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Shu Proteins Promote the Formation of Homologous Recombination Intermediates That Are Processed by Sgs1-Rmi1-Top3[D]

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***CSM2*, *PSY3*, *SHU1*, and *SHU2* (collectively referred to as the *SHU* genes) were identified in *Saccharomyces cerevisiae* as four genes in the same epistasis group that suppress various *sgs1* and *top3* mutant phenotypes when mutated. Although the *SHU* genes have been implicated in homologous recombination repair (HRR), their precise role(s) within this pathway remains poorly understood. Here, we have identified a specific role for the Shu proteins in a Rad51/Rad54-dependent HRR pathway(s) to repair MMS-induced lesions during S-phase. We show that, although mutation of *RAD51* or *RAD54* prevented the formation of MMS-induced HRR intermediates (X-molecules) arising during replication in *sgs1* cells, mutation of *SHU* genes attenuated the level of these structures. Similar findings were also observed in *shu1* cells in which Rmi1 or Top3 function was impaired. We propose a model in which the Shu proteins act in HRR to promote the formation of HRR intermediates that are processed by the Sgs1-Rmi1-Top3 complex.**

INTRODUCTION

Homologous recombination repair (HRR) is a well-conserved cellular process for the repair of single-strand DNA (ssDNA) gaps and double-strand DNA breaks (DSBs) that can arise during DNA synthesis or as a result of replication fork stalling during S-phase. Although many of the key proteins involved in HRR have been identified (reviewed in Paques and Haber, 1999; Sung *et al.*, 2003; West, 2003; Krogh and Symington, 2004), in some cases their precise function(s) remains to be identified. Moreover, the mechanisms for suppression of inappropriate HRR in S-phase are only poorly defined. It is likely that HRR must be carefully regulated and/or executed in dividing cells, as inappropriate or excessive HR can lead to genome rearrangements and cancer in mammals. This is exemplified by the cancer-predisposition disorder, Bloom's syndrome, which is caused by mutation in the human *BLM* gene (reviewed in German, 1993). Because the BLM protein, in conjunction with its associated proteins, hTOPOIIIα and hRMI1 (Johnson *et al.*, 2000; Wu *et al.*, 2000; Yin *et al.*, 2005), can catalyze dissolution of HRR intermediates in vitro (Wu and Hickson, 2003; Plank *et al.*, 2006; Raynard *et al.*, 2006; Wu *et al.*, 2006), it is likely that unprocessed and/or aberrantly processed HRR intermediates at least partly contribute to the cellular defects in Bloom's syndrome. Indeed, Bloom's syndrome cells classically demonstrate elevated levels of sister chromatid exchanges, mitotic recombination, and genome instability. Mutation of the *BLM*, *hTOPOIIIα*, or *hRMI1* homologues in *Saccharomyces cerevisiae* (*SGS1*, *TOP3*, or *RMI1*, respectively) similarly causes sensitivity to genotoxic agents, hyper-recombination, and synthetic lethality with mutations in other genes also implicated in HRR (e.g., *MUS81* and *SRS2*; Gangloff *et al.*, 1994; Watt *et al.*, 1996; Gangloff *et al.*, 2000; Mullen *et al.*, 2001; Fabre *et al.*, 2002; Chang *et al.*, 2005; Mullen *et al.*, 2005). Furthermore, unresolved HRR intermediates have been directly visualized by two-dimensional (2D) gel electrophoresis in cells lacking Sgs1 or in cells with impaired Top3 function (Liberi *et al.*, 2005; Mankouri and Hickson, 2006). Interestingly, many of the cellular/phenotypic defects observed in *sgs1*, *top3*, or *rmi1* cells can be suppressed by deletion of genes that control the early steps of HRR (e.g., *RAD52*, *RAD51*, *RAD55*, *RAD57*, and *RAD54*; Gangloff *et al.*, 2000; Fabre *et al.*, 2002; Oakley *et al.*, 2002; Shor *et al.*, 2002; Chang *et al.*, 2005; Mullen *et al.*, 2005). Taken together, these observations suggest that excessive, unscheduled, or incomplete HRR can create toxic DNA repair intermediates, and highlights the requirement for cells to carefully regulate HRR during S-phase.

CSM2, *PSY3*, *SHU1*, and *SHU2* (collectively referred to as the *SHU* genes) were identified recently as four novel genes within the same epistasis group that, when mutated, can suppress various defects in *sgs1* or *top3* mutants (Shor *et al.*, 2005). Interestingly, the single *csm2*, *psy3*, *shu1*, or *shu2* mutants all demonstrate similar phenotypes (a mutator phenotype and moderate sensitivity to methyl methane sulfonate (MMS) and cross-linking agents), and mutation of all four does not cause any additive effects (Shor *et al.*, 2005). Taken together with the demonstration that all four *SHU* gene products interact in a two-hybrid assay, it has been proposed that these proteins exist in a multimeric complex that fails to function when any one member is missing (Shor *et*

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Abbreviations used: DSB, double-strand break; FACS, fluorescent-activated cell sorting; HRR, homologous recombination repair; HU, hydroxyurea; MMS, methyl methane sulfonate.

al., 2005). It is likely that the Shu complex is involved in some aspect of HRR, because mutation of *RAD52*, which abolishes all types of HRR (Symington, 2002), is epistatic to *shu* mutations for MMS sensitivity and a mutator phenotype (Shor *et al.,* 2005). However, it is unlikely that the Shu proteins are bona fide members of the core HRR machinery. Unlike canonical HRR mutants (e.g., *rad52*, *rad51*, *rad54*, *rad55*, and *rad57*), the *shu* mutants are not appreciably sensitive to hydroxyurea (HU) or gamma-rays (Shor *et al.,* 2005). Therefore, unlike classic HRR proteins, Shu proteins are apparently not essential for the repair of DSBs or collapsed replication forks. Nevertheless, mutation of the *SHU* genes does affect some aspect of HRR, because DNA damage-induced Rad52 foci persist for longer in the nuclei of *shu1* cells exposed to MMS as compared with wild-type cells (Shor *et al.,* 2005).

Further investigation of the *SHU* genes in *S. cerevisiae* is likely to be relevant to all eukaryotes, because putative *PSY3* and *SHU2* homologues have recently been identified in *Schizosaccharomyces pombe* (*rld1*$^+$ and *sws1*$^+$, respectively) and human cells (*RAD51D* and *SWS1*, respectively; Martin *et al.,* 2006). *RAD51D* is one of the so-called human *RAD51* paralogs, based on some shared sequence similarity to *RAD51* (Cartwright *et al.,* 1998a; Kawabata and Saeki, 1998; Pittman *et al.,* 1998). Interestingly, like *psy3* and *shu2* mutations in *S. cerevisiae*, mutation of *rld1*$^+$ or *sws1*$^+$ in *S. pombe* also causes sensitivity to MMS (but not to other types of DNA damage) and rescues various cellular defects caused by mutation of the RecQ helicase gene, *rqh1*$^+$, in *S. pombe* (Martin *et al.,* 2006). Furthermore, Sws1 associates in vivo with Rld1 and a novel protein, Rlp1, which shows sequence similarity to another of the human *RAD51* paralogs, *XRCC2* (Cartwright *et al.,* 1998b; Liu *et al.,* 1998; Martin *et al.,* 2006). Therefore, Sws1, Rld1, and Rlp1 appear to be part of a multimeric complex in *S. pombe*, which is similar to the complex proposed to exist in *S. cerevisiae* comprising Csm2, Psy3, Shu1, and Shu2 (Shor *et al.,* 2005). Interestingly, SWS1 and XRCC2 associate with RAD51D in human cells (Braybrooke *et al.,* 2000; Martin *et al.,* 2006), and RAD51D can bind to BLM (Braybrooke *et al.,* 2003), suggesting that Shu-like complexes also exist in human cells and therefore probably perform an evolutionarily conserved role. Ablation of SWS1 in human cells reduces the number of RAD51 foci in both control and IR-treated human cells (Martin *et al.,* 2006), suggesting that SWS1, like its yeast counterparts, is involved in some aspect of HRR.

It is likely that a modulation of HRR is responsible for the suppression of *sgs1/top3* phenotypes by *shu* mutations (*S. cerevisiae*) or *rqh1* phenotypes by *rld1/rlp1/sws1* mutations (*S. pombe*). In *S. cerevisiae*, mutation of *SHU1* suppresses the increased rate of recombination and elevated (spontaneous) Rad52 foci in *sgs1* and *top3* cells (Shor *et al.,* 2005). A similar scenario exists in *S. pombe*, where *sws1* and *rlp1* mutations reduce the increased recombination rate and elevated number of nuclei containing spontaneous Rad22 foci in *rqh1* mutants, without apparently affecting the outcome of HRR (Martin *et al.,* 2006). Taken together, these data are consistent with the Shu complex in *S. cerevisiae* and the Sws1, Rld1, and Rlp1 complex in *S. pombe*, somehow modulating HRR in cells lacking RecQ helicases or Topoisomerase III. However, the molecular mechanisms underlying this suppression remain to be clarified.

