Regulation of Homologous Recombination between Divergent DNA Sequences by Mismatch Repair Proteins

Regulatie van homologe recombinatie
tussen divergente DNA moleculen door mismatch herstel eiwitten

PROEFSCHRIFT
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Chapter 1

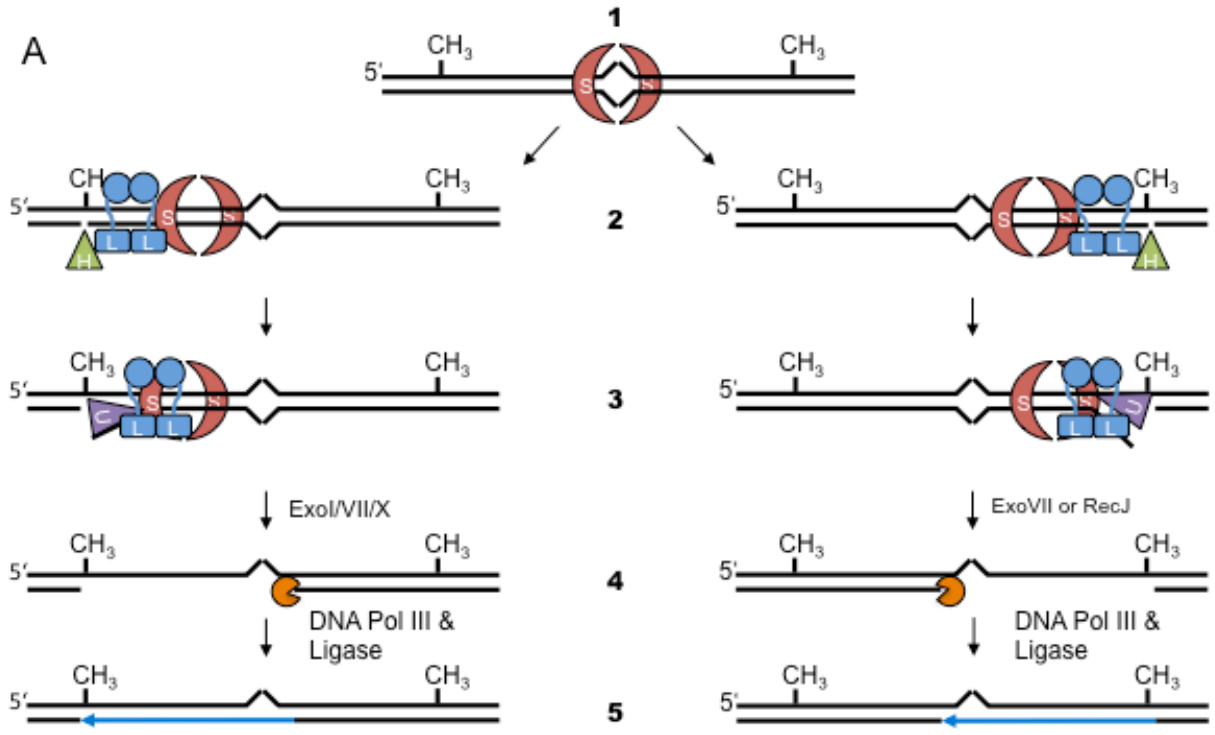
INTRODUCTION

Genomes of living organisms, from unicellular bacteria to multicellular human, are threatened by a plethora of DNA lesions. It is estimated that there are ~100,000 DNA lesions inflicted in a single cell on a daily basis (Lindahl, 1993). Both endogenous and exogenous agents induce the formation of these lesions in the genome. Internally, other than replication errors that generate DNA mismatches, reactive oxygen species, alkylating agents and spontaneous hydrolysis damage DNA. These structural alterations include oxidation, methylation, deamination, depurination and depyrimidation of DNA bases. External agents such as ultraviolet radiation, high-frequency radiations like X-rays and γ-rays, natural and synthetic toxins, can all damage DNA by changing its structure. These agents mediate the formation of intra- and interstrand crosslinks, of bulky adducts as well as of single- and double-strand breaks (DSBs) in the DNA. To ensure proper operation of DNA transactions, which are important for cellular survival, a variety of DNA-repair pathways act on these DNA lesions to preserve the integrity of the genome (Hoeijmakers 2001, 2009; Friedberg 2003; Garinis et al., 2008). These DNA-repair pathways include DNA mismatch repair (MMR; Jiricny 2013), base excision repair (BER; Goosen and Moolenaar, 2008), nucleotide excision repair (NER; Goosen and Moolenaar, 2008), non-homologous end joining (NHEJ; Shuman and Glickman, 2007) and homologous recombination (HR; Kowalczykowski et al., 1994-1; Heyer et al., 2010). Among the various types of DNA lesions, DSBs are particularly toxic. The failure to correctly process DSBs result in genome instability and has been associated with cancer predisposition, immune deficiency and infertility (Jackson and Bartek, 2009; McKinnon, 2009). Two prominent pathways that repair DSB are the error-prone NHEJ and the largely error-free HR, which uses the additional copies of homologous DNA sequences, present during the S and G2 phases of the cell cycle in eukaryotes, as repair template (Takata et al., 1998). However, DNA sequences of sister chromatids and homologous chromosomes are not always identical. Recombination between such DNA partners (hereafter referred to as homeologous recombination) generates mismatches within the heteroduplex region of the strand-exchange products. Similarly, recombination between repetitive DNA in eukaryotic genomes generates mismatches as well (George and Alani, 2012). These mismatches in turn act as DNA substrates for the activation of the MMR pathway. Depending on which strand within the heteroduplex is targeted by MMR proteins, mismatches result in gene conversion or simply restoration (Surtees et al., 2004). This could occur either during strand exchange or after the formation of crossover and non-crossover recombination products. MMR proteins additionally modulate HR to prevent recombination between divergent sequences. How the *Escherichia coli* MMR proteins impose homeologous antirecombination is the main question addressed in this thesis.

Escherichia coli DNA Mismatch Repair

DNA MMR increases the fidelity of replication by detecting and replacing misincorporated nucleotides with correct complementary nucleotides (Iyer et al. 2006; Jiricny, 2013). The MMR system of *E. coli* involves MutS (mismatch detector), MutL (mediator), MutH (GATC endonuclease), UvrD (3'-5' helicase), exonuclease I (3'-5'),

exonuclease X (3'-5'), exonuclease VII (3'-5' and 5'-3'), RecJ exonuclease (5'-3'), DNA polymerase III and DNA ligase to complete the repair of a DNA mismatch (Figure 1A). MutS exploits local distortions in base-pairing and base-stacking to kink the DNA and form a stable complex with a DNA mismatch (Lamers et al., 2000; Obmolova et al., 2000; step 1 in Figure 1A). This induces an ATP-dependent conformational change into a clamp-like structure that subsequently allows MutS to diffuse along the DNA helix (Acharya et al., 2003) and recruit MutL (Grilley et al., 1989; Selmane et al., 2003; step 2 in Figure 1A). In turn, ATP-induced rearrangements within MutL allow recruitment and activation of the endonuclease MutH and the helicase UvrD (Spampinato and Modrich, 2000; Schofield et al., 2001; steps 2 and 3 in Figure 1A). MutH is responsible for strand discrimination by recognizing the hemi-methylated status of GATC sites, cleaving the GATC sequence of the unmethylated strand (Welsh et al., 1987; Au et al., 1992). Using the energy derived from ATP hydrolysis, UvrD is coordinated to unwind double-strand (ds) DNA unidirectionally toward the mismatch, starting at the single-strand nick created by MutH located either 5' or 3' from the mismatch (Yamaguchi et al., 1998; Dao and Modrich, 1998). As a result, a stretch of single-strand (ss) DNA containing the misincorporated nucleotide with either a free 3'- or 5'-end is displaced from the template strand and thus available for degradation by exonuclease I, exonuclease VII, exonuclease X or RecJ (Cooper et al., 1993; Burdett et al., 2001; Viswanathan et al., 2001; step 4 in Figure 1A). After removal of this DNA segment including the mismatched base, DNA polymerase III copies the template strand again, and DNA ligase completes the MMR event by sealing the nick at the end of this synthesis step (Lahue and Modrich, 1989; step 5 in Figure 1A).



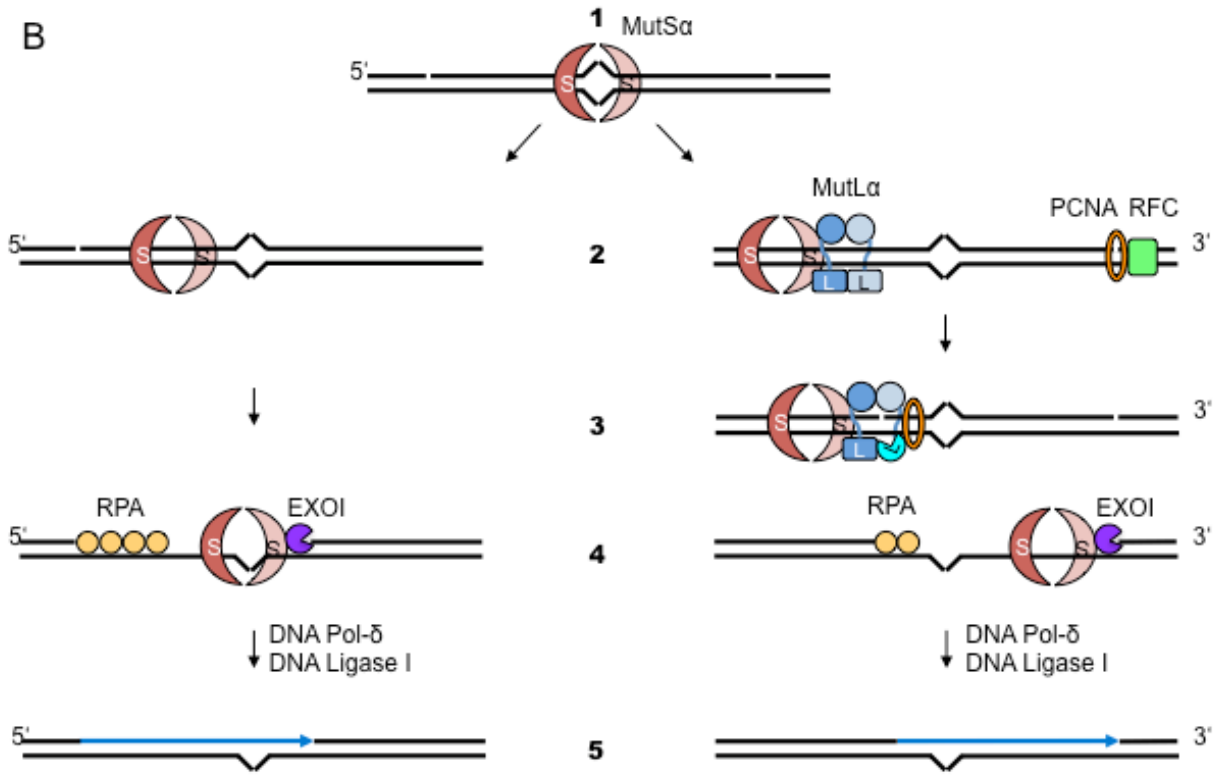


Figure 1. DNA Mismatch Repair
(A) *E. coli* MMR pathway. (Step 1) The MutS dimer (red) recognizes and binds a DNA mismatch. (Step 2) Upon ATP binding, MutS changes its conformation and diffuses bidirectionally along the dsDNA and recruits dimeric MutL (blue). MutH (green), which is responsible for strand discrimination by recognizing the hemi-methylated status of GATC sites, is then activated to cleave the unmethylated strand. (Step 3) At the nick, UvrD (purple; 3'-5' helicase) is loaded and coordinated to unwind dsDNA unidirectionally from either the 3'- or 5'-located nick toward the mismatch. (Step 4) A stretch of ssDNA containing the misincorporated nucleotide is displaced from the template strand for degradation by exonuclease I (3'-5'; orange), exonuclease VII (3'-5' and 5'-3'; orange), exonuclease X (3'-5'; orange) or RecJ (5'-3'; orange). (Step 5) Subsequently, DNA polymerase III copies the template strand again (blue arrow), and DNA ligase completes the MMR event by sealing the nick at the end of this synthesis step (blue arrow head).
(B) Mammalian MMR relies on MutSα (red), MutLα (blue), PCNA (orange), RPC (green), EXOI (purple), RPA (yellow), DNA polymerase-δ and DNA ligase I. (step 1) MMR is initiated by MutSα, which recognizes and binds to the DNA mismatch and slides along the DNA contour upon ATP binding. Nicks present in the daughter strand after replication (okazaki fragments in the lagging strand) or during processing of misincorporated ribonucleotides (in the leading strand) serve as strand discrimination signal. If the pre-existing nick is located 5' of the mismatch, this directs recruitment of Exonuclease I by MutSα, which results in degradation of the daughter strand from the nick up to and including the mismatch (steps 2 and 4 on the left side of the diagram). DNA polymerase-δ fills in the single-strand gap (blue arrow) and DNA ligase I seals the nick (blue arrow head). If the pre-existing nick is located 3' from the mismatch, the participation of MutLα, PCNA and the RFC clamp loader is also required due to the obligate nucleolytic polarity (5'-3') of EXOI. The PMS2 subunit of MutLα is activated by loaded PCNA to introduce additional nicks flanking the mismatch and thus creating the conditions for 5'-directed repair (steps 2-4 on the right side of the diagram). The single-strand gap generated by EXOI is stabilized by RPA. (step 5) Finally, DNA polymerase-δ fills in the single-strand gap (blue arrow) and DNA ligase I seals the nick (blue arrow head).

Eukaryotic DNA Mismatch Repair

Mammalian MMR relies on evolutionarily conserved homologs of MutS (MSH) and MutL (MLH) (Jiricny, 2013). MSH2 forms heterodimers with either MSH6 or MSH3, referred to as MutSα or MutSβ, respectively (Acharya et al., 1996; Palombo et al., 1996). MutSα recognizes and binds base-base mismatches and small insertion/deletion loops of 1-2 nucleotides (nt) (Acharya et al., 1996; Palombo et al., 1996). MutSβ recognizes and binds larger insertion/deletion loops (Acharya et al., 1996; Palombo et al., 1996). Similarly, MLH1 forms heterodimers with either PMS2, PMS1 or MLH3, referred to as MutLα (Li and Modrich, 1995), MutLβ (Raschle et al., 1999) or MutLγ, respectively (Cannavo et al., 2005). Unlike mammalian cells, *Saccharomyces cerevisiae* MutLα is composed of Mlh1p and Pms1p (Prolla et al., 1994), and MutLβ is composed of Mlh1p and Mlh2p (Wang et al., 1999). Importantly, mainly MutLα participates in mammalian MMR (Li and Modrich, 1995) with a partial role for MutLγ (Cannavo et al., 2005), while MutLβ functions remain unknown. The binding of MutSα to a mismatch initiates mammalian MMR (Figure 1B step 1). Upon exchange of ADP for ATP, MutSα is converted into a sliding clamp (Gradia et al., 1997, 1999; Iaccarino et al., 2000; Figure 1B step 2), which then recruits MutLα to form a ternary complex (Black et al., 2001; Plotz et al., 2002; Figure 1B step 2). Strand discrimination is achieved using nicks in the daughter strand that are (i) present after replication, especially on the lagging strand (Claverys and Lacks, 1986), or (ii) introduced by RNase H2-dependent processing of misincorporated ribonucleotides, especially on the leading strand (Ghodgaonkar et al., 2013; Lujan et al., 2013). *In vitro* reconstitution of human MMR using purified MutSα, MutLα, exonuclease I (EXOI), proliferating cell nuclear antigen (PCNA), replication factor C (RFC), replication protein A (RPA), DNA polymerase-δ and DNA ligase I shows that MMR can be achieved whether the nick is situated at the 3' or 5' of a mismatch (Figure 1B; Constantin et al., 2005; Zhang et al., 2005). Participation of MutLα is not absolutely required for 5'-directed repair of a mismatch because EXOI has an obligate 5'-3' polarity and can be loaded onto the pre-existing nick by MutSα (Genschel et al., 2002; left diagram step 4). In 3'-directed repair, a PCNA clamp, loaded by RFC at a nick 3' of the mismatch, activates the ternary MutSα-MutLα complex to create additional nicks flanking the mismatch via the latent endonuclease located in the PMS2 subunit of MutLα (Dzantiev et al., 2004; Kadyrov et al., 2006; right diagram steps 3 and 4). Then, the activated MutSα-MutLα ternary complex loads EXOI at one of the nascent nicks situated 5' to the mismatch (Genschel et al., 2002). The EXOI mediates 5'-3' single-strand degradation past the mismatch and generates a single-strand gap, which is stabilized by RPA (Genschel and Modrich, 2003; Constantin et al., 2005; Zhang et al., 2005). Finally, DNA polymerase-δ fills the single-strand gap with its complementary sequence and DNA ligase I seals the nicks (Constantin et al., 2005; Zhang et al., 2005; step 5).

Homologous Recombination

HR, which repairs DSBs and rescues replication-fork collapse, is highly conserved throughout evolution (Lin et al., 2006). The strand-exchange proteins (i.e. RecA and its homologs) that mediate the core events of homology search, base pairing, strand exchange and heteroduplex extension can be found in a wide variety of organisms

including bacteriophages, bacteria, archaea and eukaryotes (Ogawa et al., 1993; Yang et al., 2001; Lopes et al., 2010). In *E. coli*, RecA promotes HR for DSB repair via the RecBCD and RecF pathways (Spies and Kowalczykowski, 2005). The RecBCD pathway relies on single-strand DNA binding proteins (SSB), RecA, the RecBCD complex, RuvAB (branch-migration mediator) and RuvC (Holliday-junction endonuclease). The RecF pathway relies on SSB, RecQ (3'-5' helicase), RecJ (5'-3' exonuclease), RecA, RecFOR and RuvABC. In addition to DSB repair, HR repairs single-stranded DNA gaps using the RecF pathway, in which RecQ helicase and RecJ exonuclease coordinate the expansion of the single-strand gap, and RecFOR complex facilitates RecA loading to create a nucleoprotein filament (Kantake et al., 2002; Morimatsu and Kowalczykowski, 2003; Morimatsu et al., 2012). Furthermore, these HR proteins promote genome diversification in bacteria via DNA-transfer processes such as conjugation, transduction and transformation (Cotter and Thomashow, 1992; Mahan et al., 1993; Kok et al., 1997).

Repair of DSBs starts with processing of the ends of the broken DNA molecules to prepare them for DNA strand exchange. This central process consists of three crucial and sequential aspects called presynapsis, synapsis and postsynaptic heteroduplex DNA extension (Kowalczykowski and Eggleston, 1994-2). Briefly, presynapsis is the initial stage when RecA monomers polymerize onto ssDNA to form a helical nucleoprotein filament that is active in homology search. Synapsis is the subsequent stage when non-homologous and homologous contacts are made during the search for homology. Upon homologous pairing between a segment of RecA-ssDNA filament and dsDNA, a paranemic joint molecule is formed. Then, intertwining between the RecA-ssDNA filament and dsDNA generates a plectonemic joint molecule that facilitates strand exchange. DNA heteroduplex extension, as indicated by its name, is the stage when heteroduplex DNA is formed and extended upon strand exchange. These HR steps will be further described in detail below, after which we will consider at which steps mismatches are formed and might be recognized by MMR proteins, and implications for the mechanism of antirecombination.

End Processing of Double-Strand Breaks

DSBs requiring HR for repair are first processed into overhangs with 3'-terminated ends by the heterotrimeric complex of RecBCD in *E. coli*. Among the three subunits, RecB and RecD are ATPases (Hickson et al., 1985; Chen et al., 1997). RecB is both a helicase (3'→5'; Boehmer and Emmerson, 1992) and a non-specific endo/exonuclease (Yu et al., 1998-1, 1998-2; Wang et al., 2000; Sun et al., 2006). RecD is also a helicase but with the opposite polarity (5'→3'; Dillingham et al., 2003). The non-ATPase subunit RecC recognizes the asymmetric Chi sequence (crossover hotspot instigator, 5'-GCTGGTGG-3'; Handa et al., 2012). Upon binding to a DSB end, processive translocation of the RecBCD complex unwinds the dsDNA continuously (Bianco et al., 2001), which is powered by the helicase activities of the RecB and RecD subunits (Taylor and Smith, 1995). The resulting two single strands with opposite polarity are subject to different degree of nucleolysis imposed by the RecB subunit. During the early phase, the ssDNA with 3'-end is degraded much more vigorously than the complementary strand with 5'-end. When the RecBCD complex arrives at the Chi

sequence within the 3'-terminated single strand, a conformational change within the heterotrimer downregulates degradation of 3'-end ssDNA and upregulates degradation of 5'-end ssDNA (Dixon and Kowalczykowski, 1991, 1993, 1995; Anderson and Kowalczykowski, 1997-1; Handa et al., 2005). The RecBCD complex also facilitates loading RecA proteins onto the ssDNA downstream of Chi sequence (of 3'-terminated strand), a function requiring the nuclease domain of RecB (Anderson and Kowalczykowski, 1997-2; Anderson et al., 1999; Amundsen et al., 2000; Arnold and Kowalczykowski, 2000; Spies and Kowalczykowski, 2006).

Alternatively, DSBs can be processed into overhangs with 3'-ends by the coupled activities of RecQ helicase and RecJ exonuclease. RecQ mediates the unwinding of DSB ends (Umezu et al., 1990). The resulting 5'-terminated strand can be degraded by RecJ exonuclease (Spies and Kowalczykowski, 2005). Conversely, the complementary 3'-terminated strand is intact and is protected by SSB proteins. The heterotrimer RecFOR bound at the resulting ss/ds DNA junction facilitates the formation of a nucleoprotein filament by loading RecA onto the SSB-coated 3'-terminated ssDNA (Umezu et al., 1993; Hedge et al., 1996-1, 1996-2; Morimatsu and Kowalczykowski, 2003).

Presynapsis

The defining strand-exchange step of HR is catalyzed by a nucleoprotein filament consisting of RecA, ATP and ssDNA, whose assembly occurs during presynapsis. Structurally, the RecA monomer (molecular mass of 37,842 Da) consists of a major central domain and two smaller flanking subdomains at the amino and carboxy termini. The central domain contains a mostly parallel, twisted, eight-stranded β-sheet that is flanked by α-helices (Story et al., 1992). In the presence of ATP, RecA monomers bind ssDNA with high affinity (Menetski and Kowalczykowski, 1985). RecA cooperatively polymerizes onto ssDNA to form a right-handed helix around the ssDNA with ATP cofactors bound between the monomers (Figure 2; Stasiak and Egelman, 1994; Chen et al., 2008). Filament growth through monomer addition is bidirectional, but faster in the direction from 5' to 3' relative to the ssDNA the protein is bound to (Register and Griffith, 1985; Bell et al., 2012; Figure 2d-e). Pioneering work on RecA-ssDNA filaments using electron microscopy (EM) has disclosed that nucleoprotein filaments formed in the absence of nucleotide cofactor or in the presence of ADP cofactor are less extended compared to filaments formed in the presence of ATP or non-hydrolyzable ATP analogs, and these latter correlate with strand exchange activity. The DNA in those filaments is approximately 150% of the length of corresponding B-form DNA (Flory et al., 1984; Egelman and Stasiak, 1986; Heuser and Griffith, 1989; Yu and Egelman, 1992; van Loenhout et al., 2009). In agreement with the recent crystal structure of the RecA-ssDNA nucleoprotein filament (Chen et al., 2008), the average axial rise per base and helical parameter obtained by EM are 5.1 Å and 18.5 nucleotides per turn respectively. The crystal structure of RecA-ssDNA nucleoprotein filaments (Chen et al., 2008) further confirms that the bound ATP is completely buried and sandwiched at the interface of the RecA monomers. RecA binds to the ssDNA with an exact stoichiometry of three nucleotides per RecA monomer. Bases within the nucleotide triplet are spaced with an intervening 3.5-4.2 Å axial rise, while the backbone connecting the nucleotide

triplets is stretched to 7.8 Å axial rise. The RecA monomer-bound nucleotide triplet adopts a nearly B-DNA-like conformation that restricts the homology search to Watson-Crick-type base pairing. ATP cofactors are hydrolyzed at 25-30 min^{-1} in the RecA-ssDNA filament (Kowalczykowski, 1991). The resulting ADP bound cofactors cause nucleoprotein filament disassembly by reducing the ssDNA-binding affinity of RecA proteins (Menetski and Kowalczykowski, 1985). Thus, maintaining a high ATP/ADP molar ratio is important for formation of a RecA-ssDNA nucleoprotein filament that is active in homology search and strand invasion. An ATP/ADP molar ratio lower than 2-3 is able to shift the ssDNA-binding affinity state of RecA monomers from high to low (Menetski et al., 1988), and complete disassembly from ssDNA occurs when the ATP/ADP molar ratio is lower than 1 (Lee and Cox, 1990-1, 1990-2). As a positive cofactor in nucleoprotein filament formation, homotetrameric SSB melts the secondary structures formed within the ssDNA to promote assembly of contiguous RecA filaments (Muniyappa et al., 1984; Kowalczykowski et al., 1987-1; Kowalczykowski and Krupp, 1987-2; Roman et al., 1991; Figure 2b-c). Recent single-molecule studies show that RecA can displace SSB bound to ssDNA if a nucleation cluster of RecA is preformed adjacent to bound SSB (Joo et al., 2006; Figure 2d). In addition, a RecA dimer is proposed to nucleate on SSB-coated ssDNA if mobilization of SSB, by sliding or transient unwrapping and rewrapping, exposes at least six nucleotides of ssDNA (Lohman and Kowalczykowski, 1981; Kuznetsov et al., 2006; Roy et al., 2009; Bell et al., 2012). RecA loading onto SSB-coated ssDNA can be facilitated by RecBCD and RecFOR complexes (Anderson and Kowalczykowski, 1997-2; Morimatsu and Kowalczykowski, 2003). Upon replacing SSB proteins bound to the ssDNA by RecA, the filament is competent for homology search (Figure 2e).

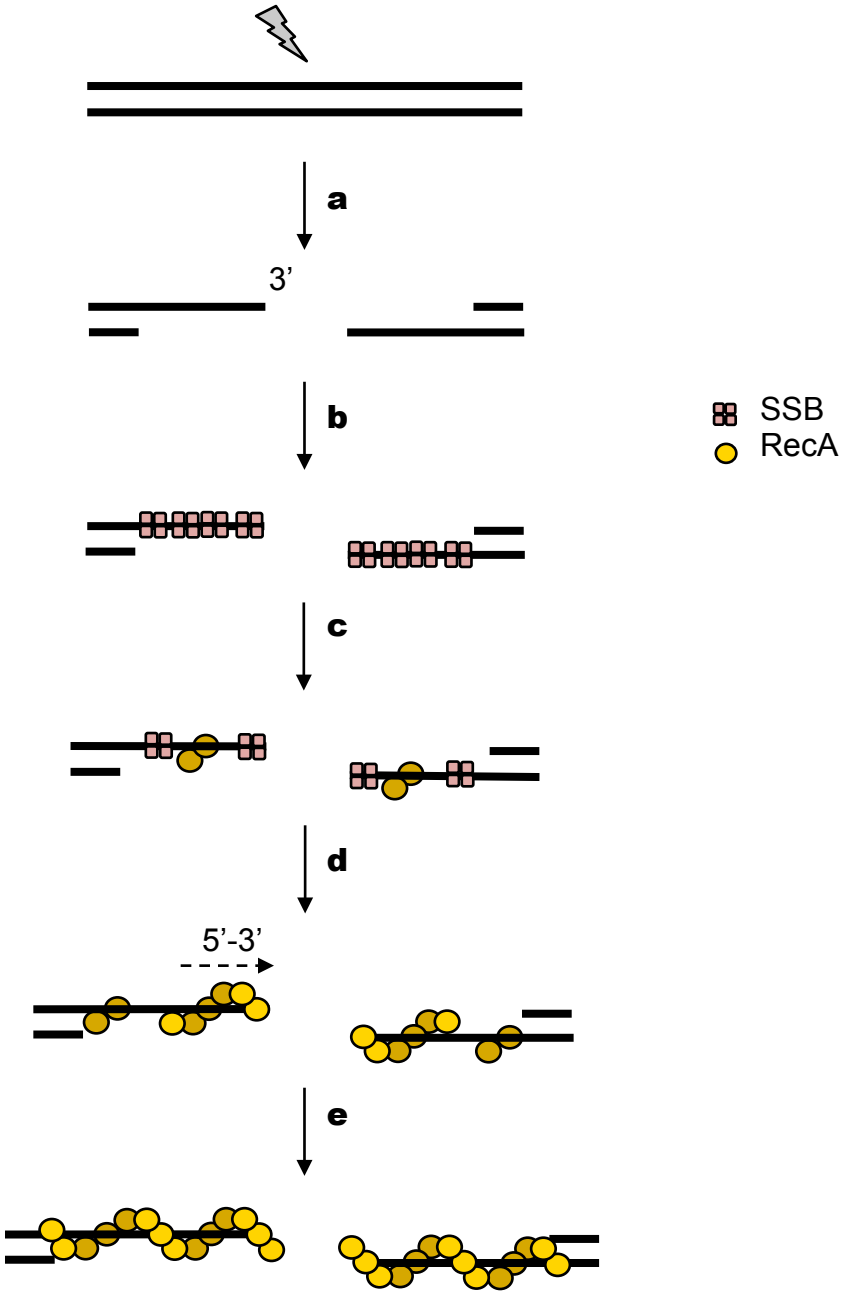


Figure 2. Presynapsis
(a) The DNA ends at a DSB are processed into 3’ single stranded termini by the heterotrimeric complex RecBCD. (b) The exposed ssDNA is bound by homotetrameric SSB, which removes and prevents the formation of secondary structures. (c-d) RecA filament growth is bidirectional, but monomer addition is faster in the 5’ to 3’ direction. (e) The ssDNA within the ATP bound RecA filament is stretched approximately 150% relative to B-form DNA. This form of the nucleoprotein filament is active for the subsequent event of homology search.

Synapsis

The helical RecA filament includes primary and secondary DNA binding sites (Mazin and Kowalczykowski, 1996). Within the nucleoprotein complex of RecA and ssDNA, the primary DNA-binding site interacts with the ssDNA. The secondary DNA-binding site binds transiently to dsDNA, either homologous or non-homologous, as part of the homology-search process. Because ATP hydrolysis is not required for strand exchange (Menetski et al., 1990; Rosselli and Stasiak, 1990), homology search is thought to be a passive process of diffusion and equilibrium thermal fluctuations (Savir and Tlusty,

2010). Recent single-molecule studies indicate that the RecA-ssDNA filament searches and pairs with the homologous sequence via the intersegmental contact mode of sampling (Figure 3; Forget and Kowalczykowski, 2012) that may combine with the sliding of nucleoprotein filament (Ragunathan et al., 2012). Mechanistically, the intersegmental model begins with non-specific interactions between a RecA nucleoprotein filament and dsDNA (Figure 3a-c). The secondary DNA-binding sites bind weakly and simultaneously with the non-contiguous heterologous segments of dsDNA for homology sampling (Mazin and Kowalczykowski, 1999). During this event, homology can be discriminated from heterology through fast exchange of bases via Watson-Crick recognition between the nucleotide triplets within the presynaptic filament and one, potentially complementary, strand of the dsDNA (Bazemore et al., 1997; Folta-Stogniew et al., 2004; Chen et al., 2008). Iterative attempts, of nucleoprotein filament being transferred intersegmentally on dsDNA for homology sampling, would eventually lead to homologous pairing (Figure 3d). The three-dimensional (3D) conformation of dsDNA is crucial for the repeated events of intersegmental transfer (Forget and Kowalczykowski, 2012). The required 3D conformation includes an increased local concentration of dsDNA, such as occurs when it is able to form a random coil in solution. For instance, linear dsDNA of around 48 kbp (~16 μm) with a manipulated end-to-end distance of 2 μm, creating a high local concentration of DNA, forms a larger fraction of homologously paired molecule with 430-nt ssDNA than DNA with a manipulated end-to-end distance of 6 μm. In addition, the efficiency of intersegmental transfer depends on the length of the invading ssDNA, presumably correlated with the effectiveness of simultaneous contacting of two non-contiguous regions of dsDNA (Forget and Kowalczykowski, 2012). For instance, homologous ssDNA of 430 and 1762 nucleotides in length pair with the homologies within the coiled-like dsDNA, but a 162 nt fragment does not. Homology search and pairing is further enhanced by the negatively-supercoiled conformation of dsDNA (Shibata et al., 1979; Wong et al., 1998; De Vlaminck et al., 2012). Negative supercoiling, but not positive supercoiling, promotes DNA-breathing characterized by the local opening of duplex structure that occurs preferably at AT-rich region (Strick et al., 1998; Jeon and Sung, 2008; Jeon et al., 2010). This dynamic feature of negative supercoiling facilitates homologous pairing with the presynaptic nucleoprotein filament of RecA-ssDNA.

Upon homologous pairing between a segment of RecA nucleoprotein filament and dsDNA, the initial joint molecule is referred to as paranemic (Christiansen and Griffith, 1986; Figure 3e). By definition, a joint molecule that is paranemic contains at least a segment of ssDNA homologously paired with its complementary strand within the dsDNA partner but not intertwined. Starting from the first paranemic region that often occurs at internal sites of the nucleoprotein filament and the dsDNA, homologous pairing could be further extended bidirectionally in increment of three base pairs (bp) as suggested by recent single-molecule studies (Ragunathan et al., 2011). During the extension of homologous pairing, the invading helical strand rotates around and spools onto the helical dsDNA bidirectionally (Howard-Flanders et al., 1984; Register et al., 1987; Honigberg and Radding, 1988; West, 1992; Ragunathan et al., 2011; Figure 3f) and leads to the structural transition of joint molecule from paranemic to plectonemic, in which the recombining DNA strands are intertwined (Bianchi et al., 1983; Figure 3g).

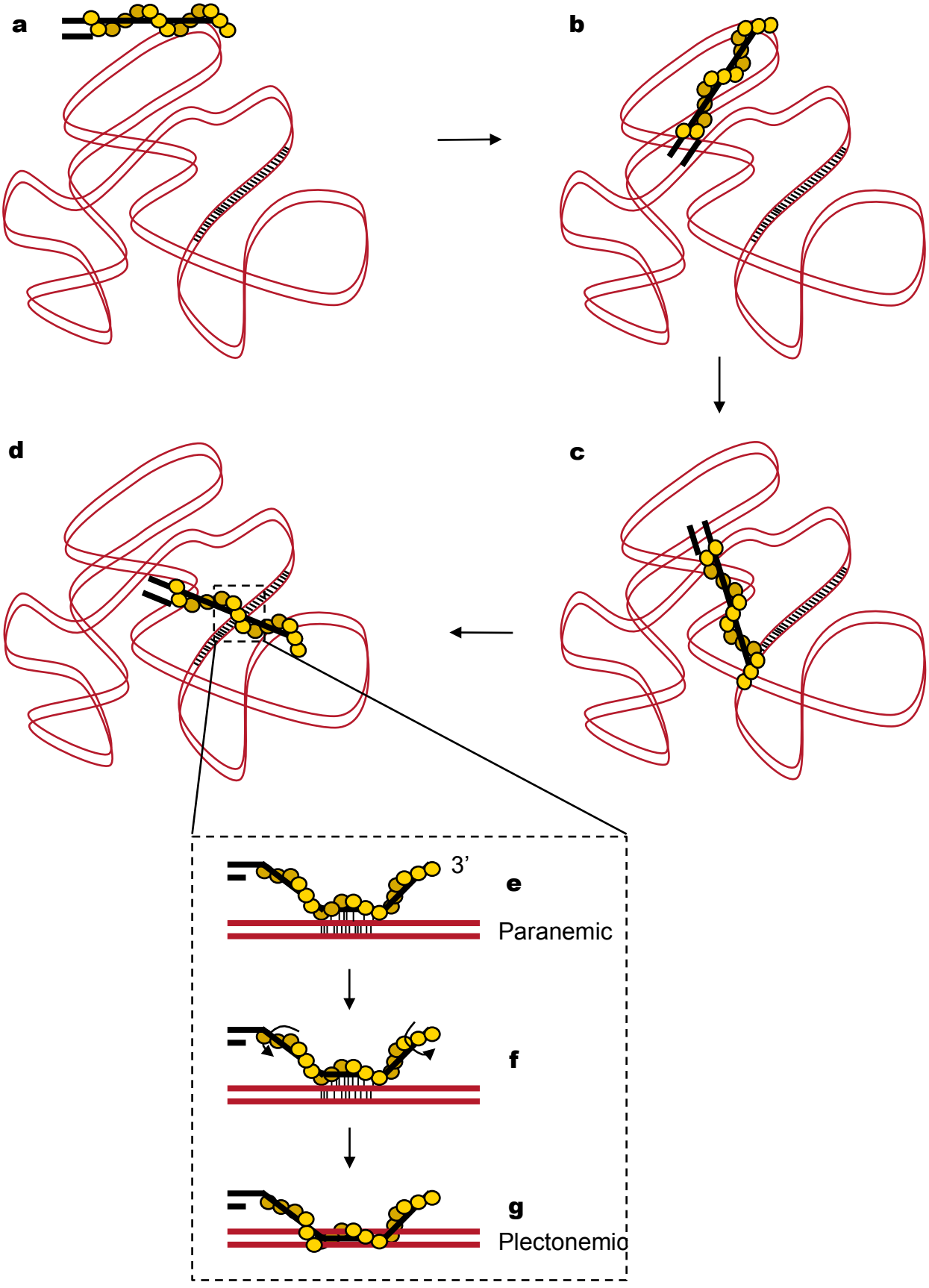
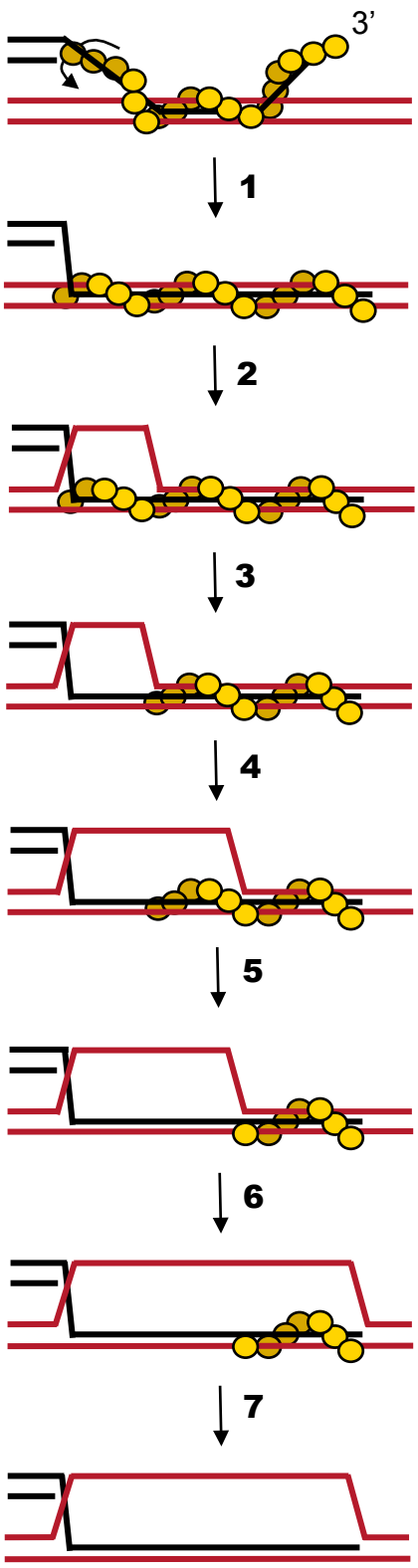

a
b
c
d
3’
e
Paranemic
f
g
Plectonemic

Figure 3. Synapsis
(a-b) RecA-ssDNA nucleoprotein filament (yellow balls and black line) passively searches for homology (black dashes) on the randomly-coiled dsDNA (red lines) here illustrated via intersegmental contact sampling. Most of the time initial contacts between the non-contiguous segments of the RecA filament and the dsDNA are heterologous. Due to diffusion and equilibrium thermal fluctuation, the RecA filament is transferred iteratively and intersegmentally among different regions of the random coiled dsDNA. (c) Upon encountering a homologous sequence, pairing occurs between a segment of the RecA filament and a segment of the dsDNA resulting in joint molecule formation. (e) Initially, the joint molecule is not topologically linked and thus is unstable, referred to as the paranemic structure. (f and g) The joint molecule becomes stable when the dsDNA and RecA filament become intertwined, referred to as a plectonemic joint.

