Rad54 protein possesses chromatin-remodeling activity stimulated by the Rad51–ssDNA nucleoprotein filament

Andrei Alexeev[1], Alexander Mazin[1,2] and Stephen C. Kowalczykowski[1]

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In *Saccharomyces cerevisiae*, the Rad54 protein participates in the recombinational repair of double-strand DNA breaks together with the Rad51, Rad52, Rad55 and Rad57 proteins. *In vitro*, Rad54 interacts with Rad51 and stimulates DNA strand exchange promoted by Rad51 protein. Rad54 is a SWI2/SNF2-related protein that possesses double-stranded DNA–dependent ATPase activity and changes DNA topology in an ATP hydrolysis–dependent manner. Here we show that Rad54 catalyzes bidirectional nucleosome redistribution by sliding nucleosomes along DNA. Nucleosome redistribution is greatly stimulated by the Rad51 nucleoprotein filament but does not require the presence of homologous single-stranded DNA within the filament. On the basis of these data, we propose that Rad54 facilitates chromatin remodeling and, perhaps more generally, protein clearing at the homology search step of genetic recombination.

The eukaryotic genome is compacted by chromatin organization, which restricts the accessibility of DNA to the basal processes of transcription, replication, recombination and DNA repair[1–3]. To overcome these barriers, cells possess several ATP-dependent chromatin-remodeling factors, which are multiprotein complexes containing the ATPases of the SWI2/SNF2 superfamily[2,3]. Four groups of these complexes containing the SWI2/SNF2, ISWI, MI-2 or INO80 ATPases have been described[2–4]. Chromatin-remodeling complexes function by increasing nucleosome mobility, thereby providing access to DNA-binding factors[2–4]. *In vitro* studies using purified complexes from the SWI2/SNF2, ISWI and MI-2 groups showed that these complexes can facilitate nucleosome 'sliding', defined as the movement of the histone octamer in *cis*, without displacement from DNA[5–7]. The observation that homologous recombination is sensitive to local chromatin organization[8] indicates that chromatin remodeling accompanies the process of DNA strand exchange.

Here we examine whether the Rad54 protein, a member of the SWI2/SNF2 group of ATP-dependent chromatin-remodeling factors, can indeed redistribute nucleosomes. Furthermore, because Rad54 is a protein essential to recombinational DNA repair, we asked whether Rad54 functions in concert with Rad51, the protein that has the task *in vivo* of promoting DNA strand invasion at homologous chromosomal target loci, to remodel chromatin.

Rad54 protein mediates nucleosome movement

To investigate the ability of Rad54 protein to remodel chromatin structure, we used a nucleosome-mobility assay that allows detection of nucleosome movement[5,6]. Reconstitution of mononucleosomes on short DNA fragments generates a mixture

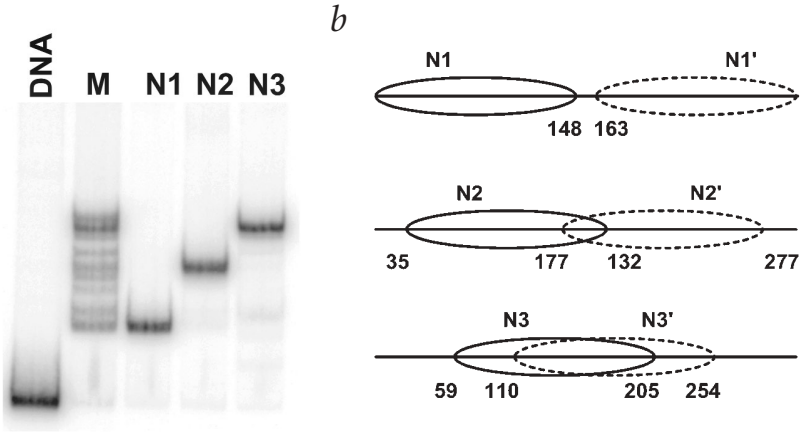

Fig. 1 Defined positions on DNA occupied by reconstituted nucleosomes. ***a***, Native gel electrophoresis showing the various nucleosome species. Nucleosomes were reconstituted on a ^{32}P–end-labeled 314 bp fragment from the *RAD51* promoter region and purified by sucrose gradient sedimentation. The resulting mixture of mononucleosome position-isomers is shown (lane indicated as 'M'). The three discrete mononucleosome species (N1, N2 and N3) were isolated by preparative gel electrophoresis, and their positions were determined by exonuclease III protection and micrococcal nuclease mapping. ***b***, Summary of the mapping experiments. Nucleosome positions are represented as ovals. Each of the three unique electrophoretically homogeneous nucleosome bands consists of two nucleosome species occupying symmetrical positions on the DNA fragment.

