Supporting Information

Hilario *et al.* 10.1073/pnas.0811965106

SI Materials and Methods

Rad51 Purification. Rad51 was purified according to the protocol published in ref. 1, except with omission of Affi-Gel Blue and Mono S chromatography columns. The final pooled fractions of Rad51 were dialyzed against Rad51 storage buffer (50 mM TrisOAc, 200 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 50% glycerol) and stored at −80 °C. Rad51 protein was found to be free of both ssDNA and dsDNA nucleases. Rad51 protein concentration was determined spectrophotometrically, using a calculated extinction coefficient of 14,900 $M^{-1}{\cdot}cm^{-1}$ at 280 nm (2).

Fluorescent End-Labeling of λ DNA. For studies with unlabeled Rad51 protein, the analysis of assembly and disassembly by single-molecule fluorescence microscopy used λ DNA end-labeled with Cy3. The fluorescent end-labeled λ DNA was prepared by annealing and ligating a 3′-biotinylated, 12-base oligonucleotide complementary to the *cosB* site and a 3′-digoxigenin, 12-base oligonucleotide complementary to the *cosA* site. Oligonucleotides (IDT) were present at ≈2,000-fold molar excess over λ DNA ([λ DNA] ≈ 1.6 nM). Ligated λ DNA products were purified by S-400 spin columns (GE Healthcare). This doubly ligated λ DNA was first reacted with 1.0 μm streptavidin-coated microspheres (Bangs Laboratories) to produce the DNA-bead complex (3), and then was reacted sequentially with primary anti-digoxigenin antibody (Roche) and Cy3-labeled anti-sheep IgG (Chemicon).

For studies with $Rad51^{FAM}$ protein, the analysis of assembly and disassembly by single-molecule fluorescence microscopy used λ DNA-bead complexes that were initially stained with 10 nM YOYO-1. DNA-bead complexes were prepared as described in ref. 3, and the YOYO-1 was removed before incubation with $Rad51^{FAM}$ by washing the trapped DNA-bead complex in the observation channel of the flow-cell (4).

Fluorescent Labeling of Human Rad51 Protein. Direct observation of Rad51 filament formation was conducted using fluorescently labeled Rad51. Rad51 was labeled with 5(6)-carboxyfluorescein succinimidyl ester (Molecular Probes/Invitrogen). To specifically label the N terminus of the protein, labeling reactions were performed in 50 mM KH_2PO_4 (pH 7.1), 200 mM KCl, 0.1 mM DTT, and 25% glycerol (4, 5). A 20-fold molar excess of FAM was reacted with ≈2.0 mg of Rad51 at 4 °C, in the dark, for ≈6 h. Labeled Rad51 was purified from unreacted FAM by gel filtration chromatography (P-10 column, 1 cm × 28 cm; Bio-Rad). Labeled protein was pooled and dialyzed against Rad51 storage buffer to further remove any remaining unincorporated dye, and the degree of labeling was determined spectrophotometrically (6) by subtracting the contribution of FAM to the protein absorbance at 280 nm to determine protein concentration. An extinction coefficient of 74,000 $M^{-1}{\cdot}cm^{-1}$ at 495 nm was used to determine the concentration of incorporated FAM. Independent labeling reactions consistently yielded a degree of labeling of 0.9–1.5 dye molecule per Rad51 molecule.

D-Loop Assay. All D-loop assays used a ^{32}P-radiolabeled single-strand 90-mer oligonucleotide that is complementary to a region in the multiple cloning site of pUC19 (7). Reactions contained 1 μM Rad51, 3 μM 90-mer (33 nM molecules), 50 μM pUC19 (9 nM molecules), where the concentrations of DNA are expressed in nucleotides. The reaction buffer contained 25 mM TrisOAc (pH 7.5), 1 mM DTT, 5 mM $CaCl_2$, and 2 mM ATP (7). All components except pUC19 were combined and incubated at 37 °C for 10 min. The reaction was initiated by the addition of pUC19 plasmid and allowed to proceed at 37 °C. Aliquots at the specified time points were removed and terminated with stop buffer (1% SDS and 1 mg/ml Proteinase K, final concentrations.). Samples were analyzed by electrophoresis in 1% agarose gels run in buffer composed of 20 mM TrisOAc (pH 8.0) and 0.5 mM EDTA). Gels were dried, imaged with a Molecular Dynamics Phosphor Imager and then quantified using ImageQuant software. Product yield is reported as the percentage of plasmid DNA.

Movies. All movies were created in ImageJ (version 1.33u) by frame averaging the original video data (in TIFF format, digitized at 30 frames/s) to 1 frame/s. These averaged movies were then converted to AVI format.