DNA Heteroduplex Extension

The bidirectional spooling of RecA-bound invading ssDNA with dsDNA (Honigberg and Radding, 1988; Stasiak et al., 1991; Stasiak and Egelman, 1994) promotes the formation of the RecA-DNA complex containing three intertwined DNA strands (step 1 in Figure 4A), hereafter simply referred to as the strand-exchange complex. EM images suggest that the spooling toward the 5' terminus relative to the invading strand is faster than spooling into the opposite direction (Stasiak et al., 1991; Stasiak and Egelman, 1994). When spooling of the invading strand reaches its 5' end, the complementary strand within the dsDNA is preferably transferred from its 3' end to the RecA-bound invading ssDNA (Cox and Lehman, 1981-1; Kahn et al., 1981; West et al., 1981; Jain et al., 1994). This transfer event is called strand exchange, in which the complementary strands in the dsDNA unpair and then one of them base pairs with the invading strand. Within the strand-exchange complex, *in vitro* strand exchange is catalyzed independent of ATP hydrolysis (Menetski et al., 1990; Rosselli and Stasiak, 1990; Kowalczykowski and Krupp, 1995). RecA mutants defective in ATPase activity, such as K72R and K250R, retain the ability to promote homologous pairing and strand exchange (Rehrauer and Kowalczykowski, 1993; Cox et al., 2008). As a result of strand exchange, nascent heteroduplex and displaced ssDNA are formed within the strand-exchange complex. The heteroduplex binds to the primary DNA-binding site in the RecA helical filament, whereas the outgoing displaced strand binds to the secondary site, which further stabilizes the newly formed heteroduplex (Mazin and Kowalczykowski, 1996). The displaced strand formed within the strand-exchange complex apparently has increased conformational entropy that possibly drives its dissociation (steps 2, 4 & 6), which can be further accelerated by SSB proteins that bind the displaced ssDNA (Lavery et al., 1992; Mazin and Kowalczykowski, 1998; Ragunathan et al., 2011). Subsequent to ssDNA displacement, RecA polymers bound to the heteroduplex undergo a conformational change due to ATP hydrolysis and the filament disassembles (Ragunathan et al., 2011; steps 3, 5 & 7). RecA release is important for extensive heteroduplex formation (Jain et al., 1994) and strand exchange through heterology (Rosselli and Stasiak, 1991). The DNA structure formed upon RecA filament disassembly is referred to as a displacement-loop (D-loop) (Shibata et al., 1979; McEntee et al., 1979) consisting of short regions of heteroduplex and displaced ssDNA within the joint molecule. The events described so far, including fast spooling (3'-5' relative to the invading ssDNA), strand exchange (3'-5' relative to the incoming complementary strand), ssDNA displacement (5'-3' relative to the displaced strand) and

A



RecA disassembly from heteroduplex, all occur at the 5' end of the strand-exchange complex relative to the invading strand (Stasiak et al., 1984), hereafter referred to as the trailing end. At the same time, the plectonemic D-loop structure is further extended at the opposite 3' end of strand-exchange complex (Cox and Lehman, 1981-2), hereafter referred to as the leading end, at which the RecA-bound invading ssDNA spools onto the dsDNA (5'-3' relative to the invading strand) at a slower rate than the opposite direction (Stasiak et al., 1991; Stasiak and Egelman, 1994). During the concurrent events of strand displacement at the trailing end and slow spooling of RecA-bound invading strand onto dsDNA at the leading end, a synaptic window of strand-exchange complex travels unidirectionally toward the 3' end relative to the invading strand, as suggested by recent single-molecule studies (van der Heijden et al., 2008).

Figure 4. Postsynapsis
(A) DNA heteroduplex extension. (Step 1) Spooling of the RecA filament into the dsDNA generates a RecA-DNA complex containing three DNA strands referred to as the strand-exchange complex. (Step 2) Within this complex, strand exchange is facilitated. The displaced strand dissociates from the strand-exchange complex due to an increase in the conferred conformational entropy. (Step 3) Upon ATP hydrolysis, RecA proteins disassemble from the heteroduplex DNA. (Steps 4-7) This generates the D-loop DNA structure consisting of a displaced strand and a heteroduplex.

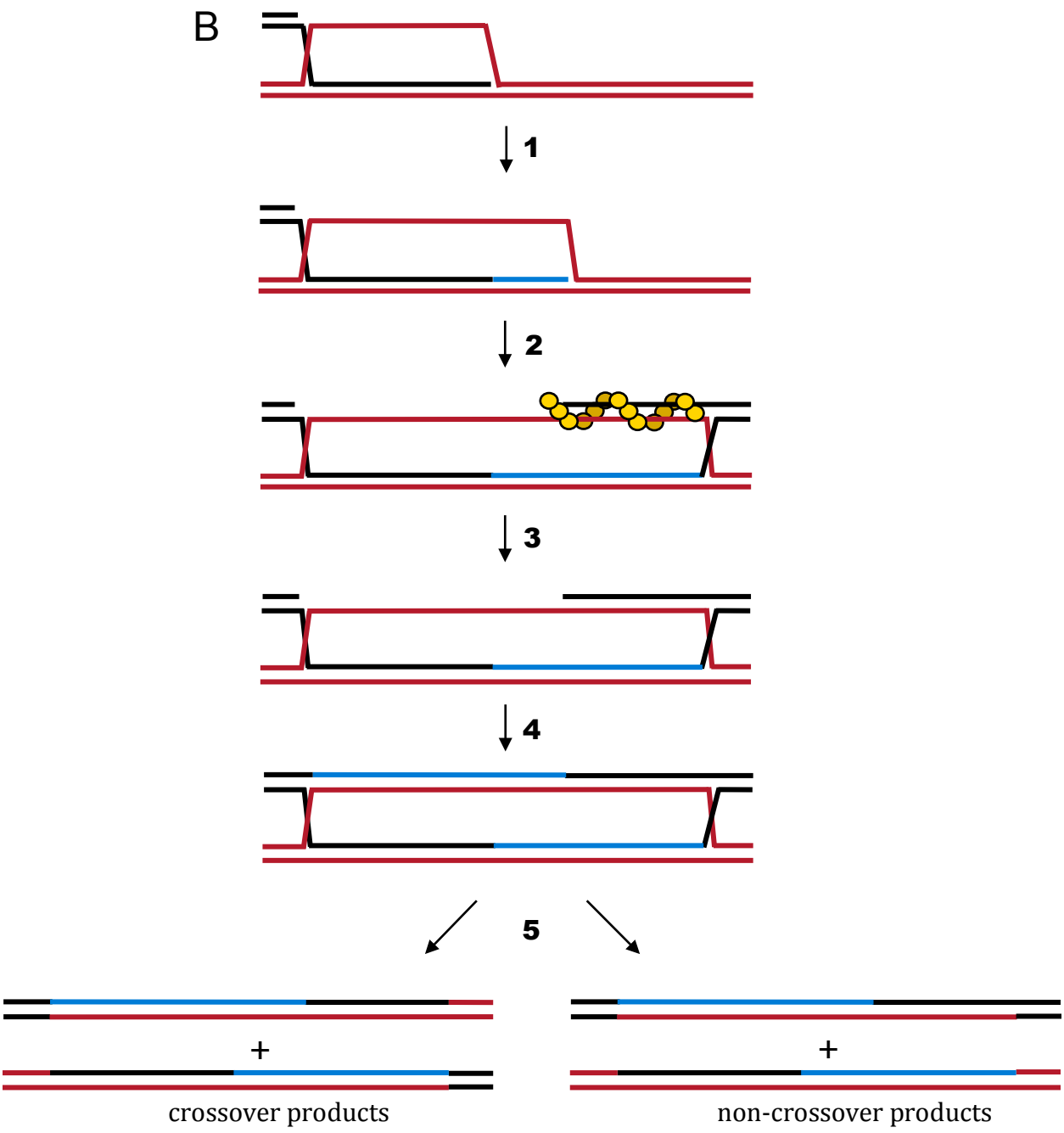


Figure 4. Postsynapsis
(B) Formation and resolution of Holliday junctions. (Step 1) Upon completion of strand exchange, the missing sequence is resynthesized on the homologous template (lower blue line). (Step 2) The unwinding of dsDNA during replication facilitates the capturing of the second 3'-end assembled with RecA filament. (Step 3) Homologous pairing generates a secondary D-loop structure. (Step 4) After RecA disassembly upon ATP hydrolysis, the heteroduplex of D-loop structure is further extended by replication, which resynthesizes the sequence resected by RecBCD complex (upper blue line). Sealing of the two 3'-ends by the DNA ligase generates a recombination intermediate with two Holliday junctions. (Step 5) Resolution of these junctions leads to the formation of crossover or non-crossover products depending on the cleavage orientation of endonuclease.

Upon completion of strand exchange (step 1 in Figure 4B), both heteroduplex and displaced ssDNA are further extended when DNA polymerase resynthesizes from the invading 3' end, thereby recovering any sequence information lost due to resection or an initial DNA break. The displaced ssDNA is then paired with complementary ssDNA within the RecA nucleoprotein filament formed with the second 3'-terminated end (step 2). Similarly, the resected region of the second 3'-terminated end is resynthesized using the displaced ssDNA as the template. Sealing of the two 3'-terminated invading strands by DNA ligase completes the production of recombination intermediate with Holliday junctions (steps 3 & 4). Resolution of these Holliday junctions by structure-specific endonucleases determines whether crossover (CO) or non-crossover (NCO) products are ultimately produced (step 5).

Eukaryotic Homologous Recombination

Similar to initiation of recombination in *E. coli*, HR in yeast and humans starts with presynapsis, in which DNA breaks are resected to form extended 3' single-strand tails for Rad51p (in yeast) and RAD51 (in human cells) filament formation (Sung, 1994; Benson et al., 1994). Limited 5'-3' single-strand resection of DNA breaks requires collaboration between the heterohexameric complex of Mre11p-Rad50p-Xrs2p, MRX (human MRE11-RAD50-NBS1, MRN), and Sae2p (human CtIP) (Mimitou and Symington, 2008; Zhu et al., 2008). More extensive resection requires ExoIp (human EXOI), or the combined activities of Sgs1p-Top3p-Rmi1p complex, STR (human BLM-TOPOIIIα-RMI1, BTR), and Dna2p (human DNA2) through the recruitment by the MRX complex (Cejka et al., 2010; Nicolette et al., 2010; Niu et al., 2010; Shim et al., 2010). Furthermore, RPA is required for extensive DNA end resection (Chen et al., 2013). In addition, RPA prevents DNA hairpins formed within the 3' ssDNA of resected ends that prevent Rad51 binding (Chen et al., 2013). To overcome the inhibitory effect of RPA on Rad51p filament formation, a variety of recombination mediators enhance the formation of presynaptic filament. The paralogs of Rad51p, Rad55p and Rad57p (human RAD51B, RAD51C, RAD51D, XRCC2, XRCC3), form heterodimers to facilitate the nucleation of Rad51p onto RPA-coated ssDNA (Sung, 1997-1) and to stabilize Rad51p-ssDNA filament from the activity of antirecombinase (Liu et al., 2011). Akin to Rad55p-Rad57p, recombination mediator Rad52 (human RAD52) counteracts the inhibitory effects of competing RPA by targeting Rad51p to RPA-coated ssDNA (Sung, 1997-2; New et al., 1998; Shinohara and Ogawa, 1998), and human BRCA2 promotes presynaptic filament formation by displacing bound RPA and blocking ATP hydrolysis within RAD51 filaments (Jensen et al., 2010; Liu et al., 2010). The multifunctional Rad54p (human RAD54) also promotes the stability of the presynaptic filament (Mazin et al., 2003), which is competent in DNA strand exchange during the next stage – synapsis. During this stage, the Rad51p-ssDNA filament searches for homology and forms a displacement loop (D-loop) constituting of a heteroduplex region and a displaced single stranded DNA. This structure is the key intermediate during HR as precursor for multiple HR subpathways: double Holliday junctions (dHJ), synthesis-depedent strand annealing (SDSA), and break-induced replication (BIR). Rad54p is a DNA-translocating motor that not only stimulates homologous pairing during synapsis (Petukhova et al., 1998) but also initiates DNA synthesis by removing Rad51p bound to

the heteroduplex of D-loop structure during the next stage – postsynapsis (Solinger et al., 2002; Sugawara et al., 2003). Finally, a variety of structure-selective endonucleases determine whether the HR outcome will be a CO or NCO event (Schwartz and Heyer, 2011).

Antirecombination Mediated by DNA Mismatch Repair Proteins

As described previously, genetic exchange between DNA molecules that share less than 100% homology is termed homeologous recombination rather than HR. Genomes of M13 and fd bacteriophages, whose sequences diverge by approximately 3%, are frequently used in *in vitro* studies of homeologous recombination (DasGupta and Radding, 1982; Bianchi and Radding, 1983). These studies demonstrate that RecA facilitates homeologous strand exchange (i.e. between M13-fd) albeit at a slower rate than the homologous reaction (i.e. between M13-M13 or fd-fd). This discrimination is due to reduced rates of both the homologous pairing and strand exchange steps during homeologous reactions compared to homologous reactions (Bazemore et al., 1997). Although sequence divergence *per se* delays HR across imperfect regions, the inherent inefficiency of homeologous recombination is not sufficient to abort the reaction. MMR proteins provide additional and more efficient regulation that is lesion-specific (i.e. dependent on mismatches and insertion/deletion loops) (Rayssiguier et al., 1989; Worth et al., 1994; Stambuk and Radman, 1998).

The involvement of MMR proteins in suppressing homeologous recombination have been verified extensively with genetic approaches in prokaryotes and eukaryotes. Suppression of homeologous recombination, mediated by MMR proteins, is activated by mismatches produced within the heteroduplex region of recombination intermediates. It has long been recognized that bacterial interspecies conjugation (Rayssiguier et al., 1991), transduction (Zahrt et al., 1994; Zahrt and Maloy, 1997) and transformation (Majewski et al., 2000), processes during which recombination intermediates containing mismatches are generated, are negatively regulated by MMR proteins. Extensive studies of interspecies conjugation between *E. coli* and *Salmonella typhimurium*, whose genomes diverge by approximately 20%, indicate that MutS, MutL, MutH and UvrD reduce the homeologous recombination frequency to different levels (Rayssiguier et al., 1989, 1991; Matic et al., 1994; Stambuk and Radman, 1998). MutS and MutL, acting at early steps of MMR, are the most potent suppressors of homeologous recombination (Rayssiguier et al., 1989; Stambuk and Radman, 1998). Similar to its role in recognition of replication errors, MutS dimers or tetramers are expected to bind to mismatches formed within the heteroduplex and interact with MutL to recruit downstream MMR proteins (Junop et al., 2003; Calmann et al., 2005-1, 2005-2). The importance of MutL is corroborated by the finding that high cellular levels of MutL are necessary for negative regulation of homeologous recombination (Elez et al., 2007). MutH and UvrD, acting downstream of MutS and MutL during MMR, have a less dramatic but still important effect suppressing homeologous recombination (Stambuk and Radman, 1998). Simultaneous loss of MutH and UvrD *in vivo* synergistically increases homeologous recombination frequency equivalent to loss of either MutS or MutL (Stambuk and Radman, 1998), indicating that MutH and UvrD may act in different subpathways or at different moments during the antirecombination process (Stambuk and Radman, 1998).

The MutH-independent mechanism, which relies on MutS, MutL, and UvrD helicase, is proposed to resolve homeologous DNA intermediates during the early stage of recombination when DNA heteroduplex extension is presumably still ongoing. The MutS-MutL complex that is assembled onto mismatches within the heteroduplex region presumably recruits UvrD and activates the helicase to unwind the recombination intermediate. Due to the participation of the UvrD helicase, this pathway is also referred to as the UvrD-dependent mechanism. During the late stage of recombination, when strand exchange has been completed and *de novo* DNA synthesis is initiated for the resynthesis of sequence lost or resected before strand exchange, the MutH-dependent mechanism is proposed to be responsible for suppression of homeologous recombination. The MutS-MutL complex bound to the heteroduplex coordinates MutH to cleave hemi-methylated GATC sites within the newly synthesized duplex DNA. Starting at this nick, an alternative helicase (rather than UvrD) is proposed to unwind the heteroduplex and resolve the homeologous recombination intermediate. In short, downstream MMR players UvrD and MutH are recruited by mismatch-activated MutS-MutL complexes at early and late stages of homeologous recombination to resolve reaction intermediates. Both UvrD- and MutH-dependent mechanisms have yet to be tested biochemically.

MMR-mediated suppression of mitotic homeologous recombination is preserved in eukaryotes. In yeast, a single mismatch in the heteroduplex is sufficient to activate the MMR system in suppressing recombination (Datta et al., 1997). Additional mismatches further increase the strength of antirecombination exerted by MMR proteins (Datta et al., 1997), which inhibit the extension of heteroduplex DNA during homeologous recombination *in vivo* (Chen and Jinks-Robertson, 1998, 1999). CO events in homeologous recombination are reported to be impeded by the yeast MMR machinery to a much greater extent than the alternative NCO events (Welz-Voegele and Jinks-Robertson, 2008). It is postulated that the formation of more extensive heteroduplex in the CO intermediate than the NCO intermediate, and thus higher amount of potential mismatches formed in the CO intermediate, targets the MMR machinery to regulate CO-NCO outcome (Mitchel et al., 2010). Like *E. coli*, yeast similarly relies on the mismatch-recognizing MutS homologs (Msh2p, Msh3p and Msh6p) and the mediator MutL homologs (Pms1p, Mlh1p) to reduce the mitotic homeologous recombination frequency (Bailis and Rothstein, 1990; Selva et al., 1995, 1997; Datta et al., 1996; Nicholson et al., 2000; Myung et al., 2001). As part of MutSα, Msh2p and Msh6p are involved in recognizing base-base mismatches and 1-nt insertion/deletion loop in the regulation of homeologous recombination (Nicholson et al., 2000). As part of MutSβ, Msh2p and Msh3p recognize 1-, 4-, 12-nt insertion/deletion loop and 18-nt palindromic hairpin but not base-base mismatches (Nicholson et al., 2000). ATPase activities of both MutLα subunits (Pms1p and Mlh1p) are important for the regulation of homeologous recombination imposed by yeast MMR machinery (Welz-Voegele et al., 2002). The homotrimeric proliferating cell nuclear antigen (PCNA) sliding clamp, which interacts directly with Msh3p, Msh6p and Mlh1p (Umar et al., 1996; Flores-Rozas et al., 2000; Hombauer et al., 2011), is indispensable for coupling MMR during DNA replication. In contrast, yeast PCNA plays a relatively minor role in the negative regulation of homeologous recombination mediated by the MMR proteins (Stone et al., 2008). Yeast Sgs1p helicase, whose homolog in humans (BLM) interacts

with MSH6 and MLH1 (Pedrazzi et al., 2001, 2003), reduces the frequency of homeologous recombination (Myung et al., 2001; Spell and Jinks-Robertson, 2004; Welz-Voegele and Jinks-Robertson, 2008; Mitchel et al., 2013). Interestingly, BLM-deficient mammalian cells suppress homeologous recombination to the same extent as wild-type cells (LaRocque and Jasin, 2010). The C-terminal 200 amino acids of Sgs1p, which may interact with MMR factor Mlh1p, are particularly important for its homeologous antirecombination (Spell and Jinks-Robertson, 2004). The UvrD-like Srs2p helicase, which interacts with MMR factor Mlh2p (Chiolo et al., 2005), also suppresses recombination between divergent DNA sequences (Welz-Voegele and Jinks-Robertson, 2008; Mitchel et al., 2013). Independent of mismatch activation, Srs2p can prevent HR by disrupting recombinase nucleoprotein filaments akin to UvrD (Veaute et al., 2003, 2005; Krejci et al., 2003). Both Sgs1p and Srs2p helicases can reverse or resolve Holliday junction-containing intermediates in homeologous recombination (Mitchel et al., 2013). In the cells of higher eukaryotes, sequence divergence also decreases the efficiency of HR during mitosis (Waldman and Liskay, 1987, 1988; te Riele et al., 1992; Deng and Capecchi, 1992; Nassif and Engels, 1993; Belmaaza et al., 1994). Relatively little is known about its mechanism but the suppression does require MSH2 (de Wind et al., 1995; Abuin et al., 2000).

Which Step of Homeologous Recombination is Modulated by MMR proteins?

Among the three steps during strand exchange: presynapsis, synapsis and postsynapsis, mismatches would be exposed only at the postsynaptic stage of DNA heteroduplex extension. During the earlier stage of HR, presynapsis or synapsis, mismatches are absent or hidden within the RecA filament, respectively. Upon RecA disassembly from the heteroduplex, the mismatches exposed during postsynapsis can be recognized by MutS, which could activate either antirecombination or MMR. We expect that if the heteroduplex is short (i.e. when strand exchange is still ongoing), the homeologous recombination intermediate may be resolved back to substrate DNAs coordinated by MMR proteins (i.e. MutS, MutL and UvrD helicase). In contrast, the mismatch-containing intermediate might be repaired rather than resolved if the heteroduplex is too long for complete unwinding (i.e. when DNA resynthesis has begun). However, the participation of MutH, which nicks the unmethylated strand of GATC site, may reduce the length of heteroduplex required for complete unwinding.

In vitro studies show that MutS by itself is capable of inhibiting homeologous strand-exchange using purified *E. coli* RecA and DNA substrates from bacteriophage M13 and fd (Worth et al., 1994). MutL, which interacts with and stabilizes MutS bound to mismatches (Grilley et al., 1989; Worth et al., 1998; Drotschmann et al., 1998; Schofield et al., 2001), further enhances the inhibition by MutS. The ATPase activities of both MutS and MutL are required for this inhibition (Worth et al., 1998; Junop et al., 2003). The presence of MutS and MutL during homeologous strand exchange effectively prevents completion of DNA heteroduplex formation, even in the presence of RuvAB (branch migration stimulator; Fabisiewicz and Worth, 2001). Because mismatches are only generated after strand exchange has occurred and probably are inaccessible until RecA disassembly from the heteroduplex, the molecular mechanism by which MutS and MutL are able to block completion of heteroduplex formation is intriguing, and is one of

the main questions addressed in this thesis. Interestingly, reduced antirecombination activity of a MutS C-terminal deletion mutant suggests that MutS tetramerization plays a role during antirecombination (Calmann et al., 2005-1, 2005-2). The importance of MutS tetramerization indicates that binding multiple locations or multiple strands may play a role in the mechanism of antirecombination imposed by MutS and MutL *in vitro*.

Another major question addressed in this thesis is the fate of the trapped homeologous recombination intermediates. If homeologous recombination is blocked by MutS and MutL, and amounts of strand exchange intermediates are building up, cells have to get rid of these potentially harmful DNA structures. Abovementioned genetic studies indicate this may be dependent on UvrD helicase (Stambuk et al., 1998). UvrD helicase is known to reverse or stimulate intermediates during homologous recombination, in varying biochemical conditions, back to substrate or forward to product DNAs (Morel et al., 1993). How UvrD helicase resolves homeologous recombination intermediates and how this depends on the presence of mismatch-activated MutS-MutL complexes is an intriguing question.

Outline of the Thesis

Similar to mice (de Wind et al., 1999), human individuals with defects in MMR have a predisposition to cancer (Fishel et al., 1993; Leach et al., 1993; Parsons et al., 1993). MMR not only reduces mutation frequencies within the genome by repairing replication errors, but also inhibits illegitimate recombination that very often involves divergent DNA sequences (Modrich and Lahue, 1996). Insight into the mechanism by which MMR regulates different DNA transactions is thus vital for the understanding and improvement of cancer studies. Knowledge of MMR-imposed suppression of homeologous recombination is particularly lacking compared to the mechanistic information that is available for MMR-mediated correction of replication errors. This thesis focuses on the mechanism of homeologous antirecombination mediated by MMR proteins.

In vitro recombination assays are the core element of most studies performed in this work. Therefore, the mechanism of HR was studied in detail before analysis of MMR effects on homeologous recombination. In Chapter 2, the stability of D-loop intermediates is examined directly using fluorescent DNAs and agarose gel electrophoresis. Previous studies on D-loop stability use nitrocellulose filter trapping that provides only limited information on the form of protein-bound DNA intermediates (Shibata et al., 1982). Understanding why and how D-loop structures dissociate is important for analyzing the effects of MMR proteins on the homeologous D-loop reaction.

In Chapter 3, the structures of DNA strand-exchange intermediates (i.e. M13-fd joint molecules), mediated by *E. coli* RecA protein, are characterized with scanning force microscopy (SFM). Our SFM images complement existing EM data on the reaction intermediates mediated by the strand-exchange proteins from yeast (Holmes et al., 2002). The SFM-obtained structures of strand exchange intermediates are crucial for analysis of the effects of MMR proteins on homeologous recombination.

The mechanistic roles of MutS, MutL and the UvrD helicase in suppressing homeologous recombination are addressed in Chapters 3 and 4. The coordinated action

of MutS and MutL is known to inhibit heteroduplex extension in homeologous recombination intermediate (Worth et al., 1994). We investigate the requirements for this inhibition in terms of the components and structural elements involved. A mechanistic model accounting for how MutS and MutL can trap the homeologous recombination intermediate, thereby inhibiting heteroduplex extension is described in Chapter 3.

MutS, MutL and UvrD helicase are hypothesized to orchestrate the resolution of DNA intermediates during the early stage of homeologous recombination *in vivo* in bacteria (Stambuk and Radman, 1998). But this hypothesis remained to be tested with purified proteins and DNAs. In Chapter 4, we reconstitute the reaction biochemically and propose a mechanistic model for the roles of UvrD helicase in the presence and absence of MutS and MutL in homeologous recombination.

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Chapter 2

TITLE

Characterization of D-Loop Dissociation during Strand-Exchange Reactions Mediated by *Escherichia coli* RecA

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SUMMARY

Homologous recombination repairs DNA breaks accurately. Using the sister chromatid as pairing template, a joint molecule (or D-loop structure) is formed upon the invasion of the RecA filament assembled on the ssDNA of a processed DNA break. The phenomenon of D-loop dissociation during RecA-mediated strand exchange reactions has been reported over three decades ago. Here we studied the mechanism of D-loop dissociation to determine the molecular details responsible for this process. Consistent with previous reports we find that the naked D-loop structure *per se* is stable indicating that the mechanism of D-loop dissociation is RecA-dependent. We use DNA-binding proteins, which specifically target different DNA strands within the D-loop structure, to examine the *in situ* requirements for the mechanism of D-loop dissociation. We find that proteins (i.e. MutS) binding to the heteroduplex DNA prevent D-loop dissociation. Similarly, increasing competition of ssDNA-binding proteins (i.e. SSB) with RecA to assemble on the displaced strand also prevents dissociation of the D-loop structure. Furthermore, the finding that unengaged RecA-ssDNA filaments are available in the D-loop reaction implies that the homologous region of the supercoiled plasmid is transiently inaccessible, which may be due to RecA binding subsequent of reinvasion events. Finally, we show that a high molar ratio of ATP/ADP and the optimal molar ratio of one RecA to three nucleotides of ssDNA are indispensable for sustaining high amounts of accumulating D-loops in the cycle, in which D-loop formation and dissociation events occur simultaneously. This finding indicates that the formation of active RecA-ssDNA filaments is important for both D-loop formation and dissociation events. Taken together, our results support a model in which the dissociation of D-loops is triggered via reinvasion of RecA-bound displaced ssDNA into the heterologous region of the joint molecules.

INTRODUCTION

DNA double-strand breaks (DSBs), which are particularly lethal to cells, occur as a result of replication-fork collapse (Cox et al., 2000) and exposure to DNA damaging agents such as ionizing radiation. In *Escherichia coli*, a DSB is processed by the multifunctional complex RecBCD to generate a 3'-terminated single-stranded (ss) DNA tail (Dixon and Kowalczykowski, 1993). In addition, RecBCD coordinates the polymerization of RecA protomers from 5' to 3' onto the ssDNA tail (Churchill and Kowalczykowski, 2000; Register and Griffith, 1985). The resulting right-handed helical nucleoprotein filament recognizes and invades the homologous region within chromosomal double-stranded (ds) DNA. Similarly, *in vitro*, RecA proteins catalyze the formation of joint molecules between linear ssDNA and negatively supercoiled dsDNA substrates (Shibata et al., 1979; McEntee et al., 1979), within which a heteroduplex and a displaced ssDNA constitute the displacement loop or D-loop structure (Kasamatsu et al., 1971). The homology at the 3'-end of the linear ssDNA is preferred for D-loop formation mediated by RecA (Konforti and Davis, 1990; Mazin et al., 2000), although no preference for homologous polarity is reported using linear ssDNA with a tailed duplex (McIlwraith et al., 2001). The formed D-loop structure can also dissociate during the reaction mediated by either RecA or Rad51, the eukaryotic RecA homolog (Mazin et al., 2000). The reaction can thus be referred to as the D-loop cycle, which consists of the events of joint-molecule formation and dissociation between the linear ssDNA and the negatively supercoiled dsDNA substrates (Shibata et al., 1982-2).

Pioneering studies propose two models that describe how RecA-dependent D-loop structures dissociate *in vitro* (Shibata et al., 1982-1; Iwabuchi et al., 1983). The first model takes advantage of the fact that RecA not only forms filaments on ssDNA, but also on the displaced strand of the D-loop structure. This nascent RecA-ssDNA filament reinvades the naked heteroduplex of the D-loop structure. Eventually, the displaced strand re-pairs with its complementary strand within the heteroduplex region, which directly resolves the joint molecule and reforms the initial DNA substrates. Hereafter, we refer to this model as the reinvasion model. The second model posits that cooperative binding of RecA proteins along the plasmid DNA initiating at one end of the D-loop structure stimulates the unidirectional unwinding of dsDNA from this end. The resulting topological stress accumulating in the supercoiled plasmid leads to the rewinding of the displaced strand with its complementary strand at the other end of the D-loop structure. Eventually this rewinding causes the resolution of the D-loop joint molecule. This model is hereafter referred to as the rewinding model. Here, using purified proteins and DNA substrates, we study the mechanism of D-loop dissociation and identify the requirements for the D-loop cycle. Our results provide support for the reinvasion model of D-loop dissociation.

MATERIALS AND METHODS

DNA Substrates

The negatively supercoiled dsDNA substrate (pUC19) was purified by non-denaturing lysis and CsCl ultracentrifugation as described (Sambrook and Russell, 2001).

Oligonucleotides were of the following sequence (named as SK3, Mazin et al., 2000): 5'-AATTCTCATTTTACTTACCGGACGCTATTAGCAGTG*GCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGT*-3'. The sequence of the 36 nucleotides at the 5'-end (non-italic) and the 54 nucleotides at the 3'-end (*italic*) of SK3 are heterologous and homologous to pUC19 respectively. Other oligonucleotides used are the heterologous 36-nt ssDNA, SS36: 5'-AATTCTCATTTTACTTACCGGACGCTATTAGCAGTG-3', and the homologous 54-nt ssDNA, SS54: 5'-*GCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGT*-3'.

Proteins

RecA protein was purified from *E. coli* strain GE1710 using a protocol based on spermidine acetate precipitation (Harmon and Kowalczykowski, 1998), and was confirmed to be nuclease-free (see below). RecA K250R mutant protein was purified as described and the kind gift of Michael Cox (Cox et al., 2008). RecA was flash-frozen in small aliquots and stored at -80°C. MutS was purified essentially as described (Natrajan et al., 2003) with the following adaptations: NaCl was replaced with KCl in the lysis and chromatography buffers. Anion exchange chromatography on the MonoQ column was performed after heparin affinity chromatography, followed by an additional POROS S column (Life Technologies) using the same buffers and salt gradient. After size exclusion chromatography, MutS was flash frozen in 25 mM HEPES-KOH pH 7.5, 250 mM KCl, 1 mM DTT, 50% glycerol and stored at -80°C. MutL was purified as described (Lebbink et al., 2010), except that KCl replaced NaCl in all buffers and the heparin gradient was developed from 0.1-1.0 M KCl. MutL was flash frozen in 20 mM Tris-HCl pH 8.0, 0.5 M KCl, 1 mM DTT, 50% glycerol and stored at -80°C. ScaI was purchased from Roche.

Nuclease Assay

The absence of contaminating endo- and/or exonuclease activity was addressed using linear ssDNA and dsDNA substrates. Synthetic oligonucleotides of arbitrary sequence (30 bases) were radioactively labeled at their 5'- and 3'- end with ^{32}P using T4 polynucleotide kinase (Roche) and terminal transferase (Roche) according to the specifications of the supplier. Blunt and sticky dsDNA fragments were created by digestion of pUC19 with HaeIII and MseI respectively, purified using phenol chloroform extraction and labeled using T4 polynucleotide kinase (5'-end labeling of blunt fragments) and Klenow (3'-end labeling of sticky fragments) according to the specifications of the supplier (Roche). Labeled DNA fragments were separated from free radioactive label using G50 size exclusion spin columns. Next, DNA substrates with equal levels of radioactivity were incubated for two hours at room temperature without any protein (negative control), with 0.25 units/µl lambda exonuclease (New England Biolabs), 0.1 ug/ul commercial RecA (New England Biolabs) and 0.1 ug/ul purified RecA. Next, 0.3 mg/ml salmon sperm DNA was added as a carrier. To precipitate all the DNA molecules except the deoxyribonucleotides resulting from nucleolysis, 7.5% trichloro acetic acid was added. The mixture was further incubated on ice for 15 minutes

and centrifuged for 15 minutes at 13000 rpm. Supernatant was transferred into 1-ml scintillation fluid and analyzed on a liquid scintillation analyzer (2500 TR; Packard).

D-Loop Reaction

RecA-ssDNA filaments were preformed at 37°C for 5 minutes with 50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT, 5% glycerol, 1 mM ATP, 45 nM 5'-labeled SK3 (linear ssDNA of 90 nucleotides tagged with Alexa Fluor 532), and 1.35 μM RecA. Then, 135 nM single-strand DNA binding proteins (SSB; Promega) was added and reaction mixtures were further incubated for 5 minutes. MutS and/or MutL were added before the D-loop reaction was initiated by adding 7 nM supercoiled pUC19. When appropriate, extra SSB, nucleotides cofactors (i.e. ATP and ADP), ATP regeneration system (6 mM phosphocreatine, 10 U/ml creatine phosphokinase), ssDNA or supercoiled (SC) plasmid was added into ongoing reaction incubated at 37°C as stated in the legends. Aliquots of 9 μl were taken at various time points and denatured with 0.1% SDS, 25 mM EDTA and 153 μg/ml proteinase K and incubated at 37°C for 10 minutes. Samples were loaded onto 1.3% agarose gels in 1x TA buffer and 10 mM magnesium acetate. Electrophoresis was carried out at 4.7 V/cm at 4°C. Gels were scanned with standard setting for Alexa Fluor 532 using Typhoon TRIO Variable Mode Imager (GE Healthcare Life Sciences).

RESULTS

Here we study the mechanism of D-loop dissociation, using a defined DNA oligonucleotide homologous to a region within the pUC19 plasmid, and extensively purified RecA (Figure 1) verified to be free of nuclease contamination (Figure 2). The main difference between our experimental design and pioneering studies addressing D-loop dissociation (Shibata et al., 1982-1, 1982-2) is the assay used for the detection of the D-loop species. We used agarose gel electrophoresis instead of retention of D-loop species by nitrocellulose filters. The latter technique is less direct because the retention of D-loop species on the filter and their subsequent elution may not be optimal. In contrast, gel electrophoresis separates the D-loop species directly from the labeled ssDNA without the risk of losing any DNA sample. Another significant difference is the ssDNA on which the RecA filaments are assembled. We used a defined synthetic ssDNA oligonucleotide that will result in the formation of one D-loop structure per plasmid circle at a predetermined position. In contrast, in the early reports (Shibata et al., 1982-1, 1982-2), several D-loop structures could form within a single plasmid because RecA filaments were formed upon a mixture of ssDNA fragments of different length and sequences homologous to multiple regions on the plasmid. Therefore, our experimental design allows studying the dissociation of a defined D-loop without the influence of additional D-loop structures being present *in cis*. We examined the mechanism of D-loop dissociation by perturbing the ongoing D-loop reaction with different DNA-binding proteins (i.e. MutS, MutL, SSB), extra DNAs (i.e. ssDNA) and extra nucleotide cofactors (i.e. ATP, ADP) and different ratios of reactants.

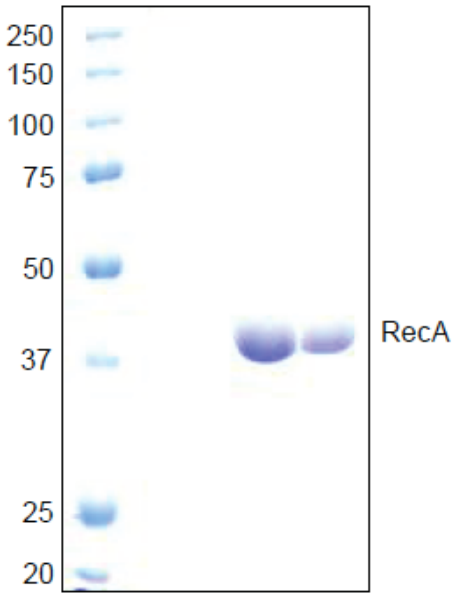

Figure 1. Gel electrophoresis of purified RecA
RecA (molecular mass 37.8 kD) was analyzed using 10% SDS-PAGE and stained with Coomassie Blue. From left to right, molecular weight standard and two fractions eluted from the MonoQ column containing approximately 10 and 3 µg RecA.

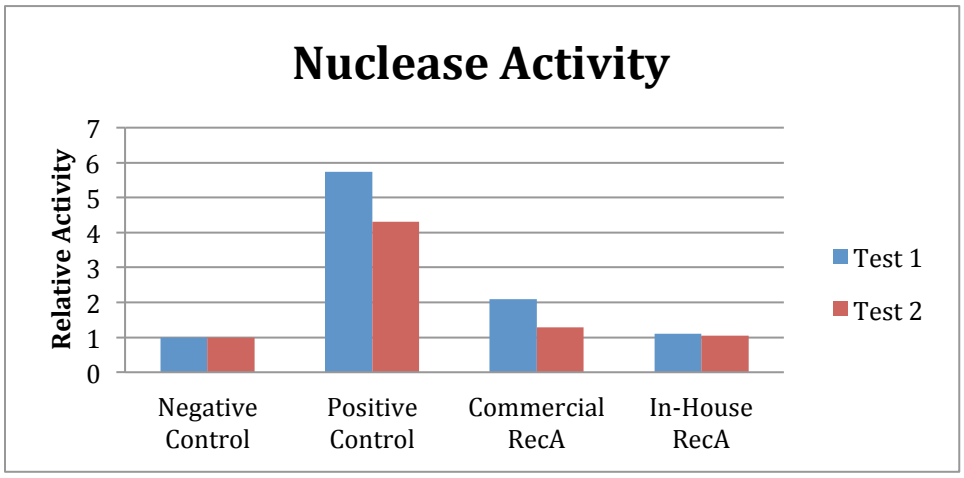

Figure 2. Purified RecA is nuclease-free Commercial RecA, in-house RecA, and λ-exonuclease (positive control) were incubated with mixtures of radiolabeled ssDNA and dsDNA. Radiolabels liberated from nucleolysis were purified and quantified to assess nuclease contamination relative to a negative control sample without added protein.

