

The Smc5/6 Complex and Esc2 Influence Multiple Replication-associated Recombination Processes in *Saccharomyces cerevisiae*

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Replication-associated recombinational repair is important for genome duplication and cell survival under DNA damage conditions. Several nonclassical recombination factors have been implicated in this process, but their functional relationships are not clear. Here, we show that three of these factors, Mph1, Mms2, and the Shu complex, can act independently to promote the formation of recombination intermediates during impaired replication. However, their functions become detrimental when cells lack the Smc5/6 complex or Esc2. We show that *mph1Δ*, *mms2Δ*, and *shu1Δ* suppress the sensitivity to the replication-blocking agent methylmethane sulfonate (MMS) in *smc6* mutants, with double deletions conferring stronger suppression. These deletion mutations also rescue the MMS sensitivity of *esc2Δ* cells. In addition, two-dimensional gel analysis demonstrates that *mph1Δ*, *mms2Δ*, and *shu1Δ* each reduce the level of recombination intermediates in an *smc6* mutant when cells replicate in the presence of MMS, and that double deletions lead to a greater reduction. Our work thus suggests that Mph1, Mms2, and the Shu complex can function in distinct pathways in replication-associated recombinational repair and that the Smc5/6 complex and Esc2 prevent the accumulation of toxic recombination intermediates generated in these processes.

INTRODUCTION

Recombinational repair provides an important means to facilitate replication when DNA lesions or other obstacles are present on the template. Several modes of replication-associated recombinational repair have been proposed. These include gap filling that repairs single-stranded DNA regions left behind by the replication machinery, template switching that entails the use of newly synthesized sister strands as templates to overcome lesions on parental strands, and replication fork regression in which the newly synthesized DNA strand anneal to each other leading to DNA synthesis and/or strand invasion (Branzei and Foiani, 2007; Lambert *et al.*, 2007; Li and Heyer, 2008; Budzowska and Kanaar, 2009; Chang and Cimprich, 2009). All modes of recombinational repair probably require core recombination proteins such as the recombinase Rad51 and mediator proteins that are essential for homology search and strand invasion (Krogh and Symington, 2004; San Filippo *et al.*, 2008). Moreover, it is increasingly clear that additional proteins are used to couple stalled or interrupted replication with recombination. Unlike core recombination proteins, some of these factors are not crucial for other recombination processes, such as the repair of double-strand breaks in a setting that is not coupled with replication (Lambert *et al.*, 2007; Branzei and Foiani, 2007; Li and Heyer, 2008; Budzowska and

Kanaar, 2009). Analyzing the genetic relationships among these factors will help to delineate the recombination pathways operating during replication.

One such group of proteins prevents the accumulation of recombination intermediates when cells replicate in the presence of the DNA-damaging agent methylmethane sulfonate (MMS) (Branzei *et al.*, 2006; Mankouri *et al.*, 2007, 2009; Sollier *et al.*, 2009). Cells lacking these proteins display high levels of Rad51-dependent X-shaped DNA molecules (X-mols) and reduced viability when grown in media containing MMS (Branzei *et al.*, 2006; Mankouri *et al.*, 2007, 2009; Sollier *et al.*, 2009). Two members of this group are the evolutionarily conserved Smc5/6 complex and Esc2. The Smc5/6 complex is composed of Smc5, Smc6, and six other subunits (Hazbun *et al.*, 2003; Zhao and Blobel, 2005; Sergeant *et al.*, 2005; Pebernard *et al.*, 2006; Taylor *et al.*, 2008). One of its subunits, Mms21, possesses SUMO ligase activity that promotes the covalent linkage of SUMO to substrates (Andrews *et al.*, 2005; Potts and Yu, 2005; Zhao and Blobel, 2005). In addition, the Smc5/6 complex may have the ability to tether DNA as similar SMC complexes have been shown to condense or co-hese DNA strands (Murray and Carr, 2008; De Piccoli *et al.*, 2009). The Esc2 protein contains SUMO-like domains and has no known enzymatic activities (Novatchkova *et al.*, 2005; Raffa *et al.*, 2006; Sollier *et al.*, 2009). The accumulation of X-mols in mutants of the Smc5/6 complex and Esc2 suggest that these two factors can facilitate the resolution and/or limit the formation of potentially toxic recombination structures.

Another group of proteins, in contrast, promotes the formation of recombination intermediates during impaired replication. One such protein is the DNA helicase Mph1,

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which has been implicated in replication fork regression (Schurer *et al.*, 2004; Prakash *et al.*, 2005; St Onge *et al.*, 2007; Sun *et al.*, 2008). Another in this category is the Shu complex, which is composed of Shu1, Shu2, Psy3, and Csm2, and has been proposed to work as a type of Rad51 paralogue acting at an early recombination step (Huang *et al.*, 2003; Shor *et al.*, 2005; Martin *et al.*, 2006; Mankouri *et al.*, 2007). In addition, proteins in the error-free branch of the postreplicative repair pathway, such as Mms2, a subunit of a ubiquitin conjugating enzyme, also play a role in the formation of recombination intermediates (Branzei *et al.*, 2008). It has been shown that the removal of Mph1 decreases the amount of X-mols in *smc6* mutants and *esc2Δ* cells, whereas the removal of Shu1 exerts a similar effect in *esc2Δ* cells (Chen *et al.*, 2009; Mankouri *et al.*, 2009). However, it is not clear whether the Shu complex contributes to the accumulation of X-mols in mutants of the Smc5/6 complex and whether Mph1, the Shu complex and Mms2 act independently or in the same pathway. Another unresolved question is whether accumulation of X-mols underlies MMS sensitivity, because *mph1Δ* has been shown to suppress the MMS sensitivity of *smc6* but not *esc2Δ* mutants (Chen *et al.*, 2009; Mankouri *et al.*, 2009).

To gain a better understanding of the replication-associated recombinational repair system, we examined genetic interactions among the aforementioned proteins that modulate recombination intermediates. Our results suggest that Mph1, the Shu complex, and Mms2 can act independently to promote the formation of recombination structures, and that the Smc5/6 complex and Esc2 prevent toxicity from unresolved recombination intermediates generated by Mph1-, Shu complex-, and Mms2-dependent processes.