[1]Division of Biological Sciences, Sections of Microbiology and of Molecular and Cellular Biology, Center for Genetics and Development, University of California, Davis, California 95616-8665, USA. [2]Present address: Drexel University College of Medicine, Department of Biochemistry, MS 497, Philadelphia, Pennsylvania 19102-1192, USA.

Correspondence should be addressed to S.C.K. *e-mail: sckowalczykowski@ucdavis.edu*

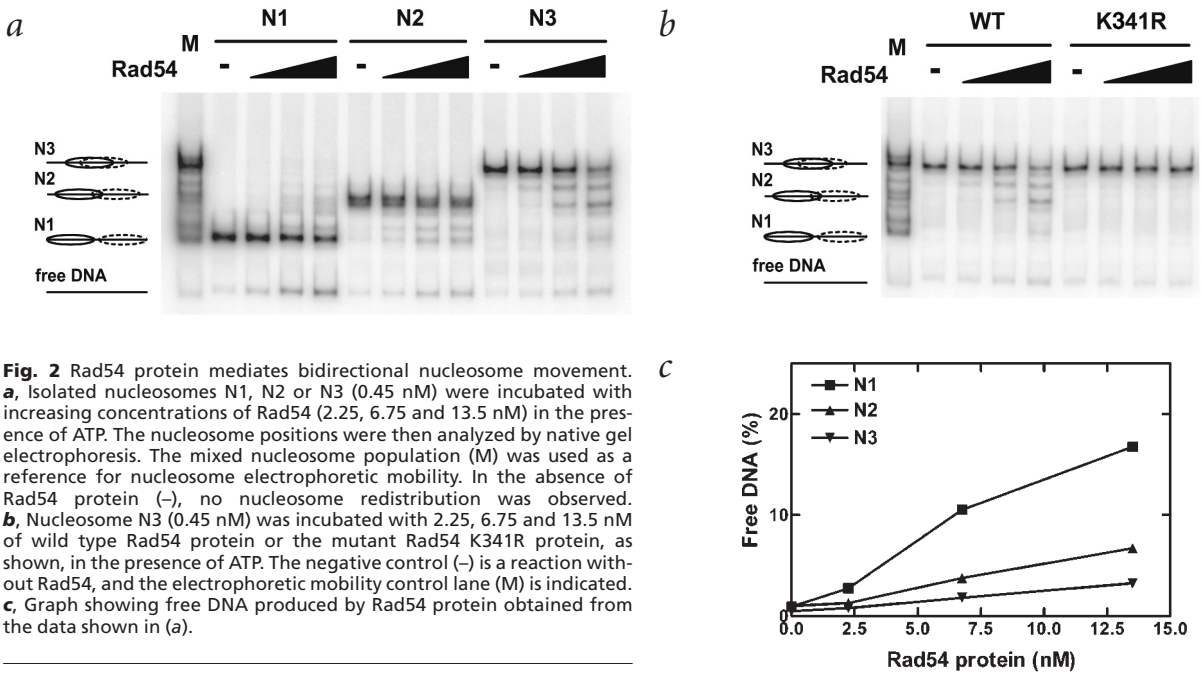

Fig. 2 Rad54 protein mediates bidirectional nucleosome movement. ***a***, Isolated nucleosomes N1, N2 or N3 (0.45 nM) were incubated with increasing concentrations of Rad54 (2.25, 6.75 and 13.5 nM) in the presence of ATP. The nucleosome positions were then analyzed by native gel electrophoresis. The mixed nucleosome population (M) was used as a reference for nucleosome electrophoretic mobility. In the absence of Rad54 protein (–), no nucleosome redistribution was observed. ***b***, Nucleosome N3 (0.45 nM) was incubated with 2.25, 6.75 and 13.5 nM of wild type Rad54 protein or the mutant Rad54 K341R protein, as shown, in the presence of ATP. The negative control (–) is a reaction without Rad54, and the electrophoretic mobility control lane (M) is indicated. ***c***, Graph showing free DNA produced by Rad54 protein obtained from the data shown in (*a*).

