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RAD54 controls access to the invading 3′-OH end after RAD51-mediated DNA strand invasion in homologous recombination in *Saccharomyces cerevisiae*

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ABSTRACT

Rad51 is a key protein in homologous recombination performing homology search and DNA strand invasion. After DNA strand exchange Rad51 protein is stuck on the double-stranded heteroduplex DNA product of DNA strand invasion. This is a problem, because DNA polymerase requires access to the invading 3′-OH end to initiate DNA synthesis. Here we show that, the *Saccharomyces cerevisiae* dsDNA motor protein Rad54 solves this problem by dissociating yeast Rad51 protein bound to the heteroduplex DNA after DNA strand invasion. The reaction required species-specific interaction between both proteins and the ATPase activity of Rad54 protein. This mechanism rationalizes the *in vivo* requirement of Rad54 protein for the turnover of Rad51 foci and explains the observed dependence of the transition from homologous pairing to DNA synthesis on Rad54 protein in vegetative and meiotic yeast cells.

INTRODUCTION

Homologous recombination is a high-fidelity DNA repair pathway that is critical for DSB repair and the recovery of stalled and broken replication forks, as well as chromosome segregation during meiosis (1). Rad51, the eukaryotic RecA homolog, catalyzes the key reaction of homology search and DNA strand invasion (2). In the yeast *Saccharomyces cerevisiae*, Rad51-deficient cells display extreme sensitivity to DSBs. In these cells recombinational repair is virtually eliminated, leaving only non-homologous endjoining and single-strand annealing as DSB repair pathways. Similar to RecA, Rad51 forms a right-handed filament on DNA and performs the same basic function of homology search and DNA strand exchange (2,3). Nucleotide cofactor binding and hydrolysis influence DNA binding of both RecA and Rad51, but with differences depending on the species (4). For both proteins, cofactor binding induces a high-affinity DNA-binding state, whereas hydrolysis lowers DNA-binding affinity. Two of the major biochemical differences between RecA and Rad51 are the kinetic impediment to RecA binding to dsDNA and the lower ATPase activity of Rad51 (200-fold with dsDNA for yeast Rad51) (2,4–6). The impact and significance of these differences remain to be elucidated but, as a consequence, Rad51 shows avid binding to duplex DNA and the resulting dsDNA nucleoprotein filaments are relatively stable even under conditions of ATP hydrolysis, in particular for yeast Rad51 (5,7). It is likely that eukaryotes evolved cofactors that influence Rad51 filament dynamics and its nucleotide cofactor cycle.

Rad54 is a eukaryotic-specific recombination factor without an obvious bacterial counterpart (1), with the exception of an archaeal homolog found in *Sulfolobus solfataricus* [(8); C. Hazeltine and S. Kowalczykowski, personal communication] that may have undergone horizontal gene transfer. Rad54-deficient yeast cells display the same array and severity of DNA-repair defects as Rad51-deficient cells (1). Mutations in *RAD51* and *RAD54* are fully epistatic, demonstrating their function in a single pathway. Rad54 protein is a dsDNA-dependent ATPase that translocates at 300 bp/s on duplex DNA (9). Rad54 protein augments the *in vitro* recombination activities of Rad51 protein by ATP-independent (Rad51 filament stabilization) and ATP-dependent mechanisms [including stimulation of DNA strand invasion, branch migration and chromatin remodeling; for reviews see (10,11)]. Previously, we have shown that the Rad54 ATPase activity is specifically stimulated up to 6-fold at the termini of Rad51 filaments on duplex DNA, leading to the dissociation of the Rad51–dsDNA filaments (7,12–14).

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We have proposed that eukaryotes utilize Rad54 for the turnover of Rad51–dsDNA filaments to compensate for the intrinsically low dsDNA-dependent ATPase activity of Rad51 and help increase the dynamic turnover of Rad51–dsDNA complexes *in vivo*.

Consistent with this idea, RecA protein is bound to the heteroduplex DNA after DNA strand exchange and requires hydrolysis of the bound ATP to lower its DNA-binding affinity to release the duplex DNA (15). Using the ATPase-deficient Walker A box mutant RecA–K72R protein, Xu and Marians (35) showed that access to the invading 3′-OH end by DNA polymerase was obstructed by RecA remaining bound to the heteroduplex DNA. Hence, the absence of ATP hydrolysis by RecA to induce filament turnover precluded the transition from strand invasion to D-loop extension. The reduced dynamics of the Rad51–dsDNA filament may pose a significant dilemma, as it may interfere with subsequent steps in recombination, namely DNA synthesis. Here we present biochemical evidence, consistent with numerous *in vivo* studies, which the Rad54 motor protein functions to turnover Rad51 from the heteroduplex DNA allowing access of DNA polymerase to the 3′-OH end.

MATERIALS AND METHODS

Proteins, DNAs

S. cerevisiae Rad51, Rad51–K191R, Rad54, Rad54–K341R, RPA, as well as human Rad51 and Rad54 proteins were purified as previously described (7,16). The pUC19 DNA (2686 bp) was prepared by Triton lysis and purified by isopycnic density centrifugation on CsCl/Ethidium bromide gradients. T4 polynucleotide kinase and DNA polymerase I Klenow fragment were purchased from New England Biolabs.