MATERIALS AND METHODS

Yeast Strains and Genetic Manipulations

Yeast strains are listed in Table 1. Strains in this study are derivatives of W1588-4C, a *RAD5* derivative of W303 (Thomas and Rothstein, 1989). Standard yeast protocols were used for strain construction, growth, and medium preparation. The construction of *smc6-56* and *smc6-P4* strains has been described previously (Chen *et al.*, 2009). To detect wild-type *RAD5* and *rad5-535*, polymerase chain reaction (PCR) reactions using primer pair RAD5-L (5'-gagcaggaccatgtaaagc-3') and RAD5-R (5'-aaactggtactactcactgcg-3') were performed followed by MnlI digestion. Wild-type *RAD5* PCR products gave rise to two fragments (155 and 182 base pairs), whereas *rad5-535* PCR products gave rise to three fragments (155, 120, and 62 base pairs). Genotyping of different alleles bearing the same selection marker was carried out by PCR as described below. For *shu1Δ::HIS3*, the primer pair Shu1 UF (5'-GTATGCGTGT GTTATAC GTGAG-3') and Shu1 DR (5'-GATGCCTCTTTTGGTTTCG-3') was used. *shu1Δ::HIS3* gave rise to a 1.6-kb PCR fragment, whereas wild-type *SHU1* gave rise to a 0.9-kb fragment. For *smc6-56* and *smc6-P4*, the primer pair Smc6-3163ntF (5'-CAGGTTAACAGGA AGATTGG-3') and Myc-TADH R (5'-TAGAAGTGGCGGAATTCAC-3') was used. Both alleles gave rise to a 750-base pair PCR fragment. For *mms2Δ::KAN*, the primer pair KAN CterF (5'-CCTATGGAAGTCCC TCGGTG-3') and Mms2 DR (5'-CAAACG-CAGAAGCAACTAAAT-3') was used. *mms2Δ::KAN* gave rise to a 630-base pair PCR fragment. For *esc2Δ::KAN*, the primer pair KAN CterF and Esc2 DR (5'-GGTAGAAGAGGG TCAGCAC-3') was used. *esc2Δ::KAN* gave rise to a 620-base pair PCR fragment.

To test whether *shu1Δ* or *mms2Δ* suppresses the lethality caused by *smc6Δ* and *mms21Δ*, diploid cells heterozygous for *smc6Δ* or *mms21Δ* and for *mph1Δ* were transformed with a PCR fragment containing the *HIS3* marker flanked by the upstream and downstream sequences surrounding the *SHU1* or *MMS2* ORF, respectively. The transformants were verified for the disruption of the *SHU1* or *MMS2* gene by PCR (see above) and sporulated. The confirmed diploids were dissected, and plates were incubated at 30°C for 7 d before being photographed. Tetrad analyses were performed as described in the text.

Spot assays for detecting DNA damage sensitivity were carried out as described previously (Chen *et al.*, 2009). In brief, early to mid-log phase cells grown in rich medium (YPD) were spotted in 10-fold serial dilutions (10^4 – 10^8 cells) on plates containing YPD with or without MMS and were grown at 30°C. Plates were photographed 2–4 d after spotting. At least two different isolates for each genotype were tested. Each figure panel shows strains spotted on the same plate, with occasional rearrangement of lanes for presentation purposes.

Two-dimensional (2D) Gel Analysis of Recombination Intermediates

Experiments were performed as described previously (Branzei *et al.*, 2006). In brief, cells were synchronized in G2 by adding nocodazole at a final concentration of 10 mg/ml together with DMSO at 1% (vol/vol) for ~2.5 h. Cells were then released from nocodazole arrest into YPD medium containing MMS at a final concentration of 0.033% (vol/vol) at 30°C. At the indicated times after release, cells were collected for fluorescence-activated cell sorting (FACS) analysis and purification of DNA intermediates. The DNA samples were digested with HindIII and EcoRV and separated by 2D gel electrophoresis followed by Southern blotting with a probe against *ARS305*. Quantification of the X-mol signals was performed using the ImageQuant software (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Regions corresponding to monomer DNA, X-mols, and background were selected. Signal intensity (S) and area (A) for each region were calculated by the software. Background (BK) value was derived as the ratio between signal intensity and area for the background region. Values of X-mols (V_x) and monomer DNA (V_m) were calculated by the following equations: $V_x = S_x - (BK \times A_x)$ and $V_m = S_m - (BK \times A_m)$, respectively. The relative amount of X-mols was derived as $V_x/(V_x + V_m)$ and was normalized against the highest values in the plot of Figure 3, C and E. Two different strains were examined for each genotype; each set of experiments was performed twice with qualitatively identical results. Results from representative experiments are shown.

RESULTS

Mph1, the Shu Complex and Mms2 Have Nonoverlapping Functions

Previous studies have shown that mutants of Mph1 and the Shu complex display moderate MMS sensitivity and have epistatic relationships with *rad51Δ* (Schurer *et al.*, 2004; Shor *et al.*, 2005; Mankouri *et al.*, 2007; Chen *et al.*, 2009). To further understand the recombination processes in which Mph1 and the Shu complex participate, we analyzed the genetic interaction between *mph1Δ* and deletion of *SHU1*, which encodes a subunit of the Shu complex. As shown in Figure 1A, *mph1Δ shu1Δ* double mutants were more sensitive to MMS than either single mutant. A similar interaction was found between *mph1Δ* and the deletion of the gene encoding another Shu complex subunit, Csm2 (Figure 1B). These results indicate that Mph1 and the Shu complex have nonoverlapping functions under conditions of replicative stress.

Next, we examined how Mph1 and the Shu complex are related to Mms2, which also contributes to the formation of recombination intermediates when cells replicate in the presence of MMS (Branzei *et al.*, 2008). As shown in Figure 1, C and D, both *mph1Δ* and *shu1Δ* enhanced the MMS sensitivity of *mms2Δ* cells, indicating that Mms2 has functions distinct from those of Mph1 and Shu1. The synthetic interactions described above suggest that Mph1, the Shu complex, and Mms2 can function in separate pathways, although they do not exclude the possibility that these proteins may work with each other under certain circumstances.

Both *shu1Δ* and *mms2Δ* Rescue the MMS Sensitivity of Two *smc6* Mutants

The Smc5/6 complex has been shown to prevent unregulated or incomplete recombination reactions involving Mph1 (Chen *et al.*, 2009). We asked whether it also affects recombinational processes involving Shu1 and Mms2. To address this, genetic interactions between two previously characterized *smc6* alleles, *smc6-P4* and *smc6-56*, and *shu1Δ* and *mms2Δ* were examined. *smc6-P4* and *smc6-56* cells display severe MMS sensitivity, which is suppressed by *mph1Δ* or its helicase mutations (Onoda *et al.*, 2004; Chen *et al.*, 2009; Sollier *et al.*, 2009). We found that the MMS sensitivity of both *smc6* mutants was also suppressed by either *shu1Δ* (Figure 2, A and B) or *mms2Δ* (Figure 2, C and D), indicating that like Mph1, the actions of the Shu complex and Mms2 also become toxic when the Smc5/6 complex is defective.

The strengths of suppression resulting from *MPH1*, *SHU1*, or *MMS2* deletion in *smc6-P4* or *smc6-56* cells were different.