We sought to identify the mechanism by which the *shu* mutations suppress *sgs1* and *top3* defects in *S. cerevisiae*. We demonstrate that, in addition to suppressing *sgs1* or *top3* phenotypes (Shor *et al.,* 2005), *shu1* mutation also suppresses the poor growth caused by the deletion of *RMI1* or the overexpression of a dominant-negative allele of *TOP3* (*TOP3*^{Y356F}). Consistent with a role for the Shu proteins in HRR (Shor *et al.,* 2005), we demonstrate that Shu1 acts in the Rad51/Rad54-dependent HRR repair of MMS lesions, upstream of the Sgs1-Rmi1-Top3 complex. Interestingly, we demonstrate that, unlike mutation of *RAD51* (or *RAD54*), mutation of *SHU* genes does not prevent unresolved HRR intermediates from persisting in cells compromised for Sgs1, Rmi1, or Top3. However, the level of unresolved HRR intermediates was attenuated to some extent by *shu* mutations. We propose that Shu proteins perform a nonessential role in HRR to promote the formation of HRR intermediates that are substrates for Sgs1-Rmi1-Top3.

MATERIALS AND METHODS

S. cerevisiae Strains and Plasmids

All strains were isogenic derivatives of T344 or BY4741. All strains carrying gene deletions were either obtained from the yeast deletion collection (EUROSCARF, University of Frankfurt, Germany) or constructed using a PCR-based gene disruption method (Wach *et al.,* 1994). The *rmi1*Δ haploid strain was obtained by sporulating a heterozygous *RMI1*$^{+/-}$ diploid strain. All haploid *rmi1* strains used for our experimental analyses were confirmed to demonstrate a slow-growth phenotype (Chang *et al.,* 2005; Mullen *et al.,* 2005). The pYES2-*TOP3* and pYES2-*TOP3*^{Y356F} plasmids have been described previously (Oakley *et al.,* 2002; Mankouri and Hickson, 2006).

Growth Conditions, Cell Synchronization, and Flow Cytometry Analysis

Strains were grown and synchronized with α-factor mating pheromone as described previously (Mankouri and Hickson, 2006). After release from α-factor arrest, all experiments were performed at 25°C. Release from MMS treatment was achieved by centrifugation, washing, and resuspension of cells in drug-free medium. Cell cycle progression was monitored using flow cytometry (fluorescent-activated cell-sorting [FACS]) as described previously (Mankouri and Hickson, 2006).

2D Gel Electrophoresis

The hexadecyltrimethylammonium bromide (CTAB) method of DNA extraction and 2D gel procedures were described previously (Brewer and Fangman, 1987; Allers and Lichten, 2000; Lopes *et al.,* 2003; Liberi *et al.,* 2006). DNA was digested with NciI and NcoI before running the first-dimension gels. Quantification of X-shaped molecules was performed using Image Quant analysis software (Molecular Dynamics, Sunnyvale, CA). "Object Average" mode of background correction was used and then each X-molecule was normalized with respect to the monomer (1N) spot, to provide an arbitrary value of X-molecule intensity.

RESULTS

SHU Mutations Are Epistatic with rad51 and rad54 for Suppression of TOP3^{Y356F}*-induced Poor Growth*

We demonstrated previously that overexpression of a catalytically dead mutant of *TOP3*, *TOP3*^{Y356F}, causes a dominant-negative *top3*-like phenotype (Oakley *et al.,* 2002; Mankouri and Hickson, 2006). Because the *SHU* genes (*CSM2*, *PSY3*, *SHU1,* and *SHU2*) were initially identified as suppressors of *top3* (Shor *et al.,* 2005), we investigated if *SHU1* mutation could also suppress the poor growth caused by overexpression of *TOP3*^{Y356F} in the T344 strain (Hovland *et al.,* 1989). Consistent with the demonstration that *shu* mutations suppress *top3* poor growth (Shor *et al.,* 2005), we found that mutation of *SHU1* also partially suppressed the poor growth caused by overexpression of *TOP3*^{Y356F} (Figure 1A). Interestingly, we noted that the partial suppression of *TOP3*^{Y356F}-induced poor growth was reminiscent of that previously caused by mutation of the HRR gene, *RAD51* (Mankouri and Hickson, 2006). We therefore asked if a combination of *shu1* and *rad51* mutations caused additive suppression of *TOP3*^{Y356F}-induced poor growth. For comparison, we also compared the effects of mutating *RAD54*, which functions in *RAD51*-dependent HRR (Rattray and Symington, 1995;

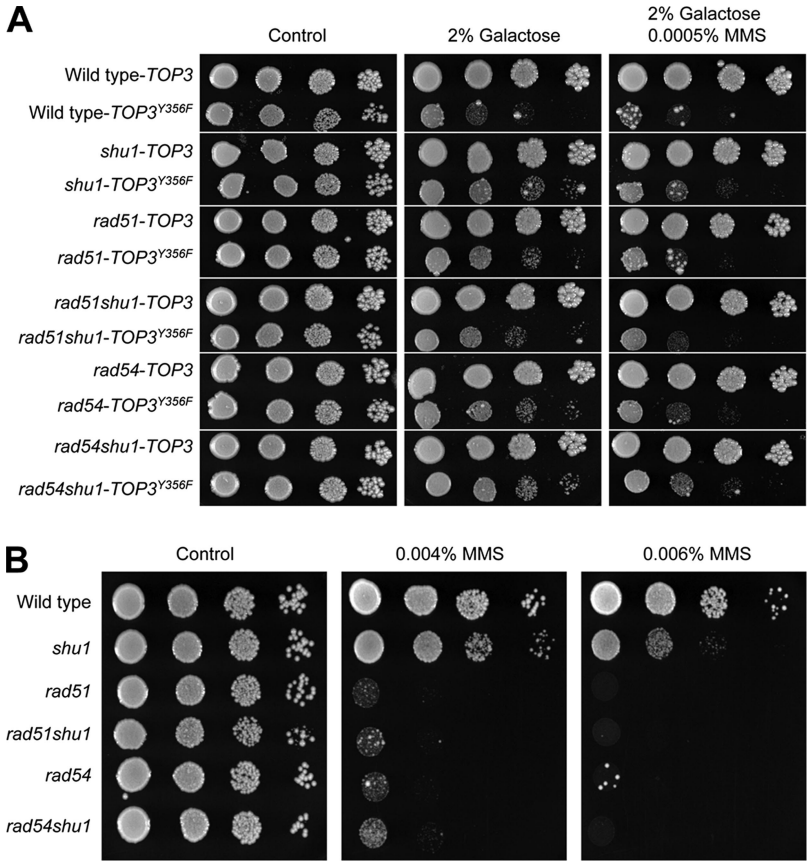

Figure 1. *SHU1, RAD51,* and *RAD54* are epistatic for suppression of $TOP3^{Y356F}$-induced poor growth and MMS sensitivity. (A) Wild-type, *shu1*, *rad51*, *rad51shu1*, *rad54,* and *rad54shu1* strains transformed with pYES2-*TOP3* or pYES2-$TOP3^{Y356F}$ were diluted to equivalent densities and spotted onto control plates or plates containing 2% galactose (to induce expression from the pYES2 *GAL1* promoter). Spots from left to right represent serial 1 in 10 dilutions of yeast cultures. Plates were grown at 30°C. The large single colonies that arise in strains overexpressing $TOP3^{Y356F}$ represent suppressors (probably *SGS1*-linked) as seen previously (Oakley *et al.,* 2002; Mankouri and Hickson, 2006). (B) Wild-type, *shu1*, *rad51*, *rad51shu1*, *rad54,* and *rad54shu1* strains were diluted to equivalent densities and spotted onto control plates (no drug) or plates containing MMS. Spots from left to right represent serial 1 in 10 dilutions of yeast cultures. Plates were grown at 30°C.

