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Rmi1 stimulates decatenation of double Holliday junctions during dissolution by Sgs1–Top3

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Abstract

A double Holliday junction (dHJ) is a central intermediate of homologous recombination which can be processed to yield crossover or non-crossover recombination products. To preserve genomic integrity, cells possess mechanisms to avoid crossing-over. Here we show that *Saccharomyces cerevisiae* Sgs1 and Top3 proteins are sufficient to migrate and disentangle a dHJ to produce exclusively non-crossover recombination products, in a reaction termed “dissolution”. Furthermore, we show that Rmi1 stimulates dHJ dissolution at low Sgs1–Top3 protein concentrations, although it has no effect on the initial rate of Holliday junction (HJ) migration. Rmi1 serves to stimulate DNA decatenation, thereby removing the last linkages between the repaired and template DNA molecules. Dissolution of a dHJ is a highly efficient and concerted alternative to nucleolytic resolution that prevents crossing over of chromosomes during recombinational DNA repair in mitotic cells, and thereby contributes to genomic integrity.

INTRODUCTION

Homologous recombination (HR) is an important pathway for the repair of double-stranded DNA breaks, single-stranded gaps, and the restart of stalled replication forks. In this pathway, a homologous DNA sequence is utilized to repair the damaged strand, often resulting in a double Holliday junction (dHJ), which can be processed to yield crossover or non-crossover recombination products (Supplementary Fig. 1). A number of endonucleases have been isolated from various organisms ranging from *E. coli* to mammals that can specifically cleave, or “resolve” HJs to yield equal numbers of crossover and non-crossover recombination products¹. However, crossing-over can have deleterious consequences during the mitotic cell cycle by promoting loss of heterozygosity between homologous

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Author contributions P.C., J.L.P., and S.C.K. conceived the general ideas for this study. All authors planned experiments and interpreted data; J.L.P. prepared DHJs; P.C. and C.Z.B. performed experiments. P.C., J.L.P., and S.C.K. wrote the manuscript and all authors provided editorial input.

chromosomes, a recognized driver of tumorigenesis in multicellular organisms. Accordingly, very few (~5%) recombination events in mitotic *Saccharomyces cerevisiae* cells result in crossovers², implying that DNA breaks can be repaired either without dHJ formation or by resolving dHJs without crossing-over³ (Supplementary Fig. 1). Because a dHJ is a topologically-linked DNA structure, dHJs can be uniquely processed without endonucleolytic cleavage by coupling the unwinding activity of a helicase to the unlinking activity of a type IA topoisomerase^{4,5}. This combined activity could process a dHJ by migrating one or both HJs toward the other (*i.e.*, convergently) until the two HJs merge to form a hemi-catenane, which can then be decatenated to separate the recombining DNA molecules without them having cross over, in a process defined as dissolution⁶ (Supplementary Fig. 1).

In *S. cerevisiae*, Sgs1 helicase, DNA topoisomerase III (Top3), and Rmi1 act together to prevent chromosome exchanges^{2,7,8}. Although these observations led to the proposal that these proteins may be acting to process a dHJ⁵, direct biochemical analysis has only been performed on the human and *Drosophila* homologs of these proteins^{6,9-13}, due to the lack of full-length Sgs1 protein. Once full-length recombinant Sgs1 was purified, it was found to be a much more vigorous helicase than the human and *Drosophila* homologs¹⁴⁻¹⁶. This distinction, combined with the fact that *S. cerevisiae* Top3 and Rmi1 lack large domains present in their higher eukaryotic homologs^{7,17,18}, prompted us to ask whether the *S. cerevisiae* proteins possess this unique biochemical capability. While genetic studies of these proteins in yeast could be interpreted to support a role in dHJ dissolution, these proteins could also be acting in an alternate pathway that also leads to non-crossovers (Supplementary Fig. 1). To address these questions, we tested these proteins on a mobile, topologically-constrained dHJ substrate (DHJS).

Dissolution of a dHJ was initially defined using a model oligonucleotide-based substrate that possessed two junctions separated by 2 topological links¹⁹ and that required little or no branch migration to separate the oligonucleotides⁶. Here, we use a much larger DHJS that recapitulates many of the features of an endogenous dHJ²⁰. It is most easily envisioned as a pair of double-stranded DNA (dsDNA) rings conjoined by two HJs, with 165 base pairs of homologous dsDNA between each junction (Fig. 1a). The homology between the two HJs allows for the convergent migration of the HJs without the obligate generation of large tracts of single-stranded DNA (ssDNA), and the dissolution of this substrate requires both branch migration and DNA strand passage to separate the conjoined DNA molecules. Due to the distance between the two HJs, 30-35 strand passage events are required to separate the two DNA rings, providing a rigorous test for proteins thought to participate in dHJ dissolution.