1. Sigurdsson S, Trujillo K, Song B, Stratton S, Sung P (2001) Basis for avid homologous DNA strand exchange by human Rad51 and RPA. *J Biol Chem* 276:8798–8806.
2. Pace CN, Vajdos F, Fee L, Grimsley G, Gray T (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci* 4:2411–2423.
3. Bianco PR, *et al.* (2001) Processive translocation and DNA unwinding by individual RecBCD enzyme molecules. *Nature* 409:374–378.
4. Galletto R, Amitani I, Baskin RJ, Kowalczykowski SC (2006) Direct observation of individual RecA filaments assembling on single DNA molecules. *Nature* 443:875–878.
5. Kim YT, Tabor S, Churchich JE, Richardson CC (1992) Interactions of gene 2.5 protein and DNA polymerase of bacteriophage T7. *J Biol Chem* 267:15032–15040.
6. Haugland RP (2002) *Handbook of Fluorescent Probes and Research Products* (Molecular Probes, Eugene, OR), 9th Ed.
7. Bugreev DV, Mazin AV (2004) Ca^{2+} activates human homologous recombination protein Rad51 by modulating its ATPase activity. *Proc Natl Acad Sci USA* 101:9988–9993.

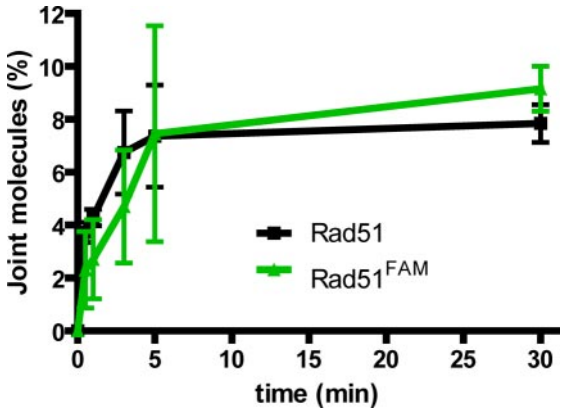


Fig. S1. Rad51^{FAM} is as active in D-loop formation as wild-type Rad51. Time course for D-loop (joint molecule) formation by Rad51 (squares) and Rad51^{FAM} (triangles). Results are the average and standard deviation of 2 experiments.

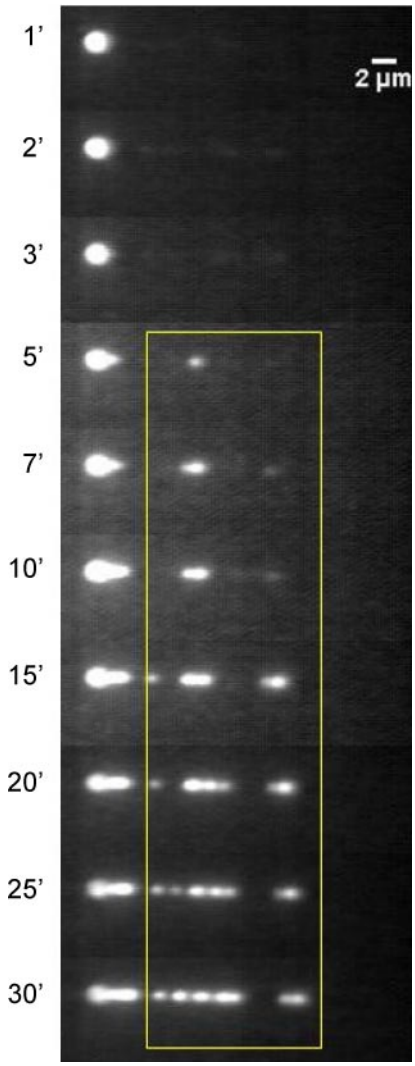


Fig. S2. Rad51 filament formation occurs via multiple nucleation events in the presence of AMPPNP. Frames from a video (time indicated in minutes) for the nucleation of a $Rad51^{FAM}$ nucleoprotein filaments on a single λ DNA molecule. The reaction contained 200 nM $Rad51^{FAM}$, 2 mM $Mg(OAc)_2$, 1 mM AMPPNP, and 150 mM NaCl. The maximum size of clusters within the indicated boxed region was 1.5 μm.