Table 1. Strains used in this study^a

Name	Genotype	Source
W1588-4C	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	R. Rothstein
W2889-19B	<i>MATa shu1Δ::HIS3</i>	Shor <i>et al.</i> (2005)
X2745-3A	<i>MATa mph1Δ::KAN</i>	This study
X2745-2A	<i>MATa mph1Δ::KAN shu1Δ::HIS3</i>	This study
T592	<i>MATa mms2Δ::KAN</i>	This study
X2739-4A	<i>MATa mph1Δ::URA3 mms2Δ::KAN</i>	This study
X2726-15C	<i>MATa shu1Δ::HIS3 mms2Δ::KAN</i>	This study
X2123-2A	<i>MATa smc6-56-13myc::HIS3</i>	Chen <i>et al.</i> (2009)
T382-P4	<i>MATa smc6-P4-13myc::HIS3</i>	Chen <i>et al.</i> (2009)
X2123-3C	<i>MATa smc6-56-13myc::HIS3 mph1Δ::KAN</i>	Chen <i>et al.</i> (2009)
X1787-4A	<i>MATa smc6-P4-13myc::HIS3 mph1Δ::KAN</i>	Chen <i>et al.</i> (2009)
X2575-5A	<i>MATa smc6-56-13myc::KAN shu1Δ::HIS3</i>	This study
X2605-12C	<i>MATa smc6-P4-13myc::KAN shu1Δ::HIS3</i>	This study
X2607-14B	<i>MATa smc6-56-13myc::KAN mph1Δ::URA3 shu1Δ::HIS3</i>	This study
X2605-19C	<i>MATa smc6-P4-13myc::KAN mph1Δ::URA3 shu1Δ::HIS3</i>	This study
X2740-4C	<i>MATa smc6-56-13myc::HIS3 mms2Δ::KAN</i>	This study
X2739-5A	<i>MATa smc6-P4-13myc::HIS3 mms2Δ::KAN</i>	This study
X2819-1A	<i>MATa smc6-56-13myc::HIS3 mms2Δ::KAN shu1Δ::HIS3</i>	This study
X2818-6A	<i>MATa smc6-P4-13myc::HIS3 mms2Δ::KAN shu1Δ::HIS3</i>	This study
X2740-6C	<i>MATa smc6-56-13myc::HIS3 mms2Δ::KAN mph1Δ::URA3</i>	This study
X2739-2B	<i>MATa smc6-P4-13myc::HIS3 mms2Δ::KAN mph1Δ::URA3</i>	This study
X2622-1-20B	<i>MATα rad5-535 LEU2 TRP1 URA3</i>	This study
X2622-1-6C	<i>MATα rad5-535 mph1Δ::KAN LEU2 TRP1 URA3</i>	This study
X2942-2-2D	<i>MATa rad5-535 shu1Δ::HIS3</i>	This study
X2752-4-2A	<i>MATα rad5-535 mms2Δ::KAN LEU2 TRP1 URA3</i>	This study
W6330-3D	<i>MATα esc2Δ::KAN ADE2 TRP1 lys2Δ leu2-ΔBstII</i>	R. Rothstein
X2853-1-3C	<i>MATa esc2Δ::KAN mph1Δ::URA3 lys2Δ</i>	This study
X2751-12B	<i>MATa esc2Δ::KAN shu1Δ::HIS3 TRP1</i>	This study
X2750-8B	<i>MATα esc2Δ::KAN mms2Δ::KAN TRP1</i>	This study
T749	<i>MATa/α smc6Δ::KAN/+ shu1Δ::HIS3/+ mph1Δ::URA3/+</i>	This study
T750	<i>MATa/α mms21Δ::KAN/+ shu1Δ::HIS3/+ mph1Δ::URA3/+</i>	This study
T768	<i>MATa/α smc6Δ::KAN/+ mms2Δ::HIS3/+ mph1Δ::URA3/+</i>	This study
T769	<i>MATa/α mms21Δ::KAN/+ mms2Δ::HIS3/+ mph1Δ::URA3/+</i>	This study
X3056-2A	<i>MATa rad5-535</i>	This study
X3054-2-6A	<i>MATa mph1Δ::KAN rad5-535</i>	This study
X3056-3B	<i>MATa shu1Δ::HIS3 rad5-535</i>	This study
X3056-2C	<i>MATa esc2Δ::KAN rad5-535</i>	This study
X3054-2-6C	<i>MATα mph1Δ::KAN esc2Δ::KAN rad5-535</i>	This study
X3056-3C	<i>MATa shu1Δ::HIS3 esc2Δ::KAN rad5-535</i>	This study
X3057-2-14A	<i>MATa mms2Δ rad5-535 TRP</i>	This study
X3057-1-1C	<i>MATα mms2Δ esc2Δ rad5-535 TRP</i>	This study
X2662-4A	<i>MATα mph1Δ::URA3</i>	This study
X2662-4C	<i>MATα csm2Δ::KAN</i>	This study
X2662-4D	<i>MATa mph1Δ::URA3 csm2Δ::KAN</i>	This study
X3053-2D	<i>MATa pol30K164R</i>	This study
X3053-8B	<i>MATa pol30-K164R smc6-P4::HIS3</i>	This study
X3005-9B	<i>MATα pol30-K164R esc2Δ::KAN</i>	This study

^a Strains in this study are derivatives of W1588-4C, a *RAD5* derivative of W303 (*MATa ade2-1 can1-100 ura3-1 his3-11,15 leu2-3,112 trp1-1 rad5-535*; Thomas and Rothstein, 1989). When applicable, a single representative of each genotype is listed.

mph1Δ conferred better suppression than *shu1Δ* (Figure 2, A and B) and *mms2Δ* (Figure 2, C and D), and suppression by *mms2Δ* was greater than that by *shu1Δ* (Figure 2, E and F). These results are consistent with the idea that Mph1, the Shu complex and Mms2 can function in different processes, and that the Mph1-dependent process causes the most toxicity when the Smc5/6 complex is defective.

Combinatorial Deletions of MPH1, SHU1, and MMS2 Confer Greater Suppression of MMS Sensitivity of *smc6* Mutants than Any Single Deletion

To further test the idea that Mph1, the Shu complex and Mms2 have independent functions, we examined whether their combinatorial deletions can result in additive effects in *smc6* mutants. As shown in Figure 2, A and B, the *mph1Δ shu1Δ* double mutant alleviated the MMS sensitivity of

smc6-P4 and *smc6-56* cells to a greater degree than either single mutant. Similarly, *mph1Δ mms2Δ* (Figure 2, C and D) and *shu1Δ mms2Δ* (Figure 2, E and F) resulted in better suppression in both *smc6-P4* and *smc6-56* cells than the corresponding single deletions. These results strongly support the notion that Mph1, the Shu complex and Mms2 can act independently and that the Smc5/6 complex is genetically linked with the processes involving these three proteins.

