

**Enhancement of ADP Release from the RAD51 Presynaptic Filament
by the SWI5-SFR1 Complex**

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Supplementary Data

Supplementary Materials and Methods

DNA substrates

For the homologous DNA pairing assay, the 80-mer Oligo 1: 5'TTATGTTTCATTTTTTATATCCTTTACTTTATTTTCTCTGTTTATTCATTTACTTATTTTGTATTATCCTTATCTTATTTA was used for assembling the presynaptic filament. To prepare the target duplex 40-mer dsDNA, Oligo 2: 5'TAATACAAAATAAGTAAATGAATAAACAGAGAAAATAAAG was 5' end-labeled with polynucleotide kinase (New England Biolabs) and [γ - 32 P] ATP (PerkinElmer). Following the removal of the unincorporated nucleotide with a Spin 6 column (Bio-Rad), the radiolabeled oligonucleotide was annealed to its exact complement, by heating the mixture of the two oligonucleotides at 85°C for 10 min and slow cooling to 23°C. The resulting duplex was purified from a 10% polyacrylamide gel by electro-elution and filter-dialyzed in a Centricon-10 concentrator (Millipore) at 4°C into TE buffer (10 mM Tris-HCl, pH 8.0, and 0.5 mM EDTA). Oligo 1 was also annealed to its exact complement to generate the duplex form, which was purified from a 10% polyacrylamide gel, as above. Oligo 1 or 80-mer Poly-dT ssDNA was used in assays to examine ATP hydrolysis by Rad51.

For the optical tweezers experiments, the 6123 bp duplex DNA substrates was prepared by the polymerase chain reaction (PCR) using two modified primers: 5'-digoxigenin-TGCCACCTTTTCAGCTCG and 5'-biotin-AGTGTAAGCCTGGGGTG, and M13mp18 single-stranded DNA (New England Biolabs) as template. The PCR product was purified using a gel extraction kit (QIAGEN).

Plasmids

SWI5, SFR1, and SWI5-SFR1 expression plasmids: The cDNAs that code for (His)₆-tagged SWI5, SFR1, and SWI5-SFR1 with a (His)₆ tag on SFR1 were introduced into *E. coli* expression plasmids as described (22).

RAD51 expression plasmid: The mouse *Rad51* cDNA was inserted into a pRSFDuet vector (Novagen) to add a (His)₆ tag to the amino-terminus of the protein (22).

Protein purification

SWI5 and SWI5-SFR1 purification: (His)₆-tagged SWI5 and SWI5-SFR1 with a (His)₆ tag on SFR1 were expressed in *E. coli* Rosetta cells and purified as described (22).

SFR1 expression and purification: (His)₆-tagged SFR1 was expressed in *E. coli* Rosetta cells. All the purification steps were carried out at 4°C. Briefly, cell extract was prepared from 36 g of cell paste and then clarified by centrifugation as described previously (22). The cleared lysate was incubated with 4 ml Talon beads (Clontech) for 12 h. The matrix was poured into a column, washed with 40 ml buffer T (25 mM Tris-HCl, pH 7.5, 10% glycerol, 0.01% Igepal, and 1 mM 2-mercaptoethanol) containing 300 mM KCl, 1 mM ATP, 8 mM MgCl₂, and 10 mM imidazole. SFR1 was eluted with 25 ml of 200 mM imidazole in buffer K (20 mM KH₂PO₄, pH 7.4, 10% glycerol, 0.5mM EDTA, 0.01%

Igepal, and 1 mM 2-mercaptoethanol) containing 50 mM KCl. The eluate was diluted with 40 ml of buffer K containing 50 mM KCl and fractionated in a 1 ml Macrohydroxyapatite column, using a 30 ml gradient of 0-350 mM KH_2PO_4 in buffer K. SFR1-containing fractions were diluted with 40 ml of buffer T and further fractionated in a 1 ml Mono Q column, using a 15 ml gradient of 75-475 mM KCl in buffer T. The peak fractions were pooled and concentrated to 3-4 mg/ml in a Centricon-10 concentrator. The concentrated preparation was divided into small aliquots and stored at -80°C .

RAD51 expression and purification: RAD51 was overexpressed and purified to near homogeneity from the RecA-deficient *E. coli* strain BLR, using our published procedure (22). Briefly, clarified cell lysate was subjected to Talon affinity step, followed by chromatographic fractionation in columns of SP Sepharose and Macrohydroxyapatite. RAD51 thus purified was $\geq 98\%$ homogeneous.

