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Genome integrity, repair and replication

During recombinase-mediated homology recognition RecQ helicases inhibit formation of toxic long-lived D-loops that could promote genomic instability

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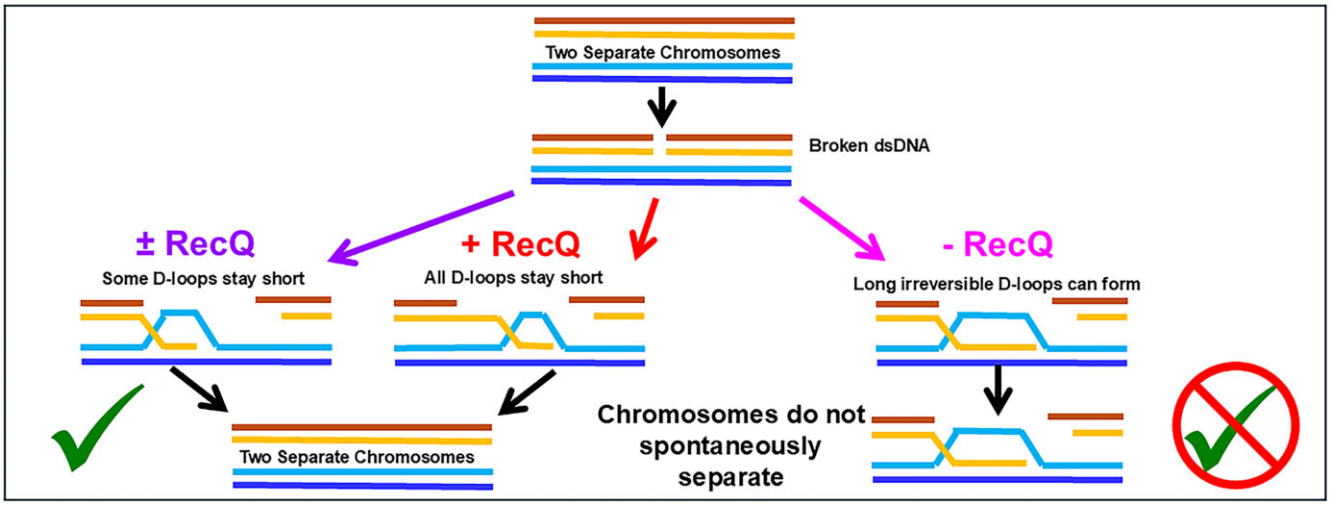
Abstract

Mutations in RecQ family helicases underlie human genetic disorders associated with genomic instability and cancer predisposition, but questions remain about how properly functioning RecQ reduces these deleterious effects. Importantly, some of the deleterious effects may result from incorrect repair of DNA double-strand breaks (DSBs) by recombinase proteins. Displacement loops (D-loops) are three-strand intermediates formed by recombinases during repair of DSB. RecQ helicases might enhance genome stability by disassembling incorrect recombinase-mediated D-loops formed between mismatched sequences and/or between short regions of accidental homology. We used bulk FRET and gel electrophoresis assays to probe the effects of RecQ family helicases in the context of ongoing recombinase-mediated D-loop formation. We found that RecQ does not differentially promote disassembly of short D-loops or D-loops that include mismatched base pairs. Thus, RecQ does not reduce genomic instability by discriminating against incorrect D-loops. In contrast, our results suggest that RecQ intervenes during D-loop formation to limit the length of recombinase-mediated D-loops. Without that intervention, D-loops can become so long that they do not spontaneously reverse. We suggest that RecQ prevents undesirable long-lived connections between chromosomes that could compromise chromosome metabolism and/or segregation and promote genomic instability.

Graphical abstract



Introduction

RecQ helicases are an important family of proteins that are conserved from bacteria to humans. *In vitro* experiments suggested that in bacteria, RecQ interacts with DNA structures formed by RecA-mediated homologous recombination [1]. Furthermore, each of the five human RecQ helicases plays critical roles in genome maintenance and stability, and all of them interact with Rad51, which is a RecA family protein [2]. Mutations in eukaryotic helicases of the RecQ family, BLM, Werner syndrome helicase (WRN), and RECQL4, are found in Bloom's, Werner's, and Rothmund–Thomson syndromes, respectively. All three syndromes are human genetic disorders associated with genomic instability and cancer predisposition [3]. Incorrect double-strand break (DSB) repair can cause chromosomal aberrations, genomic instability, and tumorigenesis [4]. DSB can be repaired by various mecha-

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nisms including homologous recombination. Importantly, human RecQ helicases are believed to affect the outcome of homologous recombination [5]. Thus, it is useful to study how RecQ proteins influence homologous recombination.

During homologous recombination, recombinase proteins form nucleoprotein filaments by binding to the two single-stranded DNA (ssDNA) tails that are formed after a DNA DSB [6, 7]. To correctly repair the DSB, each nucleoprotein filament searches the unbroken chromosome for a sequence region that is homologous to the invading ssDNA in the nucleoprotein filament [6, 7]. Recombinases probe the homology between the invading strand and a strand in the unbroken chromosome by attempting to establish Watson–Crick pairing between the strands [6, 7]. Most attempted pairings are quickly rejected because they fail a homology test [8, 9]; however, if a strand in the unbroken chromosome is sufficiently homologous to the invading strand, then the recombinase can form a D-loop that includes three DNA strands [6, 7]. Two DNA strands in the D-loop form a heteroduplex product in which the invading strand is base paired to the complementary strand in the unbroken chromosome. The third strand in the D-loop is the displaced DNA strand that was originally base paired to the complementary strand. D-loops are temporary scaffolds that align the 3′ end of the invading strand so that a polymerase can extend the invading strand past the break using the complementary strand as a template [6, 7].

Initially, the three strands in the D-loop are bound to the recombinase [6, 7]. Without ATP hydrolysis, recombinase-bound D-loops become irreversible once they extend over more than ∼20 bp [10]. Importantly, *in vivo* D-loops do not remain bound to recombinases. In bacteria, recombinase-free D-loops are created by ATP hydrolysis driven unbinding of RecA [11–15]. In eukaryotes, recombinase free D-loops are created by helper proteins that promote the unbinding of recombinases from D-loops [14, 16]. Recombinase-free D-loops as long as ∼160 bp reverse spontaneously [17, 18]; therefore, even without a helicase, recombinase-free D-loops shorter than ∼160 bp should not block chromosome separation.

In contrast, recombinase-free D-loops longer than ∼300 bp do not spontaneously reverse [11, 17]. Furthermore, invading strands *in vivo* can have lengths exceeding 300 nt [19]; however, *in vivo* implications of the irreversibility of long D-loops have not been widely considered. Importantly, long-lived D-loops could inhibit necessary chromosome separation [20] and could also compromise chromosome metabolism. Previous work suggests that some D-loops can be reversed by RecQ [1] or by the eukaryotic RecQ helicase BLM [21–23], but the effect of RecQ helicases on long D-loops formed by recombinases has not been studied.

Instead, much previous *in vitro* work focused on three-stranded "D-loop like" structures formed by annealing [24–26]. Those works showed that those "D-loop like" structures can be reversed by RecQ [24] and by eukaryotic helicases BLM and WRN [25, 26]. The observed reversal of short "D-loop like" structures by RecQ led to the proposal that *in vivo* RecQ reverses short incorrect D-loops formed between regions of accidental homology [24]. It has also been suggested that RecQ reverses incorrect D-loops that contain mismatches [27–30]. Despite these proposals regarding the function of RecQ, previous work has not studied how the length of D-loops or how the presence of mismatches in D-loops affects RecQ's interaction with those D-loops.