***shu1Δ*, *mms2Δ*, and *mph1Δ* Each Reduces the Levels of X-mols in *smc6-P4* Cells, with Double Deletions Conferring Greater Reduction**

The alleviation of MMS sensitivity in *smc6* mutants by *mph1Δ*, mutations in its helicase domain, and *rad51Δ* have been attributed to their ability to reduce the levels of potentially toxic recombination intermediates (Branzei *et al.*, 2008; Chen *et al.*,

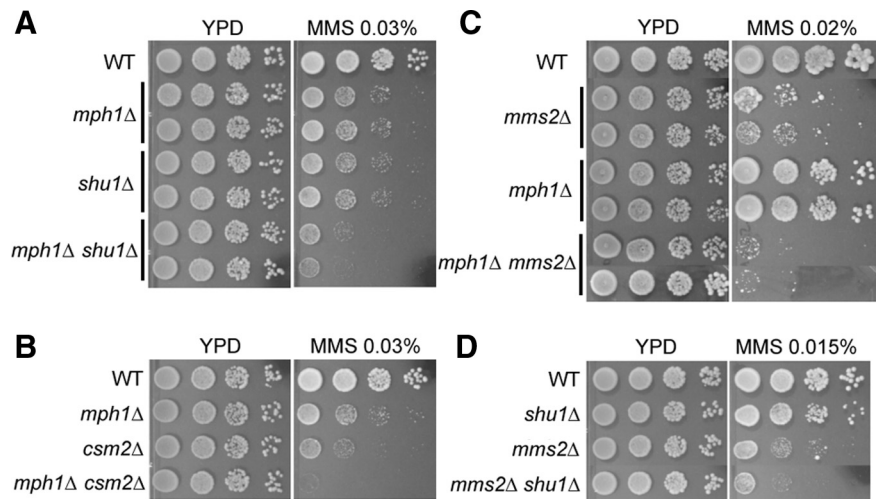


Figure 1. Mph1, the Shu complex, and Mms2 have nonoverlapping functions in DNA repair. Wild-type (WT) and mutant cell cultures were diluted and spotted onto YPD plates with or without the indicated concentration of MMS. *mph1Δ* enhances the MMS sensitivity of *shu1Δ* (A), *csm2Δ* (B), and *mms2Δ* (C) cells. *shu1Δ* enhances the MMS sensitivity of *mms2Δ* cells (D).

2009). Here, we examined whether *shu1Δ* and *mms2Δ* can also attenuate X-mol accumulation in *smc6-P4* cells. Synchronized cells were released into the cell cycle and allowed to replicate in the presence of sublethal doses of MMS. DNA from *smc6-P4* and *smc6-P4 shu1Δ* cells was extracted at intervals and analyzed by 2D agarose gel electrophoresis (2D gel) using a probe for the early firing replication origin *ARS305* (Figure 3A). As shown in Figure 3, B and C, *shu1Δ*, like *mph1Δ*, reduced the

levels of X-mols in *smc6-P4* cells. Similarly, *mms2Δ* also decreased the amounts of X-mols in *smc6-P4* cells (Figure 3, D and E). These results correlate well with the observed genetic suppression shown in Figure 2 and indicate that the Smc5/6 complex is required to prevent the accumulation of X-mols generated by Shu1- and Mms2-dependent processes, in addition to those generated by the Mph1-dependent process. Because Mms2 catalyzes proliferating cell nuclear antigen (PCNA;

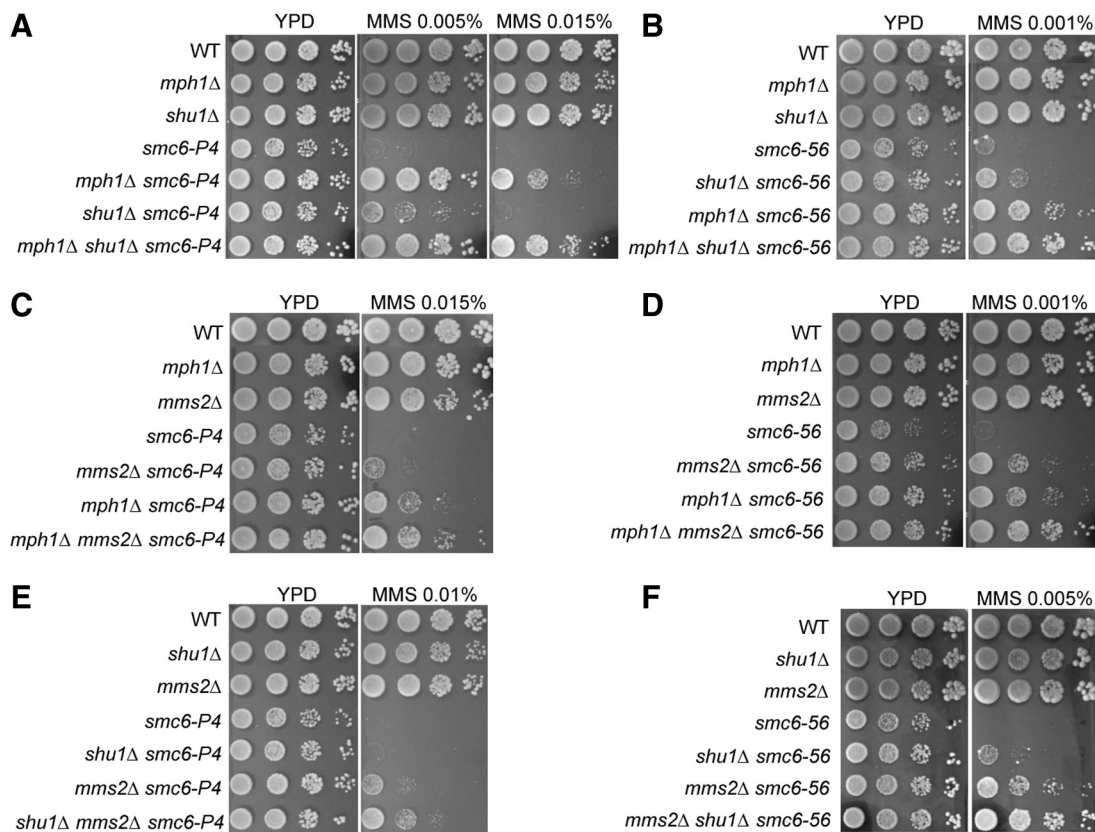


Figure 2. Suppression of MMS sensitivity in *smc6* mutants by *mph1Δ*, *shu1Δ*, and *mms2Δ*. (A and B) *mph1Δ shu1Δ* confers greater suppression of MMS sensitivity of *smc6-P4* (A) and *smc6-56* (B) cells than either *mph1Δ* or *shu1Δ* alone. (C and D) *mph1Δ mms2Δ* confers greater suppression of MMS sensitivity of *smc6-P4* (C) and *smc6-56* (D) cells than either *mph1Δ* or *mms2Δ* alone. (E and F) *shu1Δ mms2Δ* confers greater suppression of the MMS sensitivity of *smc6-P4* (E) and *smc6-56* (F) cells than either *shu1Δ* or *mms2Δ* alone.

