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Supplemental Information

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

Clare L. Fasching, Petr Cejka, Stephen C. Kowalczykowski, and Wolf-Dietrich Heyer

Figure S1 (associated with Figure 1)

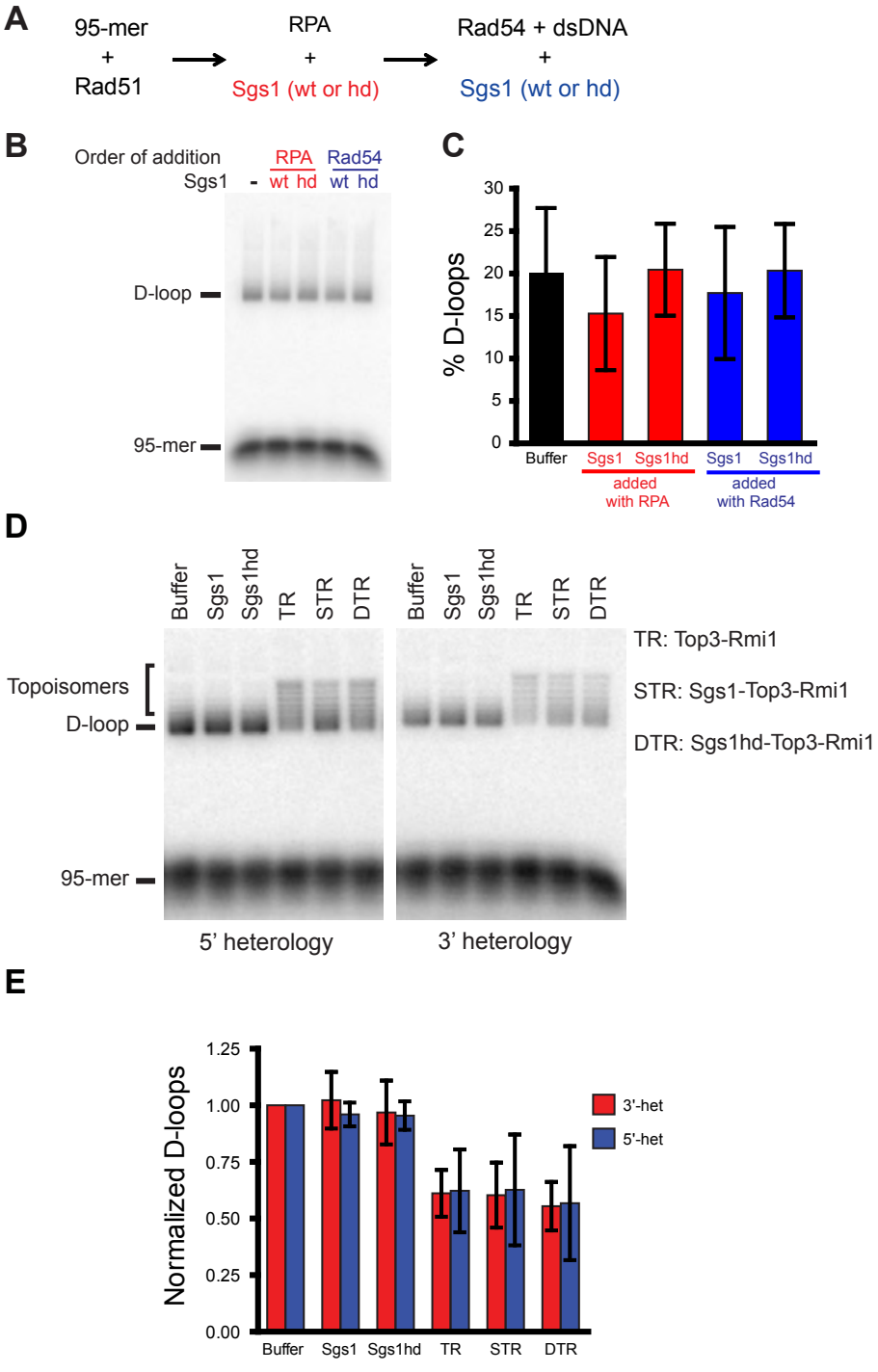

Figure S1. Sgs1 does not disrupt Rad51-mediated D-loops regardless of reaction order, and Top3-Rmi1 but not Sgs1 efficiently disrupts D-loops containing 5' or 3' heterology. A, Sgs1 does not disrupt Rad51-mediated D-loops regardless of reaction order. Reaction scheme with order of additions of Sgs1. **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)

