Supplemental Information

Human RAD52 Captures and Holds DNA Strands, Increases DNA Flexibility, and Prevents Melting of Duplex DNA: Implications for DNA Recombination

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Figure S1. Functionality of GFP-RAD52 and experimental assay, related to Figures 1, 2, 3, and 4.

A) SDS-PAGE and Coomassie Blue staining analysis of purified wild-type RAD52 and N-terminally tagged GFP-RAD52. Lane 1: molecular size marker. Lanes 2 and 3: 0.5 µg of RAD52 and GFP-RAD52, respectively.

B) Kinetics of single-strand annealing reactions mediated by RAD52, eGFP-RAD52 or in the absence of protein using a FAM-labeled 50-mer oligonucleotide and its unlabeled complementary strand as described below in Supplemental Experimental Procedures. Progressive annealing was detected by taking samples at different time points. After fractionation of the samples by native PAGE, fluorescence signal was collected using a CCD camera.

C) Quantification of annealing activities detected as in (B). The extent of annealing is assessed as the percentage of dsDNA formed over time for RAD52 (black dataset), GFP-RAD52 (red dataset), and spontaneous annealing in the absence of protein (grey dataset). Error bars: standard error of the mean (SEM) from four independent experiments.

D) Schematic of a dsDNA molecule (purple) tethered between two optically trapped micrometer-sized polystyrene beads (grey) with GFP-RAD52 complexes (green) bound to the dsDNA molecule. By manipulating the position of the beads the extension of the DNA molecule can be controlled while the tension in the molecule is monitored. At the same time, the proteins can be directly visualized with single-fluorophore resolution using wide-field fluorescence microscopy.

E) Schematic, such as shown in (D), but for GFP-RAD52 complexes bound to ssDNA complex.

F) Typical fluorescence image emitted by GFP-RAD52 complexes bound to dsDNA such as schematically shown in (D). Scale bar: 2 µm.

G) Typical fluorescence image emitted by GFP-RAD52 complexes bound to ssDNA such as schematically shown in (E). Scale bar: 2 µm.
Schematic of the 5-channel flow cell used (top panel), indicating the solutions in the different channels. For the dsDNA binding experiments, the solutions in the channels were as follows: channel 1, beads; channel 2, DNA; channels 3 and 4, experiment buffer (buffers indicated in the text/captions of the manuscript); and channel 5, GFP-RAD52 solution. For the ssDNA experiments, they were as follows: channel 1, beads; channel 2, DNA; channel 3, melting buffer (20 mM Tris-HCl pH 7.6); channel 4, experiment buffer (buffers indicated in the text/captions of the manuscript); and channel 5, GFP-RAD52 solution. The imaging experiments were done deep into channel 4 (blue rectangle: “Imaging”) after stopping the flow in all channels. The mixing there is very slow (longer than the timescale of the experiments). As can be seen using red and blue colored aqueous solutions, when all channels are flowing (middle panel) the fluids in the 5 channels do not mix. Next, channels 1 and 2 were closed, and after 30 s of flow all channels were closed. 180 s after stopping the flow there is no mixing deep into channel 4 in the imaging area (bottom panel). Scale bar: 2 cm.

Schematic explaining the imaging assays. A typical experiment comprises the following steps: step 1, capture of 2 beads; step 2, capture of a single dsDNA-molecule between these beads; step 3, probing the mechanical properties of the tethered dsDNA molecule, to ensure that it is a single molecule with the expected mechanical properties; then either step 4, the dsDNA is incubated in a channel with the GFP-RAD52 and step 5 the construct is brought back to the buffer channel where it can be imaged in the absence of fluorescence protein in the background; or step 4’, the tension on the dsDNA molecule is increased to generate an ssDNA molecule by force-induced melting; step 5’, the ssDNA is incubated in the protein channel; and step 6’, the construct is brought into the buffer channel for imaging. Imaging of the GFP-RAD52 complexes bound to DNA was always performed in the absence of flow as indicated in panel (H).
Figure S2. GFP-RAD52 binding to ssDNA, related to Figure 1.

A) Relative position along the DNA of all detected GFP-RAD52 complexes on ssDNA (red, N = 282) and on dsDNA (blue, N = 720). Because the orientation of the DNA molecule between the two beads is unknown in the experiments, the distance to the closest bead was used as a measure for the position. Error bars: statistical errors in the number of counts.

B) Relation between the average number of patches and the force on ssDNA at the indicated GFP-RAD52 concentrations and given cationic conditions.

C) Relation between the average patch size and the force on ssDNA at the indicated GFP-RAD52 concentrations and given cationic conditions. In contrast to what was observed on dsDNA (Figure 2C), GFP-RAD52 patch size on ssDNA is independent of force. Error bars: SEM.