Zhang *et al.,* 2007) and also partially suppresses *top3* poor growth when mutated (Oakley *et al.,* 2002; Shor *et al.,* 2002). Interestingly, we observed that *rad51*, *rad54* and *shu1* mutations all demonstrated a similar partial ability to suppress $TOP3^{Y356F}$-induced poor growth (Figure 1A). Furthermore, this suppression was not additive in *rad51shu1* or *rad54shu1* double mutants. Therefore, we propose that *SHU1*, *RAD51,* and *RAD54* are epistatic for suppression of $TOP3^{Y356F}$-induced poor growth. These data are consistent with a proposed role for the Shu proteins in *RAD52*-dependent HRR (Shor *et al.,* 2005). However, because mutation of *RAD52* abolishes all types of HRR repair (Symington, 2002), some of which are Rad51-independent, our data implies that, more specifically, Shu1 is involved in Rad51- and Rad54-dependent branch of HRR.

Shu1 Functions in RAD51- and RAD54-dependent Homologous Recombination Repair of MMS-induced Lesions

We investigated further the genetic relationship between *SHU1* and the HRR genes, *RAD51* and *RAD54*. Rad51 catalyzes the early strand invasion step of HRR, whereas Rad54 likely acts at multiple stages during HRR (reviewed in Sung *et al.,* 2003; Tan *et al.,* 2003; Heyer *et al.,* 2006). However, Rad54 probably acts alongside Rad51 early in HRR, because Rad54 stimulates Rad51 in DNA pairing reactions (Petukhova *et al.,* 1998; Zhang *et al.,* 2007). We confirmed that *rad51* and *rad54* mutants demonstrate a similar level of sensitivity to MMS (Figure 1B). We also confirmed that, in agreement with Shor *et al.* (2005), all four *shu* mutants were sensitive to MMS, but were not sensitive to HU in our (T344) strain background (see Figure 3A). Interestingly, we observed that *rad51shu1* and *rad54shu1* mutants did not demonstrate any additive sensitivity to MMS (Figure 1B and see Figure 3C). Rather, if anything, mutation of *SHU1* produced a very slight suppression of the MMS sensitivity of *rad51* or *rad54* mutants. Similar results were also observed in the BY4741 genetic background (data not shown; St. Onge *et al.,* 2007). Because *rad51shu1* or *rad54shu1* strains were no more sensitive to MMS than *rad51* or *rad54* strains, we conclude that the Shu proteins act in the same pathway as Rad51 and Rad54 for repair of MMS-induced DNA lesions.

Our previous data demonstrated that the mutation of *RAD51* causes an impaired ability to traverse S-phase in the presence of MMS (Mankouri and Hickson, 2006). This phenotype is a consequence of a persistent activation of the DNA damage checkpoint, because this phenotype can be overridden by addition of caffeine (Mankouri and Hickson, 2006). Interestingly, mutation of *RAD52* also impairs S-phase progression in the presence of MMS (Oakley *et al.,* 2002), and substitution of phosphorylation sites targeted by checkpoint kinases in Rad55 causes defects in cell cycle resumption after MMS treatment (Herzberg *et al.,* 2006). Taken together, these findings suggest that impaired S-phase progression in the presence of MMS may be a general property of HRR-defective mutants. To explore this possibility further, we examined if mutation of *RAD54* or *SHU1* affects S-phase progression in the presence of MMS. The strains used in Figure 1B were synchronously released from G1 arrest into fresh medium and DNA content was analyzed at regular intervals using flow cytometry (FACS). Under these unperturbed conditions, all strains progressed through S-phase (as measured by a doubling of DNA content) by ~60 min (Figure 2A), with only a very modest increase in S-phase duration ob-

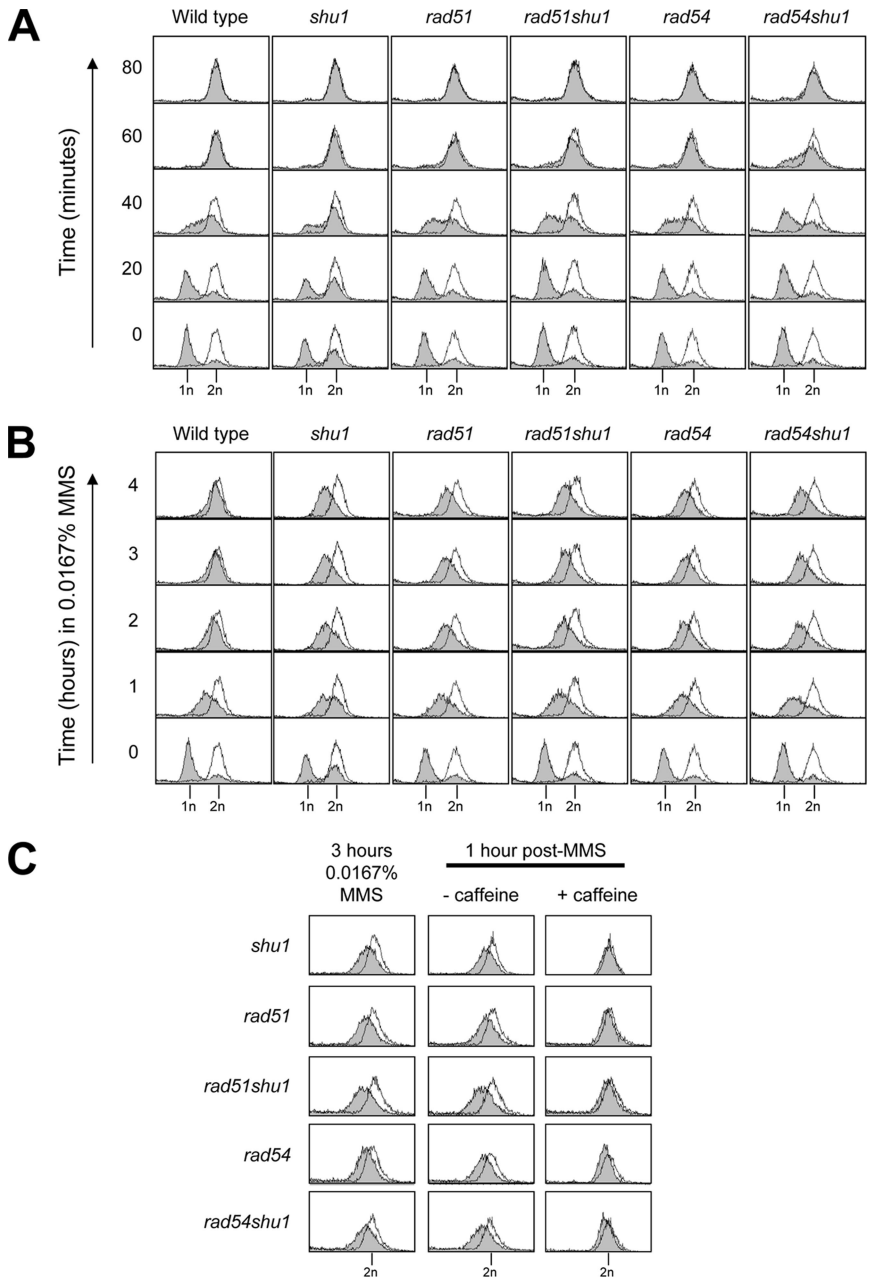

Figure 2. Shu1 functions in a Rad51/Rad54-dependent pathway to repair/tolerate MMS-induced lesions during S-phase. (A and B) Wild-type, *shu1*, *rad51*, *rad51shu1*, *rad54*, and *rad54shu1* strains were released from G1 arrest into fresh medium (A) or medium containing 0.0167% MMS (B). DNA content was analyzed by flow cytometry at the indicated times. The shaded peaks represent experimental data, whereas the unshaded peak is a reference to indicate a normal G2/M peak (at 2 h release from G1 arrest). The positions of the 1n (G1) and 2n (G2/M) peaks are indicated below. (C) After 3 h treatment with 0.0167% MMS, 1-ml aliquots of cells were taken, and caffeine was added to a final concentration of 5 mg/ml, or an equivalent amount of fresh medium was added. Samples were taken 1 h later for analysis of DNA content by flow cytometry.

