted acidic region between the helicase and Top3/Rmi1 interaction domains (Figure 1D) (Weinstein and Rothstein, 2008). Upon mutating the hydrophobic residues within these motifs to alanine (*sgs1-sim*; Figure 1D), the Y2H SUMO interaction was lost, suggesting that these SIMs mediate Sgs1 interaction with SUMO (Figure 1E). Importantly, the sgs1-sim mutant protein maintained Top3 interaction, indicating that the mutations did not grossly affect protein functions (Figure 1E).

Interestingly, when we tested for Smc5 interaction, a defect was observed for the sgs1-sim mutant (Figure 1E). In conjunction with previous findings that several subunits of the Smc5/6 complex are sumoylated (Bermúdez-López et al., 2015; Zhao and Blobel, 2005), these results suggest that SUMO moieties on these subunits and SIMs in Sgs1 likely form multi-valent interactions. Detailed assessment of which SUMO moieties interact with Sgs1 SIMs requires mapping sumoylation sites on the Smc5/6 complex and is an ongoing effort. Herein, we focused on understanding the biological functions and significance of the SIM-mediated interactions of Sgs1 with Smc5/6 and SUMO.

Top3 and Rmi1 Are Sumoylated

Considering that the Smc5/6 complex contains the SUMO E3 ligase Mms21 (Zhao and Blobel, 2005), a possible function of the Sgs1-Smc5 interaction might be to promote the sumoylation of the STR complex (Figure 1F). Sumoylation of Sgs1, but not Top3 or Rmi1, has been documented (Branzei et al., 2006; Lu et al., 2010) (also discussed later). Therefore, we first examined whether Top3 and Rmi1 are sumoylated after treatment with MMS (0.03%). We chose this condition for most of the experiments herein because mutants of both Mms21 and STR exhibit increased JM levels under this condition (Branzei et al., 2006; Liberi et al., 2005). Using a well-established method to examine protein sumoylation (Ulrich and Davies, 2009), we enriched proteins containing His_8-tagged SUMO (H-SUMO) on nickel-nitrilotriacetic acid (Ni-NTA) resin and probed the sumoylated protein pool for specific targets by western blotting (referred to as Ni-PD). We observed several modified forms of Top3 and Rmi1 (Figures 2A and 2B). Control tests verified that these were sumoylated Top3 and Rmi1 species, since they were absent when Top3 or Rmi1 was not tagged, when untagged SUMO was used, and in SUMO E2 mutant strains (Figures 2A, 2B, S2A, and S2B). The patterns of Top3 and Rmi1 sumoylated forms are suggestive of modification with multiple SUMO molecules.

Optimal Top3/Rmi1 Sumoylation Relies on Sgs1 Interaction, Sgs1 SIMs, and Mms21

The hypothesis that Sgs1-Smc5 interaction promotes Top3 and Rmi1 sumoylation by Mms21 predicts that their sumoylation would be eliminated or reduced under three conditions: (1) when Top3/Rmi1 no longer binds Sgs1, (2) when Sgs1 fails to interact with Smc5, or (3) when Mms21 is inactive. We expected a partial defect in the latter two situations, because the Mms21 E3 function partially overlaps with those of the two Siz SUMO E3s (Johnson, 2004; Reindle et al., 2006; Silver et al., 2011). We tested these predictions using specific mutations to generate each situation without affecting Top3 or Rmi1 protein levels (Figures S2C–S2F).

To test the first prediction, we used a previously characterized *sgs1-ΔN* mutant, in which the N-terminal 82 amino acids of Sgs1 required for Top3 binding are deleted (Weinstein and Rothstein, 2008). We found that *sgs1-ΔN* completely abolished Top3 and Rmi1 sumoylation (Figures 2C and S2C). In contrast, Top3 and Rmi1 sumoylation remained robust when Sgs1 helicase activity was abolished by mutating a key ATP binding residue (*sgs1-hd*; Figures 2D and S2D) (Weinstein and Rothstein, 2008). Thus, Top3 and Rmi1 sumoylation requires their binding to Sgs1 but not Sgs1 helicase activity.

In testing the second prediction, we found that *sgs1-sim*, which diminished Smc5 interaction (Figure 1E), reduced Top3 and Rmi1 sumoylation (Figures 2E and S2E). To discern whether these effects were specific to Top3 and Rmi1, we did control tests examining the sumoylation of Smc5 and Rfa1. Smc5 is a known Mms21 substrate (Zhao and Blobel, 2005), and its association with Sgs1 requires the SIM region as we have shown earlier. Rfa1, a subunit of the single-stranded DNA binding protein RPA, binds to the SIM region of Sgs1 and is sumoylated after MMS treatment (Burgess et al., 2007; Hegnauer et al., 2012). We found that *sgs1-sim* did not affect sumoylation of either Smc5 or Rfa1 (Figures 2F and 2G).

These results suggest that the SIM cluster of Sgs1 contributes specifically to Top3 and Rmi1 sumoylation.

To evaluate the last prediction, we used an *mms21* allele containing mutations in two key residues for SUMO E3 activity (Branzei et al., 2006; Zhao and Blobel, 2005). As in the case for *sgs1-sim*, we found a reduction of Top3 and Rmi1 sumoylation in the *mms21* mutant (Figures 2H and S2F). In contrast, Top3 and Rmi1 sumoylation did not change in cells lacking the other SUMO ligases, Siz1 and Siz2 (Figures 2H and S2F). We note that the residual Top3 sumoylation seen in the *mms21* mutant was due to Siz2 but not Siz1 (Figure S2G). Thus, Top3 and Rmi1 sumoylation largely depends on Mms21 SUMO ligase function, with a minor contribution from Siz2. These data are consistent with the predictions made by our proposed model (Figure 1F) and support the notion that the Sgs1-Smc5 interaction brings the Mms21 SUMO ligase into proximity with the STR complex to mediate Top3 and Rmi1 sumoylation.