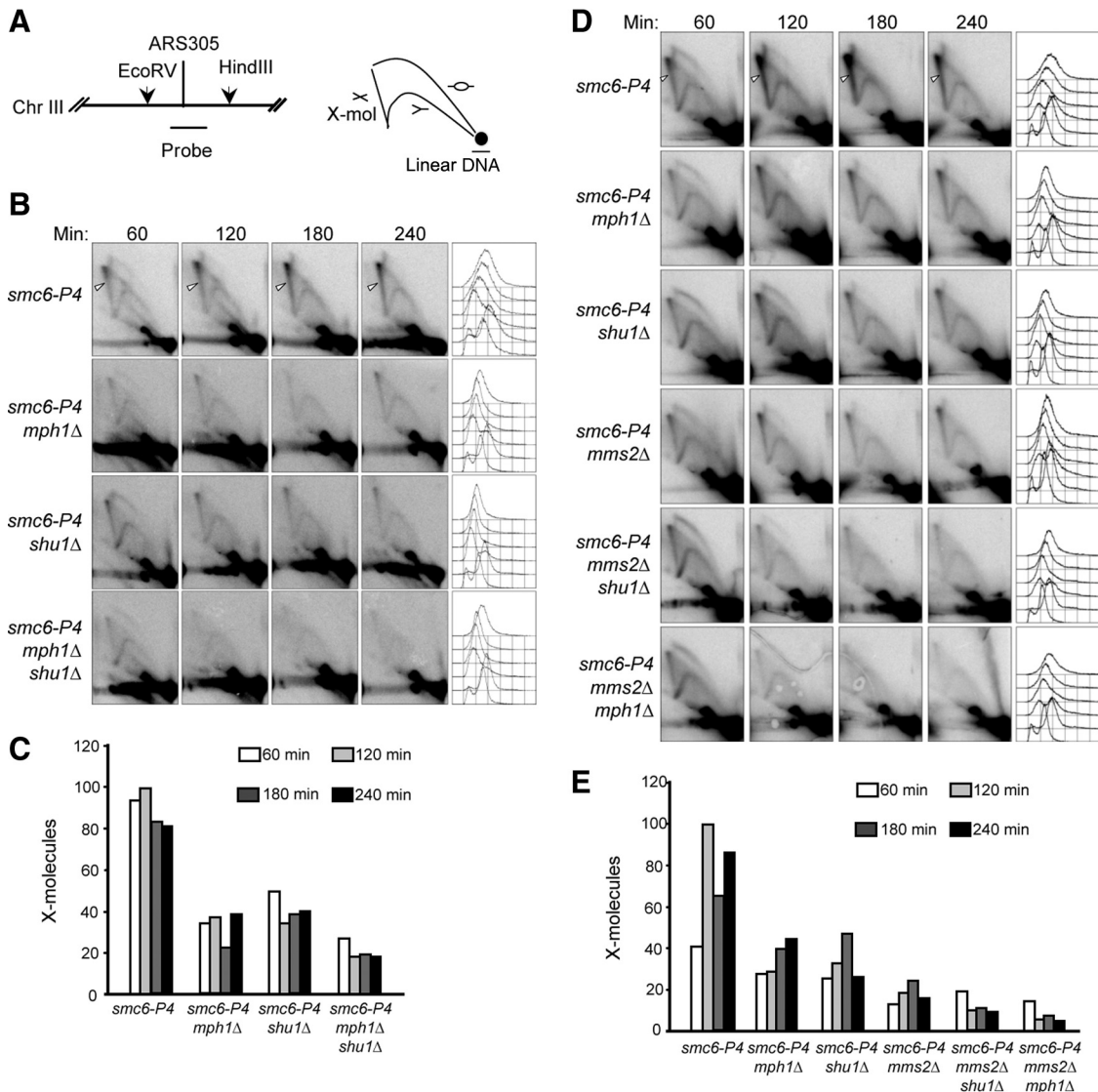


Figure 3. *shu1Δ*, *mms2Δ*, and *mph1Δ* each reduces the levels of X-mols in *smc6-P4* cells with double deletions conferring greater reduction. Cells were arrested using nocodazole and then released into YPD medium containing 0.033% MMS. The replication and recombination intermediates at the ARS305 region 60, 120, 180 and 240 min after release were analyzed by 2D gel electrophoresis followed by Southern blotting (see *Materials and Methods*). (A) Cartoons indicating the position of the probe and the replication structures. (B and D) Results of 2D gel analyses. X-shaped DNA structures are indicated by arrowheads in *smc6-P4*. FACS analyses are presented to the right of the gel image; and displayed from bottom to top are the profiles for asynchronous cultures, G2 arrested cells, released cultures at 60, 120, 180, and 240 min. Quantification of the 2D gel results in B and D are plotted in C and E, respectively.

Pol30) polyubiquitination at lysine 164, promoting template switching, we examined whether mutating this residue to arginine can mimic *mms2Δ* in the suppression of X-mol levels. As shown in Supplemental Figure 1, *pol30-K164R*, like *mms2Δ*, reduced the X-mol levels in *smc6-P4* cells. This result is in line with previous findings (Moldovan *et al.*, 2007; Branzei *et al.*, 2008) and suggests that Mms2-mediated PCNA polyubiquitination is partly responsible for X-mol accumulation in *smc6-P4* cells.