Other protein reagents

S. cerevisiae Rad51 and Strep-tagged mouse RAD51, used for the affinity pulldown and homologous DNA pairing experiments, were purified as described (9, 22). Mouse RAD51AP1 was expressed in *E. coli* Rosetta cells and purified as described for human RAD51AP1 (26). We note that mouse RAD51AP1 physically interacts with mouse RAD51 and stimulates mouse RAD51-mediated homologous DNA pairing just as what has been reported for the human ortholog (25, 26; and our unpublished data). Mouse DMC1 with an amino terminal (His)₆ tag was expressed and purified from RecA-deficient *E. coli* cells (our unpublished results). *E. coli* RecA protein was purchased from New England Biolabs.

Affinity pulldowns

Strep-tagged mouse RAD51 or *S. cerevisiae* Rad51 (ScRad51; 3.2 μg) was incubated with 5 μg SWI5-SFR1 containing a (His)₆ tag in SFR1's amino-terminus in 30 μl buffer C (25 mM Tris-HCl, pH 7.5, 10% glycerol, 0.01% Igepal, 100 mM KCl, 1 mM 2-mercaptoethanol and 5 mM imidazole) for 30 min at 4°C . After being mixed with 10 μl of Talon resin for 30 min at 4°C to capture the (His)₆-tagged protein and associated RAD51, the resin was washed three times with 30 μl buffer C and then treated with 25 μl 2% SDS to elute proteins. The supernatant, last wash, and SDS eluate, 10 μl each, were analyzed by SDS-PAGE.

DNA strand exchange assay

All the reaction steps were carried out at 37°C . The 80-mer Oligo 1 (4.8 μM nucleotides) was incubated with mouse RAD51, DMC1 or *S. cerevisiae* Rad51 (ScRad51; 1.6 μM) in 10.5 μl buffer A containing 1 mM ATP for 5 min. The indicated amount of SWI5-SFR1 complex was then added in 1 μl , followed by a 5-min incubation. The reaction was initiated by adding homologous ^{32}P -labeled 40-mer duplex (2.4 μM base pairs) to 12.5 μl of final volume. After 20 min incubation, a 5 μl aliquot was removed and mixed with an equal volume of 1% SDS containing proteinase K (1 mg/ml) and incubated at 37°C for 10 min. The samples were subjected to gel electrophoresis and phosphorimaging analysis.

Exonuclease I protection assay

DMC1 (1.3 μM) was incubated with 5'- ^{32}P -labeled 80-mer Oligo 1 ssDNA (3 μM nucleotides) in 18 μl buffer A containing 0.1 mM ATP at 37°C for 5 min. Following the incorporation of the indicated amount of SWI5-SFR1 and a 5-min incubation, exonuclease I (1.5 units; New England Biolabs) was added to the reaction mixture (20 μl final volume). After 20 min of incubation at 37°C, a 10 μl aliquot was removed and mixed with 2.5 μl of 60 mM EDTA, 0.5 % SDS, and proteinase K (1 mg/ml) and incubated at 37°C for 10 min. The reaction mixtures were resolved in 10% polyacrylamide gels in TBE buffer (89 mM Tris, 89 mM borate, and 2 mM EDTA, pH 8.0) and the DNA species were quantified by phosphorimaging analysis.

Nitrocellulose filter binding assay to monitor ADP release from RAD51 filament

Determining the ratio of [α - ^{32}P]ADP/[α - ^{32}P]ATP

To monitor the conversion of ATP to ADP within the RAD51 presynaptic filament (**Supplementary Figure S4B**), 1.6 μM RAD51 were incubated in 12.5 μl of buffer A containing the final concentration of 1.6 μM ATP with 0.05 μCi [α - ^{32}P] ATP and 80-mer Oligo 1 ssDNA (4.8 μM nucleotides) at 37°C. 1.5 μl aliquots of the reactions were removed at the indicated time point and mixed with an equal volume of 500 mM EDTA to halt the reaction. The level of [α - ^{32}P] ADP to [α - ^{32}P] ATP was determined by thin layer chromatography in polyethyleneimine cellulose sheets (Fluka) in 0.3 M KH_2PO_4 (pH 7.5) and with phosphorimaging analysis.

Measuring [α - ^{32}P] ADP amounts within the RAD51 filament

Reaction mixtures were assembled in 12.5 μl buffer A containing 1.6 μM RAD51, 80-mer Oligo 1 ssDNA (4.8 μM nucleotides), and 1.6 μM ATP with 0.05 μCi [α - ^{32}P] ATP. Reactions were incubated at 37°C for 10 min, followed by the incorporation of the indicated amount of SWI5-SFR1 and an additional 5-min incubation. Reaction mixtures were filtered through a nitrocellulose filter and the level of [α - ^{32}P] ADP was determined by phosphorimaging analysis (**Supplementary Figure S4C**).