of nucleosomes positioned at different locations on the fragment, which can be separated electrophoretically[9,10]. The electrophoretic mobility of these position-isomers is a function of the distance of the nucleosome core from the center of the DNA fragment and is minimal for centrally positioned nucleosomes[9,10]. Native electrophoresis of nucleosomes reconstituted on a 314 base pair (bp) DNA fragment (from the *RAD51* promoter region) showed ~9 different complexes, each representing a nucleosome at a defined position (Fig. 1*a*). Three nucleosome species N1, N2 and N3 were isolated (Fig. 1*a*), and the nucleosome positions for each were mapped by two methods: exonuclease III protection and micrococcal nuclease sensitivity (data not shown). All three isolated nucleosome species consist of a mixture of two nucleosome populations occupying symmetrical positions on the DNA fragment (Fig. 1*b*). The slowest migrating nucleosome, N3, occupies positions proximal to the center of the DNA fragment. The fastest migrating species, N1, is located at the fragment ends. The nucleosome of intermediate mobility, N2, is located between the positions of nucleosomes N1 and N3.

To test whether Rad54 could alter nucleosome positioning, we incubated the isolated nucleosomes N1, N2 and N3 with Rad54 in the presence (Fig. 2*a*) or absence of ATP. In the presence of ATP and Rad54, the distribution of nucleosome positions was altered for each of the mononucleosome isomers. The nucleosome at position N1 was redistributed towards more central positions on the DNA fragment; the nucleosome N2 was relocated primarily towards DNA-end proximal positions, but a smaller, clearly defined fraction was observed at more central positions, and nucleosome N3 was redistributed to multiple positions towards the DNA fragment ends. In all cases, the extent of nucleosome repositioning was dependent on the concentration of Rad54. In addition to the repositioning of nucleosomes, free DNA that was devoid of nucleosomes was produced (nonspecific binding of dissociated histones and Rad54 protein was prevented by the presence of unlabeled scavenger DNA) (Fig. 2*a*). These results demonstrate that Rad54 can move nucleosomes in either of the two possible directions and actually move them off the DNA fragment. Rad54-mediated nucleosome movement requires ATP, because no alteration of nucleosome positions was observed in the control experiment where ATP was omitted (data not shown). Furthermore, ATP hydrolysis is needed, because replacement of Rad54 by the ATPase-deficient Rad54 K341R mutant[11] abolishes nucleosome redistribution (Fig. 2*b*).

Rad54 could relocate nucleosomes either by dissociating histone octamers from DNA followed by re-association of the histones at the new position or by sliding nucleosomes along DNA. Because we observed that redistribution proceeded successively to vicinal positions rather than to random positions for each nucleosome species used (Fig. 2*a*), we inferred that nucleosome movement occurs by sliding of the histone octamer along DNA. In addition, we observed that the amount of free DNA produced by Rad54 increased in inverse proportion to the distance of nucleosome from the ends of DNA and was maximal for the nucleosomes positioned at the ends (Fig. 2*c*). Given that remodeling reactions were terminated by the addition of an excess of unlabeled scavenger DNA, these data indicate that histone octamers relocated to the DNA fragment ends before dissociation. Collectively, these results suggest that Rad54 protein can 'slide' nucleosomes along DNA.

Rad51–ssDNA complexes enhance remodeling

Recently, interaction of Rad54 with a complex of Rad51 and ssDNA was shown to stimulate both the ATPase and dsDNA topological unwinding activities of the Rad54 protein[11,12]. To investigate the effect of the Rad51–ssDNA nucleoprotein complex on Rad54-dependent chromatin remodeling activity, nucleosome N3 was incubated with Rad54 and increasing amounts of a pre-assembled Rad51–ssDNA complex (using a heterologous 90-nucleotide oligomer). The Rad51–ssDNA nucleoprotein complex (Fig. 3*a*) enhances nucleosome repositioning mediated by Rad54 protein in a concentration-dependent manner (compare lane 3 with lanes 4–9). No stimulation was observed with ssDNA alone (Fig. 3*c*). The observed