D) For the ssDNA molecule shown in the figure, the probability of a new RAD52 patch to bind to a location of a previously bound one is on average $7.5 \pm 3\%$ (SEM) over the 5 subsequent 2 s-long dipping. Globally, we observed 21 co-localizing patches over the 144 detected patches on 8 different ssDNA constructs with an overall average patch grow probability of about $14 \pm 4\%$ (SEM). Scale bar: 5 µm.

E) Histogram of contour length changes observed in the rupture events for ssDNA. Data were generated by applying a step-fitting algorithm (Kerssemakers et al., 2006) to the traces such as the red curve shown in Figure 1D. On average, the contour length changes in step of $0.35 \pm 0.02 \mu m$ (SEM, N = 143).
Figure S3. Intensity calibration and bleaching time of individual GFP-RAD52 proteins, related to Figures 1 and 2.

A) Example of fluorescence intensity trace of an individual GFP-RAD52 complex as a function of time. Step values were obtained from such traces using a step fitting algorithm (Kerssemakers et al., 2006).

B) Histogram of step intensities (N = 8188). Lorentzian fit (blue curve) was used to determine the most likely value giving 345 ± 10 intensity a.u., which we assume as the average intensity value of a single GFP.

C) Histogram of photobleaching times from the dataset shown in (B). The bleaching time obtained from an exponential fit (blue curve) to the data is 2.17 ± 0.03 s. Error bars: statistical errors in the number of counts.
Figure S4. GFP-RAD52 binding to dsDNA, related to Figure 2 and Table 1.

A) Average patch size as a function of the incubation time, shown for the data measured on dsDNA at a GFP-RAD52 concentration of 20 nM, in the presence of CaCl$_2$ and with a DNA tension of 50 pN. For the other conditions, similar results were obtained. Data display no dependence of the patch size on the incubation time, showing no evidence for a mechanism of cooperative patch growth. Error bars: SEM.

B) Force-extension curves of 11 different GFP-RAD52-dsDNA complexes shows irregular force-extension behavior, caused by nonspecific sticking of DNA-bound RAD52 to the beads. This made it impossible to obtain reliable eWLC fits to this data. Nevertheless, the data do not show the typical plateau at around 65 pN indicating the melting of the duplex DNA. Instead the curves show a continuous raise of the force during extension.

C) Force-relaxation curves of 8 different GFP-RAD52-dsDNA complexes. These curves are more regular and reliable eWLC fits can be performed on them. Fit parameters are reported in Table 1.

D) Example of force-extension data recorded during a stretching-relaxation cycle in the absence of GFP-RAD52. Blue curve shows extension curve and red curve relaxation data. Up to 30 pN, the curves are well described by a fit to the eWLC model. Fits are shown in light grey (extension data) and dark grey (relaxation data). Fit parameters for extension and relaxation are reported in Table 1.
Figure S5. Analysis of GFP-RAD52 diffusion, related to Figures 1, 2, and 3.

A) Typical example of a kymograph of diffusive GFP-RAD52 particles. Data were recorded at 20 nM GFP-RAD52 in the presence of CaCl$_2$ on dsDNA with a tension of 5 pN. Scale bars: 2 µm, 10 s.

B) Typical example of a kymograph of static GFP-RAD52 particles. Data were recorded at 20 nM GFP-RAD52 in the presence of MgCl$_2$ on dsDNA with a tension of 50 pN. Scale bars: 2 µm, 10 s.

C) Displacement from the initial position for a mobile particle (red dataset, corresponding to the particle indicated by the red dot in (A)) and static particle (black dataset, corresponding to the particle indicated by the black dot in (B)).

D) Mean-squared displacement analysis of the particles in (C). Linear fits to the first 5 points of the MSD reveal a diffusion coefficient of (4.4·10$^4$) nm$^2$/s (red dataset) and 91 nm$^2$/s (black dataset). Error bars: SEM.

E) GFP-RAD52 diffusive fraction as a function of cationic conditions for dsDNA at different forces and for ssDNA. Error bars: statistical errors in the number of counts.

F) Effect of the tension on ssDNA constructs on the diffusion coefficients of GFP-RAD52 complexes in CaCl$_2$ or MgCl$_2$.

