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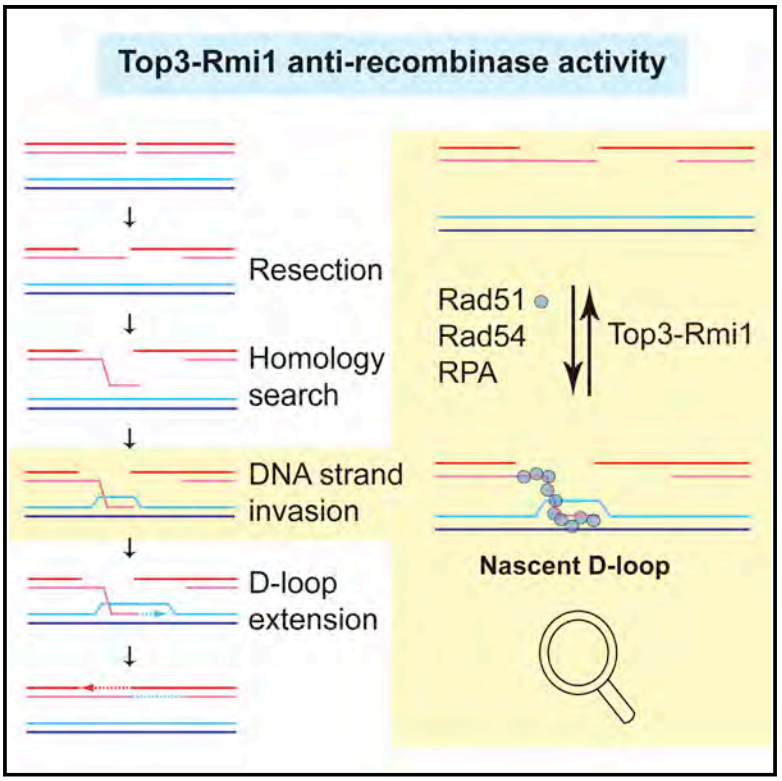
Molecular Cell

Top3-Rmi1 Dissolve Rad51-Mediated D Loops by a Topoisomerase-Based Mechanism

Graphical Abstract



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In Brief

Mutations in the topoisomerase Top3 lead to an extreme hyper-recombination phenotype, but a mechanistic explanation remained elusive. Fasching et al. provide in vitro biochemical evidence that Top3 has anti-recombination activity that it uses to dissolve D loops that form during recombination.

Highlights

- Yeast Top3 and human TOPOIIIα dissolve D loops
- Top3-mediated D loop dissolution is dependent on its topoisomerase activity
- D loop dissolution by yeast Top3 is specific for the cognate system
- The results explain the different hyper-rec phenotypes of *top3* and *sgs1* mutants

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Top3-Rmi1 Dissolve Rad51-Mediated D Loops by a Topoisomerase-Based Mechanism

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SUMMARY

The displacement loop (D loop) is a DNA strand invasion product formed during homologous recombination. Disruption of nascent D loops prevents recombination, and during synthesis-dependent strand annealing (SDSA), disruption of D loops extended by DNA polymerase ensures a non-crossover outcome. The proteins implicated in D loop disruption are DNA motor proteins/helicases that act by moving DNA junctions. Here we report that D loops can also be disrupted by DNA topoisomerase 3 (Top3), and this disruption depends on Top3's catalytic activity. Yeast Top3 specifically disrupts D loops mediated by yeast Rad51/Rad54; protein-free D loops or D loop mediated by bacterial RecA protein or human RAD51/RAD54 resist dissolution. Also, the human Topoisomerase IIIa-RMI1-RMI2 complex is capable of dissolving D loops. Consistent with genetic data, we suggest that the extreme growth defect and hyper-recombination phenotype of Top3-deficient yeast cells is partially a result of unprocessed D loops.

INTRODUCTION

Homologous recombination (HR) is a highly conserved and ubiquitous mechanism for the repair or tolerance of complex DNA damage such as double-stranded breaks or interstrand cross-links (Li and Heyer, 2008). HR is essential for meiotic chromosome segregation and crossover formation involving the formation and resolution of double Holliday junctions (dHJs) (Hunter, 2007). In addition, HR is required for the recovery of blocked or broken replication forks. Filaments of the Rad51 protein on ssDNA perform the signature reactions of HR: homology search and DNA strand invasion (Heyer et al., 2010). The product of strand invasion is the displacement loop (D loop), a joint molecule in which the invading strand primes DNA synthesis on a donor template. In yeast, the Rad54 protein is required for D loop formation and displaces Rad51 from the heteroduplex (hDNA) giving the DNA polymerase access to the invading 3′ end (Li and Heyer, 2009). In somatic cells, HR is heavily skewed toward using the sister chromatid as a template and favors a non-crossover (NCO) outcome (Johnson and Jasin, 2000; Kadyk and Hartwell, 1992). This avoids the potential for loss of heterozygosity, a process known to be involved in tumorigenesis (LaRocque et al., 2011). To ensure an NCO outcome, the D loop is disrupted after DNA polymerase extension, and the extended strand is annealed to the second end of the original DSB in a process termed synthesis-dependent strand annealing (SDSA).

D loops constitute reversible, metastable intermediates of the HR pathway (Heyer et al., 2010). The nascent D loop (i.e., the D loop before extension by DNA polymerase) can be reversed to its component DNA molecules to abort HR. This mechanism of anti-recombination has been implicated in a process termed hDNA rejection, where mismatches between invading strand and the donor template trigger abortion of HR (Hombauer et al., 2011). Disruption of extended D loops (i.e., D loops after extension by DNA polymerase) is an integral part of SDSA and a mechanism of anti-crossover. The mechanisms involved in D loop disruption are not fully understood. A number of genes/proteins have been implicated in this process either by genetic, biochemical, or cell biological evidence. These include *Saccharomyces cerevisiae* Srs2 and Mph1 as well as the Mph1 homologs, FANCM, and Fml1 in plants and fission yeast, respectively (Crismani et al., 2012; Ira et al., 2003; Lorenz et al., 2012; Prakash et al., 2009; Robert et al., 2006). In addition, the human RecQ-like helicases BLM and RECQ1, as well as the helicase RTEL1, have been implicated in D loop dissociation (Bachrati et al., 2006; Barber et al., 2008; Bugreev et al., 2008; van Brabant et al., 2000). Specifically, Srs2, Mph1/FANCM/Fml1, and RTEL1 have been implicated in crossover avoidance. RECQ1, instead, has been implicated in the disruption of dead-end D loops, where the 5′ end has invaded a donor template. Also Rad54 protein, which is required for D loop formation by yeast Rad51, disrupts D loops depending on the specific structure of the joint molecule (Bugreev et al., 2007a; Wright and Heyer, 2014). Common to all reported mechanisms of D loop disruption is the involvement of DNA helicase/motor proteins that disrupt D loops by an ATP-driven mechanism involving translocation on ssDNA or dsDNA.

Sgs1 is the single RecQ helicase in the budding yeast *S. cerevisiae* and represents the homolog to human BLM, one of five RecQ helicases in mammals (Bernstein et al., 2010; Chu and Hickson, 2009). Sgs1 is a 3′-5′ DNA helicase that associates

with a topoisomerase (Top3) and an OB-fold protein (Rmi1). The yeast Sgs1-Top3-Rmi1 complex is considered homologous to the human BLM-TOPOIIIα-RMI1-RMI2 complex. Sgs1/BLM is a potent DNA helicase active on a variety of substrates. Top3 and its human homolog TOPOIIIα are type IA DNA topoisomerases that introduce a transient nick in one DNA strand (cut strand or C-strand), allowing a second unbroken ssDNA (transfer strand or T-strand) to be transferred reversibly through the nick. The C-strand is cut in a reversible transesterification mechanism involving the formation of a covalent linkage between the 5′-end of the C-strand and the active site tyrosine, Y356, of Top3. In order to act, Top3 needs access to ssDNA; in order to relax dsDNA, high temperature and specific reactions conditions such as high glycerol concentrations are required (Chen and Brill, 2007). Rmi1 projects as a loop into the Top3/TOPOIIIα gate, stabilizing the open conformation to favor decatenation over relaxation (Bocquet et al., 2014). As a result, Rmi1 enhances the decatenation activity of Top3 while slowing DNA relaxation (Cejka et al., 2012). The function of Top3 as an ssDNA decatenase is consistent with genetic data in combination with mutations in Top1 and Top2 that led to the conclusion that Top3 does not act as a relaxase of negatively supercoiled DNA in vivo (Kim and Wang, 1992).

The phenotypes of Sgs1/BLM-deficient cells are exceedingly complex and reflect an involvement in several aspects of DNA metabolism, including DNA replication, DNA checkpoint signaling, and HR (Bernstein et al., 2010; Chu and Hickson, 2009). Both yeast Sgs1-Top3-Rmi1 and human BLM-TOPOIIIα-RMI1-RMI2 complexes are involved at various steps throughout HR. In addition, they also process structures generated during replication fork stalling or collapse and have been implicated in the resolution of late replication intermediates (Bernstein et al., 2009; Chan et al., 2009; Liberi et al., 2005; Wang, 1991). The specific DNA structures and mechanisms involved are only partly understood, but they may be the consequence of a single mechanistic defect in the decatenation of DNA (Cejka et al., 2012; Hickson and Mankouri, 2011). During HR, Sgs1 and its catalytic activity are required for long-range DSB resection to initiate HR (Cejka et al., 2010a; Mimitou and Symington, 2008; Niu et al., 2010; Zhu et al., 2008). Interestingly, while Top3 protein is required for this function, the Top3 catalytic activity is not (Niu et al., 2010). Seminal work on the human BLM-TOPOIIIα complex established a mechanism to process dHJs, a late HR intermediate, into NCO products, which had been termed dissolution to distinguish the process from endonucleolytic resolution (Wu and Hickson, 2003). Both the human and yeast complexes collapse the dHJ into a hemi-catenane intermediate by joint catalytic action of BLM/Sgs1 and Top3/TOPOIIIα, such that Top3/TOPOIIIα can dissolve the final hemi-catenane to separate the two parent molecules into an NCO outcome (Cejka et al., 2010b; Wu and Hickson, 2003). Both end resection and dHJ dissolution require Sgs1 catalytic activity, but genetic data indicate that Sgs1 also performs helicase-independent functions, which have not been defined yet (Lo et al., 2006; Mullen et al., 2000). Top3 catalytic activity has been demonstrated to be required for dHJ dissolution, but surprisingly, the slow growth phenotype of Top3-deficient cells is significantly more pronounced than the phenotype of Sgs1-deficient cells (Mullen et al., 2000; Onodera et al., 2002; Shor et al., 2002; Wallis et al., 1989). The phenotype of Rmi1-deficient cells appears to be indistinguishable from Top3 deficiency and strongly suggests that Top3-Rmi1 form an obligatory functional complex in cells (Mullen et al., 2005). Current models cannot provide a mechanistic explanation for the differential phenotype of *sgs1* and *top3/rmi1* mutants. It has been suggested that Sgs1 generates DNA intermediates whose resolution requires Top3 (Wallis et al., 1989). However, it is also possible that, in the absence of Top3, DNA intermediates accumulate that are then processed by Sgs1 in a pathological manner. Both models are consistent with the observed partial suppression of the *top3* growth defect by *sgs1* (Wallis et al., 1989).

In this study, we set out to evaluate the role of Sgs1 and the Sgs1-Top3-Rmi1 complex in reversing the D loop intermediate in HR. As expected based on experiments with purified human BLM protein (Bachrati et al., 2006; van Brabant et al., 2000), Sgs1 was found to dissociate protein-free D loops in a manner that was dependent on its helicase activity. Surprisingly, Sgs1 was unable to dissociate D loops in a reconstituted D loop reaction with the cognate Rad51, Rad54, and RPA proteins. Unexpectedly, we found that yeast Sgs1-Top3-Rmi1 as well as human TOPOIIIα-RMI1-RMI2 dissolve D loops in such reconstituted reactions. Specifically, Top3 and its catalytic activity were required for D loop dissolution dependent on the presence of a single-stranded DNA (ssDNA) binding protein. This reaction proceeds with significant specificity and does not occur on protein-free D loops or D loops generated by bacterial RecA protein or human RAD51/RAD54. Results from several control experiments suggest that Top3 does not act by relaxing the negatively supercoiled duplex substrate, consistent with previous biochemical and genetic results that Top3 is inefficient as a DNA relaxase. Sgs1 moderates the activity of Top3, whereas Rmi1 stimulates Top3 in D loop dissolution. Taken together, we show D loop reversal by a Top3-based mechanism that may share mechanistic similarities with dHJ dissolution catalyzed by the Sgs1-Top3-Rmi1/BLM-TOPO3α-RMI1-RMI2 complexes. We discuss genetic data that are consistent with a specific role of Top3 in reversing HR intermediates ensuring an NCO outcome in addition to its known HR roles in DSB end resection and dHJ dissolution.