served in *rad51shu1* and *rad54shu1* double mutants (note DNA content at 40 min). In the presence of 0.0167% MMS, wild-type cells successfully traversed S-phase by ~2 h (Figure 2B). This prolongation of S-phase by MMS (relative to unperturbed cells) is a consequence of MMS-induced replication fork stalling and subsequent checkpoint activation (Paulovich and Hartwell, 1995; Santocanale and Diffley, 1998; Shirahige *et al.*, 1998; Tercero and Diffley, 2001). In agreement with our previous data (Mankouri and Hickson, 2006), *rad51* cells failed to completely traverse S-phase by 4 h in the presence of 0.0167% MMS, demonstrating a mid-S-phase DNA content at 4 h (Figure 2B). Interestingly, we observed that *rad54* and *shu1* mutants resembled *rad51* mutants and also failed to traverse S-phase by 4 h in 0.0167% MMS (Figure 2B). Furthermore, like *rad51* cells (Mankouri and Hickson, 2006), the impaired S-phase progression phenotype of MMS-treated *rad54* or *shu1* mutants could be overridden by addition of caffeine (Figure 2C). Therefore, these data imply that a more persistent or robust checkpoint activation occurs after MMS treatment in cells lacking Rad51, Rad54, or Shu1. Similar results were also observed for the other *shu* mutant strains (data not shown), consistent with the similar phenotypes caused by mutation of any one of the *SHU* genes (Figure 3A; Shor *et al.*, 2005). Interestingly, *rad51shu1* or *rad54shu1* double mutants did not demonstrate additive impaired S-phase progression phenotypes in the presence of MMS, consistent with the proposed epistasis between these genes (Figure 1). Furthermore, the impaired S-phase progression in MMS-treated *rad51shu1* or *rad54shu1* double mutants could be overridden by caffeine (Figure 2C). We propose that Rad51, Rad54, and Shu1 all act at a similar step in a common pathway for the repair of MMS-induced DNA lesions during S-phase and that perturbation/inactivation

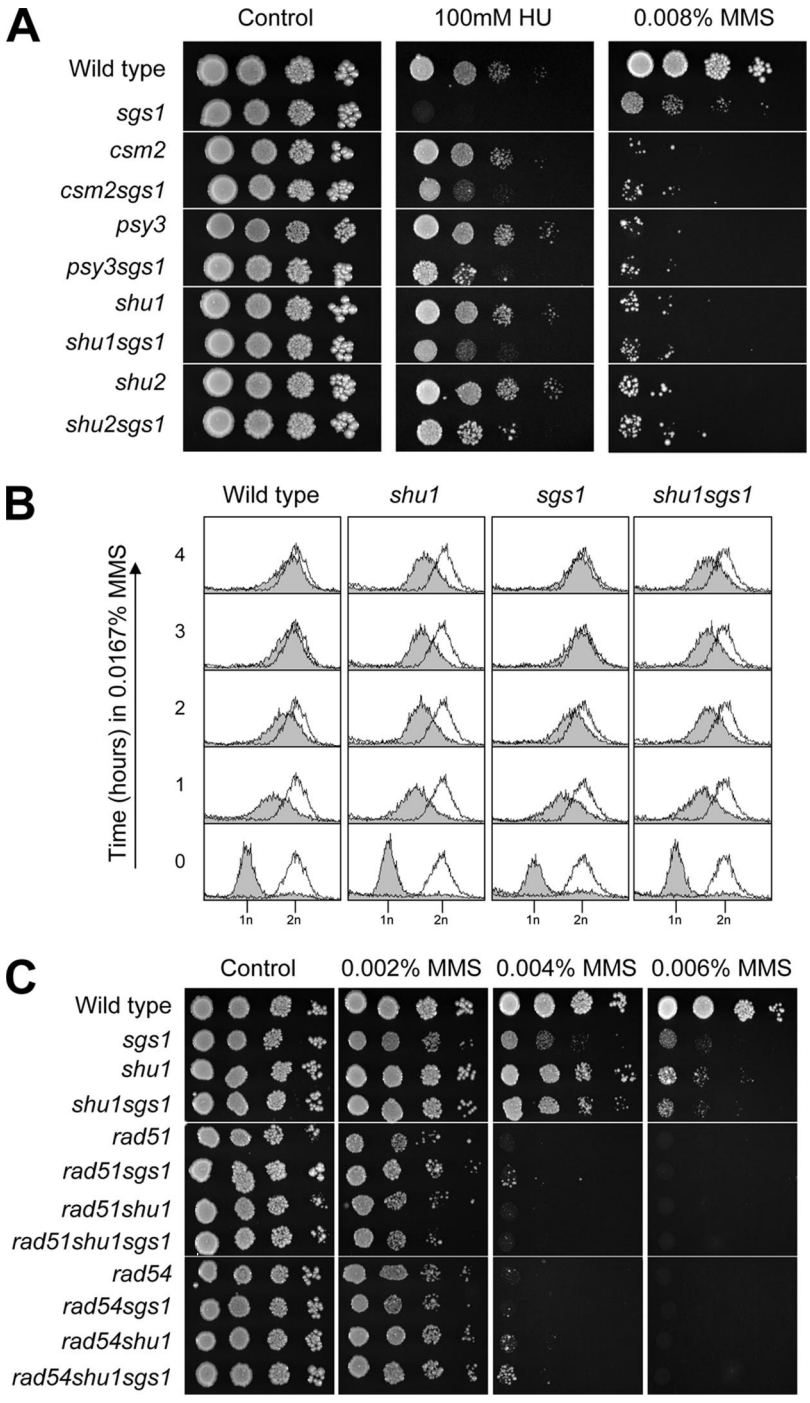

Figure 3. *shu1* mutation suppresses *sgs1* HU sensitivity and is epistatic to *sgs1* for MMS sensitivity. (A) Wild-type, *sgs1*, *csm2*, *csm2sgs1*, *psy3*, *psy3sgs1*, *shu1*, *shu1sgs1*, *shu2*, and *shu2sgs1* strains were diluted to equivalent densities and spotted onto control (no drug) plates or plates containing indicated doses of MMS or HU. Spots from left to right represent serial 1 in 10 dilutions of yeast cultures. Plates were grown at 30°C. (B) Wild-type, *shu1*, *sgs1*, and *shu1sgs1* strains were released from G1 arrest into fresh medium containing 0.0167% MMS. DNA content was analyzed by flow cytometry at the indicated times, as in Figure 2. (C) Wild-type, *sgs1*, *shu1*, *shu1sgs1*, *rad51*, *rad51sgs1*, *rad51shu1*, *rad51shu1sgs1*, *rad54*, *rad54sgs1*, *rad54shu1*, and *rad54shusgs1* strains were diluted to equivalent densities and spotted onto control (no drug) plates or plates containing MMS. Spots from left to right represent serial 1 in 10 dilutions of yeast cultures. Plates were grown at 30°C.

of this pathway leads to accumulation of an HRR substrate that causes persistent checkpoint activation.

shu Mutations Suppress sgs1 HU Sensitivity and Are Epistatic to sgs1 for MMS Sensitivity

Because *shu* mutations suppress various phenotypic defects caused by mutation of *SGS1* (Shor *et al.*, 2005), we further investigated the relationship between the *SHU* genes and *SGS1*. Previous data demonstrated that mutation of *SHU* genes suppresses the HU sensitivity of *sgs1* cells (Shor *et al.*, 2005). However, in the study of Shor *et al.* (2005) it was shown that *csm2sgs1* and *shu1sgs1* mutants exhibited additive sensitivity to MMS, suggesting that Sgs1 and the Shu proteins function in separate pathways for repair of MMS-induced DNA lesions. We verified that our *sgs1* strain demonstrated sensitivity to both HU and MMS, whereas mutation of *SHU* genes conferred sensitivity to MMS alone (Figure 3A). We also verified that, in agreement with Shor *et al.* (2005), mutation of all four *shu* genes (similarly) suppressed the HU sensitivity of *sgs1* cells (Figure 3A). However, in contrast to Shor *et al.* (2005), we observed that *shu* mutations were epistatic to *sgs1* for MMS sensitivity, because *shu sgs1* double mutants resembled the *shu* single mutants on plates containing MMS (Figure 3A). Although

shu1 mutation appears to suppress *sgs1* MMS sensitivity at low doses of MMS (0.0040%; see Figure 3C), we observed that *shu* (and *shu sgs1* double) mutants were more sensitive to MMS than *sgs1* mutants at higher doses (0.0080%; Figure 3A).

To further investigate this apparent discrepancy, and to test for any strain-dependent variations, we investigated the genetic relationship between *SHU1* and *SGS1* in a third strain background, BY4741. In this background, mutation of *SHU1* again suppressed *sgs1* HU sensitivity (data not shown). However, in agreement with a recent independent study (St. Onge *et al.*, 2007), we observed that mutation of *SHU1* suppressed *sgs1* MMS sensitivity in the BY4741 genetic background (data not shown). We propose that this apparent inconsistency between our T344 and BY4741 *shu1sgs1* strains can be reconciled by the fact that *shu* mutants are more sensitive to MMS than are *sgs1* strains in the T344 background, whereas *sgs1* mutants are more sensitive to MMS than are *shu* mutants in the BY4741 background. In both of the strain backgrounds that we analyzed, the phenotype of the *shu sgs1* double mutant mimics the phenotype of the corresponding *shu* single mutant. Therefore, the additive sensitivity of *shu1sgs1* double mutants to MMS as reported by Shor *et al.* (2005) likely reflects a strain-specific effect. Taken together, our data implies that, despite apparent strain-dependent differences in MMS sensitivity, *shu* mutations are epistatic to *sgs1* for MMS sensitivity. Moreover, our data are consistent with the Shu proteins acting upstream of Sgs1.