Crosslinking of linear D-loops

Reactions were carried out at 30°C in SEB buffer (30 mM Tris–acetate pH 7.5, 1 mM DTT, 50 μg/ml BSA, 2.5 mM ATP, 4 mM Mg^{2+}-acetate, 20 mM phosphocreatine). A 95-mer (olWDH760 5′-TgCAggCATgCAAgCTTggCgTA ATCATggTCATAgCTgTTTCCTgTgTgAAA TTgTTAT CCgCTCACAATTCCACACAACATACgAgCCggAAg-3′ referred to as *Pst*I-95-mer; olWDH848 5′-ACgTCTAA gAAACCATTATTATCATgACATTAACCTATAAAA ATAggCg TATCACgAggCCCTTTCgTCTCgCgCgTTT CggTgATgACggTgAA-3′ referred to as *Aat*II-95-mer) labeled at its 5′-end (2 μM nt, 21 nM molecules for 1:1 ratio; 6 μM nt, 63 nM molecule for 3:1 ratio) by T4 polynucleotide kinase was incubated with Rad51 (0.67 or 2 μM) for 10 min to assemble nucleoprotein filaments. pUC19 plasmid (56.6 μM bp; 21 nM molecule; *Pst*I- or *Aat*II-linearized) was added to initiate the formation of linear D-loop in the absence or presence of Rad54 (72 or 144 nM). At each time point, aliquots of sample were mixed with psoralen (100 ng/ml), placed on the surface of a piece of parafilm in an ice container, and crosslinked by UV-irradiation at 365 nm (UVP, model B-100A) for 5 min at about 4 cm from the light source. After crosslinking, samples were incubated with stop buffer (10% SDS, 0.5 M EDTA, 20 mg/ml Proteinase K) for 10 min at 30°C, and separated on 1% agarose gels at 7 V/cm for 3 h. The gel was dried, and analyzed by a phosphoImager.

Linear D-loop extension

Reactions were carried out at 30°C in SEB buffer and the four dNTPs were added to a final concentration of 100 μM each. 95-mer labeled at its 5′-end (2 or 6 μM nt) was incubated with Rad51 (0.67 or 2 μM) for 10 min to assemble nucleoprotein filaments. Plasmid pUC19 (56.6 μM, *Pst*I- or *Aat*II-linearized) was added to initiate the formation of linear D-loop for another 10 min. Rad54 (72 or 144 nM) and DNA-polymerase I (Klenow fragment, 24 or 72 nM) were added to the reaction to initiate the extension. At each time point, aliquots of sample were mixed with stop buffer for 10 min at 30°C, and separated on 1% agarose gels at 7 V/cm for 3 h. The gels were dried and analyzed by a phosphoImager.

α-^{32}P-dGTP incorporation assay

For the extension with α-^{32}P-dGTP, the experimental procedure is as described, except that the 95-mer was unlabeled and the dNTP pool consisted of three dNTPs at 14 μM each and α-^{32}P-dGTP at 70 nM. For the analysis of extension products on denaturing gels, after mixing with the stop buffer for 10 min, the samples were heated at 95°C for 5 min with 2× formamide loading buffer (10 mM EDTA pH 8.0, 98% v/v formamide, 0.025% w/v Bromophenol Blue). Samples were separated by 6% PAGE containing 8 M urea at 20 V/cm for 60 min.

RESULTS

The progression from strand invasion to strand extension by DNA synthesis is a key transition in homologous recombination. Like its bacterial counterpart RecA protein, Rad51 protein is expected to remain bound to the heteroduplex DNA product after DNA strand invasion, but this has never been directly demonstrated. Using a DNA topology-based ligation assay (Supplementary Figure 1), we show that yeast Rad51 remains bound to the heteroduplex DNA after DNA strand exchange. This occurred even under conditions that allowed ATP hydrolysis, where RecA is known to release dsDNA. This poses a significant problem, as Rad51 may interfere with the subsequent step in recombination, DNA synthesis.

Assay system

To study the transition between DNA strand invasion and DNA repair synthesis, we used a linear D-loop assay (Figure 1A), in which Rad51 forms a presynaptic filament on a 95-mer to catalyze DNA strand invasion into linear pUC19 dsDNA. Subsequent addition of Rad54 and DNA polymerase allowed the detection of D-loop extension products (Figure 1D). As a model polymerase to probe the access to the invading 3′-OH end, we employed the Klenow fragment of DNA polymerase I. The linear D-loop extension assay affords the advantage of avoiding potential topological problems for DNA synthesis on closed circular DNA (35). In addition, linear