Considering our genetic results showing that *mph1Δ shu1Δ*, *mph1Δ mms2Δ*, and *shu1Δ mms2Δ* yielded greater suppression of *smc6* MMS sensitivity than each single deletion, we asked whether these double deletions have additive effects on X-mol levels in *smc6* mutants. As shown in Figure 3, B and C, *mph1Δ shu1Δ* reduced the levels of X-mols in *smc6-P4* cells to a greater degree than either single deletion. Similarly, when *mph1Δ* or *shu1Δ* was combined with *mms2Δ*, they conferred a larger

reduction in X-mol levels than each single deletion at times when the majority of the cells were undergoing replication (Figure 3, D and E). These results suggest that Mph1, Shu1, and Mms2 can act independently to promote the formation of recombination structures. The similar suppression patterns observed for MMS sensitivity and X-mol levels in all cases strongly indicate that unresolved recombinational structures contribute to the MMS sensitivity of *smc6* mutants.

mph1Δ, but Not *shu1Δ* or *mms2Δ*, Rescues the Lethality of *smc6Δ* and *mms21Δ*

The Smc5/6 complex is not only required for replication under DNA damage conditions but is also essential for normal growth (Murray and Carr, 2008; De Piccoli *et al.*, 2009). Although its essential functions are not completely understood, they are linked with the Mph1-dependent recombi-

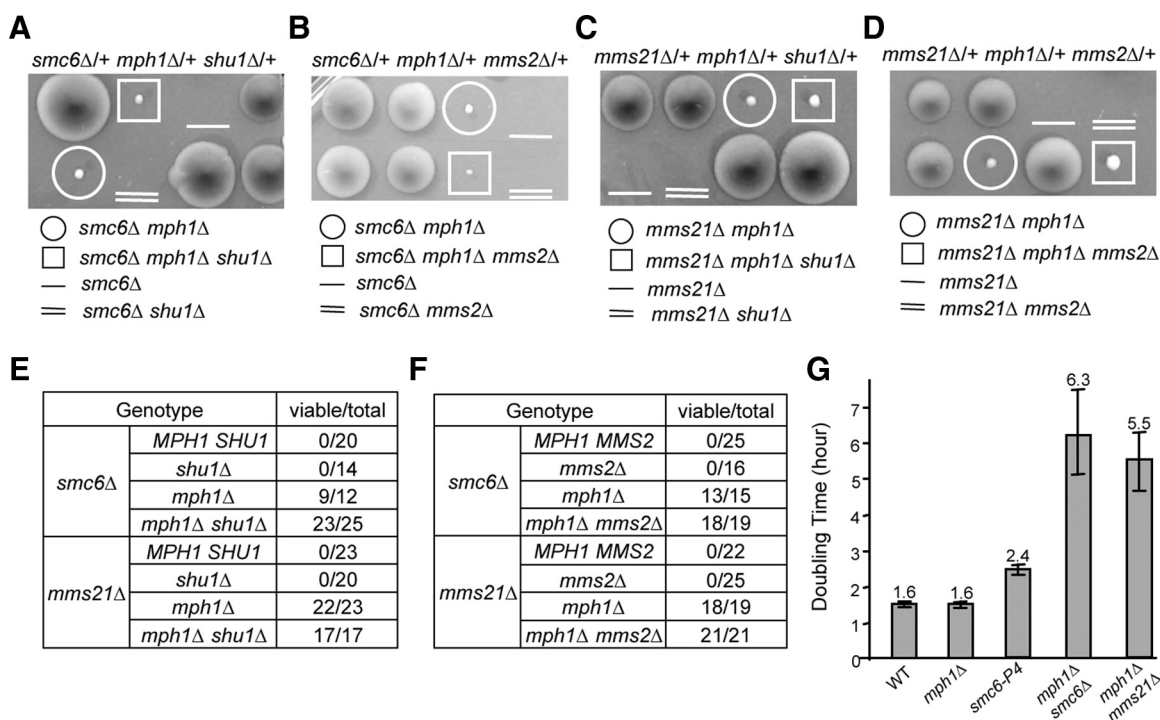


Figure 4. *mph1Δ*, but not *shu1Δ* or *mms2Δ*, rescues the lethality of *smc6Δ* and *mms21Δ*. (A–D) Representative tetrads from diploid strains with the indicated genotypes are shown. Spore clones with relevant genotypes are labeled with indicated symbols. Genotypes for spore clones containing *smc6Δ* and *mms21Δ* were deduced from sibling spore clones. (E–F) Summary of tetrad analysis for the diploids depicted in A–D. The numbers of viable spores versus total spores for each genotype are given (viable/total) for diploids shown in A and C (E) and for the diploids shown in B and D (F). (G) The doubling time of strains with indicated genotype.

national process, because *mph1Δ* rescues the lethality caused by deleting the genes encoding Smc5/6 complex subunits (Chen *et al.*, 2009). Considering the observed suppression effects of *shu1Δ* and *mms2Δ* in *smc6* mutants, we asked whether those deletions can also rescue the inviability of cells lacking the Smc5/6 complex. To do this, a copy of *SHU1* or *MMS2* was deleted in diploid cells that are heterozygous for *mph1Δ* and for either *smc6Δ* or deletion of the gene encoding the Mms21 subunit of the Smc5/6 complex. As summarized in Figure 4, E and F, and shown as examples in Figure 4, A–D, neither *shu1Δ* nor *mms2Δ* conferred viability to *smc6Δ* or *mms21Δ* cells. Control *mph1Δ smc6Δ* and *mph1Δ mms21Δ* cells were viable (Figure 4, A–F) and grew with a doubling time of 6.3 and 5.5 h, respectively (Figure 4G). Furthermore, deletion of either *SHU1* or *MMS2* did not improve the growth of *smc6Δ mph1Δ* or *mms21Δ mph1Δ* cells (Figure 4, A–F). These results suggest that the role of the Smc5/6 complex in the Mph1- but not Shu1- or Mms2-dependent processes is essential for cell viability, further illustrating the difference in the functions of these three proteins.

shu1Δ, *mph1Δ*, and *mms2Δ* All Alleviate the MMS Sensitivity of *esc2Δ* Cells

In addition to the Smc5/6 complex, Esc2 is also required to prevent the accumulation of X-mols. *esc2Δ* cells exhibit increased levels of these DNA structures and are moderately sensitive to MMS. It was recently found that both *mph1Δ* and *shu1Δ* decrease the levels of X-mols in *esc2Δ* cells when replication takes place in MMS-containing media, but neither improves *esc2Δ* cell survival in MMS-containing media (Mankouri *et al.*, 2009). This lack of correlation in the suppression of two ostensibly related defects in *esc2Δ* cells is in contrast with our observations in *smc6* mutants. Although both studies used strains in the W303 background, we noticed that the previous study used strains containing the *rad5-535* mutation (Mankouri

et al., 2009). Rad5 is a multifunctional protein that can work with Mms2 as a ubiquitin ligase to polyubiquitinate PCNA, and as a DNA translocase potentially promoting replication fork regression (Johnson *et al.*, 1994; Ulrich and Jentsch, 2000; Hoege *et al.*, 2002; Ulrich, 2003; Blastyak *et al.*, 2007). These two functions of Rad5 are independent of each other, because mutations impairing its ATP binding and translocase activity exhibit synergistic relationship with *mms2Δ* (Chen *et al.*, 2005). Rad5-535 contains a glycine-to-arginine change at amino acid 535 within the ATP-binding consensus sequence required for its translocase activity (Fan *et al.*, 1996). This change results in mild MMS sensitivity, presumably due to a partial defect in the Rad5 translocase function (Fan *et al.*, 1996). This observation prompted us to examine the genetic relationship of *rad5-535* with *mph1Δ*, *shu1Δ*, and *mms2Δ*, and its potential effects on *esc2Δ* cells.