Sumoylation of Sgs1 Partially Relies on Its SIMs and Mms21

We proceeded to test whether the aforementioned notion also applies to Sgs1 sumoylation. First, we confirmed previous findings that Sgs1 is sumoylated and that lysine 621 is the major sumoylation site, as mutating this residue to arginine (*sgs1-KR*) greatly reduced Sgs1 sumoylation (Figures 1D, 3A, and S3A) (Branzei et al., 2006; Lu et al., 2010). Extending these findings, we found that Sgs1 sumoylation was reduced in the *mms21* E3 mutant but not in the Siz1 and Siz2 double-deletion mutant (Figure 3B). The effect of the *mms21* mutation on Sgs1 sumoylation appeared to be less severe than what we observed for the sumoylation of Top3 and Rmi1. On the other hand, as was the case for Top3 and Rmi1, Sgs1 sumoylation largely depended on its SIMs (Figures 3C and S3A). Together with the data pertaining to Top3 and Rmi1 sumoylation, these findings show that optimal sumoylation of the three STR subunits has a shared requirement for Mms21 and Sgs1 SIMs.

A requirement of SIMs for the sumoylation of Sgs1 family proteins appears to be evolutionarily conserved, since BLM sumoylation also relies on its SIMs, which lie in close proximity to its sumoylation site, as is the case for Sgs1 (Eladad et al., 2005; Zhu et al., 2008a). Furthermore, similarly to the direct association observed between BLM and poly-SUMO chains (Zhu et al., 2008a), we found that purified full-length Sgs1 interacted with SUMO chains formed by in vitro sumoylation using SUMO, E1, and E2 enzymes in the presence of ATP (Figure 3D). This result is consistent with the presence of multiple SIMs on Sgs1 and is congruent with our model that SUMO can mediate the interaction of Sgs1 with sumoylated proteins.

STR Sumoylation Correlates with Recombination Activities

Our data, thus far, support the model that Sgs1 SIMs bring the Smc5/6 SUMO E3 complex into proximity with STR to promote the sumoylation of its three subunits. Next, we addressed whether these sumoylation events underlie the roles of SUMO and Smc5/6 in JM metabolism. To this end, we first investigated when STR sumoylation occurs and how it affects JM removal. If STR sumoylation occurs more robustly when JM levels are high, it would support its involvement in JM metabolism. Indeed, sumoylation levels of Sgs1, Top3,

and Rmi1 were upregulated upon treatment of cells with 0.03% MMS, a condition known to increase JM levels (Figure 3E). Next, we tested whether the increased STR sumoylation requires JM formation. The Rad51 recombinase and the Rad54 DNA motor protein are key factors to generate JMs. We found that *rad51Δ* and *rad54Δ* cells exhibited strong reductions of Sgs1, Top3, and Rmi1 sumoylation levels (Figures 3F, S3B, and S3C). Together, these findings suggest that bulk STR sumoylation occurs in response to JM accumulation. We suspect that the residual STR sumoylation in cells lacking Rad51 or Rad54 may reflect the involvement of STR in other processes, such as DNA end resection, that do not require these two recombination factors.

Genetic Evidence that SIMs and Sumoylation of Sgs1 Contribute to JM Removal

To further probe the role of STR sumoylation in JM removal, we examined *sgs1-sim*, which reduces sumoylation of all STR subunits and impairs Smc5/6 interaction (Figures 1E, 2E, and 3C), in JM-related functions. We also examined *sgs1-KR* as a representative sumoylation mutant of STR (Figure 3A), as the sumoylation sites on Top3 and Rmi1 remain elusive, despite our mapping efforts. If STR sumoylation contributes to JM removal, one would predict *sgs1-sim* and *sgs1-KR* mutant cells to have difficulty coping with high JM burdens and to accumulate JMs. In addition, since *sgs1-sim* is defective in sumoylation of all three STR subunits, it should have stronger defects than *sgs1-KR*.

To test how *sgs1-sim* and *sgs1-KR* mutants cope with high JM burdens, we first examined situations where proteins involved in JM resolution, such as Mms4 and Slx4, were absent. We found that both *sgs1-sim* and *sgs1-KR* mutants had poorer survival on MMS-containing media when Mms4 was removed (Figure 4A). In this test, *sgs1-sim mms4Δ* cells were ~1,000-fold more MMS sensitive than *sgs1-KR mms4Δ* cells (Figure 4A). In addition, *sgs1-sim* cells could not survive in the absence of Slx4, and *sgs1-KR slx4Δ* cells grew slowly, in contrast to the normal growth of relevant single-mutant cells (Figure 4B). Moreover, when examined in the presence of JM nucleases, both *sgs1-sim* and *sgs1-KR* cells were moderately sensitive to MMS, with *sgs1-sim* exhibiting a stronger defect (Figure 4C). The stronger defects of *sgs1-sim*, compared to *sgs1-KR* in all three tests, particularly in the absence of Mms4 and Slx4 (Figures 4A–4C), are consistent with its broader effect on STR sumoylation (Figures 2E, 3A, 3C, and S3D). Taken together, these data provide genetic evidence that STR subunit sumoylation contributes to JM removal.

2D Gel Analyses Show Increased JM Levels in *sgs1-SIM* and *-KR* Mutants

Next, we directly visualized JM levels using two-dimensional agarose gel (2D gel) electrophoresis, a high-resolution method for detecting DNA structures. As shown previously, *sgs1Δ* cells accumulated JMs when replicated in the presence of MMS (Figure 4D) (Liberi et al., 2005). Moreover, *sgs1-sim* and *sgs1-KR* had higher JM levels than wild-type cells (Figure 4D). Quantification of JM levels showed that *sgs1-sim* reproducibly exhibited more JMs than *sgs1-KR* (Figure 4D). Taken together, these 2D gel results are consistent with our genetic findings, suggesting that *sgs1-sim* and *sgs1-KR* compromise JM removal, with the former exhibiting a stronger defect. These findings support the notion that Sgs1 SIMs and sumoylation contribute to JM removal.

