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The resistance of DMC1 D-loops to dissociation may account for the DMC1 requirement in meiosis

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Abstract

The ubiquitously expressed Rad51 and the meiosis-specific Dmc1 recombinases promote the formation of strand invasion products (D-loops) between homologous molecules. Strand invasion products are processed by either the double strand break repair (DSBR) or synthesis-dependent strand annealing (SDSA) pathway. D-loops destined to being processed by SDSA need to dissociate producing noncrossovers (NCOs) and those destined for DSBR should resist dissociation to generate crossovers (COs). The mechanism that channels recombination intermediates into different HR pathways is unknown. Here we demonstrate that D-loops in a DMC1 driven reaction are substantially more resistant to dissociation by branch migration proteins such as RAD54, than those formed by RAD51. We propose that the intrinsic resistance to dissociation of DMC1 strand invasion intermediates may account for why DMC1 is essential to ensure the proper segregation of chromosomes in meiosis.

Keywords

Homologous recombination; branch migration; D-loop; DNA strand exchange; meiosis; RAD54

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AUTHOR CONTRIBUTIONS

D.V. B., R.J.P., A.V.M., and R.D.C.-O. conceived the general ideas for this study. All authors planned experiments and interpreted data. D.V. B., R.J.P., O.N.V., and O.M. M. performed experiments. A.V.M. and R.D.C.-O. wrote the manuscript and all authors provided editorial input.

In eukaryotes, homologous recombination (HR) performs important but different functions in the repair of DSBs in both mitosis and meiosis. In mitosis most DSBs are repaired through the non-homologous end joining pathway and those that are repaired by HR are mostly in the context of repairing from a sister chromatid often in a stalled replication fork 1,2. In this scenario the inter-chromosomal interactions involved should be transient, as genetic exchange between homologous (non-sister) chromosomes in mitosis may lead to loss of heterozygosity (LOH) 3. The opposite is true in meiosis. As far as we know DSBs are repaired generally by HR and stable interactions between non-sisters (homologs) are required for the homologous chromosome alignment and spindle assembly at metaphase I that are the hallmark of meiosis in that they ensure the proper segregation of the chromosome homologs 4–6.

The DNA breaks that initiate HR are produced as a result of DNA damage or generated by specialized meiosis-specific enzymes 3,7. The initial steps of HR in both mitotically and meiotically dividing cells involve processing of the DNA ends by exonucleases to generate 3′-ssDNA tails 8–10. Then, a specialized HR protein of the RecA family binds to this ssDNA and promotes invasion of the DNA ends into the homologous duplex DNA 11,12. As a result, joint molecules (D-loops) are formed that provide both a template and a primer for the DNA synthesis that is required for retrieving the information lost at the site of the break and for the consequent restoration of a contiguous DNA structure (Fig. 1). It is currently thought that the joint molecules continue down one of two pathways (Fig. 1). They can proceed through the DSBR mechanism that includes the formation of stable double Holliday junctions (DHJ) 13–18 (Fig. 1), which are later resolved by structure-specific endonucleases (Fig. 1, left) 3. Alternatively, the joint molecules dissociate leading to re-joining of the broken chromosome through the SDSA mechanism (Fig. 1, right) 3,13,19 or double-D-loop dissociation (DDD, not shown in Fig 1) 20. Whereas DSBR takes place primarily in meiosis, the SDSA pathway occurs in both mitotically and meiotically dividing cells 5. The DHJ intermediates formed in meiosis, that are ultimately resolved as COs, play a critical role in the proper segregation of chromosomes. These COs which involve chromosome-size exchanges of genetic information give rise to the genetic diversity that is characteristic of meiosis (Fig. 1). In contrast, the DNA recombinational repair that occurs during mitotic cell growth involves primarily unstable inter-chromosomal interactions and the formation of NCO recombinants products, which result in the exchange of limited genetic information (Fig. 1).