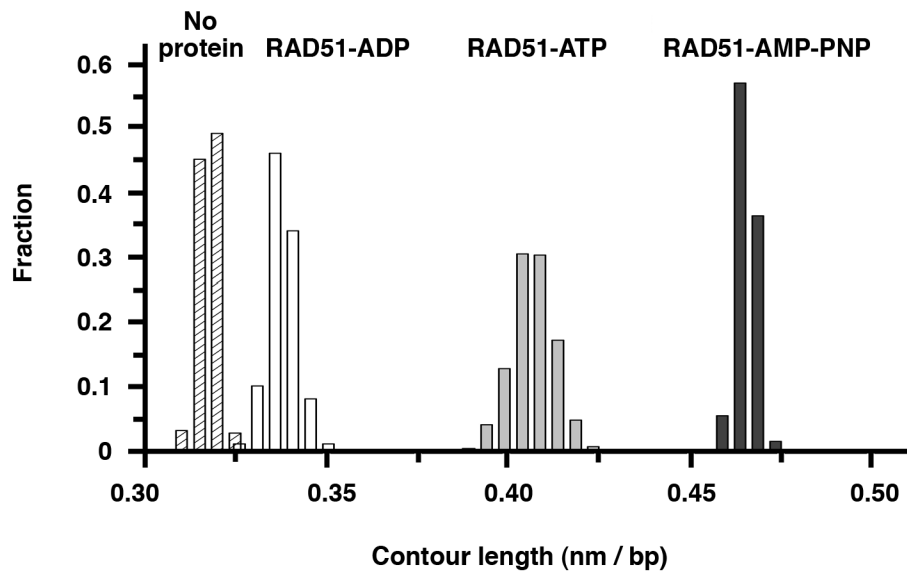
Nucleotide binding assay to measure ADP/ATP binding activity of SWI5-SFR1

To examine the ATP binding activity (**Supplementary Figure S3A**), 1.6 μM SWI5, SFR1, SWI5-SFR1 or RAD51, were incubated in 25 μl of buffer A containing 1.6 μM ATP- γ -S with 0.08 μCi [^{35}S] ATP- γ -S with or without 80-mer Oligo 1 ssDNA (4.8 μM nucleotides) or of buffer B containing 10 mM CaCl_2 , 20 μM ATP with 80-mer Oligo 1 ssDNA (1.6 μM ATP without ssDNA) and with 0.08 μCi [γ - ^{32}P] ATP at 37°C for 5 min. For measuring ADP binding ability (**Supplementary Figure S3C**), we incubated 3.2 μM RAD51, SWI5, SFR1, or SWI5-SFR1 with 3.2 μM [^{14}C] ADP in the presence of Oligo 1 ssDNA (9.6 μM nucleotides) or with 16 μM [^{14}C] ADP without ssDNA at 37°C for 30 min. Reaction mixtures were filtered through a nitrocellulose filter and the level of ADP determined by phosphorimaging analysis.

UV cross-linking assay

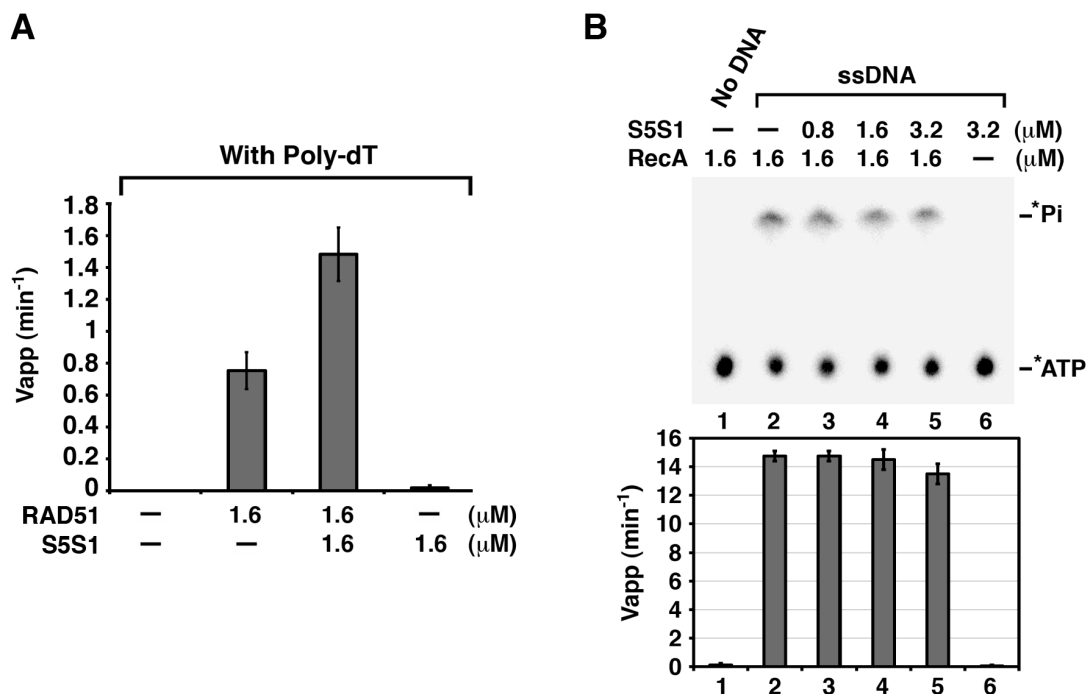
3.2 μM BSA, SWI5, SFR1, SWI5-SFR1 or RAD51, were incubated in 12.5 μl buffer A containing 0.064 μM [α - ^{32}P] ATP (3000 Ci/mmol) at 37°C for 5 min (Supplementary Figure S3B). The reaction was then irradiated at 2 cm from the 254 nm UV source (Handheld UV Lamp; UVP) for 15 min. Samples were resolved by 15% SDS-PAGE and the gel was dried and radiolabel visualized with a phosphorimager.