To further examine the relationship between *SHU1* and *SGS1* in the repair of MMS-induced DNA lesions, we examined S-phase progression in wild-type, *sgs1*, *shu1* and *shu1sgs1* cells in the presence of 0.0167% MMS. We observed that all strains demonstrated similar S-phase kinetics in unperturbed cells (data not shown). In the presence of 0.0167% MMS, however, wild-type and *sgs1* cells completed DNA replication by 3 h, whereas *shu1* cells failed to complete S-phase by 4 h (Figure 3B). Therefore, in agreement with previous data (Mankouri and Hickson, 2006), *sgs1* cells do not demonstrate impaired S-phase progression in the presence of 0.0167% MMS. Consistent with the genetic relationship observed between *SHU1* and *SGS1* (Figure 3A), we observed that *shu1sgs1* cells resembled *shu1* cells in failing to complete DNA replication by 4 h in 0.0167% MMS (Figure 3B). Similar results were also observed in the BY4741 strain background (data not shown), consistent with our proposal that *shu1* is epistatic to *sgs1* for MMS sensitivity in both the T344 and BY4741 strain backgrounds. These findings were not limited to *shu1*, because *csm2*, *csm2sgs1*, *psy3*, *psy3sgs1*, *shu2,* and *shu2sgs1* mutants all demonstrated a similar degree of impaired S-phase progression in the presence of MMS to that of *shu1* or *shu1sgs1* mutants (data not shown). Furthermore, in each *shu* single mutant or *shu sgs1* double mutant, the impaired S-phase progression in the presence of MMS could be overridden by addition of caffeine (Figure 2C and data not shown). Therefore, loss of any one of the Shu proteins causes persistent checkpoint activation after treatment with MMS, regardless of whether Sgs1 is present or not. Taken together with the fact that *shu* mutations are epistatic to *sgs1* for MMS sensitivity (Figure 3A), these findings are consistent with a role for the Shu complex upstream of Sgs1 in the same pathway to repair MMS-induced DNA lesions during S-phase.

To further investigate the relationship between Shu1, Sgs1, and the HRR pathway, we examined the effects of deleting *RAD51* and *RAD54*. Interestingly, we observed that, similar to what was observed when *SHU1* was mutated (Figure 3B), mutation of *RAD51* or *RAD54* impaired the S-phase progression of *sgs1* mutants (Supplementary Figure 1). Consistent with our proposal that the impaired S-phase progression phenotype of a *shu1* mutant is not additive with *rad51* or *rad54* mutations, we observed that *rad51shu1sgs1* and *rad54shu1sgs1* triple mutants demonstrated rates of (impaired) S-phase progression in the presence of MMS similar to that of *rad51sgs1*, *rad54sgs1,* or *shu1sgs1* double mutants (Supplementary Figure 1). Furthermore, *rad51shu1sgs1* and *rad54shu1sgs1* triple mutants were no more sensitive to MMS than *rad51* or *rad54* single mutants, respectively (Figure 3C). This apparent lack of additive phenotypes is consistent with the proposal that Rad51/Rad54, Shu1, and Sgs1 are all epsitatic for MMS sensitivity. We conclude that Rad51, Rad54, Shu1, and Sgs1 all function in the same (HRR) pathway to repair MMS lesions during S-phase and that Sgs1 acts at a later stage than Rad51, Rad54, or Shu1.

Formation of MMS-induced X-Molecules in Cells Lacking SGS1 Is Abolished by Mutation of RAD51 or RAD54, and Is Attenuated by Mutation of SHU Genes

Previous studies indicated that *sgs1* cells exhibit persistent, Rad51-dependent, HRR intermediates on 2D gels after exposure to MMS (Liberi *et al.*, 2005). On the basis of our proposal that the Shu proteins act upstream of Sgs1 in the same HRR pathway to repair MMS-induced lesions, we examined whether mutation of the *SHU* genes prevents the accumulation of HRR intermediates in MMS-treated *sgs1* cells. To confirm that *sgs1* mutants demonstrate Rad51-dependent X-shaped molecules after exposure to MMS in the T344 strain background, we analyzed DNA replication intermediates on 2D gels originating from an early firing origin, *ARS305*, in wild-type, *sgs1,* and *rad51sgs1* strains. Additionally, we also analyzed whether persistent X-shaped molecules were present in MMS-treated *shu1* or *rad54sgs1* cells. Strains were released from G1 arrest into fresh medium containing 0.033% MMS, and cells were harvested at early (30 min) or late (180 min) time points. Genomic DNA was prepared using the CTAB method of DNA extraction to restrain branch migration of joint (X-shaped) molecules, as described previously (Lopes *et al.*, 2003). We observed that origin firing at *ARS305* was detectable after 30 min in all strains, as evidenced by the appearance of bubbles, Y-molecules and the so-called origin-associated X-structures (Figure 4A). Previous studies have indicated that the origin-associated X-structures are normal DNA replication intermediates that are not dependent on Rad51 or Rad52 for their formation and are not, therefore, HRR intermediates (Lopes *et al.*, 2003). In each strain, the relative ratio between bubbles, Y-molecules, and X-structures was similar at 30 min, suggesting that origin firing occurs normally in *shu1*, *sgs1*, *rad51sgs1,* and *rad54sgs1* mutants. After 180 min, all of the *ARS305* replication intermediates detectable at 30 min were substantially diminished, or had disappeared, in wild-type cells, consistent with replication fork progression beyond the boundaries of this genomic region by this time. Similar results were observed in *shu1* cells, suggesting that mutation of *SHU1* does not noticeably affect DNA replication at *ARS305* (Figure 4A). In contrast, we observed that, although bubbles and Y-molecules had largely disappeared from the *ARS305* region at 180 min, abnormal DNA replication intermediates (X-molecules) persisted in MMS-treated *sgs1* cells (Figure 4A). In agreement with previous data (Liberi *et al.*, 2005), we also observed that these X-molecules were not detectable in *rad51sgs1* cells (Figure 4A). Interestingly, we also observed a similar effect when *RAD54* was mutated, because *rad54sgs1* mutants also did not exhibit persistent MMS-induced X-molecules (Figure 4A). There-