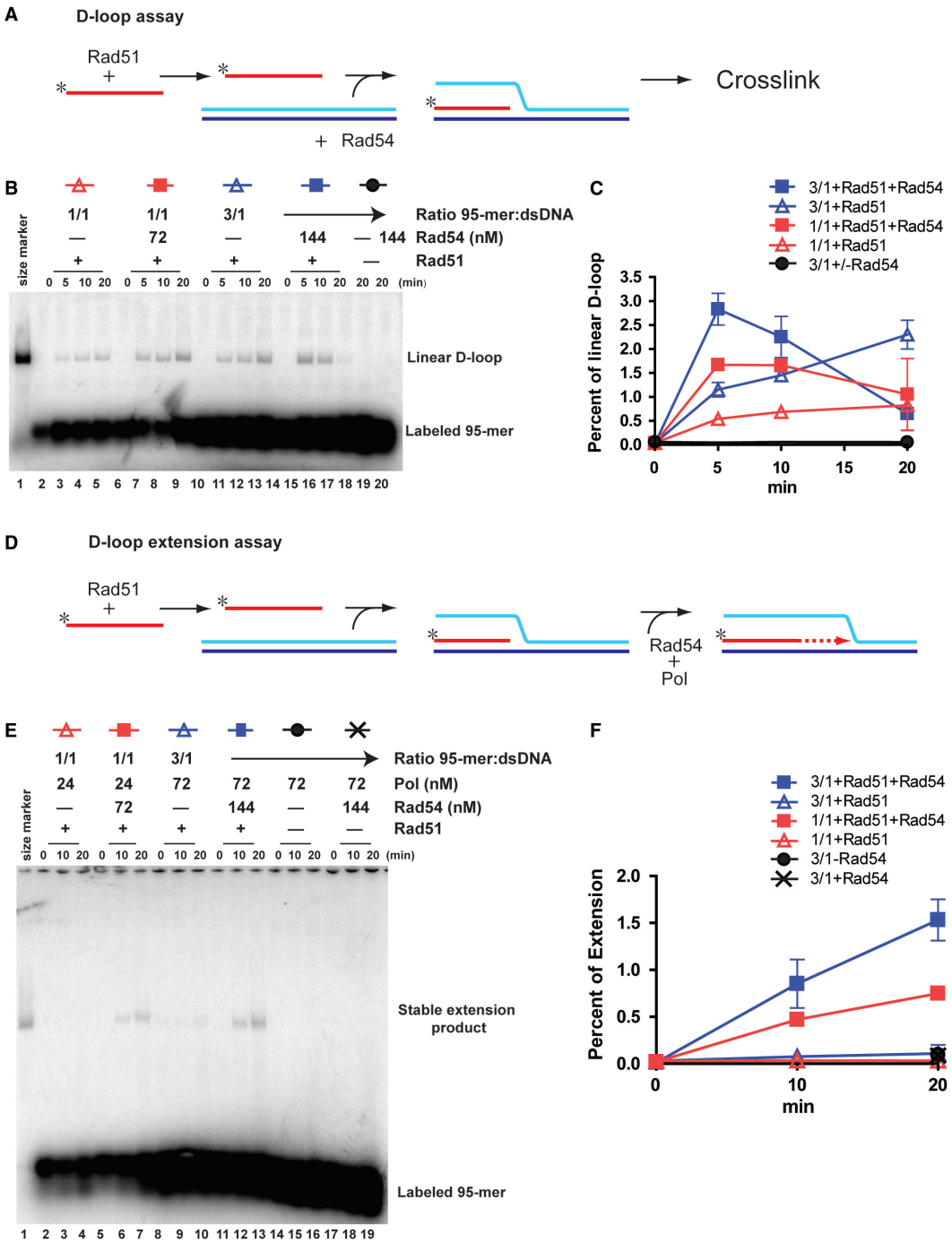

Figure 1. Rad54 is required for D-loop extension. (**A**) Schematic representation of the linear D-loop assay. The *Aat*II-95-mer is homologous to the terminal sequence of the *Aat*II-linearized pUC19 DNA (2686 bp). Reaction products are identified by 5′-end-labeling the 95-mer. (**B**) D-loop assay. End-labeled *Aat*II-95-mer was incubated with Rad51 (1 monomer:3 nt) and then with *Aat*II-linearized pUC19 dsDNA at molecular ratios of oligonucleotide to dsDNA of 1:1 (lanes 2–9) or 3:1 (lanes 10–20). Reactions contained 72 or 144 nM Rad54 (lanes 6–9, 15–18, 20) and Rad51 (lanes 2–18). End-labeled linearized pUC19 served as size marker (lane 1). Products were crosslinked before electrophoresis. (**C**) Quantitation of results in (B). (**D**) Schematic representation of the linear D-loop extension assay. (**E**) D-loop extension assay. Reaction schemes as in (A), except that they also contained 24 or 72 nM polymerase (lanes 2–19). Stable extension products are D-loops of sufficient length to be stable under electrophoresis conditions. Products were not crosslinked before electrophoresis in the D-loop extension assay. (**F**) Quantitation of results in (E). Shown are means from three determinations; error bars represent 1 SD.

D-loop formation by yeast Rad51 is less dependent on Rad54 than the traditional D-loop reaction (linear ssDNA and supercoiled dsDNA) [Figure 1, (17)], allowing us to better address the role of Rad54 after DNA strand invasion. Linear D-loops are unstable and required psoralen-crosslinking to be analyzed by gel electrophoresis (Figure 1A–C, data not shown). In the D-loop extension assay no cross-linking was used and two products were