RESULTS

Sgs1 Disrupts Protein-Free D Loops but Fails to Disrupt D Loops in Reconstituted Reactions with Rad51-Rad54

Disruption of nascent D loops is a potential mechanism of anti-recombination, and disruption of extended D loops is an integral part of the SDSA pathway of HR leading to an NCO outcome. The BLM helicase has been implicated in D loop disruption, and biochemical experiments have shown that purified BLM disrupts D loops assembled from oligonucleotide substrates or D loops produced by bacterial RecA protein from an invading oligonucleotide and a supercoiled target duplex DNA after deproteinization of the substrate (Bachrati et al., 2006; van Brabant et al., 2000). The yeast BLM homolog Sgs1 is a potent DNA helicase active at sub-nanomolar concentrations (Cejka and Kowalczykowski, 2010), but its activity on D loops has never been tested. Following the approach used with BLM (Bachrati et al.,

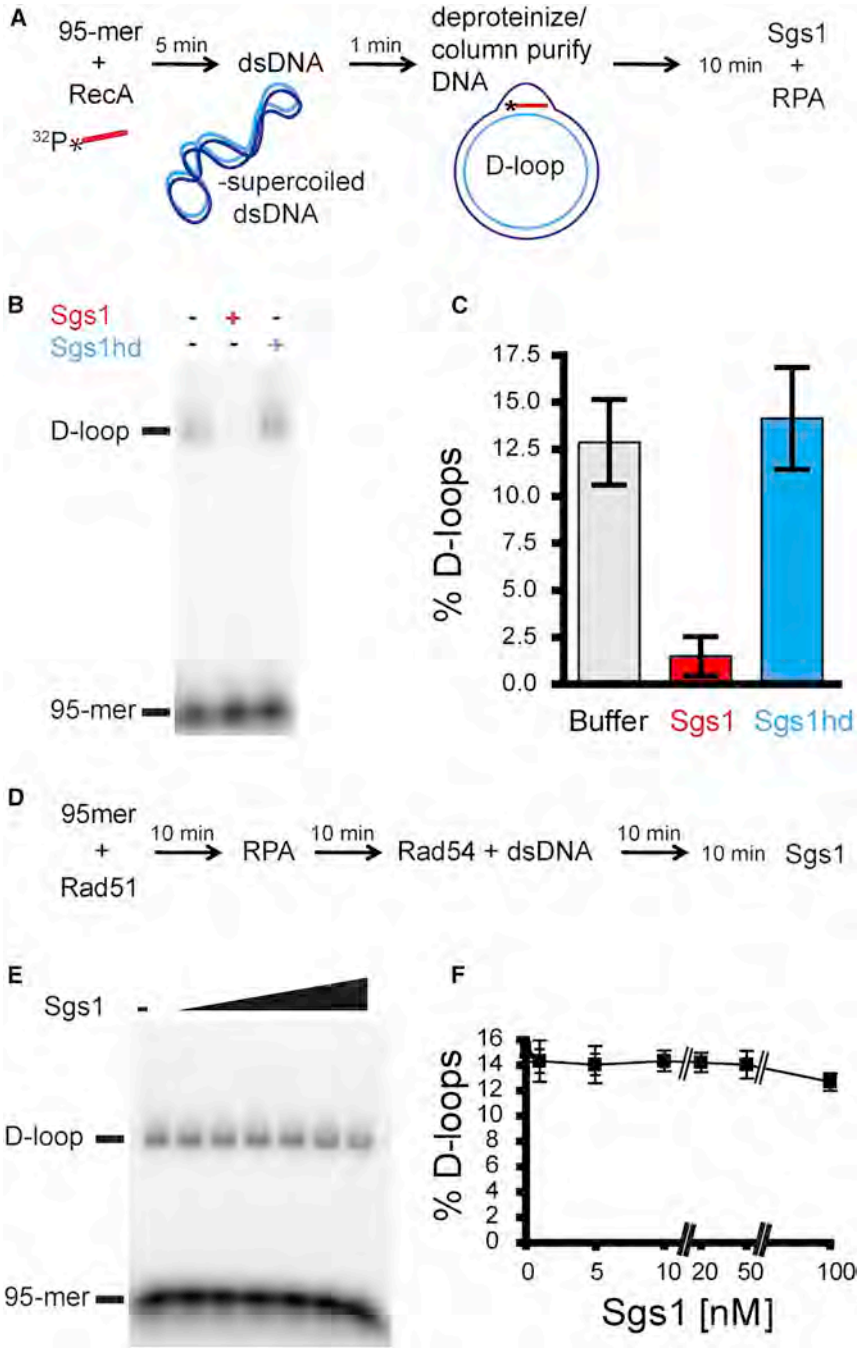

Figure 1. Sgs1 Disrupts Protein-Free but Not Rad51-Mediated D Loops

(A) Reaction scheme for deproteinized purified D loops.

(B) Purified protein-free D loops (~1 nM) containing a 5′-end-labeled 95-mer were incubated with 0.5 nM Sgs1 or Sgs1hd (Sgs1-K706A) or reaction buffer for 10 min and the reaction products resolved on agarose gels.

(C) Quantitation of D loops. Shown are means ± SD of three independent experiments.

(D) Scheme for Rad51/Rad54-mediated D loop reaction.

(E) Representative gel of products from reactions containing 20 nM 5′-end-labeled 95-mer, 0.67 μM Rad51 (1 Rad51: 3 nt), 100 nM RPA, 112 nM Rad54, 20 nM supercoiled plasmid DNA, and Sgs1 (0, 1, 5, 10, 20, 50, and 100 nM).

(F) Quantitation of D loops. Shown are means ± SDs of three independent experiments.

2006), we tested the activity of yeast Sgs1 on deproteinized D loops produced by the bacterial RecA protein (Figure 1A). Using near equimolar amounts of Sgs1 (0.5 nM) and D loop substrate (~1 nM), we show that Sgs1, like human BLM, efficiently disrupts protein-free D loops (Figures 1B and 1C). While it has been assumed that the BLM helicase activity is responsible for D loop disruption, this had not been formally demonstrated. Consistent with this expectation, Sgs1hd, the helicase-deficient Sgs1-K706A protein, is completely deficient in disrupting protein-free D loops (Figures 1B and 1C). The data show that Sgs1 disrupts D loops by a mechanism that depends on its ATPase activity that is required for its helicase function.

In cells, D loops are unlikely to be protein free and rather represent different species of protein-DNA complexes. Nascent D loops likely still have proteins bound to the substrate that performed homology search and strand invasion (e.g., RPA, Rad51, and Rad54) (Solinger et al., 2002). To test whether Sgs1 can disrupt nascent D loops, we reconstituted D loop formation with the yeast RPA, Rad51, and Rad54 proteins. After an initial 2 min incubation, about 15% D loops were formed, which represents about 3 nM substrate (input 20 nM dsDNA). Then Sgs1 was added, and the amount of D loops was determined after an additional 10 min of incubation (Figure 1D), which was sufficient for complete disruption of protein-free D loops (Figures 1B and 1C). A titration of up to 100 nM of Sgs1, representing 30-fold excess of protein over substrate, failed to show any D loop disruption activity in this assay (Figures 1E and 1F).

As a member of the RecQ family of helicases, Sgs1 translocates along ssDNA with 3′-to-5′ polarity. During the D loop reaction, Rad54 stimulates formation of the D loop and removes Rad51 exposing the 3′ end of the heteroduplex (Li and Heyer, 2009). To determine if exposing the 3′ end of the heteroduplex DNA was required for its removal by Sgs1, we added the helicase at different times after initiation of D loop formation. Sgs1 does not dissolve D loops even when added up to 20 min post-D loop initiation (data not shown), a time at which the 3′ end is accessible to extension by Polδ (Li and Heyer, 2009). To

determine if Sgs1 blocks formation of D loops by interfering with the Rad51 filament stability or prevents Rad54-mediated joint molecule formation, we added Sgs1 to the reaction with RPA, which is prior to D loop initiation or with Rad54 at the time of D loop initiation (Figure S1A). We found that Sgs1 or Sgs1hd were unable to block formation of D loops when added at early times during their formation (Figures S1B and S1C). Our standard D loop is formed with 95 bp of fully homologous hDNA (Figure 1A). As Sgs1 is a 3′-to-5′ helicase, it may require a portion of unpaired filament to recognize the D loop as a substrate (Cejka and Kowalczykowski, 2010). To evaluate such substrate requirements that more closely emulate invasion of ssDNA into a dsDNA molecule, we formed D loops containing 25 nt of heterology 5′ of the 95 nt of homology. However, such 5′-tailed substrates were also refractory to disruption by Sgs1 in the reconstituted D loop reaction (Figures S1D and S1E). It has been proposed that Sgs1 acts to remove erroneous joint molecules such as those formed by 5′ strand invasion events (Bernstein et al., 2010). BLM was found to disrupt such protein-free 3′-tailed D loops faster than any other D loop substrate (Bachrati et al., 2006). We formed D loops with 25 nt of 3′-heterology emulating a 5′ invasion and found that similar to the 3′ invasions, neither wild-type Sgs1 nor Sgs1hd were able to disrupt such joint molecules (Figures S1D and S1E).

We conclude that yeast Sgs1, like human BLM, efficiently disrupts protein-free D loops but cannot directly act on the formation or turnover of D loops in reconstituted reactions with yeast RPA, Rad51, and Rad54. Human BLM was reported to disrupt D loop in reactions reconstituted with human RPA and RAD51 (Bugreev et al., 2007b). This activity depended on activating the RAD51 ATPase activity by chelation of the Ca^{2+} ions present in the reaction to inhibit the RAD51 ATPase. No D loop disruption by BLM was evident when RAD51 was maintained in the active ATP-bound form (Bugreev et al., 2007b; Nimonkar et al., 2008). Activation of the RAD51 ATPase activity lowers its affinity to DNA (Ristic et al., 2005; van Mameren et al., 2009). Hence, it is possible that D loop disruption by BLM after Ca^{2+} chelation reflects activity on protein-free substrates.

Top3 Dissolves Rad51-Rad54-Mediated D Loop with a Topoisomerase-Dependent Mechanism

Sgs1 forms a conserved complex with Top3 and Rmi1 and acts together with these proteins during HR in DSB end resection and dHJ dissolution (Chu and Hickson, 2009; Symington and Gautier, 2011). The availability of purified Sgs1-Top3-Rmi1 (STR) complex (Cejka and Kowalczykowski, 2010; Cejka et al., 2010b; Cejka et al., 2012) afforded us the opportunity to test the entire STR complex in our reconstituted D loop system (Figure 2A). Unlike Sgs1 (Figures 1D–1F and 2C), the STR complex efficiently removed Rad51-Rad54-mediated D loops (Figures 2B and 2C). In reactions containing 2 nM D loops (20 nM dsDNA input), up to 80% of the D loops were eliminated (Figures 2B and 2C). Unexpectedly, this activity by the STR complex was independent of the Sgs1 ATPase activity, as the complex of Sgs1hd-Top3-Rmi1 (DTR) was as efficient as the wild-type complex (Figures 2B and 2C). This suggests that the mechanism active in the reconstituted reaction is fundamentally different from the Sgs1-mediated disruption of protein-free D loops observed in Figure 1. In fact, Top3 alone efficiently eliminated Rad51-Rad54-mediated D loops (Figures 2D and 2E). This activity depended on the topoisomerase activity of Top3, as the Top3 catalytic mutant (Top3cd) affecting the active site tyrosine (Y356F) (Figure S2) was completely devoid of this activity even at up to 12-fold excess protein over substrate. We term this novel Top3 activity "D loop dissolution" to acknowledge the similarity to dHJ dissolution by Sgs1-Top3-Rmi1 and BLM-TOPOIIIα-RMI1/2 (Cejka et al., 2010b; Wu and Hickson, 2003). In these reactions, we observed not only an overall decrease in the D loop signal but also indication of topological activity leading to slower migration of D loops labeled as topoisomers in Figures 2B and 2D. This topological activity is specific and not seen with the negatively supercoiled substrate DNA or with D loop formed by human RAD51/RAD54 (see below).

Top3 is a ssDNA-specific topoisomerase (Kim and Wang, 1992) that is stimulated by its cognate ssDNA binding protein, RPA, but also by non-cognate ones such as *E. coli* SSB (Cejka et al., 2012). We tested the role of RPA in the D loop dissolution reaction and found a mild stimulation of Top3 or Top3-Rmi1-mediated D loop dissolution with no apparent preference for yeast RPA over human RPA or bacterial SSB (Figures S2B–S2D).