In most eukaryotes, there are two RecA homologous, Rad51 and Dmc1, that promote the search for homologous DNA sequences and DNA strand exchange that leads to formation of joint molecules 7,21. Rad51 and Dmc1 share 54% amino acid identity in humans and 45% amino acid identity in yeast. Whereas Rad51 acts in both meiotic recombination and during DNA repair in mitotically dividing cells; Dmc1 is expressed only during meiosis. In Saccharomyces cerevisiae, the rad51 mutants show severe deficiency in both mitotic DNA repair and meiotic recombination 6, but the dmc1 mutants are deficient only in meiotic recombination 22. Again, in S. cerevisiae, a physical analysis of meiotic joint molecules by 2D gel electrophoresis indicates that Dmc1 specifically promotes inter-homolog recombination 23. In mice, the *Rad51*$^{-/-}$ knockouts are embryonically lethal 24. Mouse

Dmc1$^{-/-}$ knockouts are viable, but are sterile and do not synapse their chromosomes 25; a hypomorphic mouse *Dmc1*^{mei11} allele (A272P) causes male-specific sterility 26.

In vitro, Rad51 and Dmc1 show similar biochemical activities; both proteins form helical nucleoprotein filaments on ssDNA and promote DNA strand exchange with homologous dsDNA, which results in the formation of DNA joint molecules (D-loops) 27,28. Immediately after completion of DNA strand exchange, Rad51 and Dmc1 likely remain associated with joint molecules protecting them against dissociation 29. Previously it was found that human helicase-like proteins RAD54 and BLM can promote dissociation of DNA joint molecules formed by human RAD51 20,30–32 through their DNA branch migration activity 33,34. It was suggested that by promoting D-loop dissociation RAD54 and BLM can channel recombination into the SDSA mechanism 20,35. Several lines of genetic evidence indicate that helicases such as orthologs of BLM, WRN, and Mph1 direct recombination intermediates away from COs and toward NCOs, and that the abrogation of these helicases greatly increase COs 14,36–38. The importance of this regulation in ensuring genomic stability is highlighted by the redundancy of these helicases, even though each on its own can have an important effect on the balance of COs versus NCOs. The regulation of COs products is of extreme importance for both mitotic and meiotic processes. In mitosis, COs result in LOH and chromosome rearrangement 2,37,39. Indeed, LOH is one of the most common genetic alterations observed in sporadic tumors, and it is also anobligate step in carcinogenesis in several familial cancer syndromes involving tumor suppressor genes 40. Equally important, during meiotic cell growth, COs stabilizes the temporal association between the homologous chromosomes necessary for their proper segregation.

Formation of D-loops marks an important bifurcation point in HR; their further processing through either the DSBR or SDSA pathway may lead to either CO or NCO recombinants. Even though considerable genetic data and molecular analyses of in vivo intermediates have been mustered in support of the view that the CO/NCO decision is made at least by the stage of D-loop formation, if not earlier 13,15, there are still no biochemical underpinnings for such a model. An important question is how the choice between these mechanisms is made. A possible obvious difference is that D-loops destined to being processed by SDSA need to dissociate and those destined for DSBR should be resistant to dissociation. Here we demonstrate that D-loops formed by human DMC1 but not RAD51 resist disruption by branch migration proteins. This difference in stability may reflect differences in the structure of DMC1 and RAD51 presynaptic filaments. To our knowledge our results are the first to reveal that the recombination intermediates catalyzed by RAD51 and DMC1 are biochemically distinguishable. Therefore, the high stability of joint molecules formed by Dmc1 may help to promote HR through the DSBR mechanism, leading to the formation of stable inter-homolog chromosome interactions that ultimately are resolved as COs.

RESULTS

Native D-loops formed by DMC1 resist dissociation by RAD54

We previously found that human RAD54 protein can dissociate D-loops through its DNA branch migration activity 20. RAD54 can dissociate “native” (non-deproteinized) D-loops with RAD51 bound to them, a substrate which mimics in vivo recombination intermediates.

However, native D-loops formed by RAD51 were sensitive to dissociation by RAD54 only after Ca^{2+} depletion 20, because Ca^{2+} stabilizes the RAD51–DNA filament preserving it in an active ATP-bound form 41. Here, we tested the ability of RAD54 to dissociate native D-loops formed by DMC1 (Fig. 2). D-loop formation was carried out in the reaction mixture containing Ca^{2+} that stimulates DNA strand exchange activity of DMC1 42. We found that in the presence of Ca^{2+} DMC1-bound D-loops were resistant to dissociation by RAD54 (Fig. 2b; 2c). We then tested the effect of Ca^{2+} depletion on the ability of RAD54 to dissociate D-loops. We found that, in contrast to D-loops formed by RAD51 (Fig. 2d; 2e), native D-loops formed by DMC1 resisted dissociation by RAD54 (Fig. 2b; 2c).

