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

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A double Holliday junction (dHJ) is a central intermediate of homologous recombination that can be processed to yield crossover or non-crossover recombination products. To preserve genomic integrity, cells possess mechanisms to avoid crossing over. 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." 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, 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.

Homologous recombination (HR) is an important pathway for the repair of double-stranded DNA breaks and single-stranded gaps and for restart of stalled replication forks. In this pathway, a homologous DNA sequence is used 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 *Escherichia coli* to mammals that can specifically cleave, or "resolve," HJs to yield equal numbers of crossover and non-crossover recombination products[1]. However, crossing over can have deleterious consequences during the mitotic cell cycle by promoting loss of heterozygosity between homologous chromosomes. Such loss is a recognized driver of tumorigenesis in multicellular organisms. Accordingly, very few (~5%) recombination events in mitotic *S. cerevisiae* cells result in crossovers[2], implying that DNA breaks can be repaired either without dHJ formation or by resolving dHJs without crossing over[3] (**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 (that is, convergently) until the two HJs merge to form a hemicatenane, which can then be decatenated to separate the recombining DNA molecules without crossover formation, in a process defined as dissolution[6] (**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 dHJ[5], direct biochemical analysis has only been performed on the human and *Drosophila melanogaster* homologs of these proteins[6,9–13] because of 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* homologs[14–16]. 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. Although 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 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 two topological links[19] and that required little or no branch migration to separate the oligonucleotides[6]. Here we use a much larger DHJS that recapitulates many of the features of an endogenous dHJ[20]. It is most easily envisioned as a pair of double-stranded DNA (dsDNA) rings conjoined by two HJs, with 165 base pairs (bp) 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. Owing 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

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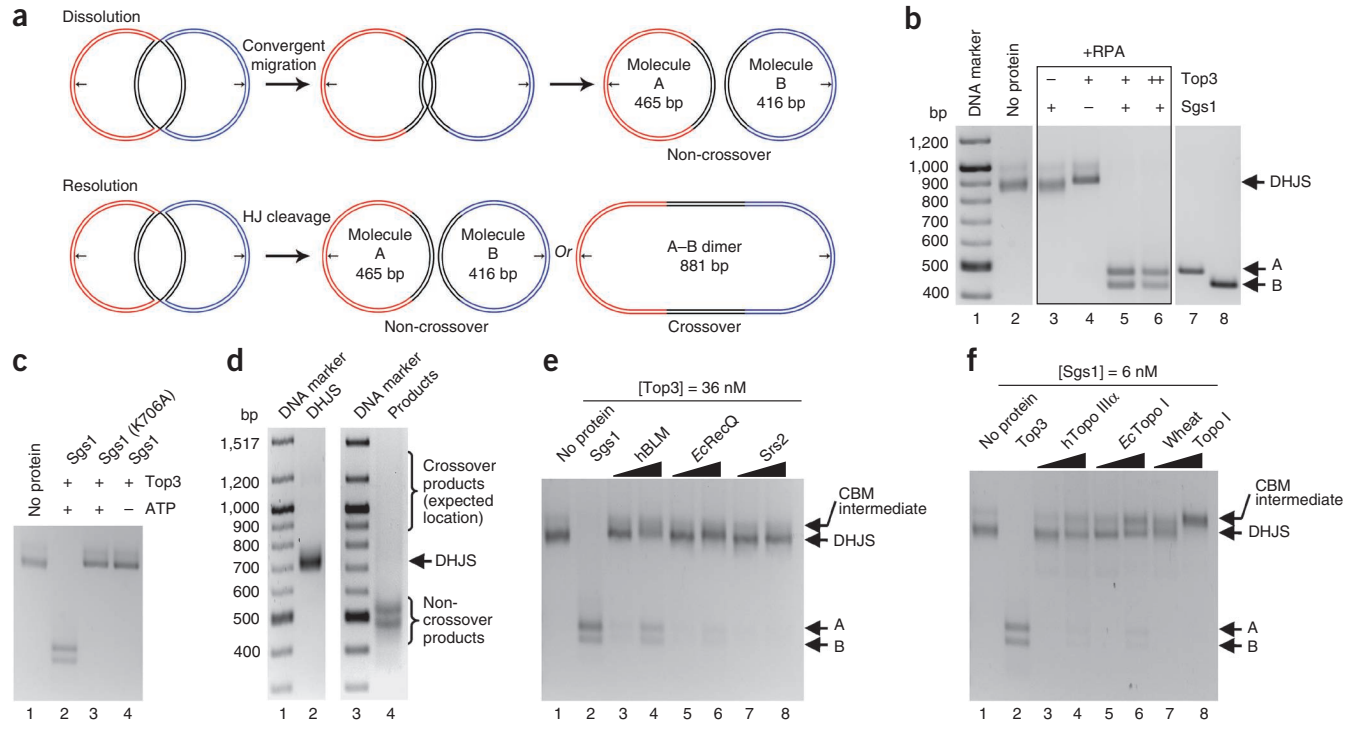