Here we report that *S. cerevisiae* Sgs1 and Top3 are capable of dissolving the DHJS in a reaction that is largely species-specific, implying that specific protein-protein interactions are important for this activity. At low protein concentrations, Rmi1 stimulates dissolution of the DHJS, although it does not stimulate the rate of convergent branch migration of the HJs. Further studies using an oligonucleotide-based dHJ substrate confirmed that Rmi1 more strongly stimulated the dissolution reaction when the two HJs were in close proximity. We go on to show that Rmi1 stimulates the decatenation activity of Sgs1-Top3, and is likely stimulating the dissolution reaction at the final decatenation step. These results confirm that

dHJ dissolution is an evolutionarily conserved process and they define a novel role for Rmi1 in this pathway.

RESULTS

Sgs1 and Top3 dissolve dHJs

The full-length Sgs1 protein is a potent DNA helicase that preferentially unwinds an immobile four-way junction over duplex DNA₁₄ (Supplementary Fig. 2a,b), implying that it is preferentially targeted to a HJ. When assayed using the mobile, topologically-linked dHJ substrate (DHJS, Fig. 1a), Sgs1 and Top3 clearly produce DNA products (Fig. 1b, lanes 5 and 6) that comigrate with the expected sizes of the unlinked monomeric DNA markers (lanes 7 and 8). Product formation requires catalytically active Sgs1 and ATP (Fig. 1c). The reaction is stimulated ~10-fold by either Replication protein-A (RPA) (Compare Fig. 1b with Supplementary Fig. 2c) or the *E. coli* ssDNA binding protein (SSB) (Supplementary Fig. 2d), implying that species-specific interaction of Sgs1 and/or Top3 with RPA is not essential. In addition, the gels reveal the presence of a reaction intermediate whose electrophoretic mobility is reduced relative to the substrate (Fig. 1 and Supplementary Fig. 2c,e); this intermediate, as will be established below, is substrate that has undergone partial convergent branch migration. A time course shows that the intermediate and the monomeric products are present after 1 minute; and the monomeric products gradually accumulate over a period of 30 minutes resulting in complete disappearance of the DHJS (Supplementary Fig. 2e). Omitting the final restriction enzyme cleavage step shows that Sgs1 and Top3 produce exclusively non-crossover monomeric products (Fig. 1d). In addition, we used a mismatch-containing DHJS (MM-DHJS) which was designed such that the intervening DNA between the HJs is restored to a homoduplex only after branch migration through the region, thereby creating DNA products that are uniquely susceptible to cleavage by product-specific restriction endonucleases⁹. Indeed, the products of reactions utilizing the MM-DHJS possessed the expected novel restrictions sites (Supplementary Fig. 3a,b), verifying that the HJs were indeed being branch migrated to produce the dissolved circular DNA products. Finally, the HJs migrated exclusively through the homologous, and not heterologous, region between the junctions because if the junctions had migrated in the opposite directions (from the center outward), then the migration would have restored the A/B and B/A DNA heterodimers. These DNA molecules are the building blocks of DHJS₂₀ and one is shown as a marker (both heterodimers have almost identical mobilities) in Supplementary Fig. 3c (lane 3); it is evident that these theoretical products of dissolution are not detected in our enzyme-catalyzed reactions (lane 2). These findings demonstrate that Sgs1 and Top3 process dHJs by convergent branch migration through the homologous region and unlinking of the duplex DNA resulting in dissolution exclusively, rather than by nucleolytic resolution of the HJs.

To determine the specificity of the dissolution reaction, non-cognate partner proteins were examined. Replacing yeast Sgs1 with the same concentration of human BLM, *E. coli* RecQ, or yeast Srs2 proteins results in little or no dissolution (Fig. 1e), demonstrating that species-specific protein-protein interactions are important. Similarly, dissolution product formation is reduced or undetectable when the non-cognate type IA topoisomerases, human

Topoisomerase III α and *E. coli* Topoisomerase I, or the type IB topoisomerase, wheat germ Topoisomerase I, are used (Fig. 1f). Addition of *S. cerevisiae* Rmi1 to these heterologous reactions did not markedly improve dissolution efficiencies (Supplementary Fig. 4a,b). However, more dissolution product appears upon raising the concentrations of the non-cognate RecQ helicases and type IA topoisomerases (Supplementary Fig. 4c), indicating either that the weaker non-cognate protein-protein interactions can be overcome at higher protein concentrations, or that dissolution can occur in an uncoupled manner without a direct helicase-topoisomerase interaction²¹. Interestingly, very high concentrations of Top3 (360 nM) can support limited dissolution (< 5%) after a 30-minute incubation in the absence of Sgs1 (Supplementary Fig. 4d), likely resulting from thermal HJ movement and the concomitant random unlinking by Top3. These results indicate that topoisomerase activity *per se* is sufficient for the dissolution of dHJs, but that spontaneous convergent migration is slow (due to the non-processive, random-walk nature of this mechanism) and requires the ATP-dependent activity of the Sgs1 helicase for efficient dHJ processing (Fig. 1b,c).