These data show an important difference in the behavior of the two human recombinases, RAD51 and its meiosis-specific homologue DMC1. Joint molecules formed by DMC1 are substantially more resistant to dissociation by RAD54 than those formed by RAD51. These data agree with a greater resistance of the DMC1 nucleoprotein complexes to disruption by BLM than that of the RAD51–ssDNA filament32.

D-loops formed by DMC1 in the presence of HOP2/MND1 resist dissociation by RAD54

The DNA strand exchange activity of both RAD51 and DMC1 is stimulated by the HOP2/MND1 heterodimer, which plays an important role in meiotic recombination 43,44. Here we tested the ability of RAD54 to dissociate D-loops formed by RAD51 and DMC1 in the presence of HOP2/MND1 (Fig. 3). Because HOP2/MND1 enables RAD51 and DMC1 to efficiently promote D-loop formation in the absence of Ca^{2+}, the reactions were performed in the presence of Mg^{2+}. Under our experimental conditions, both DMC1 and RAD51 form synaptic complexes (see below) of similar length (Supplementary Fig. 1) and showed comparable yields of D-loops (Fig. 3). We found that RAD54 rapidly dissolved D-loops formed by RAD51 in the presence of HOP2/MND1 (Fig. 3b; 3c, closed circles), even though HOP2/MND1 is known to increase equally well the stability of both RAD51 and DMC1 nucleoprotein filaments 45,46. In contrast, D-loops formed by DMC1 in the presence of HOP2/MND1 resisted RAD54 dissolution even after 30 minutes (Fig. 3b; 3c open circles).

We tested the effect of altering the RAD54 concentration on D-loop dissociation. At concentrations 90 nM and higher, RAD54 dissociated more than 70% of the RAD51-formed non-deproteinized D-loops (Fig. 3d, closed circles). In contrast, even at the highest concentration tested (1.4 μM) RAD54 failed to dissociate native D-loops formed by DMC1 protein in the presence of HOP2/MND1 (Fig. 3d, open circles).

The difference in the RAD54-mediated destabilization of native D-loops promoted by DMC1 and RAD51might be a direct consequence of the differential ability of RAD54 to physically interact with RAD51 but not DMC1. In order to test this hypothesis, we performed surface plasmon resonance experiments to show that there is efficient binding of RAD54 to both DMC1 and RAD51 nucleoprotein filaments (Supplementary Fig. 2, Panel a).

We also tested whether the relative insensitivity of the DMC1 nucleoprotein filaments to the branch migration activity of RAD54 might reflect an inhibitory effect of DMC1 on the hydrolysis of ATP promoted by RAD54, an activity essential for its ability to dissociate D-

loops 20. We analyzed the ATPase activity of RAD54 in the presence of DMC1 and RAD51. We observed that the level of ATP hydrolyzed by RAD54 increased in the presence of both DMC1 and RAD51, although at low protein concentrations RAD51 stimulated the RAD54 ATPase activity more strongly than DMC1 (Supplementary Fig. 2, Panel b). These results indicate that the difference in the stability observed for DMC1 versus RAD51 promoted D-loops is not a consequence of a less active RAD54 in the presence of DMC1.

We conclude that D-loops formed by DMC1 in the presence of HOP2/MND1 are more resistant for dissociation by RAD54 than D-loops formed by RAD51.

We used an independent approach to confirm the greater resistance of DMC1 nucleoprotein filaments to dissociation by RAD54. We used the synaptic complex protection assay 46,47 (Supplementary Fig. 3) in which an oligonucleotide spanning a restriction endonuclease site is used to form a homology-dependent ternary complex of three strands and a recombinase, thereby rendering the duplex resistant to cleavage by the restriction endonuclease. For D-loops formed by the RAD51–ssDNA filament, we observed a gradual increase of SspI-dependent DNA cleavage with an increase in RAD54 concentration (Supplementary Fig. 3d, lanes 10–16; 3e, closed circles). In contrast, for the D-loops produced by the DMC1–ssDNA filament, substantially smaller amounts of DNA cleavage is observed in the presence of RAD54 (Supplementary Fig. 3d, lanes 2–8; 3e, open circles).