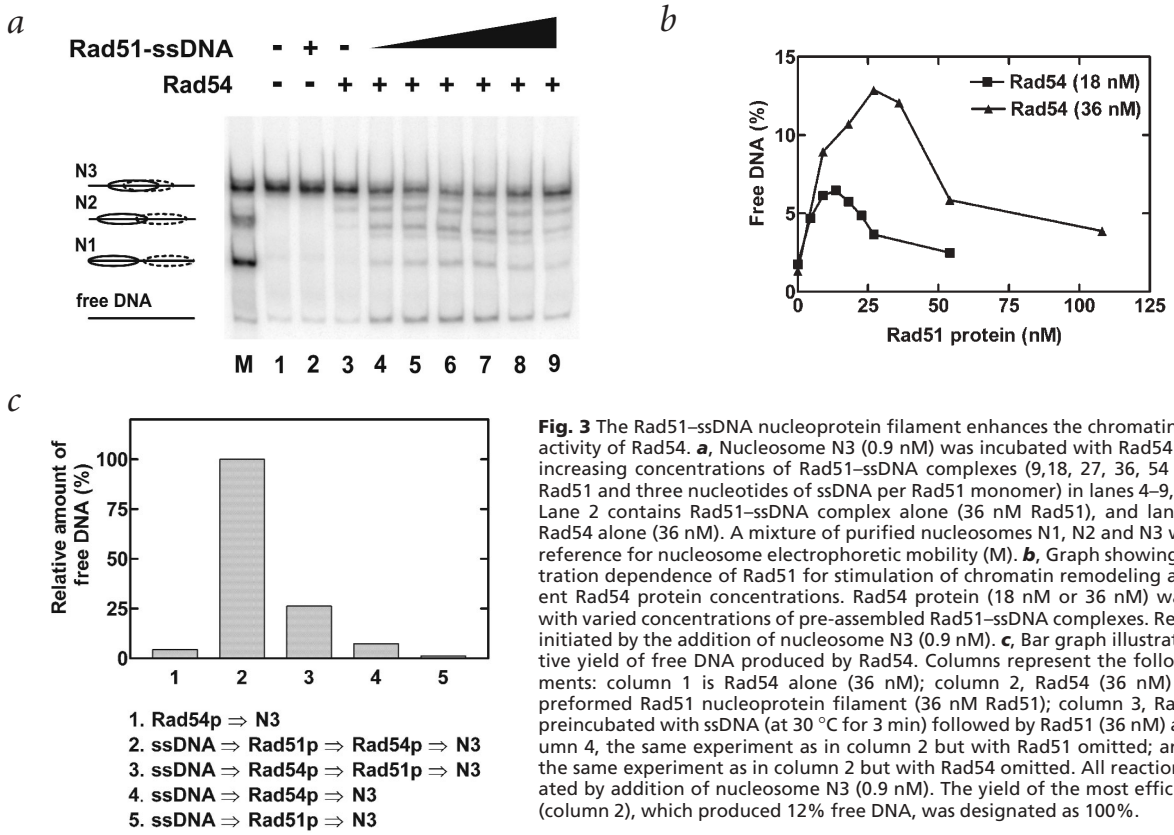

Fig. 3 The Rad51–ssDNA nucleoprotein filament enhances the chromatin-remodeling activity of Rad54. ***a***, Nucleosome N3 (0.9 nM) was incubated with Rad54 (36 nM) and increasing concentrations of Rad51–ssDNA complexes (9,18, 27, 36, 54 and 108 nM Rad51 and three nucleotides of ssDNA per Rad51 monomer) in lanes 4–9, respectively. Lane 2 contains Rad51–ssDNA complex alone (36 nM Rad51), and lane 3 contains Rad54 alone (36 nM). A mixture of purified nucleosomes N1, N2 and N3 was used as a reference for nucleosome electrophoretic mobility (M). ***b***, Graph showing the concentration dependence of Rad51 for stimulation of chromatin remodeling at two different Rad54 protein concentrations. Rad54 protein (18 nM or 36 nM) was incubated with varied concentrations of pre-assembled Rad51–ssDNA complexes. Reactions were initiated by the addition of nucleosome N3 (0.9 nM). ***c***, Bar graph illustrating the relative yield of free DNA produced by Rad54. Columns represent the following experiments: column 1 is Rad54 alone (36 nM); column 2, Rad54 (36 nM) added to a preformed Rad51 nucleoprotein filament (36 nM Rad51); column 3, Rad54 (36 nM) preincubated with ssDNA (at 30 °C for 3 min) followed by Rad51 (36 nM) addition; column 4, the same experiment as in column 2 but with Rad51 omitted; and column 5, the same experiment as in column 2 but with Rad54 omitted. All reactions were initiated by addition of nucleosome N3 (0.9 nM). The yield of the most efficient reaction (column 2), which produced 12% free DNA, was designated as 100%.