Sgs1 SIMs and Sumoylation Do Not Affect Its Checkpoint Function or Top1 Interaction

Aside from a role in JM removal, STR has additional functions, such as supporting cell growth in the absence of Top1 and DNA end resection (Mimitou and Symington, 2008; Mullen et al., 2000; Zhu et al., 2008b). Sgs1 also has functions independent of Top3/Rmi1, such as in DNA replication checkpoint activation through association with Rfa1 (Hegnauer et al., 2012). We tested these functions in order to understand whether *sgs1-sim* and *sgs1-KR* show specificity in their effects. First, we examined the Top3/Rmi1-independent Sgs1 function in replication checkpoint activation by assessing levels of Rad53 phosphorylation, a well-established indicator of checkpoint function. The ratios of phosphorylated to unmodified Rad53 were similar among *sgs1-sim*, *sgs1-KR*, and wild-type cells (Figure 4E). In addition, the sgs1-sim mutant protein retained the ability to interact with Rfa1 in Y2H analysis (Figure S3E). Consistent with a proficient checkpoint, *sgs1-sim* and *sgs1-KR* cells were not sensitive to hydroxyurea (HU), a condition requiring checkpoint function for cell survival (Figure 4C). We note that, although MMS and HU both generate replication stress, JMs accumulate more prominently in *sgs1Δ* cells upon MMS treatment, presumably because recombination is inhibited by the checkpoint under HU conditions (Barlow and Rothstein, 2009; Liberi et al., 2005). In addition, we found that, unlike *sgs1Δ*, which shows synthetic lethality with *top1Δ*, *sgs1-sim* and *sgs1-KR* did not affect cell growth when Top1 was removed (Figure 4F) (Mullen et al., 2000). Moreover, we found that, unlike *sgs1Δ*, which is synthetic lethal with the deletion of the gene encoding the DNA end resection factor Sae2, *sgs1-sim sae2Δ* mutants showed wild-type growth (Figure S3F). In conjunction with the effects of *sgs1-sim* and *-KR* in assays pertaining to JM removal (Figures 4A–4D), the lack of defects in the aforementioned assays suggests that sumoylation primarily promotes STR-mediated JM removal.

Top3 and Rmi1 Foci Induced by MMS Require Sumoylation

Having established a positive effect of STR sumoylation in JM removal, we proceeded to test how this effect is achieved. Sumoylation has recently been shown to promote protein accrual at DNA damage sites in mammalian cells (reviewed in Jentsch and Psakhye, 2013; Sarangi and Zhao, 2015). Interestingly, STR subunits form subnuclear foci that colocalize with the recombination factor Rad52 (Tkach et al., 2012; Yimit et al., 2016). These observations raised the possibility that STR sumoylation may enhance its accrual at subnuclear repair centers. To test this idea, we first examined whether STR foci levels were upregulated upon MMS treatment and whether such a change could be affected by sumoylation. Using GFP-tagged STR subunits, we observed an increased occurrence of STR nuclear foci upon MMS treatment (Figure 5A; Figure S4A). Furthermore, the SUMO E2 mutant *ubc9-10* reduced the levels of MMS-induced Top3 and Rmi1 foci without affecting STR subunit foci in untreated cells or MMS-induced Sgs1 foci (Figures 5A and S4A). The persistence of Sgs1 foci in *ubc9-10* mutants might be related to Top3/Rmi1-independent Sgs1 functions, such as checkpoint activation, which is not influenced by its sumoylation (Figure 4E).

As mutating Ubc9 reduces global sumoylation, we further tested the effect of specifically reducing STR sumoylation using the *sgs1-sim* allele. Again, fewer MMS-induced Rmi1 foci were observed in *sgs1-sim* cells, and this effect was strongest in S phase, when Rmi1 foci

were most abundant (Figure 5B). Taken together, these results suggest that sumoylation and Sgs1 SIMs promote DNA damage-induced accrual of Rmi1 and Top3 at nuclear foci.

Top3 Associates with Sumoylated Sgs1 More Avidly

Sumoylation promotes protein accrual at DNA damage sites, in part through SUMO-mediated protein-protein interactions (reviewed in Jentsch and Psakhye, 2013; Sarangi and Zhao, 2015). We found that, aside from Sgs1, Top3 also showed Y2H interaction with SUMO, thus raising the possibility that Top3 may interact preferentially with sumoylated Sgs1 (Figure 5C). To test this idea, we examined whether sumoylated Sgs1 showed greater association with Top3 than unmodified Sgs1. Because sumoylated forms are generally low in abundance and prone to desumoylation in non-denaturing protein preparations, we strived to increase Sgs1 sumoylation levels for co-immunoprecipitation experiments. To this end, we used the SuOn tagging strategy, which utilizes a unique high-affinity SUMO interaction domain to promote sumoylation of its fusion partner, presumably by increasing local SUMO concentration (Almedawar et al., 2012; Wei and Zhao, 2016). This domain is different from a SIM in that it binds the SUMO C-terminal tail through a large interface, while SIMs recognize a small surface area located far from the SUMO C terminus (Hannich et al., 2005; Mossessova and Lima, 2000). As expected, Sgs1-SuOn increased the levels of the Sgs1 sumoylated form in an E2-dependent manner so that it was readily detectable in cell extracts (Figures 5D and S4B). We also confirmed that the increased sumoylation levels mainly depended on K621, as sgs1-K621R-SuOn reduced sumoylation levels 3-fold compared with Sgs1-SuOn (Figure S4C). The partial effect is consistent with the fact that Sgs1 contains additional sumoylation sites (Lu et al., 2010). Importantly, the ratio of sumoylated to unmodified Sgs1 was higher in the Top3 pull-down fraction relative to the extract (Figure 5D), and the increase was largely dependent on K621 (Figure S4C). These data suggest that, although Top3 and Sgs1 interact without sumoylation, sumoylated forms of Sgs1, particularly those containing K621 conjugates, are preferentially enriched in complexes with Top3.

DISCUSSION

Recombination intermediates are carefully managed to prevent their accumulation in cells. This process requires not only STR/BTR and JM nucleases but also their regulators. While kinases and phosphatases control JM nuclease functions (Blanco et al., 2014; Eissler et al., 2014; Gallo-Fernández et al., 2012; Matos et al., 2011; Szakal and Branzei, 2013), how the STR dissolution functions are regulated has not been elucidated. In this study, several approaches were used to reveal a molecular connection of SUMO and Smc5/6 with STR. Our results suggest that Sgs1 interacts with SUMO and Smc5/6 and that these interactions promote the sumoylation of Sgs1, Top3, and Rmi1. Importantly, we have shown that mutants reducing STR sumoylation compromised JM removal and cell survival under high JM burdens. As *sgs1-sim* exhibited stronger defects than *sgs1-KR*, we infer that, in addition to Sgs1 sumoylation, Top3 and Rmi1 sumoylation also facilitate JM removal. Finally, our findings that sumoylation and Sgs1 SIMs aid Top3 and Rmi1 accrual at nuclear foci and that Top3 acquires enhanced association with sumoylated Sgs1 either directly or indirectly provide possible mechanisms for the effects of SUMO and Smc5/6 in JM removal.