In this work, we performed experiments that probe how RecQ helicases interact with D-loops formed by RecA family protein-mediated homologous recombination. In the experiments, the invading strands in the nucleoprotein filaments included an N-nucleotide region that was either homologous or nearly homologous to the double-stranded DNA (dsDNA). During homologous recombination, we followed the D-loop population using FRET and a gel electrophoresis assay. In the first set of FRET experiments, the bacterial helicase RecQ or the eukaryotic helicase WRN was added after homologous recombination created a significant amount of D-loop product. We also compared the RecQ effect on those D-loops to the effect of RecQ on "D-loop like" structures and flapped dsDNAs. Interestingly, we found the RecQ discriminates against "D-loop like" structures that contain mismatches as well as flapped dsDNA that contains mismatches; however, neither RecQ nor WRN discriminate against recombination mediated D-loops that include mismatches. In addition, we studied the effect of RecQ helicase on D-loops of increasing length using both bulk FRET and a gel electrophoresis assay. Both assays indicated that if RecQ was present when D-loop formation began, then the formation of long irreversible D-loops was suppressed; however, both assays also suggest that RecQ cannot reverse D-loops longer than ∼300 bp after they have formed. In sum, our experiments indicate that RecQ does not have a strong influence on short D-loops or moderate length D-loops that contain mismatches, suggesting that RecQ does not enhance rejection of incorrect recombinase-mediated DSB repairs; however, RecQ acts during homologous recombination to block formation of toxic DSB repair products that could compromise chromosome metabolism and/or segregation and promote genomic instability.

Materials and methods

Reagents

RecA, T4 DNA ligase, and PhiX174 RfI were obtained from New England Biolabs (NEB); single-stranded binding protein (SSB) and recombinant RecQ helicase were obtained from Abcam; recombinant human WRN was obtained from BellBrook Labs; and pyruvate kinase and phosphoenolpyruvate were obtained from Sigma. All the oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Strand exchange assay

Strand exchange between ssDNA–RecA filaments and labeled dsDNA was detected *in vitro* using 53 nM 180 or 562 bp dsDNAs and 53 nM ssDNAs of varying lengths of homology from $N = 0$ (98-nt heterologous ssDNA) up to $N = 320$. RecA and nucleotides at a 1:3 ratio [typically 5.3 μM (in bases) = 53 nM 98-nt ssDNA and 1.8 μM RecA] were mixed in RecA buffer (70 mM Tris–HCl, 10 mM $MgCl_2$, and 5 mM dithiothreitol, pH 7.6) containing 1 mM ATP, a regeneration system with 10 U/ml of pyruvate kinase and 3 mM phosphoenolpyruvate, and 0.4 μM SSB. Filament formation proceeded at 37°C for 10 min.

Strand exchange followed by addition of helicase protein

The ssDNA/RecA filaments and dsDNA were placed in a quartz cuvette for initial strand exchange, and typically emission was followed for 800 s. An aliquot of recombinant *Escherichia coli* RecQ helicase or recombinant human WRN was

added, resulting in a final concentration of 40 nM; emission was followed for another 1000 s.

Preparation of dsDNA samples

To prepare 180 bp rhodamine and fluorescein labeled dsDNA (Supplementary Table S1), a 90 nt ssDNA containing an internal rhodamine label on base 58 (O1) from the 5′ end and a 5′ end phosphorylated oligonucleotide (82 bases) containing an internal fluorescein label (position 57 from the 3′ end (O2) were first annealed together. Another solution containing a 90-base 5′-end phosphorylated oligonucleotide (O3) and a 98-nt oligonucleotide (O4) were annealed to obtain an unlabeled dsDNA fragment. These two dsDNA fragments (labeled and unlabeled) were annealed and ligated overnight at 16°C with T4 DNA ligase in ligase reaction buffer [50 mM Tris, 10 mM $MgCl_2$, 1 mM ATP, and 10 mM dithiothreitol, pH 7.5 (NEB)]. The 180-bp labeled dsDNA product was purified by running a 3% agarose gel in TBE (Tris/Borate/EDTA) buffer for 3 h (6 V/cm). The 180 bp band was visualized with a midrange UV transilluminator, cut out, and extracted from agarose using a Nucleospin kit (Machery and Nagel, Bethlehem, PA). The concentration was measured with a photometer at 260 nm.

For the preparation of 562-bp dsDNA labeled with rhodamine and fluorescein (Supplementary Table S2), complementary oligonucleotides were initially annealed: O5 and O6, O7 and O8, O9 and O10, O11 and O12, O13 and O14, and O15 and O16. The annealed dsDNA fragments: first and second, third and fourth, and fifth and sixth were ligated overnight at 16°C with T4 DNA ligase in ligase reaction buffer yielding ∼180 bp partial products. After purification and concentration of the products using Millipore YM100 filters, 180 bp fragments containing O9 + O10 and O11 + O12 and fragments with O13 + O14 and O15 + O16 were ligated overnight, and the resulting ∼380 bp fragment was purified. This fragment was finally ligated to the ∼180 fragment (O5 + O6 plus O7 + O8), yielding 562 bp labeled dsDNA.

Constructs used in the D-loop like experiments were prepared by annealing the corresponding oligonucleotides O17, O18, O19 (or O20 for four mismatches) (Supplementary Table S3) starting at 90°C and then cooling back to 40°C at 1°C per step with 1-min equilibration at each step. Similarly, constructs used for flapped DNA experiments were prepared by annealing three oligonucleotides (Supplementary Table S3): O21, O22, and O23 (or O24 for four mismatches). The products were checked by gel electrophoresis. Additional D-loop like constructs and flapped dsDNA were prepared with oligonucleotides in Supplementary Tables S4 and S5.

The oligonucleotide sequences used for the ssDNA–RecA filaments are listed in Supplementary Tables S6–S12.

Fluorescence measurements

The fluorescent signal of the strand exchange reactions resulting when the ssDNA/RecA filament and dsDNA mixture was placed in a quartz cuvette was measured using FluorEssence spectroscopy software and the automated FluoroMax spectrofluorometer (Horiba, Edison, NJ). Emission of the fluorescein label in counts per second was read using 493 nm excitation wavelength and a 2 nm slit and detected at 518 nm with a 2 nm slit. The reaction was run for 30 min, and emission measurements were collected every 1 s with an integration time of 0.5 s. Temperature was kept constant at 37°C.

Gel electrophoresis experiments

The 444-nt ssDNA sample (Supplementary Table S12) was initially incubated with 0.5 mM dTTP and 0.5 mM fluorescein-labeled dUTP and terminal transferase for 3′ end labeling. After purification with Millipore YM100 filters, filament formation was performed in 1 mM ATP, a regeneration system containing 10 U/ml of pyruvate kinase and 3 mM phosphoenolpyruvate, and SSB (Abcam) in RecA buffer (70 mM Tris–HCl, 10 mM $MgCl_2$, and 5 mM dithiothreitol, pH 7.6) at 37°C for 15 min. After filament formation, dsDNA PhiX174 supercoiled was added and incubated for 30 min. RecQ was added either after strand exchange proceeded for 800 s or at the beginning of strand exchange. After strand exchange took place, the reaction products were deproteinized with SDS and proteinase K, and blue gel loading dye was finally added before loading each well. All the aliquots were run on 1% agarose gel for 2 h at 80 V and 4°C using Tris-acetate EDTA buffer and scanned at 488 nm on a Sapphire Biomolecular Imager (Azure Biosystems). In order to identify the position of the unlabeled dsDNA PhiX174 bands, the gel was finally transferred into a 1:10 000 ethidium bromide (10 mg/ml) solution for 20 min and scanned at 302 nm using a gel documentation system Azure c200.