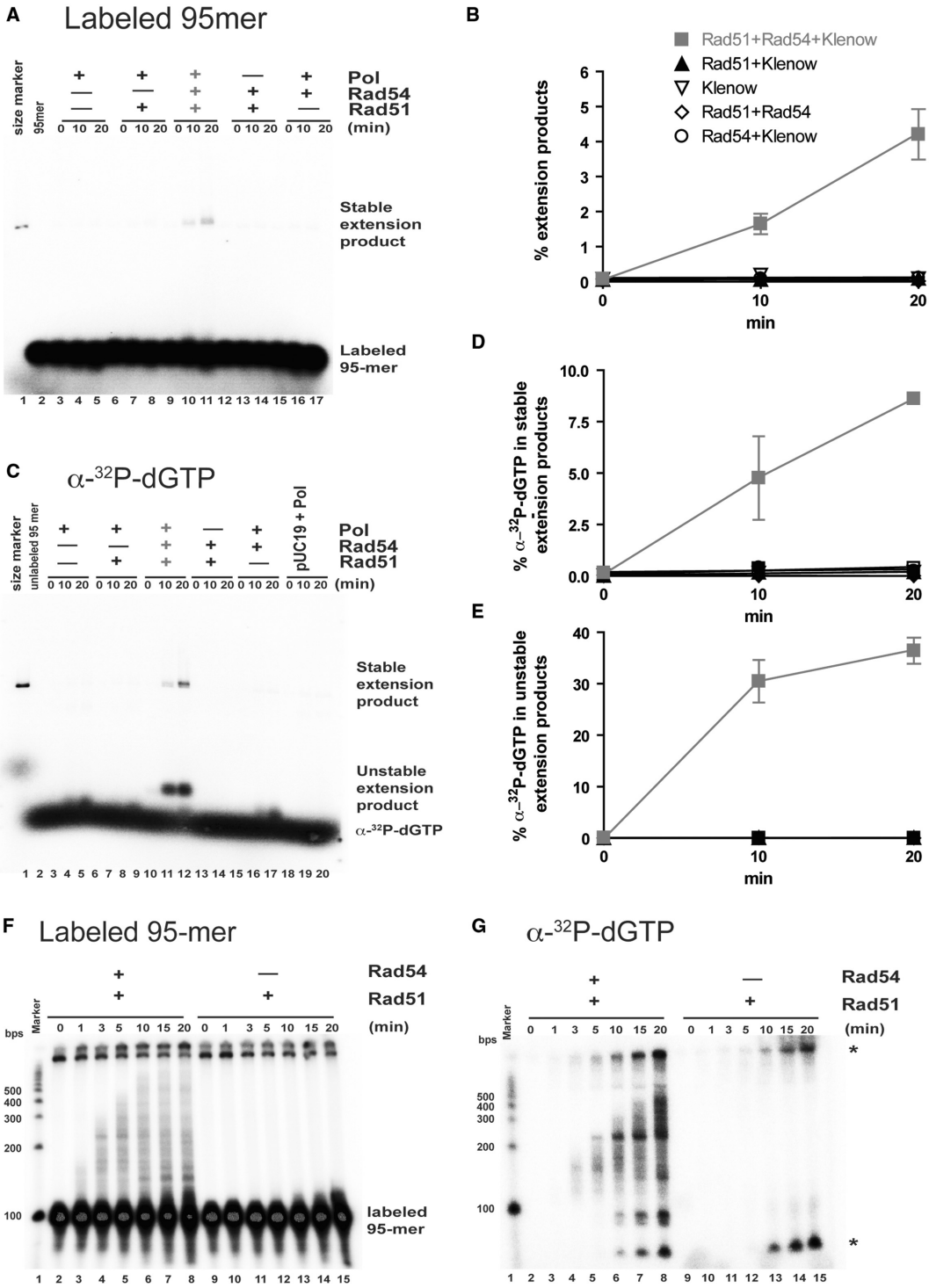

Figure 2. Rad54 is required for D-loop extension. D-loop extension assays. The *Pst*I-95-mer is homologous to the terminal sequence of the *Pst*I-linearized pUC19 DNA (2686 bp). Reaction products are identified either by end-labeling the 95-mer (**A**, **B**, **F**) or by incorporation of α-^{32}P-dGTP (**C**–**E**, **G**). (A) D-loop extension assay with end-labeled *Pst*I-95-mer. Rad51 nucleoprotein filaments were incubated either in the presence of Rad54 (72 nM, lanes 9–11), or absence of Rad54 (lanes 6–8), or absence of DNA polymerase I (Klenow fragment 24 nM, lanes 12–14). Protein-free 95-mer was also incubated either in the presence of Rad54 (72 nM, lanes 3–5), or absence of Rad54 (lanes 15–17). Lane 1 shows end-labeled *Pst*I-linearized pUC19 as a size marker, and lane 2 the end-labeled 95-mer. (B) Quantification of the results for D-loop extension in (A). (C) D-loop extension assay with α-^{32}P-dGTP and unlabeled *Pst*I-95-mer. Reactions were as in (A), except lane 2 contains unlabeled *Pst*I-95-mer and lanes 18–20 show *Pst*I-linearized pUC19 with DNA polymerase I (Klenow fragment, 24 nM). (D) Quantification of the stable extension product from (C). Stable extension products are D-loops of sufficient length to be stable under electrophoresis conditions. (E) Quantification of the unstable extension product from (C). Unstable extension products are extended 95-mers with insufficient length to result in stable D-loops under electrophoresis conditions. For (B)–(E) shown are means from three determinations; error bars represent 1 SD. (F) Analysis of extension products on denaturing gel from reactions with end-labeled 95-mer and (G) with α-^{32}P-dGTP. The signals labeled by asterisk are due to a combination of 3′–5′ exonuclease (proofreading) and/or polymerase activity of Klenow polymerase on the 95-mer or the linear dsDNA.

observed. First, unstable extension products represented short extensions of the D-loop that dissociated during gel electrophoresis and migrated just slightly slower than the labeled 95-mer. These products are overshadowed by the end-labeled primer (Figure 1E), but are readily identified when using incorporation of α-^{32}P-dGTP to label extension products (Figure 2C and E). These unstable extension products are represented by the Rad54-dependent signal migrating just around the 100 nt marker in Figure 2G. Second, stable extension products represent longer DNA synthesis products with sufficient length to survive electrophoresis (Figures 1E, F, 2A and B; Supplementary Figure 2D and F).