G) Effect of the tension on ssDNA constructs on the diffusive fraction of GFP-RAD52 complexes in CaCl$_2$, MgCl$_2$ or without divalent cation.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Proteins purification
Plasmid pET28a-eGFP-polyHis-hRAD52 for the expression of N-terminally tag GFP-RAD52 was constructed by modification of pET28a-polyHis-hRAD52 (de Jager et al., 2001). RAD52 and GFP-RAD52 expression was performed in Rosetta(DE3)pLysS cells (Novagen) grown in LB medium containing kanamycin at 25 µg/ml and chloramphenicol at 34 µg/ml. Two liters of the same medium was inoculated with 20 ml of a saturated overnight pre-culture and incubated at 37°C with shaking. Expression was induced with 0.5 mM IPTG when the optical density at 600 nm reached 0.5. After overnight growth at 16°C, the cells were harvested by centrifugation (4,500 × g, 15 min, 4°C), resuspended with 15 ml PBS and stored at -20°C. The cell paste was thawed and mixed with one volume of lysis buffer (40 mM Tris-HCl pH 7.5, 1600 mM NaCl, 4 mM 2-Mercaptoethanol, 20 mM Imidazole, 10% glycerol) supplemented with 1 tablet of protease inhibitors without EDTA (Pierce), 1 mM PMSF, 10 mg lysozyme and 1 ml of 10% Triton X-100. After resuspension the lysate was treated by sonication to reduce the viscosity. The lysate was clarified by centrifugation (25,000 × g, 60 min, 4°C). The supernatant was collected and passed through a 5 ml HisTrap FF Crude column (GE Healthcare) equilibrated with 20 mM Tris-HCl pH 7.5, 800 mM NaCl, 2 mM 2-Mercaptoethanol, 10 mM Imidazole and 10% glycerol. The column was washed extensively with 20 column volumes of the same buffer and proteins were eluted with 25 ml of the same buffer but containing 300 mM NaCl. The eluate was dialyzed against 2 liters of 20 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol overnight at 4°C and applied to a 5 ml Heparin column (GE Healthcare) equilibrated with the dialysis buffer, washed by raising the salt to 100 mM and eluted with 0.1-1 M KCl gradient. The peaks of RAD52 and GFP-RAD52 eluting around 300 mM KCl (Figure S1A), were collected, divided in aliquots and flash frozen in liquid nitrogen before storage at -80°C.

Annealing assay
Two complementary 50-mer oligonucleotides, oligo 1 (5'-TAA ATG CCA ATG CTG ATA CGT ACT CGG ACT GAT TCG GAA CTG GTA GTG ATA CGT ACT CGG ACT GAT TCG GAA CTG TAA CG-3'), and oligo 2 (5'-CTG TAC AGT TCC GAA TCA GTC GGA ATT GGC ATT TA-3') were purchased from Eurogentec. Oligo 1 was 5'-end labeled with FAM. FAM-labeled oligo 1 (final concentration 10 nM), complementary oligo 2 (10 nM), RAD52 protein (final concentration 100 nM) or GFP-RAD52 (final concentration 100 nM) were added in reaction buffer (final volume 90 µl) containing 0.25 mM EDTA, 5 mM Tris-HCl pH 8.0, 40 mM KCl, 2.5% Glycerol, 50 µg/ml BSA, and 2.5 mM MgCl₂, kept at room temperature. At the temperature indicated, 10 µl of reaction mixture were taken out and quenched by addition of 2 µl of stop buffer containing 5 µM unlabeled oligo 1, 5% SDS, 250 mM EDTA and 2.5 mg/ml proteinase K for 15 min at room temperature. DNA products were fractionated by native polyacrylamide gel electrophoresis using a Tris-phosphate buffer (pH 8.0, 2.5 mM MgCl₂, 150 mM NaCl, 10 mM phosphate buffer, 15 mM sodium chloride, 15% glycerol, 50% (Spherotech). The preparation of the construct which can be used for ssDNA experiments upon annealing and ligation of an oligonucleotide of 48 nt-long λ phage DNA were used in combination with streptavidin-coated beads with a diameter of 4.5 µm (Spherotech). The preparation of the construct for the dsDNA experiments was described previously (van den Broek et al., 2005). In brief, it consists of using Klenow DNA Polymerase in combination with biotin-14-dATP and biotin-14-dCTP to label the 5' overhangs of both strands of the dsDNA. The preparation of the construct which can be used for ssDNA experiments upon force-induced melting was described previously (Candelli et al., 2013). In brief, biotinylation of both the 3' and 5' end of the same DNA strand is achieved by sequential annealing and ligation of oligonucleotides (5'-GGG CGG CGA CCT GGA CAA-3' and 5'-AGG TCG CCG CCC TTT TTT TTT TTT TTT-3') to first biotinylate the 5' end and subsequently the annealing and ligation of an oligonucleotide (5'-TTT TTT TTT TTT AGA GTA CTG TAC GAT CTA GCA TCA ATC TTG TCC-3') to the 3' end of a linearized λ DNA molecule.