We asked whether the observed difference in the ability of the D-loops formed by DMC1 and those formed by RAD51 to resist dissociation is specific for RAD54 or does it reflect a distinction in the intrinsic properties of the nucleoprotein complexes formed by RAD51 and DMC1. We then tested the ability of BLM to dissociate D-loops formed by RAD51 30–32 and DMC1 in the presence of HOP2/MND1. We found that BLM efficiently dissociated D-loops formed by RAD51–HOP2/MND1 but not by DMC1–HOP2/MND1 complex (Supplementary Fig. 4). These data indicate that the high resistance to dissociation may represent an intrinsic property of DMC1 D-loops.

The ability of RAD54B to disrupt D-loops was also examined. RAD54B failed to dissolve native D-loops produced by either RAD51 or DMC1, although it showed disrupting activity on deproteinized D-loops (data not shown).

Presynaptic filaments formed by RAD51 and DMC1 have different structures

The ability of RAD54 to dismantle D-loops formed by RAD51, but not ones produced by DMC1, may reflect differences in the structure of the nucleoprotein complexes formed by these recombinases. In order to address this possibility we probed the structure of DMC1 or RAD51 complexes formed on a 3′-tailed duplex by treating them with $KMnO_4$ (Supplementary Fig. 5). The patterns of modifications changed drastically when the RAD51 and DMC1 were added to DNA. The ssDNA oligonucleotide and ssDNA portion of the partial duplex became much more reactive as is evident from the increase in the intensity of the bands corresponding to thymine bases (Supplementary Fig. 5b, lanes 2, 3, 6 and 7 and corresponding densitograms in Supplementary Fig. 5c). The increase in the reactivity of the ssDNA has been previously observed for bacterial RecA protein 48,49. While an increase in the modification of the thymines was observed for both RAD51 and DMC1 complexes, an

important difference in the fine patterns of modification for these two proteins was clearly seen (Supplementary Fig. 5b, lane 2 vs lane 3 and lane 6 vs lane 7; 4c). For example, in ssDNA, the reactivity of the bases in the block T21–T26 gradually increases in 3′ to 5′ direction in the presence of RAD51, however, in the complex with DMC1 two bases, T22 and T25, are much more reactive compared to T21, T23, T24 and T26 (Supplementary Fig. 5b, lanes 2 and 3, Supplementary Fig. 5c, top panel). The difference in the patterns of modifications induced by the two recombinases is also evident through the rest of the ssDNA. This dramatic and consistent difference in the reactivity of the ssDNA was also observed when RAD51 and DMC1 formed complexes with the partial duplex (Supplementary Fig. 5b, lanes 6 and 7; Supplementary Fig. 5c, bottom panel). Similar experiments performed under conditions not involving ATP hydrolysis (in the presence AMPPNP–Mg^{++} and ATP–Ca^{++}) also showed similar differences in the DMC1 and RAD51 induced reactivity of thymines in the ssDNA of the partial duplex (data not shown).

The dramatic difference in unstacking of DNA bases and, consequently in the reactivity towards $KMnO_4$, reflects a dissimilarity in the structure of the nucleoprotein complexes formed by RAD51 and DMC1. Such dissimilarity may be responsible for the different ability of D-loops to resist dissociation by DNA translocases.

DISCUSSION

Both RAD51 and DMC1 are essential for HR during meiosis; but only RAD51 operates in mitotically dividing cells. Previous data indicate that RAD51 and DMC1 play different functions in meiosis, for example, and most importantly in the present context, Dmc1 specifically promotes inter-homolog recombination 6,23. Most likely the evolution of a meiosis-specific RecA homologue, Dmc1, reflects the need for a protein to fulfill this unique role.

Since, Rad51 and Dmc1 show similar properties in vitro, it has been so far unclear how these proteins differ in their functions. The in vivo activities of Rad51 and Dmc1 are regulated by accessory proteins. It is thought that distinct sets of accessory proteins may affect the functions of Dmc1 and Rad51 50. The activity of yeast Dmc1 is influenced by its interactions with Hop2/Mnd1, Mei5–Sae3, and Tid1/Rdh54 proteins 51–53. The activity of mammalian DMC1 is stimulated by HOP2/MND2 43,44,46 or RAD54B 28. Whereas activities of yeast or mammalian Rad51 are affected by interactions with Rad52, Rad54 and other proteins12,50. However, specific mechanisms that differentiate the functions of Dmc1 and Rad51 have yet to be elucidated. Moreover, at least some of the Dmc1 accessory proteins, e.g., Hop2/Mnd1 43,45, Rdh54 54, or RAD54B 28 are known to interact with Rad51 affecting its activity in a similar fashion to that of Dmc1.