Intermediates of convergent branch migration can be detected

Interestingly, in reactions containing Sgs1 and wheat germ topoisomerase I, most or all of the substrate is converted into the slower-migrating intermediate species but no dissolution of the DHJS is detected (Fig. 1f, lane 8). We suspected that this slower migrating species, which is also apparent in most Sgs1–Top3 reactions, comprised molecules in which the two HJs have converged, but did not fully dissolve, resulting in intermediates with more of an X-shaped structure that would display reduced electrophoretic mobility²². We took advantage of a feature of the MM-DHJS to test this hypothesis. The site of the mismatch in the MM-DHJS is located 35 nucleotides away from the nearest HJ position. Consequently, migration of the HJ past the mismatch site would generate the unique SphI restriction site, which would render any such convergent branch migration intermediates sensitive to restriction digestion, even in the absence of full dissolution (Fig. 2a). Intermediates were not formed by Sgs1 alone and, furthermore, the substrate remained refractory to restriction digestion (Fig. 2b), indicating that Sgs1 alone is incapable of migrating a HJ under topological constraint. This is expected, as any attempted migration of a HJ in the DHJS by Sgs1 alone would result in positive supercoiling in the homologous region between the HJs, thus inhibiting branch migration. The same result was obtained with Top3 alone (at a low concentration of 36 nM; Fig. 2b) demonstrating that thermal branch migration does not detectably proceed beyond 35 bp under these conditions. We could, however, generate a slower-migrating species by the action of Sgs1 and wheat germ Topoisomerase I on MM-DHJS. This species was sensitive to SphI endonuclease (Fig. 2c), demonstrating that convergent branch migration had occurred, but not dissolution, and that this species is indeed the predicted intermediate of dissolution.

Rmi1 stimulates late, but not early, steps in dHJ dissolution

We next investigated the role of the Rmi1 protein in the dissolution of the DHJS by Sgs1 and Top3. Rmi1 forms a complex with Top3 and Sgs1 *in vivo*^{7,8}, and was originally proposed to stimulate the Sgs1–Top3 complex through DNA binding and targeting to specific DNA structures^{8,23}. When Rmi1 was added to a reaction containing Sgs1 and Top3, initially no stimulation of dissolution was observed, with or without RPA

(Supplementary Fig. 5a,b). However, when a detailed analysis of MM-DHJS dissolution by Sgs1–Top3–RPA with and without Rmi1 was performed, a subtle but reproducible stimulation by Rmi1 was observed, which suggested that Rmi1 stimulates the dissolution of the branch migrated intermediate (Supplementary Fig. 5c-f).

A marked stimulation of dissolution by Rmi1, however, was seen when the Sgs1 and Top3 protein concentrations were lowered to 1 nM (Fig. 2d). Early in the reaction, the amount of intermediate that is rendered susceptible to SphI endonuclease (Fig. 2d, compare lanes 5 and 7 (black arrows)) in reactions either with or without Rmi1 is the same, indicating that Rmi1 plays no substantial role in the initial convergent branch migration phase of dHJ dissolution (see Fig. 2a). However, the final dissolution products (both A and B linear DNA, in the BamHI-only digest) were generated only in the presence of Rmi1 (Fig. 2d, compare lanes 8 and 10 (white arrows)), indicating that Rmi1 functions in the conversion of convergent branch-migrated intermediates into fully dissolved molecules (Fig. 2e), *i.e.*, in the last stage of dissolution. These reactions also demonstrate the remarkable dissolution capability of the Sgs1–Top3–Rmi1 complex. At only 1 nM of complex, this heterotrimer converts an approximately equimolar amount of DHJS into dissolved products. Since there are 165 base pairs of homologous DNA between the two HJs, each Sgs1–Top3–Rmi1 complex must remove 30-35 DNA linkages during the dissolution process. Moreover, at 1 nM Rmi1, the product yield is almost saturated (Supplementary Fig. 5g,h), indicating that dHJ dissolution is a highly concerted reaction under these conditions.

The analyses described above indicated that Rmi1 stimulated the late events of DHJS dissolution catalyzed by Sgs1 and Top3. To verify this conclusion, we used a dHJ substrate that is constructed from oligonucleotides and that contains only 14 bp of DNA between the two HJs, corresponding to 2 DNA linkages total²⁴ (Fig. 3a). Thus, this substrate resembles a “nearly dissolved” dHJ, and can be used to study the last steps prior to full dissolution. Both Sgs1 and Top3 were needed to dissolve this substrate (Fig 3a); Rmi1 strongly stimulated the dissolution when the concentrations of Sgs1 and Top3 were closer to physiological levels, in the low nM range (Fig. 3b,c and Supplementary Fig. 6a,b). Our results with the yeast proteins parallel those for the human homologues¹⁰⁻¹³. This finding further supports a role for Rmi1 in the late stage of dHJ dissolution, although at this point it is not clear if Rmi1 is stimulating the removal of the last handful of topological linkages between the HJs, or if it is only stimulating decatenation of the hemi-catenane, the last predicted intermediate of dHJ dissolution where the two respective DNA molecules are conjoined only by a single DNA link.