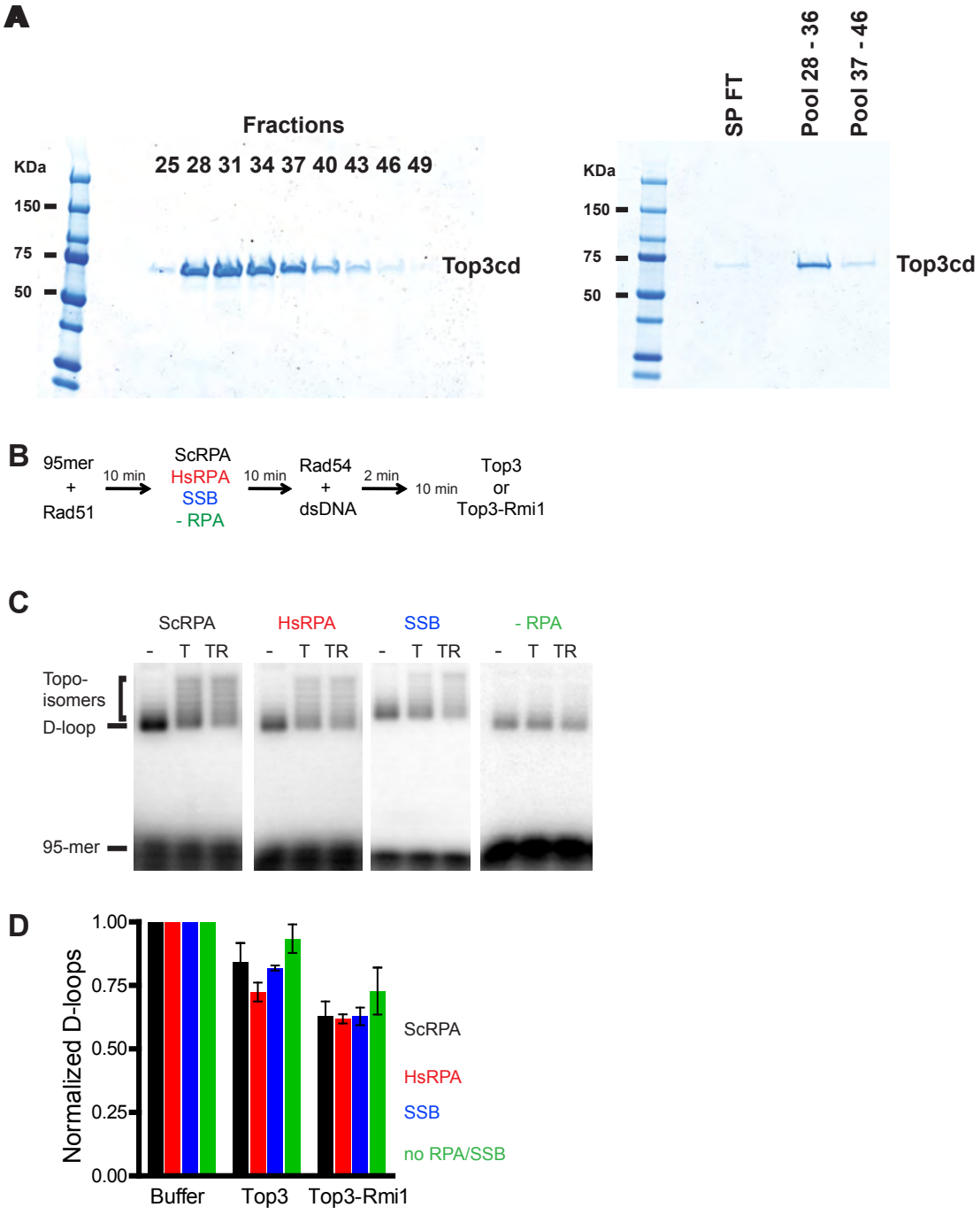

Figure S2. Purification of Top3cd (Top3-Y356F) and effect of different ssDNA binding proteins. A, Top3-Y356F samples from fractions 25 – 46 collected from the SP-sepharose column separated on a 4 – 20% gradient gel (left). Top3-Y356F samples from the SP sepharose column flow through (FT), the high concentration 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)

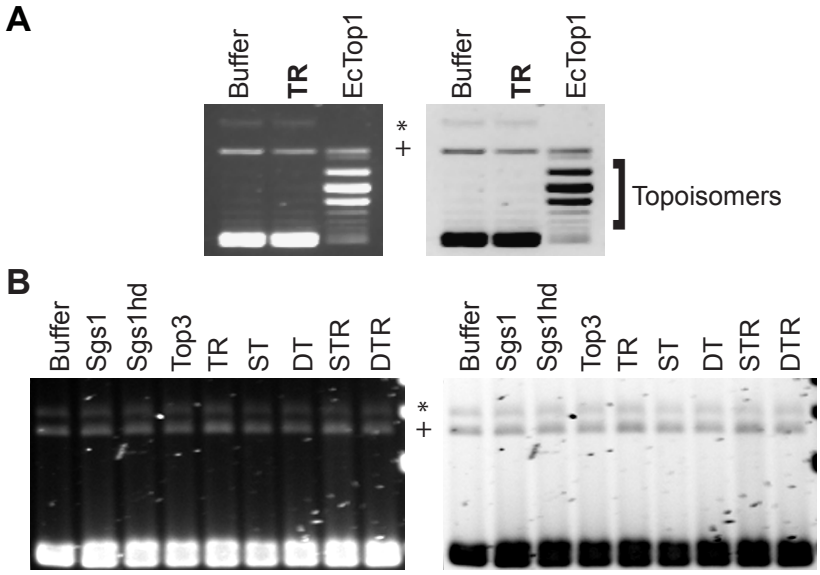

Figure S3. *S. cerevisiae Top3* does not relax supercoiled substrate under D-loop reaction conditions. A, Direct comparison of topological activity of Top3-Rmi1 (TR, 500 nM) and *E. coli* Top1 500 nM). All reactions were separated on 1.0% agarose gels and stained afterwards with ethidium bromide (left: 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)