Rad54 is required for extension of linear D-loop by DNA polymerase

As expected, yeast Rad51 catalyzes linear D-loop formation (Figure 1A–C). Rad54 stimulates D-loop formation early in the time course. Consistent with previous observations (18), Rad54 also destabilizes D-loops, as is evident from the loss of the D-loop signal at later time points, in particular in reactions using a ratio of three oligonucleotides per dsDNA substrate. Importantly, the generation of stable extension products from linear D-loops by polymerase was almost entirely dependent on the presence of Rad54 protein (Figure 1D–F). The 5′-end-labeled 95-mer allowed accurate quantitation of D-loop formation (Figure 1C) and D-loop extension (Figure 1F). At a substrate ratio of 3:1, the presence of Rad54 stimulated D-loop extension by over 15-fold. At the 1:1 substrate ratio, extension was entirely dependent on Rad54. These results clearly demonstrate a role for Rad54 after DNA strand invasion. D-loops formed at comparable levels, albeit at different times, in reactions with or without Rad54 protein, but extension was greatly or completely dependent on the presence of Rad54. This conclusion was verified independently with a second substrate pair, using a different 95-mer and dsDNA linearized at a different site (Figure 2). This substrate pair showed greater dependence of D-loop formation on Rad54 (about 5-fold stimulation, Supplementary Figure 2A and B), but reconstruction experiments demonstrated the complete dependence of D-loop extension by DNA polymerase on Rad54 (compare Supplementary Figure 2B and D).

To further enhance the sensitivity of the D-loop extension assay and to formally demonstrate DNA synthesis, we used incorporation of α-^{32}P-dGTP to label the extension products in reactions containing unlabeled 95-mer. For both substrate pairs label incorporation was greatly or completely dependent on Rad54 (Figure 2D and E, and data not shown). Analysis of the extension products with the labeled 95-mer or after α-^{32}P-dGTP incorporation (Figure 2F and G), showed rapid and progressive extension of the invading strand by DNA polymerase. The D-loop extension reaction reached an optimum at a substoichiometric protein ratio of 1/12 to 1/6 of Rad54 to Rad51 (Supplementary Figure 3). This corresponds to three to five molecules of Rad54 per Rad51-ssDNA filament, compatible with the possible action of a Rad54 homo-multimer in Rad51 dissociation.

The extension reaction is quite efficient, as a majority of the D-loops formed are extended by DNA polymerase (~50% for Figure 1C and F; ~80% for Figure 2B, Supplementary Figure 2C). The analysis of the extension on denaturing gels (Figure 2F and G) revealed a heterogeneity of the extension products that is not evident from the analysis by native gel electrophoresis (Figure 2A and C) due to the limits of resolution. In total, consistent with previous observations, these data show that the yeast Rad54 motor protein stimulates D-loop formation by yeast Rad51 protein in a somewhat substrate-dependent fashion. Importantly, the results also provide compelling evidence for a novel role of Rad54 after DNA strand invasion to modulate access of DNA polymerase to the invading 3′-OH end.

D-loop extension by DNA polymerase requires Rad54 motor activity

D-loop formation and extension was entirely dependent on sequence homology (Figure 3A, Supplementary

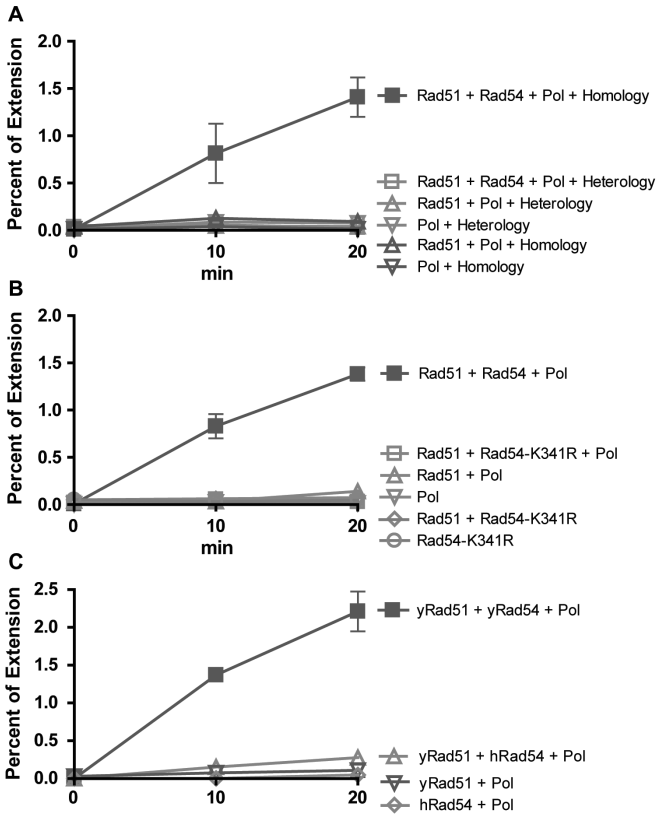

Figure 3. D-loop extension depends on sequence homology, species-specific protein interaction, and Rad54 ATPase activity. (**A**) D-loop extension depends on sequence homology. Linear D-loop extension assays were performed as in Figure 1 either with the *Aat*II-95-mer or a heterologous 95-mer (olWDH640). (**B**) Rad54 ATPase activity is required for D-loop extension. Linear D-loop extension assays were performed as in Figure 1 in the presence of wild-type Rad54 (72 nM) or Rad54–K341R mutant protein (72 nM). (**C**) D-loop extension requires species-specific interaction between Rad51 and Rad54. Linear D-loop extension assays with either *S. cerevisiae* Rad54 (yRad54, 72 nM) or human Rad54 (hRad54, 72 nM) were performed as in Figure 1. Shown are means from three determinations; error bars represent 1 SD.