Sumoylation is emerging as an important means for regulating early stages of DNA break repair in mammals (reviewed in Jackson and Durocher, 2013). Our work here presents an example of how SUMO also controls a key late step in recombination (Figure 5E). These findings extend the previously noted enrichment of sumoylated substrates and SUMO interactors among recombination proteins (Cremona et al., 2012; Psakhye and Jentsch, 2012). Together, they support the idea that multi-valent SUMO-SIM interactions constitute an important means for regulating both early and late steps of recombination. Such regulation can be achieved by modulating dynamic protein-protein interactions, local protein concentrations, and subnuclear compartments for DNA repair. Future efforts will be needed to delineate the rules governing the precise connections between specific SIMs and particular SUMO moieties.

Our findings on SUMO-based regulation of JM dissolution, in conjunction with phosphorylation-based control of JM resolution, suggest a bifurcated regulatory pathway attuned to the intracellular environment through distinct protein modifications. Since the nucleases generate both non-crossover and crossover products, with the latter more likely to embody deleterious genetic changes, while STR/BTR exclusively generates non-crossover products, STR/BTR plays a prominent role in JM removal during mitotic growth. Our observed SUMO and Smc5/6-mediated aid of the STR dissolution function likely plays an indispensable role in enabling this preference.

We have shown that sumoylation of Top3 and Rmi1 requires Sgs1 interaction and partially relies on the Sgs1 SIMs that are involved in Smc5 association and the Mms21 SUMO ligase activity. Considering the overlap among SUMO E3 activities (Johnson, 2004; Reindle et al., 2006; Silver et al., 2011), the partial effects in the latter two situations are expected. Indeed, we found that Siz2 makes a minor contribution to Top3 sumoylation, at least in *mms21* mutants. Sgs1 sumoylation follows principles that are similar to those of Top3/Rmi1 sumoylation, though the requirement for Mms21 is more relaxed. A lesser requirement of Mms21 for Sgs1 sumoylation under low MMS concentrations is reminiscent of the lack of such a requirement under lethal dosage of MMS (Branzei et al., 2006). Therefore, it is possible that, aside from Mms21, Sgs1 SIMs could contribute to Sgs1 sumoylation by other means, such as recruiting Ubc9 or other E3s (Figure 5E). Biochemical tests to provide further insights into the roles of Sgs1 SIMs are underway. Regardless, our data demonstrate a shared contribution of Sgs1 SIMs and Mms21 to the sumoylation of all three subunits of STR, connecting Smc5/6 and SUMO with STR at a molecular level.

These molecular connections also extend our understanding of the roles of Smc5/6, a poorly understood SMC complex. Findings here add to the previously noted function of Smc5/6 in preventing JM formation through inhibiting the DNA helicase Mph1 in replication fork regression and branch migration (Chen et al., 2009, 2013; Xue et al., 2014). Together, they suggest that, through regulating two DNA helicases, Smc5/6 is a master regulator of JM metabolism, with dual roles in JM formation and dissolution. Our findings are also consistent with previous studies showing that Smc5/6 is required for completing DNA replication and segregation, such as those analyzing late replication progression of *smc6* mutants, examining temperature-sensitive *smc6* alleles, and utilizing Smc5 fusion to a Clb2 module (e.g., Hang et al., 2015; Menolfi et al., 2015)

While our work focuses on understanding how Smc5/6 and SUMO affect STR dissolution function, the results do not exclude possible roles of STR sumoylation in other processes. The residual STR sumoylation in the *rad51Δ* and *rad54Δ* mutants is consistent with this view. Also, an effect of BLM sumoylation at an early step in recombination has been noted (Ouyang et al., 2013). Future studies will clarify other potential roles of STR sumoylation and establish whether the SUMO-mediated molecular effects uncovered here are generally applicable or whether there are additional effects, such as modulating STR interactions with DNA. Through these future endeavors, we will gain a comprehensive understanding of the regulation of STR/BTR functions and their relevance in human health and disease.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and General Procedures

Standard procedures were used for cell growth, medium preparation, epitope-tagging at endogenous loci, mutagenesis, dissection, spotting, and Y2H assays. Strains are isogenic to W1588-4C, a *RAD5* derivative of W303 (*MATa ade2-1 can1-100 ura3-1 his3-11,15 leu2-3,112 trp1-1 rad5-535*) (Zhao and Blobel, 2005). Strains and plasmids are listed in Table S1. At least two biological replicates were performed for each experiment. For details, see the Supplemental Experimental Procedures.

Detection of Protein Sumoylation

A well-established method wherein denaturing conditions throughout protein extract preparation minimize desumoylation (Ulrich and Davies, 2009) was used; details are given in the Supplemental Experimental Procedures. In each case, Ponceau S stain was shown to ensure equal loading. All tests used cells treated with 0.03% MMS for 2 hr, unless indicated otherwise. Note that, during this short-term treatment at low MMS concentration, wild-type cells achieve high viabilities.

Biochemical Methods

Protein extraction, immunoprecipitation, protein purification, in vitro sumoylation, and pull-down assays were performed as previously described (Chen et al., 2009; Zhao and Blobel, 2005; Xue et al., 2014). Details are described in the Supplemental Experimental Procedures. Antibodies used are: α-HA (F-7, Sc-7392, Santa Cruz Biotechnology; 3F10 Roche), α-V5 (R960-25, Invitrogen), α-myc (9E10, Bio X Cell), TAP (P1291, Sigma), α-Flag (M2, Sigma), α-Rad53 (yC-19, sc-6749, Santa Cruz Biotechnology), α-SUMO (Zhao and Blobel, 2005), and α-Rfa1 (a gift from S. Brill).