Rmi1 strongly stimulates DNA decatenation by Sgs1 and Top3

To determine whether Rmi1 can stimulate Sgs1–Top3 mediated decatenation, we used purified kinetoplast DNA (kDNA), which exists as large catenated complexes primarily consisting of ~2.5 kb circular DNA molecules²⁵. This complex catenane is trapped in the wells of an agarose gel, but treatment with Topo II, which has a well-defined decatenation activity^{25,26}, released monomer DNA rings from the complex, allowing them to enter the gel (Fig. 4a, lane 3). Incubation with Sgs1 or Sgs1–Top3 modestly increased the amount of nicked monomers released, likely through Sgs1-mediated unwinding of doubly nicked

circles present in the substrate (note that the majority of circular DNA in the kDNA catenane contains at least one nick (Fig. 4a, lane 3)). However, Rmi1 addition resulted in nearly complete decatenation of the kDNA catenane by Sgs1–Top3 (Fig. 4a, lanes 7 and 8), with the product distribution being similar to that of a Topo II-catalyzed decatenation reaction. Decatenation mediated by Sgs1–Top3–Rmi1 was dependent on the Rmi1 concentration, with maximal stimulation occurring when Rmi1 was approximately equimolar with Top3 (Fig. 4b, Supplementary Fig. 7a), and was apparent across a wide range of Top3 and Sgs1 protein concentrations (Supplementary Fig. 7b). Taken together, these data indicate that Rmi1 is stimulating decatenation to promote dHJ dissolution by Sgs1–Top3.

DISCUSSION

Our results demonstrate that Sgs1, Top3, and Rmi1 coordinate the dissolution of dHJs. The helicase activity of Sgs1 is needed to migrate the HJs and create ssDNA for the DNA strand passage activity of the type IA topoisomerase, Top3. Yeast RPA and *E. coli* SSB stimulate this reaction by binding and stabilizing the Sgs1-generated ssDNA. This concerted reaction results in the convergent migration of the HJs, and Rmi1 stimulates this process in the late stages (Fig. 5). It was recently discovered that dHJs formed during mitotic homologous recombination were not processed in the absence of Sgs1, further supporting the role of this complex in the dissolution of dHJs *in vivo*²⁷.

Dissolution activity was first demonstrated *in vitro* with human BLM helicase and topoisomerase III α 6 using an oligonucleotide-based dHJ²⁴ and later for the *Drosophila* homologues⁹ using the larger DHJS²⁰. Seminal genetic experiments revealed that yeast *sgs1* mutation rescues the slow growth phenotype of *top3* mutants, suggesting that Sgs1 creates toxic DNA intermediates that require processing by Top3⁴. We imagine that in the *top3* mutant background, the Sgs1 protein may catalyze HJ migration in an uncoupled reaction with Top1, but the pair cannot fully unlink the dHJ, as we showed herein. These nearly converged HJs might represent the “toxic intermediates” that affect chromosome segregation. Furthermore, an *rmi1* deletion closely phenocopies a *top3* deletion⁸, indicating that Rmi1 is as essential as Top3 for decatenation *in vivo*. In agreement, *in vitro*, we observed that Rmi1 is indispensable for dissolution when the concentrations of Top3 and Sgs1 are low (in the nM range). Similarly to yeast *sgs1* mutants², BLM-deficient human cells display a large increase in sister chromatid exchanges²⁸. These aberrant chromosomal exchanges may result from the inability of BLM-deficient cells to catalyze normal dHJ dissolution. In such cells, HJs or their precursors can be resolved only by alternative pathways that involve nucleolytic cleavage by enzymes such as GEN1/Yen1, MUS81–EME1/Mms4, or SLX1–SLX4, and that would conceptually lead to crossover products in half of recombinational DNA repair events^{1,3} (Supplementary Fig. 1). Alternatively, prevention of chromosomal crossovers could result from the ability of BLM/Sgs1 to disrupt the joint molecule intermediates (D-loop structures) that precede HJ formation²⁹. Given the prominence of dHJs as intermediates in dsDNA break and ssDNA gap repair, our findings show that dissolution by Sgs1–Top3–Rmi1 is a highly concerted and efficient process, supporting the genetic observations that the 95% of recombinational DNA repair events avoid the crossover of chromosomes². The remaining events, which lead to crossovers, would be produced by one of the nucleolytic enzymes listed above, perhaps resolving those

recombination intermediates which have only one HJ due to loss of the other and which consequently cannot be dissolved by Sgs1–Top3–Rmi1. Individuals lacking the BLM helicase are highly predisposed to the development of a wide spectrum of cancers³⁰, demonstrating the importance of these proteins in processes that prevent genome rearrangements and instability.

METHODS

DNA substrates and recombinant proteins

The double Holliday junction substrate (DHJS), the mismatch-containing double Holliday junction substrate (MM-DHJS) and other constructs used as DNA markers throughout this study were prepared as described previously^{9,20}. Catenated kinetoplast DNA and the corresponding linear DNA were purchased from Topogen. Sgs1 (wild-type and the helicase- and ATPase-dead mutant (K706A)) were purified as recently described¹⁴. The preparation of Top3, Rmi1, and Top3–Rmi1 heterodimer is detailed in Supplementary Methods, and is summarized in Supplementary Fig. 8. RPA, *E. coli* SSB, *E. coli* RecQ and human BLM were prepared as described^{15,31–33}. Human Topo III α , *S. cerevisiae* Srs2 and *D. melanogaster* Topo II were generous gifts from Pavel Janscak (University of Zurich, Switzerland), Xavier Veaute (Institute of Cellular and Molecular Radiation Biology, France), and Tao-shih Hsieh (Duke University), respectively. *E. coli* Topo I and wheat germ Topo I were purchased from New England Biolabs and Promega, respectively.