nucleosome repositioning is attributable to the Rad54, because the Rad51 nucleoprotein complex alone does not move nucleosomes (Fig. 3*a*, lane 2).

Stimulation of Rad54 by Rad51–ssDNA is specific

The stimulation of Rad54 activity by the Rad51 nucleoprotein filament has an optimum at approximately one Rad54 protein monomer per Rad51 protein monomer. To verify that the optimum of the stimulation is defined with respect to the concentration of Rad51, we reduced the concentration of Rad54 two-fold and repeated the titration with the Rad51 nucleoprotein filament (Fig. 3*b*). In this case, maximum enhancement of chromatin remodeling by Rad51 also occurred at approximately stoichiometric amounts of Rad54 and Rad51. A similar behavior was seen for stimulation of both the DNA pairing activity of Rad51 by Rad54 and the DNA topological unwinding activity of Rad54 by the Rad51 nucleoprotein complex[12]. Stimulation requires the interaction of Rad54 protein with a pre-assembled Rad51–ssDNA complex; when Rad54 protein was incubated with ssDNA before the introduction of Rad51 protein, the stimulatory effect was reduced by 75% (Fig. 3*c*). Stimulation of chromatin remodeling is specific to Rad51, because control reactions

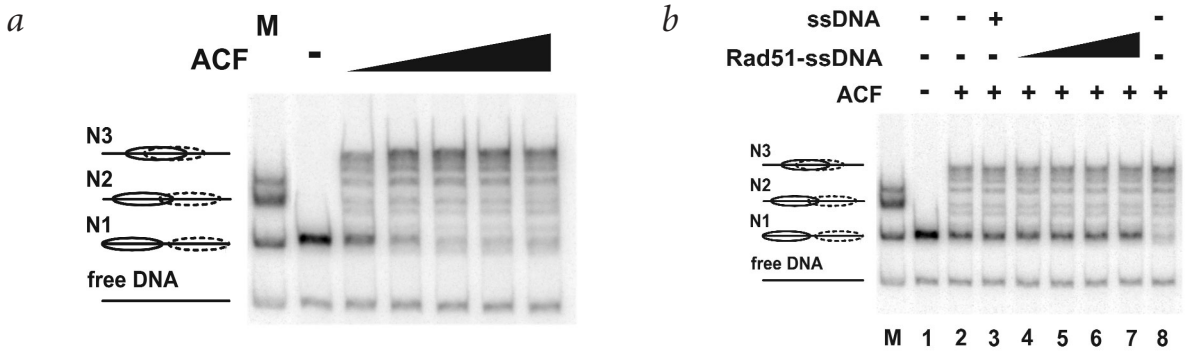

Fig. 4 The Rad51–ssDNA nucleoprotein filament does not affect chromatin remodeling by ACF. ***a***, Isolated nucleosome N1 (0.2 nM) was incubated with increasing concentrations of ACF (0.05, 0.1, 0.2, 0.4 and 0.8 nM). The nucleosome positions were then analyzed by native gel electrophoresis. A mixture of purified nucleosomes N1 and N2 was used as a reference for nucleosome electrophoretic mobility (M). In the absence of ACF (–), nucleosome redistribution was not observed. ***b***, Nucleosome N1 (0.2 nM) was incubated with ACF (0.05 nM) and increasing concentrations of Rad51–ssDNA complexes (2, 4, 8 and 16 nM Rad51 protein) in lanes 4–7. Lane 2 contains ACF alone (0.05 nM); lane 3 contains ssDNA (48 nM, nt) and ACF, but no Rad51 protein; and lane 8 contains ACF alone (0.4 nM). A mixture of purified nucleosomes N1 and N2 was used as a reference for nucleosome electrophoretic mobility (M).