2D Gel and Live-Cell Imaging

2D gel analysis was performed as previously described (Branzei et al., 2006). *ubc9-10*, *sgs1-sim*, and wild-type cells were imaged on confocal fluorescence Leica or Zeiss wide-field microscopes using standard methods. Both procedures are described in detail in the Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Sgs1 interacts with the Smc5/6 SUMO ligase complex
- Smc5/6 promotes STR sumoylation upon recombination intermediate formation
- STR sumoylation aids recombination intermediate removal
- Sumoylation favors STR inter-subunit interactions and accumulation at DNA repair foci

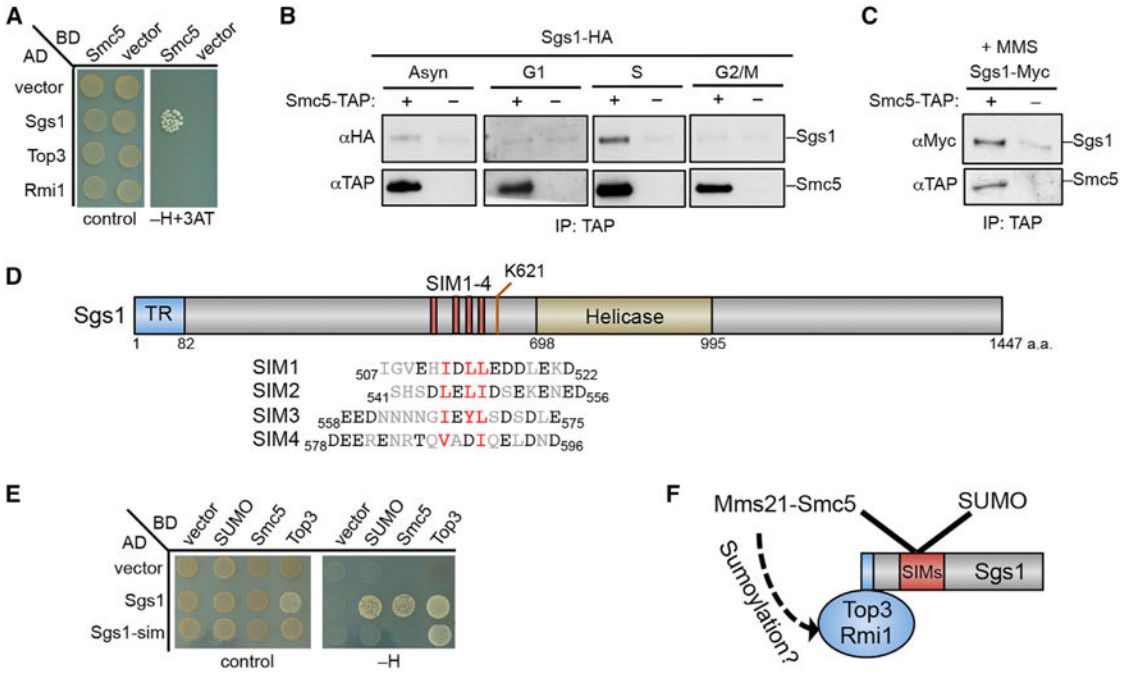

Figure 1. Sgs1 Associates with Smc5, and This Interaction Is Facilitated by Sgs1 SIMs

(A) Smc5 interacts with Sgs1 in Y2H assays. Reporter strains contained the DNA binding domain (BD) and DNA activation domain (AD) constructs. Cells were spotted onto SC-Trp-Leu (control) media for plasmid selection and on SC-Trp-Leu-His+3mM 3AT (−H+3AT) media to detect reporter activation.

(B and C) Smc5 interacts with Sgs1 in co-immunoprecipitation assays. Protein extracts from cells containing untagged (−) or TAP-tagged (+) Smc5 were subjected to immunoprecipitation (IP) using anti-TAP antibody. In (B), results for asynchronous cultures (Asyn) and cells arrested in G1 phase (alpha-factor treated), S phase (HU treated), and G2/M phase (nocodazole treated) are shown. Fluorescence-activated cell sorting (FACS) profiles indicating proper arrests are included in Figure S1C. In (C), the result for cells treated with 0.03% MMS for 2 hr is shown. Protein levels in the cell extracts are shown in Figures S1B and S1D.

(D) Schematic representation of Sgs1. Top: three features of Sgs1 relevant to the study: the N-terminal Top3/Rmi1 binding domain (TR), the middle region containing four putative SIMs and the major sumoylation site lysine 621, and the helicase domain. Bottom: the sequences of the four SIMs. Hydrophobic residues within the SIM consensus sequences (red) were mutated to alanine in *sgs1-sim* mutant. Acidic residues are indicated in black.

(E) Mutation of Sgs1 SIMs (sgs1-sim) reduces Y2H interactions with SUMO and Smc5. Experiments were performed and are presented as in (A).

(F) A model based on data in this figure. SIM-mediated interaction between Sgs1 and Smc5 may bring the Mms21 E3 subunit of the Smc5/6 complex in close proximity to the STR subunits and lead to sumoylation (see text for details).

See also Figure S1.

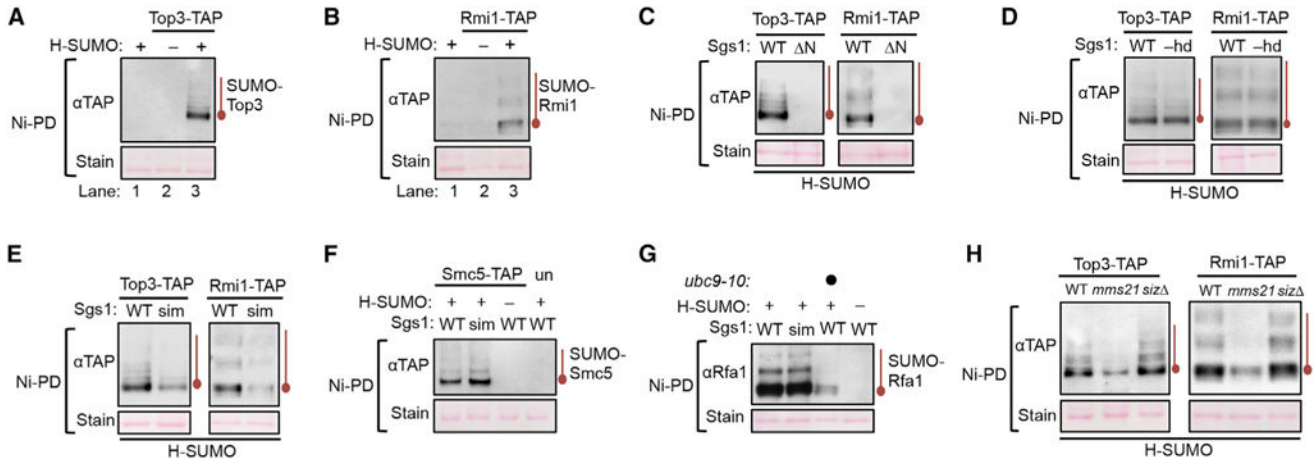

Figure 2. Optimal Top3 and Rmi1 Sumoylation Relies on Sgs1 Interaction, Sgs1 SIMs, and the Mms21 SUMO Ligase