Figure 1 Sgs1 and Top3, stimulated by RPA, dissolve double Holliday junctions (dHJ) to yield non-crossover products. (**a**) A schematic representation of the dHJ substrate (DHJS). Top, dissolution of the DHJS by convergent branch migration leads exclusively to monomeric non-crossover products (A and B). Bottom, resolution by nucleolytic cleavage leads to either monomeric non-crossover products (A and B) or a dimeric crossover product (A–B dimer). Small arrows indicate BamHI restriction sites. (**b**) Action of Sgs1 (6 nM) and/or Top3 (36 or 360 nM) and RPA on the DHJS (see Online Methods for details). 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 before electrophoretic analysis. (**c**) Processing of DHJS requires catalytically active Sgs1 and ATP. Wild-type or ATPase-dead Sgs1 (K706A) mutant (both 6 nM) were incubated with Top3 (36 nM) 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, and the reaction products were analyzed by electrophoresis without digestion by BamHI. (**e**) The dissolution of dHJs is optimal with the cognate Sgs1 and Top3 pair. Dissolution reactions were carried out with RPA, Top3 (36 nM), and either yeast Sgs1 (6 nM), human BLM (6 or 36 nM), *E. coli* RecQ (6 and 36 nM) or another *S. cerevisiae* helicase with 3′–5′ unwinding polarity, Srs2 (6 and 36 nM). "CBM-intermediate" denotes the convergently branch-migrated intermediate with a reduced mobility relative to the DHJS; the CBM-intermediate appears in some preparations owing to the action of reverse gyrase (which contains a type IA topoisomerase domain) in the final step of substrate synthesis[20]. (**f**) Dissolution reactions were carried out with RPA, Sgs1 (6 nM), and yeast Top3 (36 nM), human Topo IIIα (36 and 100 nM), *E. coli* Topo I (2.5 and 10 units) or wheat germ Topo I (2.5 and 10 units).

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 previously unknown 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 DNA[14] (**Supplementary Fig. 2a**,**b**), implying that it is preferentially targeted to an 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 lower than that of 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 min; and the monomeric products gradually accumulate over a period of 30 min, 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) that 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 endonucleases[9]. Indeed, the products of reactions using the MM-DHJS possessed the expected branch migration–dependent restriction sites (**Supplementary Fig. 3a**,**b**), verifying that the HJs were being branch migrated to produce the