Top3 Dissolves Rad51-Rad54 Reconstituted D Loops in a Species-Specific Manner

The key steps in HR and their catalysts are well conserved in evolution. Specifically, the central reactions of homology search and DNA strand invasion are catalyzed by a highly conserved nuclear protein filament composed of a RecA protein family homolog bound to ssDNA and ATP. While these proteins, archaeal RadA, bacterial RecA, or eukaryotic Rad51, form structurally and functionally highly similar filaments, they engage in species-specific protein interactions (Heyer, 2007). These characteristics allow testing of the specificity of Top3-mediated D loop dissolution. First, we employed protein-free D loops (Figure 3A) that can readily be disrupted by yeast Sgs1 (Figures 1A–1C). To not confound the analysis with Sgs1, we only tested Top3 and the Top3-Rmi1 complex, but not the Sgs1-Top3-Rmi1 heterotrimer. Neither Top3 nor Top3-Rmi1 in the presence or absence of RPA was able to dissolve protein-free D loops (Figures 3B and 3C). In reactions with yeast Rad51-Rad54, topological isoforms of the D loops (Figures 2B and 2D) were generated during D loop dissolution that indicate that Top3 was able to topologically relax the D loop, leading to slower migration on agarose gels. Protein-free D loops, however, showed no evidence of Top3 topological activity (Figure 3B). This behavior of yeast Top3 is in contrast to *Drosophila* TopIIIβ, which has been shown to act on deproteinized D loops by nicking the displaced strand, which leads to accumulation of nicked product on a gel (Wilson-Sali and Hsieh, 2002). Next, we reconstituted the D loop reaction with bacterial RecA protein using either yeast or human RPA as the ssDNA binding protein (Figure 3D). RecA-mediated D loops were also refractory to Top3-mediated dissolution (Figures 3E and 3F). However, unlike with protein-free D loops, there was some evidence of topological activity to relax D loops in the reaction (Figure 3E), which depended on the presence of RPA (not shown). The total amount of D loops did not change significantly. Finally, we reconstituted the D loop

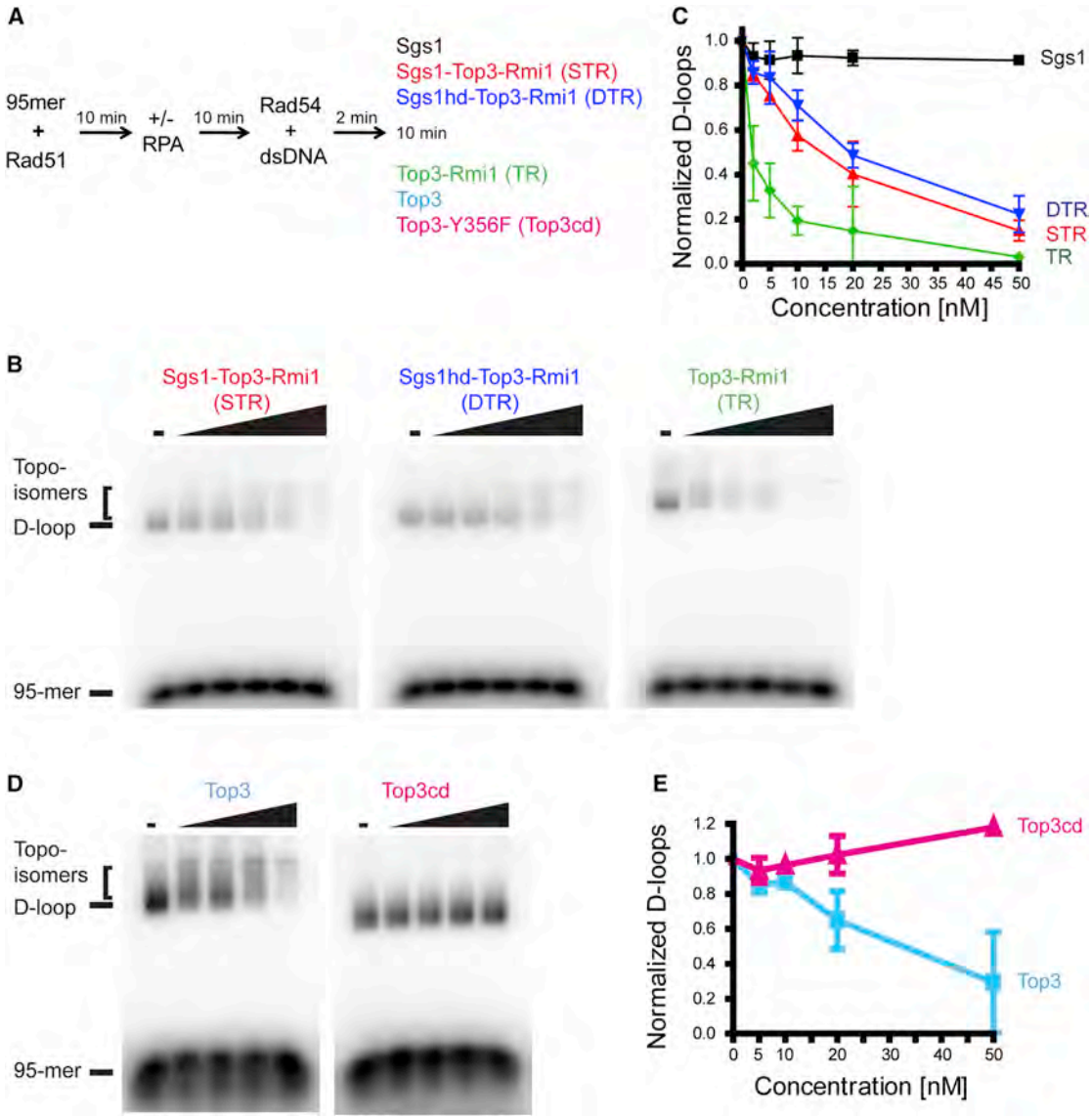

Figure 2. Topoisomerase Activity Is Necessary and Sufficient for Dissolution of Rad51-Rad54 Reconstituted D Loops by Sgs1-Top3-Rmi1

(A) Reaction scheme and proteins.

(B) D loop dissolution by Sgs1, Sgs1-Top3-Rmi1 (STR), Sgs1-K706A-Top3-Rmi1 (DTR), and Top3-Rmi1 (TR) (0, 2, 5, 10, 20, and 50 nM).

(C) Quantitation of D loops. Shown are normalized means ± SDs of three independent experiments. The absolute values corresponding to maximal D loop levels are Sgs1: 14%, DTR: 12%, STR: 11%, and TR: 18%. The Sgs1 data were taken from Figure 1.

(D) D loop dissolution by Top3 and Top3cd (Top3-Y356F) (0, 5, 10, 20, and 50 nM).

(E) Quantitation of D loops. Shown are normalized means ± SDs of three independent experiments. The absolute values corresponding to maximal D loop levels are Top3 22% and Top3cd 19%.

reaction with human RAD51 and human RPA in the presence and absence of human RAD54 (Figure 3G; see also Figures 5A and 5B). Human RAD51/RAD54-mediated D loops were refractory to Top3-mediated D loop dissolution and Top3-mediated relaxation of D loops. We conclude that D loop dissolution by yeast Top3 is highly species specific, which indicates that this activity is likely to be of biological significance.

The stability of D loops with an invading 95-mer depends on negative supercoiling of the duplex DNA (Wright and Heyer, 2014). A possible mechanism of D loop disruption would the relaxation of the negative supercoils. To confirm that *Sc*Rad51-Rad54-reconstituted D loops are a specific template for Top3 activity rather than D loop dissolution by topoisomerase-mediated relaxation of the negatively supercoiled template, we directly determined the ability of Top3 to relax the negatively supercoiled duplex substrate under our experimental conditions. Bacterial Top1 readily relaxed negatively supercoiled dsDNA, as expected (Figure S3A). However, 500 nM Top3 did not relax

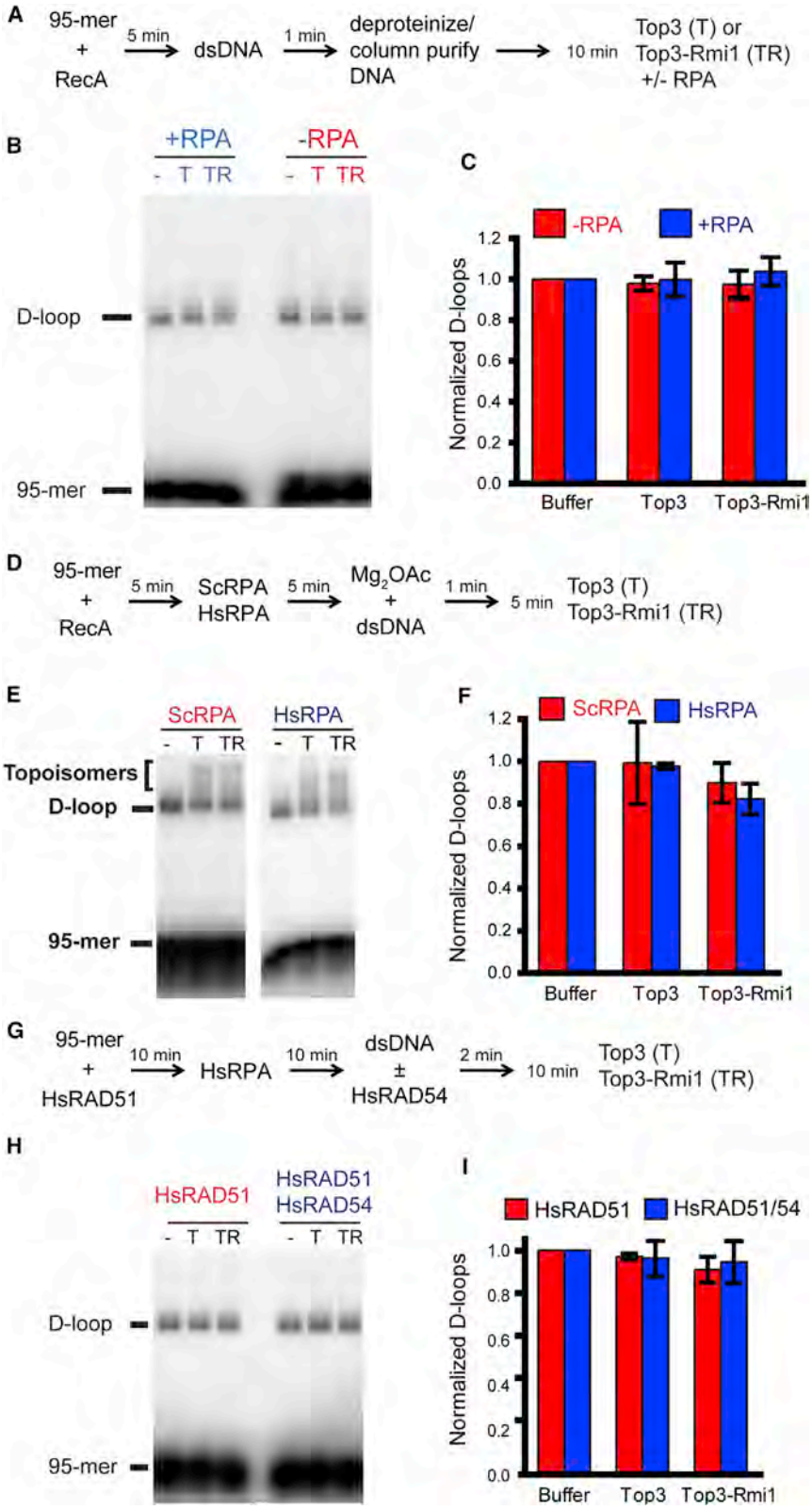

Figure 3. Top3-Mediated D loop Dissolution Is Highly Specific

Top3 does not dissolve protein-free D loops.

(A) Reaction scheme for deproteinized D loops.

(B) Deproteinized D loops (~1 nM) were incubated with 0.5 nM Top3 (T) or Top3-Rmi1 (TR) in the presence or absence of 100 nM RPA.

(C) Quantitation of D loops. Shown are normalized means ± SDs of three independent experiments. The absolute values corresponding to maximal D loop levels were buffer 11.9%, Top3 12.6%, and Top3-Rmi1 12%. Top3 does not dissolve RecA-mediated D loops.

(D) Reaction scheme for RecA-mediated D loops.

(E) RecA D loop reactions were incubated with 0.5 nM Top3 (T) or Top3-Rmi1 (TR) in the presence or absence of 100 nM yeast RPA (ScRPA) or human RPA (HsRPA).

(F) Quantitation of D loops. Shown are normalized means ± SDs of three independent experiments. The absolute values corresponding to maximal D loop levels were buffer (ScRPA 5.8%, HsRPA 7.4%), Top3 (ScRPA 5.5%, HSRPA 6%), and Top3-Rmi1 (ScRPA 5.1%, HsRPA 5.7%). Top3 does not dissolve human RAD51-mediated D loops.

(G) Reaction scheme for human RAD51- or RAD51/RAD54-mediated D loops.

(H) RAD51 D loop reactions were incubated with 2 nM Top3 (T) or Top3-Rmi1 (TR) in the presence or absence of 100 nM human RPA (HsRPA).

(I) Quantitation of D loops. Shown are normalized means ± SDs of three independent experiments. The absolute values corresponding to maximal D loop levels were buffer (RAD51 7.8%, RAD51/RAD54 7.5%), Top3 (RAD51 7.6%, RAD51/RAD54 7.2%), and Top3-Rmi1 (RAD51 7.1%, RAD51/RAD54 7.1%).

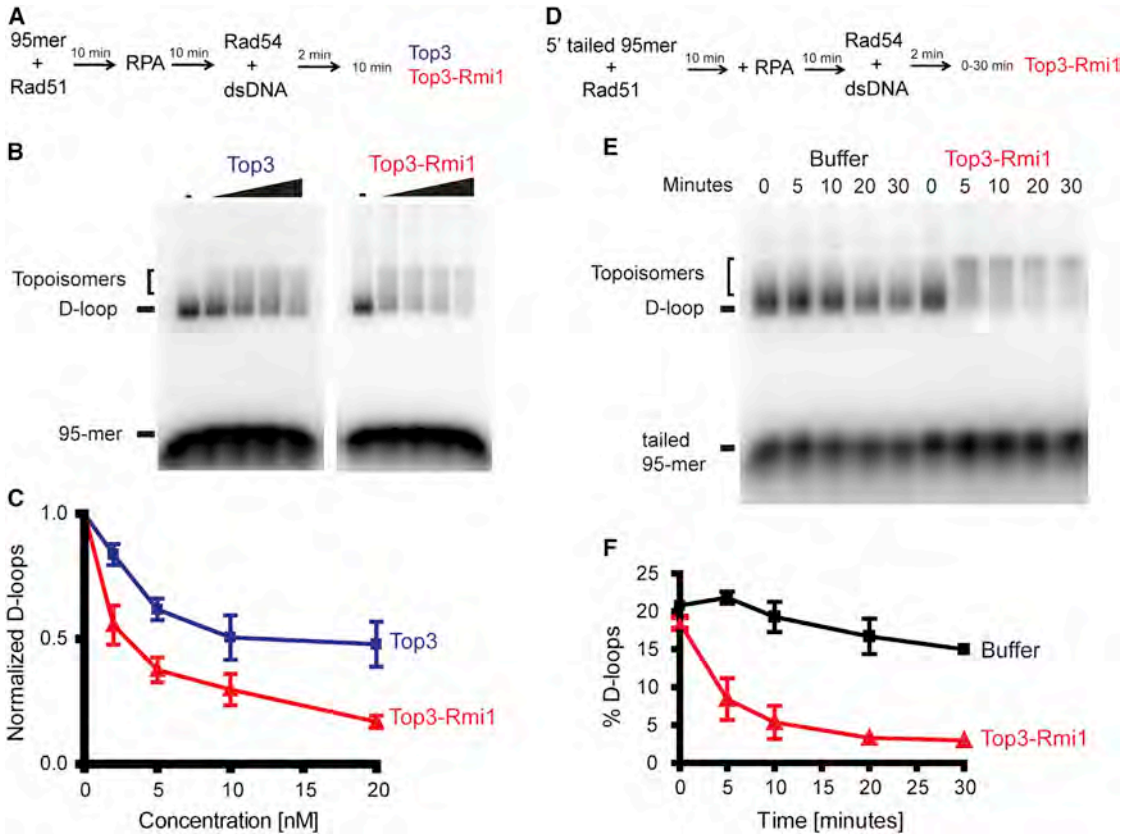

Figure 4. Rmi1 Stimulates D Loop Dissolution by Top3

(A) Reaction scheme for Rmi1-stimulated reactions.