(A and B) Top3 (A) and Rmi1 (B) are sumoylated upon treatment with 0.03% MMS. Sumoylated proteins were enriched with Ni-NTA resin in the presence of His8-tagged SUMO (H-SUMO, lane 3) but not untagged SUMO (lane 2). Sumoylated forms of TAP-tagged Top3 or Rmi1 were detected by immunoblotting against the tag and were not detected when untagged protein was used (lane 1). Mono-sumoylated band is denoted by a dot, and the bands containing more SUMO moieties are denoted by a line. Loading is shown by Ponceau S stain (stain). Similar methods and annotations are used in subsequent figure panels.

(C) The Top3-interacting region of Sgs1 is required for Top3 and Rmi1 sumoylation. The Sgs1-ΔN protein lacks the N-terminal Top3 interaction domain. WT, wild-type.

(D) Sgs1 helicase activity is not required for Top3 and Rmi1 sumoylation. The Sgs1-hd protein (−hd) contains the point mutation K706R in the helicase domain.

(E) Sumoylation of Top3 and Rmi1 is reduced in *sgs1-sim* mutant cells.

(F) The *sgs1-sim* mutant does not affect Smc5 sumoylation. As in (A), TAP-Smc5 sumoylation was examined by the Ni-PD method. Control tests using untagged SUMO (−) or untagged Smc5 (un) did not yield the modified bands.

(G) The *sgs1-sim* mutant does not affect Rfa1 sumoylation. Same as in (A), except Rfa1 was detected by anti-Rfa1 antibody, and the *ubc9-10* mutant was used to verify that the detected bands were sumoylated species.

(H) Sumoylation of Top3 and Rmi1 is reduced in the *mms21* mutant lacking SUMO ligase activity but not in cells lacking the Siz1 and Siz2 (*sizΔ*) SUMO E3 ligases.

See also Figure S2.

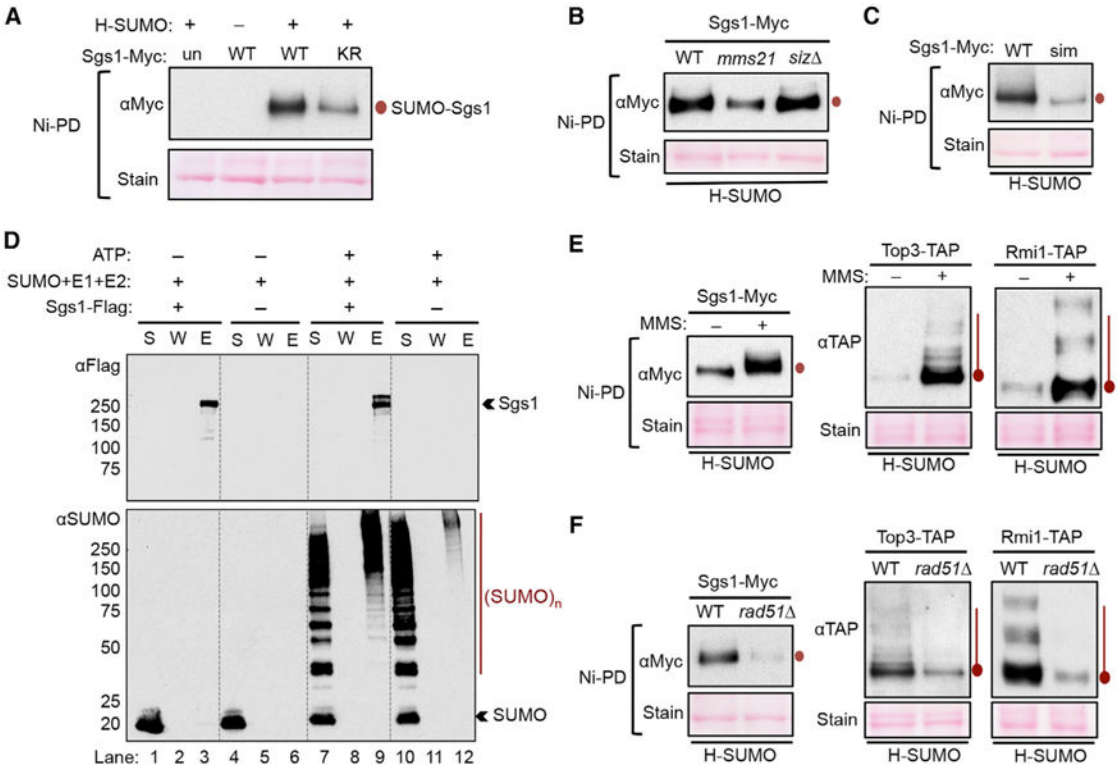

Figure 3. Optimal Sgs1 Sumoylation Requires Its SIMs and Mms21, and STR Sumoylation Largely Depends on Recombinase

(A) Sgs1 sumoylation is reduced when the major sumoylation site K621 is mutated. As in Figure 2A, sumoylated Myc-tagged Sgs1 was detected. No signal was detected in lanes with untagged Sgs1 (un) or untagged SUMO (−). Mono-sumoylated Sgs1 band is denoted by a dot. The same annotation is used in subsequent figure panels. WT, wild-type. KR, *sgs1-KR*.

(B) Sgs1 sumoylation is reduced in the *mms21* SUMO E3 mutant but is maintained in cells lacking Siz1 and Siz2 E3s (*siz Δ*).