Here we demonstrate that human DMC1 and RAD51 have intrinsically different properties. The joint molecules formed by DMC1 show much greater resistance to dissociation by two human branch migration proteins, RAD54 and BLM, than those formed by RAD51 (Fig. 2 and 3 and Supplementary Fig. 4). This property of joint molecules formed by DMC1 and RAD51 parallels a substantially greater resistance of the DMC1–ssDNA filament to disruption by BLM than that of the RAD51–ssDNA filament 32. Taken together our current

and previous data indicate that distinct structural features of the DMC1–DNA complexes are responsible for the resistance of joint molecules formed by DMC1 to dissociation by branch migration proteins. This conclusion is supported by chemical probing of the DMC1 and RAD51 nucleoprotein complexes (Supplementary Fig. 5) and by recent EM study showing a marked difference in the architecture of the DMC1 and RAD51 nucleoprotein filament 55.

While in this work we have used RAD54 and BLM as molecular tool to demonstrate intrinsic differences between DMC1 and RAD51 strand invasion intermediates, our data reinforce previous genetic proposals for a role of helicases and branch migration proteins in preventing excess recombination by down regulating COs in both mitosis and meiosis 14,36–38.

The high stability of DMC1-formed D-loops and their resistance to dissociation by dissolvases may have an important impact on HR in mammalian cells. In meiotic cells, the formation of intermediates required for stable interactions between chromosome homologs that are essential for accurate chromosome segregation result in COs. In contrast, formation of COs in mitotic cells may lead to LOH and account for some human disorders 39,40. Not surprisingly, specific mechanisms have evolved to ensure that in mitotically dividing cells COs occur much less frequently than in meiotic cells 2,3,5. Remarkably, coincident with the absence of Dmc1 in their genomes 56, flies and worms have developed alternative ways of stabilizing inter-homolog pairing interactions, in which synapsis of homologous chromosomes does not depend on the formation of strand invasion intermediates 57–59. Altogether, these facts might reflect the functional differences between DMC1 and RAD51 described in this paper. Thus, RAD51, absent regulation of its function by modulators or posttranslational modifications, forms D-loops that can be easily dissociated to enter the SDSA pathway. On the other hand, DMC1 forms the more stable D-loops required for its meiosis-specific role, through the DSBR pathway, a prerequisite in establishing the stable inter-homolog interactions (DHJ) that are required for the proper segregation of chromosome homologs in meiosis and that result in genetic diversity.

METHODS

Proteins and DNA

Human RAD54 60, BLM 30, RAD51 41, DMC1 43, and murine HOP2/MND1 43 were purified as described previously. Supercoiled pUC19 dsDNA was purified as described 41. All oligonucleotides (IDT, Inc.) used in this study (see Table 1) were purified, labeled, and stored as described previously 33. Tailed DNA substrates (oligonucleotides 209/199) were formed by the annealing of equimolar amounts of oligonucleotides followed by purification in native polyacrylamide gels. The supercoiled pUC19 dsDNA was purified by anion exchange (Qiagen) followed by CsCl banding.

Preparation of native D-loops in the presence of Ca^{2+} and their dissociation by RAD54

To form D-loops, ^{32}P-labeled tailed DNA (#209/#199; 30 nM, molecules) was preincubated with DMC1 (1 μM) or RAD51 (1 μM) protein in buffer containing 25 mM Tris-acetate, pH 7.5, 1 mM ATP, 1 mM magnesium acetate, 2 mM calcium chloride, 2 mM DTT, BSA (100