DNA constructs for optical trapping experiments
For optical trapping experiments with both ss and dsDNA, biotinylated DNA constructs based on the 48 517 nt-long λ phage DNA were used in combination with streptavidin-coated beads with a diameter of 4.5 µm (Spherotech). The preparation of the construct for the dsDNA experiments was described previously (van den Broek et al., 2005). In brief, it consists of using Klenow DNA Polymerase in combination with biotin-14-dATP and biotin-14-dCTP to label the 5' overhangs of both strands of the dsDNA. The preparation of the construct which can be used for ssDNA experiments upon force-induced melting was described previously (Candelli et al., 2013). In brief, biotinylation of both the 3' and 5' end of the same DNA strand is achieved by sequential annealing and ligation of oligonucleotides (5'-GGG CGG CGA CCT GGA CAA-3' and 5'-AGG TCG CCG CCC TTT TTT TTT TTT TTT-3') to first biotinylate the 5' end and subsequently the annealing and ligation of an oligonucleotide (5'-TTT TTT TTT TTT AGA GTA CTG TAC GAT CTA GCA TCA ATC TTG TCC-3') to the 3' end of a linearized λ DNA molecule.

Experimental conditions for optical trapping experiments
Catching of the beads and the DNA were performed in PBS buffer (10 mM phosphate, 150 mM NaCl, pH 7.3–7.5). DNA melting for generation of ssDNA templates was performed in 20 mM Tris-HCl pH 7.6. Buffer conditions in the protein and imaging channels were 20 mM Tris-HCl pH 7.6, 100 mM KCl and either 1 mM MgCl₂, 1 mM CaCl₂, or no divalent cations.

Experimental apparatus combining optical trapping, fluorescence microscopy, and microfluidics
The custom-built instrument was described in detail elsewhere (Gross et al., 2010). Briefly, it is built around a Nikon inverted microscope equipped with a 1064 nm trapping laser, where the two traps which can be manipulated independently using steerable mirrors are generated by splitting the laser into two perpendicularly polarized beams using a half-wave plate.
and polarizing beam splitter cube. Using a second polarizing beam splitter, the two trapping beams are recombined and coupled into a water-immersion objective on the microscope. By collecting the transmitted light using an oil-immersion condenser and rejection of the unwanted light by a third polarizing beam splitter, the force can be detected on a position-sensitive diode. The bead-to-bead distance was measured using real time template matching of bright field images obtained by blue LED illumination. For fluorescence imaging, 491 nm and 639 nm excitation lasers were simultaneously coupled into the microscope and imaged on an EMCCD camera. A multichannel laminar flow cell was used to enable fast buffer exchange between beads, DNA, buffer, and protein solutions.

Quantification of fluorescence intensity
To obtain the stoichiometry of the DNA-bound GFP-RAD52 complexes, we applied a step-fitting algorithm (Kerssemakers et al., 2006) to the photobleaching traces of all individual fluorescent patches and determined the average intensity of a single GFP from a Lorentzian fit to the histogram of step intensities (Figure S3). From this, the total number of eGFP molecules in each patch could be inferred from the initial fluorescence intensity of each patch.

Quantification of protein diffusion
The diffusion of GFP-RAD52 complexes along dsDNA and ssDNA was quantified by tracking the proteins for a large number of frames (on average 29 ± 2 s). The corresponding diffusion coefficient was calculated using 1-dimensional mean square displacement (MSD) analysis (Heller et al., 2014b). Because the pixel size of our camera is 130 nm, we estimate that the minimal diffusion we could observe would be if the particle moves a distance of 1 pixel during the average interaction time. Therefore, we estimate the minimum detectable diffusion of RAD52 interactions using our system to be 583 nm²/s. We therefore assume that a particle is static if its diffusion coefficient is lower than this value and that it is diffusive when its diffusion coefficient is higher than this value.

Experiments of ssDNA oligonucleotide capture in trans by RAD52
The sequence of the 60-mer fluorescent oligonucleotide was Atto647N-5’-ACA-GCC-AGA-CGA-CGC-TGA-CGC-TCG-ACC-GTA-AAA-TCA-GCG-TGC-CAT-CCT-CGG-GTA-CGG-3’. Experiments were performed in a flow cell with six channels. DNA molecules with GFP-RAD52 bound were incubated for 30 s in a 10 nM solution of the oligonucleotides in 20 mM Tris-HCl pH 7.6, 100 mM KCl and 1 mM CaCl₂ and visualized in the same buffer in the absence of oligonucleotides in solution.

SUPPLEMENTAL REFERENCES
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