(B) D loop dissolution by Top3 or Top3-Rmi1 (0, 2, 5, 10, and 20 nM).

(C) Quantitation of D loops. Shown are normalized means ± SDs of three independent experiments. The absolute values corresponding to maximal D loop levels are Top3 21.1% and Top3-Rmi1 18.7%.

(D) Reaction scheme with tailed 95-mer.

(E) D loop dissolution time course by 2 nM Top3-Rmi1.

(F) Quantitation of D loops. Shown are means ± SDs of three independent experiments.

negatively supercoiled DNA under the same reaction conditions used in D loop assays. This compares to dissolution of 70% of the D loops by 100-fold less Top3-Rmi1 (5 nM; Figure 2C). This finding is consistent with Top3 being a single-strand-specific DNA topoisomerase with poor activity on negatively supercoiled duplex DNA (Kim and Wang, 1992; Wang, 1996). Top3 readily relaxes hyper-negatively supercoiled DNA or supercoiled DNA containing a single-stranded bubble (Chen et al., 2013). The negatively supercoiled duplex DNA used in our experiments has been prepared to avoid potential denaturation by alkali and is not hyper-negatively supercoiled, which explains why Top3 does not relax this substrate. Second, testing Sgs1, Top3, and Rmi1 as assemblies or as individual components under D loop reaction conditions showed no evidence for relaxation of negatively supercoiled DNA (Figure S3B). Importantly, these control experiments support our proposal that D loop dissolution by yeast Top3 is a distinct mechanism that does not involve relaxation of the negatively supercoiled substrate DNA.

Top3-Mediated D Loop Dissolution Is Moderated by Sgs1, Stimulated by Rmi1, and Unlikely Mediated by Rad54

Top3-Rmi1 acts in a complex with Sgs1, and we noted that the presence of Sgs1 consistently mitigated the activity Top3-Rmi1-mediated D loop dissolution (Figure 2C). This effect was independent of the Sgs1 ATPase activity, as the helicase-dead Sgs1hd protein exerted a near identical effect as wild-type Sgs1 (Figure 2C). These data suggest that Top3 activity on D loops is controlled by Sgs1. A structural role for Sgs1 has been also reported for Top3-Rmi1-mediated catenation of bubbled dsDNA (Cejka et al., 2012). RMI1 provides the decatenation loop for the TOPIIIα gate (Bocquet et al., 2014) and stimulates decatenation while inhibiting the relaxation activity of Top3 by stabilizing the nicked intermediate (Cejka et al., 2012). We found that Rmi1 significantly stimulates D loop dissolution by Top3-mediated D loop (Figures 4A–4C).

Finally, we sought to exclude the possibility that Top3-mediated D loop dissolution involves the dsDNA motor protein

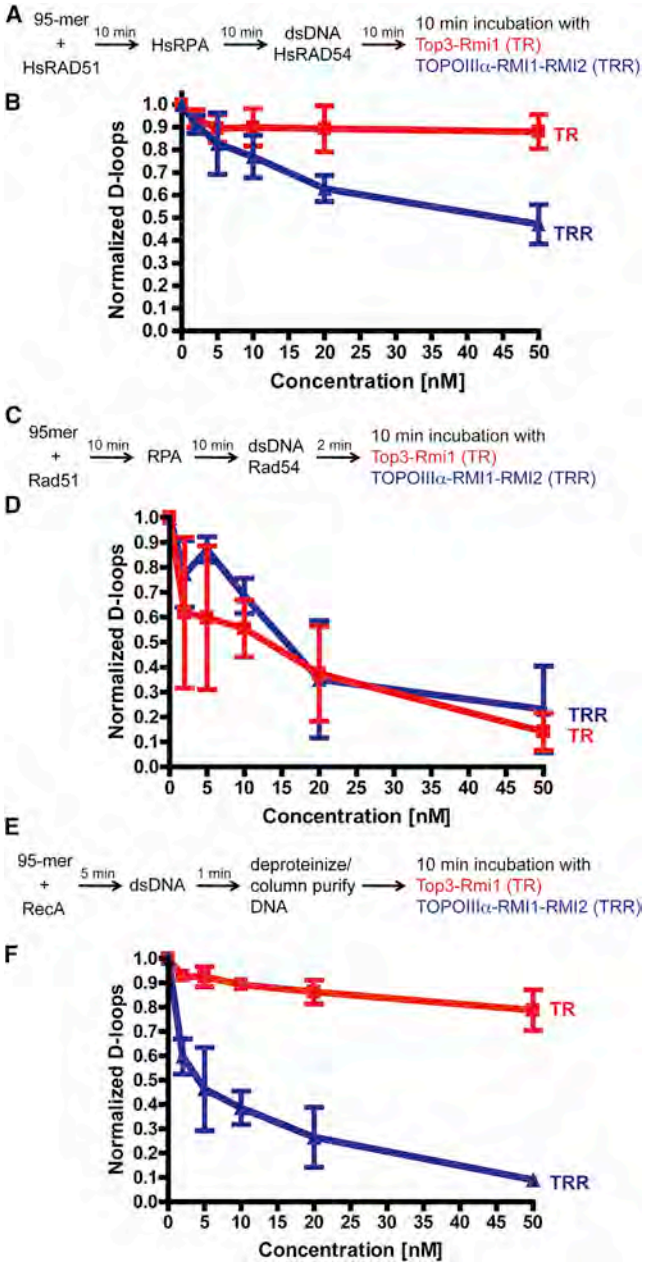

Figure 5. Human TOPOIIIα-RMI1-RMI2 Dissolves D Loops

(A) Reaction scheme for human RAD51/RAD54-mediated D loops.

(B) Quantitation of D loops. The absolute values corresponding to maximal D loop levels were TR 8% and TRR 8%.

(C) Reaction scheme for yeast Rad51/Rad54-mediated D loops.

(D) Quantitation of D loops. The absolute values corresponding to maximal D loop levels were TR 12% and TRR14%.

(E) Reaction scheme for deproteinized D loops. Deproteinized D loops (~1 nM) were incubated with TR and TRR.

(F) Quantitation of D loops. The absolute values corresponding to starting D loop levels were TR 39% and TRR 37%. Shown are normalized means ± SDs of three independent experiments.

Rad54. Rad54 has been found to dissociate D loops in vitro (Bugreev et al., 2007a), and we have recently shown that this activity depends on the specific structure and length of the invading ssDNA (Wright and Heyer, 2014). While Rad54 easily displaces a perfectly homologous oligonucleotide after D loop formation, a heterologous extension at either end endows such D loop with some stability against disruption by Rad54 (Wright and Heyer, 2014). Using the same 95-mer but tailed with 25 bp heterology at its 5′ end, we show in a time course experiment that the resulting D loops are essentially stable over the reaction time against Rad54-mediated dissociation (Figures 4D–4F). As Rad54 is a potent ATPase, the D loop assays conditions include an ATP regeneration system to ensure ample supply of ATP during the course of the reaction. Addition of Top3-Rmi1 to such 5′-tailed D loop resulted in robust dissolution eliminating over 70% of the initial D loop in the first 10 min of the reaction. The results suggest that Top3-mediated D loop dissolution differs from Rad54-mediated D loop dissociation. In concordance with these data, Top3-Rmi1 dissolves D loops with 3′- or 5′-tailed invading strands in the presence or absence of wild-type or helicase-dead Sgs1 (Figure S1E).

Human TopoIIIα-RMI1-RMI2 Is More Promiscuous in Dissolving D Loops

Human TopoIIIα-RMI1-RMI2 is homologous to the yeast Top3-Rmi1 complex. Human TopoIIIα-RMI1-RMI2 also was able to dissolve D loops in the reconstituted D loop reaction with human RAD51, RAD54, and RPA (Figures 5A, 5B, and S4). Surprisingly, human TopoIIIα-RMI1-RMI2 was found to be much more promiscuous than the yeast Top3-Rmi1 complex and able to efficiently dissolve D loops made by yeast Rad51, Rad54, and RPA or protein-free D loops (Figures 5C–5F and S4). Side-by-side titrations with yeast Top3-Rmi1 confirmed the previously determined specificity of the yeast complex (Figures 3 and 5). Control experiments showed that like Top3-Rmi1, human TopoIIIα-RMI1-RMI2 also does not relax negatively supercoiled DNA under D loop reaction conditions (Figure S5). We conclude that also human TopoIIIα-RMI1-RMI2 is endowed with the ability of dissolving D loops. The data suggest that in the human system there may exist additional factors that impart specificity to the human complex. This could be BLM, which is known to interact with RAD51, or additional novel factors (Braybrooke et al., 2003).

DISCUSSION

Here we report an activity of Top3 in specifically dissolving D loops generated by DNA strand invasion with the cognate Rad51 and Rad54 proteins in reconstituted in vitro reactions. We term this reaction D loop dissolution, because it shares essential features with other dissolution reactions performed by Top3, including the dissolution of dHJs, which depend on Top3 catalytic activity (Figure 2). Compared to Sgs1 (Figure 1), which dissociates protein-free D loops, and Mph1 (Prakash et al., 2009), which dissociates protein-free D loops and D loops generated by yeast or human Rad51, Top3 exerts surprising specificity in D loop dissolution. Indeed, neither Top3 alone nor Top3-Rmi1 can dissolve protein-free D loops or D loops generated by RecA or human RAD51 (Figure 3). Sgs1 moderates

Top3-mediated D loop dissolution in a way that is independent of the Sgs1 ATPase activity. This may provide a potential explanation for a structural role of Sgs1 in functions independent of its ATPase activity (Cejka et al., 2012; Lo et al., 2006; Mullen et al., 2000). Finally, our control experiments eliminate a simple mechanism, by which Top3 relaxes the duplex substrate. Instead, it appears that the yeast Rad51-mediated D loop is specifically targeted for topological unlinking by Top3. Deciphering the mechanism and regulation of Top3 activity in this reaction requires additional mechanistic work, but D loops fit nicely into the range of substrates for ssDNA-specific decatenation reactions previously identified for Top3 (Cejka et al., 2012; Hickson and Mankouri, 2011).

The specificity of Top3-mediated D loop dissolution prompted us to examine specific protein interactions between Top3 and Rad51 or Rad54. Despite intensive efforts, we were unable to demonstrate significant interactions by immunoprecipitation experiments from yeast whole-cell extracts (data not shown). Likewise, despite numerous approaches, we were unable to see species-specific interactions between the purified proteins in vitro, although we detected consistent above background association between Top3 with both yeast and human Rad51 (data not shown). We believe that the suspected interactions may occur between the DNA bound forms of the proteins.

The key question is whether the Top3 D loop dissolution activity is of biological relevance. Significant genetic data with *top3* and *sgs1* single and double mutants are consistent with a Top3-based mechanism of anti-recombination targeting an early HR intermediate such as nascent D loops. Top3 plays established roles in HR in long-range end resection and dHJ dissolution in conjunction with Sgs1 and Rmi1. However, these two roles cannot explain the much stronger phenotypes of *top3* mutants compared to *sgs1* mutants for slow growth and hyperrecombination (Onodera et al., 2002; Shor et al., 2002; Wallis et al., 1989). The observation that expression of the Sgs1-hd protein suppresses the *top3* slow growth phenotype only partially suggests that the Sgs1 helicase activity is not entirely responsible for the slow growth of *top3* mutants (Mullen et al., 2000). Moreover, *top3* mutants enhance the frequency of crossover 177-fold and NCO 69-fold in the *SUP4-o* system, which led to the original genetic discovery of *TOP3* (Shor et al., 2002; Wallis et al., 1989). This increase is entirely dependent on HR and eliminated in *rad51*, *rad52*, or *rad54* mutants (Shor et al., 2002). This result is unexpected for a defect affecting only dHJ dissolution. Moreover, there is additional evidence for Sgs1-independent roles of Top3. Recent analysis in *sgs1* cells demonstrated a role of Top3 in eliminating recombination-dependent template switch intermediates accumulating during replication (Glineburg et al., 2013). Consistent with the biological relevance of this observation, Top3 expression suppressed some of the MMS sensitivity of *sgs1 top3* double mutants dependent on Top3 catalytic activity (Glineburg et al., 2013; Onodera et al., 2002). Finally, recent results from detailed analyses of meiotic recombination demonstrate Sgs1-independent roles for Top3 in meiotic recombination (Kaur et al., 2015; Tang et al., 2015) that are very consistent with a role of Top3 in dissolving D loops in vivo. Specifically, the Top3 catalytic activity is required late in meiosis at the exit of pachytene to process Spo11-dependent HR intermediates that impede meiotic chromosome segregation (Tang et al., 2015). Absence of Top3 leads to loss of about 25% of the NCO products and persistence of single end invasions, previously identified as D loop intermediates (Hunter and Kleckner, 2001), and other types of joint molecules. It is unclear whether all accumulating joint molecules represent various forms of D loops. These phenotypes are not present in Sgs1-deficient cells and uncover a role for Top3 in meiotic recombination that is consistent with Top3-mediated dissolution of D loops and potentially other recombination-dependent joint molecules.