Results

RecQ has a larger effect on sequence-matched D-loops than on D-loops containing mismatches

To monitor the effect of RecQ helicases on D-loops formed by RecA family protein homologous recombination, we created nucleoprotein filaments by allowing RecA to bind to the invading ssDNA. As illustrated in Fig. 1Ai, the invading strand included 98 nt. The 79 nt at the 3′ end were homologous to the central region in a 180 bp dsDNA. The 19 bases at the 5′ end of the invading ssDNA were heterologous to the dsDNA and were included to increase the stability of the filaments [31]. The complementary strand in the dsDNA was labeled with rhodamine, and the displaced strand was labeled with fluorescein. The 180 bp heteroduplex dsDNA (Supplementary Table S1) formed by the complementary and displaced strands was perfectly sequence-matched. When the nucleoprotein filament was combined with the dsDNA, the nucleoprotein filament formed D-loops. *In vivo*, after a DSB, the recombinase-mediated D-loops formed by the invading strands are flanked by homoduplex dsDNA tails in which the displaced and complementary strands remain base-paired. Thus, in our experiments the recombinase-mediated D-loops were also flanked by homoduplex tails (Fig. 1Aii).

To monitor the D-loop population, we measured the emission of the fluorescein label. Initially, the fluorescein emission was quenched by rhodamine due to base pairing between the displaced and complementary strands. The fluorescein emission increased when D-loop formation separated the displaced strand from the complementary strand (Fig. Aii). ATP hydrolysis can drive unbinding of RecA from the DNA, creating a protein free three strand structure (Fig. 1Aiii) [11–15]. That protein-free structure can spontaneously reverse without a helicase, or a helicase could promote reversal. In either case, the result would be a RecA-free invading ssDNA and a RecA-free dsDNA in which the displaced and complementary strands are again base paired along their entire lengths (Fig. 1Aiv). Free RecA can bind to the RecA-free invading strand, forming a

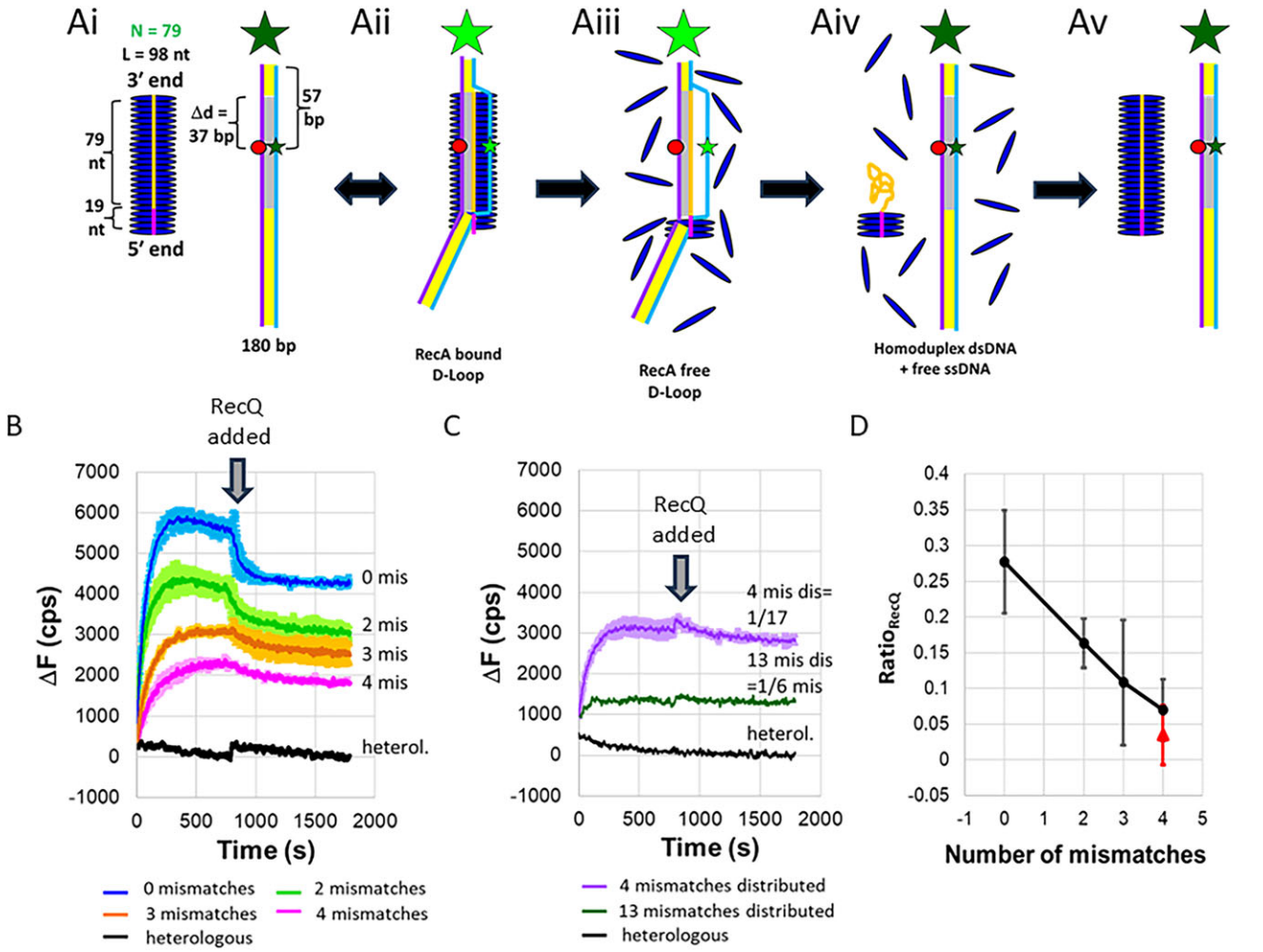

Figure 1. Effect of RecQ on D-loops formed by homologous recombination by RecA for matched or mismatched invading strands. (**A**) The nucleoprotein filament is formed by combining RecA (blue ovals) and a 98 nt ssDNA (Supplementary Table S6). The orange line indicates the 79 nt sequence region at the 3′ end of the ssDNA that is homologous to the dsDNA. The magenta line at the 5′ end indicates the 19 bases that are heterologous to the dsDNA. The purple and light blue lines indicate the complementary and displaced strands in the 180-bp dsDNA (Supplementary Table S1), respectively. The base pairing in the homologous region is indicated in gray, whereas the base pairing in the flanking heterologous tails is indicated in yellow. The red circle indicates the rhodamine label. Dark green and bright green stars indicate quenched fluorescein and unquenched fluorescein, respectively. (i) Initial conditions. (ii) Strand exchange forms a D-loop in which the complementary and invading strands are base-paired, and the displaced strand is not base-paired. The fluorescein label is not quenched. (iii) ATP hydrolysis drives unbinding of RecA, forming a RecA free three-strand product in which the fluorescein is not quenched. (iv) The heteroduplex reverses with or without the help of RecQ, restoring the homoduplex dsDNA in which the fluorescein is quenched. (v) A new nucleoprotein filament can form on the invading strand. (**B**) ΔF (calculated as the difference between each emission value and the heterologous emission at 1800 s) versus time curves for a completely heterologous invading strand, an invading strand with 79 contiguous homologous bases at the 3′ end, or an invading strand in which the 79 nt at the 3′ end include mismatches (mis) positioned 36 nt from the 3′ end: two grouped mismatches (two mis-grouped), three grouped mismatches (three mis-grouped), and four grouped mismatches (four mis-grouped). No RecQ was present initially. The arrow indicates the time when RecQ was added. The curves represent averages of at least two independent runs. (**C**) ΔF versus time curves for a completely heterologous invading strand or an invading strand with single mismatches evenly distributed (mis dis) along the 79-nt invading strand: four mismatches distributed (four mis dis), which is one mismatch every 17 bp, and 13 mismatches distributed (13 mis dis), which is 1 mismatch every 6 bp. (**D**) $\text{Ratio}_{\text{RecQ}}$, the fractional decrease in emission that occurs in the first 250 s after adding RecQ as a function of the number of mismatches (see text for description). The black curve shows the results for the grouped mismatches [curves shown in panel (B)]. The red triangle shows the result for four distributed mismatches [purple curve in panel (C)].

new nucleoprotein filament (Fig. 1Av). That filament can perform strand exchange with RecA-free dsDNA in which the displaced and complementary strands are base paired (Fig. 1Aii); therefore, the cycle can continue indefinitely. Thus, after some time our bulk samples can include a mixture of the structures shown in Fig. 1A.