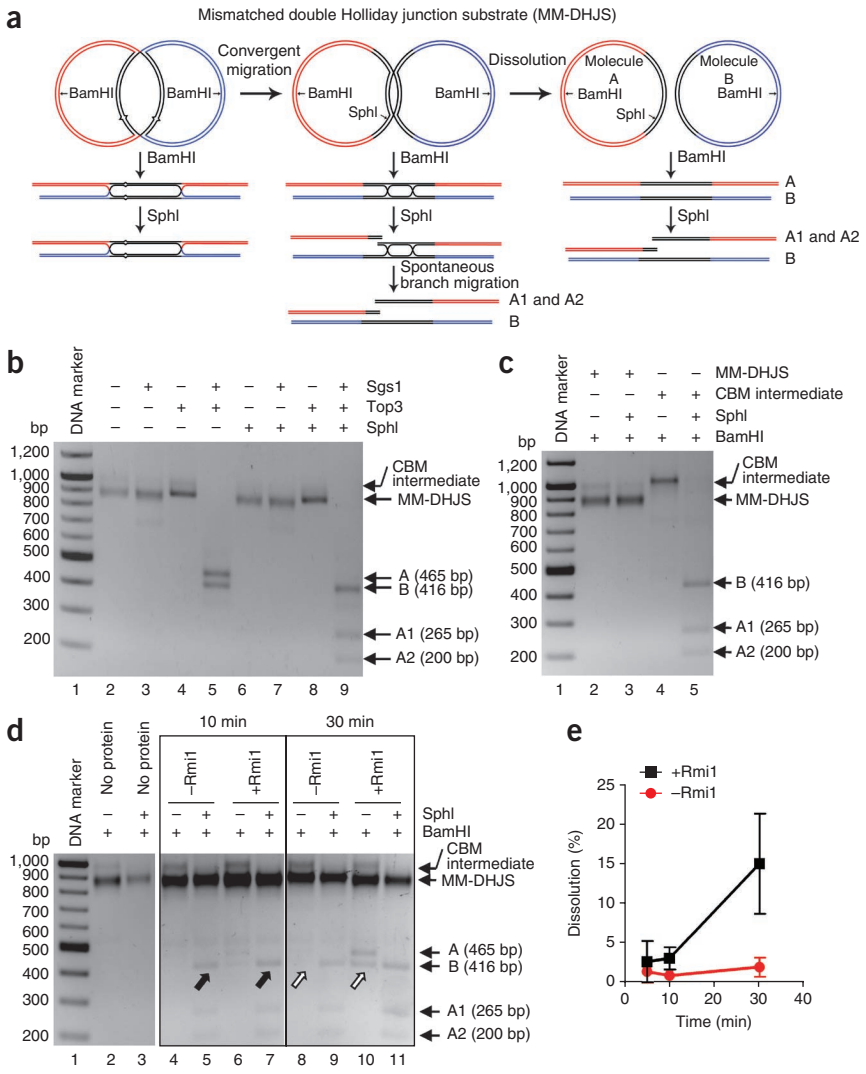

Figure 2 Rmi1 stimulates a late step of dHJ dissolution catalyzed by Sgs1 and Top3. (**a**) Schematic representation of dissolution of the mismatched double Holliday junction substrate (MM-DHJS), which contains a mismatch 35 bp from the nearest HJ position, such that HJ migration past the mismatch site generates a unique SphI restriction site. Left, substrate cleavage by BamHI results in a molecule that is not cleaved by SphI and migrates between the 800- and 900-bp linear markers. Middle, branch migration of the left HJ past the mismatch restores the SphI site; digestion with BamHI produces a single DNA molecule migrating at approximately 900 bp, and additional digestion with SphI produces fragments A1 (265 bp) and A2 (200 bp), which are derived from A DNA (465 bp), and the linearized B DNA (416 bp), which lacks an SphI site; the A2 and B fragments are initially linked by the HJs, but quickly dissociate due to spontaneous branch migration. Right, BamHI digestion of the final products of the dissolution reaction yields A and B DNA fragments, and subsequent digestion with SphI generates A1 and A2 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). All reactions contained RPA. Sgs1 alone did not produce dissolution products (lane 3), nor did it allow for HJ migration past the mismatch site (lane 7). "CBM-intermediate" indicates the convergently branch-migrated intermediate. (**c**) The MM-DHJS was incubated with Sgs1, wheat germ Topo I and RPA to produce the CBM intermediate, which was then digested with BamHI and SphI. The MM-DHJS is resistant to SphI (lane 3), whereas the CBM intermediate is sensitive to SphI (lane 5), showing that branch migration occurred. (**d**) Restriction analysis of the time course for dissolution of MM-DHJS. The reactions were carried out with Sgs1 (1 nM), Top3 (1 nM), Rmi1 (10 nM) and RPA. Branch migration of the HJ past the SphI site is initially identical without and with Rmi1, as detected by the SphI-dependent B fragment (black arrows). Final dissolution products appear almost exclusively in reactions containing Rmi1 at later time points (white arrows). The contrast of this image was enhanced, compared to other images in this study, to allow visualization of the faint product DNA bands. (**e**) Kinetics of MM-DHJS dissolution, based on three experiments such as that shown in **d**. Error bars, s.e.m.