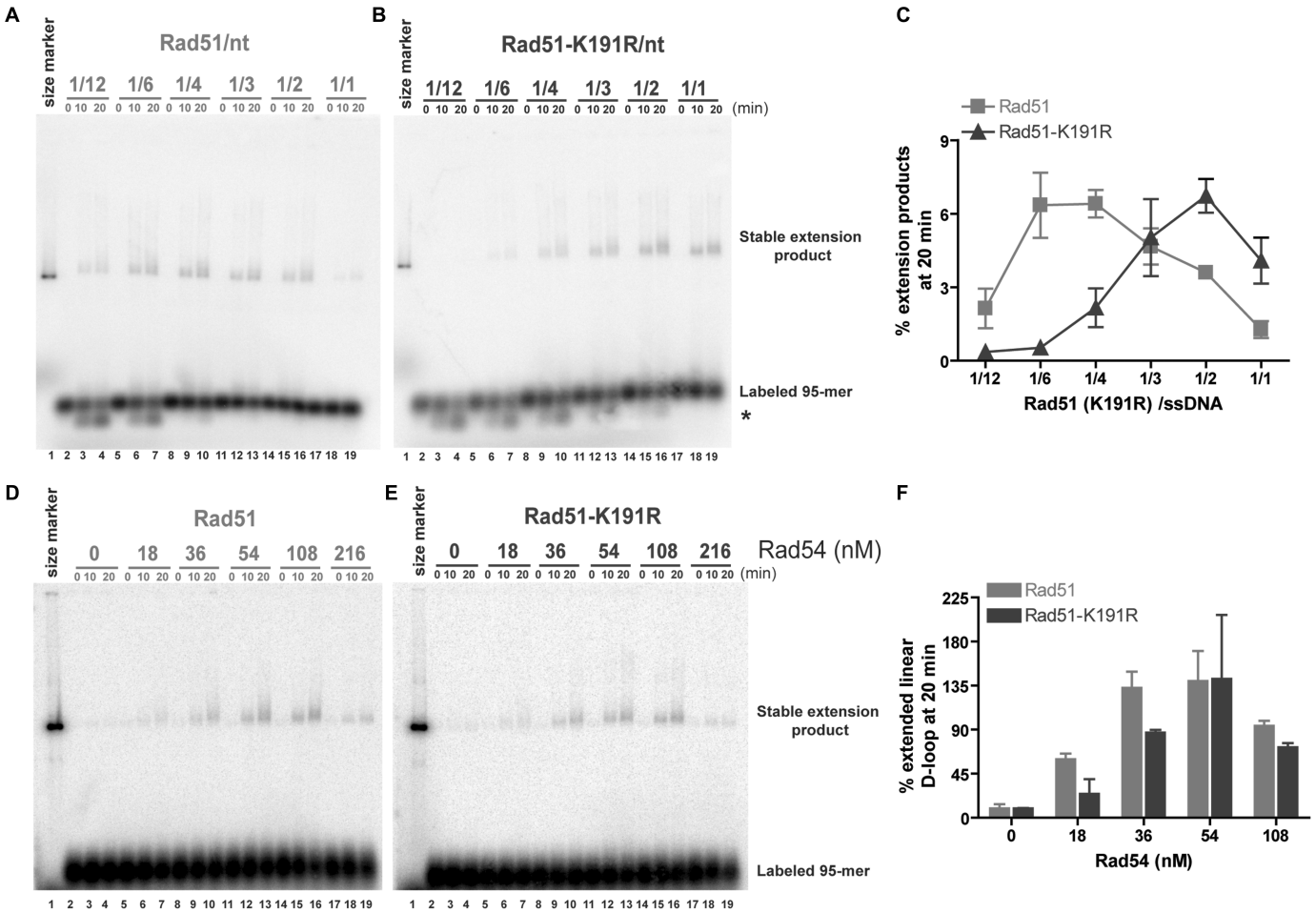

Figure 4. Rad51–K191R requires higher Rad54 concentrations for efficient D-loop extension. (**A**) Rad51 titration in the D-loop extension assay with *Pst*I-95-mer: 0.17 μM (lanes 2–4), 0.34 μM (lanes 5–7), 0.5 μM (lanes 8–10), 0.67 μM (lanes 11–13), 1 μM (lanes 14–16), and 2 μM (lanes 18–20). Reactions contain 72 nM Rad54, as determined to be the optimum in Supplementary Figure 4. Lane 1 shows end-labeled *Pst*I-linearized pUC19 as a size marker. (**B**) Rad51–K191R titration, otherwise as in (A). The signals labeled by asterisk in A and B are generated by the proofreading activity (3′–5′ exonuclease) of Klenow polymerase as determined in reconstruction experiments and verified using a proofreading-deficient version of Klenow polymerase (data not shown). This signal disappears when Rad51 fully occupies the 95-mer, further validating that Klenow polymerase has no access to the 3′-OH end with Rad51 bound to it. (**C**) Quantitation of the Rad51 and Rad51–K191R protein titration results in (a, b; 20 min time points). A higher stoichiometry (1/2 nt) is optimal for the Rad51–K191R protein compared to the 1/4 nt stoichiometry for the wild-type Rad51 protein. This was expected from previous results showing a DNA-binding defect for the Rad51–K191R protein, requiring higher protein to DNA ratios to assemble saturated protein filaments (7). (**D**) Titration of Rad54 in D-loop extension assay with Rad51 at optimal 1/4 stoichiometry. (**E**) Titration of Rad54 in D-loop extension assay with Rad51–K191R at optimal 1/2 stoichiometry. (**F**) Quantitation of results in (D, E; 20 min time points). The results were normalized for the amount of linear D-loops captured by psoralen crosslinking under each assay condition. D-loop extension with wild-type Rad51 reaches an optimum at 36 nM Rad54, whereas the optimum with Rad51–K191R is reached at 54 nM. Shown are the means from three determinations; error bars represent 1 SD.