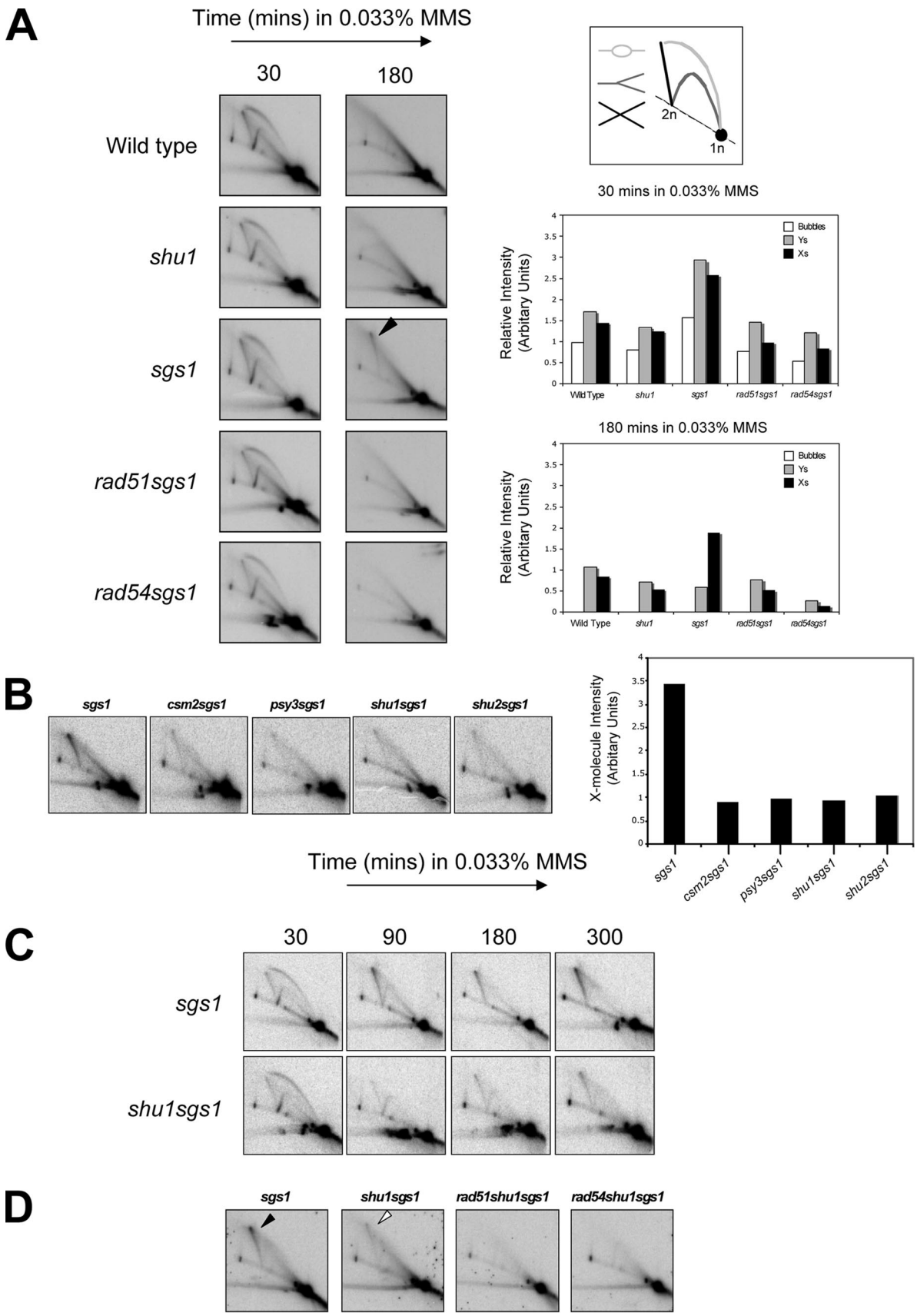

Figure 4. Mutation of *SHU* genes attenuate persistent X-molecules in MMS-treated *sgs1* cells. (A) Wild-type, *shu1*, *sgs1*, *rad51sgs1*, and *rad54sgs1* strains were released from G1 arrest into fresh medium containing 0.033% MMS. DNA replication intermediates were analyzed by

fore, we conclude that persistent X-molecules in MMS-treated *sgs1* cells are both Rad51- and Rad54-dependent. This finding is consistent with Rad54 acting at an early stage in Rad51-dependent HRR upstream of Sgs1 (Rattray and Symington, 1995; Petukhova *et al.*, 1998; Zhang *et al.*, 2007).

Because *sgs1*, but not wild-type (or *shu1*), strains exhibit persistent MMS-induced X-molecules after 180 min in 0.033% MMS (Figure 4A), we examined the effects of deleting *SHU* genes on X-molecule signal intensity in *sgs1* strains at this time point. We verified that, similar to what was observed in *shu1* mutants (Figure 4A), all replication intermediates apparently disappeared from *ARS305* in *csm2*, *psy3*, or *shu2* single mutants by 3 h after release from G1 arrest into medium containing MMS. Therefore, DNA replication appears normal, and MMS-induced X-molecules are not detectable at *ARS305* in *csm2*, *psy3*, or *shu2* single mutants after a 3-h exposure to MMS (data not shown). However, we observed that MMS-induced X-molecules were detectable at *ARS305* in *csm2sgs1*, *psy3sgs1*, *shu1sgs1*, or *shu2sgs1* mutants (Figure 4B). However, these X-molecules were diminished in intensity by ~70% in all *shu sgs1* double mutants, relative to those observed in an *sgs1* single mutant (Figure 4B). This effect was not simply a consequence of altered DNA replication kinetics at *ARS305*, because origin firing (bubble formation at 30 min) and replication fork progression (disappearance of bubbles and Y-arcs at 90–180 min) at *ARS305* were indistinguishable in *sgs1* and *shu1sgs1* cells (Figure 4C). Therefore, we conclude that mutation of *SHU* genes does not abolish, but does attenuate, the level of MMS-induced X-molecules at *ARS305* in MMS-treated *sgs1* cells.

To determine if the low level of MMS-induced X-molecules in *shu1sgs1* cells were nevertheless HRR intermediates, we investigated the effects of deleting *RAD51* or *RAD54* in *shu1sgs1* cells. We found that origin firing at *ARS305* and S-phase progression were indistinguishable in MMS-treated *shu1sgs1*, *rad51shu1sgs1*, and *rad54shu1sgs1* strains (data not shown and Supplementary Figure 1). After 180 min of MMS treatment, we again observed that persistent X-molecules were detectable in MMS-treated *sgs1* cells and that these were attenuated in *shu1sgs1* cells (Figure 4D). However, we found that the MMS-induced X-molecules were absent from *rad51shu1sgs1* or *rad54shu1sgs1* mutants, indicating that the attenuated X-molecules in *shu1sgs1* cells are *RAD51*- and *RAD54*-dependent HRR structures. (Figure 4D). Taken together, the data presented in Figure 4 indicate that, whereas acute formation of MMS-induced persistent X-molecules in *sgs1* cells is abolished by mutation of *RAD51* or *RAD54*, these structures are not completely abolished by mutation of *SHU* genes. However, because the level of MMS-induced X-molecules is attenuated in *shu sgs1* double mutants, we propose that mutation of the *SHU* genes affects either the rate of formation/removal, or the nature, of the X-molecules in *sgs1* cells.

Figure 4 (cont.). 2D gel electrophoresis at the times indicated. DNA samples were analyzed with a probe for the early-firing *ARS305* replication origin. The diagrammatic representation on the top right of the panel denotes DNA structures that can be identified by the 2D gel technique. For quantification of replication intermediates (middle and bottom panels on the right), each signal (bubbles, Ys, and Xs) was normalized to its corresponding monomer (1N) spot. The arrowhead indicates the position of MMS-induced persistent X-molecules. (B) DNA replication intermediates at *ARS305* were analyzed in *sgs1*, *csm1sgs1*, *psy3sgs1*, *shu1sgs1*, and *shu2sgs1* mutants after 3-h treatment with 0.033% MMS after release from G1 arrest. For quantification of X-molecules (right), each X-molecule signal was normalized to its corresponding monomer (1N) spot. (C) A time course of DNA replication at *ARS305* is shown for *sgs1* and *shu1sgs1* mutants after release from G1 arrest into fresh medium containing 0.033% MMS. (D) DNA replication intermediates at *ARS305* were analyzed in *sgs1*, *shu1sgs1*, *rad51shu1sgs1*, and *rad54shu1sgs1* mutants after 3-h treatment with 0.033% MMS after release from G1 arrest. The filled arrowhead indicates the position of MMS-induced persistent X-molecules, whereas the white arrowhead indicates the position of attenuated X-molecules.

Mutation of shu1 Attenuates Persistent MMS-induced X-Molecules in Cells Overexpressing TOP3^{Y356F}

We demonstrated previously that overexpression of *TOP3*^{Y356F} causes Rad51-dependent X-molecules to persist during S-phase after exposure to MMS. To determine if the attenuation of *sgs1* MMS-induced X-molecules by *shu* mutations was specific for *sgs1* X-molecules, or general for both *sgs1* and *TOP3*^{Y356F}-induced X-molecules, we examined if mutation of *SHU1* affects MMS-induced X-molecule persistence in cells overexpressing *TOP3*^{Y356F}. For this, wild-type and *shu1* strains overexpressing *TOP3*^{Y356F} were released from G1 arrest into medium containing 0.033% MMS. In agreement with our previous data (Mankouri and Hickson, 2006), wild-type cells overexpressing *TOP3*^{Y356F} demonstrated persistent MMS-induced X-molecules at 180 min after G1 release (Figure 5, A and B). These structures were absent in MMS-treated *rad51* or *rad54* cells overexpressing *TOP3*^{Y356F}, verifying that Rad51 and Rad54 are required for their formation (data not shown; Mankouri and Hickson, 2006). Persistent MMS-induced X-molecules were detected in *shu1* cells overexpressing *TOP3*^{Y356F}, although their level was attenuated relative to those observed in wild-type cells overexpressing *TOP3*^{Y356F} (Figure 5, A and B). Furthermore, no MMS-induced X-molecules were detectable in *rad51shu1 TOP3*^{Y356F} strains, suggesting that these X-molecules are also Rad51-dependent HRR intermediates (Figure 5B). Because these results are qualitatively very similar to those observed in *shu1sgs1* cells (Figure 4, B and C), we propose that mutation of *SHU1* attenuates MMS-induced X-molecule formation when Top3 function is impaired by a similar mechanism to that occurring in *shu1sgs1* cells.