The 5'-Heterologous ssDNA Tail is not Important for D-Loop Dissociation

D-loop species are formed when RecA-bound ssDNA invades negatively supercoiled plasmid DNA, and subsequently decrease over time (left panel of Figure 3; Mazin et al., 2000; Tham et al., 2013). The ssDNA substrate that we have been using previously in the D-loop reaction (Tham et al., 2013) has 5'-heterology (36 nucleotides) and 3'-homology (54 nucleotides) to the negatively supercoiled dsDNA substrate. To find out if the 5'-heterologous end of the ssDNA substrate plays a role during D-loop dissociation, we used homologous ssDNA substrate (54 nucleotides; SS54) lacking the 5'-heterology (right panel of Figure 3). We found that in the absence of 5'-heterology in ssDNA substrate, D-loop dissociation was equally efficient as in the presence of 5'-heterology. This excludes the possibility that the 5'-heterologous tail participates in the mechanism of D-loop dissociation and emphasizes the intrinsic role of the D-loop structure in the mechanism.

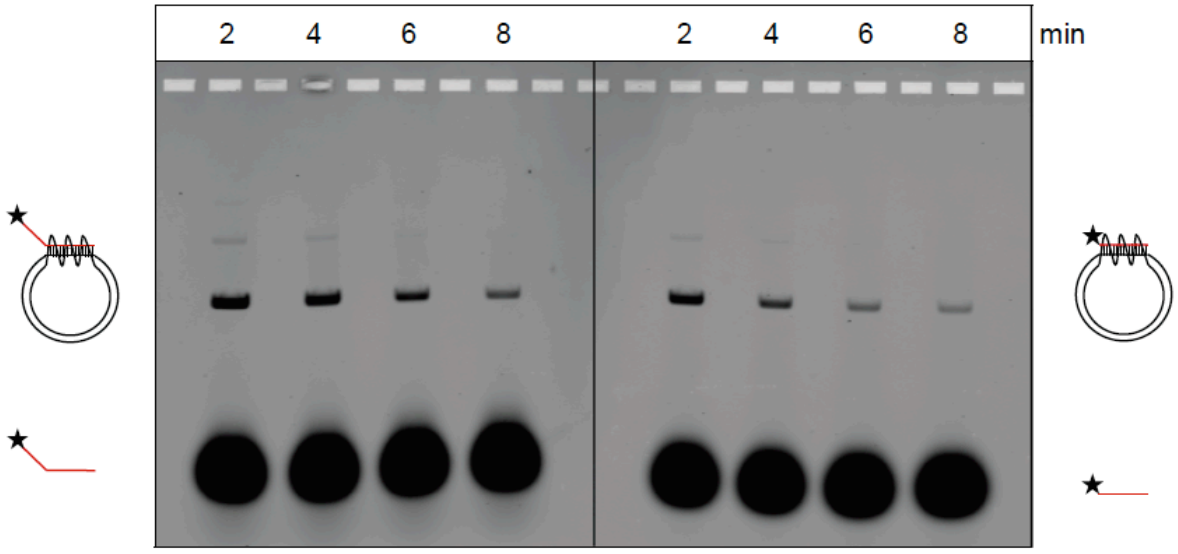

Figure 3. The heterologous tail is not required for D-loop dissociation
D-loop reactions reveal rapid formation of joint molecules that gradually dissociate over the time course of the reaction. Reactions were performed using negatively supercoiled dsDNA and labeled linear ssDNA with 5'-heterologous end (SK3; left panel) and without5'-heterologous end (SS54; right panel).

The Naked D-Loop Species *per se* does not Dissociate

To test if D-loop dissociation depends on RecA, we tested whether the D-loop structure would dissociate by itself during extended incubation upon denaturation of RecA. We thus divided the D-loop reaction mixture of a single time point into two aliquots, and deproteinized them at 37°C for 10 minutes and 60 minutes. As shown in Figure 4A, the deproteinized D-loop species was still stable when incubation at 37°C was extended for another 50 minutes as indicated by the equal amount of D-loop species in the two lanes. Furthermore, the stability of deproteinized D-loop species during extended incubation was not influenced by the absence of the 5'-heterologous tail (Figure 4B). These results indicate that D-loop dissociation is RecA-dependent.

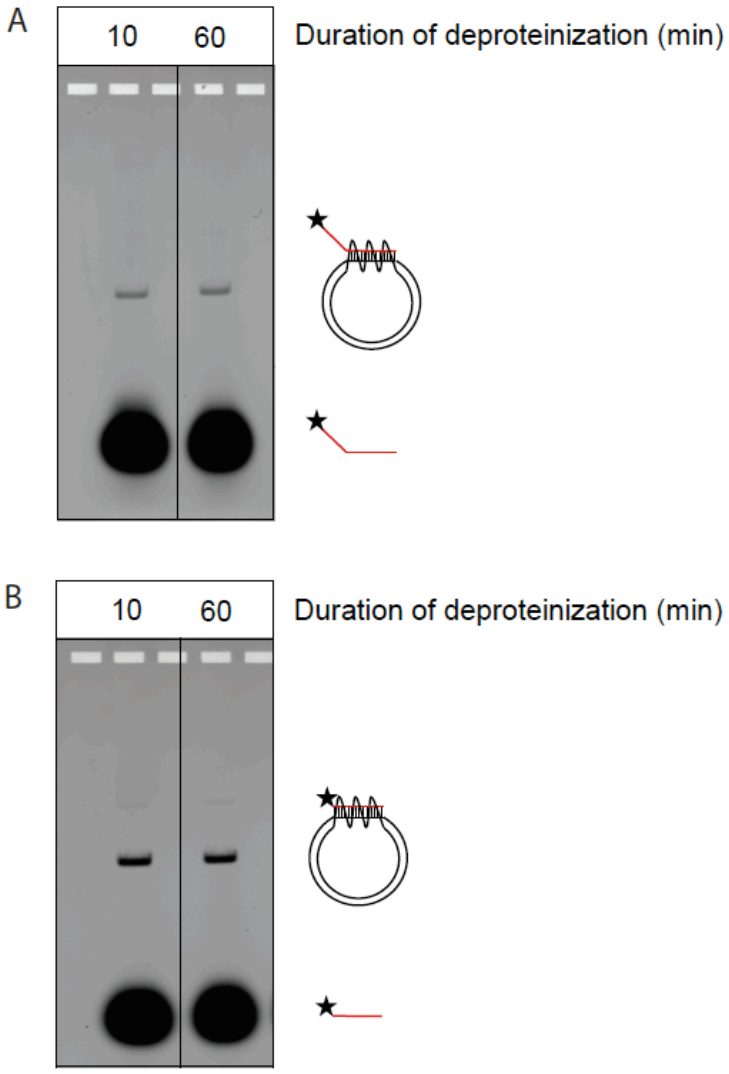

Figure 4. The naked D-loop species *per se* does not dissociate
(A) The D-loop reaction mixture was incubated for 6.5 minutes at 37°C and subsequently divided into two aliquots. Before loading into the gel for electrophoresis, one aliqout was deproteinized for 10 minutes and the other aliqout was deproteinized for 60 minutes at 37°C.
(B) Similar as panel (A), but the reaction was incubated for 2 minutes at 37°C before deproteinization and linear ssDNA without 5'-heterologous end was used.

RecA-Bound Heteroduplex Prevents D-Loop Dissociation

We tested whether D-loop dissociation would be prevented, in case RecA proteins fail to disassemble from the heteroduplex region of the D-loop structure. Indeed, when we used the RecA ATPase defective mutant K250R (Cox et al., 2008), D-loop dissociation, but not D-loop formation was inhibited as indicated by the constant amount of D-loop species over time (Figure 5A; reproduced from Tham et al., 2013). RecA K250R is defective in disassembly from DNA because we managed to crosslink larger amounts of this protein on D-loop structures, compared to wild-type RecA (Figure 5B; reproduced from Tham et al., 2013). These results suggest that RecA disassembly upon ATP hydrolysis, from the heteroduplex region of the D-loop structure is important for D-loop dissociation.

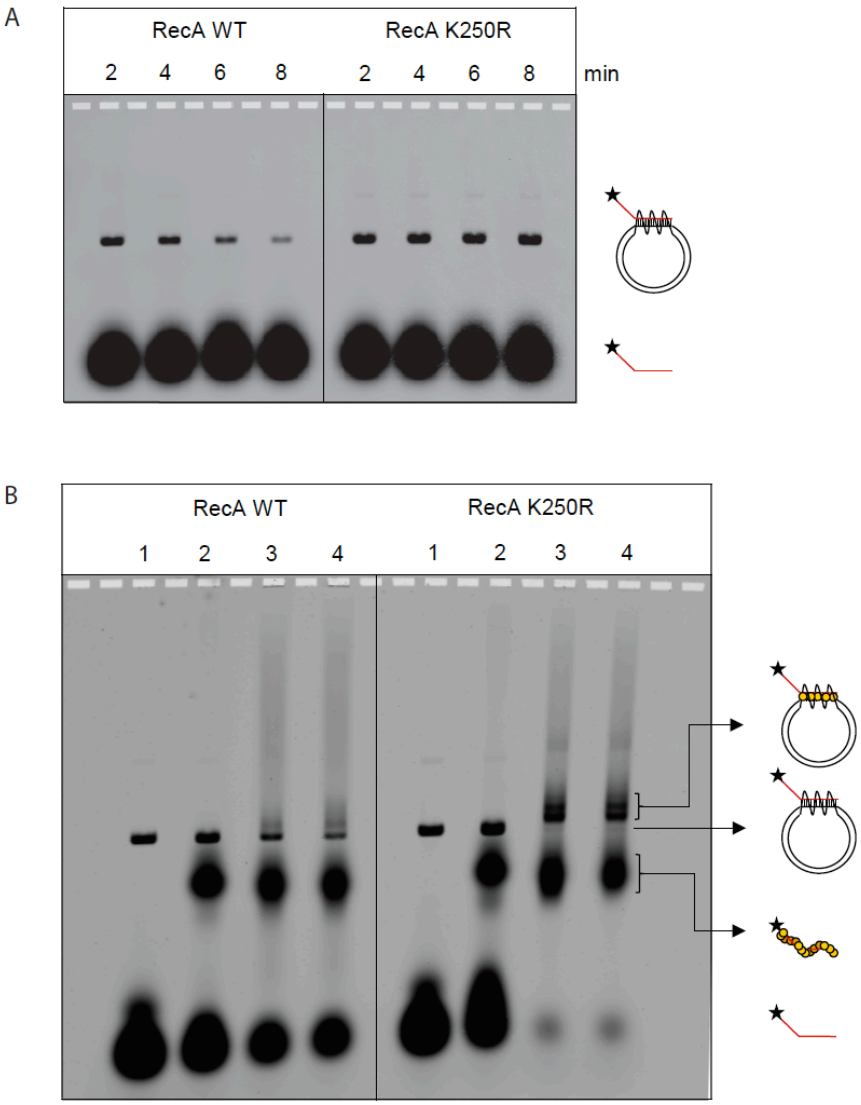

Figure 5. RecA disassembly from the heteroduplex region within the D-loop structure allows joint-molecule dissociation
(A) D-loops formed with wild-type RecA (left panel) disappear over time, while D-loops formed with ATPase mutant K250R (right panel) do not dissociate (Tham et al., 2013). Same as Figure 2D in Chapter 3.
(B) D-loop samples, of wild-type RecA (left panel) and ATPase mutant of RecA K250R (right panel), of identical time point (2 min) were deproteinized with SDS buffer (lane 1), not treated (lane 2), crosslinked with 0.3% glutaraldehyde (lane 3) or 0.6% glutaraldehyde (lane 4) (Tham et al., 2013). Same as Figure 2C in Chapter 3.

Formation of a Mismatch-MutS/MutSL Complex on the Heteroduplex region Prevents D-Loop Dissociation

We learnt from the RecA K250R studies that proteins bound to the heteroduplex within the D-loop prevent D-loop dissociation. We predicted, that under conditions that allow ATP hydrolysis by RecA, proteins other than RecA that bind to the heteroduplex DNA might also delay the dissociation of the D-loop. Indeed, we reported previously that in the presence of a single mismatch within the heteroduplex region and the mismatch-binding protein MutS (Su and Modrich, 1986), D-loop dissociation was delayed (Figure 6A; reproduced from Tham et al., 2013). This delay was exclusively mismatch dependent as we did not observe this inhibition in the absence of a mismatch (Figure 6B; reproduced from Tham et al., 2013). MutL (Grilley et al., 1989), which physically interacts with MutS and stabilizes the mismatch-MutS complex, enhanced the delay of D-loop dissociation exerted by MutS (Figure 6A; reproduced from Tham et al., 2013). This enhanced delay was dependent on both the concentrations of MutS and MutL as shown in Figures 6C and 6D. Consistent with the effect of RecA K250R, these results

show that the formation of the mismatch-MutS/MutSL complex on the heteroduplex prevents the D-loop structure from dissociating upon RecA disassembly.

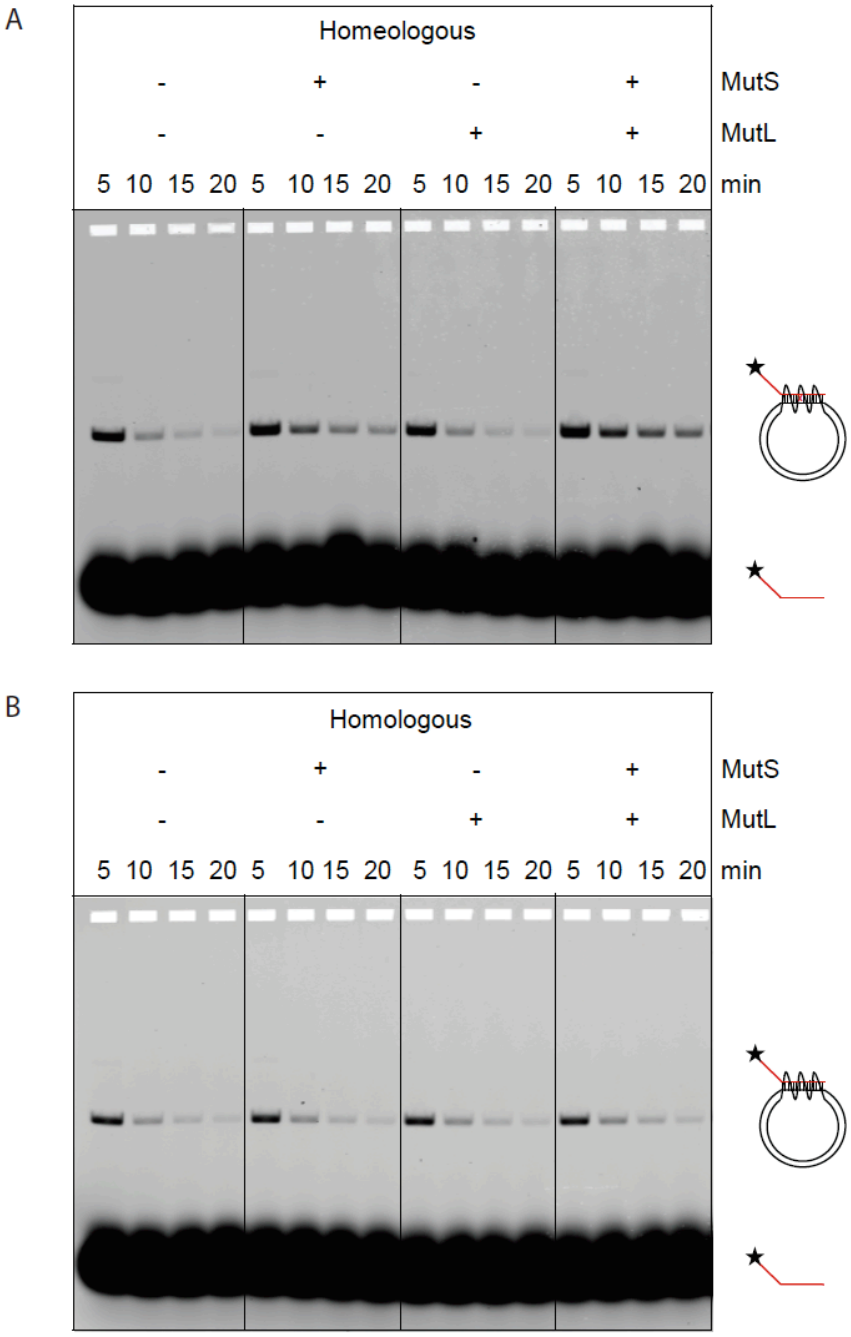

Figure 6. MutS and the MutSL complex prevent D-loop dissociation
(A) MutS (50 nM), MutL (50 nM) and MutS-MutL (50, 50 nM) were included in the respective D-loop reactions, in which a single DNA mismatch is generated within the heteroduplex. This is termed as the homeologous reaction (Tham et al., 2013). Same as Figure 2B in Chapter 3.
(B) Similar as panel (A) except that there is no mismatch generated within the heteroduplex upon D-loop formation. This is termed as the homologous reaction (Tham et al., 2013). Same as Figure 2A in Chapter 3.

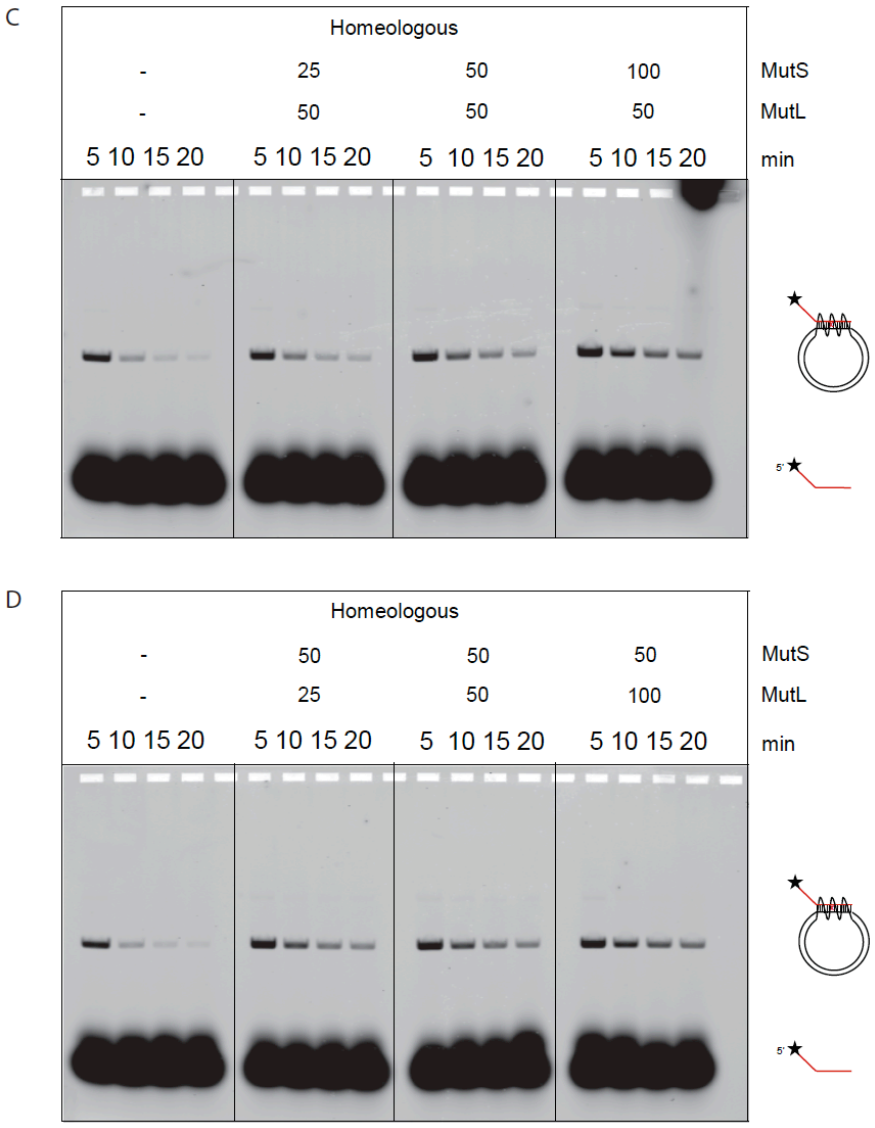

Figure 6. MutS and the MutSL complex prevent D-loop dissociation
(C) Titration of MutS (25, 50, 100 nM) in the presence of MutL (50 nM) in the homeologous reaction.
(D) Titration of MutL (25, 50, 100 nM) in the presence of MutS (50 nM) in the homeologous reaction.

Extra Single-Strand DNA Binding Proteins Prevent D-Loop Dissociation

We next tested the role of the displaced strand for D-loop dissociation. If RecA is able to bind to this displaced strand it might catalyze strand exchange with the previously formed heteroduplex region and thereby induce D-loop dissociation. We postulated that addition of extra single-strand DNA binding protein (SSB) to the ongoing reaction would delay the dissociation of D-loop species, because increasing the competition between RecA and SSB binding to the displaced strand would reduce the efficiency of forming a contiguous RecA filament onto the displaced ssDNA. Thus we carried out an experiment in which extra SSB was added one minute after initiation of strand exchange. At this time point, we assume that D-loop dissociation has already started as

suggested by the slightly lower amount of D-loop species in a reaction mixture containing ATP as compared to the one containing a non-hydrolyzable ATP analog, ATPγS (Figure S1) in which the amount of D-loop species is maximal because it does not dissociate (Mazin et al., 2000). We observed a delay of D-loop dissociation upon addition of extra SSB to 540 nM (Figure 7A). These results suggest that a contiguous RecA nucleoprotein filament formed with the displaced strand of the D-loop structure is important for the dissociation of the joint molecule.

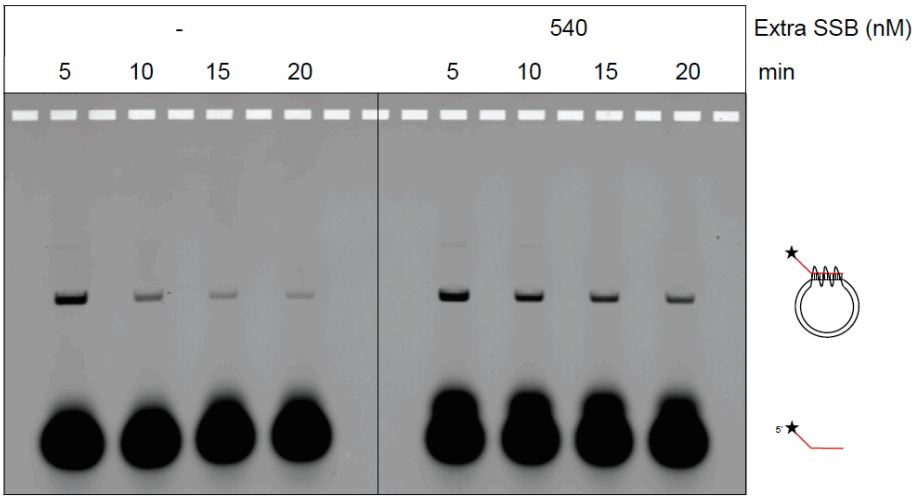

Figure 7. SSB delays D-loop dissociation
Additional SSB (540 nM, in addition to 135 nM in the initial reaction mixture) was added into the ongoing D-loop reaction one minute after initiation.

High Molar Ratio of ATP/ADP Increases the Accumulated Amount of D-Loop Species

During the D-loop cycle, DNA substrates and D-loop products are recycled because D-loop species are created from fresh DNA substrates, DNA substrates reform due to the dissociation of D-loops and new D-loops are created again from the reformed DNA substrates. The rates of both D-loop formation and dissociation thus determine the accumulated amount of D-loop molecule during the reaction cycle. Disruption of either event will affect the accumulated amount of D-loop species. Thus, the RecA K250R mutant, which prevents D-loop dissociation, generated the highest accumulated amount of D-loop (Figure 5A). Conversely linearization of the negatively supercoiled dsDNA substrate within the accumulated D-loop species completely prevented the accumulation of D-loops at later time points (Figure S2). The D-loop cycle requires RecA and a hydrolysable form of ATP. Since we did not use an ATP regeneration system, it is possible that ATP consumption and ADP accumulation in the circular reaction suppressed the formation of active RecA-ssDNA filaments that promote strand exchange (Menetski et al., 1990) for D-loop formation and dissociation. These filaments are ATP-bound and extended between the nucleotide triplets along the DNA backbone (Heuser and Griffith, 1989; Chen et al., 2008). It is reported that a molar ratio of ATP/ADP higher than 2-3 is required for RecA binding to ssDNA (Mazin et al., 1988), and complete disassembly from ssDNA occurs when the molar ratio is lower than 1 (Lee and Cox, 1990). We thus predicted that the shift from high to low molar ratio of ATP/ADP throughout the reaction reduces the accumulated amount of D-loop species

during the cycle due to the reduction of active RecA filaments assembled on both the linear ssDNA substrate and the displaced strand of D-loop structure. To test this idea, we added extra ATP or ADP to separate reactions one minute after initiation. Additionally, another reaction included an ATP regeneration system from the beginning of the reaction. As expected, addition of extra ATP to the ongoing reaction increased the accumulated amount of D-loop species, and addition of extra ADP to the ongoing reaction decreased the accumulated amount of D-loop species (Figure 8). The conversion of accumulated ADP to ATP by the regeneration system again increased the accumulated amount of D-loop species to a level higher than with addition of ATP alone (Figure 8). In short, these results suggest that a high molar ratio of ATP/ADP is required in order to maintain high accumulated amounts of D-loop species. This finding indicates that the increased fraction of active RecA filaments assembled on the linear ssDNA substrates and possibly on the displaced strand, due to the high molar ratio of ATP/ADP, differentially affects the D-loop formation and dissociation rates such that more D-loop molecules accumulate in the reaction.

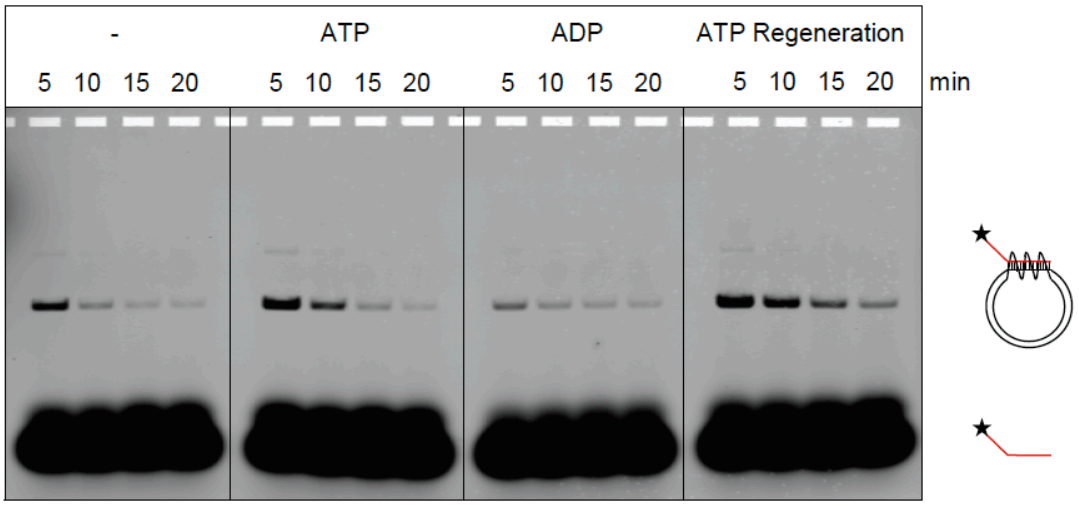

Figure 8. High molar ratio of ATP/ADP is required to maintain the high accumulated amount of D-loop species in the cycle
Additional ATP (1 mM) and ADP (1 mM) were added one minute after initiation of the D-loop reaction at 37°C. The ATP regeneration system (6 mM phosphocreatine and 10 U/ml creatine phosphokinase) was included from the start of the reaction.

Extra Plasmid DNA Increases the Accumulated Amount of D-Loop Species

Based on the finding that the homologous region of the reformed plasmid is transiently inaccessible due to residual RecA binding immediately after D-loop dissociation (Register and Griffith, 1988), we deduced that the reaction might contain unengaged active RecA-ssDNA. If this is the case, adding additional plasmid DNA to an ongoing reaction might increase the accumulated amount of D-loop species. At a low molar ratio of ATP/ADP, we expected a slight increase in the amount of D-loop species. In contrast, a large increase in the amount of D-loop species is expected when the molar ratio of ATP/ADP is high. Indeed, when we added additional plasmid to the reaction that had been ongoing for eight minutes and was low in the molar ratio of ATP/ADP, a small

increase in the amount of D-loop species was observed (Figure 9A). When we added a combination of extra ATP and additional plasmid to the ongoing reaction, the accumulated amount of D-loop species increased significantly (Figure 9B). These results suggest that unengaged RecA filaments formed on linear ssDNA substrates do exist in the D-loop reaction mixture. The presence of unengaged RecA-ssDNA filaments indicates that the homologous region within the negatively supercoiled dsDNA substrate is transiently inaccessible possibly due to RecA binding subsequent of reinvasion event.

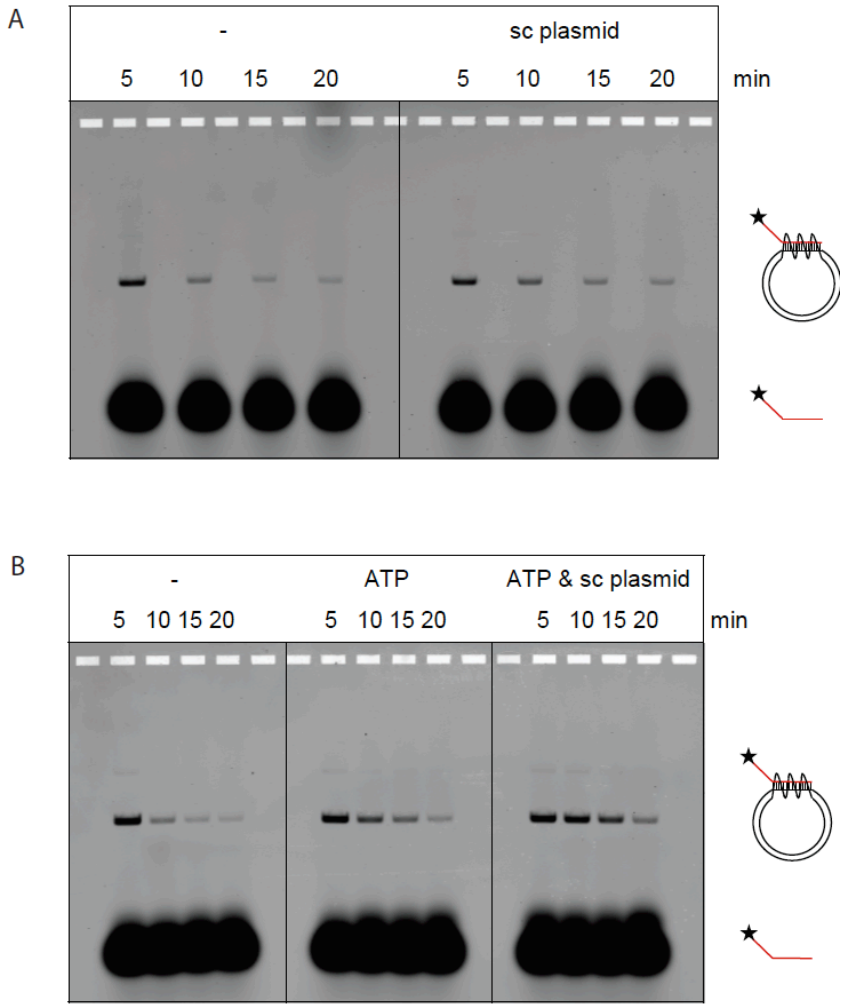

Figure 9. Unengaged RecA filaments, assembled on the linear ssDNA, are present in the D-loop reaction
(A) Additional negatively supercoiled dsDNA substrate (5.5 nM) was added to the ongoing D-loop reaction 8 minutes after initiation of the reaction.
(B) Additional ATP (1 mM) and the combination of additional ATP (1 mM) and negatively supercoiled dsDNA substrate (5.5 nM) were added 8 minutes after initiation of the D-loop reaction.

A Molar Ratio of One RecA to Three Nucleotides of ssDNA is Optimal for a High Accumulated Amount of D-Loop Species

It is well established that one RecA monomer binds to three nucleotides within filaments of RecA-ssDNA and RecA-dsDNA (Flory et al., 1984; Egelman and Stasiak, 1986; Chen et al., 2008). Thus we set the molar ratio of one RecA to three nucleotides of linear ssDNA substrates in our experiments. This molar ratio will not change throughout the reaction because the generation of displaced strand is offset by the formation of heteroduplex within the D-loop structure. Under this constant molar ratio, active RecA-

ssDNA filaments form and initiate both D-loop formation and dissociation events. As changing the molar ratio of ATP/ADP (that is important for forming active RecA-ssDNA filaments) changes the amount of D-loop species, we addressed whether varying the molar ratio of RecA to nucleotide of ssDNA would affect the accumulated amount of D-loop species during the cycle as well. Therefore we added additional ssDNA of 90 nucleotides (SK3) or 36 nucleotides (SS36) to ongoing D-loop reactions one minute after initiation with negatively supercoiled dsDNA. This decreased the molar ratio of RecA to nucleotide of ssDNA from 1:3 to 1:9 and 1:5.4, respectively. Similar to the result of adding additional ADP (Figure 8), addition of extra ssDNA of either 90 nucleotides (Figure 10A) or 36 nucleotides (Figure 10B) decreased the accumulated amount of D-loop species formed. The accumulated amount of D-loop species formed was lower in the reaction with the molar ratio of 1 RecA to 9 nucleotides of ssDNA than the reaction with the molar ratio of 1 RecA to 5.4 nucleotides of ssDNA. Thus, these results suggest that decreasing the molar ratio of one RecA to three nucleotides of ssDNA reduces the accumulated amount of D-loop species in the cycle. The larger the deviation, the lower the accumulated amount of D-loop species. These findings indicate that the reduction of active RecA filaments assembled on the linear ssDNA substrates and possibly the displaced strand, due to suboptimal molar ratio of RecA to nucleotides of ssDNA, result in low amounts of D-loop over time.

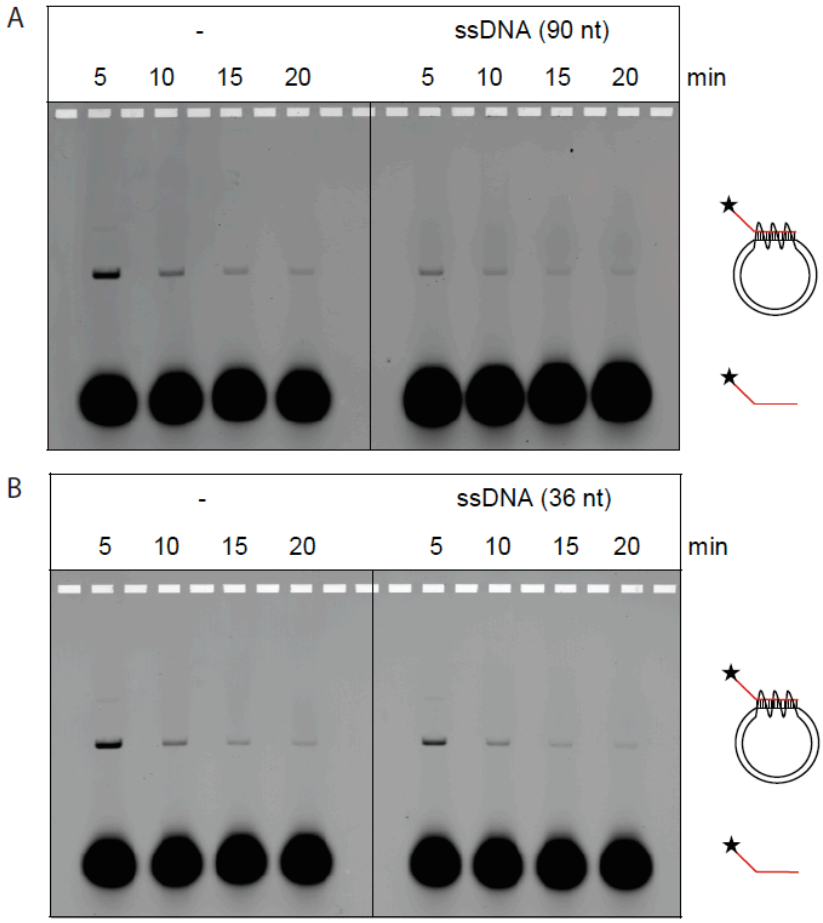

Figure 10. Optimal molar ratio of one RecA to three nucleotides of ssDNA is required to maintain the high accumulated amount of D-loop species in the cycle
(A) Labeled additional homologous ssDNA substrate of 90 nucleotides in length (SK3; 90 nM) was added 1 minute after initiation of the reaction.
(B) Unlabeled additional heterologous ssDNA of 36 nucleotides in length (SS36; 90 nM) was added 1 minute after initiation of the reaction.

DISCUSSION

The D-loop structure is the initial joint molecule formed during homologous recombination. D-loops can be extended either via branch migration or via *de novo* DNA synthesis, or dissociate. The stability of a D-loop thus determines the fate of DNA damage and the accuracy of information restoration (Kanaar et al., 2008). The mechanism of D-loop dissociation, as an integral part of the D-loop reaction cycle (Shibata et al., 1982-2) is thus of interest to understand the regulation of homologous recombination. Here, we show that the heterologous region of the invading linear ssDNA substrate is not involved in the mechanism of D-loop dissociation. This finding emphasizes the intrinsic role of D-loop structure, which consists of a heteroduplex and a displaced ssDNA, in the mechanism of D-loop dissociation. Similar to previous studies (Bachrati et al., 2006), we find that the naked D-loop species *per se* does not dissociate. Consistent with the stabilizing effect of the RecA K250R mutant that is unable to disassemble from the heteroduplex region within the D-loop, MutS and MutSL binding to a mismatch within the heteroduplex prevents D-loop dissociation. Furthermore, the dissociation of the D-loop can be prevented by increased competition of SSB with RecA to assemble onto the displaced strand of the D-loop structure. The addition of extra plasmid to the ongoing reaction (regardless of whether the molar ratio of ATP/ADP is low or high) reveals the presence of unengaged active RecA-ssDNA filaments in the reaction. A high molar ratio of ATP/ADP and an optimal molar ratio of one RecA to three nucleotide of ssDNA are required to maintain a high accumulated amount of D-loop species during the reaction.