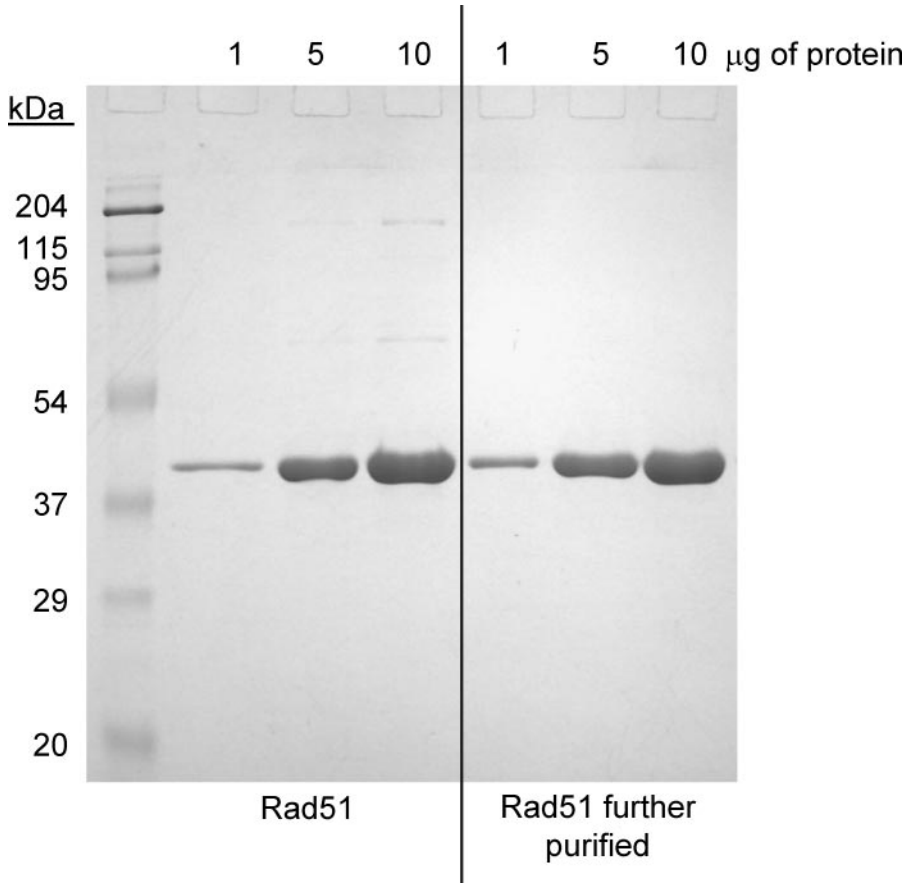
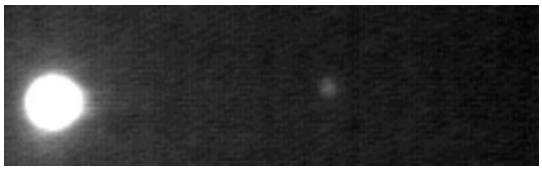
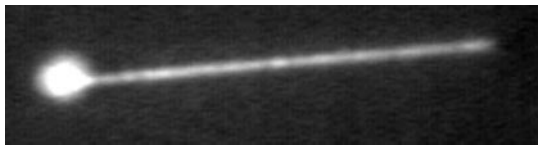


Fig. S3. SDS/PAGE analysis shows the purity of the Rad51 preparation that was used for both DNA end-labeled and fluorescent Rad51 experiments. Lanes labeled ''Rad51'' contain 1, 5, and 10 μg of protein. Protein is estimated to be $>$98% pure as determined by band intensities in the 10 μg of protein lane. Rad51 was further purified by ssDNA cellulose chromatography and then again by MonoQ chromatography (labeled as ''Rad51 further purified,'' last 3 lanes). The trace amounts of higher molecular weight contaminants detectable in the ''Rad51'' lanes were removed by these additional procedures; however, the properties of the protein were unaltered.



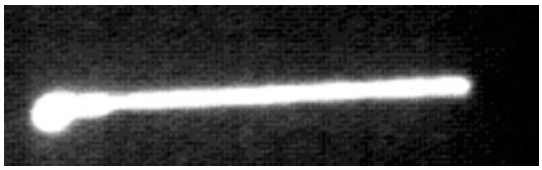
Movie S1. Wild-type Rad51 binding to a Cy3, end-labeled λ DNA molecule. This movie shows the real-time assembly of wild-type Rad51 protein binding to a single, Cy3 end-labeled λ DNA molecule. The movie is the same data from which the kymograph in Fig. 1*B* was obtained. Experimental conditions are indicated in the main text and in the legend to Fig. 1.

Movie S1 (AVI)



Movie S2. Visualization of Rad51^{FAM} binding to a single λ DNA molecule. This movie shows the binding of Rad51^{FAM} to a single λ DNA molecule. The beginning of the movie (0:00–0:05 sec) shows the removal of YOYO-1 dye from the DNA molecule, followed by repeated dipping of the DNA molecule in the Rad51^{FAM} reaction channel. Blank frames in between images occur as a result of suspending data collection during dipping periods. Still images of this movie are shown in Fig. 4*A*. Experimental conditions are indicated in the main text and in the legend to Fig. 4.

Movie S2 (AVI)



Movie S3. The disassembly of Rad51^{FAM} filaments visualized on a single λ DNA molecule. This movie shows the shortening of a complete Rad51 nucleoprotein filament under ATP hydrolyzing conditions. Still images and a kymograph of this movie are presented in Fig. 7*B Left*. The trajectory of this movie is given in Fig. 7*D*. Experimental conditions are indicated in the manuscript and in the legend to Fig. 7.

Movie S3 (AVI)