Double Holliday junction dissolution assay

The reaction buffer contained 20 mM Tris-acetate (pH 7.5), 2 mM magnesium acetate, 1 mM dithiothreitol, 100 $\mu\text{g ml}^{-1}$ bovine serum albumin (New England Biolabs), 80 U ml^{-1} pyruvate kinase (Sigma), 1 mM phosphoenolpyruvate (Sigma), and 1 mM ATP. Reactions also contained 2.5 ng μl^{-1} (4.37 nM molecules) DHJS or MM-DHJS, and the indicated concentrations of Sgs1, Top3, Rmi1, and Top3–Rmi1 heterodimer. Unless indicated otherwise, the reactions also contained RPA (578 nM), which corresponds to a 1.5-fold molar excess (with regard to potential ssDNA saturation, if all of the DNA became single-stranded, and a DNA-binding site size for RPA of 20 nt). The reaction was assembled in a final volume of 20 μl on ice, initiated by addition of Sgs1 and incubated at 30 °C for 30 min. The reaction was terminated by addition of SDS to 0.5% (w/v), EDTA to 25 mM, and 1 μl proteinase K (14–22 mg ml^{-1} , Roche) and incubating at 37 °C for 60 min. Unless indicated otherwise, the reaction products were then purified by phenol and phenol-chloroform extraction using Phase-Lock GelTM (Eppendorf) and were precipitated by ethanol in the presence of linear polyacrylamide as a co-precipitant³⁴. The precipitated DNA was then resuspended in water at 4 °C overnight and, unless indicated otherwise, digested with 10 U BamHI in a 20 μl reaction for 30 min. The digestion with BamHI improved resolution of the assay products; also, because the DNA species were linearized prior to gel electrophoresis, the intermediates cannot represent simple topoisomers of the covalently-closed DHJS. In case of the MM-DHJS, a fraction of the reaction products was also digested sequentially with SphI (5 U, 45 min) and BamHI (5 U, 45 min) at 37 °C. The reaction products were then separated by native gel electrophoresis in 1.9% (w/v) agarose in TAE buffer with 0.05 $\mu\text{g ml}^{-1}$ ethidium bromide, at $\sim 4 \text{ V cm}^{-1}$ for ~ 3 hours. The gel was photographed using

AlphaInnotech imaging station, and quantified using ImageQuant software (GE Healthcare). The percent dissolution was calculated as the amount of dissolution products divided by the sum of remaining DHJS plus the dissolution products. The preparation of the oligonucleotide-based dHJ and dissolution reactions were carried out and analyzed as described previously^{10,29,35}.

Decatenation of kinetoplast DNA

Reactions (20 μ l) contained 25 mM Tris-acetate pH 7.5, 1 mM magnesium acetate, 75 mM NaCl, 0.1 mM dithiothreitol, 1 mM phosphoenolpyruvate, 100 μ g ml⁻¹ bovine serum albumin (New England Biolabs), 80 U ml⁻¹ pyruvate kinase (Sigma), 1 mM ATP, 5 ng μ l⁻¹ kDNA (Topogen), 340 nM RPA and purified Sgs1, Top3 and Rmi1, as indicated. The reactions were carried out at 30 °C for 60 min; terminated by the addition of SDS to 0.5% (w/v), EDTA to 25 mM, and proteinase K (1 μ l of 14-22 mg ml⁻¹, Roche); and then incubated at 37 °C for 60 min. Decatenation of kDNA with *D. melanogaster* Topo II was carried out in 50 mM Tris-acetate pH 7.5, 60 mM KCl, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 0.1 mM EDTA, 30 μ g ml⁻¹ bovine serum albumin (New England Biolabs), 5 ng μ l⁻¹ kDNA and 20 U Topo II. The reaction products were separated by electrophoresis in 1.3% (w/v) agarose in TAE buffer with 0.5 μ g ml⁻¹ ethidium bromide, destained in TAE buffer for 1 hour, and analyzed as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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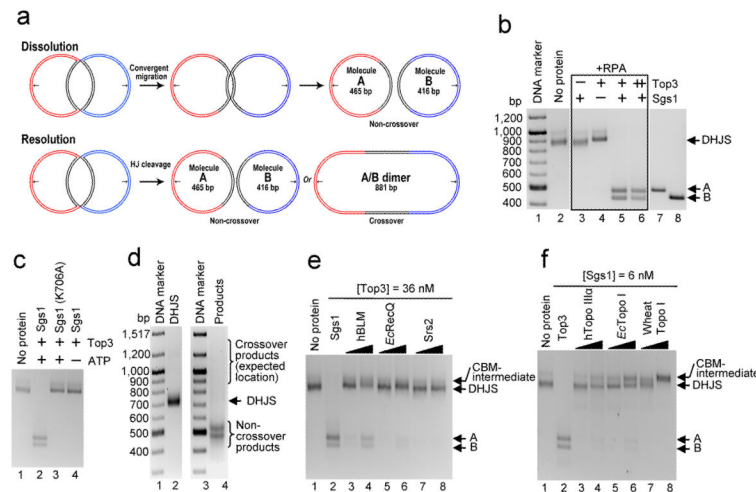


Figure 1.