(C) Sgs1 sumoylation is reduced in the *sgs1-sim* mutant.

(D) Purified Sgs1 interacts with high-molecular-weight poly-sumoylated species in vitro. Lanes 1–3 and 7–9: resin-bound FLAG-tagged recombinant Sgs1 was incubated with the indicated sumoylation reaction mixture with ATP (lanes 7–9) or without ATP (lanes 1–3). In both cases, Sgs1 was recovered from the resin in the eluted fraction (lanes 3 and 9), but poly-SUMO species, denoted as $(SUMO)_n$, were only recovered when the sumoylation reaction contained ATP (lane 9). In the control lanes (lanes 4–6 and 10–12), resin alone (no Sgs1) retained a small amount of poly-SUMO species in the presence of ATP (lane 12), due to non-specific binding of these molecules. Supernatant (S), wash (W), and elute (E) fractions were examined. All lanes are in the same gel; lines demarcate four groups of samples.

(E) Increased levels of STR subunit sumoylation are seen after treatment with 0.03% MMS for 2 hr, compared to untreated conditions.

(F) Maximal sumoylation of STR subunits is dependent on Rad51. Cells were treated with 0.03% MMS for 2 hr.

See also Figure S3.

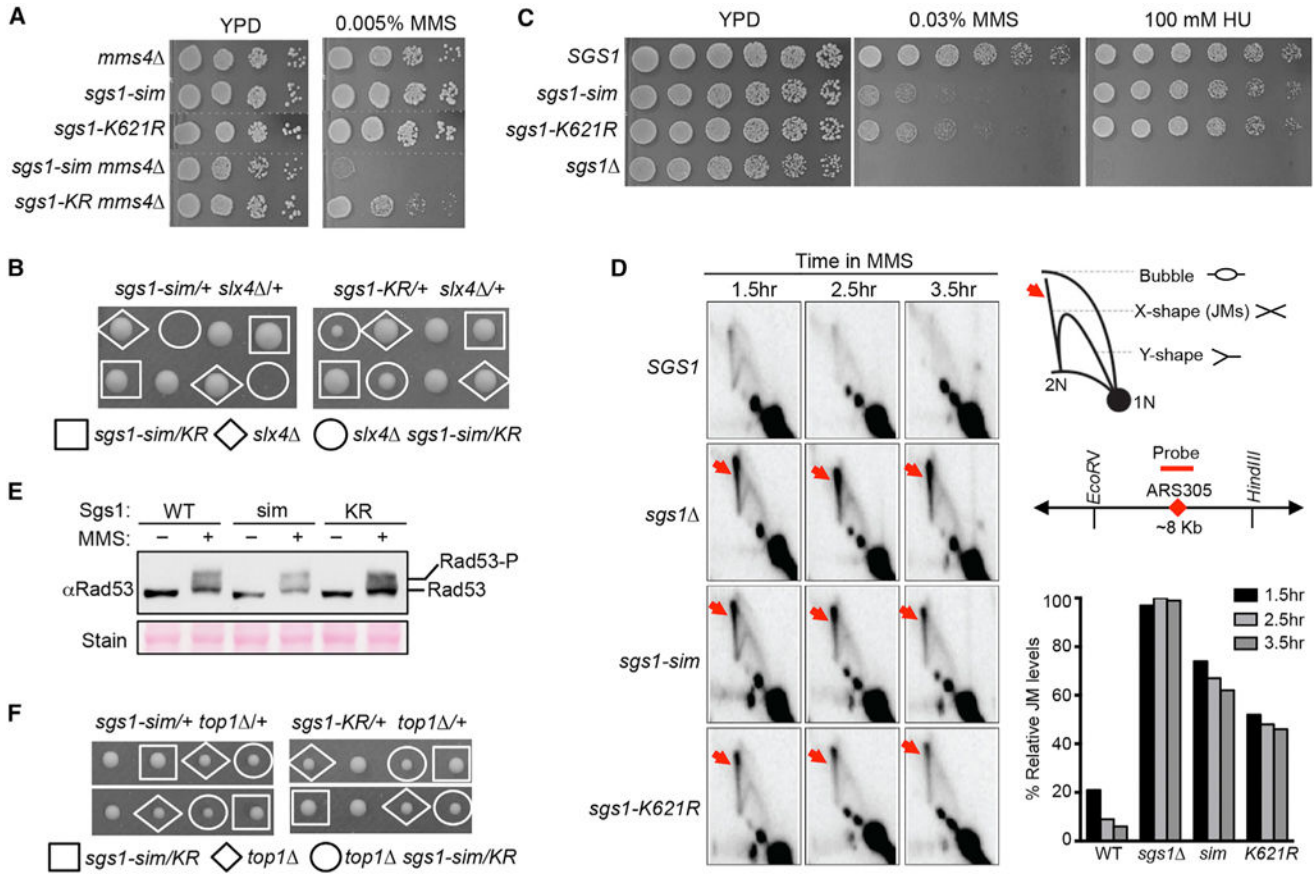

Figure 4. Sgs1 SIMs and Sumoylation Are Required for Coping with High JM Burdens and for Preventing JM Accumulation

(A) *sgs1-sim* and *sgs1-KR* sensitize cells lacking Mms4. Cells were spotted in 10-fold serial dilutions. The lines indicate the removal of superfluous rows. YPD, yeast peptone dextrose.

(B) Slx4 removal causes lethality and sickness in *sgs1-sim* and *sgs1-KR* cells, respectively. Representative tetrads from diploid strains with the indicated genotype are shown. Spore clones were grown at 30°C for 3 days, and genotypes are indicated. Note that *slx4Δ sgs1-sim* spore clones are inviable, and the sizes of spore clones of *slx4Δ sgs1-KR* are smaller than those of *slx4Δ* or *sgs1-KR* single mutants.

(C) *sgs1-sim* and *sgs1-KR* cells exhibit sensitivity to MMS but not to HU. Cells were spotted in 3-fold serial dilutions.