It is presently unclear whether Top3-Rmi1 acts independent of Sgs1 in a different protein pool or in a manner that is not dependent on Sgs1 protein/activity but still in the same complex. Sgs1 and Top3 are largely stable in cells lacking either binding partner, suggesting that Top3-Rmi1 may exist outside a complex with Sgs1 (Mullen et al., 2005). In sum, the existing and new emerging genetic evidence points to a role of Top3 in dissolving HR-dependent intermediates in addition to the established roles in DSB end resection and dHJ dissolution. Our discovery of a Top3-based mechanism of D loop dissolution provides a satisfying biochemical mechanism for these genetic observations.

Considering the multitude of enzymes implicated in D loop disruption (see Introduction), it is important to realize that each enzyme may have overlapping substrate specificity for a variety of different D loop substrates. In Figure 6, we sketched several different types of nascent and extended D loop based on known characteristics of DSB or gap repair. The D loops differ not only in structure and length of the hDNA but also in the type and extent of bound proteins. Anti-crossover enzymes, such as Srs2, RTEL, and Mph1, are likely targeting extended D loops, whereas anti-recombinases are expected to target the nascent D loops, which has the DNA strand invasion machinery still bound to it. Top3 showed exquisite specificity for such nascent D loops, strongly suggesting that it acts as an anti-recombinase in addition to its well-established anti-crossover function in dHJ dissolution. This is consistent with the genetic data showing a strong hyper-rec phenotype for Top3-deficient cells in gene conversion events not associated with crossovers (Bailis et al., 1992; Shor et al., 2002). The observation that Top3 deficiency specifically (Top1 or Top2 defects had no effect) enhances homeologous gene conversion between ectopic *SAM* genes with a resultant increase in hDNA length spanning many mismatches (Bailis et al., 1992) may lead to the speculation that Top3-mediated D loop dissolution is connected to hDNA rejection triggered by Msh2-Msh6.

In summary, we demonstrate that nascent D loops are a substrate for dissolution by yeast Top3-Rmi1 and human TopoIIIα-RMI1-RMI2, consistent with an in vivo role as an anti-recombinases targeting the nascent D loop to abort attempted HR events.

EXPERIMENTAL PROCEDURES

DNA Substrates

olWDH566 was used as the standard invading oligonucleotide in D loops and is referred to as 95-mer (see Table S1). The heterologous 95-mer (olWDH1613) is referred to as het 95-mer. The 120-mer consisting of the 95-mer olWDH566

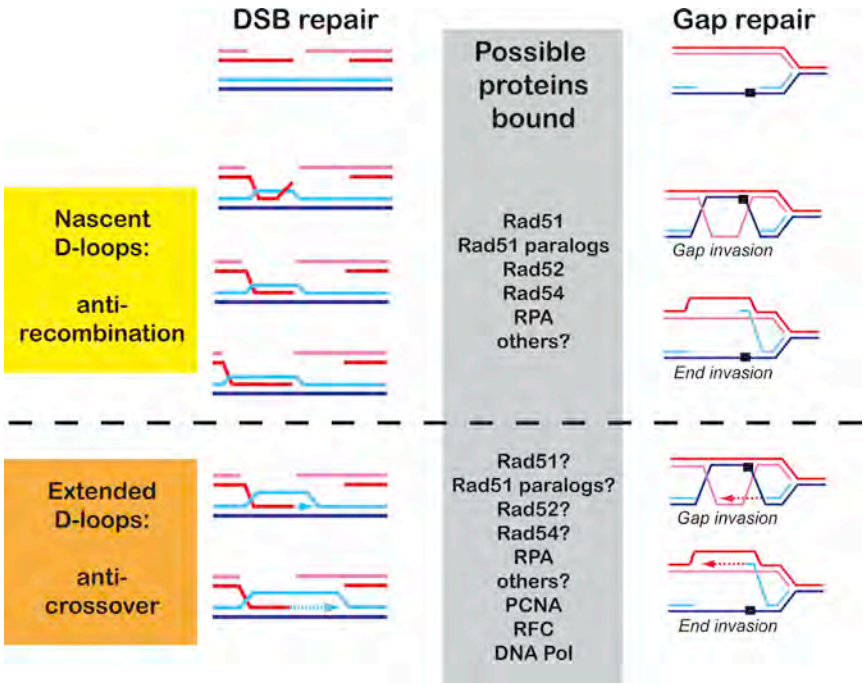

Figure 6. Different D Loops Species during HR-Mediated DSB and Gap Repair

D loops are a collection of different recombination joint molecules with different DNA junction architecture (3′-end, length, gap invasion) and different HR proteins bound to the individual DNA intermediates. D loops can form during DSB repair (left) or replication-fork-associated gap repair (right) and include nascent D loops (before extension by DNA polymerase: 3′ end not incorporated or ± branch migration), where proteins involved in strand invasion (e.g., Rad51, Rad51 paralogs, Rad54, RPA, Rad52?, others?) are likely still bound to at least parts of the D loop (top) and extended D loops (bottom), where instead or in addition to HR proteins, replication proteins (PCNA, RFC, DNA polymerase, and RPA) will be present in the D loop.

sequence with 25 nt 5′ heterology (olWDH1614) is referred to as 5′-het 120-mer. The 120-mer consisting of the 95-mer olWDH566 sequence with 25 nt 3′ heterology (olWDH1615) is referred to as 3′-het 120-mer. The 25-mer complementary to the 3′-and 5′-heterologous region (olWDH1616) was used to create the double-strand tailed substrates referred as 5′-tailed or 3′-tailed 95-mers. All oligonucleotides were purchased from Sigma. The dsDNA is a derivative plasmid (pBSder; 3,000 bp) with a pBSK backbone and 1,200 bp of phiX174 replacing 1,200 bp of pBSK (Wright and Heyer, 2014).

Proteins

Proteins were purified to apparent homogeneity, and the absence of relevant contaminating activities was experimentally established as described in the Supplemental Information.

D Loops Assays

D loop reactions were performed as previously described (Li et al., 2009), and detailed conditions are described in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2015.01.022.

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Supplemental Information

Top3-Rmi1 Dissolve Rad51-Mediated D Loops by a Topoisomerase-Based Mechanism

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Figure S1 (associated with Figure 1)

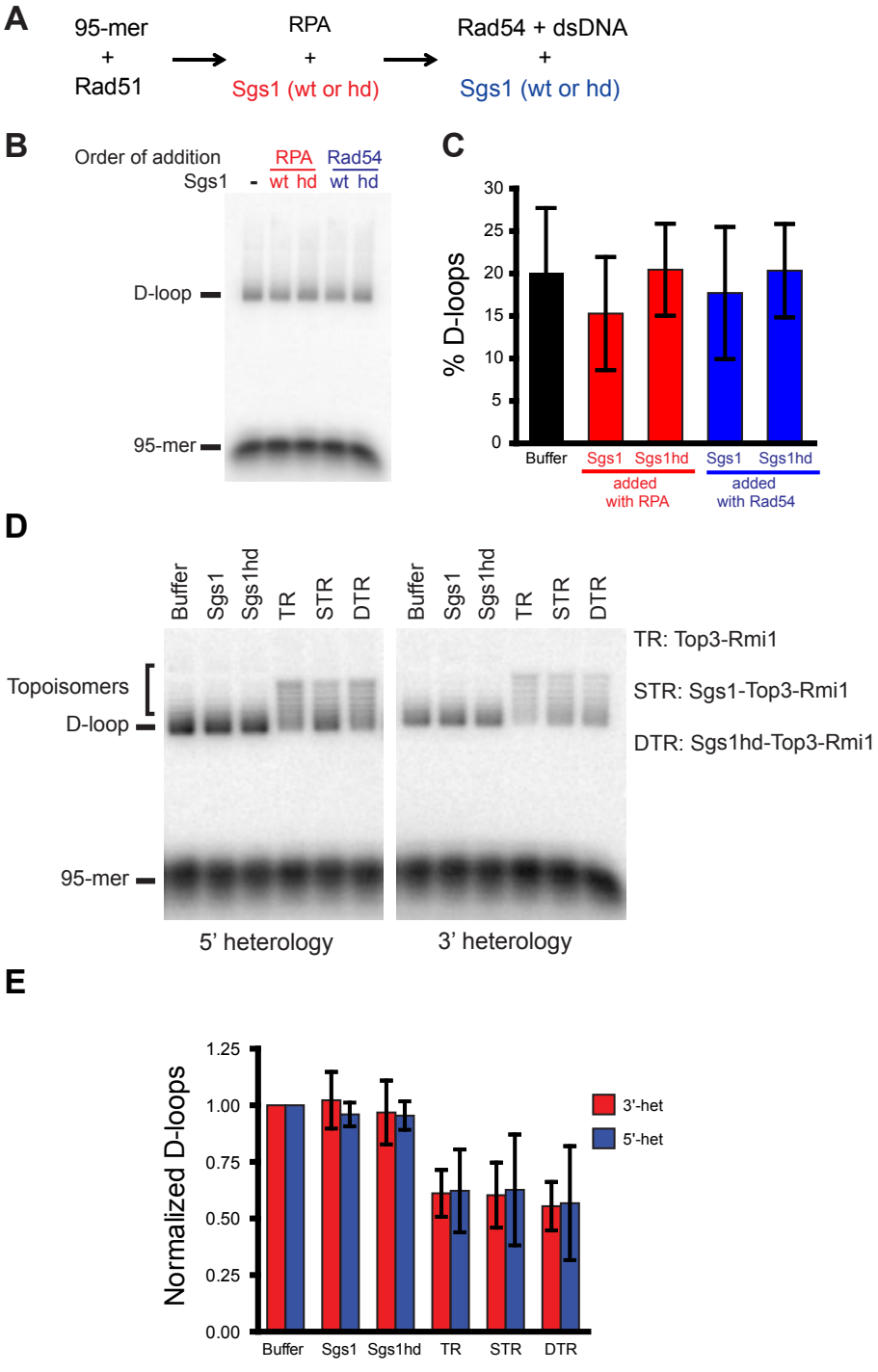

Figure S1. Sgs1 does not disrupt Rad51-mediated D-loops regardless of reaction order, and Top3-Rmi1 but not Sgs1 efficiently disrupts D-loops containing 5' or 3' heterology. A, Sgs1 does not disrupt Rad51-mediated D-loops regardless of reaction order. Reaction scheme with order of additions of Sgs1. **B**, Reactions and analysis as in Figure 1 D-F with 2 nM Sgs1, 2 nM Sgs1hd (Sgs1-K706A), or reaction buffer. **C**, Quantitation of D-loops. Shown are means ±

standard deviations of three independent experiments. **D**, Top3-Rmi1 but not Sgs1 efficiently disrupts D-loops containing 5' or 3' heterologies. Reactions and analysis as in Figure 1 D-F with 2 nM Sgs1, 2 nM Sgs1hd (Sgs1-K706A), 2 nM Top3-Rmi1 (TR), 2 nM Sgs1-Top3-Rmi1 (STR), 2 nM Sgs1hd-Top3-Rmi1 (DTR), or reaction buffer with a 95-mer with either 25 nt heterology at the 3' end (3'-het 120-mer) or 5'-end (5'-het 120-mer). E, Quantitation of D-loops. Shown are normalized means ± standard deviations of three independent experiments. Maximal D-loop levels for the 5'-het 120-mer are buffer: 27.5%, Sgs1: 27%, Sgs1hd: 27%, TR 16%, STR 21%, and DTR: 16%. Maximal D-loop levels for the 3'-het 120-mer substrate are buffer: 17%, Sgs1: 17%, Sgs1hd: 16%, TR 10%, STR 11%, and DTR: 9%.