Sgs1 and Top3, stimulated by replication protein A (RPA), dissolve double Holliday junctions to yield non-crossover products. (a) A schematic representation of the double Holliday junction substrate (DHJS) showing dissolution by convergent branch migration or resolution by nucleolytic cleavage. Dissolution of the dHJ leads exclusively to monomeric non-crossover products (denoted as A and B). Resolution leads to both monomeric non-crossover products (A and B) and a dimeric crossover product (A/B dimer). The arrows indicate positions of a BamHI restriction site. (b) Sgs1 (6 nM) and/or Top3 (36 or 360 nM) and RPA (578 nM) were incubated with the DHJS as described in Methods. The expected reaction products, A and B markers, are loaded in lanes 7 and 8, respectively. To improve resolution, the assay products were digested with BamHI prior to electrophoretic analysis. (c) Processing of DHJS requires catalytically active Sgs1 and ATP. Wild type Sgs1 or ATPase-dead Sgs1 (K706A) mutant (both 6 nM) were incubated with Top3 (36 nM) with or without ATP, as indicated, in the presence of RPA. (d) DHJS dissolution catalyzed by Sgs1, Top3, and RPA leads exclusively to non-crossover products. The DHJS was incubated with Sgs1 (36 nM), Top3 (77 nM), and RPA. The reaction products were purified, and analyzed by electrophoresis without prior digestion by BamHI. (e) The dissolution of dHJs is optimal with the cognate Sgs1 and Top3 protein pair. Dissolution reactions were carried out with RPA (578 nM), Top3 (36 nM), and either yeast Sgs1 (6 nM, lane 2), human BLM (6 and 36 nM, lanes 3 and 4, respectively), *E. coli* RecQ (6 and 36 nM, lanes 5 and 6, respectively), or another *S. cerevisiae* helicase with 3'-5' unwinding polarity, Srs2 (6 and 36 nM, lanes 7 and 8, respectively). The "CBM-intermediate" denotes the convergently branch-migrated intermediate with a reduced mobility relative to the DHJS. Some preparations of DHJS possess a small fraction of this intermediate after synthesis due to the action of reverse gyrase (which contains a type IA topoisomerase domain) in the final step of substrate synthesis²⁰. (f) Dissolution reactions were carried out with RPA (578 nM), Sgs1 (6 nM), and either yeast Top3 (36 nM, lane 2), human Topo III α (36 and 100 nM, lanes 3 and 4, respectively), *E. coli* Topo I (2.5 and 10 units, lanes 5 and 6, respectively), or wheat germ Topo I (2.5 and 10 units, lanes 7 and 8, respectively). The "CBM-intermediate" denotes the convergently branch-migrated intermediate with a reduced mobility relative to the DHJS.

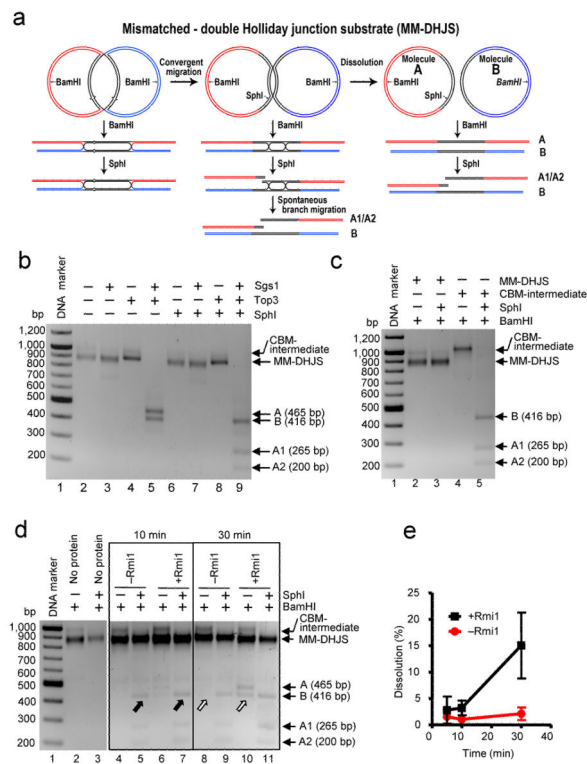


Figure 2.