D-Loop Dissociation Occurs via Reinvasion of RecA-Bound Displaced ssDNA into the Heteroduplex Region of the D-Loop Molecule

Two mechanistically distinct models for D-loop dissociation have been presented (Shibata et al., 1982-1). In the reinvasion model, after the first strand-exchange event between invading ssDNA and a supercoiled plasmid DNA, RecA proteins assemble into a filament on the displaced ssDNA of the D-loop structure. This nucleoprotein filament stimulates reinvasion into the D-loop heteroduplex region via strand exchange, resulting in displacement of the initial invading ssDNA fragment from the plasmid DNA. By contrast, in the rewinding model RecA proteins cooperatively bind along the dsDNA plasmid backbone, thereby inducing topological stress that drives dsDNA unwinding at one end of the D-loop structure, rewinding at the other end and displacement of the ssDNA oligonucleotide. The observation that the deproteinized D-loop species are stable (Figure 4; Bachrati et al., 2006) indicates that active RecA is required to induce D-loop dissociation, which is in agreement with both models. However, we find it highly unlikely that the D-loop dissociation observed in our system is occurring according to the mechanism proposed in the rewinding model. We used a 1:3 molar ratio of RecA to nucleotides to preform RecA filaments on a short and defined oligonucleotide. This amount of RecA proteins is exactly sufficient to cover the 54-bp heteroduplex region within the formed D-loop, and there is no additional RecA available to further extend the protein filament along the dsDNA plasmid backbone. Any extension of the filament into the dsDNA region would only be possible after dissociation of RecA from the heteroduplex region. Owing to the presence of merely a single and short D-loop

structure (54-bp) in the supercoiled plasmid, the dissociating amount of RecA (at maximum only 30 molecules per D-loop) may not be sufficient to induce the topological stress required to promote the unwinding and the rewinding events. However, the amount of dissociating RecA from a single D-loop would be exactly sufficient to allow rebinding to the displaced ssDNA strand and drive D-loop dissociation via reinvasion of the heteroduplex. Furthermore, considering the extent of base-paring, the reinvasion model (no net loss of base-pairs) is energetically more favorable than the rewinding model (loss of base-pairing in the RecA-unwound region of plasmid DNA adjacent to the D-loop). Taken together, our results suggests that the D loop cycle is established via RecA-driven reinvasion rather than RecA-mediated rewinding.

The observations, that the RecA K250R mutant (Figure 5) prevents, and that the MutS/MutSL complex (Figure 6) and SSB (Figure 7) delay D-loop dissociation, are in agreement with the reinvasion model. In the context of this model, the prevention of D-loop dissociation could be due to the RecA K250R mutant binding to the heteroduplex region or the presence of the MutS/MutSL complex on the heteroduplex that prevents reinvasion by the RecA-bound displaced strand. Similarly, the delay of D-loop dissociation exerted by SSB could be due to the competition of extra SSB that delays the formation of contiguous RecA filament on the displaced strand and thus the reinvasion event. The observation, that there are unengaged RecA filaments assembled onto the linear ssDNA substrates present in the D-loop reaction (Figure 9), implies that the homologous region of supercoiled plasmid is transiently inaccessible. In the context of the reinvasion model, this inaccessibility may be due to the binding of RecA proteins on the homologous region subsequent of the strand exchange events. The observation, that a high molar ratio of ATP/ADP increases the accumulated amount of D-loop species (Figure 8), implicates that the active RecA filaments assembled onto the linear ssDNA substrates and possibly the displaced strand of the D-loop structure in the D-loop cycle. Similarly the observation, that a suboptimal molar ratio of RecA to nucleotide of ssDNA decreases the accumulated amount of D-loop species (Figure 10), indicates the importance of forming contiguously active RecA filaments on both the linear ssDNA substrates and possibly the displaced strand of the D-loop structure in the D-loop cycle. These two implications are in agreement with the reinvasion model, in which the assembly of active RecA filament on the displaced strand of the D-loop structure requires high molar ratio of ATP/ADP and optimal molar ratio of one RecA to three nucleotides of ssDNA.

Model of RecA-Mediated D-Loop Cycle

Taking our results and existing data into account, we propose a coherent model for the cycle of D-loop formation and dissociation between the linear ssDNA and the negatively supercoiled dsDNA substrates (Figure 11). In the presence of ATP, RecA binds via its primary site (Mazin et al., 1996) to ssDNA and forms an active right-handed helical filament that is stretched ~150% relative to B-form DNA and extended between the nucleotide triplets (Flory et al., 1984; Chen et al., 2008) (step 1). A high molar ratio of ATP/ADP and an optimal molar ratio of one RecA to three nucleotides of ssDNA are desirable for the formation of active RecA-ssDNA filament. Upon finding the homologous region, this nucleoprotein filament invades and spools into the plasmid (step 2). The resulting D-loop intermediate I is in the state where the RecA secondary

DNA binding site of the filament is in contact with the homologous dsDNA (Mazin et al., 1996). Then, strand exchange is facilitated, independent of ATP hydrolysis, within the synaptic complex of RecA proteins and the three strands of DNA (Menetski et al., 1990; Rosselli and Stasiak, 1990) (step 3). The resulting D-loop intermediate II is in the state where the secondary DNA-binding site of RecA filament is bound with the displaced ssDNA (Mazin et al., 1996). However, the displaced strand may be sequestered by SSB (Mazin et al., 1998). When the ATP molecules bound between the interfaces of RecA monomers are hydrolyzed, RecA undergoes a conformational change and disassembles from the heteroduplex of D-loop (Ragunathan et al., 2011; step 4). The resulting naked D-loop intermediate III, which consists of a heteroduplex and a displaced ssDNA, is stable by itself. The displaced strand of this intermediate may be coated with SSB, but RecA dimers could nucleate and grow on the transiently exposed ssDNA that is released from SSB through sliding or unwrapping (Bell et al., 2012; step 5). The resulting D-loop intermediate IV is in the state where the primary site of the RecA filament is interacting with the displaced strand and the secondary DNA-binding site of the filament is in contact with the heteroduplex (Mazin et al., 1996). High molar ratio of ATP/ADP and optimal molar ratio of one RecA to three nucleotides of ssDNA are desirable for forming an active RecA filament with the displaced strand of the D-loop structure. Then, the nascent RecA filament assembled on the displaced strand reinvades the heteroduplex region of the D-loop by catalyzing strand exchange independent of ATP hydrolysis (Menetski et al., 1990; Rosselli and Stasiak, 1990; step 6). The resulting D-loop intermediate V is in the state where the displaced ssDNA (which was the invading strand) is bound to the secondary DNA-binding site of RecA filament (Mazin et al., 1996). However, the displaced strand (which was the invading strand) may be sequestered by the SSB proteins to reform active RecA-ssDNA filament (Mazin et al., 1998). The reinvasion event thus causes the D-loop structure to dissociate and reforms the linear ssDNA and RecA-bound negatively supercoiled dsDNA substrates (step 7). The resulting ssDNA substrate is released for another round of D-loop cycle. However, the homologous region of RecA-bound dsDNA substrate (intermediate VI) is inaccessible until RecA disassembly, which is catalyzed by RecA-dependent ATP hydrolysis (step 8). When the molar ratio of ATP/ADP has become low later in the reaction, the cycle is blocked and the remaining D-loop species (intermediate III) escape from being dissociated. This is supported by the detection of low amount of D-loop species up to 160 minutes of the reaction (Figure S3).

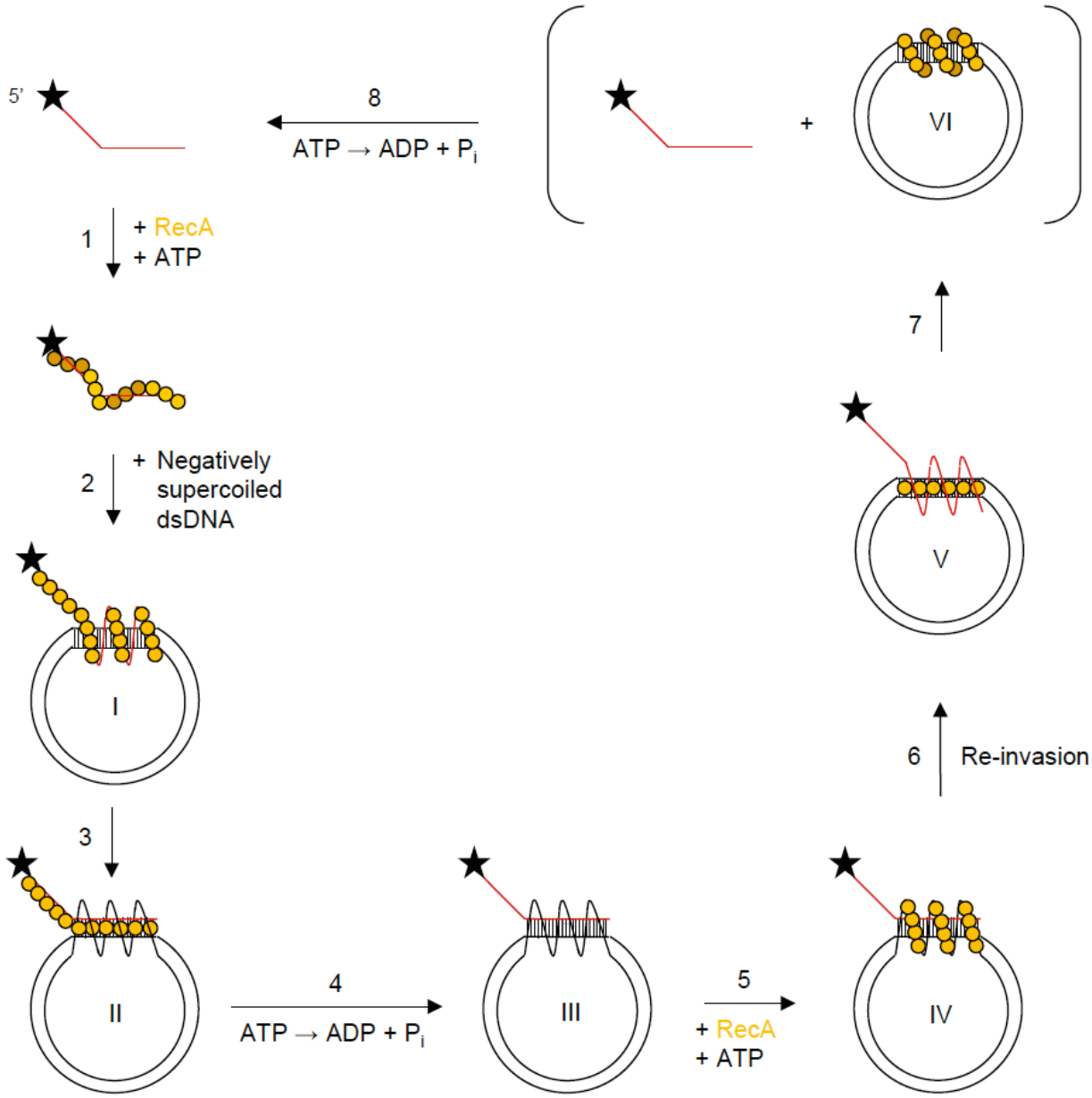

Figure 11. Model of D-loop cycle mediated by *Escherichia coli* RecA recombinase
In the presence of ATP, linear ssDNA substrate and RecA proteins form an active right-handed helical nucleoprotein filament (1). Upon localizing the homologous region, the RecA-ssDNA filament invades and spools into the negatively supercoiled dsDNA substrate (2). Within the synaptic complex of RecA protein and three DNA strands, strand exchange is facilitated independently of ATP hydrolysis (3). When ATP that is bound between interfaces of RecA monomers is hydrolyzed, RecA disassembles from the heteroduplex region of the D-loop (4). The resulting protein-free D-loop structure, which consists of a heteroduplex and a displaced ssDNA, is stable by itself. However, as long as ATP is present, a new RecA filament can assemble onto the displaced strand (5). This nascent RecA filament formed onto the displaced ssDNA reinvades the heteroduplex region of the D-loop structure (6). As a result, the linear ssDNA (which was the invading strand) is displaced and the homologous region of dsDNA substrate is bound with the RecA filament that mediated the reinvasion (7). The linear ssDNA substrate may enter another round of D-loop formation and dissociation after its displacement. However the homologous region of the negatively supercoiled dsDNA substrate does not become accessible again until the

occurrence of RecA disassembly, which is catalyzed by RecA-dependent ATP hydrolysis (8). See discussion in the main text for the description of the D-loop intermediates. Note that the helical and supercoiled nature of the dsDNA substrate is omitted from the illustration for clarity.

The Effect of ATP/ADP Molar Ratio in the D-Loop Reaction

We find that when the molar ratio of ATP/ADP becomes low later in the reaction, the accumulated amount of D-loop species in the cycle decreases dramatically. This finding implies that the formation of active RecA filaments with the linear ssDNA substrates and the displaced strand of D-loop structure is vital for maintaining the high accumulated amount of the D-loop species in the cycle. We calculated the molar ratio of ATP/ADP throughout the D-loop reaction. The ATP hydrolysis rate of RecA in the presence of ssDNA, and in the absence of dsDNA and SSB, is reported between 25-30 min^{-1} (Kowalczykowski, 1991). Using the mean rate of 27.5 min^{-1}, and not taking into account possible effects of supercoiled dsDNA and SSB on the ATP hydrolysis rate of RecA, we found that the ATP/ADP molar ratio is 1.45, 1.24, 0.92, 0.68, 0.5 after 1, 2, 4, 6 and 8 minutes of the reaction respectively (Figures 3, 5A and S1). It is reported that the ATP/ADP molar ratio lower than 2-3 is able to shift the DNA-binding affinity state of RecA proteins from high to low (Menetski et al., 1988), and complete disassembly from ssDNA occurs when the ATP/ADP molar ratio is lower than 1 (Lee and Cox, 1990). The decrease in the accumulated amount of D-loop over time, may be due to the decrease of the ATP/ADP molar ratio in our system affecting the assembly of active RecA filaments on ssDNA.

Implications Derived from the Detection of Relaxed D-Loop Species

In general, during the early time points (such as the second and the fifth minutes of the reaction, Figures 3, 4B, 5, 7, 8, 9, 10, S1, S2, S3, and S4), we detected a faint DNA band above the DNA band representing the negatively supercoiled D-loop species. Both DNA bands diminish in intensity at late time points. The faint band above the D-loop species on negatively supercoiled DNA is most likely the relaxed D-loop species (Shibata et al., 1979) because the purified dsDNA substrate contains low amount of both nicked circular dsDNA and linear dsDNA as shown by the ethidium-bromide staining in Figure S4B. Consistent with the finding that the formation of D-loop species with linear dsDNA is highly inefficient (Figure S4A), we did not detect an extra band below the faint band of the relaxed D-loop species in all the experiments. Single-molecule studies reveal that decreasing the end-to-end distance of linear dsDNA increases the rate of intersegmental homology search mediated by the RecA nucleoprotein filament (Forget and Kowalczykowski, 2012). Conformationally, a nicked circular dsDNA is similar to a linear dsDNA with its two ends being held close to each other as performed in their experiments. Thus the observation, that D-loop structure forms in the nicked circular dsDNA but not the linear variant, could be explained by the more constrained 3D conformation in the former that facilitates intersegmental homologous sampling by the RecA-ssDNA filament. In addition, RecA filaments assembled on ssDNA of 1762-nt and 430-nt, but not 162-nt, are found pairing with the homologous regions within the manipulated dsDNA (48502 bp) with the smallest bead distance (2 μm; Forget and Kowalczykowski, 2012). Although the ssDNA used in our experiments is 90 nucleotides in length, the significantly shorter nicked circular dsDNA (2686 bp) may compensate for the requirement of longer ssDNA used in the single-

molecule experiments by having shorter heterologous DNA segments that may facilitate the search for the homologous region.

Conclusions

Taken together, we propose a model for the D-loop cycle that emphasizes the reinvasion of the heteroduplex region by a RecA filament assembled onto the displaced strand of the D-loop. In addition, an optimal molar ratio of one RecA to three nucleotides (of ssDNA) and a high molar ratio of ATP/ADP promote D-loop accumulation during the cycle. The phenomenon of D-loop dissociation has also been reported in the reaction mediated by yeast Rad51 (Mazin et al., 2000). Whether the mechanism of D-loop dissociation in the eukaryotic reaction is similar to the RecA-mediated reaction remains to be studied. The phenomenon of *in vitro* D-loop dissociation suggests that D-loop structures formed *in vivo* with short invading ssDNA may be inherently unstable and might dissociate before they can be extended via branch migration or *de novo* DNA synthesis. D-loops are key intermediates in all DNA transaction involving HR and as such constitute a possible vital regulatory point (Heyer et al, 2010). The reversibility of D-loops allows quality control because of constant building up and reversal of both appropriate and inappropriate reaction intermediates that may be differentially controlled using regulatory proteins (Kanaar et al, 2008). For example, XRCC3 is proposed to stabilize heteroduplex DNA (Brenneman et al., 2002) and RAD51AP1 binds to a variety of branched DNA structures of HR intermediates (Modesti et al., 2007; Wiese et al., 2007). Our model may thus lay the foundation for future studies on the stabilization of D-loops mediated by accessory proteins.

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SUPPLEMENTAL FIGURES

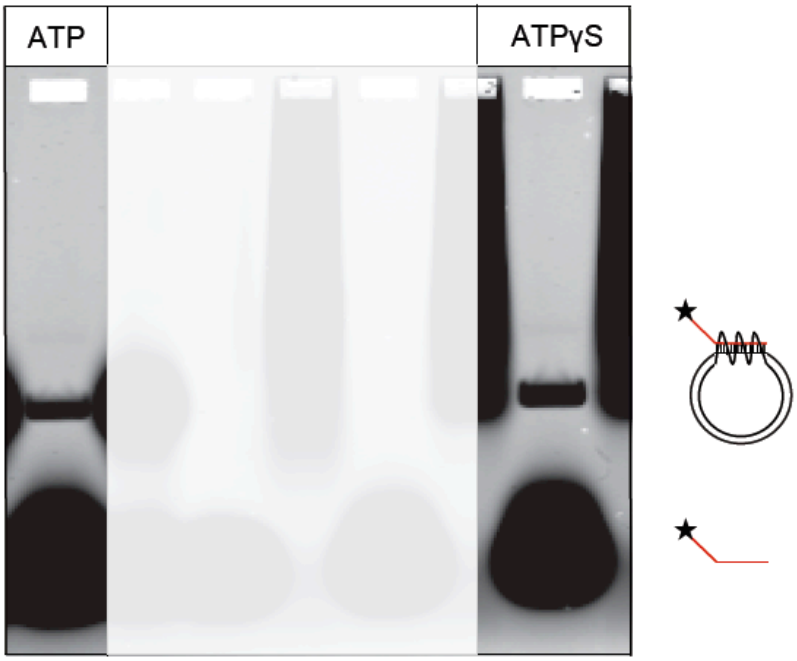


Figure S1. D-loop dissociation starts after 1 minute of the reaction
D-loop reactions were incubated for 1 minute at 37 °C in the presence of ATP (first lane) and ATPγS (last lane). The amount of observed D-loop product formed in the presence of ATP is approximately 80% of that formed with ATPγS, suggesting D-loop dissociation is already taking place at this time point.

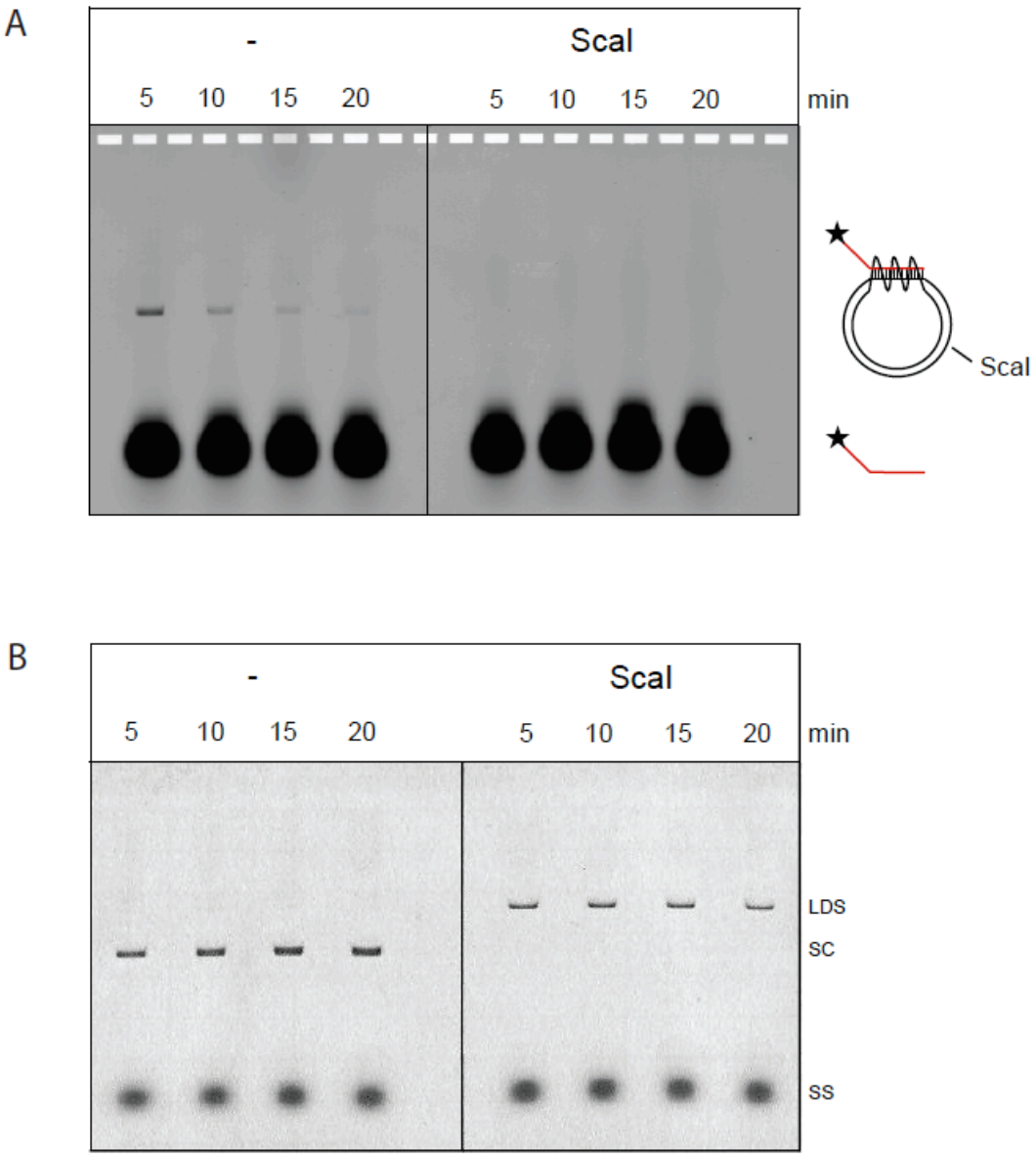


Figure S2. Linearization of supercoiled dsDNA in the ongoing reaction prevents D-loop formation
(A) Endonuclease ScaI (0.5 U/µl) was added to the ongoing D-loop reaction 1 minute after initiation. The unique ScaI site is located about 700 bp away from the end of the D-loop structure.
(B) The same gel was stained with ethidium bromide. LDS, linear double-strand; SC, supercoiled; SS, single-strand DNA.

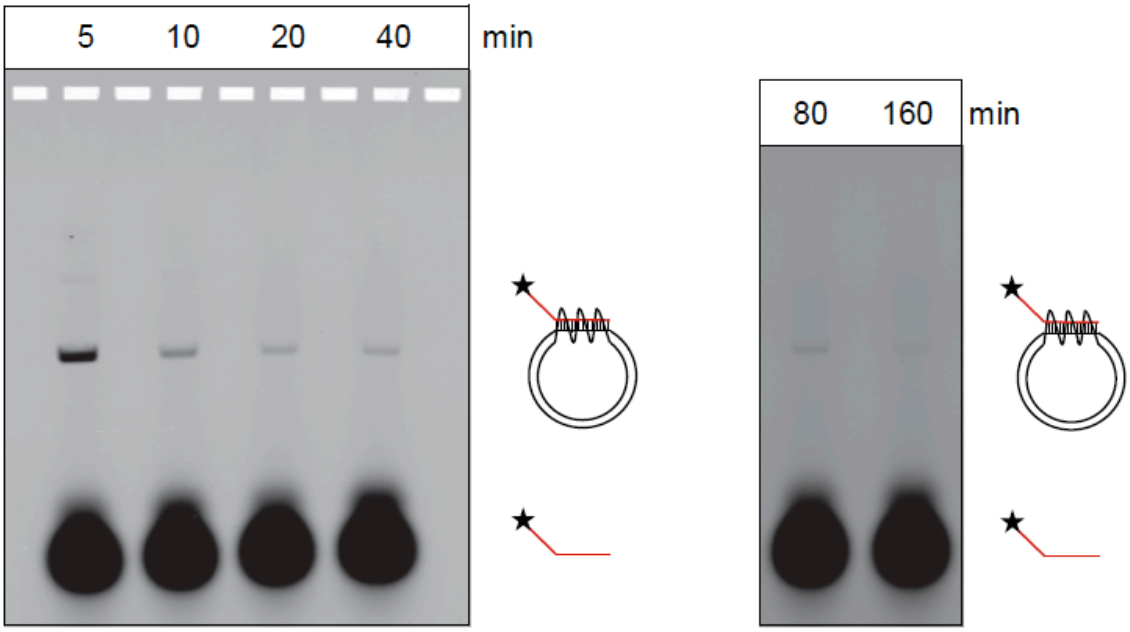
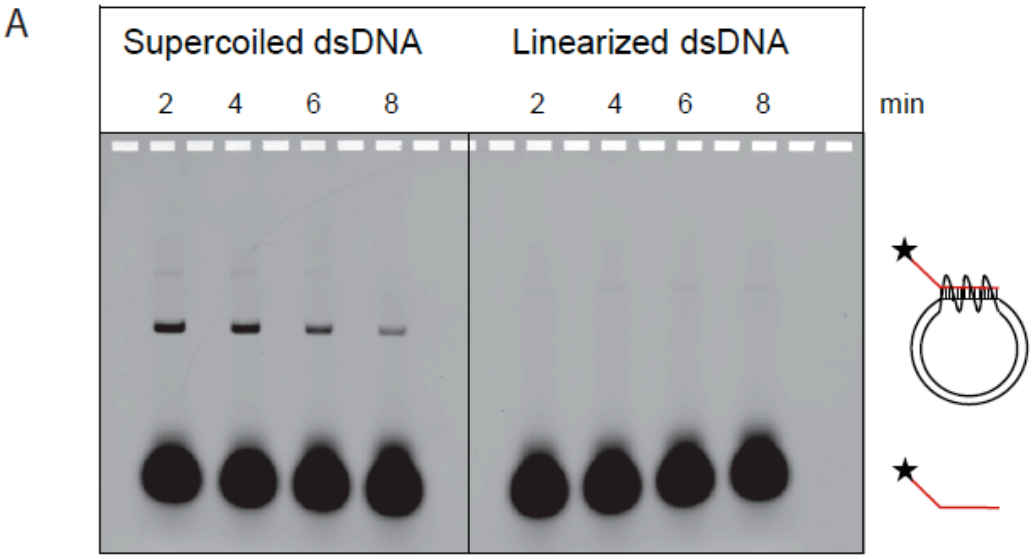


Figure S3. D-loop species is detected up to 160 minutes into the reaction
D-loop reaction was performed for 40 minutes (left panel) and 160 minutes (right panel).



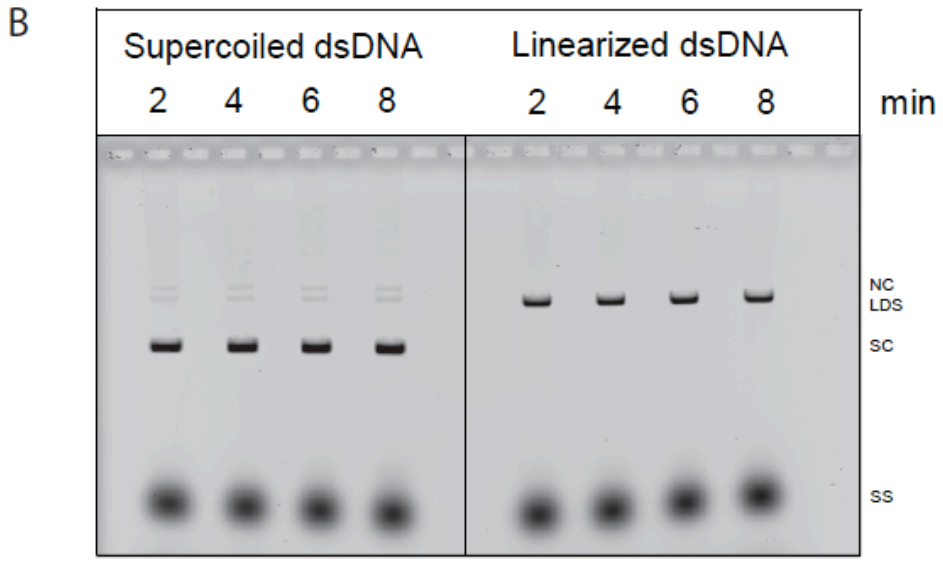


Figure S4. D-loop formation with linear dsDNA substrate is highly inefficient
(A) Blunt-ended linear dsDNA substrate (pUC19 digested with ScaI) was used to initiate the reaction.
(B) The same gel was stained with ethidium bromide. NC, nicked circular; LDS, linear double-strand; SC, supercoiled; SS, single-strand DNA.

Chapter 3

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TITLE

The Mechanism of Antirecombination Mediated by *Escherichia coli* MutS and MutL

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ABSTRACT

Homeologous recombination between divergent DNA sequences is inhibited by DNA mismatch repair. In *Escherichia coli,* MutS and MutL respond to DNA mismatches within recombination intermediates and prevent the recombination intermediates from heteroduplex formation by an unknown mechanism. Here, using purified proteins and DNA substrates, we find that in addition to mismatches within the heteroduplex region, secondary structures formed within the displaced ssDNA of the recombination intermediates are involved in the trapping mechanism. Furthermore, we find that the activity of MutS tetramerization plays a role in the inhibition of recombination by MutS and MutL. We present a model that explains how higher-order complex formation of MutS, MutL and DNA blocks branch migration by preventing rotation of the DNA strands within the recombination intermediate.

INTRODUCTION

DNA mismatch repair (MMR) is one of several important DNA repair processes conserved from bacteria to mammals. MMR repairs base-base mismatches and insertion/deletion loops generated during replication (Iyer et al., 2006; Jiricny, 2006, 2013). In addition, MMR proteins prevent illegitimate recombination between diverged (homeologous) sequences in order to promote genome stability. Thus, inactivation of MMR genes not only results in mutator but also in hyperrecombination phenotypes. The increased frequency (up to 1000 fold) of interspecies conjugation between *Escherichia coli* and *Salmonella typhimurium* in *mutS*, *mutL*, *mutH* and *uvrD* recipients indicates that MMR acts as a barrier to RecABCD-dependent homeologous recombination (Rayssiguier et al., 1989, 1991; Stambuk and Radman, 1998). Similar findings are obtained for interspecies transduction (Rayssiguier et al., 1989; Zahrt and Maloy, 1997) and transformation (Majewski et al., 2000). Likewise, in eukaryotes, individual MMR proteins have roles of varying magnitude in the prevention of homeologous recombination (Selva et al., 1995; Myung et al., 2001; Datta et al., 1996; Nicholson et al., 2000; Spell and Jinks-Robertson, 2004; Welz-Voegele and Jinks-Robertson, 2008; de Wind et al., 1995; Abuin et al., 2000). In higher eukaryotes, abortion of homeologous reactions is thought to protect the genome from recombination events between diverged repeats (de Wind et al., 1999). This is important in humans in which the amount of repetitive DNA is as high as 50%.

An intriguing question is how the MMR and recombination pathways are integrated at the molecular level. Common events during MMR and antirecombination reactions include MutS binding to a DNA mismatch (Su and Modrich, 1986; Su et al., 1988), complex formation between MutS and MutL (Grilley et al., 1989; Galio et al., 1999; Spampinato and Modrich, 2000; Schofield et al., 2001; Acharya et al., 2003; Selmane et al., 2003), and MutL-mediated orchestration of downstream MutH (GATC endonuclease) (Welsh et al., 1987; Au et al., 1992) and UvrD (3'-5' helicase) (Matson, 1986; Yamaguchi et al., 1998; Dao and Modrich, 1998). MutS and MutL, as the most upstream players of MMR, are potent in preventing homeologous recombination *in vivo* (Rayssiguier et al., 1989). Biochemical studies demonstrate that MutS and MutL block RecA-mediated homeologous strand exchange through prevention of branch migration within the recombination intermediates (Worth et al., 1994). However the molecular mechanism of this inhibition remains unknown. DNA mismatches do not occur within recombination filaments until after strand exchange has taken place, and somehow MMR proteins are able to inhibit the ongoing branch migration into a region where mismatches are not yet present.

Here, we investigated the mechanism of MutS and MutL-induced trapping of the homeologous recombination intermediates. Interestingly, we found that the displaced ssDNA of the recombination intermediates and higher-order protein complex formation are required for the MutS-MutL antirecombination activity. We present a model that explains how simultaneous binding of mismatches within the heteroduplex and the secondary structures of displaced ssDNA by MutS and MutL blocks branch migration by preventing rotation of the DNA strands within the recombination intermediate.

MATERIALS AND METHODS

DNA Substrates

Viral ssDNAs from bacteriophage M13 and fd and closed circular replicative form (RF) DNA from M13 were prepared as described (Sambrook and Russell, 2001). The M13 linear dsDNA was obtained by digestion of M13 RF DNA with HpaI restriction endonuclease (New England Biolabs). These circular ssDNAs from M13 and fd, and HpaI-linearized dsDNA from M13 bacteriophage were further purified from agarose gels using electroelution with D-Tube Dialyzer (Novagen).

Oligonucleotides were of the following sequence. SK3 (Mazin et al., 2000): 5'-AATTCTCATTTTACTTACCGGACGCTATTAGCAGTG*GCAGATTGTACTGAGAGTGC ACCATATGCGGTGTGAAATACCGCACAGATGCGT*-3'. The sequence of 54 nucleotides at the 3'-end (shown in italic) is homologous to pUC19. The underlined nucleotide is the position for G/T mismatch manipulation. The SK3 with one mismatch is named as SK3-1mm. SK3-rev is the complementary oligonucleotide to SK3. HJ01: 5'-GACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCACCTGCAGGTT CACCC-3'. For D-loop reactions, SK3 was labeled with Alexa Fluor 532. For EMSA, HJ01 and poly-dT were labeled with Alexa Fluor 488. For strand exchange using short substrates, SK3-rev was labeled with Cy5. All oligonucleotides were ordered from Eurogentec. Secondary structures of sequences shown in Figures 4C and D were predicted using the Mfold server (http://mfold.rna.albany.edu) with conditions set at 25 or 150 mM NaCl, 10 mM $MgCl_2$ and 37°C.

Proteins

RecA WT was purified from *E. coli* strain GE1710 using a protocol based on spermidine acetate precipitation (Harmon and Kowalczykowski, 1998). The RecA K250R mutant was purified as described (Cox et al., 2008). RecA was flash-frozen in small aliquots and stored at -80°C. MutS was purified essentially as described (Natrajan et al., 2003) with the following adaptations: NaCl was replaced with KCl in the lysis and chromatography buffers. Anion exchange chromatography on the MonoQ column was performed after heparin affinity chromatography, followed by an additional POROS S column (Life Technologies) using the same buffers and salt gradient. After size exclusion chromatography, MutS was flash-frozen in 25 mM HEPES-KOH pH 7.5, 250 mM KCl, 1 mM DTT, 50% glycerol and stored at -80°C. MutL was purified as described (Lebbink et al., 2010), except that KCl replaced NaCl in all buffers and the heparin gradient was developed from 0.1-1.0 M KCl. MutL was flash frozen in 20 mM Tris-HCl pH 8.0, 0.5 M KCl, 1 mM DTT, 50% glycerol and stored at -80°C.

Scanning Force Microscopy

DNA intermediates purified with electroelution were incubated with 1.6 M EtBr for 5 minutes at 37°C to reduce DNA aggregation. Then, the DNA was mixed with 20 mM Tris-HCl pH 7.5, 20 mM NaCl, 50 µM spermidine and 200 nM of *E. coli* SSB (Promega) and incubated for 5 minutes at 37°C prior to the deposition on freshly cleaved mica. After 1 minute of deposition at room temperature, the mica surface was washed with milliQ water and dried with streams of filtered air. Images were obtained with the NanoScope IIIA (Digital Instruments; Santa Barbara, CA, USA) operating in tapping

mode in air with a type E scanner. Uncoated silicon Pointprobe tips were NHC-W type, resonance frequency 310-372 kHz, force constant *C* = 29.0-52.0 N/m, (Nanosensors supplied by Veeco Instruments, Europe). NanoScope images were analyzed using IMAGE SXM 1.62 (NIH-IMAGE version modified by Steve Barrett, Surface Science Research Centre, University of Liverpool, Liverpool, U.K.). The contour lengths of SSB-coated ssDNA gaps and dsDNA tails of branched intermediates were traced manually and reported in μm, uncorrected for the apparent shortening of the ssDNA due to wrapping around SSB. This does not affect the relative comparison between intermediates 4 and 5.

Strand Exchange Assay Using M13 and fd Bacteriophage DNA

Circular single-stranded M13 or fd phage DNA (1.5 nM) was incubated for 5 minutes at 70°C to remove secondary structures. Recombinase filaments were formed by addition of 50 mM HEPES-KOH pH 7.5, 12 mM $MgCl_2$, 0.5 mM DTT, 6 mM phosphocreatine, 10 U/ml creatine phosphokinase, 5% glycerol, 3 mM ATP and 3.8 μM RecA and incubated at 37°C for 10 minutes. The ratio of RecA to ssDNA nucleotide used was approximately 1:2.5. Samples were incubated at 37°C for 10 minutes, 0.4 μM SSB was added and incubation was continued for another 10 minutes. When appropriate, MutS, MutL and/or S1 nuclease (Roche) were added at varying concentrations. Next, strand exchange was initiated with 0.6 nM of M13 linear dsDNA at 37°C. Final reaction volumes amounted up to 50 μl. At various time points 9 μl samples were taken and stopped with 0.1% SDS, 25 mM EDTA and 153 μg/ml proteinase K at 37°C for 15 minutes. These deproteinized DNA samples were subjected to electrophoresis for 12 hours at 1.4 V/cm on 0.8% agarose in 1x TAE at room temperature. The gels were stained for 45 minutes at room temperature with 0.3 μg/ml ethidium bromide. These stained gels were scanned and visualized with Typhoon TRIO Variable Mode Imager (GE Healthcare) using the standard settings for ethidium bromide.

D-Loop Reaction

RecA-ssDNA filaments were pre-formed at 37°C for 5 minutes with 50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT, 5% glycerol, 1 mM ATP, 45 nM SK3-AF532 (90 nt) or SK3-1mm-AF532 (90 nt), and 1.35 μM RecA. Then, 135 nM SSB was added and reaction mixtures were further incubated for 5 minutes. MutS and/or MutL were added before the D-loop reaction was initiated by adding 7 nM supercoiled pUC19. The mixture was then incubated at 37°C and 9 μl aliquots were taken at various time points and denatured with 0.1% SDS, 25 mM EDTA and 153 μg/ml proteinase K and incubated at 37°C for 10 minutes. Samples were loaded onto 1.3% agarose gels in 1x TA buffer and 10 mM magnesium acetate. Electrophoresis was carried out for 3 hours at 4.7 V/cm at 4°C. Gels were scanned and visualized with Typhoon TRIO Variable Mode Imager using the standard setting for Alexa Fluor 532.

Crosslink of D-Loop Reaction

RecA-ssDNA filaments were pre-formed at 37°C for 5 minutes with 50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT, 5% glycerol, 1 mM ATP, 45 nM SK3-AF532 (90 nt) and 1.35 μM RecA. Then, 135 nM SSB was added and reaction mixtures were further incubated for 5 minutes. The D-loop reaction was initiated by adding 7 nM

supercoiled pUC19 and incubated at 37°C for 2 minutes. Then, 9 µl aliquots samples were denatured with 0.1% SDS, 25 mM EDTA and 153 µg/ml proteinase K or not treated or treated with glutaraldehyde. Non-treated samples were kept on ice. Denatured and glutaraldehyde-treated samples were further incubated at 37°C for 10 minutes. Samples were loaded onto 1.3% agarose gels in 1x TA buffer and 10 mM magnesium acetate. Electrophoresis was carried out for 5 hours at 4.7 V/cm at 4°C. Gels were scanned and visualized with Typhoon TRIO Variable Mode Imager using the standard setting for Alexa Fluor 532.