Figure 4A and B), as expected. The ATPase activity of Rad54 is essential for its biological function and is required for D-loop extension, as demonstrated by the inability of ATPase-deficient Rad54–K341R protein to support formation of stable D-loop extension products (Figure 3B, Supplementary Figure 4C and D). Our previous work had shown that the ATPase-deficient Rad51–K191R protein forms exceedingly stable complexes with dsDNA and is poorly dissociated by Rad54 (7). Rad51–K191R formed linear D-loops, but relatively more Rad54 was required for optimal D-loop extension compared to wild-type Rad51 protein (Figure 4; data not shown). This suppression of a Rad51–K191R defect by elevated levels of Rad54 recapitulates the *in vivo* situation, where Rad54 overexpression can suppress the phenotypes caused by the *rad51–K191R* mutation (19).

The specificity of the D-loop extension reactions was demonstrated in experiments using human and yeast recombination proteins (Figure 3C, Supplementary Figure 4E and F). Yeast Rad54 specifically stimulates extension of linear D-loop formed by yeast Rad51 protein, whereas human Rad54 does not significantly stimulate extension of D-loops formed by yeast Rad51, suggesting that species-specific protein interaction between Rad51 and Rad54 is required. This result also excludes a potential indirect effect of the Rad54 motor protein on linear

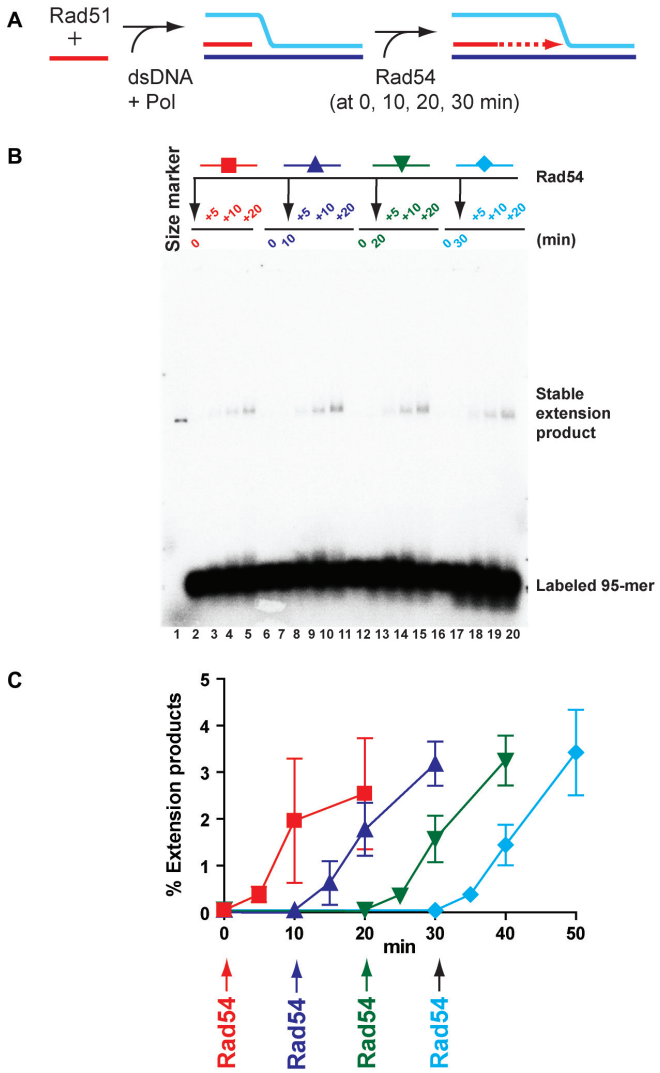

Figure 5. Time of Rad54 addition determines D-loop extension. (**A**) Schematic representation of the timed addition experiment. (**B**) D-loop extension assays were performed with the *Pst*I-95-mer and *Pst*I-linearized pUC19 as before with the exception that Rad54 was added as the last component at 0 min (lanes 2–5), 10 min (lanes 6–10), 20 min (lanes 11–15), or 30 min (lanes 16–20). After Rad54 addition, time courses (5, 10 and 20 min) were performed for every protocol. Lane 1 contains end-labeled pUC19 as size marker. (**C**) Quantification of the results in (B), shown are means from three determinations; error bars represent 1 SD.

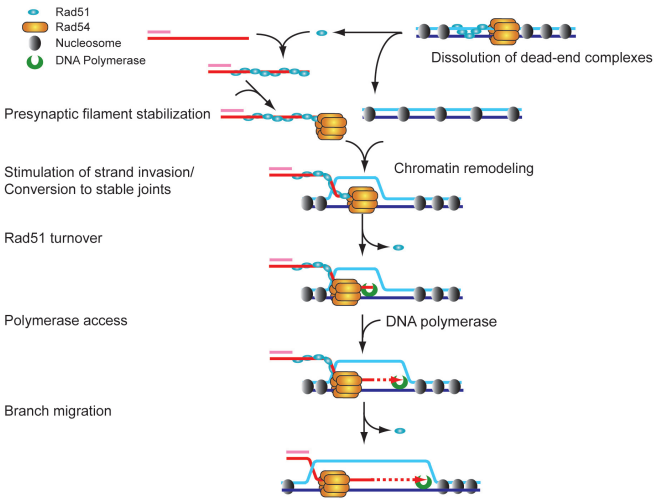

Figure 6. Model for Rad54 functions in homologous recombination. Rad54 associates with the Rad51–ssDNA filament (40), stabilizing the presynaptic filament in an ATPase-independent fashion (21,22). Rad54 stimulates DNA strand invasion (17), but the mechanisms remain to be determined and may involve the conversion of unstable (paranemic?) to stable (plectonemic?) joints (30,41). Rad54 dissociates Rad51 from dsDNA (14) at the terminus of the Rad51–dsDNA filament (13), allowing access of DNA polymerase to the invading 3′-OH end (this work). The Rad54 motor may act as hexameric (shown) or double-hexameric ring; a double ring could explain the observed reversibility of the translocation direction (9). Rad54 has also been shown to catalyze branch migration of DNA junctions and D-loop dissociation *in vitro*, which may depend on the orientation of Rad54 approaching a junction (16,18,42). Rad54's ability to slide nucleosomes *in vitro* (43–45) might help DNA strand invasion and stable joint formation in chromatinized regions *in vivo* (41,44,45). Similar to Rdh54/Tid1 dissociating dead-end Dmc1–dsDNA complexes (46), Rad54 possibly counteracts stable association of Rad51 with undamaged chromosomal DNA.