μg ml^{-1}), 20 mM phosphocreatine and creatine phosphokinase (30 units ml^{-1}) for 15 min at 37 °C. D-loop formation was initiated by addition of pUC19 scDNA (50 μM, nucleotides) followed by a 15-min incubation. When indicated, Ca^{2+} was depleted by the addition of 2 mM of EGTA followed by a 5-min incubation. D-loop dissociation was initiated by the addition of RAD54 (200 nM) and carried out for the indicated periods of time at 37 °C. The products of D-loop dissociation were deproteinized by adding 0.5 % (w/v) SDS and 1 mg ml^{-1} proteinase K for 10 min at 37 °C and analyzed by electrophoresis in 1 % (w/v) agarose gels. Gels were dried on DEAE paper and visualized and quantified using a Storm 840 PhosphorImager (GE Healthcare)

Preparation and dissociation of native non-deproteinized D-loops in the presence of HOP2/MND1

Tailed ^{32}P-labeled DNA (oligonucleotides 209/199, 14.3 nM molecules) was preincubated with DMC1 (1 μM) or RAD51 (1 μM) proteins in a 15 μl mixture containing 25 mM Tris-acetate, 25 mM NaCl, pH 7.5, 2.5 mM MgAc, 1 mM ATP, 2 mM DTT, 100 μg ml^{-1} BSA and 20 mM phosphocreatine and 30 U ml^{-1} creatine phosphokinase for 10 min at 37 °C. HOP2/MND1 (0.2 μM) were included in the reaction and incubated for an additional 10 min at 37°C, then D-loop formation was initiated by the addition of pUC19 supercoiled dsDNA (18 μM base pairs) followed by a 10-min incubation. Dissociation of protein-coated D-loops was initiated by addition of RAD54 (at indicated concentrations) and carried out for 9 min at 37 °C. Reactions were stopped by the addition of 0.5 % (w/v) SDS followed by deproteinization (1 mg ml^{-1} proteinase K) for 10 min at 37°C. The DNA products were analyzed by electrophoresis in 1 % (w/v) agarose gels. Gels were dried on DEAE paper; the DNA products were analyzed using a BAS 2500 Bio-imaging Analysis System (Fuji Medical System). Image quantification was carried out using FujiFilm Multi Gauge V3.0 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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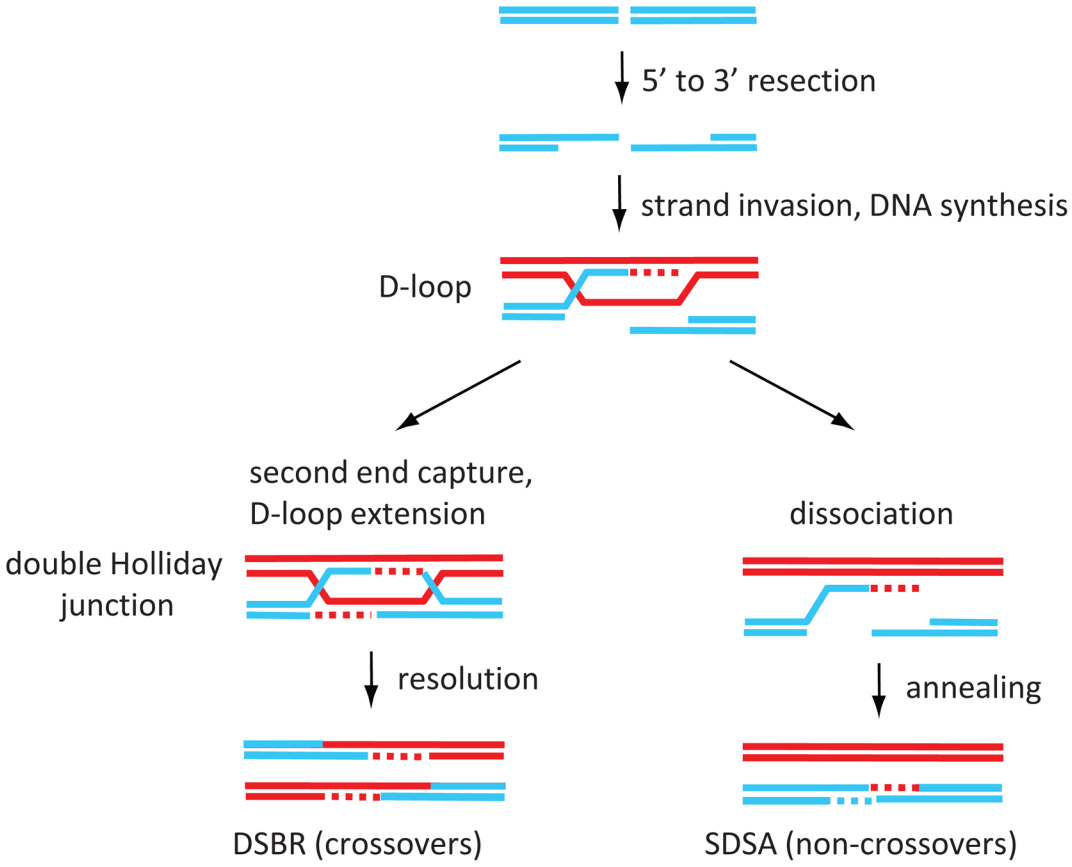