(D) Like *sgs1Δ*, *sgs1-sim* and *sgs1-KR* lead to increased levels of JMs as revealed by DNA 2D gel analysis. Left: nocodazole-arrested cells were released into media containing 0.033% MMS; samples were examined at the indicated time points. Arrows indicate JMs. Right: (top) schematic that depicts DNA structures visualized by 2D gel; (middle) schematic for ARS305, probe position, and the restriction enzyme sites used; (bottom) quantification of relative JM levels. WT, wild-type.

(E) *sgs1-sim* and *sgs1-KR* mutant cells show normal checkpoint response as indicated by Rad53 phosphorylation (Rad53-P). Cells of indicated genotypes were examined before and after treatment by 0.03% MMS for 2 hr.

(F) *sgs1-sim* and *sgs1-KR* do not sensitize cells lacking Top1. Same as in Figure 4B, but spore clones were grown at 30°C for 2 days. Note that the sizes of spore clones of *top1Δ sgs1-sim* or *top1Δ sgs1-KR* are similar to those of *top1Δ*.
See also Figure S3.

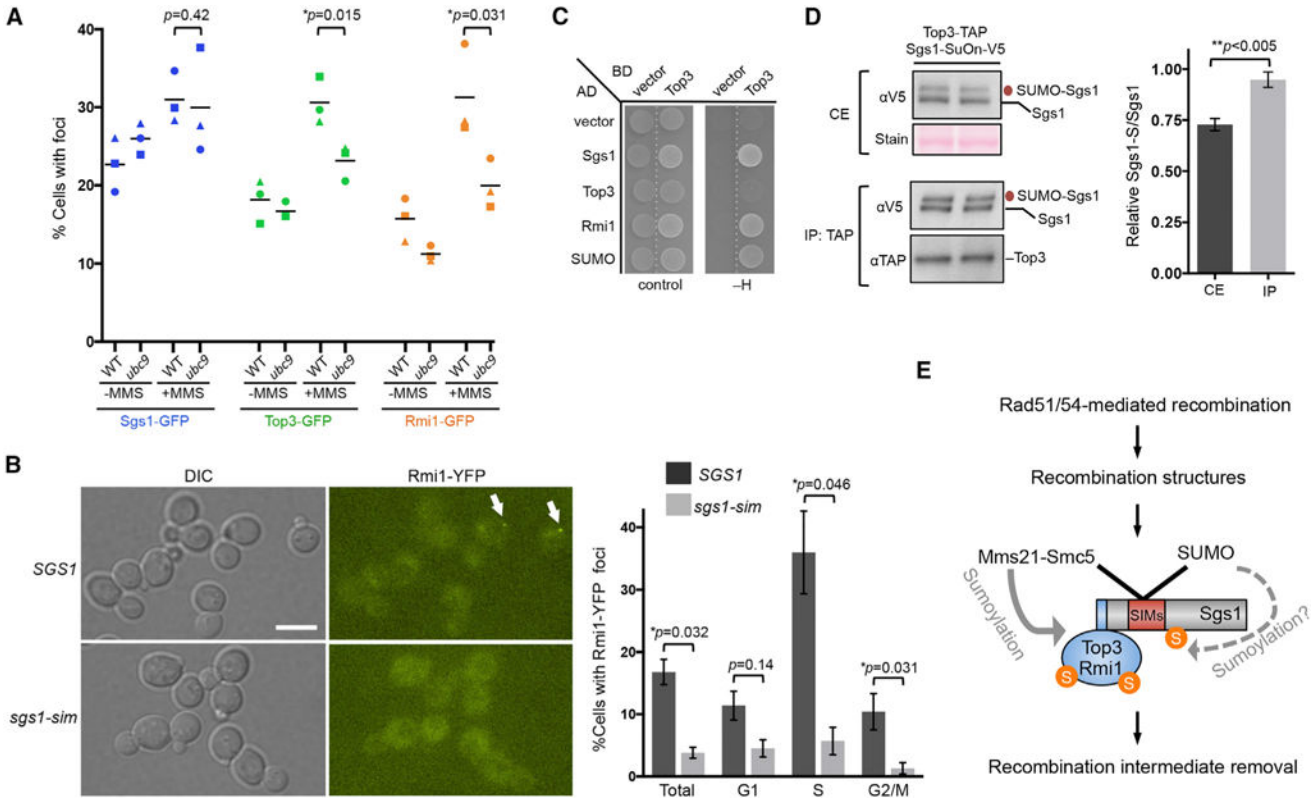

Figure 5. Sumoylation Positively Affects Top3 and Rmi1 Foci Levels and Sgs1-Top3 Association

(A) MMS-induced increase of Top3 and Rmi1 foci levels requires Ubc9. Wild-type (WT) and *ubc9-10* mutant cells were examined with or without treatment with 0.03% MMS for 2 hr. Percentages of cells containing foci in three independent trials are plotted. Horizontal bars denote the medians. The p values are from the Student's t test.

(B) Rmi1 foci levels are lower in *sgs1-sim* cells. YFP (yellow fluorescent protein)-tagged Rmi1 was examined upon MMS treatment. Left: representative images, arrows indicate foci. Scale bar, 5 μm. Right: the percentages of cells with Rmi1 foci were quantified from two trials and shown as mean ± SEM. The p values are from the Student's t test. Cell-cycle stages were based on bud size. DIC, differential interference contrast.

(C) Top3 interacts with SUMO in the Y2H assay. As in Figure 1A, Top3 showed interaction with Sgs1 and Rmi1 as expected and also with SUMO. The lines indicate the removal of superfluous rows. BD, DNA binding domain; AD, DNA activation domain.

(D) Top3 shows enhanced association with sumoylated Sgs1. Sgs1-SuOn exhibits increased sumoylation in cell extracts (CE). After immunoprecipitation (IP) of TAP-tagged Top3, the ratio of sumoylated to unmodified Sgs1 increases, compared to that in CE. Analyses from two trials are shown as mean ± SEM. The p values are from the Student's t test.

(E) Working model. During recombinational repair, Sgs1 interacts with SUMO and Smc5/6-Mms21 SUMO ligase, leading to increased STR sumoylation by Mms21. Sgs1 SIMs may also contribute to Sgs1 sumoylation through Mms21-independent means. Sumoylation of STR subunits promotes Sgs1-Top3 association and protein accrual at DNA repair foci and efficient JM removal.

See also Figure S4.