D-loop extension by locally unwinding the duplex DNA during translocation, as both yeast and human Rad54 have near identical ATPase activity (data not shown) and, by extension, motor activity.

To further support the assertion that Rad54 determines the transition to DNA synthesis from the invading end, we designed a multi-step time course experiment (Figure 5A), which demonstrated that the time of Rad54 addition determined the beginning of D-loop extension (Figure 5B and C). Although D-loops were formed during the first stage of the reaction (0–30 min), D-loop extension products were only evident in the second stage of the reaction after addition of Rad54 (Figure 5C, data not shown). These data are consistent with a model (Figure 6) that dissociation of Rad51 from the heteroduplex DNA by the Rad54 motor allows the transition to DNA synthesis.

DISCUSSION

Rad54 is an exceptionally versatile motor protein that acts at multiple stages during homologous recombination *in vitro* (Figure 6) (10,11). The ATPase activity, and by implication dsDNA translocation, is essential for Rad54 function *in vivo*, as shown by the genetic analysis of *rad54–K341R* ATPase-deficient mutant (20). Early function of Rad54 in stabilizing the Rad51 filaments has been documented *in vivo* and *in vitro* (21,22). This function is independent of its ATPase activity. Hence, it is evident that a later, ATPase-dependent function defines a critical contribution of Rad54 to recombinational repair in yeast. Genetic analysis placed the execution point of Rad54 function at or after the Rad51-dependent step, i.e. at or after D-loop formation (10,23,24). Cytological data in budding yeast, chicken DT40 cells and mouse are consistent with this notion, showing that Rad54 is not required for Rad51 focus formation after DNA damage or in meiosis and those Rad51 foci attain a longer life span in *rad54* mutants (25–29). Further critical information about

Rad54 *in vivo* function was provided by two comprehensive ChIP analyses, documenting that Rad54 was dispensable for homology search but absolutely required to initiate DNA synthesis at the recombination target site (30,31). In total, genetic and biochemical evidence from *S. cerevisiae* documents the essential role of Rad54 during recombinational repair in this organism at a step that precedes DNA synthesis emanating from the invading 3′-end.

Recent work with human recombination proteins described a role of Rad54 in branch migration of joint molecules, occurring after extension of the invading 3′-end (18). The apparent lack of a need for Rad54 to initiate DNA synthesis may be explained by a difference between the yeast and human proteins or by different experimental design. In Bugreev *et al.* (18), Ca^{2+} is used to stabilize the hRad51–DNA filament. After DNA strand invasion and before the addition of Rad54, Ca^{2+} is replaced by Mg^{2+} which is known to activate the Rad51 ATPase (32), leading to disruption of Rad51–DNA filaments as observed by AFM (33). Hence, the requirement for Rad54 in the transition to DNA synthesis may have been sidestepped by the cation change. This clever experimental design allowed the analysis of the function of Rad54 in the later stages of the reaction and showed that the Rad54 motor can migrate DNA junctions leading to second end capture (18). In another study, extension of D-loops by human DNA polymerase Eta occurred in the absence of hRad54 (34), but the D-loops used in that study were not created by Rad51-mediated DNA strand invasion, alleviating the need for a turnover factor. The human recombination machinery may differ from the yeast one, as indicated by the difference of severity in phenotypes of *RAD54* mutants in yeast *versus* mouse and chicken DT40 cells (10).

RecA is the archetypical homologous pairing and DNA strand exchange protein (4). Release of RecA from the heteroduplex DNA after DNA strand exchange driven by ATP hydrolysis is required for DNA polymerase extension (35). A similar requirement for Rad51 turnover is evident from our biochemical experiments presented here and from *in vivo* experiments in meiotic budding yeast cells, showing that removal of Rad51 from recombination foci preceded the onset of meiotic recombination-related DNA synthesis (36). A role of Rad54 in the turnover of Rad51–dsDNA filaments (7,14) rationalizes the significant differences between RecA and Rad51 in their ATPase activity and dsDNA binding. We suggest here (Figure 6) that active dissociation by the Rad54 motor protein of a key recombination intermediate, the Rad51–heteroduplex DNA complex, controls the transition between D-loop formation and DNA synthesis during homologous recombination in *S. cerevisiae*. Such a mechanism might also contribute to avert undesired extension of strand invasions at not fully homologous sites, providing an additional time window for other mechanisms, like mismatch repair or DNA helicases, to disrupt the imperfect pairing. The requirement for such a mechanism probably arose with the evolution of the complex eukaryotic genomes with more repetitive DNA.

The experiments in this study used the Klenow fragment of bacterial DNA polymerase I as a generic DNA polymerase to demonstrate the role of Rad54 in allowing access to the invading 3′-OH end. Further studies are required to recapitulate the significant complexity of the *in vivo* situation in yeast, where multiple DNA polymerases and their co-factors (PCNA) have been implicated in homologous recombination (37–39).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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