Mutation of SHU1 Suppresses Growth Defects and MMS Sensitivity Caused by Mutation of RMI1 and Attenuates X-molecules in MMS-treated rmi1 Cells

The yeast and human Rmi1 proteins have been proposed to be integral components of multienzyme complexes containing Sgs1 and Top3, and BLM and hTOPOIIIα, respectively (Johnson *et al.*, 2000; Wu *et al.*, 2000; Chang *et al.*, 2005; Mullen *et al.*, 2005; Yin *et al.*, 2005). We examined, therefore, if mutation of *SHU1* also suppresses *rmi1* phenotypes. Because *rmi1* mutants demonstrate a *top3*-like poor growth phenotype that is readily suppressed by mutations in *SGS1*, we generated haploid *rmi1* mutants by sporulating a BY4741 *RMI1*$^{+/-}$ diploid strain (see *Materials and Methods*). We verified that mutation of *RMI1* caused poor growth that was completely suppressed by mutation of *SGS1* (data not shown) or partially suppressed by *RAD51* (Figure 5C). Interestingly, we also found that mutation of *SHU1* also partially suppressed the poor growth, and MMS sensitivity of *rmi1* mutants (Figure 5C).

On the basis of its interactions with Sgs1 and Top3 (Chang *et al.*, 2005; Mullen *et al.*, 2005), we examined if *rmi1* mutants also demonstrate persistent X-molecules in the presence of MMS. We observed that, similar to cells lacking *SGS1*, or overexpressing *TOP3*^{Y356F} (Figures 4 and 5; Liberi *et al.*, 2005; Mankouri and Hickson, 2006), *rmi1* cells demonstrated persistent X-molecules at *ARS305* after exposure to MMS (Figure 5D). We also observed that, although mutation of *RAD51* eliminated the MMS-induced X-molecules at *ARS305* in *rmi1*

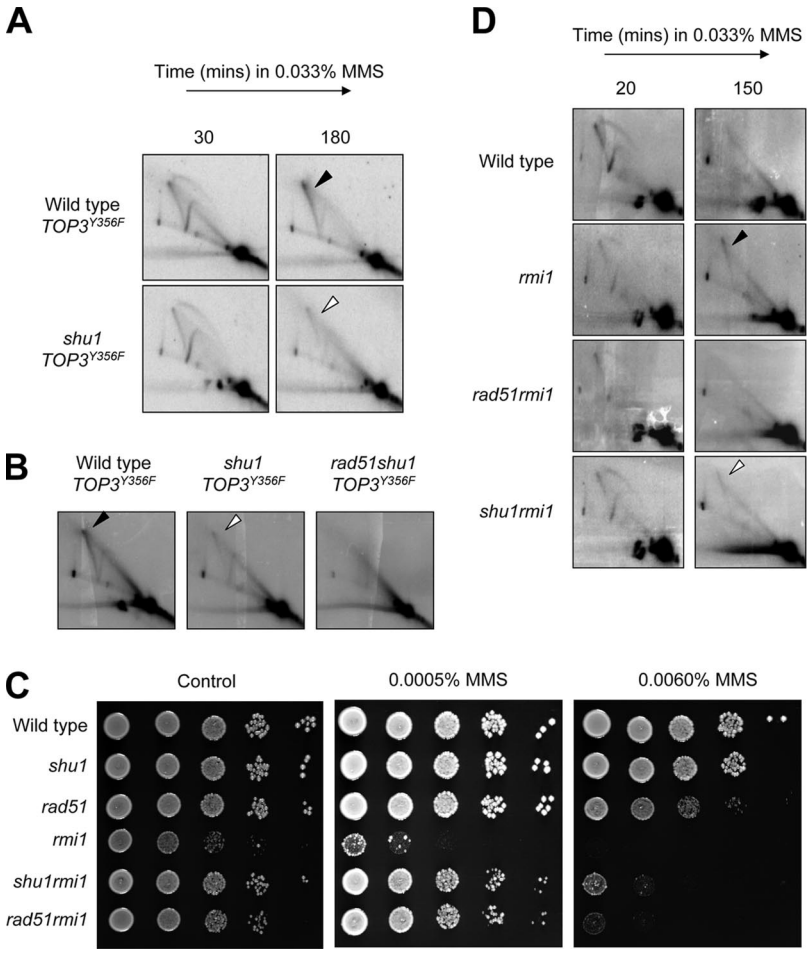

Figure 5. Mutation of *SHU1* attenuates Rad51-dependent, MMS-induced X-molecules in *rmi1* cells and in cells overexpressing $TOP3^{Y356F}$. (A) Wild-type and *shu1* strains transformed with pYES2-$TOP3^{Y356F}$ were arrested in G1 with α-factor and simultaneously treated with 2% galactose (to induce overexpression from the pYES2 *GAL1* promoter). Cultures were released into fresh medium containing 0.033% MMS, and DNA replication intermediates were analyzed by 2D gel electrophoresis at the times indicated. DNA samples were analyzed with a probe for the early-firing *ARS305* replication origin. The filled arrowhead indicates the position of MMS-induced persistent X-molecules, whereas the white arrowhead indicates the position of attenuated X-molecules. (B) DNA replication intermediates at *ARS305* were analyzed in wild-type, *shu1,* and *rad51shu1* cells overexpressing $TOP3^{Y356F}$ after 3-h treatment with 0.033% MMS after release from G1 arrest. (C) Wild-type, *shu1*, *rad51*, *rmi1*, *rad51rmi1,* and *shu1rmi1* strains were diluted to equivalent densities and spotted onto control plates (no drug) or plates containing MMS. Spots from left to right represent serial 1 in 10 dilutions of yeast cultures. Plates were grown at 30°C. (D) Wild-type, *rmi1*, *rad51rmi1*, and *shu1rmi1* strains were released from G1 arrest into fresh medium containing 0.033% MMS. DNA replication intermediates were analyzed by 2D gel electrophoresis at the times indicated. DNA samples were analyzed with a probe for the early-firing *ARS305* replication origin.

cells, mutation of *SHU1* again only attenuated their level (Figure 5D). Therefore, the persistent X-molecules detected in *rmi1* cells likely represent Rad51-dependent HRR intermediates, similar or identical to those identified in cells lacking Sgs1 or in cells overexpressing $TOP3^{Y356F}$ (Figures 4 and 5; Liberi *et al.,* 2005; Mankouri and Hickson, 2006). Furthermore, because mutation of *SHU1* only attenuated these MMS-induced X-molecules, we propose that the Shu proteins normally promote the formation of Rad51/Rad54-dependent HRR intermediates that are ultimately resolved by the Sgs1-Rmi1-Top3 complex.

DISCUSSION

CSM2, *PSY3*, *SHU1,* and *SHU2* (collectively referred to as the *SHU* genes) were recently identified in *S. cerevisiae* as four genes in the same epistasis group that, when mutated, cause sensitivity to MMS and suppression of various *sgs1* and *top3* mutant phenotypes (Shor *et al.,* 2005). *RMI1* encodes a protein that associates with Sgs1 and Top3 in vivo, and *rmi1* mutants phenotypically resemble *top3* mutants (Chang *et al.,* 2005; Mullen *et al.,* 2005). This physical interaction is also evolutionarily conserved, because the human Rmi1 homologue, hRMI1, is an integral component of the BLM-hTOPOIIIα complex in human cells (Yin *et al.,* 2005). In this study, we have demonstrated that mutation of *SHU1* suppresses poor growth caused by overexpression of $TOP3^{Y356F}$ or deletion of *RMI1*, indicating that the Shu complex actively contributes to the cellular defects seen in cells lacking Sgs1, Top3, or Rmi1.