Rmi1 stimulates a late step of dHJ dissolution catalyzed by Sgs1 and Top3. **(a)** Schematic representation for the progression of dissolution of the mismatched double Holliday junction substrate (MM-DHJS), showing that branch migration of 35 bp or more can be detected by digestion with restriction endonucleases. Cleavage of the substrate by BamHI results in a molecule that is resistant to further cleavage by SphI, and which migrates between the 800 and 900 bp linear markers. Branch migration of the left Holliday junction past the mismatch restores complementarity at the SphI site; digestion with BamHI produces a single DNA molecule migrating at approximately 900 bp (indicated as intermediate) and additional digestion with SphI cleaves this DNA to produce fragments A1 (265 bp) and A2 (200 bp), which are derived from A DNA, and also the linearized B DNA (416 bp), which lacks an SphI site. This structure is initially conjoined by the HJs; however, upon digestion the linked DNAs quickly dissociate due to spontaneous branch migration of the dHJ. Digestion of the final products of the dissolution reaction by BamHI yields linear bands 465 and 416 bp in length, corresponding to the linearized A and B markers, and subsequent digestion with SphI cleaves the A marker into A1 and A2 DNA fragments as above. **(b)** The helicase activity of Sgs1 is not sufficient for HJ migration in DHJS. MM-DHJS was incubated with Sgs1 (6 nM) and/or Top3 (36 nM), as indicated. All reactions contained RPA. The combined activity of Sgs1 and Top3 led to the full dissolution of the MM-DHJS (lanes 5 and 9). The helicase activity of Sgs1 alone did not produce dissolution products (lane 3), nor did it allow for HJ migration past the mismatch site (35 nucleotides distant from the nearest HJ position), as indicated by the resistance to SphI cleavage (lane 7). “CBM-intermediate” indicates the position of the DHJS with convergent branch of the HJs. **(c)** The MM-DHJS was incubated with Sgs1 (6 nM), wheat germ Topo I (10 U) and RPA (578 nM), as in Fig. 1f, to produce

the reaction intermediate with a reduced electrophoretic mobility, denoted as a CBM-intermediate; this intermediate was then digested with BamHI and SphI, where indicated. The MM-DHJS is resistant to SphI (lane 3), whereas the Topo I-generated intermediate is sensitive to SphI (lane 5), showing that branch migration occurred. **(d)** Restriction analysis of the time course dissolution of MM-DHJS. The reactions were carried out with Sgs1 (1 nM), Top3 (1 nM), Rmi1, where indicated (10 nM, denoted as “+Rmi1”), and RPA, and terminated at the indicated times. Branch migration of the Holliday junction past the SphI site is initially identical in reactions without and with Rmi1, and is detected by the SphI-dependent B fragment (indicated by black arrows). Final dissolution products appear almost exclusively in reactions containing Rmi1 at later time points (indicated by white arrows). The contrast of this image was enhanced, compared to other images in this study, to enhance visualization of the faint product DNA bands. The “CBM-intermediate” denotes the convergently branch-migrated intermediate with a reduced mobility relative to the MM-DHJS. **(e)** Kinetics of MM-DHJS dissolution, based on 3 experiments such as that shown in panel **d**. Error bars, s.e.m.

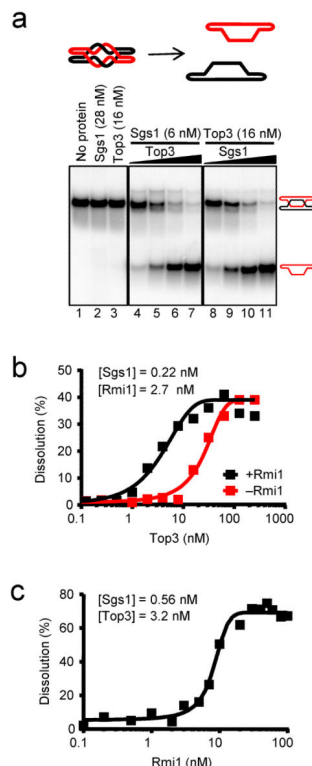


Figure 3.

Rmi1 greatly stimulates dissolution of dHJ-containing DNA that mimics the final steps of dHJ dissolution. **(a)** Sgs1 and Top3 can dissolve the oligonucleotide-based dHJ. The concentrations of Top3 in lanes 4-7 were 4, 8, 16 and 32 nM, and the Sgs1 concentrations in lanes 8-11 were 0.4, 0.9, 1.8 and 3.5 nM, respectively. The position of the oligonucleotide-based dHJ and the dissolution product are indicated on the right. The oligonucleotide-based dHJ and the respective dissolution products are schematically represented above the lanes. **(b)** Dissolution of the oligonucleotide-based dHJ is greatly stimulated by Rmi1 at low Top3 concentrations. Quantification of experiments such as in panel **a**, with Top3 concentration plotted on a logarithmic scale. The reaction contained Sgs1 (0.22 nM) and, where indicated, Rmi1 (2.7 nM). **(c)** Stimulation of dissolution by Rmi1. Quantification of experiments such as in panel **a**, with Rmi1 concentration plotted on a logarithmic scale. The reactions contained Sgs1 (0.56 nM) and Top3 (3.2 nM).