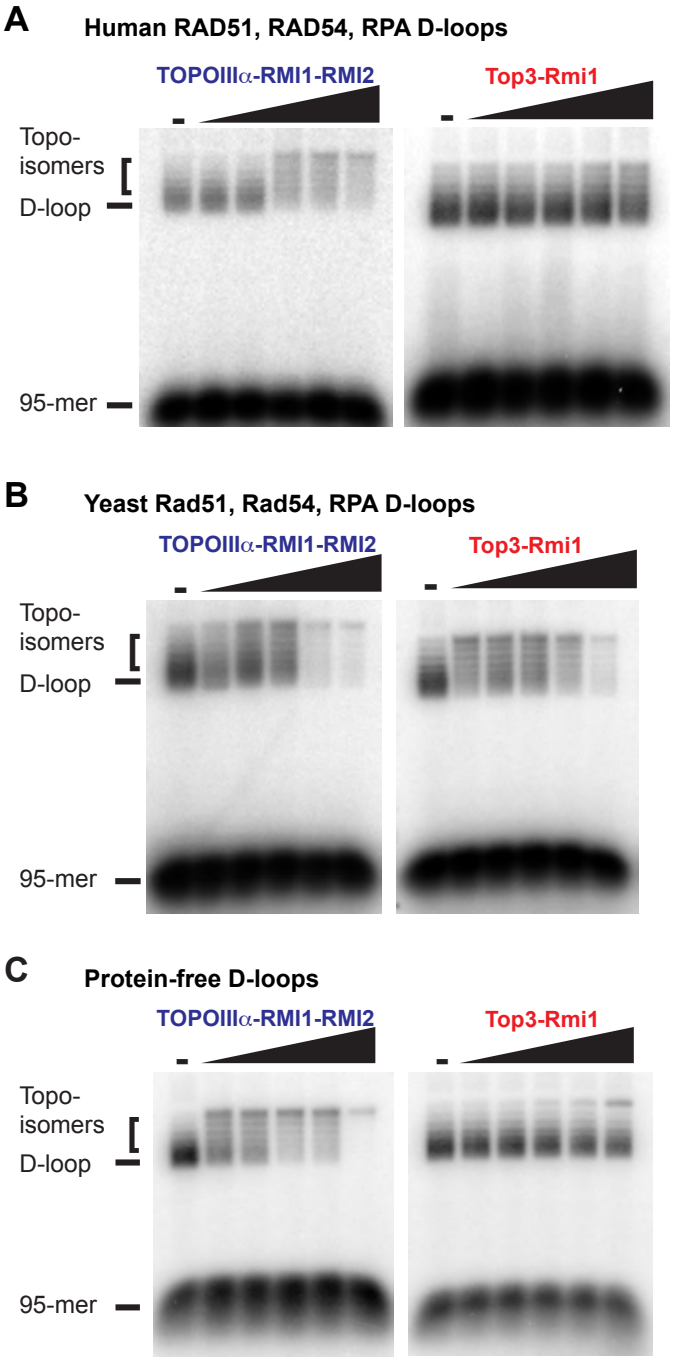

Figure S4. Representative gels for Figure 5. A. Human reconstituted D-loop system. **B.** Yeast reconstituted D-loop system. **C.** Protein-free D-loops.

Figure S5 (associated with Figure 5)

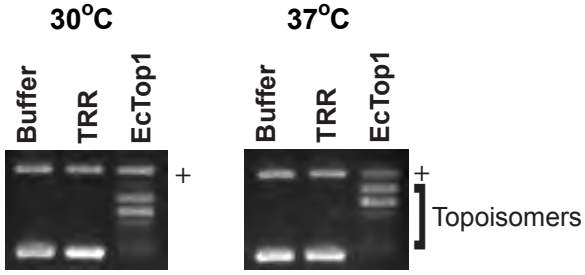

Figure S5. Human TOPOIIIalpha-RMI1-RMI2 does not relax supercoiled substrate under D-loop reaction conditions. Direct comparison of topological activity of TOPOIIIα-RMI1-RMI2 (TRR, 50 nM) and *E. coli* Top1 (500 nM) under D-loop conditions used for the yeast (30^0C) and human (37^0C) system. All reactions 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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