Fig. 1. Pathways of homologous recombination

Repair of DSB proceeds through the double-stranded break repair (DSBR) mechanism (left column), resulting in COs, more common in meiosis compared to mitosis; or the synthesis dependent strand annealing (SDSA) (right column) mechanism, resulting in NCOs. Red and blue represent DNA copies of two different homologous chromosomes.

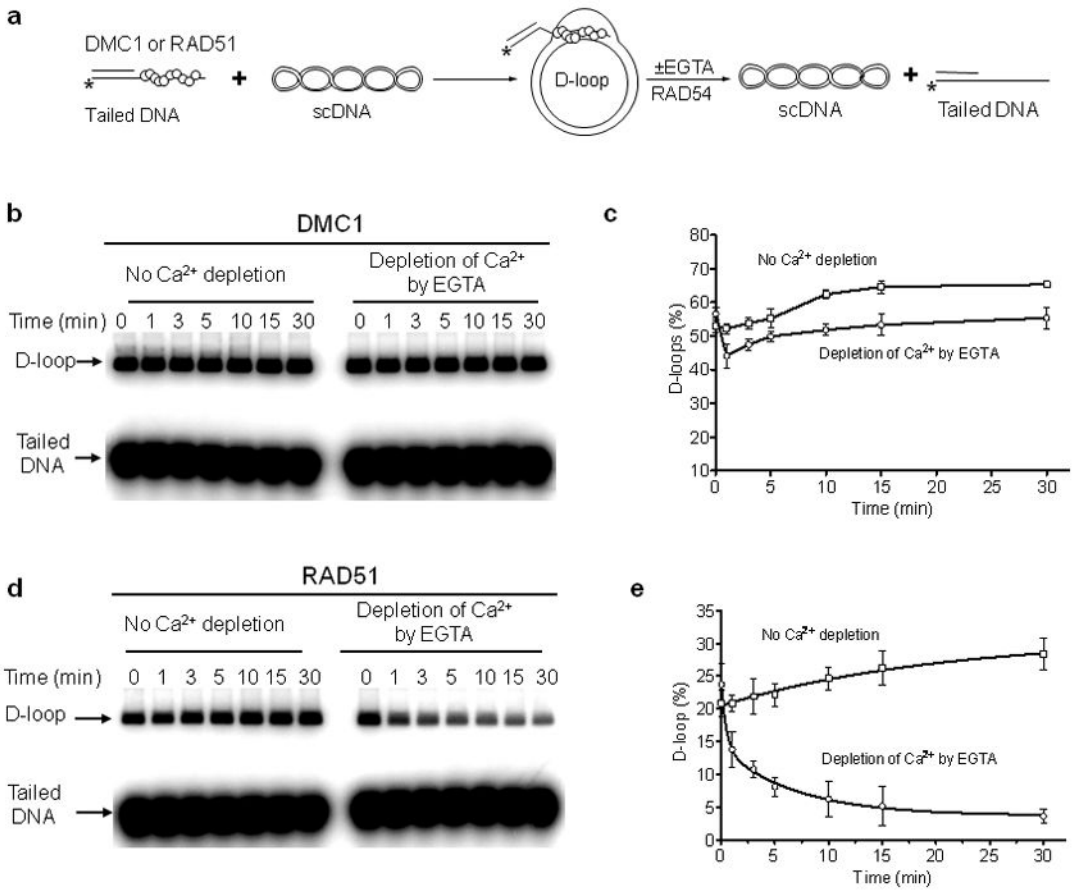

Fig. 2. RAD54 does not dissociate non-deproteinized joint molecules (D-loops) formed by DMC1