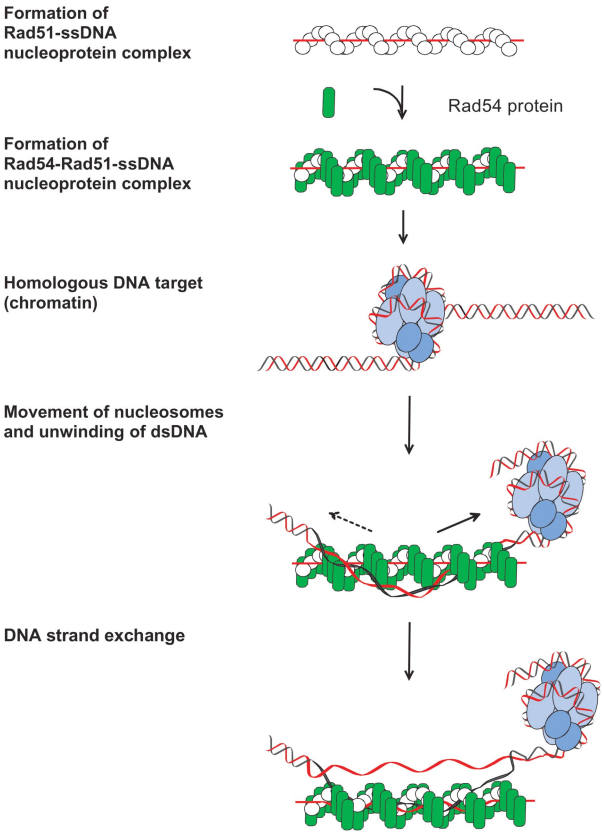

Fig. 5 Scheme illustrating the stimulatory role of Rad54 protein in DNA strand exchange. Rad54 binds to the Rad51 nucleoprotein filament, forming a tertiary Rad54–Rad51–ssDNA nucleoprotein complex. Rad54, as a part of the nucleoprotein complex, eliminates a constraint to DNA strand exchange by removing nucleosomes and other DNA-binding proteins from the DNA-target site during the homology search, in preparation for DNA strand exchange.

with *E. coli* RecA nucleoprotein filaments had no effect on Rad54 protein activity (data not shown). Similarly, the stimulation of Rad54 by the Rad51 nucleoprotein filament is not nonspecific, because chromatin remodeling by the *Drosophila* ATP-dependent chromatin assembly and remodeling factor (ACF) was unaffected by the Rad51 protein–ssDNA complex (Fig. 4). Collectively, these results indicate that a stoichiometric co-complex of Rad54 protein and the Rad51 nucleoprotein filament is the most active species in chromatin remodeling *in vivo*.

Chromatin remodeling in DNA recombination

Recombinational DNA repair requires that DNA strand exchange occur with a homolog. This process needs to overcome the barrier imposed by chromatin structure to the homology search, DNA strand invasion and the further extension of the dsDNA heteroduplex product[13,14]. The ability of Rad54 protein to remove nucleosomes from the homologous DNA target site, in a process that is enhanced by co-assembly with the Rad51 nucleoprotein filament, suggests that nucleosome sliding is the mechanism by which recombination overcomes the inhibition imposed by nucleosomes and other proteins that might be bound at homologous loci (Fig. 5). The finding that this stimulation by the presynaptic filament does not require DNA sequence complementarity with the target locus argues that Rad54 can remodel chromatin at the stage of the homology search, before the DNA pairing and DNA strand exchange steps and well before the formation of stable heteroduplex DNA. Thus, we suggest that chromatin remodeling and protein clearing by Rad54 protein occur during the search for DNA sequence homology. When the DNA is homologous, we imagine that association of the Rad54 protein with the Rad51 nucleoprotein filament also facilitates targeting of Rad54 to the DNA-pairing target site, to direct localized change in chromatin structure during DNA strand exchange and to limit generalized disruption of chromatin elsewhere. In fact, Alexiadis and Kadonaga[15] recently showed that the Rad54 protein from *Drosophila* was needed for Rad51-promoted joint molecule formation with chromatin and for the alteration of chromatin structure that accompanied pairing. Finally, because of its association with the Rad51 nucleoprotein filament, Rad54 is delivered to the homologous locus where it can facilitate both synaptic and post-synaptic events. Interestingly, recent work also shows that Rad54 can clear Rad51 from duplex DNA[16]. This clearing can serve to remove components of the DNA strand exchange machinery from the heteroduplex DNA after DNA strand exchange is complete and, thus, represents a post-synaptic remodeling function of Rad54 protein. Thus, by incorporating a DNA-remodeling protein as part of the nucleoprotein complex that mediates the homologous pairing step, efficient repair of DNA by recombination is ensured by the remodeling action of Rad54 protein at several steps in the process.