The blue curve in Fig. 1B shows ΔF, the change in fluorescein emission, as a function of time for a strand exchange reaction in which the 79 nt at the 3′ end of the invading strand were perfectly sequence-matched to the complementary strand. For the first 800 s, D-loop formation took place without RecQ. After 800 s, RecQ was added. The addition of RecQ significantly lowered the emission, suggesting that RecQ reduced the D-loop population.

The black curves in Fig. 1B and C show the results for a heterologous filament (Supplementary Table S3). Those results indicate that when filaments are heterologous, we do not detect D-loop formation. Furthermore, though there is a small abrupt jump in emission just after the addition of RecQ, the ΔF versus time slope after RecQ addition is the same as the

slope before the jump, showing that there is no significant dsDNA unwinding due to 40 nM RecQ. In contrast, at higher RecQ concentrations RecQ can unwind blunt ended dsDNA [32], resulting in a large increase in emission that continues for hundreds of seconds (Supplementary Fig. S1). We performed additional controls to follow the complete strand exchange curve (Supplementary Fig. S1), and we checked for the effect of 40 nM RecQ on spontaneous strand exchange products that result from the interaction between ssDNA and dsDNA in the absence of RecA (Supplementary Fig. S1). In sum, Fig. 1B and C and Supplementary Fig. S1 show that under our *in vitro* conditions, DNA RecQ unwinding starting from a dsDNA end did not reach the fluorophores that are 57–58 bp from the nearest dsDNA end; therefore, in Fig. 1, the decrease in emission following RecQ addition represents a decrease in D-loop population rather than RecQ unwinding of dsDNA from the ends.

In vivo, the 5′ side of the invading strand is flanked by dsDNA, but the constructs in Fig. 1 did not include dsDNA on the 5′ side of the invading strand. Importantly, we found that the presence of such a dsDNA tail does not influence the effect of RecQ (Supplementary Fig. S2). Furthermore, the constructs in Fig. 1 also included heterologous 5′ ssDNA tails that would not be present during correct DSB repair *in vivo*, but RecQ has a similar effect on invading strands without heterologous tails (Supplementary Fig. S2). Thus, the results in Fig. 1 also correspond to the results for a completely homologous invading strand with a dsDNA tail at the 5′ end, which is the configuration of invading strands *in vivo*. Finally, we note that under our experimental conditions, the effect of RecQ saturates at a RecQ concentration of ∼40 nM, and the effect of RecQ becomes small below 10 nM (Supplementary Fig. S3), consistent with the previously observed RecQ concentration dependence of dsDNA unwinding [33]. Thus, the rest of the experiments in this work were done at 40 nM RecQ, suggesting that the results shown are insensitive to small variations in RecQ concentration.

The results shown so far are compatible with RecQ acting on RecA-bound D-loops or RecA-free D-loops, so we pursued additional experiments to obtain more information on the structure acted on by RecQ. Without ATP hydrolysis, RecA cannot form RecA-free D-loops, and RecA-free D-loops do not form in the presence of both ATP and ATPγS [34]; therefore, to test whether RecQ acts on RecA-bound D-loops, we performed experiments in 1 mM ATP and 1 mM ATPγS.

There are two reasons that RecQ could have no effect on D-loops in ATP and ATPγS: (i) RecQ cannot unwind dsDNA in a solution containing both ATP and ATPγS; (ii) RecQ cannot act on RecA-bound D-loops. To eliminate the first possibility, we showed that RecQ can unwind dsDNA in the presence of 1 mM ATP and 1 mM ATPγS (Supplementary Fig. S4). In contrast, in the presence of both ATP and ATPγS after 800 s of D-loop formation, the addition of RecQ has no effect on the D-loop (Supplementary Fig. S4), even though RecQ had a large effect when only ATP was present (Fig. 1). In sum, Fig. 1 and Supplementary Fig. S4 suggest that RecQ acts on protein-free D-loops but not on D-loops bound to RecA family proteins.

So far, we have considered interactions involving fully homologous invading strands, but we also considered interactions involving invading strands that contain mismatches. The yellow, orange, and red curves in Fig. 1B show results when the 79 nt invading strand included 2, 3, or 4 mismatches that are grouped together. Results for periodically spaced single mismatches are shown in Fig. 1C. Both Fig. 1B and C show that mismatches reduced D-loop formation before RecQ was added. Thus, consistent with previous work [9, 12, 35–37], RecA-mediated homologous recombination provides some discrimination against mismatched D-loops but does not completely block formation of mismatched D-loops.

Mismatches also reduce $\Delta\Delta F_{\mathrm{RecQ}}$, the change in emission during the first 250 s after RecQ was added; however, since mismatches also reduce D-loop formation, the fractional reduction in D-loop population might still increase with the number of mismatches. Thus, to measure the influence of mismatches on RecQ function, we calculated $\mathrm{Ratio}_{\mathrm{RecQ}}$. $\mathrm{Ratio}_{\mathrm{RecQ}}$ is the ratio of 1- $\Delta\Delta F_{\mathrm{RecQ}}/\Delta F_{750}$, where ΔF_{750} is the ΔF value just before the addition of RecQ. A $\mathrm{Ratio}_{\mathrm{RecQ}}$ that increases with the number of mismatches would indicate that RecQ preferentially reverses mismatched D-loops. Importantly, Fig. 1 suggests that $\mathrm{Ratio}_{\mathrm{RecQ}}$ is larger for sequence-matched D-loops than for D-loops that contain mismatches. This result also holds for fluorophores in other positions in the invading strand (Supplementary Fig. S5). In sum, RecQ does not preferentially reverse mismatched D-loops, suggesting that RecQ does not reduce genomic instability by discriminating against incorrect D-loops that include mismatches.

WRN also has a larger effect on sequence-matched D-loops than on D-loops containing mismatches

We performed additional experiments similar to those shown in Fig. 1, except that RecQ was replaced by WRN (Fig. 2). Since WRN is a eukaryotic protein, we first created D-loops using Dmc1, which is a eukaryotic recombinase. Adding WRN after strand exchange proceeded for 800 s had no effect on the D-loops formed by Dmc1 (Fig. 2A). Unlike RecA, Dmc1 can stably bind to dsDNA [14, 16] and does not unbind from D-loops after strand exchange [22]; therefore, Dmc1 does not create protein-free D-loops. Thus, it is possible that like RecQ, WRN only acts on protein-free D-loops. To test whether WRN can act on protein-free D-loops under our *in vitro* conditions, we formed D-loops using RecA in 1 mM ATP (Fig. 2B). We then added WRN after 800 s and observed that WRN reduced the D-loop population, suggesting that WRN can reverse protein-free D-loops; however, it does not reverse protein-bound D-loops. Furthermore, like RecQ, WRN reduced mismatched D-loops less than sequence-matched D-loops (Fig. 2B). Additional controls for WRN are shown in Supplementary Fig. S6.