Strand Exchange Assay Using Short DNA Substrates

RecA filaments on ssDNA oligonucleotides (90 nM SK3 or SK3-1mm) were formed in 50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT, 5% glycerol, 3 mM ATP and 2.7 µM RecA at 37°C for 5 minutes. The ratio of RecA to ssDNA nucleotide was 1:3. Next, 270 nM SSB was added followed by further incubation at 37°C for 5 minutes. Mismatch repair proteins MutS and MutL were added and strand exchange was initiated with 10 nM of AF532-Cy5 doubly-labeled linear dsDNA at 37°C. Reactions were stopped at various time points with 0.1% SDS, 25 mM EDTA and 153 µg/ml proteinase K at 37°C for 10 minutes. Samples were subjected to electrophoresis on 10% polyacrylamide gels in 1x TA buffer and 10 mM magnesium acetate. The gels were scanned and visualized with Typhoon TRIO Variable Mode Imager using the standard settings for Alexa Fluor 532 and Cy5.

Electrophoretic mobility shift assay using ssDNA

The reaction mixture of 50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT, 150 mM NaCl, 5% glycerol, 25 µM ADP per 100 nM MutS, 1 mM ATP, and various concentrations of MutS was incubated at 37°C for 5 minutes. SSB (3.1 nM, 6.3 nM, 12.5 nM or 25 nM) was added when appropriate. Then, 5 nM of AF488-HJ01 ssDNA (61 nt) or AF488-poly-dT ssDNA (60 nt) was added and further incubated at 37°C for 2 minutes. Samples were put on ice before loading onto 3% polyacrylamide gels in 1x TA. After electrophoresis at 4°C, the gels were scanned and visualized with Typhoon TRIO Variable Mode Imager using the standard setting for Alexa Fluor 488.

Electrophoretic mobility shift assay using mismatched DNA

The reaction mixture of 50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT, 150 mM NaCl, 5% glycerol, 100 µM ADP, 1 mM ATP, MutS and/or MutL was incubated at 37°C for 5 minutes. Then, 2.5 nM of Cy5-SK3 dsDNA with 1 mismatch (90 bp) was added and further incubated at 37°C for 2 minutes. Samples were put on ice before loading onto 3.7% agarose gels in 1x TA buffer. After electrophoresis at 4°C, the gels were scanned and visualized with Typhoon TRIO Variable Mode Imager using the standard setting for Cy5.

S1 Nuclease Activity

To compare S1 nuclease activity on ssDNA versus dsDNA, reaction mixtures containing 50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT, 20 mM NaCl, 5% glycerol, 1 mM ATP, 2.5 nM Cy5-SK3-revssDNA (90 nt) or Cy5-SK3 dsDNA (90 bp) and 0.32 U/ul S1 nuclease were assembled and incubated at 37°C. Samples (9 µl) were

taken at various time points and denatured with 1 µl 10% SDS. Then, samples were loaded onto 10% polyacrylamide gels in 1x TAE and gels were scanned and visualized with Typhoon TRIO Variable Mode Imager using the standard setting for Cy5.

To study S1 nuclease activity on ssDNA circles under strand exchange conditions, circular single-stranded M13 phage DNA (1.5 nM) was incubated for 5 minutes at 70°C to remove secondary structures. Recombinase filaments were formed by addition of 50 mM HEPES-KOH pH 7.5, 12 mM $MgCl_2$, 0.5 mM DTT, 6 mM phosphocreatine, 10 U/ml creatine phosphokinase, 5% glycerol, 3 mM ATP and 3.8 µM RecA and incubated at 37°C for 10 minutes. Samples were incubated at 37°C for 10 minutes, 0.4 µM SSB was added and incubation was continued for another 10 minutes. Next, reaction was initiated with 0.04 U/µl of S1 nuclease (Roche) at 37°C. Final reaction volumes amounted up to 50 µl. At various time points 8 µl samples were taken and stopped with 0.1% SDS, 25 mM EDTA and 153 µg/ml proteinase K at 37°C for 15 minutes and 65°C for 7.5 minutes. These deproteinized DNA samples were electrophoresed on 1.5% agarose in 1x TA at room temperature. The gels were stained for 30 minutes at room temperature with 1x Sybr Green (Invitrogen). These stained gels were scanned and visualized with Typhoon TRIO Variable Mode Imager (GE Healthcare) using the standard settings for Sybr Green.

RESULTS

Characterization of Recombination Intermediates Formed during RecA-Mediated Homeologous Strand Exchange

To mechanistically study MMR-directed inhibition of homeologous recombination, we established and characterized RecA-mediated strand exchange reactions using the 6.4 kb genomes of bacteriophage fd and M13 as DNA substrates. In the homologous reaction, linear dsDNA (designated as species 1 in Figure 1A) and circular ssDNA substrates (species 2) from the bacteriophage M13 were used. The homeologous reaction was performed between fd circular ssDNA and M13 linear dsDNA, whose DNA sequences diverge by approximately 3% (Table 1). Both homologous and homeologous reactions form DNA intermediates (species 3, 4 and 5) before products of nicked circular dsDNA (species 6 in Figure 1A) and linear ssDNA (species 7) are generated.

Based on structural analysis of DNA intermediates formed during strand exchange with RecA and ATPγS (Menetski et al., 1990) and Rad51 (Holmes et al., 2002), intermediates 3, 4 and 5 observed in our system are expected to be joint molecules. Knowledge of the exact structure and stoichiometry of these intermediates is essential for a mechanistic explanation of the effect of MMR proteins on strand exchange, thus we examined the structures of DNA intermediates 4 and 5 from the homeologous reaction (red and blue box, Figure 1A) with scanning force microscopy (SFM). We incubated purified DNA intermediates with *E. coli* ssDNA binding protein (SSB) before deposition, allowing us to distinguish ssDNA from dsDNA in the SFM images. Intermediates 4 and 5 are joint molecules consisting of one circular ssDNA and one linear dsDNA substrate (Figure 1B), as was shown for the corresponding joint molecules observed with RecA in the presence of ATPγS (Menetski et al., 1990) and those categorized as JM1 generated with Rad51 (Holmes et al., 2002). Next, we

measured the contour length of the protein-free linear dsDNA tail and the SSB-bound ssDNA gap in the circle of the intermediates. Histograms of the measurements (Figure 1C) revealed that DNA intermediate 4 is the precursor of intermediate 5 because it has a longer ssDNA gap in the circle and a longer linear dsDNA tail. Unlike the RecA-ATPγS and Rad51-generated intermediates (Menetski et al., 1990; Holmes et al., 2002), joint molecules containing multiple linear dsDNA molecules are not observed as distinct species. Thus we conclude that DNA intermediates 4 and 5 are sequential on-pathway reaction intermediates that can be used as a read-out for strand exchange progress in addition to the appearance of nicked circular dsDNA product.

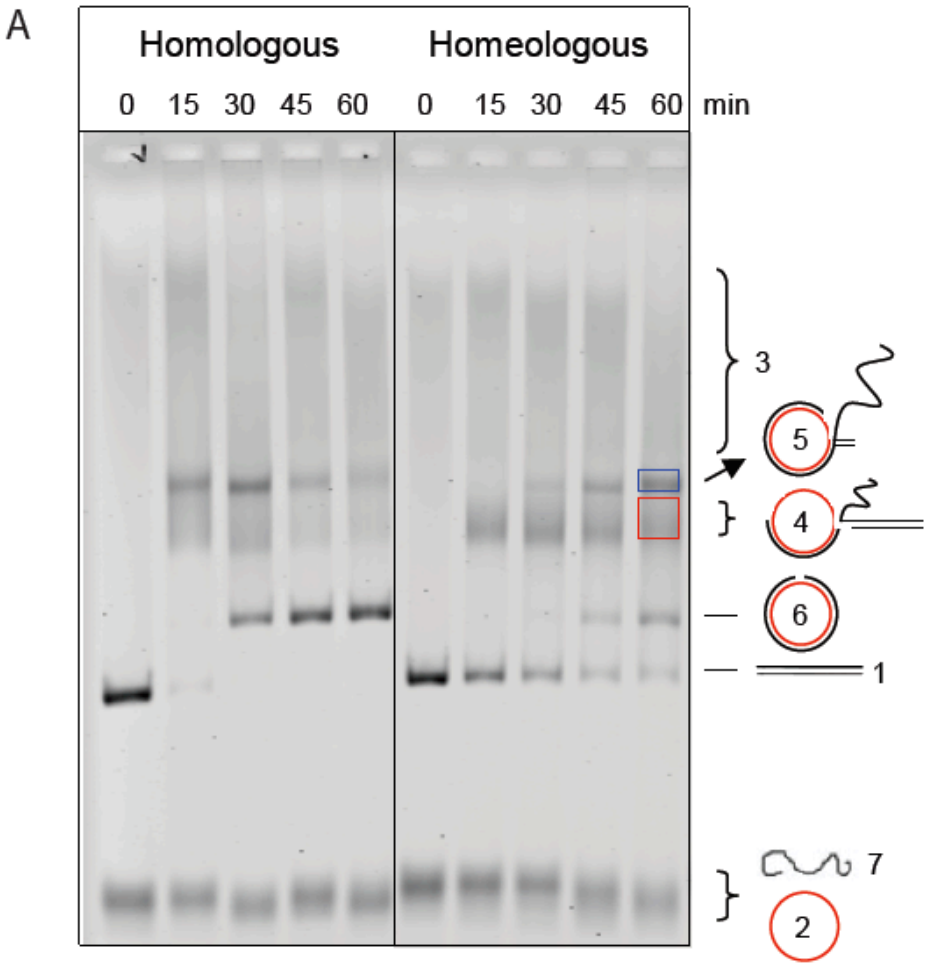

Figure 1. MutS-MutL Inhibits Progression of DNA Strand Exchange during Homeologous Recombination by Acting on Defined Intermediates
(A) Time courses of homologous and homeologous strand exchange using bacteriophage DNA (~6.4 kb). The numbering system of the DNA species, schematically depicted on the right of the gel, reflects their order of appearance.

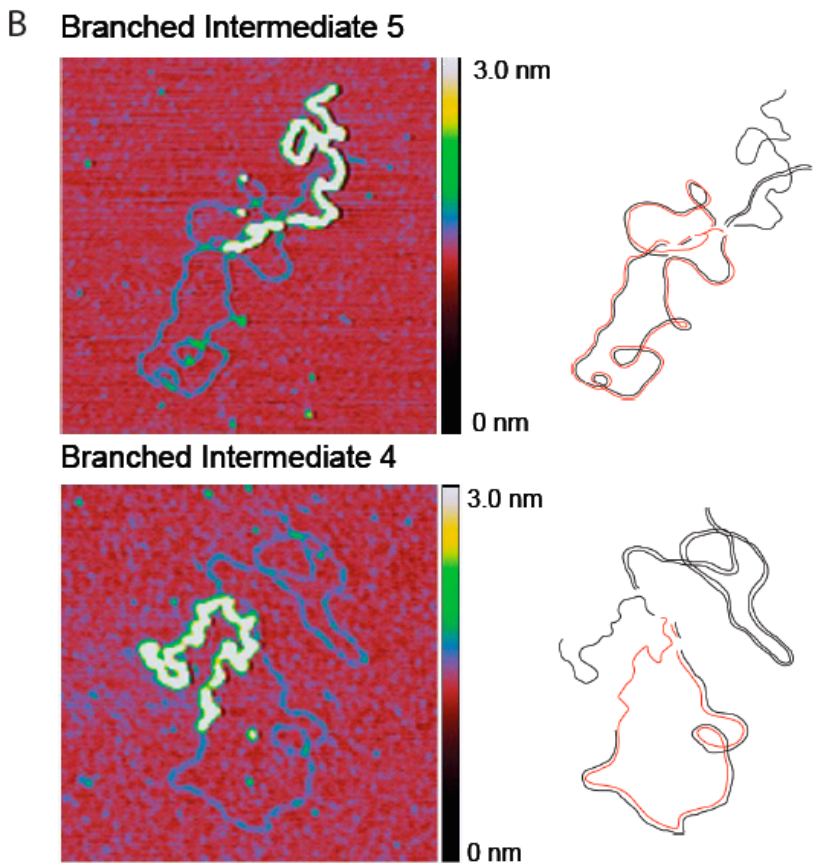

Figure 1. MutS-MutL Inhibits Progression of DNA Strand Exchange during Homeologous Recombination by Acting on Defined Intermediates

(B) Defined reaction intermediates consist of one ssDNA and one dsDNA molecule. Gel purified DNA intermediates 4 and 5 from the homeologous reaction were incubated with SSB to mark ssDNA regions and were analyzed with scanning force microscopy. SFM images are 0.6 x 0.6 µm and the z dimension is indicated by the color of the bar. Regions of dsDNA appear as a thin blue line, SSB-coated ssDNA appears as a thick yellow line. Drawings next to the SFM images are interpretations of the DNA intermediates between one circular fd ssDNA (red) and one linear M13 dsDNA (black).

MutS and MutS-MutL Block Heteroduplex Formation in Homeologous Strand Exchange

MutS and MutS-MutL inhibit homeologous strand exchange *in vitro* (Worth et al., 1994, 1998). Here we recapitulated these results. During a one-hour time course we achieved weak inhibition of homeologous recombination by MutS while the homologous reaction remained unaffected (Figure S1A). In addition, we reproduced the observation that MutL functions as an enhancer of MutS-mediated inhibition while affecting neither the homologous nor the homeologous reaction on its own (Figures S1B and S1C). Interestingly, this inhibition depended on ATP hydrolysis by MutL, because ATPase deficient MutL E29A (Ban et al., 1999) was unable to enhance the MutS-mediated inhibition (Figure S1D). To further ensure that MutS-MutL is not just delaying homeologous strand exchange, but really inhibits the reaction, we prolonged the reaction incubation time to 3 hours. We still observed accumulation of DNA intermediates and inhibition of product formation (Figure 1D). Thus, mismatch-specific inhibition of recombination by MutS-MutL suggests that mismatches in the heteroduplex region activate MutS and MutL to prevent DNA intermediates from extensive heteroduplex formation.

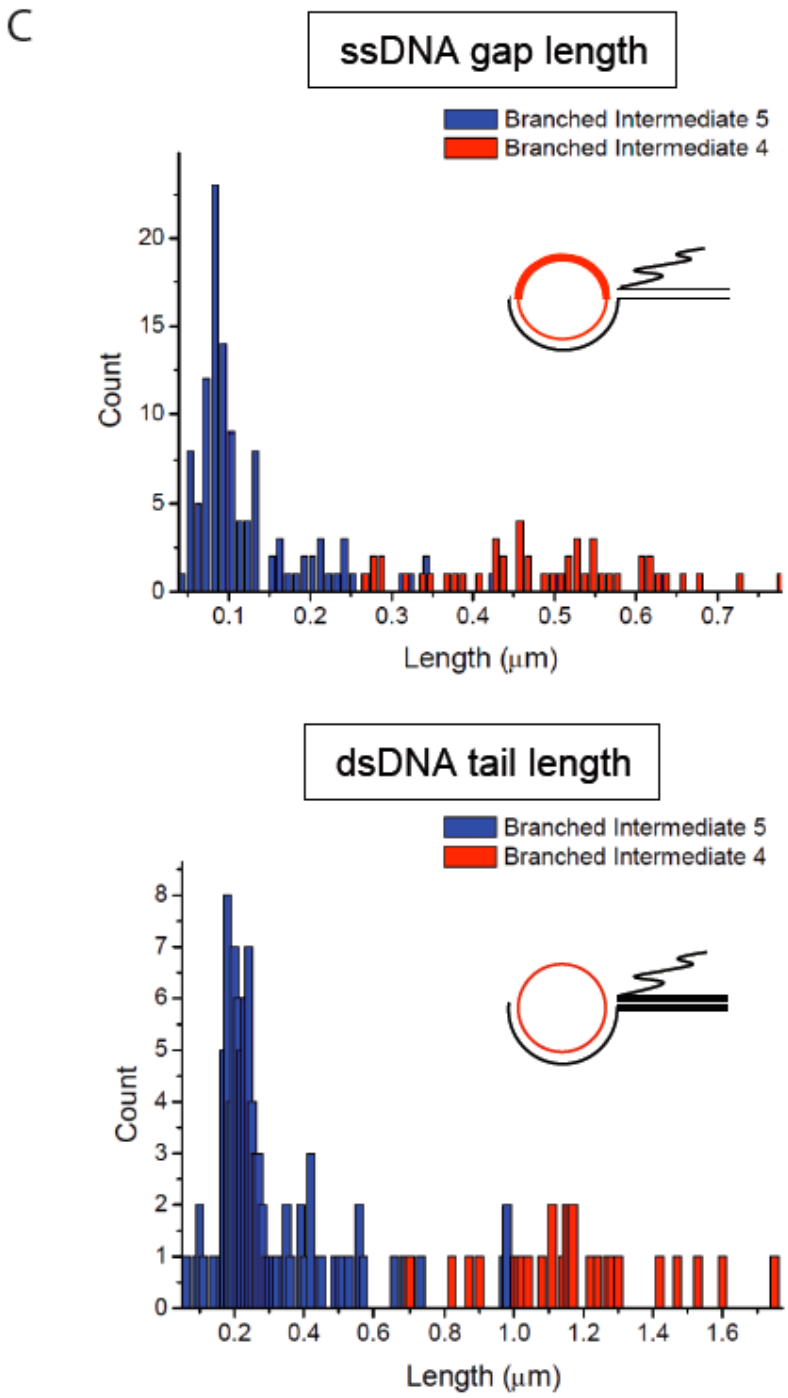

Figure 1. MutS-MutL Inhibits Progression of DNA Strand Exchange during Homeologous Recombination by Acting on Defined Intermediates

(C) DNA intermediate 4 has a shorter heteroduplex region than DNA intermediate 5. Upper panel, histogram showing the contour length measurement of the SSB-coated ssDNA gap (bold line in cartoon) from DNA intermediates 4 (n = 48, red bars) and 5 (n = 116, blue bars); lower panel, histogram showing the contour length measurement of the linear dsDNA tail (bold line in cartoon) from DNA intermediates 4 (n = 28, red bars) and 5 (n = 96, blue bars).

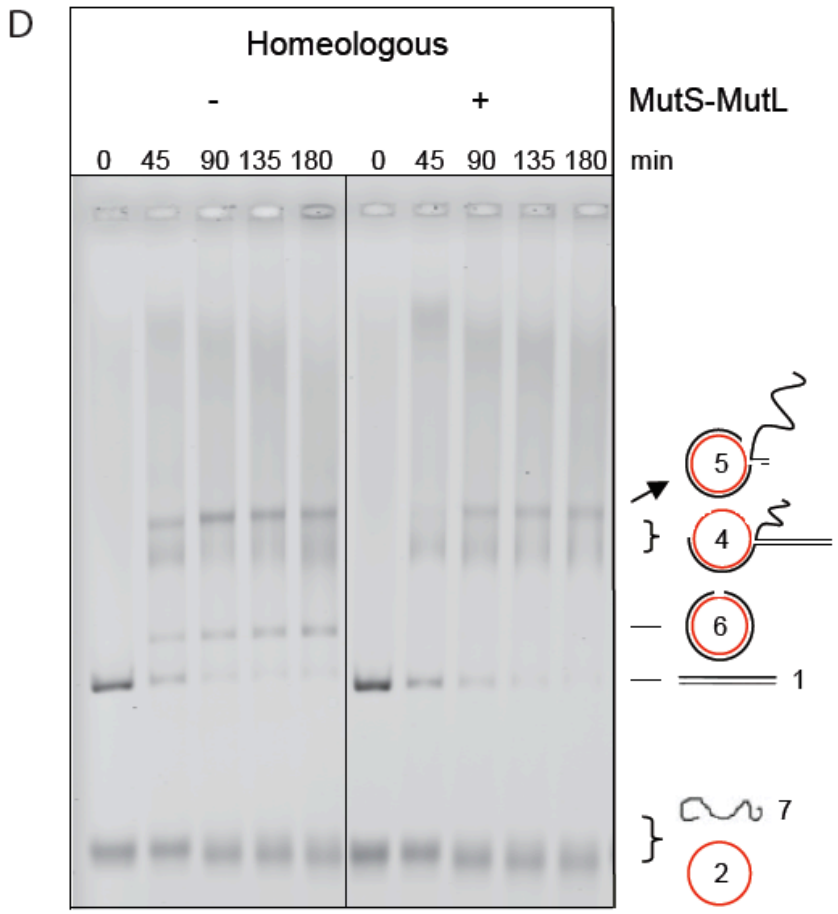

Figure 1. MutS-MutL Inhibits Progression of DNA Strand Exchange during Homeologous Recombination by Acting on Defined Intermediates
(D) Time courses of the effect of MutS (75 nM) and MutL (75 nM) on homeologous reactions using bacteriophage DNA.

MutS and MutS-MutL Prevent Mismatch-Containing D-Loop Molecules from Dissociation

Because MutS and MutL do not seem to inhibit the formation of joint molecules (Figure 1D and Worth et al., 1994), the inhibition of strand exchange likely occurs by blocking branch migration. However, it is at present difficult to envisage how MutS and MutL are able to block this strand exchange reaction because mismatches do not arise until after the strand exchange has already occurred. It is possible that MMR proteins are able to recognize mismatches within the heteroduplex region directly upon formation, thus within the recombinase filament at the site of synapsis. We therefore decided to test the effect of MutS and MutL on RecA-mediated D-loop formation, a well-established assay to address strand invasion, homologous pairing and strand exchange in the absence of extensive branch migration. We used 5'-labeled linear ssDNA (90 nt), of which 54 nucleotides at the 3' end are homologous to the supercoiled recipient plasmid DNA, as well as a variant with a single base change that will introduce a DNA mismatch upon invading the supercoiled plasmid. MutS, MutL or MutS-MutL did not affect the efficiency

of joint molecule formation in homologous (Figure 2A) or homeologous (Figure 2B) reactions as indicated by the amount of D-loop at the first time point (5 minutes). Thus, MutS and MutS-MutL may not inhibit strand exchange in the absence of extensive branch migration.

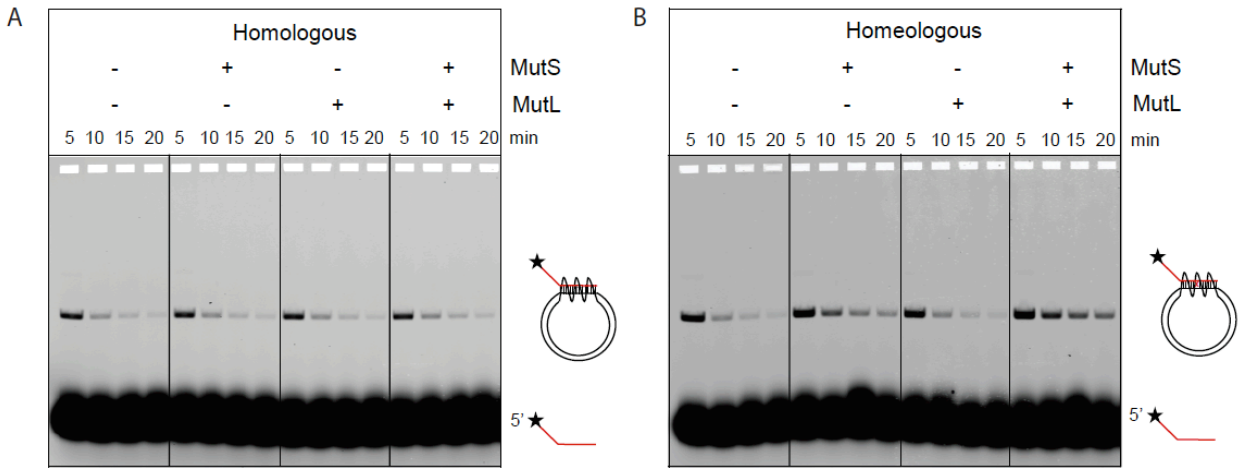

Figure 2. MutS and MutS-MutL Prevent Mismatch-Containing D-Loops from Dissociation
(A) MutS and MutL do not influence the homologous D-loop reaction. Time courses (20 minutes) show the formation and dissociation of D-loops in the absence and presence of 50 nM MutS, MutL or both. Diagrams on the right indicate the position of the labeled ss oligonucleotides and its joint molecule (D-loop) with a homologous supercoiled plasmid DNA.
(B) Upon formation of a mismatch in the heteroduplex region, MutS and MutS-MutL, but not MutL, inhibit D-loop dissociation. Time courses (20 minutes) show the formation and dissociation of D-loops in the absence and presence of 50 nM MutS, MutL or both.

Because we performed the experiments under conditions that permit ATP hydrolysis, we also addressed D-loop dissociation, a well-established phenomenon that occurs possibly via re-invasion of the heteroduplex region by the RecA-bound displaced ssDNA of the D-loop (Shibata et al., 1982). The presence of MutS, MutL or MutS-MutL did not influence the rate of D-loop dissociation in the homologous reaction (Figure 2A). Interestingly, MutS significantly decreased the rate of D-loop product dissociation in the homeologous reaction (Figure 2B). In addition, MutL further delayed D-loop dissociation but only in the presence of MutS (Figure 2B) similar to its role in enhancing MutS inhibition during homeologous strand exchange using bacteriophage DNAs (Figure S1). Clearly, MutS and MutL are able to recognize mismatches formed in recombination intermediates, a single mismatch being sufficient to significantly influence progression of the D-loop cycle.

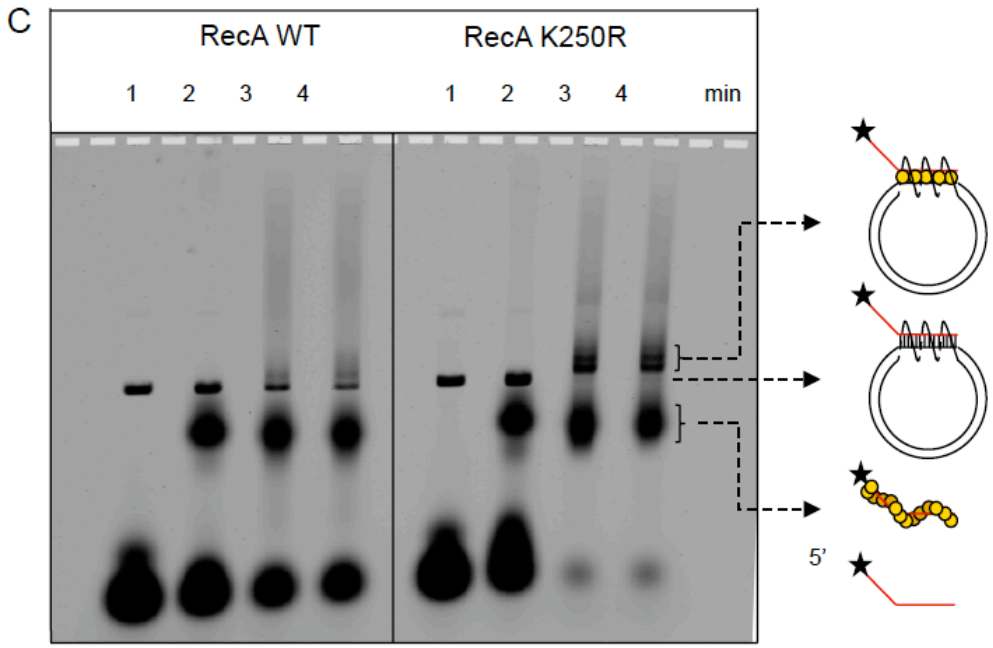

Figure 2. MutS and MutS-MutL Prevent Mismatch-Containing D-Loops from Dissociation
(C) RecA K250R ATPase mutant is defective in disassembly from the heteroduplex. D-loop samples of identical time points (2 min) were deproteinized with SDS buffer (lane 1), not treated (lane 2), crosslinked with 0.3% glutaraldehyde (lane 3) or 0.6% glutaraldehyde (lane 4).

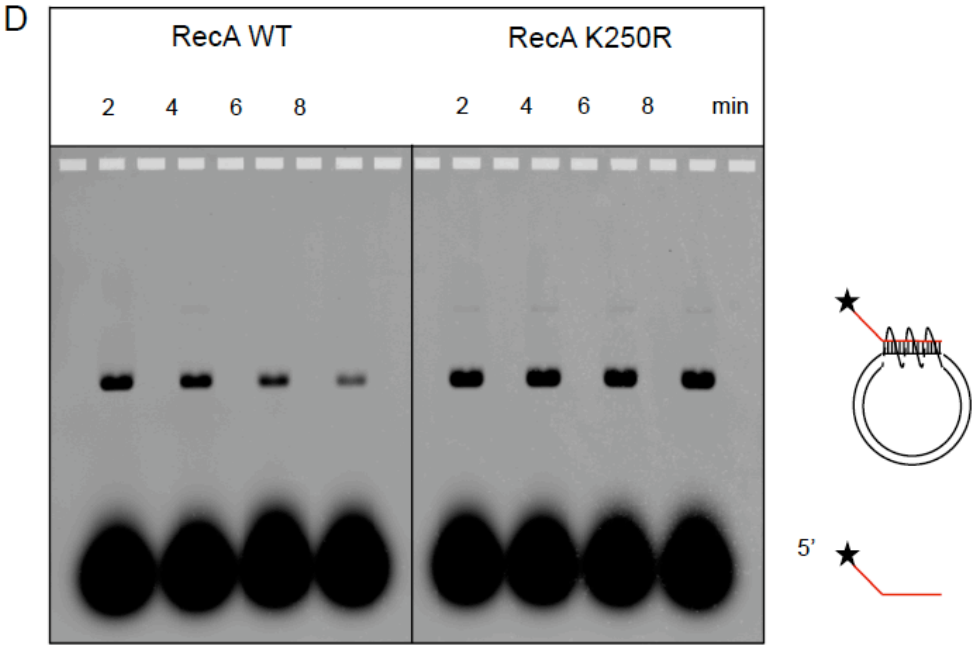

Figure 2. MutS and MutS-MutL Prevent Mismatch-Containing D-Loops from Dissociation
(D) D-loop dissociation is correlated to ATP hydrolysis activity by RecA. Time course (8 minutes) of homologous D-loop reactions mediated by wild-type RecA (left) and RecA ATPase mutant K250R (right).

RecA induced D-loops are stable when formed in the presence of a non-hydrolyzable ATP analog (Shibata et al., 1979). Using the ATP hydrolysis-deficient RecA K250R variant (Cox et al., 2008), we tested whether the ATP-hydrolysis-coupled disassembly of RecA from the heteroduplex region allows D-loop dissociation. Upon crosslinking of protein-bound D-loops, we detected more K250R RecA associated with the DNA than wild-type RecA (Figure 2C), confirming that the K250R RecA mutant is defective in disassembly from DNA. Using this mutant RecA, D-loops indeed did not dissociate over the course of the reaction (Figure 2D). These results demonstrate that when RecA is bound to the heteroduplex region of the joint molecule, re-hybridization of the supercoiled dsDNA is prevented (Figure 2E). Interestingly, binding of MutS and MutL to a single mismatch formed in the heteroduplex region also stabilized D-loops even under conditions that permit ATP hydrolysis by RecA (Figure 2F). It is well established that MutS is able to move away from the mismatch as a sliding clamp upon activation by ATP (Acharya et al., 2003). In this case however, because the recombination intermediate consists of multiple intertwined DNA strands, the diffusing complex will be retained on the heteroduplex region, thereby preventing re-invasion by the RecA-bound displaced ssDNA.

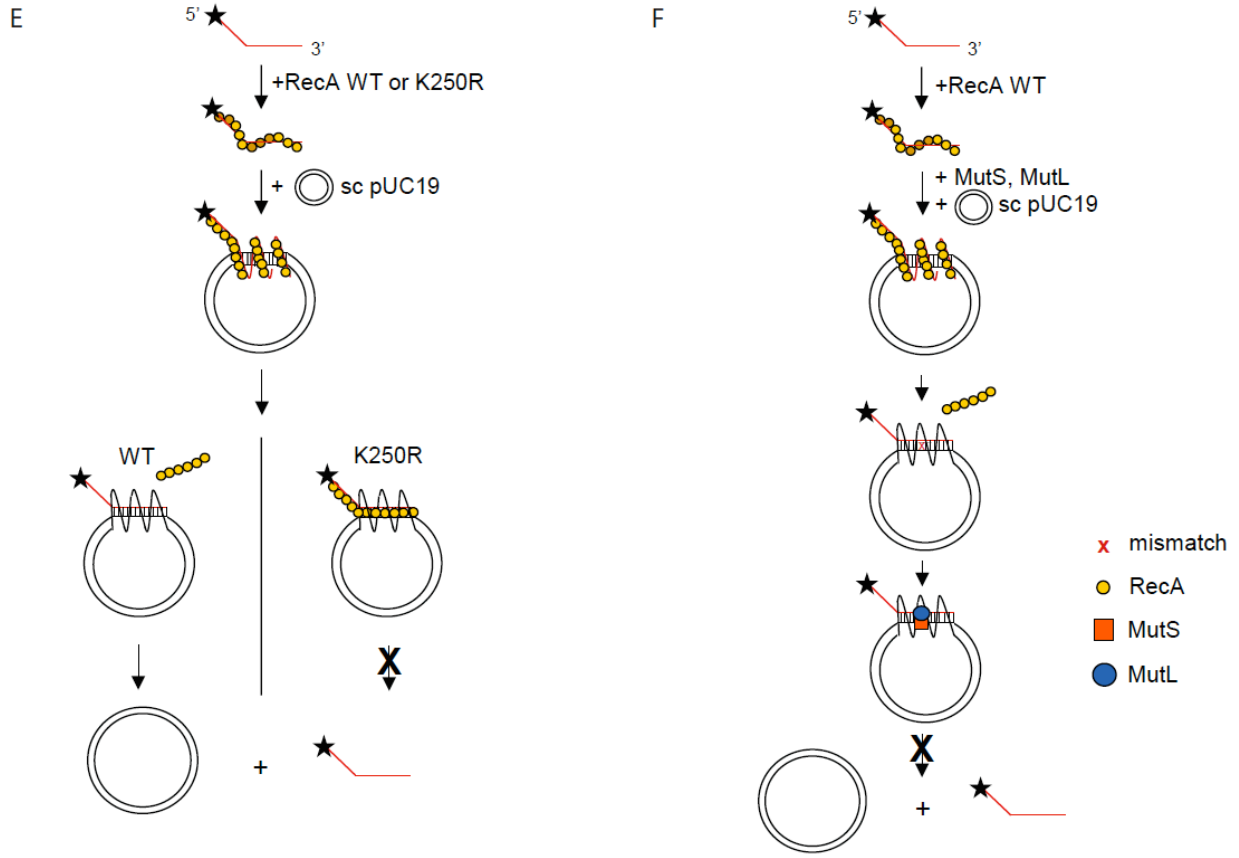

Figure 2. MutS and MutS-MutL Prevent Mismatch-Containing D-Loops from Dissociation
(E) Schematic diagram of D-loop dissociation. RecA forms a filament with ssDNA which invades and pairs with the homologous region within pUC19 supercoiled DNA. At the homologous region, a D-loop structure consisting of a heteroduplex and a displaced ssDNA is formed. After ATP hydrolysis, wild-type RecA dissociates from the D-loop structure, allowing dissociation of the D-loop structure. In contrast, the RecA

K250R mutant, which lacks ATPase activity, remains bound to the D-loop structure and prevents its dissociation.
(F) Schematic diagram of the suppression of D-loop dissociation by MutS-MutL. Similar as Figure 2E, the D-loop structure is formed by RecA-ssDNA filament. After ATP hydrolysis and RecA dissociation, the mismatch in the heteroduplex becomes available for MutS binding. MutL forms a complex with MutS and further stabilizes MutS on the heteroduplex DNA. As a result, the dissociation of the D-loop structure is attenuated.

MutS and MutL Have No Effect on Homeologous Strand Exchange Using Short DNA Substrates

Because the analysis of D-loop formation suggested that MutS or MutS-MutL do not inhibit strand exchange in the absence of extensive heteroduplex formation, we confirmed this by analyzing early steps of the recombination reaction using short DNA oligonucleotides. This assay is well established for studying DNA pairing and strand exchange without re-invasion of the displaced strand into the heteroduplex region taking place (Bazemore et al., 1997). MutS and MutS-MutL were unable to inhibit homeologous strand exchange between these short substrates (Figure 3) even though a mismatch was formed in the heteroduplex region. This finding is different from MutS and MutS-MutL inhibiting strand exchange using non-identical bacteriophage DNA (Figures 1D, S1A and S1C). A possible explanation for this difference is the shorter lifetime of the DNA intermediates formed by oligonucleotides. Thus, the displaced ssDNA is only transiently available. These considerations prompted us to test whether a long displaced ssDNA in the DNA intermediate might be required for MutS-MutL-mediated antirecombination.

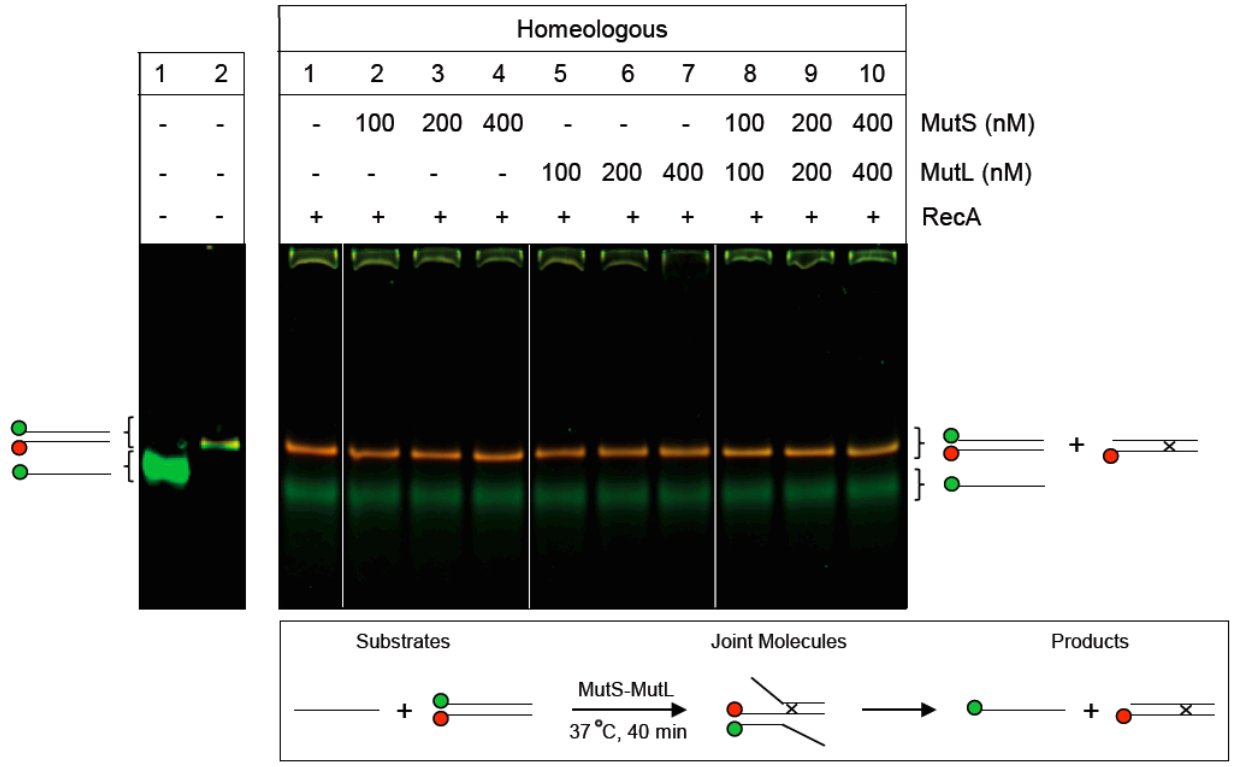

Figure 3. MutS and MutL Have No Effect on Homeologous Strand Exchange Using Short DNA Substrates

Titration of MutS, MutL and MutS-MutL in homeologous strand exchange using short DNA substrates. Upon completion of strand exchange, unlabeled ssDNA hybridizes with the complementary strand of the double-labeled dsDNA substrate (yellow) and forms a single-labeled dsDNA product (red), releasing labeled ssDNA (green). Although different in labeling, both dsDNA substrate and product migrate electrophoretically to the same place in the gel, resulting in an orange band in the gel during homeologous strand exchange. Left panel shows the DNA controls for AF532-labeled displaced ssDNA product (lane 1) and AF532-Cy5-labeled dsDNA substrate (lane 2). Right panel shows that RecA-mediated strand exchange was performed for 40 minutes at 37°C (lane 1) in the presence of increasing amount of MutS (lanes 2-4), MutL (lanes 5-7) or MutS-MutL (lanes 8-10).