Figure S2 (associated with Figure 2)

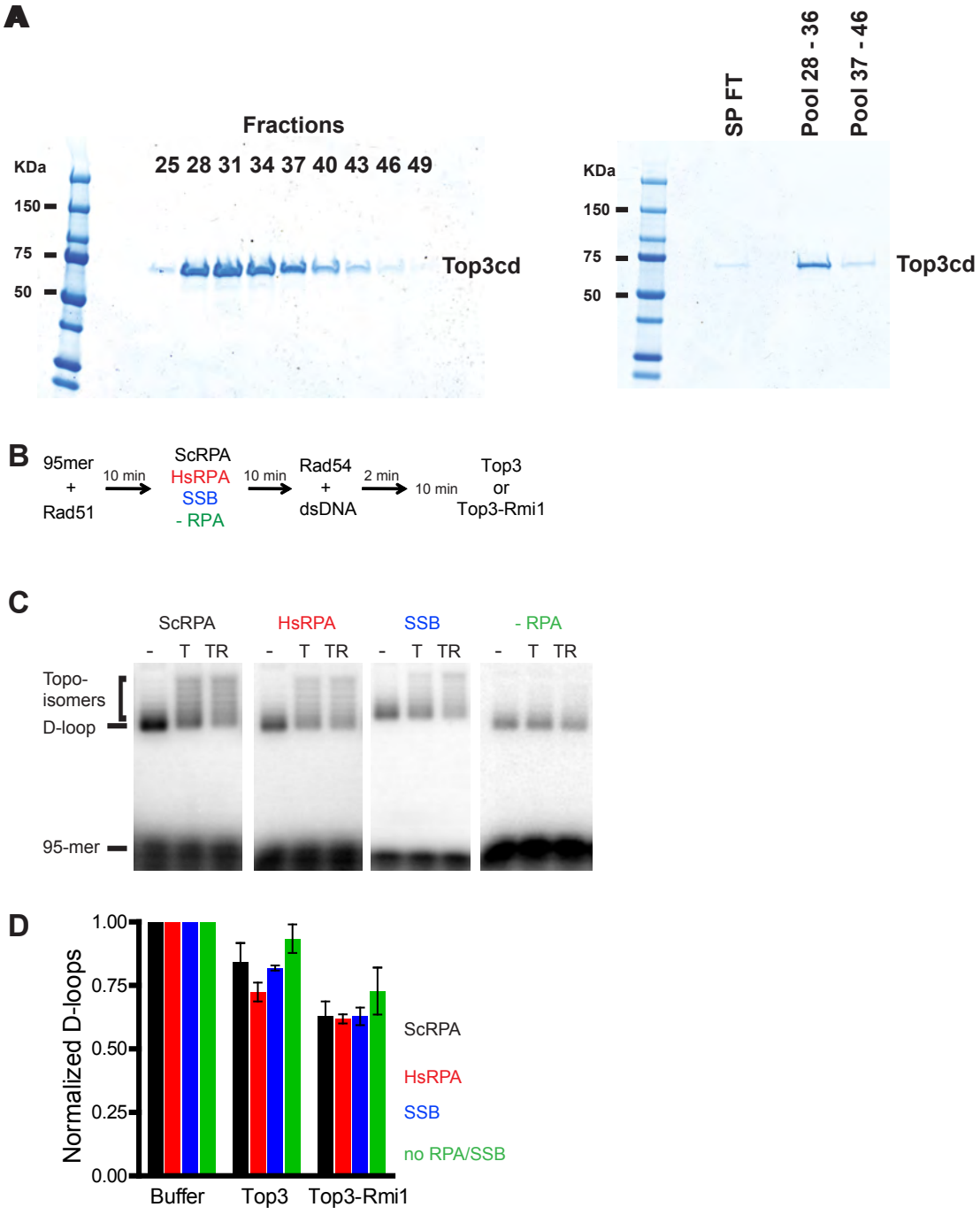

Figure S2. Purification of Top3cd (Top3-Y356F) and effect of different ssDNA binding proteins. A, Top3-Y356F samples from fractions 25 – 46 collected from the SP-sepharose column separated on a 4 – 20% gradient gel (left). Top3-Y356F samples from the SP sepharose column flow through (FT), the high concentration pool (fractions 28-36) and the low concentration pool (fractions 37-46) (right). **B**, Reaction scheme and proteins. **C**, D-loop

dissolution by Top3 (T) and Top3-Rmi1 (TR) in the presence of yeast RPA (ScRPA; 100 nM), human RPA (HsRPA; 100 nM), *E. coli* SSB (SSB; 125 nM), or no ssDNA binding protein. **D**, Quantitation of D-loops. Shown are normalized means ± standard deviations of three independent experiments. The absolute values corresponding to maximal D-loop levels are ScRPA: 23%, HsRPA: 12%, SSB: 17%, no ssDNA binding protein: 8 %

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Figure S3 (associated with Figure 3)

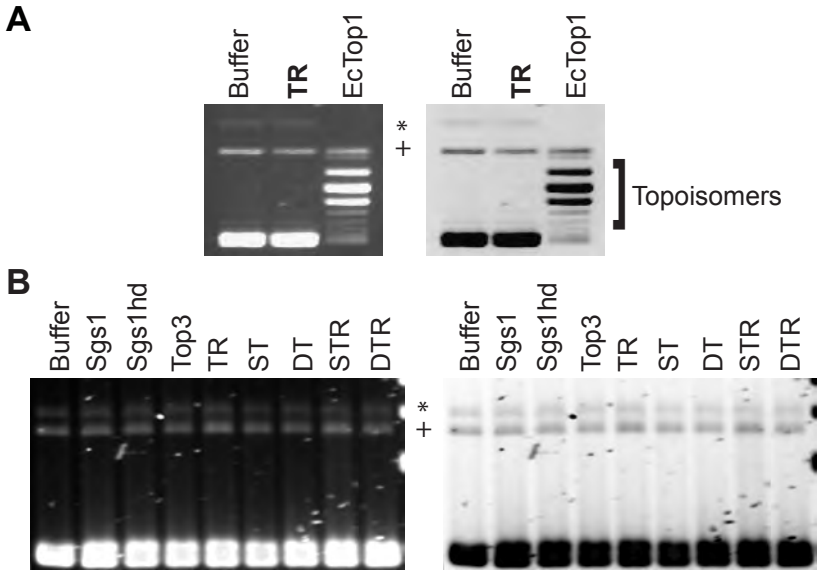

Figure S3. *S. cerevisiae Top3* does not relax supercoiled substrate under D-loop reaction conditions. A, Direct comparison of topological activity of Top3-Rmi1 (TR, 500 nM) and *E. coli* Top1 500 nM). All reactions were separated on 1.0% agarose gels and stained afterwards with ethidium bromide (left: positive image; right: negative image). * = relaxed plasmid DNA, + = linear plasmid DNA. **B**, Topological activity of Sgs1 (50 nM), Sgs1hd (Sgs1-K706A, 50 nM), Top3 (50 nM), Top3-Rmi1 (TR, 50 nM), Sgs1-Top3 (ST, 50 nM) and Sgs1hd-Top3 (DT, 50 nM), Sgs1-Top3-Rmi1 (STR, 50 nM) and Sgs1hd-Top3-Rmi1 (DTR, 50 nM) on supercoiled DNA (20 nM molecules). * = relaxed plasmid DNA, + = linear plasmid DNA.

Figure S4 (associated with Figure 5)

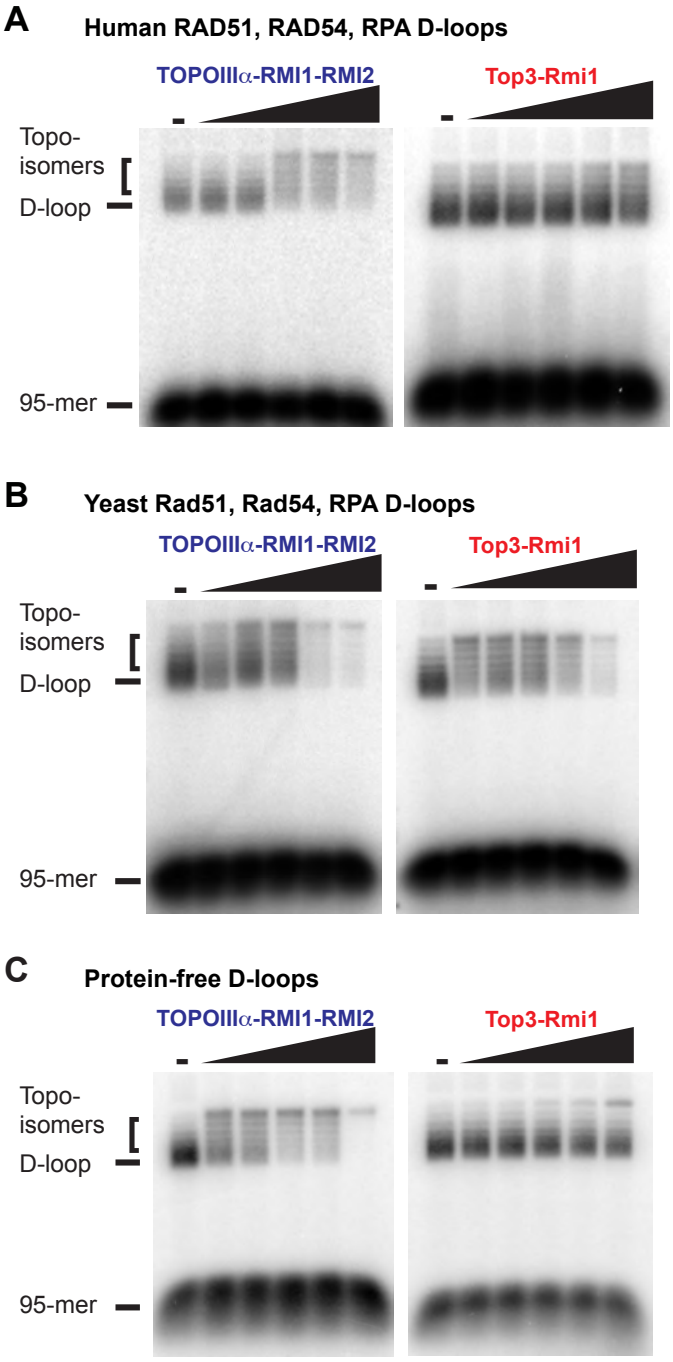

Figure S4. Representative gels for Figure 5. A. Human reconstituted D-loop system. **B.** Yeast reconstituted D-loop system. **C.** Protein-free D-loops.

Figure S5 (associated with Figure 5)

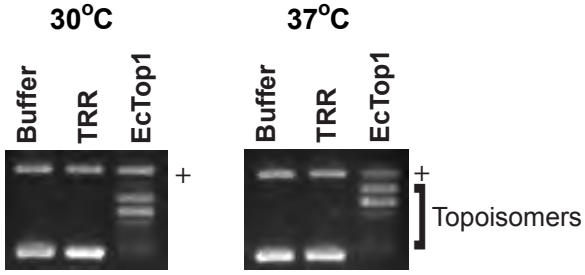

Figure S5. Human TOPOIIIalpha-RMI1-RMI2 does not relax supercoiled substrate under D-loop reaction conditions. Direct comparison of topological activity of TOPOIIIα-RMI1-RMI2 (TRR, 50 nM) and *E. coli* Top1 (500 nM) under D-loop conditions used for the yeast (30^0C) and human (37^0C) system. All reactions were separated on 1.0% agarose gels and stained afterwards with ethidium bromide. + = linear plasmid DNA.

Table S1: Oligonucleotides used in this study.

olWDH	Sequence 5'-> 3'	Text name
566	ATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTG	95-mer
1613	GACCTGATAAAGCTGTATCCGAGCATTTGTGAATTCTCCGAGTCAGCTTCTTACTCCCAAGAAGTTCGTTGGATTCGTATTCCGAATGTTAAGAC	het 95-mer
1614	ATTACTGTCCGTGCACGTTATTCTAATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTG	5'-het 120-mer
1615	ATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGATTACTGTCCGTGCACGTTATTC TA	3'-het 120-mer
1616	TAGAATAACGTGCACGGACAGTAAT	25-mer
1617	CTGTTCTTGGAAACGATATGAAC	Top3-Y356F-1
1618	GTTCATATCGTTTCCAAGAACAG	Top3-Y356F-2

Experimental Procedures

DNA substrates. The plasmid DNA was purified using TritonX-100/SDS lysis and density gradient centrifugation in CsCl/Ethidium bromide as described (Sambrook et al., 1989).

Proteins. Rad51 (Van Komen et al., 2006), Rad54 (Kiianitsa et al., 2002), RPA (Binz et al., 2006), RecA (Morimatsu et al., 1995), Sgs1 and Sgs1hd (Cejka and Kowalczykowski, 2010), Sgs1-Top3-Rmi, Sgs1hd-Top3-Rmi1 Top3-Rmi1, Top3 (Cejka et al., 2010; Cejka et al., 2012) were purified as described. The *TOP3* wild type gene was mutagenized using primers olWDH1617 and olWDh1618 to generate the *top3-Y356F* catalytic mutant as described (Oakley et al., 2002). The DNA sequence of the entire Top3-Y356F open reading frame was confirmed by DNA sequencing. Top3-Y356K was purified as previously described for wild type (Cejka et al., 2012). Briefly, 8 g of Sf9 insect cells were collected from a 1.6 L culture transfected with a recombinant virus expressing Top3-Y356. All further steps were performed at 4^{o}C or on ice. The pellet was incubated in 24 ml of lysis buffer (50 mM Tris pH7.5, 1 mM DTT, 1 mM EDTA) containing protease inhibitors (Sigmafast). 16 ml of glycerol and 3.12 ml 5 M NaCl was added followed by centrifugation of the lysates at 10,000 rpm in a J2-21 Beckman centrifuge. The lysate was incubated with 10 mL Glutathion S-transferase Sepharose beads (GE Healthcare) overnight. The beads were poured into a column, washed in a high salt buffer (50 mM Tris pH7.5, 5 mM β-mercaptoethanol, 1 M NaCl, 1 mM EDTA, 10 % glycerol, 1 mM PMSF, 10 μg/ml Leupeptin) then a low salt buffer (50 mM Tris pH8, 5 mM β-mercaptoethanol, 150 mM NaCl, 1 mM EDTA, 10 % glycerol, 1 mM PMSF, 10 μg/ml Leupeptin). Precision protease was loaded onto the column and incubated for 2h prior to elution with the low salt buffer. The eluate was diluted to lower the pH to 6.8 and 25 mM NaCl and loaded onto an SP-sepharose column. The column was washed with SP buffer (50 mM Tris pH6.8, 1 mM DTT, 50 mM NaCl, 10 % glycerol, 1 mM PMSF, 10 μg/ml Leupeptin) and loaded onto an FPLC and fractions were collected using an NaCl gradient from 50 to 500 mM. The fractions containing Top3-Y356F eluted at the expected salt concentration ~250 mM NaCl, which corresponds to the elution of wild type Top3 (Cejka et al., 2012). The purification yielded ~500 μg protein at 1.5 μM, which was aliquoted, snap frozen in liquid nitrogen and stored at -80^{o}C. The purity of the Top3cd protein is documented in Figure S2. The absence of relevant contaminating activities was experimentally established (Zhang and Heyer, 2011). Purified human TOPOIIIα-RMI1-RMI2 complex was kindly provided by Dr. Ian Hickson and Dr. Kata Sarlós (University of Copenhagen, Denmark), and the purification procedure will be published elsewhere.