Previous work has shown that eukaryotic helicases can disrupt nucleoprotein filaments formed on ssDNA by recombinase proteins [22, 38–40]. Thus, the observed reduction in D-loop populations could be due to the disruption of filaments by RecQ and/or the reversal of D-loops by RecQ. If the observed reduction was dominantly due to disruption of filaments, then the effect of RecQ on mismatched ssDNA–RecA filaments should be similar to the effect on sequence-matched filaments; however, $\mathrm{Ratio}_{\mathrm{RecQ}}$ was larger for sequence-matched filaments than for mismatched filaments, suggesting that filament disruption does not dominate the RecQ effects shown in Figs 1 and 2. Furthermore, if the effect of RecQ on D-loops was dominantly due to filament disruption, then an increase in ssDNA–RecA filament concentration should reduce the effect of RecQ; however, the reduction in D-loop population was insensitive to the concentration

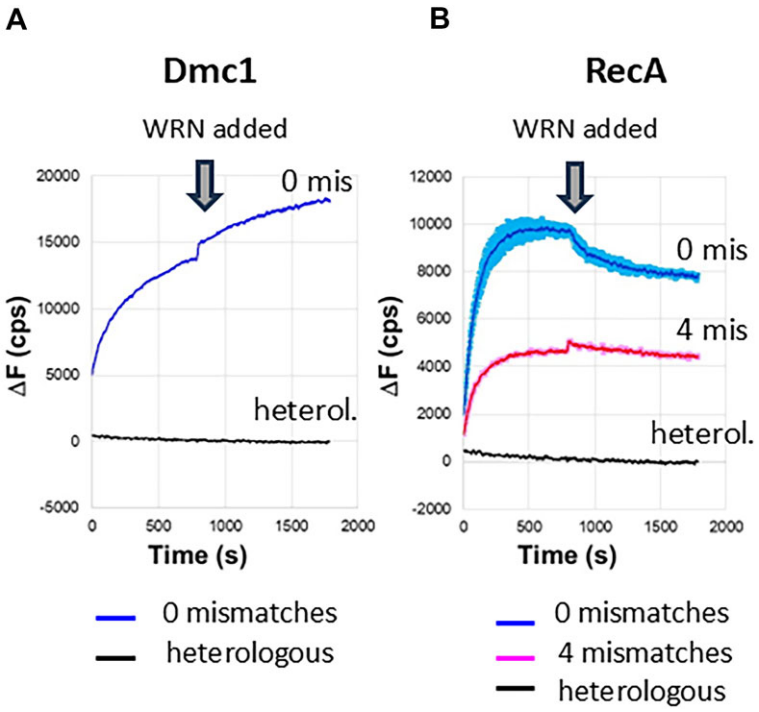

Figure 2. Effect of WRN on D-loops formed by homologous recombination for matched or mismatched invading strands. (**A**) ΔF versus time curves for homologous recombination performed by DMC1, which is a eukaryotic homolog of RecA. WRN was added at 800 s, as indicated by the arrow. The results for a completely heterologous invading strand and for an invading strand with 79 contiguous homologous bases at the 3′ end (Supplementary Table S6) are shown. Schematics are the same as in Fig. 1A and B. (**B**) Same as A except the homologous recombination was performed by RecA. The result for a filament with four grouped mismatches is also shown. The conditions are the same as those for Fig. 1B, except in Fig. 1 RecQ helicase was used. The curves represent averages of at least two independent runs. The lighter regions surrounding the curves indicate the range for the runs.

of ssDNA–RecA filaments (Supplementary Fig. S7), suggesting that filament disruption is not the dominant mechanism through which RecQ influences D-loops. In sum, the mismatch dependence and filament concentration dependence of the effect of RecQ on D-loops suggest that RecQ induced disruption of ssDNA–RecA filaments is not the dominant source of the observed reduction in D-loop population due to addition of RecQ.

For "D-loop like" structures or flapped dsDNA, RecQ unwinds mismatched structures more effectively than sequence-matched structures

RecQ could have less influence on mismatched D-loops if RecQ unwinds mismatched dsDNA less effectively than sequence-matched dsDNA. To test this possibility, we performed two sets of experiments on RecQ interactions with mismatched dsDNA that was not created by homologous recombination. In the first set of experiments, we considered "D-loop like" structures that are formed without a recombinase since the effect of RecQ on such structures has been studied previously [24–26]. We prepared a "D-loop like" structure by annealing two ssDNAs that included homologous regions at each end (Fig. 3A). The two ssDNAs combined to form dsDNA tails that flank a heterologous bubble in which the two ssDNA strands are not base paired. One of the ssDNA strands was labeled with rhodamine (Fig. 3Ai and Ci). We then added the fluorescein-labeled ssDNA that can bind to the rhodamine-labeled ssDNA in the bubble region (Fig. 3Aii and Cii). This third strand can create a three-strand structure in which the fluorescein- and rhodamine-labeled strands are base-paired within the heterologous bubble in the dsDNA. That three-strand structure in the heterologous bubble is flanked by dsDNA tails, so the overall structure is "D-loop like" and protein free.

In such three-strand "D-loop like" structures, proximity of the fluorophores in the base-paired strands decreased fluorescein emission (Fig. 3Aii and 3Cii). As a result, the initial emission from the "D-loop like" structures is low. In our experiments, we measured the initial low emission for 50 s and then added RecQ. If RecQ separates the third strand from its base pairing partner, the distance between fluorescein and rhodamine labels will become larger, and the fluorescein emission will increase (Fig. 3Aiii and Ciii). Thus, we detect RecQ unwinding of the dsDNA within the bubble by measuring the increase in fluorescein emission with time after addition of RecQ.

Previous work has shown that the presence of ssDNA tails can affect the efficiency of RecQ [1, 41]; therefore, we considered constructs with either a 5′ ssDNA tail or a 3′ ssDNA tail (Fig. 3A and C). The blue curves in Fig. 3B and D show the result when the third ssDNA strand contained no mismatches. The red curve shows the results when the third strand contained four separate single mismatches positioned 8, 16, 34, and 42 nt from the 5′ end of the fluorescein-labeled ssDNA. The curves clearly show that for "D-loop like" structures RecQ unwinds mismatched dsDNA more effectively than sequence-matched dsDNA. In sum, consistent with previous results, 3′ ssDNA tails promote RecQ activity >5′ ssDNA tails, but in either case mismatches increase the efficiency of RecQ unwinding (Fig. 3B and D).