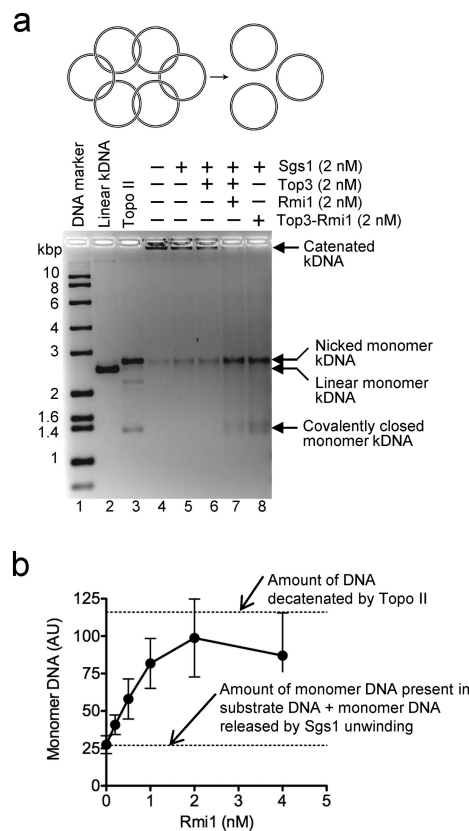


Figure 4.

Rmi1 promotes decatenation of kinetoplast DNA (kDNA). **(a)** The reactions (lanes 4-8) were carried out with Sgs1, Top3, Rmi1 and Top3–Rmi1 heterodimer, as indicated, and RPA (340 nM); kDNA was decatenated with *D. melanogaster* Topo II as a positive control (lane 3). The positions of interlinked kDNA and the various monomer forms are indicated on the right. The kDNA, a network of dsDNA rings, is schematically represented above the lanes. **(b)** Decatenation of kDNA is dependent on Rmi1 concentration. The amount of monomeric kDNA (the sum of pixel intensity values, in arbitrary units, based on experiments as shown in Supplementary Fig. 7a) was plotted against Rmi1 concentration. The reactions contained RPA (340 nM), Sgs1 (2 nM), Top3 (2 nM), and the indicated amount of Rmi1. Error bars, s.e.m., based on 4 independent experiments. The lower dashed line indicates the amount of monomer kDNA present in the absence of Rmi1 (mainly monomer DNA present in the substrate DNA, and/or released by Sgs1 unwinding, see panel **a**), and the upper dashed line indicates the amount of monomer kDNA released by *D. melanogaster* Topo II. The stimulation of kDNA decatenation is saturated at 2 nM Rmi1, a concentration equimolar to that of Top3 and Sgs1.

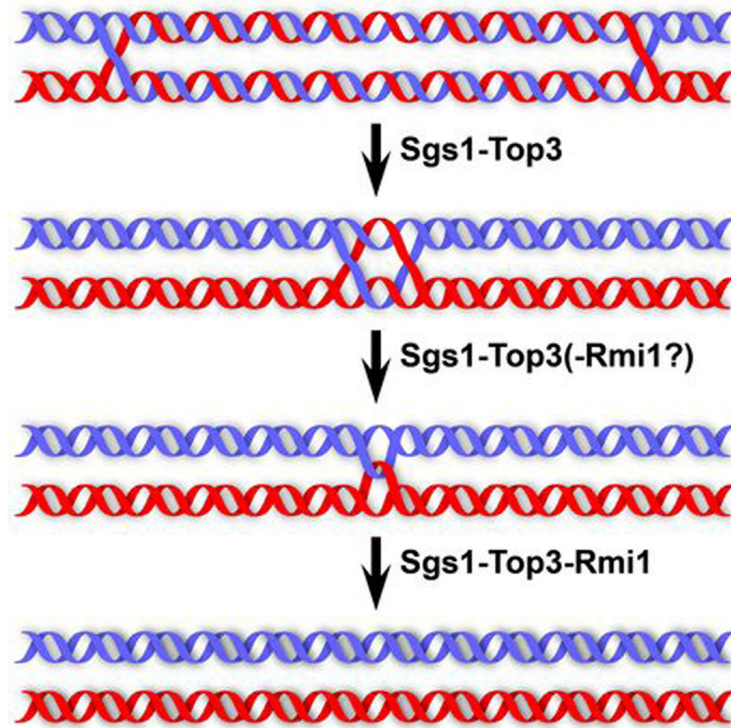


Figure 5.

Model of Sgs1–Top3–Rmi1 complex function in the dissolution of double Holliday junctions (dHJs). Sgs1 and Top3 catalyze convergent HJ migration. Rmi1 is likely an integral part of the Sgs1–Top3 complex, but its function is dispensable during this initial branch migration phase. Rmi1 stimulates dHJ dissolution at a later stage when both junctions are in close proximity. We cannot distinguish whether Rmi1 promotes dissolution of just the hemi-catenane, or of an intermediate that has several topological linkages. Rmi1 then stimulates the dissolution of a hemi-catenane, the anticipated last intermediate of dHJ dissolution.