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), the migration would have restored the A–B and B–A DNA heterodimers. These DNA molecules are the building blocks of DHJS[20], and one is shown as a marker (both heterodimers have almost identical mobilities) in **Supplementary Figure 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, noncognate 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 noncognate type IA topoisomerases human topoisomerase IIIα 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 noncognate RecQ helicases and type IA topoisomerases (**Supplementary Fig. 4c**), indicating either that the weaker noncognate protein-protein interactions can be overcome at higher protein concentrations or that dissolution can occur in an uncoupled manner without a direct helicase-topoisomerase interaction[21]. Notably, very high concentrations of Top3 (360 nM) can support limited dissolution (<5%) after a 30-min 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 nonprocessive, random-walk nature of

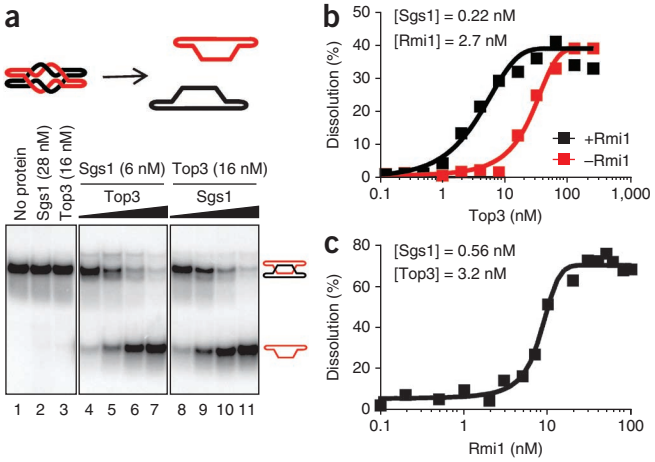

Figure 3 Rmi1 greatly stimulates dissolution of DNA that contains dHJs separated by two topological links and that mimics a nearly dissolved dHJ. (**a**) Sgs1 and Top3 can dissolve the oligonucleotide-based dHJ. The concentrations of Top3 in lanes 4–7 were 4, 8, 16 and 32 nM, and the Sgs1 concentrations 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. (**b**) Dissolution of the oligonucleotide-based dHJ is greatly stimulated by Rmi1 at low Top3 concentrations. Shown is a quantification of experiments such as those in **a**, with Top3 concentration plotted on a logarithmic scale. The reaction contained Sgs1 and, where indicated, Rmi1. (**c**) Stimulation of dissolution by Rmi1. Shown is a quantification of experiments such as those in **a**, with Rmi1 concentration plotted on a logarithmic scale.