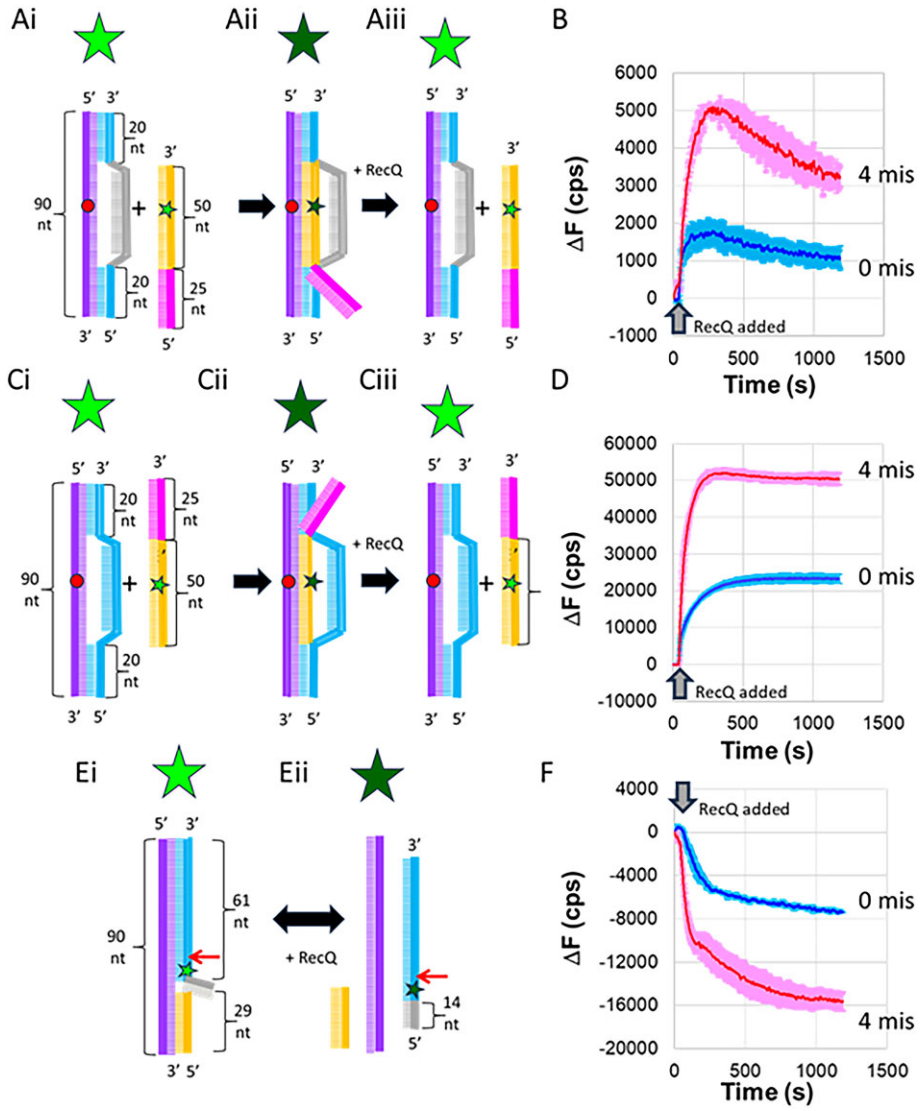

Figure 3 Effect of RecQ on D-loop-like structures and flapped DNAs A. dsDNA is formed by combining two 90-nucleotide ssDNAs (Supplementary Table S3). (**A**) (i) The two ssDNAs include 50 mismatched bases flanked on both sides by 20-bp matched dsDNA. The mismatched bases in the blue strand are shown in gray. The annealed ssDNA forms a dsDNA with a 50-bp ssDNA bubble. One of the strands has a rhodamine label (red circle) in the bubble. That strand can base pair with the 50 sequence-matched bases (orange line) at the 3′ end of a fluorescein (green star) labeled ssDNA that also includes a 25-nt 5′ tail (magenta line). (ii) When the ssDNA base pairs with the rhodamine labeled strand, fluorescein emission is quenched. (iii) Adding RecQ increases fluorescein emission if the helicase unwinds and separates the rhodamine- and fluorescein-labeled strands. (**B**) ΔF versus time curves when the 50 nt in the ssDNA are completely sequence matched (0 mis) or contain four mismatches (4 mis) for the structure shown in panel (A). The arrows indicate the time when RecQ was added. (**C**) Same as A, but the ssDNA has a 25-nt heterologous tail at the 3′ end. (**D**) ΔF versus time curves when the 50 nt in the ssDNA are completely sequence-matched (0 mis) or contain four mismatches (4 mis) for the structure shown in panel (C). The arrows indicate the time when RecQ was added. (**E**) (i) Flapped dsDNA where fluorescein label is bright if strands are annealed. The light blue strand includes a 14-nt 5′ tail shown in gray that is heterologous to the purple strand. The red arrow indicates the position of the mismatch in the structure where the mismatch is near the fluorophore. (ii) If RecQ unwinds and separates the fluorescein-labeled strand, emission decreases in the fluorescein-labeled ssDNA product. We cannot detect whether the orange strand separates, but previous work suggests that it would [1]. (**F**) ΔF versus time curves when the fluorescein-labeled strand is sequence matched (0 mis) or contains four mismatches (4 mis) in a flapped DNA. For B, D, and F, the curves represent averages of at least two independent runs. The lighter regions surrounding the curves indicate the ranges for the runs.

To further probe the effect of RecQ on mismatched dsDNA, we performed a second set of experiments using dsDNA with a 5′ flapped tail (Fig. 3E). This construct is similar to a replication fork, which is a natural substrate for RecQ helicases [42]. In these experiments, one of the strands had a fluorescein label on an internal base. Emission from this internal fluorescein is larger when the labeled strand is base-paired at the beginning of the experiment (Fig. 3Ei) than when the labeled strand is not base-paired once RecQ unwinds and separates the dsDNA (Fig. 3Ei). Thus, RecQ unwinding and separation of the labeled dsDNA decreases fluorescein emission (Fig. 3F). The blue curve shows fluorescein emission versus time when the dsDNA is sequence-matched, and the red curve shows the result when it contains four mismatches, which are 54–57 nt from the 5′ end of the 90-nt oligonucleotide. Similar curves were obtained when the mismatches were more distant from the flapped ssDNA tail (19–21 nt from the 5′ end of the 90-nt oligonucleotide) (Supplementary Fig. S8). The curves show that the change in emission due to adding RecQ was larger for the mismatched case than for the sequence-matched

Table 1. Comparison results for D-loops formed by homologous recombination, D-loop like structures, and DNA flapped DNAs

Property	D-loop from homologous recombination	D-loop like structures	Flapped DNA
Mismatches enhance the effect of RecQ	No	Yes	Yes
Position of mismatch with respect to fluorophore matters	No	No	No
RecQ unwinds beyond D-loop into the homoduplex dsDNA tail	No	Yes	Not applicable

case. Thus, the unwinding and separation of flapped DNAs by RecQ was enhanced by mismatches (Fig. 3). This enhancement is consistent with previous single-molecule results on DNA hairpins containing a few mismatches [43]. In sum, RecQ unwinding is not inhibited by mismatches in dsDNA since mismatches in dsDNA enhance the effect of RecQ on "D-loop like" structures and flapped dsDNA. Thus, the diminished effect of RecQ and WRN on mismatched D-loops cannot be attributed to a difficulty in unwinding mismatched dsDNA.

Of course, the heteroduplex dsDNA within a recombinase-free D-loop almost certainly has a different structure than flapped dsDNA or the dsDNA in "D-loop-like structures." Additional evidence for structural differences between D-loops and "D-loop like" structures is provided by the influence of ssDNA tails on RecQ. Such tails influence the effect of RecQ on "D-loop like" structures (Fig. 3A–D), but not the effect of RecQ on D-loops (Supplementary Fig. S2). Furthermore, RecQ easily opens the dsDNA tails in "D-loop like" structures but does not affect the dsDNA tails in D-loops (Supplementary Fig. S9). Table 1 lists the effects of RecQ on D-loops, "D-loop like" structures, and flapped dsDNA. In sum, the effect of RecQ on "D-loop like" structures is quite different than the effect of RecQ on D-loops. Thus, experimental observations of the influence of RecQ on "D-loop like" structures may not reflect the influence of RecQ on recombinase-mediated D-loops *in vivo*.

RecQ unwinding of D-loops decreases with N for $N > 82$ nt

In Figs 1 and 2, the heteroduplex product included ~80 sequence-matched bp ($N \sim 80$) after strand exchange with a 180-bp dsDNA construct. To probe whether the influence of RecQ depends on the number of sequence-matched bp in the heteroduplex product, we compared results for different N (Fig. 4A). Figure 4B shows emission versus time curves analogous to those in Fig. 1, but Fig. 4B includes results for $N = 82$, 100, and 140. $N = 82$ showed the largest fractional change in emission, and the effect decreased with increasing N. Results for $N = 320$ are shown in Fig. 4D, which shows that $\text{Ratio}_{\text{RecQ}}$ for $N = 320$ is even lower than for $N = 140$. Interestingly, like the results for $N = 82$ (Supplementary Fig. S2), the results for $N = 320$ (Fig. 4C) show that the effect of RecQ was not strongly influenced by ssDNA heterologous tails flanking the homologous region in the invading strand (Fig. 4D). In sum, Fig. 4 suggests that when RecQ is added after a significant D-loop population has formed, the effect of RecQ on D-loops decreases with increasing N for $N \geq 82$.

If RecQ is included when strand exchange starts, RecQ enhances D-loop reversal for $N \geq 82$, but not $N = 50$

In vivo, RecQ may be present at the time that D-loops begin to form, so we also did experiments in which RecQ was added when nucleoprotein filaments with $N = 50$, 82, 140, or 320 were combined with the dsDNA (Fig. 5). The blue curves show the emission as a function of time when RecQ was added initially to the nucleoprotein filament and dsDNA, whereas the green curves show emission for the filament and dsDNA in the absence of RecQ. With RecQ, for all N shown in Fig. 5, emission reached equilibrium within ~100 s.