The Displaced ssDNA is Involved in MutS-MutL-Mediated Blocking of Homeologous Strand Exchange

To test the role of the long displaced ssDNA, we used S1 nuclease to specifically degrade the displaced strand of the joint molecules during homeologous strand exchange (Figure S2). Indeed, as shown by Figure 4A, inhibition by MutS and MutL was reduced in the presence of S1 nuclease indicated by the appearance of DNA intermediate 5 at 90 and 120 minutes (panel 4). MutS-MutL in the absence of S1 nuclease blocked DNA intermediate 4 from extensive heteroduplex formation and prevented the formation of DNA intermediate 5 at 90 and 120 minutes (panel 3). S1 nuclease hardly affected the homeologous reaction (panel 2). Therefore, upon removal of the displaced ssDNA, MutS and MutL were unable to efficiently block strand exchange, indicating that the displaced ssDNA is involved mechanistically in the inhibition of homeologous strand exchange.

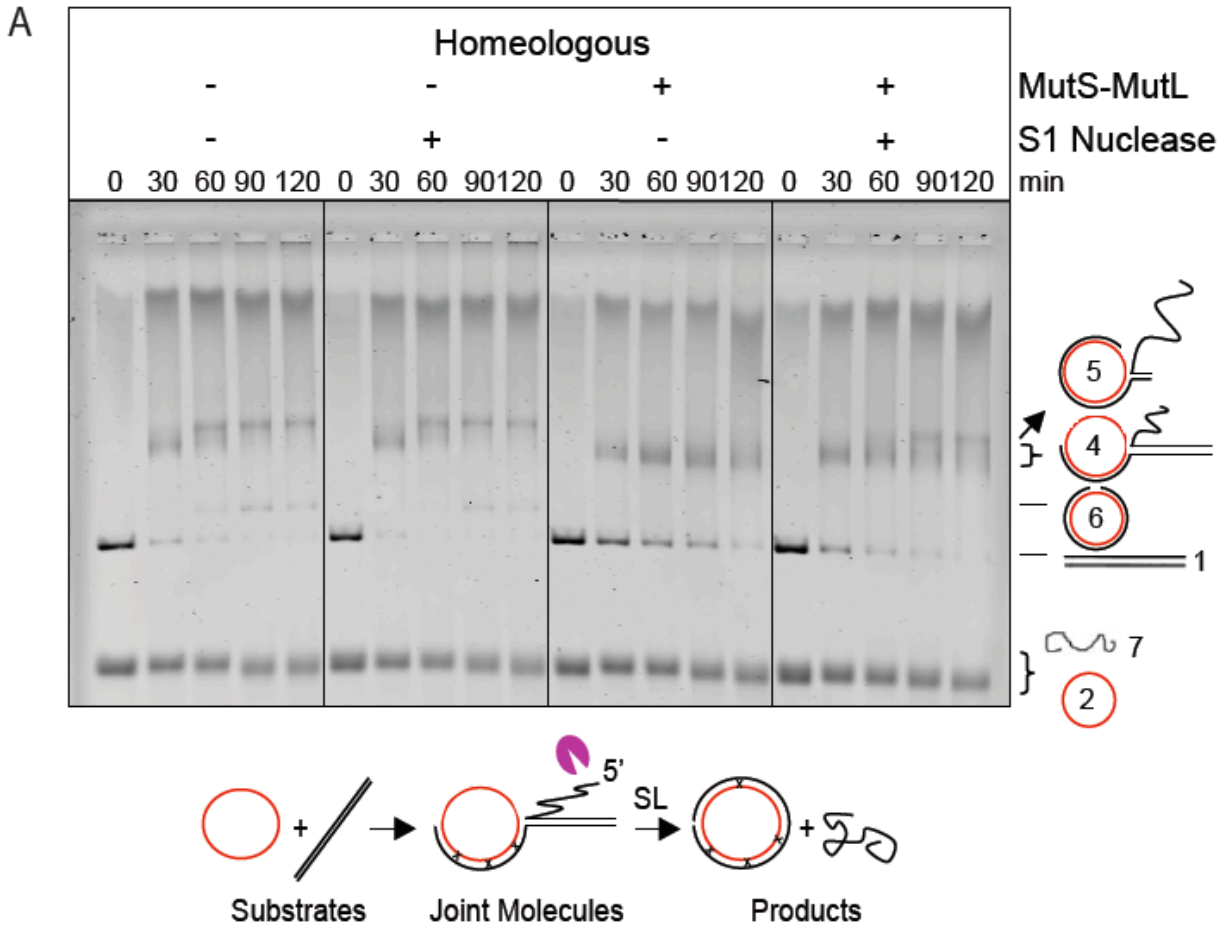

Figure 4. The Long Displaced ssDNA Tail is Required for MutS-MutL-Mediated Antirecombination
(A) S1 nuclease-mediated degradation of the 5' displaced ssDNA reduces the MutS-MutL-induced inhibition of homeologous strand exchange. Time course (120 minutes) of homeologous recombination reaction using M13-fd substrates. From left to right: control reaction; reaction in the presence of S1 nuclease (0.04 U/μl); reaction in the presence of MutS-MutL (100, 100 nM); reaction in the presence of S1 nuclease (0.04 U/μl) and MutS-MutL (100, 100 nM). DNA intermediates are schematically depicted to the right of the gels. Substrates, intermediates and products are shown below the gels. The purple pacman symbol represents the S1 nuclease.

MutS Binding to the Secondary Structures of ssDNA

Because *S. typhymurium* MutS was reported to cosediment with ssDNA (Pang et al., 1985), we investigated whether *E. coli* MutS could bind to ssDNA. Using an electrophoretic mobility shift assay (EMSA), we titrated MutS with ssDNA of either arbitrary or poly-dT sequence. This analysis showed that MutS was indeed able to bind ssDNA but only if this ssDNA is capable of forming secondary structures (Figures 4B and C). Similar secondary structures were also predicted to form in the first 60 nucleotides of the 5' displaced ssDNA of the fd-M13 DNA intermediate (Figure 4D). These results suggest that the importance of 5' displaced ssDNA during MMR-mediated

antirecombination is due to the ability of MutS to bind to secondary structures in the displaced strand.

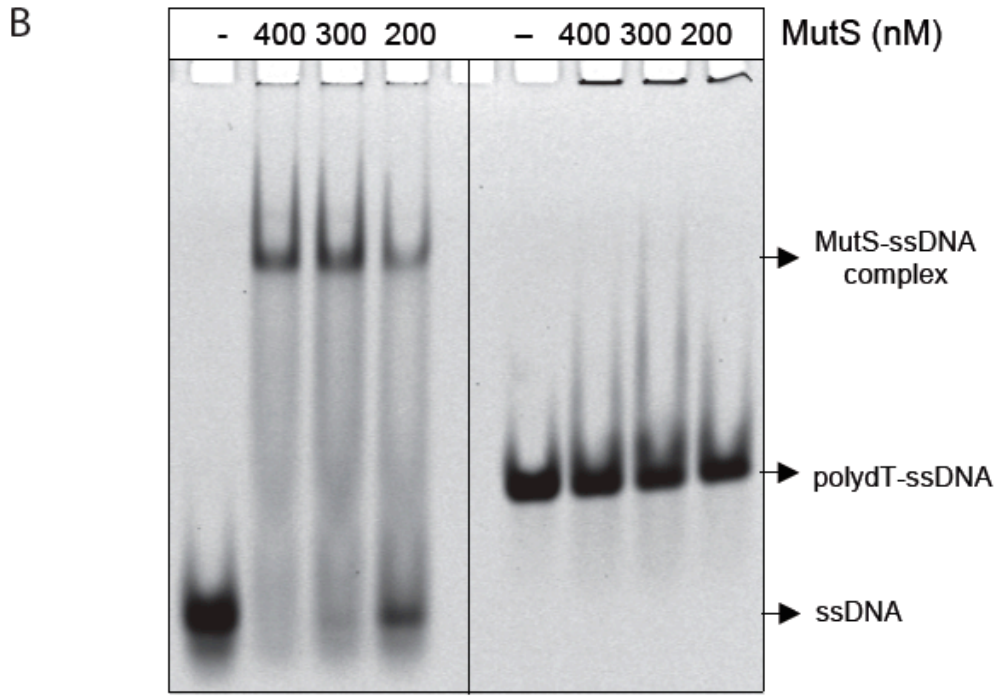

Figure 4. The Long Displaced ssDNA Tail is Required for MutS-MutL-Mediated Antirecombination
(B) Electrophoretic mobility shift assay of increasing concentration of MutS binding to ssDNA containing secondary structures and poly-dT.

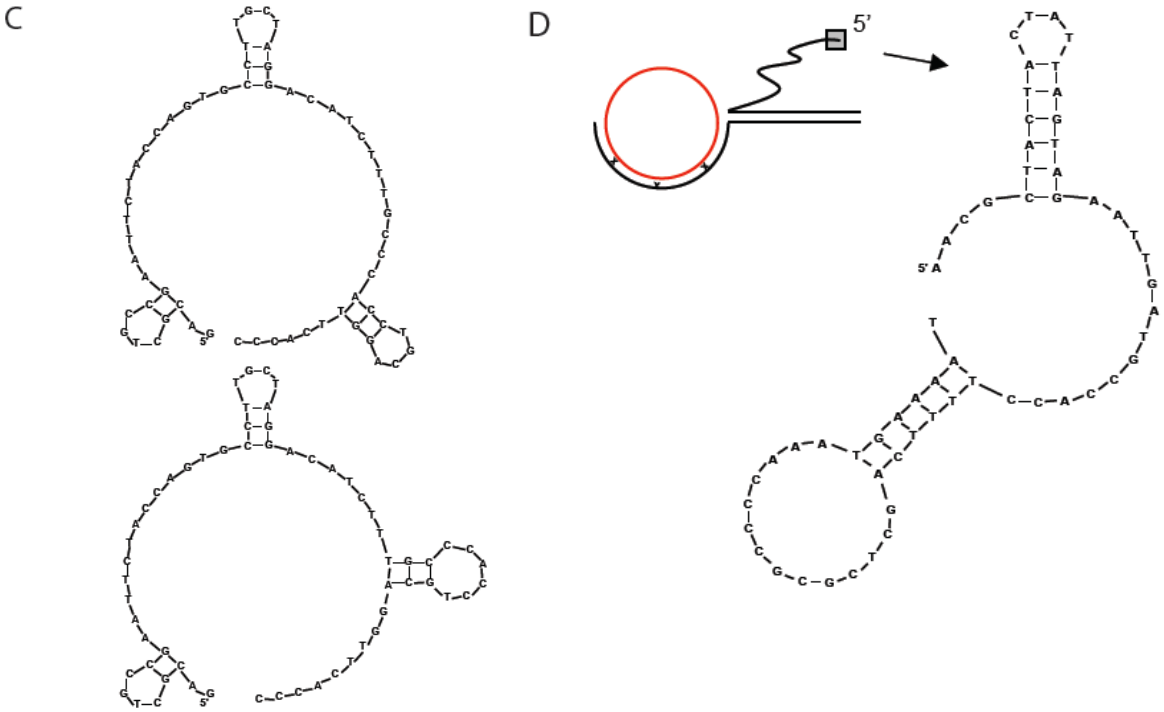

Figure 4. The Long Displaced ssDNA Tail is Required for MutS-MutL-Mediated Antirecombination
(C) Predicted secondary structures formed in the ssDNA with arbitrary sequence used in panel (B).
(D) Predicted secondary structures formed in the first 60 nucleotides of the 5' displaced strand of M13.

MutS Tetramerization Activity is Involved in the Mechanism of MutS-MutL-Imposed Antirecombination

MutS dimers can form tetramers that are able to simultaneously bind multiple mismatch-containing DNA molecules (Monti et al., 2011) or different segments of DNA creating α-shaped loops (Jiang and Marszalek, 2011). Our findings suggest that simultaneous binding of MutS to mismatches within the heteroduplex region and to secondary structure elements of the displaced ssDNA within recombination intermediates might play a role in blocking strand exchange. Consistent with this notion, *in vivo* and *in vitro* evidence indicates that MutSΔC800, which lacks the C-terminal tetramerization domain and therefore can only form monomers and dimers, is compromised in preventing homeologous recombination (Calmann et al., 2005-1, 2005-2). Because of varying reports about mismatch affinity and concentration effects for this mutant (Bjornson et al., 2003; Calmann et al., 2005-1; Lamers et al., 2000), we decided to revisit its mismatch binding and strand exchange inhibition capacities. We were able to demonstrate under our experimental conditions that in the presence of MutL, MutSΔC800 binds to mismatched dsDNA (Figure 5A), even at subsaturating SSB (Figure 5B), and imposes a less stringent block to homeologous recombination than MutS (Figure 5C). This is different from previously reported strand exchange data in which a full block was observed when MutSΔC800 was combined with MutL (Calmann et al., 2005-1). Taken together, these results indicate that the strength of the strand exchange inhibition during

homeologous recombination depends on tetramerization of MutS and possibly also on higher order complex formation with MutL.

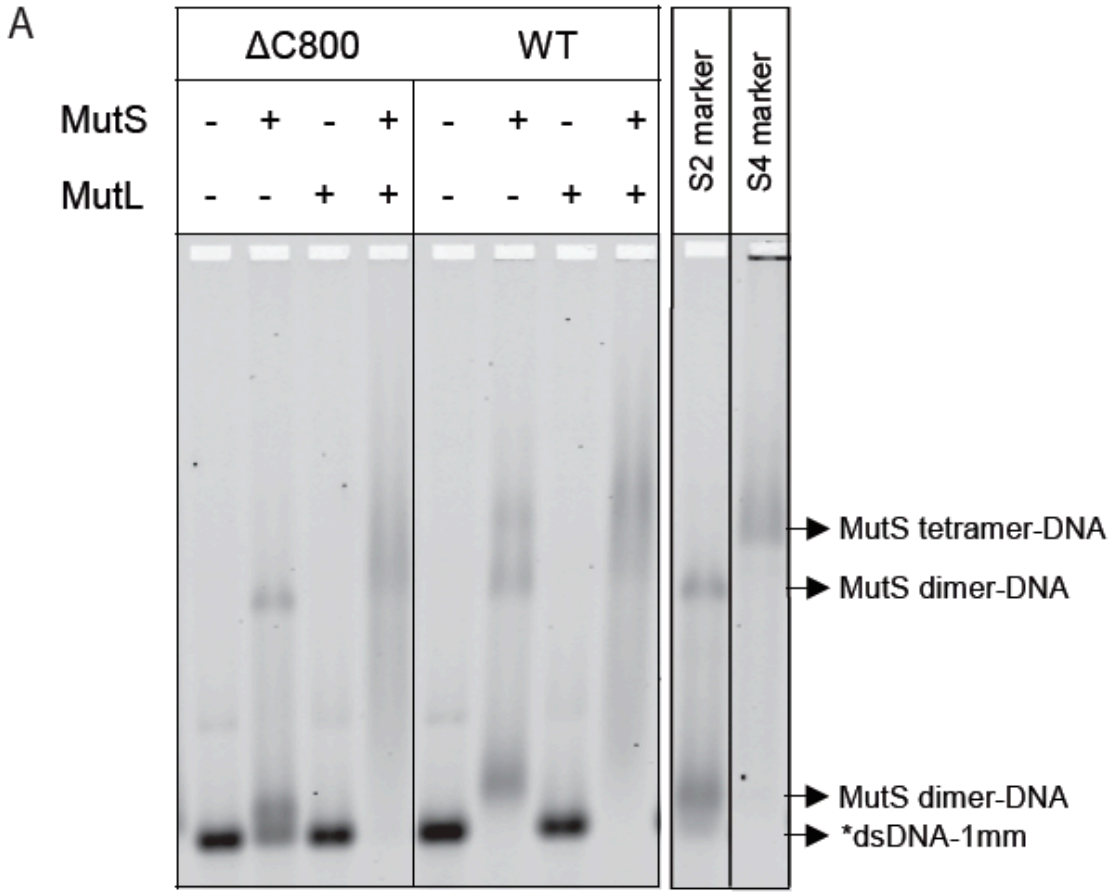

Figure 5. EMSA and Antirecombination of MutS ΔC800 Tetramer Mutant
(A) EMSA of MutS binding to the mismatched dsDNA. MutS ΔC800 and wild-type (400 nM), MutL (400 nM) and MutS ΔC800/wild-type/-MutL (400, 400 nM) were incubated with labeled 90-bp dsDNA with 1 mismatch (*dsDNA-1mm). Markers for DNA-bound MutS dimer (S2) and tetramer (S4) (Groothuizen et al., 2013) are indicated in the panel on the right.

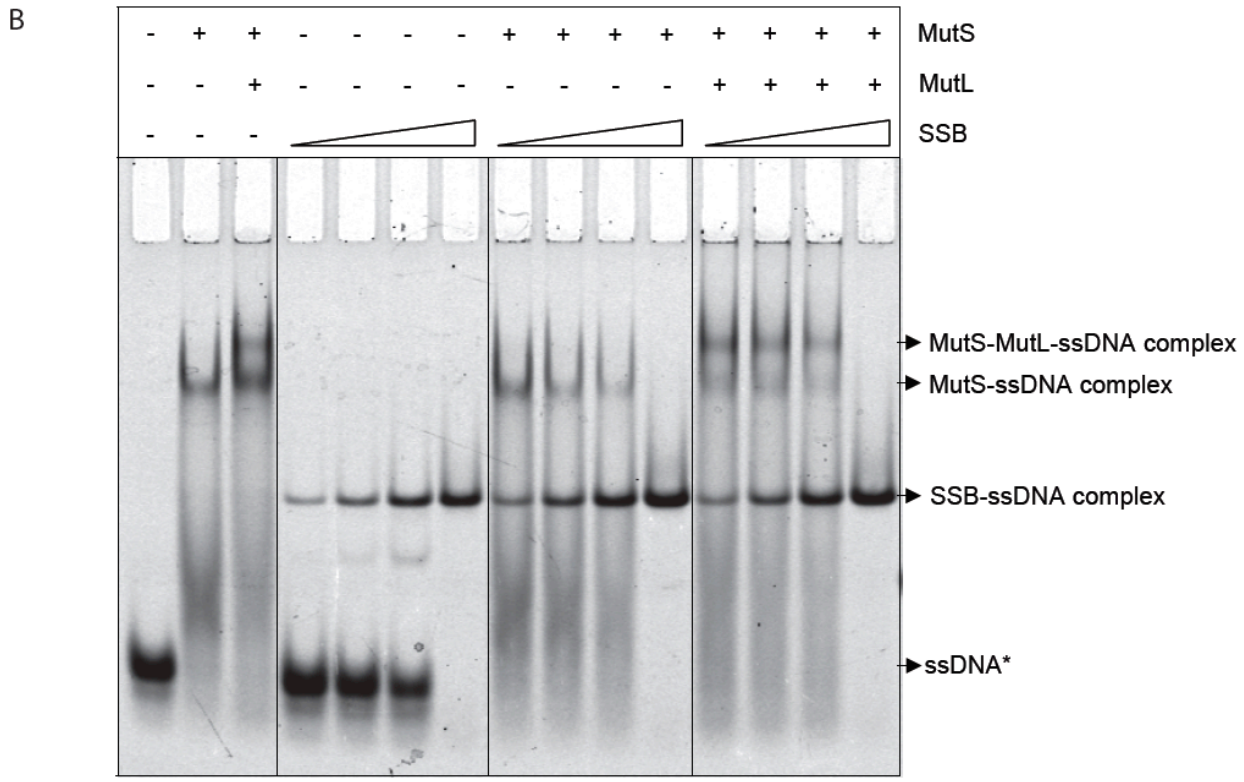

Figure 5. EMSA and Antirecombination of MutS ΔC800 Tetramer Mutant
(B) MutS and MutS-MutL bind to ssDNA with secondary structures at non-saturating level of SSB. Linear ssDNA (5 nM) was incubated with MutS (400 nM), MutL (400 nM) and increasing SSB concentrations below saturation (3.125 nM, 6.25 nM, 12.5 nM) and above saturation (25 nM).

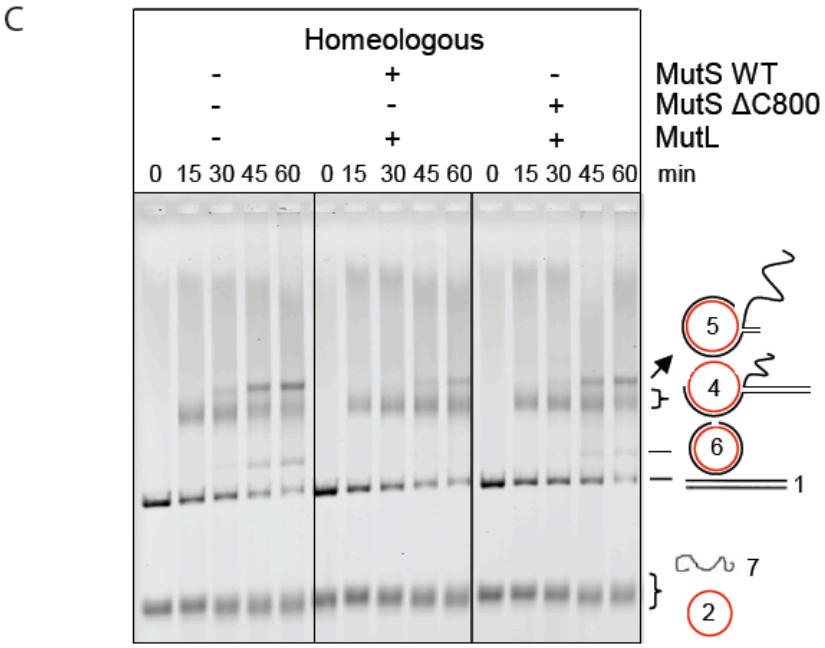

Figure 5. EMSA and Antirecombination of MutS ΔC800 Tetramer Mutant
(C) MutS tetramerization is involved in the mechanism of MutS-MutL-imposed antirecombination. MutSΔC800 only slightly inhibits homeologous M13-fd strand exchange. From left to right time courses (1 hour) of homeologous strand exchange reactions without MutS-MutL, with MutS-MutL (75, 75 nM) and with MutSΔC800-MutL (75, 75 nM).

DISCUSSIONS

Mitotic homologous recombination, which is involved in repairing damaged DNA and stalled replication forks, favors perfectly homologous sequences to maintain genome stability from one cell generation to the next (Heyer et al., 2010). The DNA MMR machinery has a vital role in preventing homeologous recombination between divergent DNA sequences (Iyer et al, 2006). This mismatch-directed antirecombination is important, because inappropriate recombination may have serious consequences for genome stability, species separation and cancer development. However, the mechanistic aspects of MMR-dependent antirecombination remain unknown. Here, we reproduced previous observations of MutS and MutL inhibiting RecA-mediated homeologous strand exchange using DNA substrates from bacteriophage M13 and fd (Worth et al., 1994). To determine which steps of the reaction are inhibited and the requirements for this inhibition we investigated the effects of MutS and MutL during RecA-mediated strand exchange reactions using different types of DNA substrates. Interestingly, we found that both the displaced ssDNA in the recombination intermediate and the tetramerization activity of MutS are required for MutS-MutL-imposed antirecombination. These data represent a significant advance in our understanding of the early-step molecular mechanism of MMR-dependent antirecombination.

Binding of MutS and MutL to Recombination Intermediates

It has been postulated that MutS and MutL bind to mismatches within the RecA-bound three-stranded DNA complex (Worth et al., 1994; Iyer et al., 2006). However, if RecA remains bound, it (i) blocks the access to these mismatches, and (ii) keeps them in a structurally inappropriate conformation for MutS binding because the RecA-dsDNA nucleoprotein filament is stretched and underwound relative to B-form DNA (Di Capua et al., 1982; Chen et al., 2008). Because of this, we would not expect MutS-MutL to be able to gain access to the mismatches imbedded within the RecA-heteroduplex filament. We therefore favor a mechanism in which mismatch recognition occurs after RecA disassembly from the heteroduplex region, rather than within the RecA bound three-stranded DNA filament.

Our data indicate that MutS not only becomes activated by mismatches in the heteroduplex formed upon strand exchange, but is also able to bind to secondary structures formed in the displaced ssDNA tail of the joint molecule. The binding of MutS tetramers to two different DNAs may be an important activity as tetramerization-deficient MutS has a strong recombination phenotype in addition to a concentration-dependent mutator phenotype (Calmann et al., 2005-1, 2005-2). MutL is detected in complex with MutS bound to the base of the loop (Allen et al., 1997). We speculate that MutL plays an important role during antirecombination by enhancing the stability of the MutS tetramer and maintaining a more stable higher-order complex of MutS, MutL and DNA strands. This is supported by *in vivo* studies indicating that low levels of MutL cause a hyperrecombination but not a mutator phenotype (Elez et al., 2007).

A Mechanistic Model for Antirecombination Mediated by MutS and MutL

Taking together our new findings describing the actions of MutS, MutL and UvrD on recombination intermediates, the polarity of RecA-mediated strand exchange (West et al., 1981; Kahn et al., 1981; Cox et al., 1981) and the requirement for rotation during the spooling of DNA into and out of the RecA-bound DNA complex (West, 1992; Honigberg and Radding, 1988; Howard-Flanders et al., 1984), we propose a coherent molecular model for the mechanism of MMR-imposed antirecombination (Figure 6). Initially, the right-handed helical filament formed between RecA and circular ssDNA (Stasiak and Egelman, 1994) pairs with the homologous region in linear dsDNA substrate in the presence of ATP (step 1). Upon intertwining of the incoming dsDNA with the RecA-ssDNA filament, strand exchange is facilitated within the RecA-bound three-stranded DNA complex (step 2) (Menetski et al., 1990; Rosselli and Stasiak, 1990). Upon heteroduplex formation, RecA proteins hydrolyze bound ATP and disassemble from the trailing end of the complex (step 3). RecA disassembly in turn allows the linear displaced ssDNA to spool out from the newly formed heteroduplex. At the same time, the incoming linear dsDNA spools into the groove of the right-handed helical RecA-ssDNA filament at the leading end of the RecA-DNA complex (step 4). These events effectively result in a window of RecA-DNA complex traveling 5' to 3' in relation to the displaced ssDNA (van der Heijden et al., 2008) causing rotation of the circular RecA-ssDNA filament around the linear dsDNA (steps 5, 6) (related to Movie S1). ATP hydrolysis and RecA disassembly is therefore important to facilitate the strand exchange reaction of long DNA substrates because the failure of RecA to disassemble would prevent the rotation of circular RecA-ssDNA filament and hinder the formation of RecA-DNA complex at the leading end. In the absence of mismatches, homologous strand exchange proceeds to completion (step 7). In contrast, mismatches formed in the homeologous reaction (step 8) activate MutS-MutL to block extensive branch migration and product formation (step 9). The rotation of RecA-ssDNA filament during strand exchange is inhibited via simultaneous binding of higher-order MutS-MutL complexes to both the heteroduplex DNA and the secondary structures DNA formed by displaced ssDNA (step 9). Throughout the course of heteroduplex formation, the increasing number of mismatches results in multiple loading of MutS-MutL complexes. Eventually, MutS-MutL exerts stronger inhibition on the rotation of the RecA-ssDNA filament circle at later stages of the homeologous strand exchange (step 10).

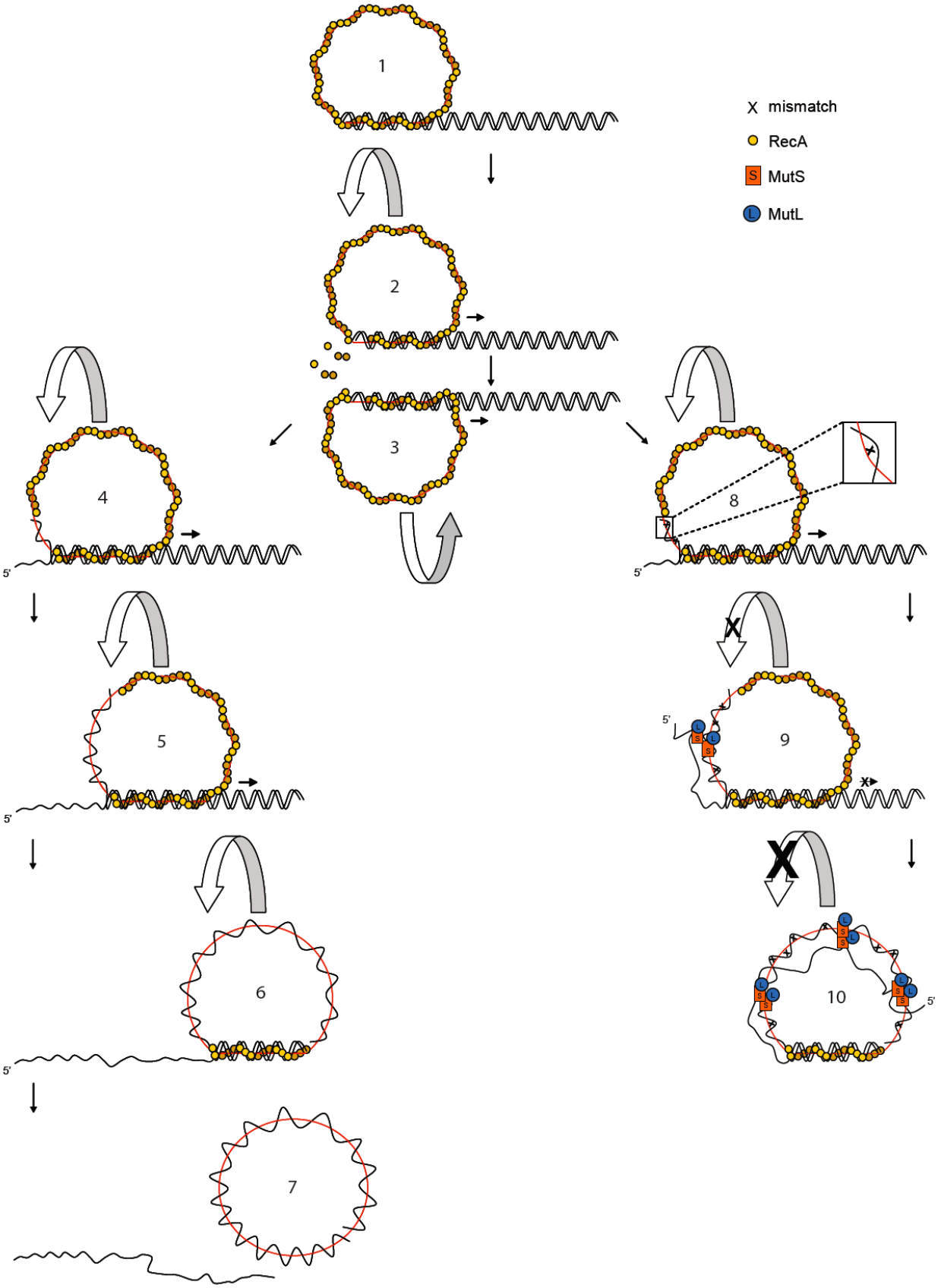
1
2
3
4
5
6
7
8
9
10
X mismatch
RecA
S MutS
L MutL
5'
5'
5'
5'
5'
5'

Figure 6. Model for MMR-Imposed Antirecombination
RecA-ssDNA filament pairs and intertwines with the homologous region of linear dsDNA and strand exchange is catalyzed within the RecA-bound three-stranded DNA complex (step 1). ATP hydrolysis of RecA-DNA complex allows dissociation of RecA from the heteroduplex, which in turn allows the rotation of RecA nucleoprotein filament. This rotation is promoted by the spooling out of displaced ssDNA from the heteroduplex and the spooling in of linear dsDNA at the trailing end and the leading end of RecA-DNA complex (steps 2,3). The rotation of circular RecA-ssDNA filament is relative to the window of RecA-DNA complex that travels from 5' to 3' in relation to the displaced ssDNA throughout the course of strand exchange (steps 4, 5, 6). Upon completion of strand exchange, a nicked circular dsDNA and a linear ssDNA are produced (step 7). The presence of mismatches in the heteroduplex of DNA intermediates activates MMR-dependent antirecombination (step 8). MutS and MutL bound to mismatches in the new heteroduplex and to secondary structures within the displaced ssDNA form higher-order complexes facilitated by MutS tetramerization. Due to the limited freedom of circular RecA-ssDNA filament during rotation, the trapped DNA intermediates are prevented from forming new synapsis at the leading end of RecA-DNA complex (step 9). Stronger inhibition on the process of strand exchange is exerted when more mismatches accumulate in the heteroduplex and multiple MutS-MutL complexes are loaded (step 10).

MMR-Dependent Antirecombination in Eukaryotes

Crossover and non-crossover events arise as a consequence of a choice between homologous recombination subpathways in double-strand break repair (DSBR). Synthesis-dependent strand annealing (SDSA) exclusively generates non-crossovers while double Holliday junctions (dHJs) intermediates of the DSBR model generate both crossovers and non-crossovers with the action of appropriate endonucleases (Schwartz and Heyer, 2011). The template within the SDSA recombination intermediate is topologically constrained due to the absence of endonuclease participation. In contrast, the endonucleolytic activities on the Holliday junctions confer some topological freedom to the DNA strands within the dHJs recombination intermediate. Thus, it is possible that the mechanism of the dHJs subpathway intrinsically involves more DNA strand rotation within the recombination intermediates during the process of branch migration than the SDSA subpathway. Interestingly, the yeast MMR machinery impedes crossover events to a much greater extent than non-crossover events in DSBR involving a divergent template (Welz-Voegele and Jinks-Robertson, 2008; Mitchel et al., 2010). This preference may be due to the topological freedom of dHJs recombination intermediate, which could be conferred by endonucleases, targeted and controlled by the MMR machinery. In the light of our finding that MutS-MutL complexes trap the recombination intermediate from branch migration by preventing rotation of the RecA-ssDNA filament, the observation by Jinks-Robertson and colleagues supports the possible relevance of our MMR-imposed antirecombination model for eukaryotic crossover inhibition mediated by the MMR system (Welz-Voegele and Jinks-Robertson, 2008).

Conclusion

MutS-MutL complexes inhibit branch migration during homeologous recombination. We propose that the link connecting mismatch binding by MutS or MutS-MutL complexes within the heteroduplex region and the prevention of branch migration is the rotation of RecA-ssDNA nucleoprotein filament. Through the formation of higher-order complexes between MutS-MutL on mismatches and secondary structures of displaced ssDNA at the trailing end of synapsis within recombination intermediate, filament rotation and branch migration at the leading end of synapsis are prevented.