We first examined whether *rad5-535* affects the MMS sensitivity of *mph1Δ* and *shu1Δ* cells. As shown in Figure 5, A and B, *rad5-535* exacerbated the MMS sensitivity of both *mph1Δ* and *shu1Δ* mutants. It also aggravated the MMS sensitivity of *mms2Δ* cells, which is consistent with the previous observation that mutations affecting Rad5 translocase activity sensitize *mms2Δ* (Figure 5C [Chen *et al.*, 2005]). These synergistic interactions suggest that the repair process hampered by *rad5-535* is distinct from those involving Mph1, Shu1, and Mms2. Second, the genetic interactions between *mph1Δ/shu1Δ/mms2Δ* and *esc2Δ* in strains containing wild-type *RAD5* were examined. As shown in Figure 6, A–C, *mph1Δ*, *shu1Δ* and *mms2Δ* all improved the survival of *esc2Δ* cells that contain wild-type *RAD5* on MMS-containing media. These results show that *shu1Δ* and *mph1Δ* can in fact rescue the MMS sensitivity of *esc2Δ* cells, in agreement with their suppression of X-mol accumulation. However, this observed effect was lost when cells contained *rad5-535* (Figure 6, D and E), suggesting that this mutation can mask the suppression of MMS sensitivity by *mph1Δ*, *shu1Δ* and *mms2Δ* in *esc2Δ* cells.

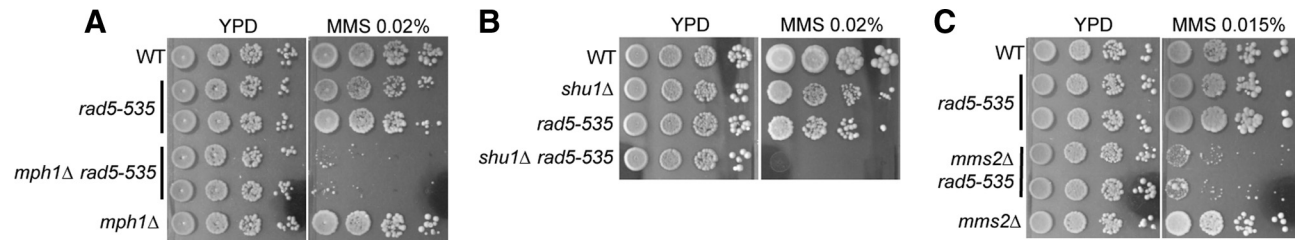


Figure 5. *rad5-535* exacerbates the MMS sensitivity of *mph1Δ*, *shu1Δ*, and *mms2Δ* cells. (A–C) Wild-type (WT) and mutant cell cultures were diluted and spotted onto YPD plates with or without the indicated concentration of MMS. *rad5-535* enhances the MMS sensitivity of *mph1Δ* (A), *shu1Δ* (B), and *mms2Δ* (C) cells.

DISCUSSION

Recombinational repair is important for genome duplication under conditions of replicative stress or DNA damage. Apart from the classical recombination proteins, additional factors that function more specifically in this process have been identified recently (Lambert *et al.*, 2007; Branzei and Foiani, 2007; Li and Heyer, 2008; Budzowska and Kanaar, 2009; Chang and Cimprich, 2009). These factors are critical for understanding the pathways coupling replication and recombinational repair. The functional relationships among a subset of these factors were analyzed in this study. Our results suggest that Mph1, the Shu complex, and Mms2, three factors involved in the formation of recombination intermediates, have nonoverlapping functions and may represent distinct pathways in replication-associated recombinational repair. This conclusion is supported by several observations. First, *mph1Δ*, *shu1Δ*, and *mms2Δ* deletion mutations exhibited additive genetic interactions. Moreover, their double deletion mutations conferred better suppression of the MMS sensitivity of *smc6-P4* and *smc6-56* cells than the corresponding single deletions. Significantly, the same suppression patterns were observed in the levels of recombination intermediates in *smc6-P4* cells by using 2D gel analyses. The disparate effects of double versus single deletions in all situations argue against

these proteins functioning in one pathway and support the idea that they promote recombination via different routes.

The presence of multiple recombinational repair processes underlines the importance and complexity of the task of rescuing impaired replication. It remains to be seen how Mph1, the Shu complex, and Mms2 differently promote recombination. One possibility is that they act on different structures generated during replication perturbation, such as collapsed forks versus single-stranded DNA gaps. Indeed, Mph1 orthologues have been proposed to catalyze replication fork regression, the Shu complex to facilitate single-strand gap repair, and the Mms2-mediated pathway to promote template switching (Komori *et al.*, 2004; Shor *et al.*, 2005; Ulrich, 2005; Martin *et al.*, 2006; Mankouri *et al.*, 2007; Branzei *et al.*, 2008; Gari *et al.*, 2008; Sun *et al.*, 2008; Unk *et al.*, 2010; Whitby, 2010). Based on the potential functions of these proteins, one plausible model for how they act independently in replication-associated recombinational repair is illustrated in Figure 7. In this model, Mph1 catalyzes fork regression when leading strand synthesis is impeded. Subsequently, the leading strand is extended using the lagging strand as template, and the resulting product can either be regressed back (not shown) or processed by nucleases and