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

Notably, 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 not fully dissolved, resulting in intermediates with more of an X-shaped structure that have reduced electrophoretic mobility[22]. 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 an HJ under topological constraint. This is expected, as any attempted migration of an 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, but not dissolution, had occurred 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**)—that is, 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. Because there are 165 bp 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 two DNA linkages total[19] (**Fig. 3a**). Thus, this substrate resembles a 'nearly dissolved' dHJ and can be used to study the last steps before 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 nanomolar range (**Fig. 3b**,**c** and **Supplementary Fig. 6a**,**b**). Our results with the yeast proteins parallel those for the human homologs[10–13]. 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 hemicatenane, 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 molecules[24]. This complex catenane is trapped in the wells of an agarose gel, but treatment with Topo II, which has a well-defined decatenation activity[24,25], released monomer DNA rings from the

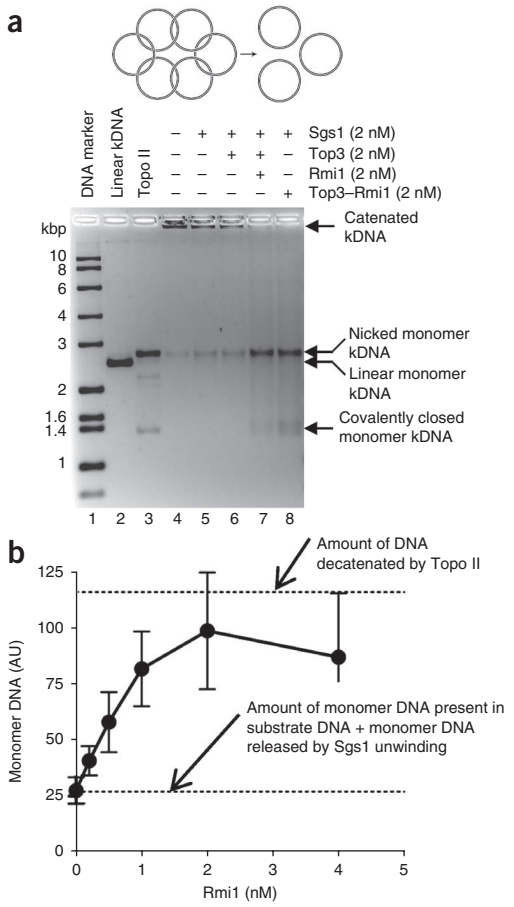

Figure 4 Rmi1 promotes decatenation of kinetoplast DNA (kDNA) by Sgs1 and Top3. (**a**) Decatenation reactions (lanes 4–8) were carried out with Sgs1, Top3, Rmi1 and Top3–Rmi1 heterodimer, as indicated, and RPA; kDNA was decatenated by *D. melanogaster* Topo II as a positive control (lane 3). The positions 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 (in arbitrary units, based on experiments as shown in **Supplementary Fig. 7a**) was plotted against Rmi1 concentration. The reactions contained RPA, Sgs1 (2 nM), Top3 (2 nM), and the indicated amount of Rmi1. Error bars, s.e.m., based on four 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 **a**), and the upper dashed line indicates the amount of monomer kDNA released by *D. melanogaster* Topo II.

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** and **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*[26].

Dissolution activity was first demonstrated *in vitro* with human BLM helicase and topoisomerase IIIα[6] using an oligonucleotide-based dHJ[19] and was later shown for the *Drosophila* homologs[9] using the larger DHJS[20]. 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 Top3 (ref. 4). We propose 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. These nearly converged HJs might represent the 'toxic intermediates' that affect chromosome segregation. Furthermore, an *rmi1* deletion closely phenocopies a *top3* deletion[8], indicating that Rmi1 is as essential as Top3 for decatenation *in vivo*. In agreement with the genetic results, we observed that, *in vitro*, Rmi1 is indispensible for dissolution when the concentrations of Top3 and Sgs1 are low (in the nanomolar range). Like yeast *sgs1* mutants[2], BLM-deficient human cells show a large increase in sister chromatid exchanges[27]. 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