Role of Shu Proteins in Rad51/Rad54-dependent Homologous Recombination Repair

The ability of *shu* mutations to suppress *rmi1*, *top3,* and $TOP3^{Y356F}$-induced poor growth, and to suppress the synthetic lethality of *sgs1srs2*, *sgs1mus81,* and *sgs1mms4* mutant combinations is very reminiscent of that achieved by deletion of genes that control the early steps of HRR (e.g., *RAD52*, *RAD51*, *RAD55*, *RAD57,* and *RAD54*; Shor *et al.,* 2005; St. Onge *et al.,* 2007). Shor *et al.* (2005) demonstrated that *rad52* is epistatic to *shu* mutations for MMS sensitivity and a mutator phenotype, suggesting that the Shu proteins are involved in *RAD52*-dependent HRR. However, because mutation of *RAD52* abolishes all types of HRR (Symington, 2002), the precise role(s) of the Shu complex in HRR was not clear. In this study, we identified a specific role for the Shu complex in Rad51/Rad54-dependent pathway(s) to repair MMS-induced lesions. This assertion is based on the following observations: 1) Mutation of *SHU1* does not cause additive sensitivity to MMS when combined with *rad51* or *rad54* mutations. 2) Mutation of *RAD51*, *RAD54,* or *SHU1* causes a similar, and nonadditive, impairment of S-phase progression in the presence of MMS, due to a more persistent or robust activation of the DNA damage checkpoint. 3) Mutation of *SHU1* is epistatic (i.e., nonadditive) with *rad51* or *rad54* for suppression of $TOP3^{Y356F}$-induced poor growth. However, it should be noted that, unlike *rad51* and *rad54* mutants, the *shu* mutants do not exhibit sensitivity to HU or ionizing radiation

(Shor *et al.,* 2005). Therefore, it is unlikely that the Shu proteins are involved in HRR repair of double-strand breaks. We propose, therefore, that the Shu proteins function at an early stage in a Rad51/Rad54-dependent HRR subpathway specifically to repair certain types of DNA lesions (probably ssDNA gaps; see below) that arise during S-phase.

Suppression of sgs1, rmi1, and top3/TOP3^{Y356F} Phenotypes by Mutation of SHU Genes

Because mutation of *SHU1* or *CSM2* suppresses hyper-recombination in *sgs1* and *top3* cells (Shor *et al.,* 2005), we examined if *shu* mutations prevent unprocessed HRR intermediates from accumulating in cells compromised for Sgs1, Top3, or Rmi1. Previous data demonstrated that unresolved Rad51-dependent HRR intermediates are detectable in MMS-treated *sgs1*, *top3,* and *TOP3^{Y356F}* cells (Liberi *et al.,* 2005; Mankouri and Hickson, 2006). In this study, we also demonstrated that *rmi1* mutants exhibit persistent, Rad51-dependent, MMS-induced X-molecules, suggesting that the accumulation of unprocessed HRR intermediates is a common property of strains mutated for *SGS1*, *TOP3,* or *RMI1*. It should be noted, however, that we do not know presently if the X-molecules arising in MMS-treated *sgs1*, *rmi1,* and *TOP3^{Y356F}* strains are identical or if they represent different types of HRR intermediates that cannot readily be distinguished by the 2D gel technique. One possibility, based on the known enzymatic functions of BLM, hTOPOIII and RMI1 (Wu and Hickson, 2003; Plank *et al.,* 2006; Raynard *et al.,* 2006; Wu *et al.,* 2006), is that X-molecules arising in *sgs1* mutants are double Holliday junctions, whereas those arising in *TOP3^{Y356F}*, or *rmi1*, mutants are hemicatenanes formed by the Sgs1-dependent convergent branch migration of double Holliday junctions.

Surprisingly, we found that although mutation of *RAD51* prevented persistent MMS-induced HRR intermediates in *sgs1*, *rmi1,* and *TOP3^{Y356F}* cells, mutation of *SHU1* did not. However, mutation of *SHU1* attenuated the level of MMS-induced HRR intermediates in *sgs1*, *rmi1,* and *TOP3^{Y356F}* cells. This finding is consistent with the report that *shu1* suppresses the elevated recombination rate in *sgs1* and *top3* cells (Shor *et al.,* 2005). Taken together with the fact that the *shu* mutations are epistatic to *sgs1* for MMS sensitivity and also suppress the poor growth caused by deletion of *RMI1* or *TOP3*, our data are consistent with an early role for the Shu proteins in HRR to promote the formation of HR intermediates that are resolved by the Sgs1-Rmi1-Top3 complex. We therefore propose that, in the absence of the Shu proteins, the maturation of Rad51-filaments into Sgs1-Rmi1-Top3 substrates (e.g., double Holliday junctions) occurs inefficiently (Figure 6). If true, this suggests that the Shu proteins likely function as nonessential HRR accessory factors that facilitate efficient and timely HRR. These findings are consistent with the proposal that Shu proteins may represent so-called "Rad51 paralogs" (Martin *et al.,* 2006), because these have been demonstrated to facilitate the action of Rad51 in HRR (Takata *et al.,* 2001).

Of wider implication, putative *SHU2* and *PSY3* homologues have recently been identified in *S. pombe* (*Sws1$^+$* and *Rld1$^+$*, respectively), and human cells (*SWS1* and *RAD51D*, respectively; Martin *et al.,* 2006), suggesting that Shu-like complexes are evolutionarily conserved. Consistent with a

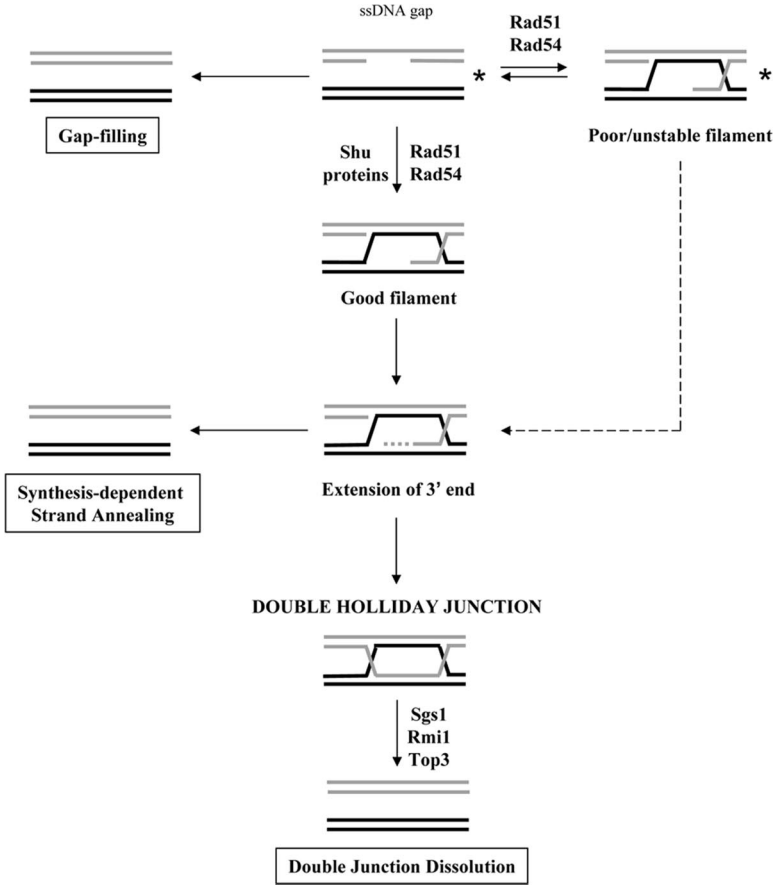

Figure 6. Proposed role of the Shu complex in promoting the formation of HRR intermediates that are processed by Sgs1-Rmi1-Top3. MMS-induced DNA lesions cause discontinuities in DNA synthesis, leading to the accumulation of ssDNA gaps during S-phase. These ssDNA gaps can be repaired by Rad51-dependent homologous recombination repair (HRR) or postreplicative gap filling by a translesion polymerase. RPA binds to ssDNA gaps and activates the DNA damage checkpoint. If HRR is initiated, then the engagement of the HRR machinery causes removal of RPA and subsequent deactivation of the checkpoint signal. However, the absence of Rad51, Rad54, or any one of the four Shu proteins results in a persistent activation of the DNA damage checkpoint, presumably due to inefficient RPA removal by the HRR machinery. DNA structures that are predicted to (persistently) activate the checkpoint are indicated by an asterisk. Rad51, Rad54, and associated proteins catalyze strand invasion and copying of genetic information from the sister chromatid to create (extended) D-Loops that either are substrates for synthesis-dependent strand annealing or else are converted into double Holliday junctions for processing by Sgs1-Rmi-Top3. In the absence of Sgs1, Rmi1, or Top3, late-stage HRR intermediates persist and are detectable as X-molecules on 2D gels. In the absence of Shu proteins, poor-quality Rad51-filaments are unstable, and maturation of these into Sgs1-Rmi1-Top3 HRR substrates occurs very inefficiently. Thus, X-molecules detectable in *sgs1*, *rmi1,* or *top3/TOP3^{Y356F}* cells are attenuated.

conserved role in HRR, ablation of *SWS1* in human cells reduces the number of Rad51 foci in both control and IR-treated cells (Martin *et al.*, 2006). It will therefore be of great interest to determine if *SWS1* or *RAD51D* ablation can prevent the defects seen in Bloom's syndrome cells.

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