D-loop assays. *S.cerevisiae proteins:* D-loop reactions were performed as previously described (Li et al., 2009). Briefly, the reaction was performed at 30^{o}C in D-loop reaction buffer (30 mM Tris-Acetate pH7.5, 1 mM DTT, 50 μg/mL BSA, 5 mM $Mg(OAc)_2$, 4 mM ATP, 100 mM NaOAc, 10 mM phosphocreatine and 10 U/ml creatine kinase). The end labeled 95-mer (20 nM molecules) was incubated with 0.67 μM Rad51 for 10 min to allow formation of the Rad51 filament. 100 nM RPA was added for an additional 10 min and the reaction was started by adding 112 nM Rad54 and plasmid pBSder (20 nM molecules). The D-loops formed for 2 min before addition of Sgs1, Top3, or complexes containing these proteins for an additional 10 min. The reactions were stopped and deproteinized by addition of Stop Buffer (final concentration 0.143 % SDS, 35.7 mM EDTA, 1.7 mg/mL Proteinase K) and incubation for 2 h at 37^{o}C. The reaction products were separated in a 0.8% agarose gel at 6 V/cm for 150 min. The gel was then dried and visualized on a phosphoimager. For the time course assays, samples were collected at each indicated time point and placed into Stop Buffer. *RecA***:** The RecA D-loops were made as previously described (McIlwraith et al., 2001). The reaction was performed at 37^{o}C in RecA reaction buffer (50 mM Tris-Acetate pH7.5, 1 mM DTT, 100 μg/mL BSA, 2.5 mM

$Mg(OAc)_2$, 2 mM ATP, 10 mM phosphocreatine and 10 U/ml creatine kinase). The end labeled 95-mer (20 nM molecules) was incubated with 0.67 μM RecA for 5 min followed by addition of a single-stranded binding protein (100 nM ScRPA, 100 nM HsRPA, 125 nM SSB) if used. The reaction continued for an additional 5 min. The D-loop reaction was initiated by addition of 12.5 mM $Mg(OAc)_2$ and 20 nM molecules plasmid dsDNA. After one minute 0.5 nM Top3 or Top3-Rmi1 was added and the reactions continued for an additional 5 min. The reactions were processed and the products were analyzed as described above. *Human proteins:* Briefly, the reaction was performed at 37°C in D-loop reaction buffer (35 mM Tris-Acetate pH7.5, 1 mM DTT, 100 μg/mL BSA, 2 mM $Mg(OAc)_2$, 2 mM $CaCl_2$, 1 mM ATP, 100 mM K^+-glutamate, 10 mM phosphocreatine and 10 U/ml creatine kinase). The end labeled 95-mer (20 nM molecules) was incubated with 0.67 μM *Hs*RAD51 for 10 min to allow formation of the Rad51 filament. 100 nM *Hs*RPA was added for an additional 10 min and the reaction was initiated by adding plasmid dsDNA (20 nM molecules) and continued for 10 min. 2 nM Top3 or Top3-Rmi1 was added and incubated for an additional 10 min. The reactions were processed and the products were analyzed as described above. The HsRAD51-RAD54 D-loop reactions were identical to the RAD51 reactions except that 112 nM RAD54 was added after RPA and incubated for 5 min prior to addition of Top3 or Top3-Rmi1. *Protein-free D-loops:* RecA D-loops were produced as described above. The DNA of the D-loop reaction was purified using a G25 spin column to remove any SDS, resulting in a yield of approximately 50%. The deproteinated D-loop reactions were performed at 30°C in reaction buffer (30 mM Tris-Acetate pH7.5, 1 mM DTT, 100 μg/mL BSA, 5 mM $Mg(OAc)_2$, 4 mM ATP, 20 mM phosphocreatine and 20 U/ml creatine kinase). 0.5 nM Sgs1, Sgs1hd, Top3 or Top3-Rmi1 was incubated with the D-loops for 10 min. The reactions were processed and the products were analyzed as described above.

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Molecular Cell
Minireview

TOPping Off Meiosis

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Double-strand breaks (DSBs) threaten chromosome integrity. The most accurate repair of DSBs is by homologous recombination (HR), catalyzed by recombination proteins such as Rad51. Three papers in this issue of *Molecular Cell* (Fasching et al., 2015; Kaur et al., 2015; Tang et al., 2015) now reveal the role of three of these proteins in budding yeast: Sgs1 (BLM homolog), Top3 (TOPIIIα homolog), and Rmi1. They demonstrate several steps where all three proteins act together, and find additional functions of the Top3-Rmi1 subcomplex that are critical for the completion of meiosis.

Double-strand breaks (DSBs) arise both spontaneously during DNA replication and from programmed expression of site-specific nucleases. Following the creation of a DSB, the broken ends are first resected to expose 3′-ended single-strand DNA (Figure 1A), which organizes the assembly of a Rad51 nucleoprotein filament. The Rad51 filament is capable of searching the entire genome to locate a region of homology that can be used to patch up the broken chromosome. Homology can be located on a sister chromatid, on a homologous chromosome, or in some ectopic location. Rad51 then pries open the intact double-stranded template to allow strand invasion and the formation of a three-stranded displacement or D-loop in which the single-stranded broken end base pairs with its complementary strand of the intact duplex. (Figure 1B) At this point the cell has several alternatives. Repair can proceed through a synthesis-dependent strand annealing (SDSA) pathway that copies the template to seal the break without an accompanying crossover (Figure 1C). Alternatively, repair can proceed through the formation of a branched intermediate known as a double Holliday junction (dHJ) that can be cut apart by resolving enzymes (HJ resolvases) to produce crossovers between the homologs (Figures 1D and 1F). Such crossovers are potentially disadvantageous in mitotic cells where an exchange between homologous chromosomes can lead to loss of heterozygosity; but in meiotic cells, crossovers are necessary to generate the tension between paired homologs to assure proper disjunction of chromosomes at the first meiotic division. To avoid a crossover outcome, dHJs can also be dissolved by unwinding until a single pair of crossing strands in a hemicatenane are removed by a topoisomerase (Figure 1E). Also, not all strand invasions are productive, especially if the invading strand encounters a homologous sequence containing mismatches; in such cases, D-loops can be dismantled to allow the broken end to search for other homologous sequences. The Sgs1-Top3-Rmi1 (STR) protein complex is involved in virtually every step along these pathways, and in mammals, the orthologous mammalian complex, BTRR, involving the BLM helicase, TOPIIIα, RMI1, and an additional RMI2, protein appears to play similar roles.

Sgs1 and BLM are members of the RecQ family of 3′ to 5′ helicases that can unwind and displace single-stranded DNA from its complementary partner. People lacking BLM exhibit Bloom's syndrome, a disease marked by a predisposition to cancer and a high level of genome instability. Top3 and its human TOPIIIα homolog are single-strand DNA cleaving enzymes that can relax supercoiled DNA, although their principal activity is in dismantling interconnected DNA molecules (Bocquet et al., 2014; Cejka et al., 2010b, 2012).

As noted above, the budding yeast STR proteins play many different roles in the completion of DSB repair. In one of its guises, STR associates with the Dna2 endonuclease to promote the 5′-to-3′ resection of the DSB ends to generate long 3′-ended single-strand ends that promote Rad51 assembly and homologous recombination (HR) (Cejka et al., 2010a; Zhu et al., 2008). STR is not required for this process, as there is a parallel activity catalyzed by the Exo1 exonuclease. In mammals, BLM and Top3α, along with Rmi1 and Rmi2, promote analogous resection in mammalian cells, but in addition BLM can also act in a second pathway with EXO1 (Nimonkar et al., 2011).

STR is also implicated in the next step, when the single-stranded DNA within the Rad51 filament invades and forms sufficient base pairs with a homologous template to form a D-loop (Figure 1B). Here STR can act to reverse this reaction, especially when the ssDNA pairs with a complementary strand containing several mismatches (Spell and Jinks-Robertson, 2004). STR also discourages annealing between two slightly mismatched ssDNA strands in the process of single-strand annealing (SSA) (Spell and Jinks-Robertson, 2004; Sugawara et al., 2004). As we will see later, removing the D-loop can occur in two ways, one requiring only Sgs1 and one needing only Top3-Rmi1(TR).

Once a D-loop has formed, repair can proceed via two major pathways: SDSA or a dHJ process. In mitotic cells Sgs1 does not appear to act in channeling repair toward SDSA, but two other 3′-to-5′ helicases, Mph1 and Srs2, are active at this step (Ira et al., 2003; Prakash et al., 2009). The dHJ pathway results in a fully ligated pair of Holliday junctions that must be resolved before chromosome segregation. In mitotic cells, where crossovers between homologous chromosomes might result in loss of heterozygosity, most dHJs are "dissolved"—producing non-crossovers (NCOs) (Figure 1E)—rather than acted on by several Holliday junctions that can cleave the HJs to yield crossovers. The dissolution of dHJs requires STR in two steps, first to unwind and migrate the strands until a single hemicatenane remains, and then to remove this last interconnection (Wu and Hickson, 2003).

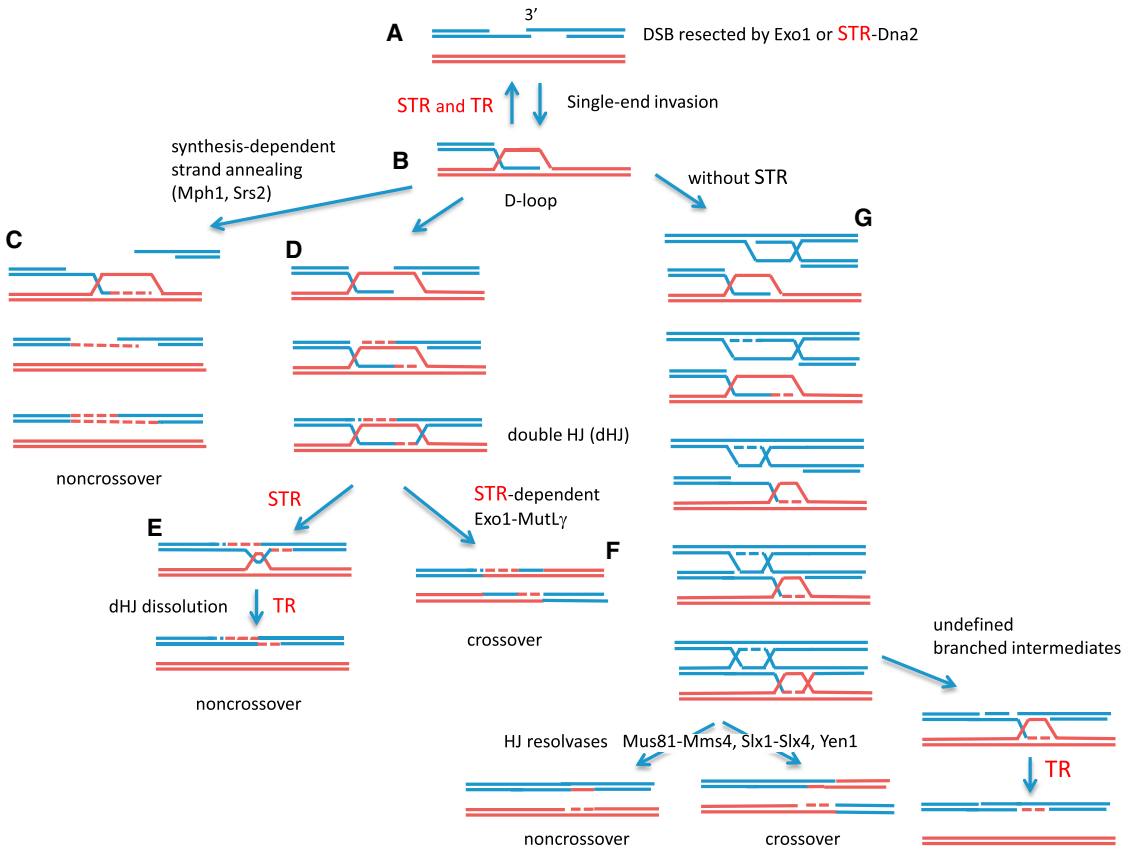

Figure 1. Roles of Sgs1, Top3, and Rmi1 in Homologous Recombination

A composite of steps in mitotic and meiotic recombination are shown, with the key steps requiring Sgs1-Top3-Rmi1 (STR) or Top3-Rmi1 alone (TR) shown in red.
(A) A double-strand break (DSB) is resected to yield 3′-ended single-strand DNA (ssDNA) tails either by the exonuclease Exo1 or by a helicase/endonuclease complex involving STR and Dna2.
(B) The ssDNA forms a nucleoprotein filament with Rad51 and engages in a search for homology, leading to single-end invasion and the formation of a D-loop. As shown by Fasching et al. (2015) in this issue, D-loop formation can be reversed in two ways: by STR or by TR alone.
(C) The D-loop can be extended and the newly synthesized strand displaced, leading to DSB repair by synthesis-dependent strand annealing that yields noncrossover outcomes.
(D) The D-loop can be extended and result in an intermediate containing a fully ligated double Holliday junction (dHJ).
(E) The dHJ can be resolved into a noncrossover by dissolution, a process requiring STR to unwind and migrate the branched HJs and then TR to remove the remaining hemicatenane.
(F) Alternatively, the dHJ can be cleaved by Holliday junction resolvase. Here, the Exo1-MutLα complex that carries out this step in meiosis is shown.
(G) In the absence of STR, recombination in meiosis is greatly altered, with the appearance of multichromatid joint molecules (mcJMs) involving more than two of the four chromatids present at meiotic prophase (here three chromatids are illustrated). These mcJMs can be largely resolved by three structure-specific nucleases (Mus81-Mms4, Slx1-Slx4, or Yen1) into either crossover or noncrossover outcomes. However, a minority of the intermediates formed without Sgs1 cannot be taken apart without the action of TR.