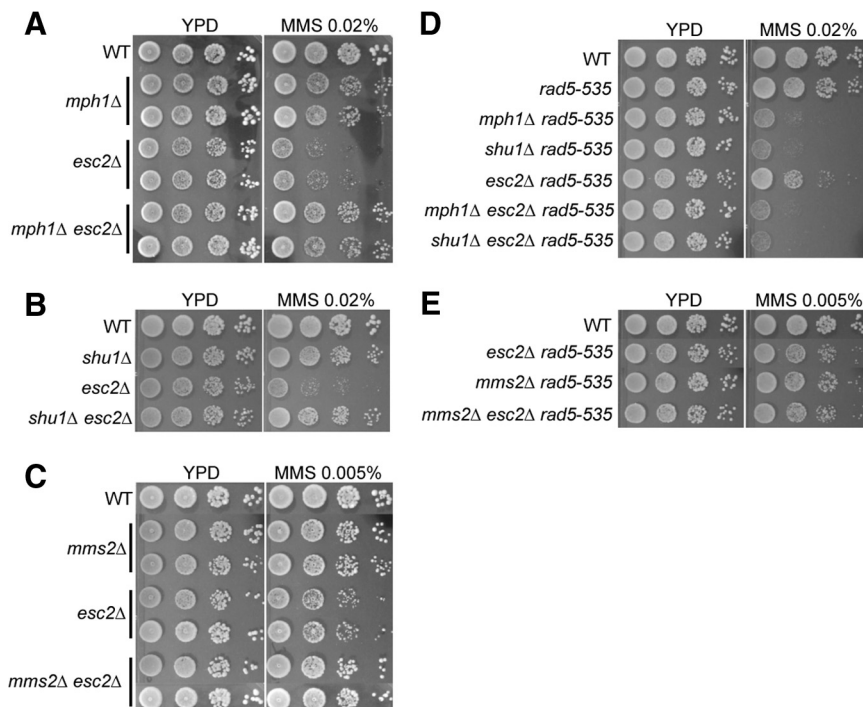


Figure 6. *mph1Δ*, *shu1Δ*, and *mms2Δ* improve *esc2Δ* cell growth on MMS-containing media in *RAD5*, but not *rad5-535*, background. Wild-type (WT) and mutant cell cultures were diluted and spotted onto YPD plates with or without the indicated concentration of MMS. In A–C, strains are in *RAD5* background, and the MMS sensitivity of *esc2Δ* is suppressed by *mph1Δ* (A), *shu1Δ* (B), and *mms2Δ* (C). In D–E, strains contain *rad5-535* as indicated.

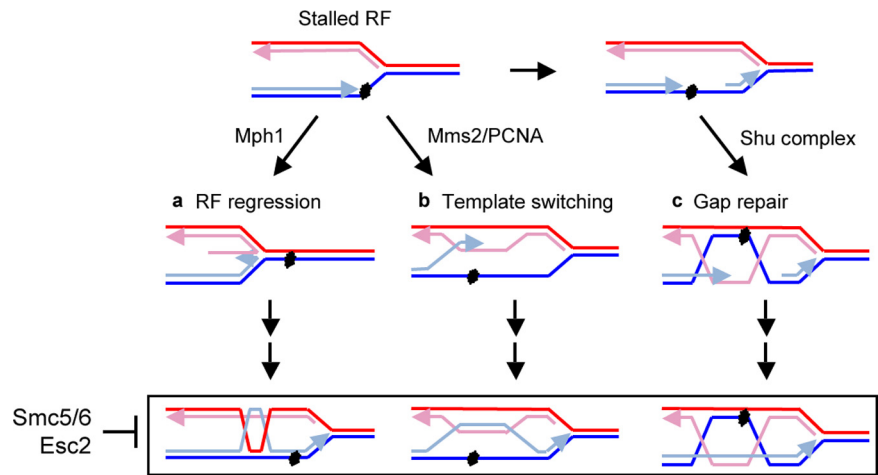


Figure 7. Candidate recombinational repair processes that require Mph1, the Shu complex, and Mms2 and the roles of the Smc5/6 complex and Esc2 in these processes. For details, see text. Note that only one of the possible modes of template switching is drawn in b.

engaged in recombination (Figure 7, a). Independently, the Mms2-mediated pathway leads to template switching, for example, the blocked leading strand invades and uses the newly synthesized lagging strand as template to synthesize DNA over the lesion (Figure 7, b). In addition, gaps on the leading or lagging strand can be repaired by Shu complex-mediated recombination, in which this complex may facilitate joint molecule formation in the absence of end invasion (a process proposed in Cunningham *et al.*, 1980) or contribute to recombination over damaged DNA (Figure 7, c). This model provides a good explanation for the observed genetic interactions and takes into consideration the known properties of the proteins. However, other possibilities also exist; for example, these protein factors may work on similar replication intermediates but in different ways depending on their unique interactions and activities. Further study of the functions of these proteins will provide a better understanding of recombinational repair pathways. Because we found that *pol30-K164R*, like *mms2Δ*, reduces X-mol levels in *smc6-P4* cells and *esc2Δ* cells (Supplemental Figure 1), the contribution of Mms2 to recombination intermediates probably requires PCNA polyubiquitination. Furthermore, because the DNA translocase activity of Rad5 functions differently from Mph1, the Shu complex, and Mms2, additional complementary routes exist for the rescue of stalled replication.

Another conclusion supported by our observations is that the Smc5/6 complex is required to prevent the accumulation of recombination structures generated by Mph1-, Shu-, and Mms2-dependent processes (Figure 7) and that this function is crucial for cell survival under replicative stress. Although Mph1 and the Shu complex are thought to function exclusively in recombination, Mms2 has additional roles. However, we favor the interpretation that the Smc5/6 complex affects its roles in recombinational repair, because the removal of Mms2 or proteins functioning with it rescues both the accumulation of X-mols and MMS sensitivity in mutants of the Smc5/6 complex (this study; Branzei *et al.*, 2008). Among the three proteins, the action of Mph1 seems to be most deleterious when the Smc5/6 complex is defective, because *mph1Δ* exhibited the strongest suppression of MMS sensitivity in *smc6* mutants and is the only mutation that rescued the lethality of cells lacking the Smc5/6 complex. Considering that the Smc5/6 complex physically interacts with Mph1 (Chen *et al.*, 2009), an attractive model is that this complex directly modulates Mph1 in fork regression or other processes. The roles of the Smc5/6 complex in the other two pathways remain to be determined, but they could involve direct modulation of protein factors and/or tethering of DNA.

Our finding that the removal of Mph1, Shu1, or Mms2 (in the *RAD5* background) resulted in better growth of *esc2Δ* cells under DNA damage conditions indicates that Esc2, like the Smc5/6 complex, also influences the processes involving these three proteins. These results, in conjunction with the reports by Mankouri *et al.* (2009), suggest a good correlation between the suppression of X-mol accumulation and MMS sensitivity in *esc2Δ* cells, similar to the situation in *smc6* cells. Therefore, the accrual of X-mols is probably a major underlying defect that accounts for the poor survival of these cells under DNA damage conditions. Although the Smc5/6 complex and Esc2 exhibit similar genetic interactions with Mph1, Shu1, and Mms2, they have at least partly different functions, as suggested by the synthetic interactions of their mutants and by the lack of physical interaction between the two factors (Sollier *et al.*, 2009). It is also noteworthy that Sgs1, a Holliday junction dissolving enzyme that also prevents the accumulation of recombination intermediates, does not affect the Mph1-dependent process (Chen *et al.*, 2009; Mankouri *et al.*, 2009). The necessity of multiple regulators of the multiple replication-associated recombinational processes underlines the need to coordinate recombination steps in dealing with different types of lesions formed when replication is perturbed. Because all the proteins investigated here are conserved in humans, it is likely that similar functional circuitries also exist in higher eukaryotic cells. Molecular dissection of these processes in yeast will thus provide important clues for understanding how recombinational repair facilitates replication in human cells.

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