For $N = 50$, RecQ did not significantly affect the emission curves, suggesting RecQ has little influence on D-loops when $N = 50$. Furthermore, for $N \geq 82$, the initial slopes of the emission curves are not affected by RecQ. The early slopes are dominated by formation of D-loops, so at least at early times D-loop formation is not affected by RecQ. In contrast, for $N \geq 82$ the curves without RecQ reached equilibrium values much later than the curves with RecQ, and the equilibrium values reached without RecQ were significantly higher than those reached with RecQ. The differences between the emission versus time curves with and without RecQ are consistent with RecQ enhancing D-loop reversal when $N \geq 82$. For $N = 82$, we also did experiments with RecA added initially and mismatches in the invading strand. For all the mismatched strands, the initial presence of RecQ reduced the time required to reach equilibrium and suppressed the emission value at equilibrium (Supplementary Fig. S10). When fewer than four mismatches were present, RecQ also significantly suppressed the emission at equilibrium. In sum, RecQ present when D-loop formation begins increases the reversal rate for D-loops when $N \geq 82$ but does not significantly affect reversal rates for D-loops when $N = 50$.

Finally, we performed gel electrophoresis experiments to probe the influence of RecQ on D-loops. In those experiments, we detected D-loops by measuring emission due to fluorescein labels. Previous work has shown that though D-loops shorter than ~160 bp reverse very rapidly, D-loops longer than ~300 bp are very long lived [11, 17]. Consistent with the FRET results shown in Fig. 4, the gel electrophoresis experiments show that adding RecQ after 800 s of D-loop formation is the same as D-loop formation without RecQ (Supplementary Fig. S11). In contrast, consistent with the FRET results shown in Fig. 5, if RecQ is present when D-loop formation starts, there is negligible emission at the position corresponding to the D-loop formed by RecA (Supplementary Fig. S11). In sum, the results in Figs 4–5 and Supplementary Fig. S11 suggest that RecQ does not efficiently reverse long-lived D-loops after they have formed, but that RecQ does block formation of long-lived D-loops.

Discussion

In vivo mutations in RecQ family helicases are linked to genetic diseases and cancer. Importantly, some genomic rearrangements are caused by incorrect repair of double-strand breaks by RecA family proteins. Thus, it has been proposed

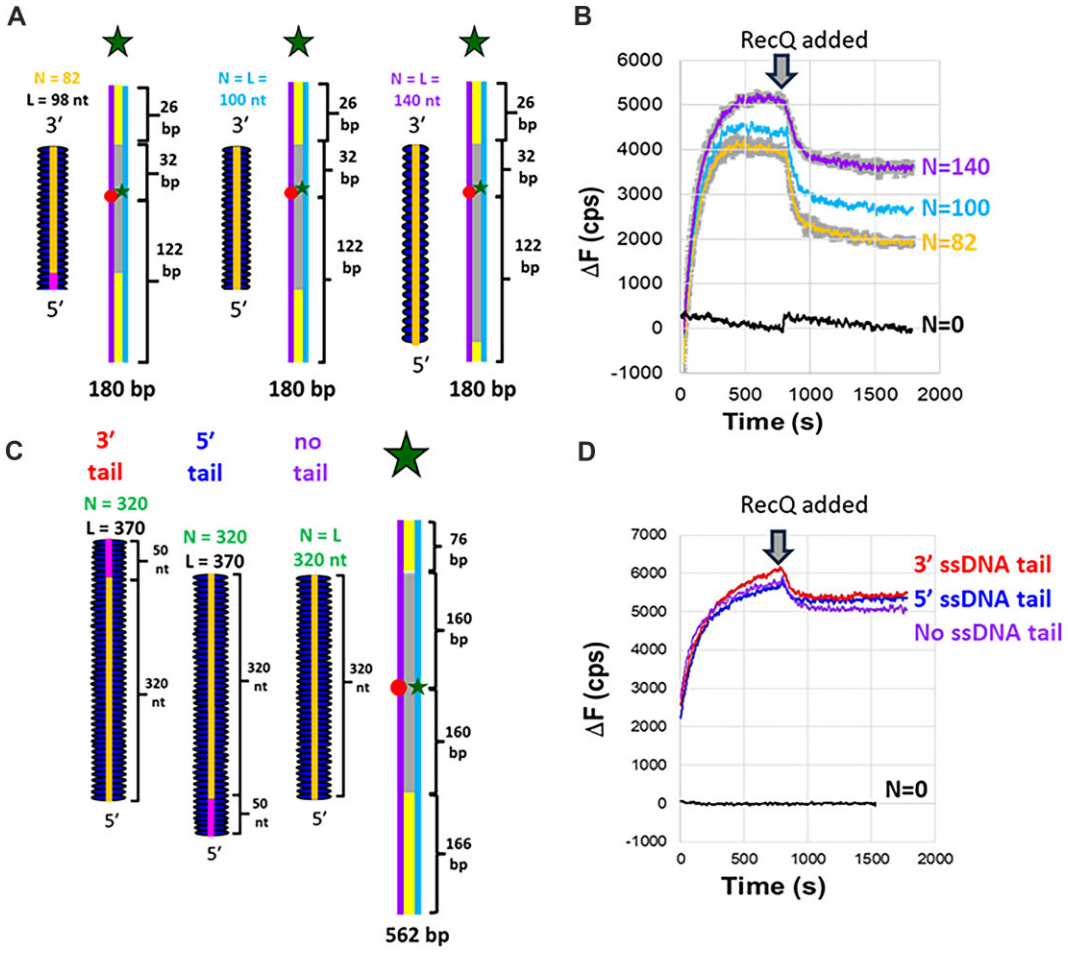

Figure 4. Effect of RecQ helicases on D-loops formed by homologous recombination for different *N* values. (**A**) Schematic of interactions between a 180 bp dsDNA (Supplementary Table S1) labeled with rhodamine (red circle) and fluorescein (green star) and filaments with homologous length $N = 82$, 100, and 140 (Supplementary Table S7) and total length $L = 98$, 100, and 140. (**B**) ΔF versus time curves for a completely heterologous invading strand and invading strands with $N = 82$, $N = 100$, or $N = 140$. The gray arrow indicates the time when the RecQ was added. The curves represent averages of at least two independent runs. The lighter regions surrounding the curves indicate the ranges for the runs. (**C**) Schematic of the interaction between a 562-bp dsDNA labeled with rhodamine (red circle) and fluorescein (green star) (Supplementary Table S2) and filaments with $N = 320$ (Supplementary Table S7). One filament included only 320 nt, but the other two filaments were 370 nt long and included 50 heterologous nt at either the 3′ or 5′ end of the invading strand. (**D**) ΔF versus time curves for filaments with $N = 320$ with or without heterologous tails and for a heterologous filament (Supplementary Table S6).

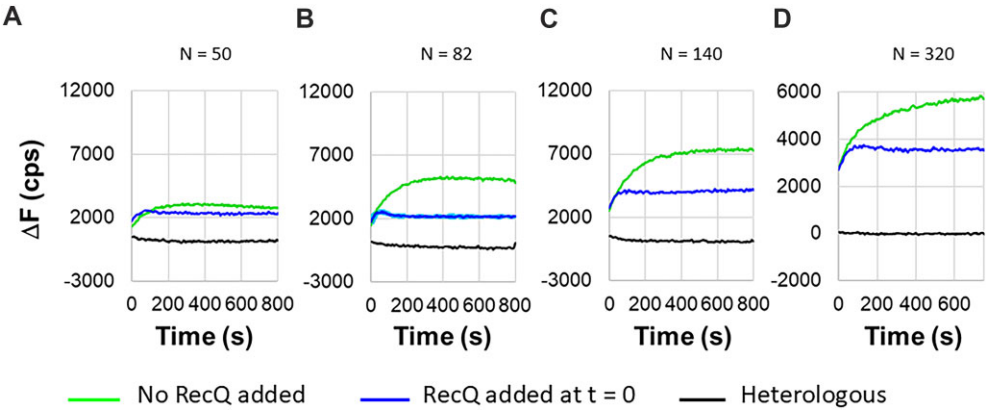

Figure 5. Effect of RecQ helicase on D-loops formed by homologous recombination for different *N* values. (**A**) ΔF versus time curves for a completely heterologous invading strand or a filament with $N = 50$. Strand exchange curve without RecQ or with RecQ added initially. (**B**) Same as A but for $N = 82$. (**C**) Same as B except for $N = 140$. (**D**) Same as C except for $N = 320$. Schematics are shown in Fig. 4.