(a) The experimental approach. The asterisk indicates ^{32}P-label. (b) DMC1-formed D-loop dissociation by RAD54 were analyzed by gel-electrophoresis. The reaction was initiated by mixing non-deproteinized DMC1-generated D-loops (containing DMC1) with Rad54 in the presence of Ca^{2+} or after Ca^{2+} depletion by EGTA and carried out for the indicated periods of time. (c) Graphical representation of the data from (b). (d) Analysis of dissociation of non-deproteinized RAD51-formed D-loop by RAD54 by gel-electrophoresis. The reaction was performed either in the presence of Ca^{2+} or after Ca^{2+} depletion by EGTA. (e) The results from b, presented as a graph. Error bars indicate (standard error of the mean) S.E.M.

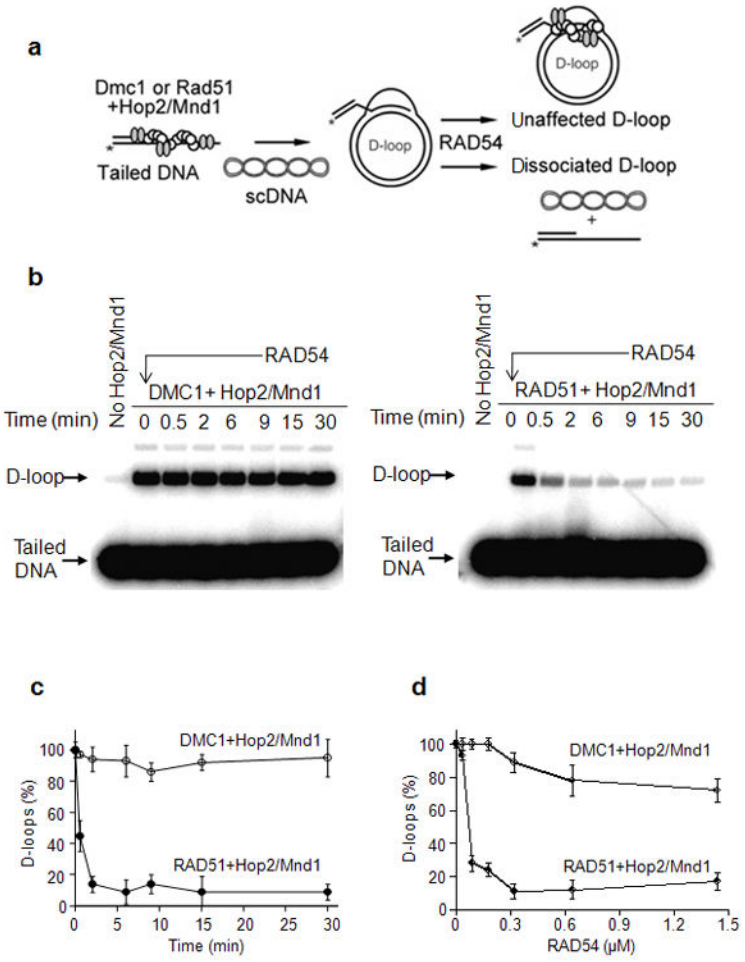

Fig. 3. RAD54 dissociates native D-loops formed by RAD51 but not DMC1 in presence of HOP2/MND1

(a) Experimental scheme. Asterisk denotes ^{32}P label. (b) The kinetics of D-loop dissociation was initiated by mixing the D-loops formed by DMC1 (1 μM) or RAD51 (1 μM) in the presence of HOP2/MND1 (0.2 μM) with RAD54 (0.32 μM). DNA products were analyzed by gel-electrophoresis. (c) The results from b are presented as a graph. (d) Effect of RAD54 concentration on D-loop dissociation. D-loops formed by DMC1 or RAD51 in the presence of HOP2/MND1 were mixed with RAD54 at the indicated concentrations. The results are shown as a graph. In c and d, 100% of the D-loop formation efficiency represents the yield of D-loop formation in the absence of RAD54. Error bars indicate S.E.M.

Table 1

Sequences of the oligonucleotides used in this study

N	Length, nt	Sequence (5′→3′)
199	36	CACTGCTAATAGCGTCCGGTAAGTAAAATGAGAATT
209	100	AATTCTCATTTTACTTACCGGACGCTATTAGCAGTGGGTGA GCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAA GGGCGACACGGAAATGTTG