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SUPPLEMENTAL FIGURES

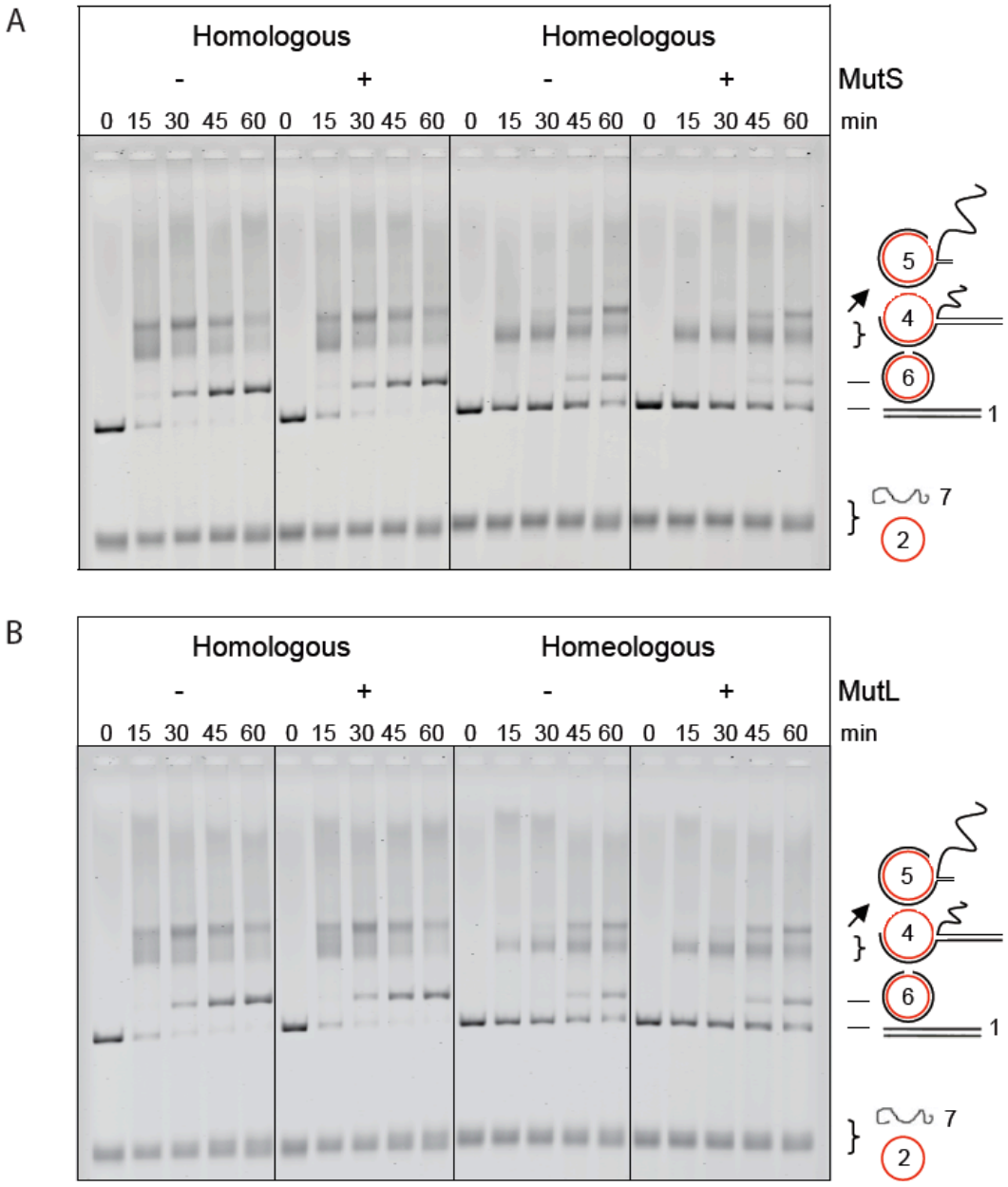
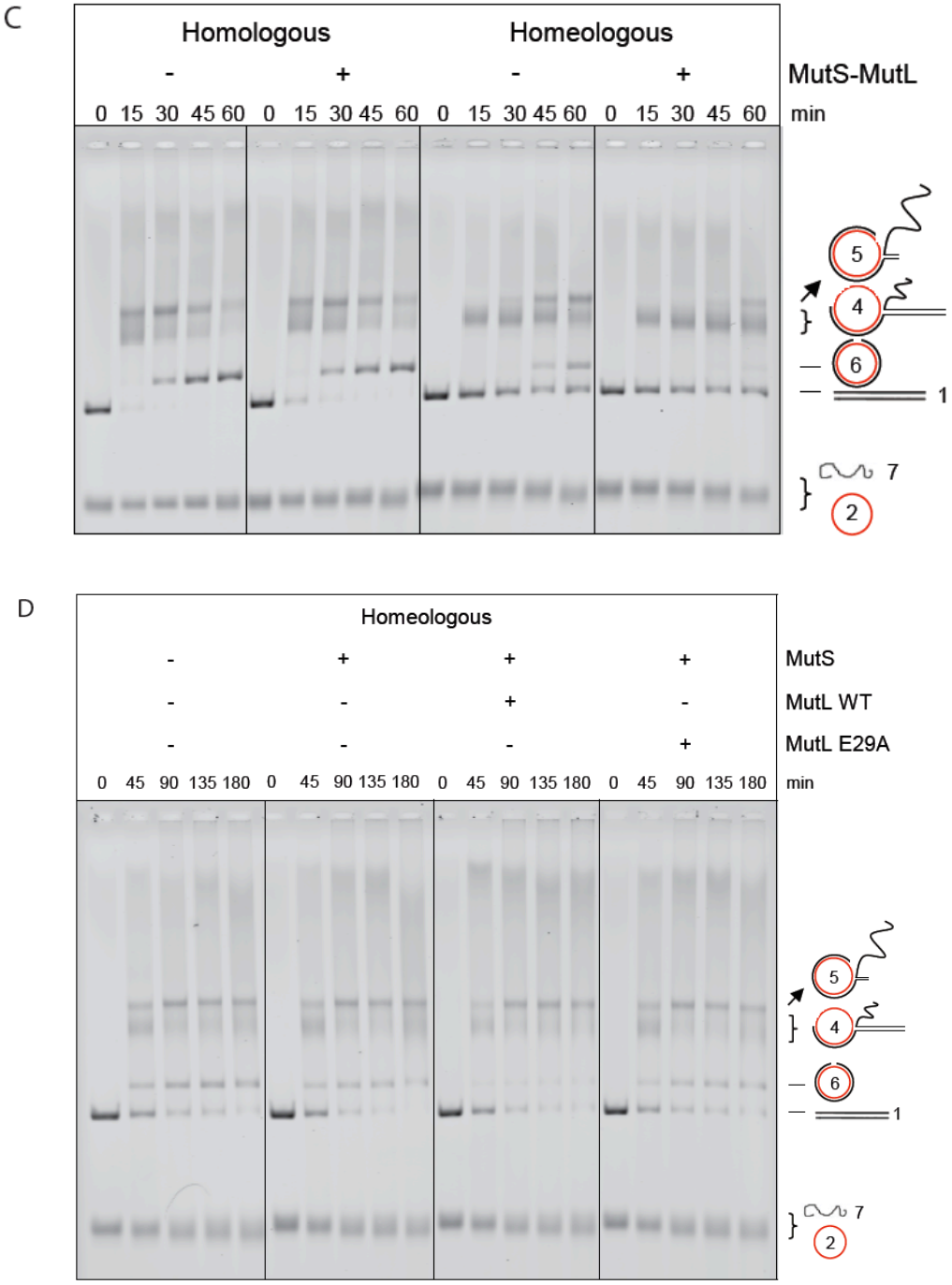


Figure S1. MutS and MutS-MutL Inhibit Homeologous Recombination
(A) One-hour time course of MutS effect on homologous and homeologous strand exchange using M13 and fd DNA substrates. MutS (100 nM) was tested in homologous and homeologous reactions.
(B) One-hour time course of MutL effect on homologous and homeologous strand exchange using M13 and fd DNA substrates. MutL (100 nM) was tested in homologous and homeologous reactions.



(C) One-hour time course of MutS-MutL effect on homologous and homeologous strand exchange using M13 and fd DNA substrates. MutS (75 nM) and MutL (75 nM) were tested in homologous and homeologous reactions.
(D) Three-hour time course of MutL ATPase mutant E29A effect in the presence of MutS on homeologous strand exchange using M13 and fd DNA substrates. MutS (75 nM), MutS-MutL (75, 75 nM) and MutS-MutL E29A (75, 75 nM) were tested in homeologous reactions.

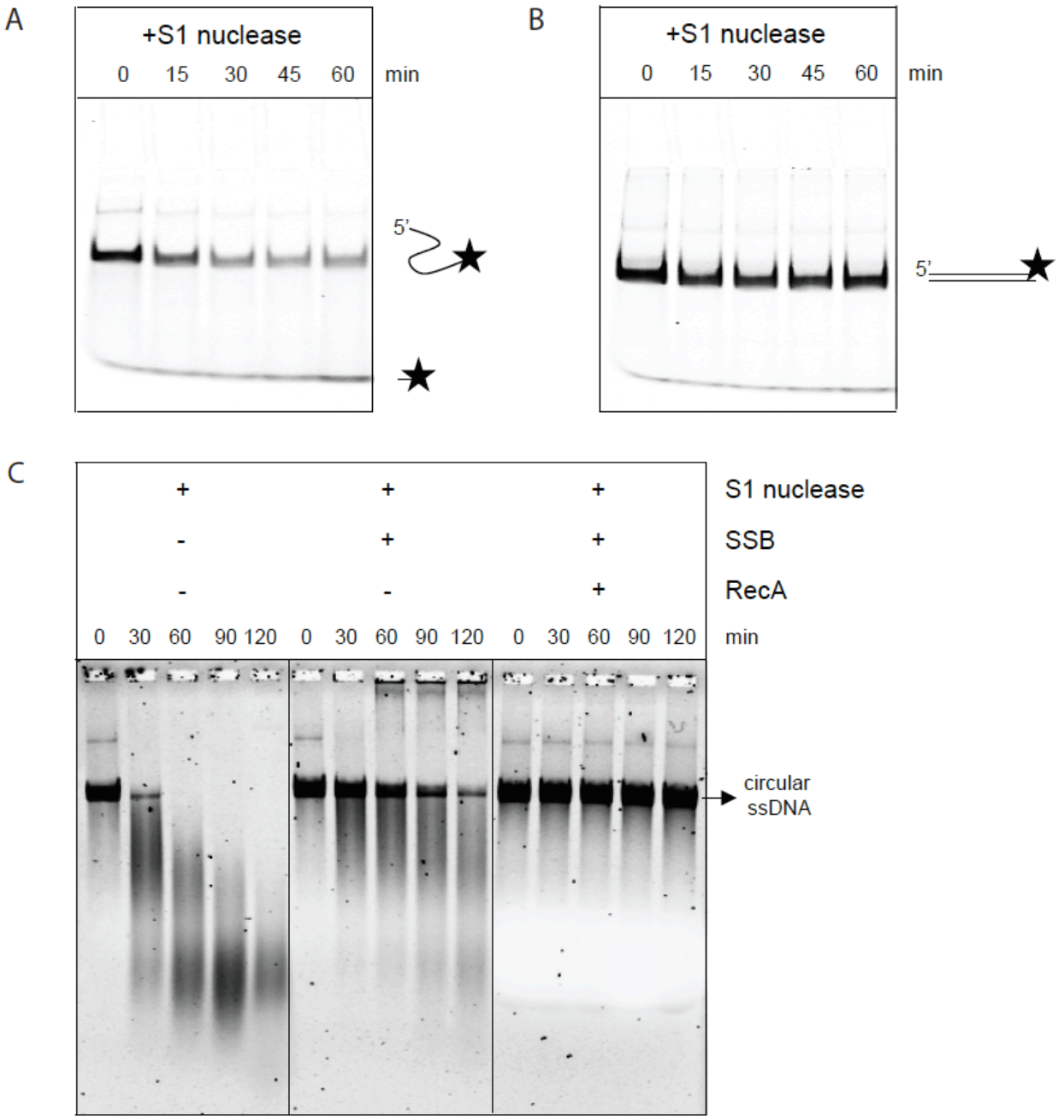


Figure S2. S1 Nuclease Activity and its Dependence on the Presence of SSB and RecA Proteins
(A) S1 nuclease digests ssDNA under strand exchange conditions. Time course (1 hour) of S1 nuclease (0.04 U/μl) incubated with 2.5 nM labeled 90-nt ssDNA.
(B) S1 nuclease does not digest dsDNA. Time course (1 hour) of S1 nuclease (0.04 U/μl) incubated with 2.5 nM labeled 90-bp dsDNA.
(C) Circular ssDNA is protected from S1 cleavage by RecA filament formation. Two-hour time courses of S1 endonuclease containing reaction using 1.5 nM M13 circular ssDNA, 0.04 U/μl S1, and 400 nM SSB and 3.8 μM RecA.

Table 1. Sequence Alignment between the Bacteriophage M13 and fd Genomes

*Types of Mismatches	Number of Mismatches
GA or AG	18
GT or TG	57
**AC or CA	54
TC or CT	8
GG	4
AA	19
TT	13
CC	3
***Insertion/Deletion	17
	193

*All mismatches and insertion/deletion are separated by at least one base pair except for two mismatches being direct neighbors

**First (C·A) and second (A·C) mismatches formed in the heteroduplex are 12 bp and 207 bp away from the ss/ds DNA junction of the recombination intermediate respectively

***of one nucleotide

Movie S1. Rotation of Circular ssDNA is Coupled with Displacement of Linear ssDNA and Uptake of Linear dsDNA

The movie shows the overall movement of DNA strands within the recombination intermediate during the process of RecA-mediated strand exchange. When the circular ssDNA rotates, the displaced linear ssDNA spools out from the heteroduplex at the trailing end, while the linear dsDNA spools into the circular ssDNA at the leading end. Between these two ends, there is a window of synapsis consisting of three-stranded DNA traveling unidirectionally toward the completion of strand exchange. Movie link: http://youtu.be/BvB4Mg2m5KQ

Chapter 4

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TITLE

Coordinated Directional Unwinding of MutS-MutL-Trapped Homeologous Recombination Intermediates by *Escherichia coli* UvrD

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ABSTRACT

DNA mismatch repair prevents homeologous recombination between divergent sequences. Previously, we showed that MutS and MutL respond to DNA mismatches within the heteroduplex region of homeologous recombination intermediates and secondary structures formed within the displaced ssDNA tail. These interactions lead to higher-order complex formation between MutS-MutL complexes bound to the heteroduplex and displaced ssDNA, thereby trapping recombination intermediates and preventing branch migration. Here, using purified proteins and DNA substrates, we find that helicase UvrD alone can simultaneously stimulate and inhibit homeologous strand exchange depending on which dsDNA within the recombination intermediate is unwound by UvrD. In contrast, in the presence of MutS and MutL, UvrD is strictly antirecombinogenic. UvrD is possibly recruited to the nearest ss/ds DNA junction by mismatch-activated MutS-MutL complexes on heteroduplex to directionally resolve the trapped intermediates and re-form DNA substrates. Thus, our results explain on a mechanistic level how the coordinated action between MutS, MutL and UvrD prevents homeologous recombination and maintains genome stability.

INTRODUCTION

DNA mismatch repair (MMR) maintains genome stability not only by repairing base-base mismatches and insertion/deletion loops generated during replication but also by preventing illegitimate recombination between divergent (homeologous) sequences (Iyer et al., 2006). The increased frequency (up to 1000 fold) of interspecies conjugation between *Escherichia coli* and *Salmonella typhimurium* in *mutS*, *mutL*, *mutH* and *uvrD* recipients indicates that MMR acts as a barrier to RecABCD-dependent homeologous recombination (Rayssiguier et al., 1989, 1991; Stambuk and Radman, 1998). Although MutS and MutL, as the most upstream players of MMR, are much more potent than MutH and UvrD in preventing homeologous recombination *in vivo* (Rayssiguier et al., 1989), more recent interspecies conjugation studies show that the homeologous recombination frequency in a recipient strain lacking both MutH and UvrD is as high as in *mutS* and *mutL* strains (Stambuk and Radman, 1998). Based on this, the fate of MutS-MutL-trapped recombination intermediates is speculated to rely on MutH and UvrD and two possible mechanisms have been proposed (Stambuk and Radman, 1998). One mechanism is MutH-independent but involves MutS, MutL, UvrD and RecBCD during the early stage of homeologous recombination. The recognition and binding of a mismatch by MutS and the MutS-MutL complex leads to the recruitment of UvrD to the nearest single strand (ss) / double strand (ds) DNA junction of the recombination intermediate. Starting at the loading junction, UvrD unwinds toward the mismatch in the heteroduplex and dissolves the recombination intermediate. Repeated strand invasions are prevented by the nuclease activity of RecBCD that destroys the invading ssDNA. Interestingly, in biochemical experiments UvrD can both stimulate and prevent RecA-mediated homologous strand exchange (Morel et al., 1993). How this activity is regulated within the context of homeologous recombination remains to be determined. The second mechanism is MutH-dependent and explains events occurring during the late stage of homeologous recombination requiring MutS, MutL, MutH, DNA helicase and *de novo* DNA synthesis. This mechanism essentially resembles the MutH-independent mechanism except for MutH nicking the nascent hemi-methylated GATC site and an unknown helicase (rather than UvrD) that needs to be recruited to dissolve the recombination intermediate. Both proposed mechanisms have yet to be tested biochemically.

In Chapter 3 we described how MutS and MutL prevent homeologous recombination intermediates from branch migration through the formation of higher-order complex between MutS-MutL complexes bound to the heteroduplex and the displaced ssDNA. These trapped homeologous strand exchange intermediates are problematic DNA structures that must somehow be disassembled. Here, we studied the effect of UvrD on trapped homeologous recombination intermediates mediated by MutS and MutL. Remarkably, in the absence of MutS and MutL, we observed higher amounts of both DNA substrate and product at the expense of DNA intermediates. This phenomenon can be further stimulated by MutL independent of its ATP hydrolysis activity. Interestingly, UvrD helicase only increased the amount of DNA substrates in the presence of MutS and MutL and this directional unwinding required MutL ATP hydrolysis activity. Thus, we successfully reconstituted *in vitro* the proposed MutH-independent pathway that rejects recombination joint molecules during the early stage

of homeologous reaction. Taken together the results in the previous and this chapter, we present a mechanistic model explaining how the action of MutS, MutL and UvrD is coordinated on recombination intermediates to prevent recombination between divergent sequences.

MATERIALS AND METHODS

DNA Substrates

Viral ssDNAs from bacteriophage M13 and fd and closed circular replicative form (RF) DNA from M13 were prepared as described (Sambrook and Russell, 2001). The M13 linear dsDNA was obtained by digestion of M13 RF DNA with HpaI restriction endonuclease (New England Biolabs). These circular ssDNAs from M13 and fd, and HpaI-linearized dsDNA from M13 bacteriophage were further purified from agarose gels using electroelution with D-Tube Dialyzer (Novagen).

Proteins

RecA WT was purified from *E. coli* strain GE1710 using a protocol based on spermidine acetate precipitation (Harmon and Kowalczykowski, 1998). RecA was flash-frozen in small aliquots and stored at -80°C. MutS was purified essentially as described (Natrajan et al., 2003) with the following adaptations: NaCl was replaced with KCl in the lysis and chromatography buffers. Anion exchange chromatography on the MonoQ column was performed after heparin affinity chromatography, followed by an additional POROS S column (Life Technologies) using the same buffers and salt gradient. After size exclusion chromatography, MutS was flash-frozen in 25 mM HEPES-KOH pH 7.5, 250 mM KCl, 1 mM DTT, 50% glycerol and stored at -80°C. MutL was purified as described (Lebbink et al., 2010), except that KCl replaced NaCl in all buffers and the heparin gradient was developed from 0.1-1.0 M KCl. MutL was flash frozen in 20 mM Tris-HCl pH 8.0, 0.5 M KCl, 1 mM DTT, 50% glycerol and stored at -80°C. UvrD was over-expressed and purified from *E. coli* as described earlier (Guarne et al., 2004), with an additional Superdex 200 gel filtration column, and a storage buffer containing 200 mM KCl, 25 mM HEPES-KOH pH 8.3, 20% glycerol and 1 mM DTT. The UvrD preparation was divided into small aliquots and stored at -80°C until use.

Strand Exchange Assay Using M13 and fd Bacteriophage DNA

Circular single-stranded M13 or fd phage DNA (1.5 nM) was incubated for 5 minutes at 70°C to remove secondary structures. Recombinase filaments were formed by addition of 50 mM HEPES-KOH pH 7.5, 12 mM $MgCl_2$, 0.5 mM DTT, 6 mM phosphocreatine, 10 U/ml creatine phosphokinase, 5% glycerol, 3 mM ATP and 3.8 μM RecA and incubated at 37°C for 10 minutes. The ratio of RecA to ssDNA nucleotide used was approximately 1:2.5; the amount of ATP in combination with the regeneration system is sufficient to still observe ATPase-dependent differences at late time points (see for example Figure 6B). Samples were incubated at 37°C for 10 minutes, 0.4 μM SSB was added and incubation was continued for another 10 minutes. When appropriate, MutS, MutL, and/or UvrD were added at varying concentrations. Next, strand exchange was initiated with 0.6 nM of M13 linear dsDNA at 37°C. Final reaction volumes amounted up to 50 μl. At various

time points 9 µl samples were taken and stopped with 0.1% SDS, 25 mM EDTA and 153 µg/ml proteinase K at 37°C for 15 minutes. These deproteinized DNA samples were subjected to electrophoresis for 12 hours at 1.4 V/cm on 0.8% agarose in 1x TAE at room temperature. The gels were stained for 45 minutes at room temperature with 0.3 µg/ml ethidium bromide. These stained gels were scanned and visualized with Typhoon TRIO Variable Mode Imager (GE Healthcare) using the standard settings for ethidium bromide. Intensities for linear dsDNA substrate and circular dsDNA product were quantified and background corrected using ImageJ (Abramoff et al., 2004). Amounts of substrate and product for each time point were calculated considering that the input substrate at t=0 corresponds to 30 fmol DNA. Changes in amount of substrate and product over time were plotted (Graphpad Prism 5) underneath corresponding gels using a segmented y-axis to visualize small amounts of product. Because many identical reactions were run on different gels in different combinations, quantifications from multiple panels from different gels were combined to be able to calculate averages +/- SEM (n>3). Unpaired t-tests (Graphpad Prism 5) of reactions containing specific MMR proteins versus reactions without MMR proteins revealed significant differences between substrate and product formation as indicated in Figure S1.

RESULTS

Bidirectional Unwinding of Homeologous DNA Intermediates Mediated by UvrD

We analyzed the effect of the UvrD helicase on strand exchange using bacteriophage DNA because UvrD dissolves recombination intermediates (Morel et al., 1993). The inclusion of 10 nM UvrD (without MutS and MutL) almost completely inhibited product formation during homologous and homeologous strand exchange (Figure 1A), most likely because the helicase disrupts the RecA-ssDNA filament (Veaute et al., 2005). Reducing UvrD to 1 nM did not affect the homeologous reaction at early time points (45 and 90 min; Figure 1B panel 2), indicating UvrD is no longer dismantling RecA-ssDNA filaments. However, UvrD caused a significant reduction of DNA intermediate 5 at later time points (135 and 180 minutes). Intriguingly, this is coupled with increases of DNA intermediate 4 and linear dsDNA substrate (indicating that the UvrD helicase is able to drive the homeologous reaction backward), as well as nicked circular dsDNA product (indicating that UvrD at the same time drives the reaction forward) (Figure 1B panel 2; Figure S1). These increases in both substrate and product also occur in the presence of MutL (Figure 1B panel 3; Figure S1) and become more prominent if MutL cannot hydrolyze ATP (Figure 1C). Processing of DNA intermediate 5 required helicase activity because UvrD mutants K35M (Figure 2A) and E221Q (Figure 2B), which are ATPase deficient but can still bind to DNA (George et al., 1994; Brosh and Matson, 1995), did not affect the homeologous reaction. Because heteroduplex regions containing mismatches cannot spontaneously branch migrate (Biswas et al., 1998), these observations imply that UvrD actively and bidirectionally unwinds strand exchange intermediate 5 (Figure 3).

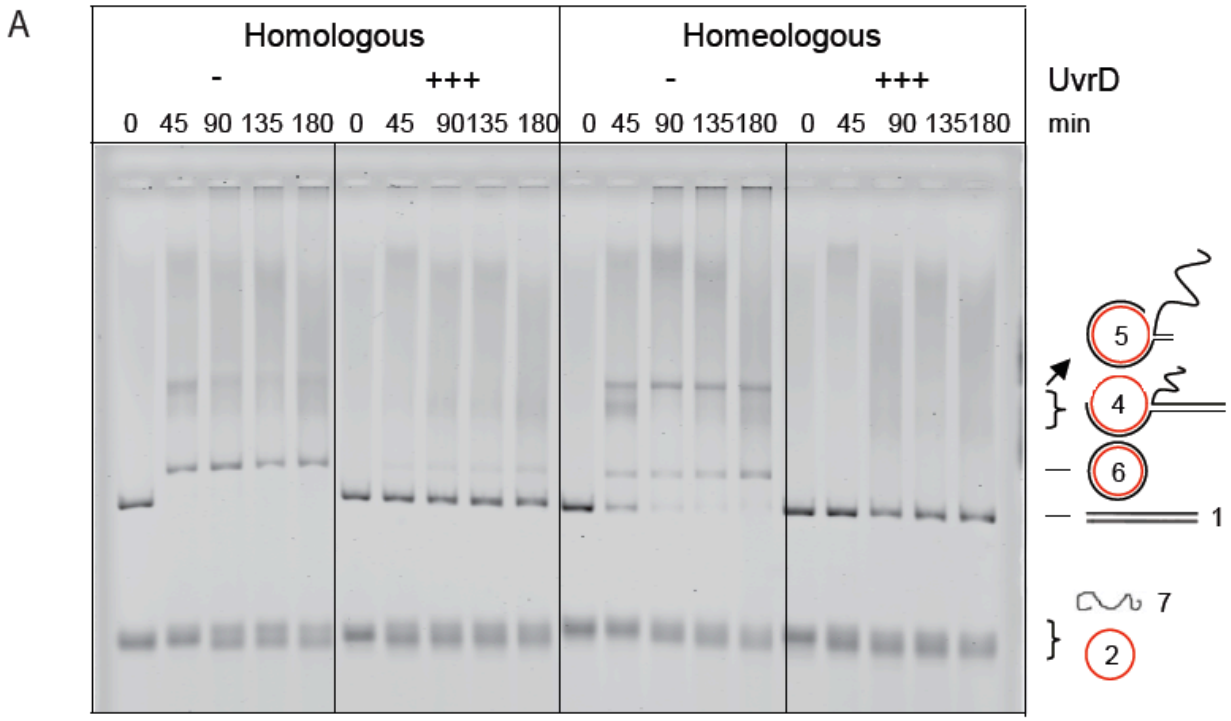

Figure 1. Bidirectional Unwinding of Homeologous DNA Intermediates by UvrD
(A) Time courses reveal that 10 nM UvrD completely inhibits homologous and homeologous strand exchange.

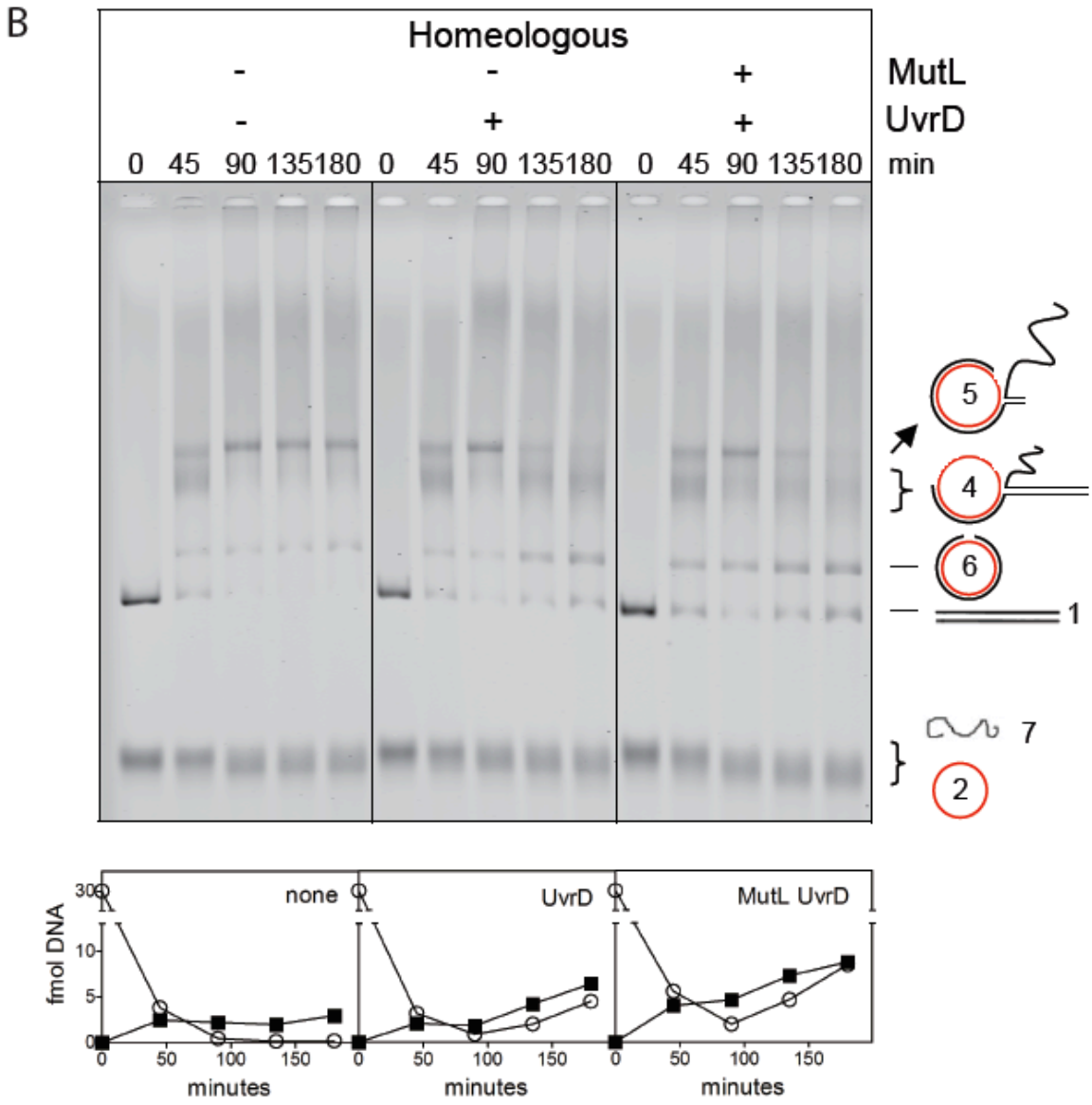

Figure 1. Bidirectional Unwinding of Homeologous DNA Intermediates by UvrD
(B) Time courses of homeologous strand exchange. From left to right: control (no additional proteins), with UvrD (1 nM) and with MutL-UvrD (75, 1 nM). The addition of UvrD and of MutL-UvrD significantly increased the amount of substrate as well as product at 135 and 180 minutes (Figure S1). Panels below the gel show quantified amounts of linear dsDNA substrate (open circles) and nicked circular dsDNA product (closed squares).

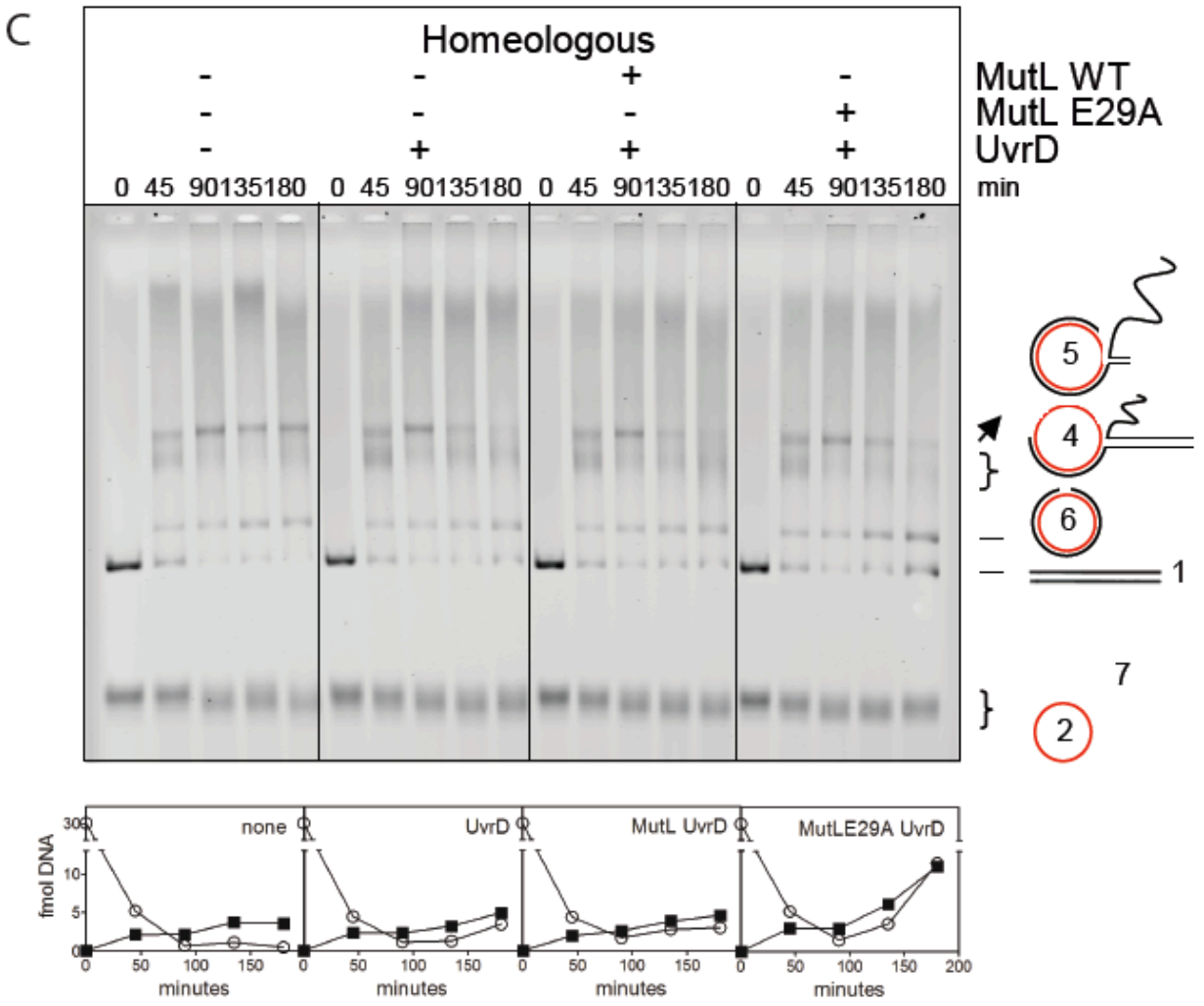

Figure 1. Bidirectional Unwinding of Homeologous DNA Intermediates by UvrD
(C) ATPase-deficient MutL E29A stimulates bidirectional unwinding of homeologous DNA intermediates by UvrD. This finding is expected because in the absence of MutS, MutL E29A also effectively stimulates UvrD unwinding rates using annealed dsDNA substrates (Guarne et al., 2004; Robertson et al., 2006). Panels from left to right contain no MMR proteins, UvrD (1 nM), MutL-UvrD (75, 1 nM) and MutL E29A-UvrD (75, 1 nM). Panels below the gel show quantified amounts of linear dsDNA substrate (open circles) and nicked circular dsDNA product (closed squares).

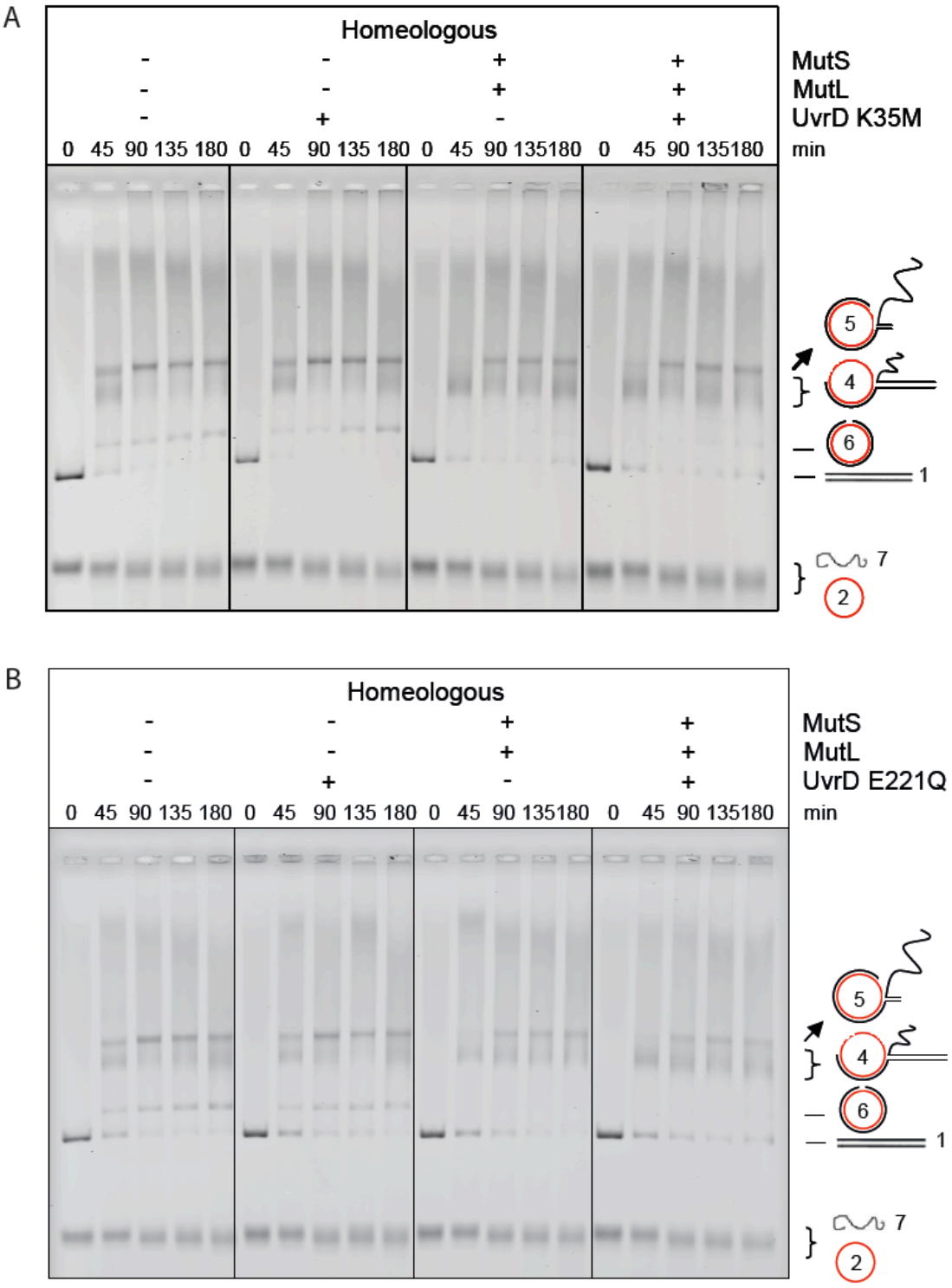

Figure 2. UvrD K35M and E221Q ATPase Mutants Studies
(A) UvrD bidirectional unwinding and MutS-MutL-directed UvrD resolution of homeologous DNA intermediates depend on UvrD helicase activity. UvrD K35M (1 nM), MutS-MutL (75, 75 nM) and MutS-MutL-UvrD K35M (75, 75, 1 nM) were tested in the homeologous reaction in three-hour time course separately.

(B) UvrD bidirectional unwinding and MutS-MutL-directed UvrD resolution of homeologous DNA intermediates depend on UvrD helicase activity. UvrD E221Q (1 nM), MutS-MutL (75, 75 nM) and MutS-MutL-UvrD E221Q (75, 75, 1 nM) were tested in the homeologous reaction in three-hour time course separately.

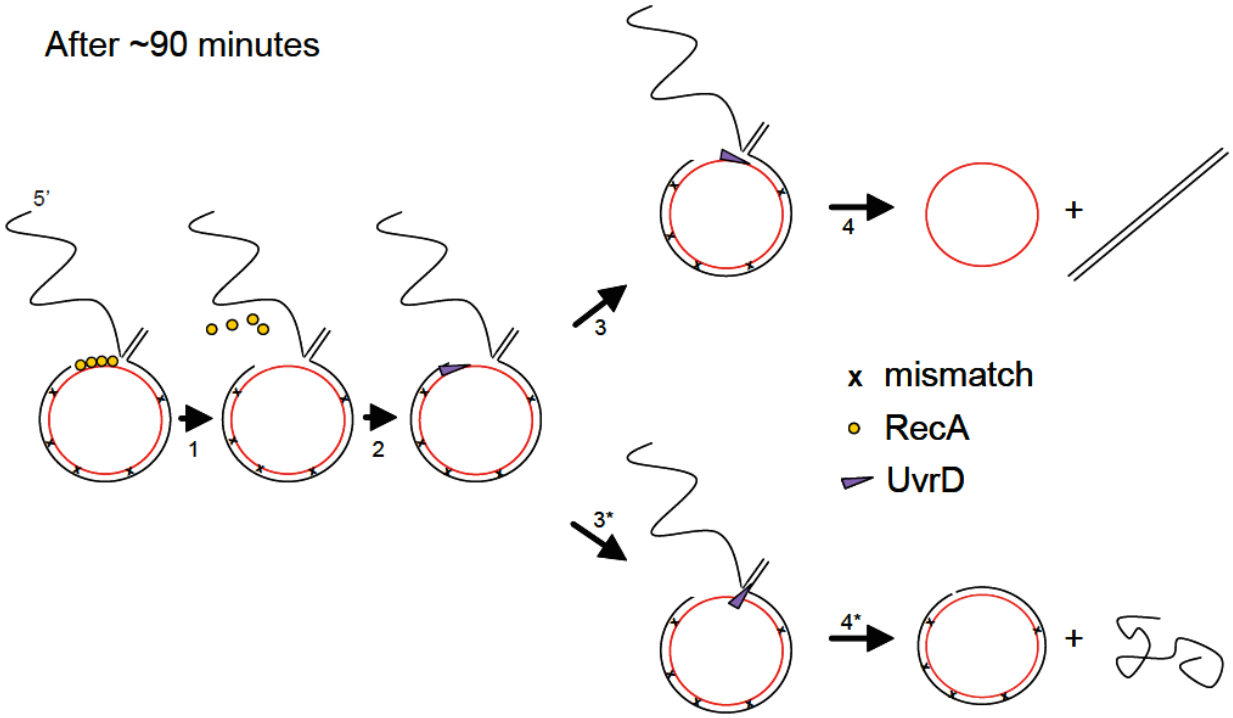

Figure 3. Model of UvrD Bidirectional Unwinding on fd-M13 DNA Intermediate
UvrD simultaneously increases both DNA substrates and products in homeologous strand exchange. RecA disassociate from the circular ssDNA gap after 90 minutes of homeologous reaction (arrow 1). The ss/dsDNA junction is thus exposed for UvrD binding (arrow 2). UvrD translocates toward the opposite DNA junction (arrows 3 and 3*). Upon reaching the three-stranded forked DNA junction, UvrD helicase either remains bound to the same strand and continues to unwind the heteroduplex (arrow 4) or switches strand and unwinds the homoduplex tail (arrow 4*). The former DNA intermediate resolution causes the re-formation of DNA substrates (arrow 4), while the latter DNA intermediate resolution leads to the formation of DNA products (arrow 4*).