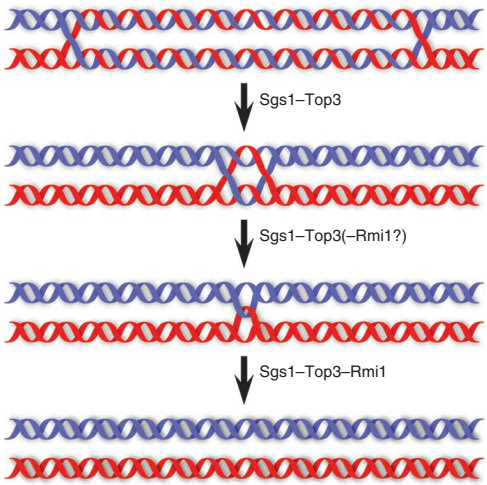

Figure 5 Model for function of Sgs1–Top3–Rmi1 complex in the dissolution of double Holliday junctions (dHJs). Sgs1 and Top3 catalyze convergent HJ migration. Rmi1 is likely an integral part of the Sgs1–Top3 complex, but its function is dispensable during this initial branch migration phase, although it may become important in later steps of this phase. Rmi1 stimulates dHJ dissolution when both junctions are in close proximity. Rmi1 subsequently stimulates the decatenation of a hemicatenane, the last anticipated intermediate of dHJ dissolution.

that involve nucleolytic cleavage by enzymes present in human and yeast cells, respectively, such as GEN1/Yen1, MUS81–EME1/Mus81–Mms4 or SLX1–SLX4/Slx1–Slx4, and that would in theory 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 human BLM, yeast Sgs1 or other homologs to disrupt the joint molecule intermediates (D-loop structures) that precede HJ formation[28]. 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 chromosomes[2]. The remaining events, which lead to crossovers, would be produced by one of the nucleolytic enzymes listed above, perhaps resolving only those residual recombination intermediates that have just one HJ owing to the loss of the other and that consequently cannot be dissolved by Sgs1–Top3–Rmi1. Individuals lacking the BLM helicase are highly predisposed to the development of a wide spectrum of cancers[29], demonstrating the importance of these proteins in processes that prevent genome rearrangements and instability.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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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.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE 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 described[14]. The preparation of Top3, Rmi1 and Top3–Rmi1 heterodimer is detailed in the **Supplementary Methods** and is summarized in **Supplementary Figure 8**. RPA, *E. coli* SSB, *E. coli* RecQ and human BLM were prepared as described[15,30–32]. Human Topo IIIα, *S. cerevisiae* Srs2 and *D. melanogaster* Topo II were generous gifts from P. Janscak (Univ. Zurich, Zurich, Switzerland), X. Veaute (Institute of Cellular and Molecular Radiation Biology, Fontenay aux Roses, France) and T. Hsieh (Duke Univ., Durham, North Carolina, USA), 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 μg 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 nucleotides). 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 incubation at 37 °C for 60 min. Unless indicated otherwise, the reaction products were then purified by phenol and phenol-chloroform extraction using Phase-Lock Gel (Eppendorf) and were precipitated by ethanol in the presence of linear polyacrylamide as a coprecipitant[33]. 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 before 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 μg ml^{-1} ethidium bromide, at ~4 V cm^{-1} for ~3 h. 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[10,28,34].

Decatenation of kinetoplast DNA. Reactions (20 μl) contained 25 mM Tris-acetate (pH 7.5), 1 mM magnesium acetate, 75 mM NaCl, 0.1 mM dithiothreitol, 1 mM phosphoenolpyruvate, 100 μg ml^{-1} bovine serum albumin (New England Biolabs), 80 U ml^{-1} pyruvate kinase (Sigma), 1 mM ATP, 5 ng μl^{-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 μl 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 $MgCl_2$, 1 mM ATP, 1 mM DTT, 0.1 mM EDTA, 30 μg ml^{-1} bovine serum albumin (New England Biolabs), 5 ng μl^{-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 μg ml^{-1} ethidium bromide, destained in TAE buffer for 1 h, and analyzed as described above.

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