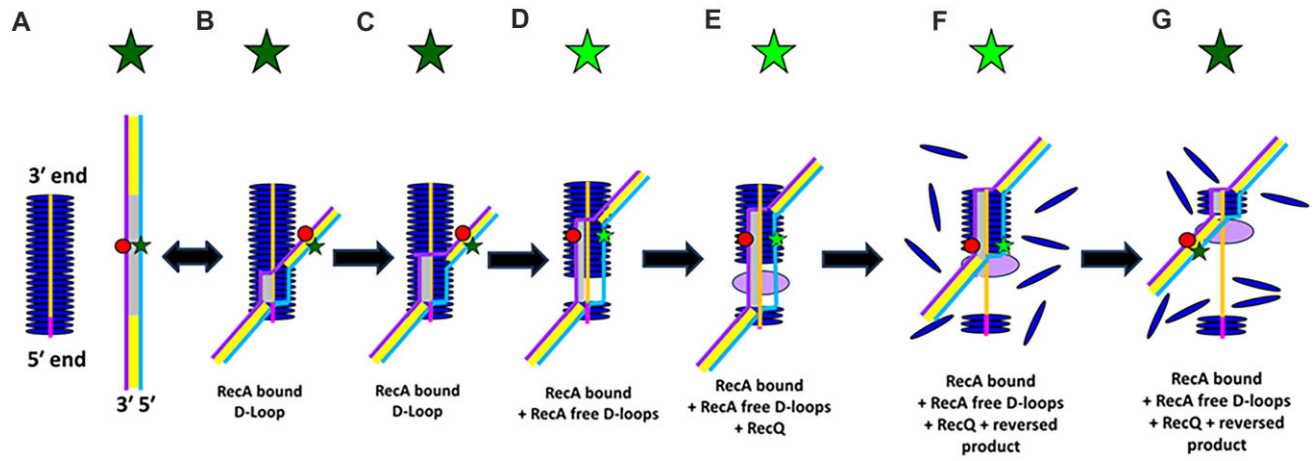

Figure 6. RecQ could block formation of long strand exchange products (**A**) initial invading strand nucleoprotein filament and fluorophore-labeled dsDNA as in Fig. 1A. (**B**) RecA-bound D-loop established at the 5′ end of the invading strand. (**C**) RecA-bound D-loop extends toward the 3′ end. (**D**) RecA begins to unbind at the 5′ end, creating a protein-free three-strand product. (**E**) RecQ binds in the RecA-free region. (**F**) RecQ begins to reverse the RecA free product at the 5′ end, but the D-loop has finally reached the 3′ end. (**G**) RecQ continues to shorten the D-loop.

that RecQ helicases discriminate against incorrect attempted repairs of double-strand breaks by recombinases. To study the roles of RecQ during recombinase-mediated homologous recombination, we studied the effect of RecQ on D-loops formed by recombinases.

Some DSB repair errors are associated with D-loops that join short regions of accidental homology. Thus, RecQ helicases might reduce incorrect DSB repairs by preferentially reversing short D-loops [24]. Other DSB repair errors create D-loops that include mismatches in the heteroduplex; therefore, RecQ helicases could reduce DSB repair errors by preferentially reversing mismatched D-loops [27–30]. Our experiments indicate that RecQ does not discriminate against D-loops that include ≤50 bp or mismatched D-loops, suggesting that RecQ does not discriminate against incorrect D-loops. In contrast, Fig. 3 shows that RecQ discriminates against "D-loop" like structures or flapped dsDNA that contain mismatches. Thus, our work suggests that RecQ unwinds mismatched DNA forks more effectively than sequence-matched forks. In sum, though RecQ may enhance the accuracy of DNA replication, our results suggest that RecQ helicases do not increase the accuracy of DSB repair that occurs via homologous recombination since most incorrect D-loops *in vivo* involve either mismatches or short regions of accidental homology.

We then considered other possible roles for RecQ during homologous recombination. Though 300 nt invading strands are present *in vivo* [19], both single molecule [11] and bulk [17] experiments indicated that protein-free D-loops longer than ∼300 bp do not spontaneously reverse. Since D-loops include three DNA strands from two different chromosomes, we probed whether RecQ might inhibit formation of long-lived D-loops that could compromise chromosome metabolism and/or segregation and promote genomic instability.

We first tested whether RecQ can reverse 320 bp D-loops after they have been formed by RecA-mediated homologous recombination. Both FRET results (Fig. 4) and gel electrophoresis results (Supplementary Fig. S11) suggest that RecQ cannot reverse long D-loops after they have formed.

We then considered how homologous recombination is influenced by RecQ when RecQ is present during D-loop formation. Fig. 5 suggests that without RecQ, D-loop formation does not reach equilibrium within 800 s. In contrast, when RecQ is present, D-loops reach equilibrium within ∼100 s. Furthermore, for $N = 50$, the equilibrium value with and without RecQ is similar, suggesting that RecQ does not play a strong role in reversing 50 bp D-loops. In contrast, for $N = 82$, 140, and 320, the equilibrium values with RecQ are much lower than the equilibrium values without RecQ. These results suggest that RecQ blocks formation of long D-loops that do not reverse spontaneously and cannot be reversed by RecQ after they have formed. Gel electrophoresis assays provide additional support for the conclusion that RecQ reverses D-loops before they become so long that they are irreversible (Supplementary Fig. S11). Importantly, RecQ (Supplementary Fig. S4) and WRN (Fig. 2) do not act on D-loops that remain bound to recombinase proteins. Thus, we propose that RecQ acts on the protein-free regions of D-loops before the D-loop reaches the 3′ end of the invading strand, as we describe in the following.

We propose that short D-loops are so short-lived that they usually reverse before RecQ can bind to the RecA-free region on the 5′ side of the filament; therefore, RecQ would not play an important role in reversing short D-loops (Fig. 5). In contrast, longer invading strands may allow ATP hydrolysis to create RecA-free regions on the 5′ side of the D-loop that last long enough for RecQ to bind to the RecA-free region, as illustrated in Fig. 6. Once RecQ binds to the RecA-free region of the D-loop, it can reverse part of the D-loop on the 5′ side of the invading strand before the D-loop reaches the 3′ end of the invading strand (Fig. 6). Thus, for invading strands with $N \geq 82$, RecQ prevents D-loops from extending over all N contiguous homologous bases in the invading strand. As a result, RecQ keeps D-loops shorter than ∼160 nt, even if N is >300 nt. Since D-loops <160 nt spontaneously reverse [17], such a mechanism could explain how RecQ could effectively block formation of long-lived D-loops even though RecQ cannot reverse long-lived D-loops once they have formed.

In sum, our results suggest that RecQ does not play a strong role in rejecting incorrect D-loops that include mismatches or are formed by short regions of accidental homology; however, interactions between RecQ and protein-free regions of

D-loops may play a significant role in preventing the formation of long irreversible D-loops that would otherwise block chromosome separation.

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Author contributions: Claudia Danilowicz: conceptualization, investigation, supervision, visualization, and writing. Athalia Meron: investigation. Mara Prentiss: conceptualization, investigation, supervision, visualization, formal analysis, funding acquisition, project administration, and writing.

Supplementary data

Supplementary data is available at NAR online.

Conflict of interest

None declared.

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Data availability

All data are included in the article and in Supplementary Material.

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