Methods

Mononucleosome purification. Mononucleosomes were assembled on a ^{32}P–end-labeled 314 bp fragment of the *RAD51* promoter region using the salt-dilution method[17]. Histones purified from chicken erythrocytes were provided by E.M. Bradbury, J.S. Siino and P.M. Yau (U.C. Davis). Reconstituted nucleosomes were separated from both dinucleosomes and free DNA by sucrose gradient centrifugation in a 5–30% (w/v) sucrose gradient[18]. The various mononucleosome position-isomers were resolved by electrophoresis through a 4% (w/v) polyacrylamide gel in 0.5× TB buffer (45 mM Tris-borate, pH 8.3). The gel slice containing the separated mononucleosome was excised, and the nucleosomes were eluted by electro-elution in 0.5× TB buffer containing 3 mM $MgCl_2$ and 100 μg ml^{-1} BSA.

Micrococcal nuclease analysis. Isolated nucleosomes (30 ng) were digested with increasing concentrations of micrococcal nuclease (0.01–0.03 U) in buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 7 mM $CaCl_2$ for 5 min at 30 °C. DNA was extracted with phenol and analyzed on 6% (w/v) polyacrylamide sequencing gel.

Exonuclease III protection assay. Isolated nucleosomes (10 ng) were digested with various concentrations of exonuclease III (50–200 U ml^{-1}) in Exo III buffer (Promega) in the presence of 50 μg ml^{-1} of phage λ DNA. The reaction mixtures were incubated 2 min at 37 °C. DNA was extracted with phenol and analyzed on 6% polyacrylamide sequencing gel.

Chromatin remodeling. Rad54 protein or the *Drosophila* ACF complex was incubated with nucleosomes in buffer A (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol and 10% (w/v) glycerol) containing 4 mM $MgCl_2$, 4 mM ATP, 3 mM phosphoenolpyruvate and 20 U ml^{-1} pyruvate kinase at 30 °C for 60 min. Reactions were stopped by the addition of 1 μl cold scavenger DNA (0.25 μg μl^{-1} each of poly(dA-dT)•poly(dA-dT) and poly(dT)). Samples were further incubated at 30 °C for 20 min and analyzed by electrophoresis through a 4% (w/v) polyacrylamide gel in 0.5× TB buffer at 4 °C. The yield of free DNA production was determined using a Storm 840 PhosphorImager (Molecular Dynamics).

To measure the chromatin remodeling activity in the presence of the Rad51 nucleoprotein filaments, Rad51 protein (at the highest

concentration indicated in the figure legends) was incubated with 90-nucleotide ssDNA (at a ratio of 3 nucleotides per Rad51 protein molecule) in buffer A containing 10 mM $MgCl_2$, 2 mM ATP, 3 mM phosphoenolpyruvate and 20 U ml^{-1} pyruvate kinase for 20 min at 37 °C. The reaction mixture was then diluted with reaction buffer to the indicated concentrations of the Rad51 nucleoprotein filament. Rad54 protein or ACF was added, followed by immediate addition of the indicated amount of nucleosome. After 60 min of incubation at 30 °C, the reaction was terminated by addition of 3 μl cold scavenger DNA. Samples were further incubated at 30 °C for 20 min and analyzed as described above.

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Competing interests statement
The authors declare that they have no competing financial interests.

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