The Mismatch-Activated MutS-MutL Complex Activates UvrD to Resolve Homeologous DNA Intermediates Unidirectionally

Because UvrD can be directed by the mismatch-activated MutS-MutL complex to unwind dsDNA toward the mismatch during MMR (Yamaguchi et al., 1998; Dao and Modrich, 1998), we tested whether UvrD can likewise be coordinated by the MutS-MutL complex during homeologous recombination (Figure 4A). As was described above, addition of UvrD alone to the homeologous reaction resulted in increased substrate and product formation at the expense of intermediate 5 (panel 2) and addition of MutS-MutL resulted in trapping of strand exchange intermediates and prevented product formation (panel 3). Addition of MutS, MutL and UvrD together (panel 4) again resulted in unwinding of trapped intermediate 5. Interestingly, in this case no additional product was formed but strand exchange intermediates were resolved to substrate (Figure S1). The increase in linear dsDNA substrate is observed at all time points indicating that the MutS-MutL complex coordinates the activity of UvrD early in the strand exchange reaction. Again, the resolution is dependent on helicase activity as UvrD ATPase mutants K35M (Figure 2A) and E221Q (Figure 2B) did not support this activity. Furthermore, this directed unwinding is largely mismatch-specific, as we did not observe a significant effect in the homologous reaction (Figure 4B). Interestingly, the UvrD-directed unwinding mediated by MutS-MutL seems to depend on ATP hydrolysis by MutL as we failed to detect resolution of the fd-M13 DNA intermediates in the presence of MutS and the MutL E29A ATPase mutant (Figure 4C). Thus the ATP-hydrolysis deficient MutL mutant is not only defective in blocking strand exchange but also unable to confer directionality to UvrD in conjunction with MutS. In conclusion, we demonstrate that the mismatch-activated MutS-MutL complex not only traps the recombination intermediates resulting in inhibition of branch migration but also stimulates UvrD helicase to resolve the DNA intermediates in a directional manner.

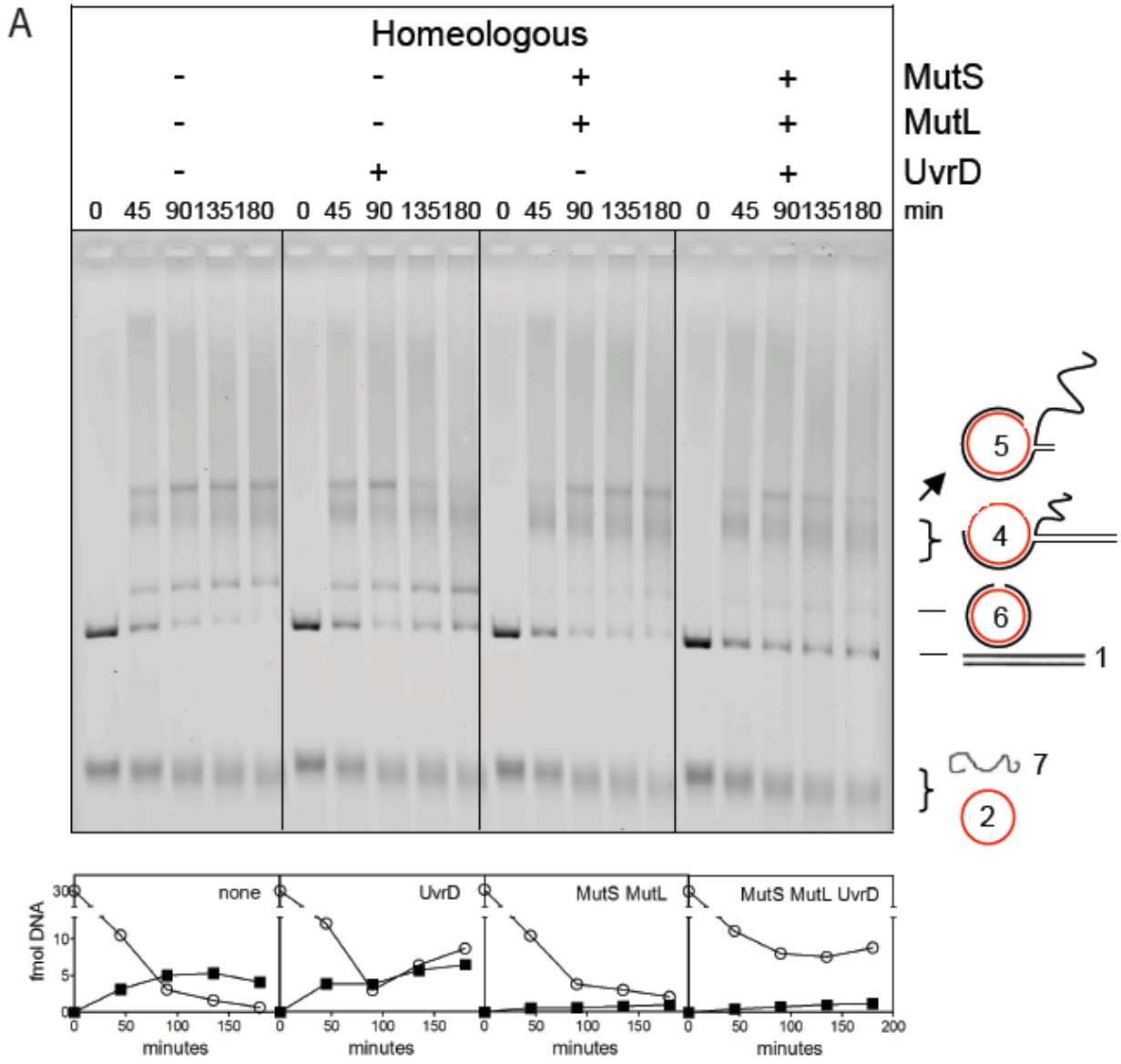

Figure 4. MutS and MutL confer directionality to UvrD-mediated unwinding of homeologous recombination intermediates

(A) Resolution of homeologous strand exchange intermediates by UvrD is bidirectional, but becomes unidirectional when guided by mismatch-activated MutS-MutL complexes. From left to right on the gel, time courses of homeologous strand exchange without MMR proteins, with UvrD (1 nM), with MutS-MutL (75, 75 nM) and MutS-MutL-UvrD (75, 75, 1 nM). Panels below the gel show quantified amounts of linear dsDNA substrate (open circles) and nicked circular dsDNA product (closed squares).

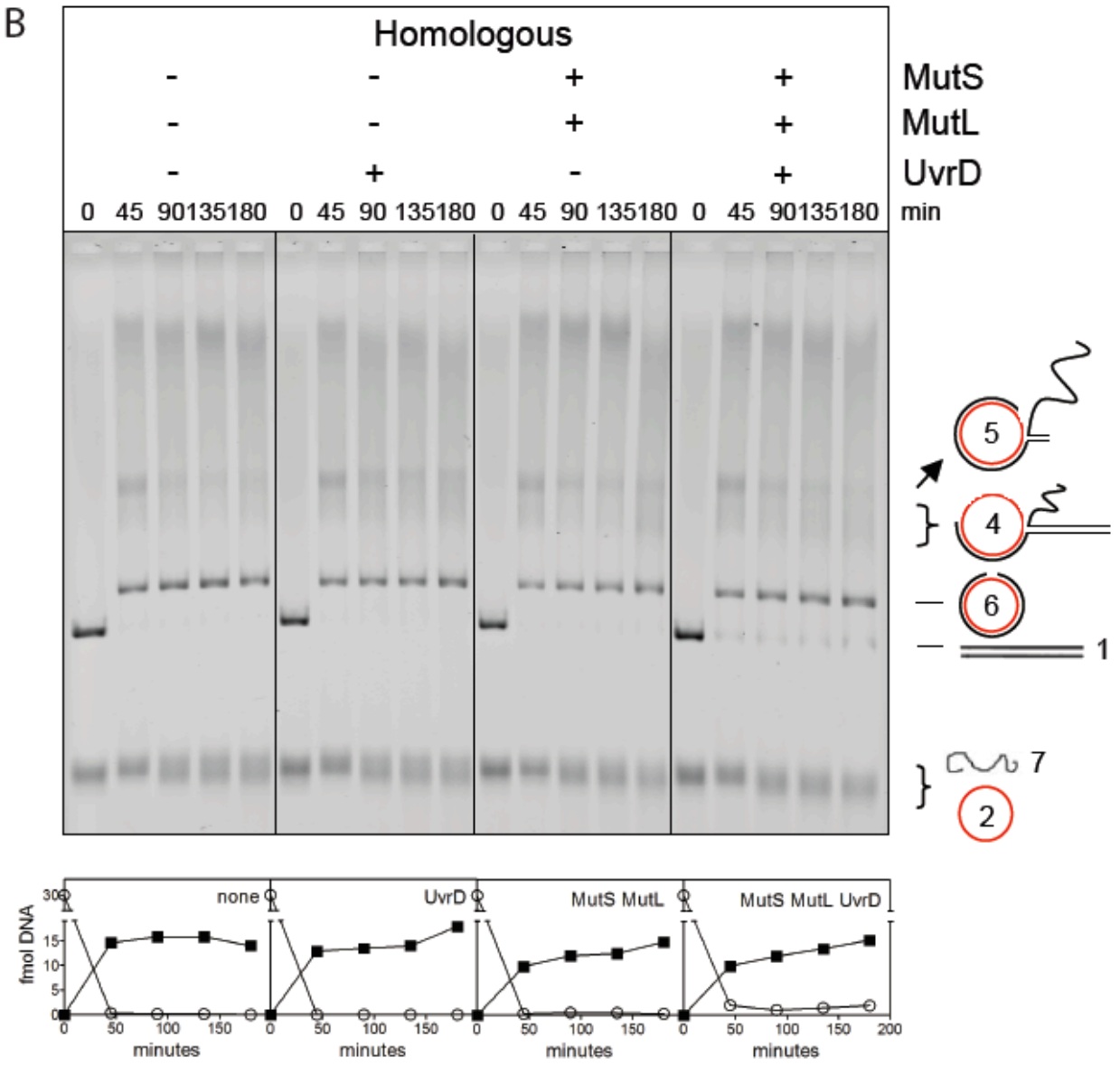

Figure 4. MutS and MutL confer directionality to UvrD-mediated unwinding of homeologous recombination intermediates

(B) MutS, MutL and UvrD do not resolve homologous strand exchange intermediates. From left to right on the gel, time courses of homologous strand exchange without MMR proteins, with UvrD (1 nM), with MutS-MutL (75, 75 nM) and MutS-MutL-UvrD (75, 75, 1 nM). Panels below the gel show quantified amounts of linear dsDNA substrate (open circles) and nicked circular dsDNA product (closed squares).

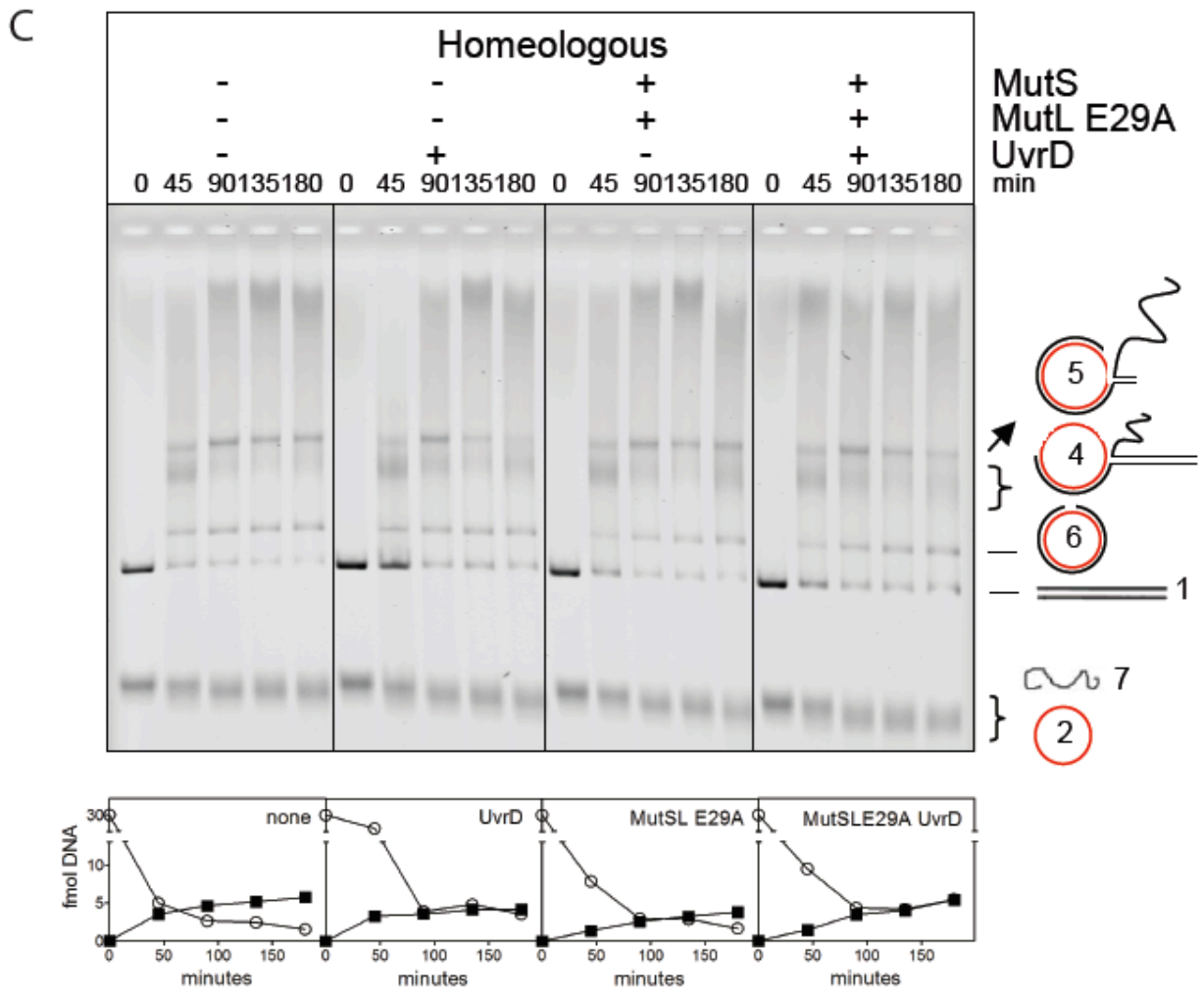

Figure 4. MutS and MutL confer directionality to UvrD-mediated unwinding of homeologous recombination intermediates

(C) MutS-MutL-directed UvrD resolution of homeologous DNA intermediates is dependent on MutL ATP hydrolysis activity. Panels from left to right contain no MMR protein, UvrD (1 nM), MutS-MutL E29A (75, 75 nM) and MutS-MutL E29A-UvrD (75, 75, 1 nM). Panels below the gel show quantified amounts of linear dsDNA substrate (open circles) and nicked circular dsDNA product (closed squares).

DISCUSSION

One of the roles of the UvrD helicase is the unwinding of DNA intermediates during MMR (Iyer et al., 2006; Jiricny, 2013). Based on genetic evidence (Rayssiguier et al., 1989; Stambuk and Radman, 1998) it also plays a role during antirecombination mediated by MMR. During MMR, UvrD is recruited by MutL, which interacts with mismatch-activated MutS, to unwind from a MutH-nicked GATC site toward a mismatch (Iyer et al., 2006). In the context of homeologous recombination, we found that UvrD alone either stimulates or inhibits the formation of DNA products depending on which dsDNA within the recombination intermediates is unwound by UvrD. In addition, we explored whether the MutS-MutL-trapped homeologous recombination intermediates

would form a substrate for UvrD. Indeed, UvrD efficiently resolves the trapped recombination intermediate in a directional manner in the presence of the mismatch-activated MutS-MutL complex.

A Mechanistic Model for the Roles of UvrD in Homeologous Strand Exchange

The observation that the UvrD-catalyzed conversion of DNA intermediate occurred late in the reaction (135 and 180 min) suggests that the loading site for the helicase is blocked by the circular RecA-ssDNA filament of the DNA intermediate during the first 90 minutes of the homeologous reaction 5 (Figure 3). After RecA dissociation (arrow 1), the two DNA junctions, that differ in structure, at the opposite ends of ssDNA gap become accessible. One is a single ss/ds DNA junction, the other is a DNA junction of three-stranded forked structure. The three-stranded forked DNA junction is considered to be the relevant UvrD loading site due to its unique conformation that can give rise to either DNA substrates or DNA products depending on the unwinding direction of UvrD. Since UvrD has a higher affinity for ss/ds DNA junctions than for ssDNA, dsDNA or Holliday junctions (Tomko et al., 2010; Carter et al., 2012) (arrow 2), translocation of UvrD begins from the ss/ds DNA junction in the 3'-5' direction toward the three-stranded forked DNA junction of the recombination intermediate (arrows 3 and 3*). Upon meeting the three-stranded forked DNA junction, UvrD either remains bound to the same DNA strand and unwinds the heteroduplex circle (arrow 4), or switches DNA strand and unwinds the linear homoduplex tail (arrow 4*) (Dessinges et al., 2004). Unwinding of the circular heteroduplex, which results in the separation of the circular heteroduplex and re-hybridization of the complementary strand with the linear displaced ssDNA, re-forms the circular ssDNA and linear dsDNA substrates (arrow 4). Unwinding of the linear homoduplex, which results in the separation of the homoduplex tail and hybridization of the complementary strand with the circular ssDNA, forms the nicked circular dsDNA and linear ssDNA products (arrow 4*). Thus, UvrD can both stimulate and inhibit homeologous strand exchange in the absence of MutS-MutL. This model of bidirectional UvrD unwinding presented in Figure S6 is consistent with previous findings (West et al., 1981; Kahn et al., 1981; Cox et al., 1981) that RecA-mediated strand exchange is strongly biased to start from the 3' end relative to the complementary strand of linear dsDNA substrate given that by having 5'-ss/ds DNA junction can UvrD translocate (3'-5' direction) to the opposite three-stranded forked DNA junction and unwind the recombination intermediate bidirectionally.

A Mechanistic Model for the Roles of UvrD in Antirecombination Mediated by MutS and MutL

UvrD acts exclusively as an antirecombinase during homeologous strand exchange mediated by RecA in the presence of MutS and MutL (Figure 5). UvrD is directed by the mismatch-activated MutS-MutL complex to resolve the trapped DNA intermediates specifically toward reformation of linear dsDNA and circular ssDNA. In the context of homeologous recombination, the interaction between MutS, MutL and UvrD is largely similar to their interactions in DNA mismatch repair (Yamaguchi et al., 1998; Dao and Modrich, 1998) in which UvrD is directed by the mismatch-activated MutS-MutL complex to unwind toward the mismatches. The entry site for UvrD unwinding is most likely the ss/ds DNA junction because it is closest to the first mismatch formed in the

heteroduplex and it is structurally similar to the MutH-incised nick that serves as the loading site of UvrD during mismatch repair. As a result of the MutS-MutL-directed UvrD unwinding, the DNA substrates are re-formed from the MutS-MutL-trapped DNA intermediates of homeologous recombination.

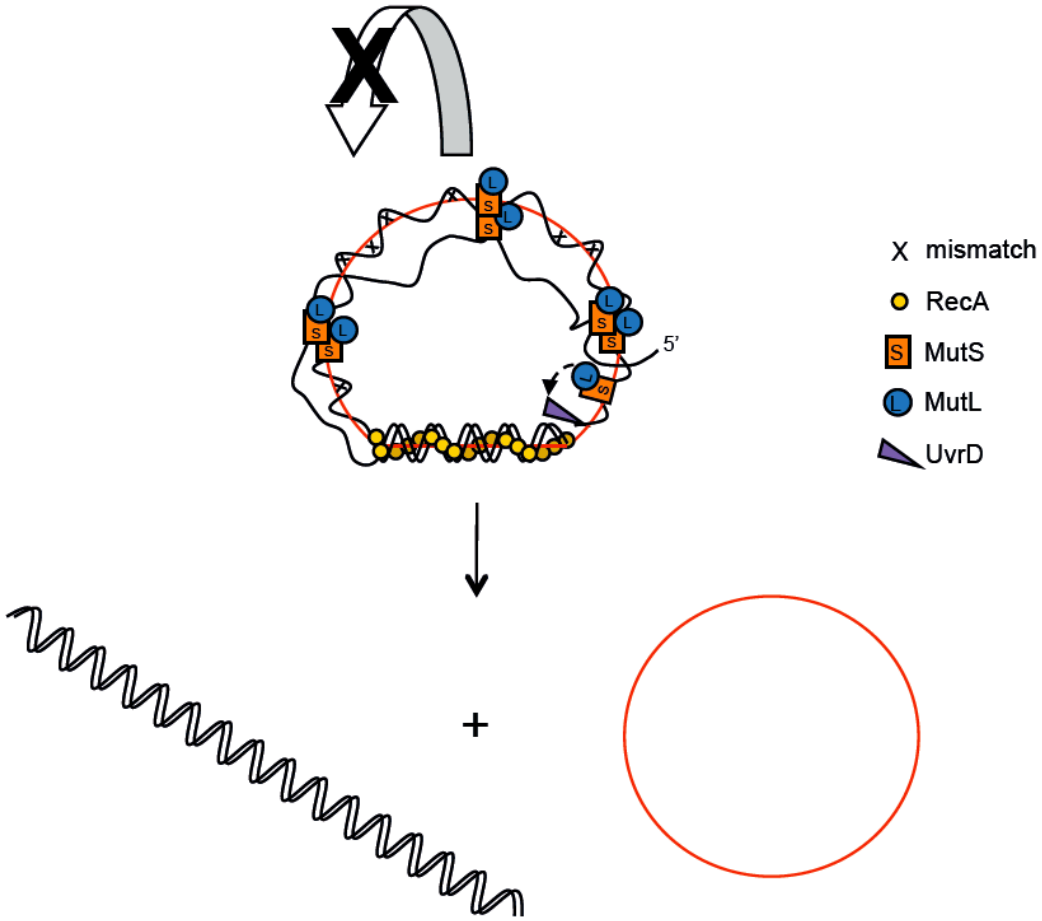

Figure 5. Model of MutS-MutL-Coordinated Unidirectional Resolution of Homeologous Recombination Intermediates by UvrD
The presence of mismatches in the heteroduplex of intermediates activates MMR antirecombination. MutS-MutL complexes bound to the mismatches and displaced ssDNA form higher-order complexes through MutS tetramerization. As a result, the freedom of heteroduplex to rotate and of displaced ssDNA to untwist from heteroduplex is hijacked, and the recombination intermediates are trapped from branch migration. UvrD is recruited specifically to the ss/ds DNA junction by MutS-MutL complexes on heteroduplex to resolve the trapped intermediates.

MMR-Dependent Antirecombination in Eukaryotes

DNA translocases confer reversibility at several steps of the homologous recombination subpathways by disrupting deadly recombination intermediates (Symington and Heyer, 2006). UvrD and its *S. cerevisiae* homolog Srs2 both disrupt recombinase-ssDNA filament and prevent strand exchange *in vitro* (Krejci et al., 2003; Veaute et al., 2003;

Veaute et al., 2005 and this study). While a specific role for Srs2 in inhibition of homeologous recombination was not observed using an inverted-repeat assay (Spell and Jinks-Robertson, 2004), recent studies indicate a role for Srs2 during gap repair involving homeologous sequences (Welz-Voegele and Jinks-Robertson, 2008; Mitchel et al., 2013). However, *S. cerevisiae* Sgs1 (a RecQ homolog) is a potent suppressor of homeologous recombination (Welz-Voegele and Jinks-Robertson, 2008; Myung et al., 2001). *sgs1* and *msh2* are epistatic in increasing homeologous recombination rates, indicating that Sgs1 may be specifically directed by mismatch-activated Msh2 to heteroduplex DNA (Spell and Jinks-Robertson, 2004). Interestingly, human BLM (also a RecQ homolog) may reverse recombination intermediates (van Brabant et al., 2000; Bachrati et al., 2006; Bugreev et al., 2007) and was shown to interact with human MSH6 both *in vivo* and *in vitro (Pedrazzi et al., 2003). As the mechanistic distinctions between the MMR systems of eukaryotes and bacteria become increasingly understood (Jiricny, 2013), it will be interesting to examine in more detail to what extent the mechanism of antirecombination is conserved throughout evolution.*

Conclusion

MutS and MutL trap homeologous recombination intermediates from branch migration involving mismatches in heteroduplex, secondary structures in displaced ssDNA and MutS tetramerization activity. In the absence of MutS, UvrD helicase acts as both inhibitor and stimulator of homeologous recombination. However, in the presence of MutS and MutL, UvrD is recruited by the mismatch-activated MutS-MutL complexes on heteroduplex to the nearest ss/ds DNA junction. As a result, UvrD reverses the trapped recombination intermediates by unwinding the heteroduplex and re-forms DNA substrates.

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SUPPLEMENTAL FIGURE

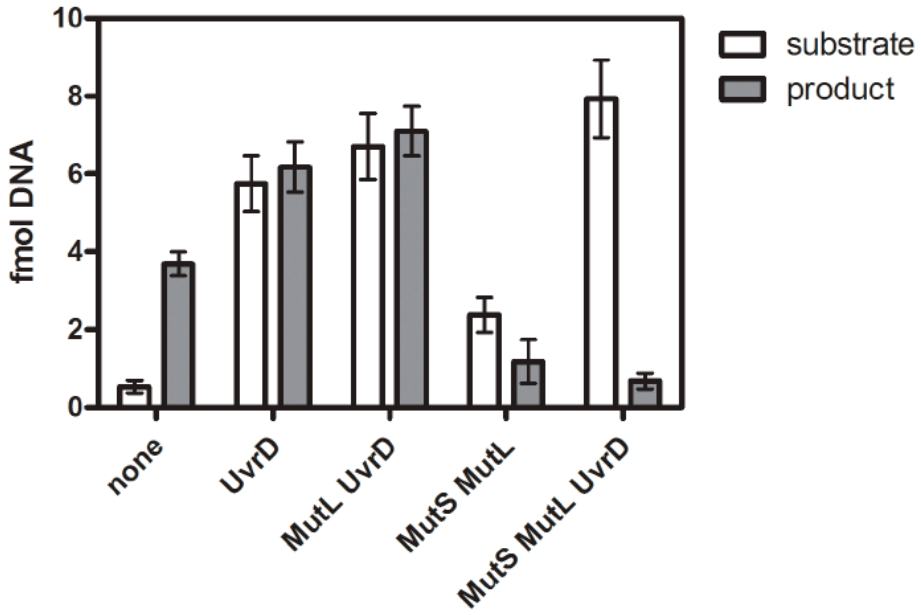

Figure S1. Quantification of Strand Exchange Reactions Performed in the Absence and Presence of Mismatch Repair Proteins

The amounts of substrate and product formed at the 180 minutes time point during homeologous strand exchange reactions in the absence of MMR proteins (none, n=9), with UvrD alone (n=8), with MutL and UvrD (n=7), with MutS and MutL (n=5), and with MutS, MutL and UvrD (n=4) from different gels were combined and averages +/- SEM are shown. UvrD in all cases significantly increased the amount of substrate (p-values < 0.0001 for conditions with UvrD, MutL and UvrD, MutS MutL and UvrD versus no MMR proteins (none); p-value 0.0009 for MutS MutL UvrD versus MutS MutL). UvrD and MutL+UvrD also significantly increased the amount of product compared to no MMR proteins added (p = 0.0024 and p= 0.0001, respectively). MutS and MutL significantly lower the amount of product (p = 0.0011 for MutS MutL versus none; p = 0.0002 for MutS MutL UvrD versus UvrD).

Summary

Homologous recombination (HR) is an evolutionarily conserved pathway involved in the repair of stalled replication forks and DNA double strand breaks (DSBs). Essentially, HR utilizes identical sequences located on sister chromatids to restart stalled forks and to resynthesize resected regions at DSBs and rejoin them. The joint molecule, which is formed between broken DNA and a homologous template, is a key intermediate of HR. The pivotal event of joint molecule formation is mediated by recombinases such as RecA in the bacterium *Escherichia coli* or its functional homologs in eukaryotic cells, including mammalian cells. The RecA recombinase, which is the core protein of HR, initially forms a nucleoprotein filament with single-strand (ss) DNA during the presynaptic phase of the recombination reaction. Next it searches for sequence homology, invades the dsDNA and forms a D-loop structure during the synaptic phase. Within the D-loop the incoming ssDNA pairs with its complementary sequence on the dsDNA while the other strand is displaced. In addition RecA allows extension of DNA heteroduplex via branch migration during the postsynaptic phase. The D-loop structure is subject to reversal via reinvasion of the displaced strand into the heteroduplex region (Chapter 2). This happens particularly in *in vitro* conditions using short invading DNA strands, suggesting that accessory proteins may regulate the stability of D-loops during recombination (Chapter 2). Sequence divergence *per se* acts as a barrier to HR, however, it is insufficient to completely inhibit recombination between divergent (homeologous) sequences. Because this could impact genome stability when HR occurs at ectopic sites, additional regulation of recombination between divergent DNA sequences is of importance. Given that recombination between homeologous sequences generates heteroduplex DNA with base mismatches, it is not surprising that proteins from the DNA mismatch repair (MMR) pathway, which are essential for the repair of replication errors, are implicated in the negative regulation of homeologous recombination. MutS and MutL, as the most upstream factors of MMR, are potent suppressors of homeologous recombination *in vivo*. The involvement of MMR proteins in homeologous antirecombination has mainly been revealed by genetic studies, but it is not clear how these proteins act at molecular level. We address mechanistically how the MutS-MutL complex and UvrD helicase suppress homeologous recombination in chapters 3 and 4, respectively. As during MMR, MutS binds to a mismatch within the heteroduplex of recombination intermediates. MutL *per se* does not inhibit homeologous recombination *in vitro*, but it interacts with and stabilizes MutS bound to the heteroduplex of the recombination intermediate. *In vitro*, MutS and MutL trap homeologous recombination intermediates and prevent heteroduplex extension. In addition, MutS tetramerization activity and MutS binding to the secondary structures formed within the displaced strand of homeologous recombination intermediate are equally important (Chapter 3). We propose a coherent model for the trapping of homeologous recombination intermediates imposed by MutS and MutL (Chapter 3). During the extension of DNA heteroduplex, a window of synaptic complex, which consists of a RecA protein filament and three DNA strands (simply referred to as RecA-DNA complex hereafter), travels unidirectionally toward the 3' end of the invading strand. At the trailing end of the RecA-DNA complex, the displaced strand unspools and RecA disassembles from the heteroduplex. At the same time, at the opposite leading

end of the RecA-DNA complex, nucleoprotein filament continuously spools into the dsDNA. Simultaneous events occurring at both ends of RecA-DNA complex are crucial for the extension of the DNA heteroduplex. During homeologous recombination, mismatches generated within the heteroduplex attract and facilitate the formation of MutS-MutL-heteroduplex DNA complexes. These events by themselves will of course not lead to antirecombination. However, we demonstrate that MutS and MutL also complex with the secondary structures formed within the displaced single strand DNA of the recombination intermediate (Chapter 3). When the MutS-MutL complexes bound to the heteroduplex and the displaced strand of the homeologous recombination intermediate form higher-order complexes aided by the MutS tetramerization domains, the freedom of the displaced strand to unspool from the heteroduplex is hijacked at the trailing end of RecA-DNA complex. Consequently the spooling of the nucleoprotein filament into the dsDNA at the leading end of the RecA-DNA complex is prevented, which in turn prevents the extension of the DNA heteroduplex. In other words, the homeologous recombination intermediate is trapped (Chapter 3). Considering the notion that trapped DNA intermediates are toxic, we investigated the possibility of intermediate resolution mediated by the UvrD helicase in chapter 4. We found that the UvrD is coordinated by mismatch-activated MutS-MutL complex to unidirectionally resolve the trapped recombination intermediate, thereby reforming the substrate DNA molecules (Chapter 4). Importantly, we demonstrate that mismatch-bound MutS-MutL complexes impart a direction on the UvrD DNA unwinding reaction, as in their absence UvrD both promotes and inhibits homeologous recombination *in vitro*, depending on whether the heteroduplex or the recombining homoduplex region of the recombination intermediate is unwound (Chapter 4). In conclusion, our findings provide a completely novel view of how MMR-mediated antirecombination works in *E. coli*. Our model lays the foundation for future research into the understanding of inhibition of recombination between homeologous sequences mediated by MMR proteins in eukaryotes, which is important because mismatch-triggered antirecombination may be crucial for preventing chromosomal translocations initiated from recombination between repetitive sequences that can cause diseases, such as cancer, in humans.

Samenvatting

Homologe recombinatie (HR) is een evolutionair geconserveerd proces dat betrokken is bij de reparatie van geblokkeerde replicatievorken en dubbelstrengsbreuken in DNA. HR gebruikt de identieke DNA volgorde die zich op de zusterchromatide bevindt om geblokkeerde vorken te herstarten, om het ontbrekende DNA rond de dubbelstrengsbreuk opnieuw te synthetiseren en de DNA fragmenten weer aan elkaar te koppelen. Een belangrijk intermediair in deze reacties is een gepaard molecuul dat gevormd wordt door het gebroken DNA en een homologe partner. De vorming van dit cruciale tussenprodukt wordt uitgevoerd door recombinatie-eiwitten zoals RecA in de bacterie *Escherichia coli*, en diens functionele homologen in eukaryote cellen waaronder zoogdiercellen. Het RecA recombinase, het centrale eiwit gedurende HR, vormt een filament op enkelstrengs DNA gedurende de presynaptische fase van de recombinatiereactie. Vervolgens zoekt het naar homologie in het dubbelstrengs DNA van de homologe partner en vormt een zogenoemde 'D-lus' (D-loop) structuur in de synaptische fase. In de D-loop structuur zit het inkomende enkelstrengs DNA gebonden aan het complementaire DNA van het dubbelstrengs molecuul, terwijl de andere streng van dit DNA verplaatst is. RecA maakt ook extensie van de heteroduplex regio mogelijk via migratie van de DNA aftakking in de D-loop structuur tijdens de postsynaptische fase. Het vormen van de D-loop structuur is omkeerbaar doordat de verplaatste DNA streng de heteroduplex regio weer kan binnendringen (hoofdstuk 2). Dit vindt in *in vitro* condities vooral plaats met korte binnenkomende DNA strengen, en suggereert dat gedurende recombinatie de stabiliteit van de D-loop gereguleerd wordt door additionele eiwitten (hoofdstuk 2). Verschillen in basevolgorde in het DNA zijn een barrière voor HR, maar dit is niet voldoende om recombinatie tussen divergente (homeologe) sequenties volledig te voorkomen. Wanneer HR tussen niet-overeenkomstige plekken op verschillende chromosomen plaatsvindt, kan dit tot genoom instabiliteit leiden en dus is het belangrijk dat recombinatie tussen niet identieke DNA fragmenten nog extra gereguleerd wordt. Omdat recombinatie tussen homeologe DNA volgordes heteroduplex DNA genereert waarin incorrecte baseparingen voorkomen is het niet verrassend dat eiwitten uit de DNA mismatch reparatie (MMR) cascade, die essentieel zijn voor het repareren van DNA kopieerfouten, betrokken zijn bij de inhibitie van homeologe recombinatie. MutS en MutL zijn sterke remmers van homeologe recombinatie in de cel. De betrokkenheid van MMR eiwitten in homeologe antirecombinatie is vooral duidelijk gebleken uit genetische studies, maar het is onbekend hoe deze eiwitten op moleculair niveau opereren in dit proces. Wij hebben in hoofdstuk 3 en 4 onderzocht hoe respectievelijk het MutS-MutL complex en het UvrD helicase bijdragen aan de remming homeologe recombinatie. Zoals het ook gedurende mismatch reparatie doet, herkent en bindt MutS een mismatch in de heteroduplex van het recombinatie tussenprodukt. MutL zelf remt de homeologe recombinatie *in vitro* niet, maar het bindt en stabiliseert MutS op de heteroduplex in het tussenprodukt. *In vitro* blokkeren MutS en MutL de homeologe tussenprodukten en voorkomen het verlengen van het heteroduplex gebied. MutS tetramerisatie en het binden van MutS aan secundaire DNA structuren binnen het verplaatste enkelstrengs DNA in het tussenprodukt zijn evenzeer belangrijk (hoofdstuk 3). Wij hebben een coherent model opgesteld voor het remmen van homeologe recombinatie door MutS en MutL

(hoofdstuk 3). Gedurende de extensie van de heteroduplex regio verschuift het synaptische complex, bestaande uit het RecA eiwit filament en drie DNA strengen (verder RecA-DNA complex genoemd) in de richting van het 3'-uiteinde van de binnenkomende DNA streng. Aan de achterkant spoelt de verplaatste DNA streng uit dit voortbewegende synaptische complex en RecA dissocieert van de heteroduplex regio. Op hetzelfde moment aan de andere kant van het RecA-DNA complex, spoelt het RecA eiwit filament continu het dubbelstrengs DNA in. Deze gelijktijdige gebeurtenissen aan beide kanten van het RecA-DNA complex zijn cruciaal voor de extensie van de heteroduplex. Gedurende homeologe recombinatie vormen zich MutS-MutL complexen op de mismatches in de heteroduplex regio. Op zichzelf kan dit niet leiden tot antirecombinatie. We hebben echter aangetoond dat MutS en MutL ook een complex vormen met de secundaire DNA structuren die gevormd worden in het verplaatste enkelstrengs DNA in het recombinatie tussenproduct (hoofdstuk 3). Als de MutS MutL complexen gebonden aan de heteroduplex regio en het verplaatste enkelstrengs DNA gezamenlijk een groter complex vormen, daarbij geholpen door MutS tetramerisatie, dan kan het verplaatste enkelstrengs DNA niet langer uit de heteroduplex regio aan het uiteinde van het RecA-DNA complex spoelen. Het gevolg hiervan is dat het RecA filament aan de andere kant van het synaptische complex niet langer het dubbelstrengs DNA in kan spoelen en dat de heteroduplex regio niet uitgebreid kan worden (hoofdstuk 3). Omdat DNA tussenprodukten waarmee niets gebeurd giftig zijn voor de cel, hebben we de mogelijkheid van ontwinden van tussenproduct door het UvrD helicase onderzocht in hoofdstuk 4. We hebben ontdekt dat UvrD door mismatch-geactiveerde MutS MutL complexen wordt gecoördineerd om de tussenproducten zodanig te ontwinden dat alleen de uitgangssubstraten worden gevormd. Mismatch-geactiveerde MutS MutL complexen geven dus richting aan UvrD, omdat in hun afwezigheid het UvrD helicase homeologe recombinatie zowel remt als stimuleert doordat het de recombinatie tussenproducten bidirectioneel kan ontwinden; naar de uitgangssubstraten of naar de producten van recombinatie. Concluderend werpen onze resultaten een compleet nieuw licht op het mechanisme van antirecombinatie door MMR in *E. coli*. Ons model legt een basis voor verder onderzoek naar de remming van homeologe recombinatie in eukaryoten, wat belangrijk kan zijn voor het voorkomen van chromosomale translocaties tussen gebieden met repeterende DNA volgordes die betrokken kunnen zijn bij het ontstaan van ziektes zoals kanker.

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-Epigenetic Regulation, Leiden, The Netherlands

-Safely Working in the Laboratory, Leiden, The Netherlands

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MGC 18th PhD Workshop, June 14-17, Maastricht, The Netherlands 2011

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