Thus, deletion of any of the STR proteins leads to a marked increase in crossovers in mitotic yeast cells during repair of a site-specific DSB. Most likely the dramatic increase in crossovers seen between sister chromatids in humans with Bloom's syndrome (lacking BLM) reflects an analogous absence of dHJ dissolving activity.

In meiosis, the basic mitotic repair machinery becomes overlaid with a number of proteins that serve to ensure that as many as half of the recombination events, initiated by DSBs created by the Spo11 enzyme, will culminate in crossovers between homologous chromosomes. These exchanges are of course important in generating genetic diversity among germ cells, but they also serve the critical function creating the necessary interconnections between chromatids that assure proper chromosome segregation. Many of these steps also counteract or modify STR function. First, STR doesn't seem to play much of a role in promoting the much more limited 5′-to-3′ resection of DSB ends; only Exo1 seems to drive this process (Zakharyevich et al., 2010). Second, the dissolving of dHJs is blocked by the "ZMM" proteins (Lynn et al., 2007), which include the Msh4-Msh5 proteins that can bind to and apparently stabilize dHJs and thus prevent STR from dissolving them. If STR isn't important for resection in meiosis and is thwarted in dHJ dissolution, one might think that removing STR from meiotic cells would have little consequence. But in fact the absence of STR prevents orderly progression through the normal pathways and creates novel meiotic phenotypes that dramatically change how intermediates of recombination are formed and processed.

The level of DSB formation at meiotic hotspots is sufficiently high to be able to identify and follow the kinetics of formation of a number of key molecular intermediates in meiotic recombination, including single-end invasion (i.e., the formation of a D-loop), the formation of dHJ intermediates, and the appearance of both NCO and crossover outcomes (Oh et al., 2009). A detailed examination of the kinetics of repair revealed that NCOs appear earlier than crossovers and that the major pathway for resolution of the dHJs involves a noncatalytic function of Exo1 and the Msh2-Msh3 (MutLγ) mismatch repair proteins, rather than any of the three identified HJ resolvases: Mus81-Mms4, Slx1-Slx4, or Yen1 (Zakharyevich et al., 2010, 2012). Surprisingly, although crossovers appear in the absence of Sgs1, they no longer require Exo1-MutLγ.

In the absence of Sgs1, there are dramatic changes in the pattern of molecular intermediates (Jessop et al., 2006; Oh et al., 2007). Normally joint molecules (JMs) form between two of the four chromatids, predominantly between nonsister DNA molecules; but in the absence of Sgs1, there are much more complex JMs, involving three and sometimes all four chromatids (Figure 1G). These results suggest that Sgs1 prevents these promiscuous strand invasions, possibly by reversing D-loop formation at one DSB end. The rejection of strand invasion is reminiscent of STR's mitotic role in rejecting heteroduplex DNA formed during strand invasion between mismatched substrates, but in meiosis these rejections occur between identical sequences. Instead it would seem that Sgs1 is needed to assure that both ends of a DSB engage the same homologous target. A similar role for Sgs1 has been suggested in establishing a recombination execution checkpoint in mitotic cells that delays recombination when the two ends of a DSB engage different partners (Jain et al., 2009).

Another surprising result that emerged from studying meiosis in the absence of Sgs1 is that the appearance of NCOs no longer precedes the advent of crossovers as seen in wild-type meiotic cells. The coincident and late appearance of both NCOs and COs suggests they could arise by alternative resolution of a dHJ and that the normally predominant SDSA and dHJ dissolution pathways that lead to NCOs are absent. Consistent with these findings, the major dHJ resolution pathway, using Exo1 and MutLγ, is absent; instead crossovers—and NCOs—depend on the three other HJ resolvases, primarily Mus81-Eme1 and Slx1-Slx4, with Yen1 playing some sort of backup role just prior to the first meiotic division (De Muyt et al., 2012; Jessop and Lichten, 2008; Oh et al., 2008). Why the normal ZMM crossover pathway deploying Exo1-MutLγ cannot deal with the multiple JMs is still unclear. Perhaps the ZMM proteins and its associated resolvase cannot find the right DNA conformations to function when there are three or four chromatids engaged in a complex intermediate; perhaps Sgs1 plays a more direct role in creating the specific geometry for ZMM proteins to act.

The two new papers from the Hunter and Lichten labs build on this foundation and uncover an unanticipated role for a subcomplex of Top3 and Rmi1 (TR), independent of Sgs1 (Kaur et al., 2015; Tang et al., 2015). These studies show that inactivating either Top3 or Rmi1 in meiosis leads to the same dramatic appearance of JMs containing three or four chromatids, as seen for the absence of Sgs1. However, whereas meiosis in the absence of Sgs1 is surprisingly complete, with quite good spore viability, the absence of either Top3 or Rmi1 results in very poor viability and the persistence of some JMs. Thus, the actions of the three HJ resolvases are insufficient to remove all the complications arising in the absence of STR. Some branched structures remain that prevent normal chromosome segregation. Thus the TR complex has an unexpected additional role in resolving branched molecules that apparently escaped the attention of the three HJ resolvases. These results may also account for the original characterization of Sgs1 mutations as *s*uppressors of the *s*low *g*rowth of *top*3Δ: in mitotic cells there must also be some Sgs1-dependent (and possibly TR-dependent) branched DNA structures that require TR for their removal.

The exact nature of the refractory structures in meiosis remains to be elucidated, but we can infer from the elegant biochemistry that has been carried out on Top3-Rmi1 that it must involve its ability to remove single-strand interconnections between different chromatids. Top3 belongs to a superfamily of enzymes that can cleave one DNA strand, which remains covalently attached via tyrosine, and pass though the other; but it differs from Top1 or bacterial homologs in that it is not efficient in relaxing supercoiled DNA by a series of rotations of the transiently broken strand. Instead, Top3 acts preferentially on structures that have some single-stranded DNA character, such as hemicatenanes. Recently Nicolas Thoma's lab, in collaboration with those of Steven Kowlaczykowski, Peter Cejka, and Ian Hickson, has provided a detailed model of the steps in this process, based on a high-resolution X-ray crystallographic study of the human TOPIIIα-RMI1 complex (Figure 2A; Bocquet et al., 2014). TOPIIIα-RMI1 binds and cleaves the C (cut) strand and opens up to accommodate the binding of the T (transfer) strand and then closes again after religating the C strand, accomplishing strand passage. Mammalian TOPIIIα and budding yeast Top3 resemble prokaryotic relaxases in overall structure, but they lack a distinctive loop that has been implicated in the decatenation process. However, in the eukaryotic Top3 enzymes this loop is provided by Rmi1 (Figure 2A). The presence of Rmi1 inhibits Top3's supercoil relaxing activity and markedly increases its decatenation activity that is key in dissolving dHJs and, as shown below, in dismantling D-loops.

The reversal of D-loops is the subject of the paper in this issue from Heyer's lab in collaboration with Cejka and Kowalczykowski (Fasching et al., 2015). Artificial D-loops can be created by in vitro recombination, either bound to RPA or to Rad51 and its associated chromatin remodeler, Rad54, after which they can be purified to be protein free. In keeping with previous results, Sgs1 by itself can dismantle a protein-free D-loop, but it fails to act on protein-bound structures (Figure 2B). In contrast, yeast TR (and STR) can take apart protein-bound D-loops, through its strand passage activity. But, surprisingly, yeast TR won't act on protein-free D-loops. In fact, yeast TR is quite fastidious: it will not work on D-loops created with human RPA or human Rad51 or Rad54.

Fasching et al. (2015) also investigated the D-loop activity of the human BTRR and TRR complexes and found that they have similar activities but are much less picky about the species origin of either RPA or Rad54. Moreover, human TRR will dismantle protein-free D-loops. It will be interesting to see how

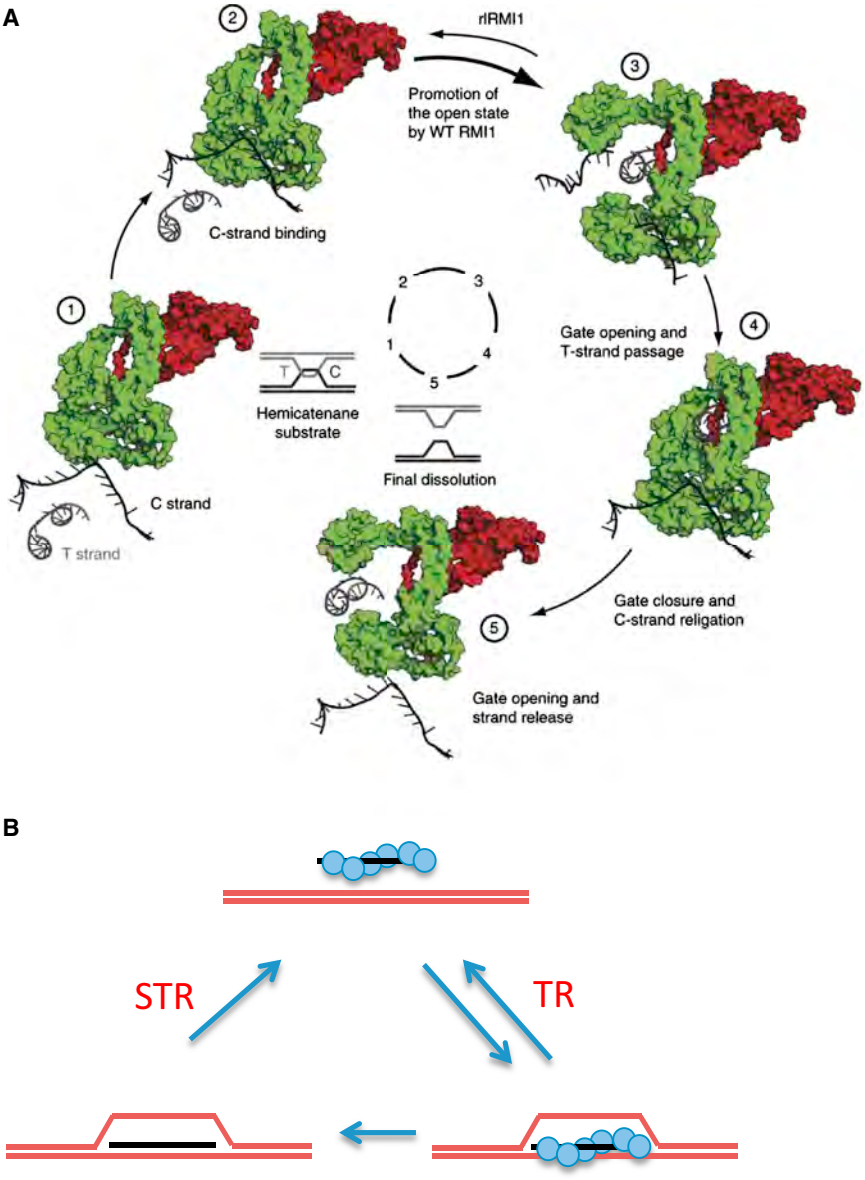

Figure 2. Roles of STR and TR in Reversing the Formation of D Loops

(A) Mechanism of strand passage carried out by mammalian TOPIIIα-RMI1 as illustrated by Bocquet et al. (2014). TopIIIα cleaves the C strand and undergoes a conformational change that allows the transfer strand (T) to pass through, after which the C strand is religated and the gate closes, with release of the C strand. This action is stimulated by a loop of RMI1 that is part of the active site. Figure reused with permission from Bocquet et al. (2014), Figure 5.

(B) Rad51 (blue circles) coating single-stranded DNA (ssDNA) facilitates strand invasion and the formation of a D-loop in the presence of the ssDNA binding protein complex, RPA, and Rad54. When the D-loop is protein free, Sgs1 alone, or STR, can take apart the D-loop, but Sgs1 alone cannot dismantle the protein bound. This protein-bound form can be taken apart by the Top3-Rmi1 complex acting alone.

mutations of TRR affect the resolution of meiotic chromosomes in mouse models to see if some of these differences will be reflected in their in vivo phenotypes. Currently little is known about how TOPIIIα mutants affect meiotic recombination and chromosome segregation, but the absence of BLM appears to reflect many of the defects seen for sgs1Δ in yeast (Holloway et al., 2010). There is no obvious defect in the early steps of recombination, but there are aberrant chromosome pairings that are reminiscent of the multichromatid JMs seen in yeast.

The biochemical studies of Top3-Rmi1 reveal a strand-passage and decatenation mechanism that can explain why TR is required for the removal of some meiotic intermediates that are left behind in the absence of Sgs1, even though there are three HJ resolvases present. But whether these intermediates are extended D-loops or some other branched structure remains to be determined. Further experiments should soon get this STRaight.

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