Supplementary Figures



Supplementary Figure S1. Stabilization of the RAD51 filament by AMP-PNP

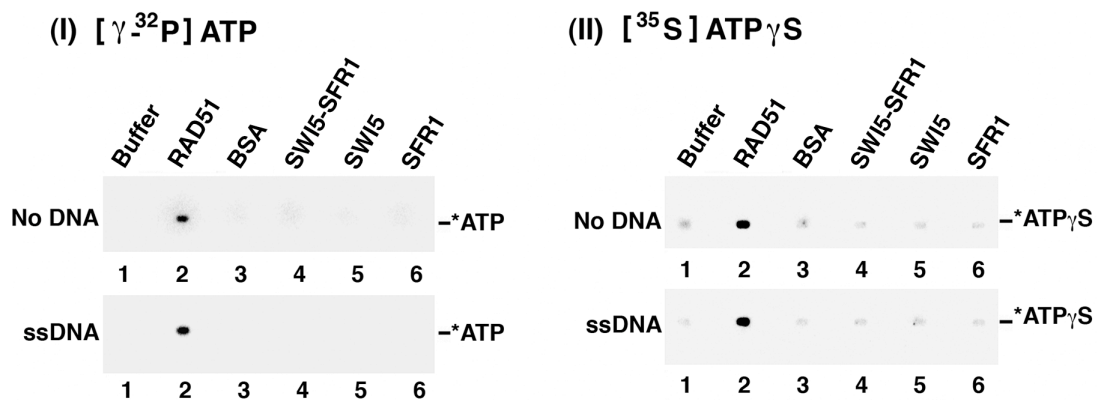
Histogram summarizing the contour length distributions of 6123 bp duplex DNA molecules without any protein bound or after incubation with RAD51 with ATP, ADP or AMP-PNP. The approximate 1.5 fold increase of the DNA contour length seen in the presence of AMP-PNP resulted from full coverage of the DNA by RAD51.



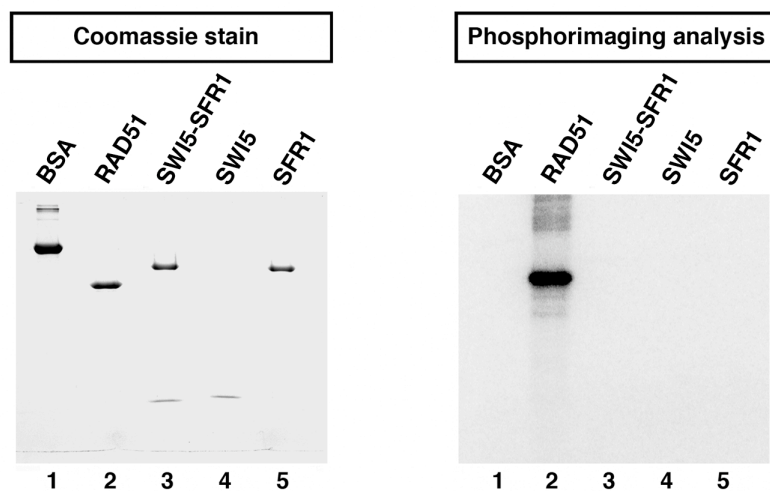
Supplementary Figure S2. The effect of SWI5-SFR1 on ATP hydrolysis by the presynaptic filament is specific for RAD51

(A) Stimulation of the RAD51 ATPase activity by SWI5-SFR1 also occurred when poly-dT was used to assemble the presynaptic filament. (B) SWI5-SFR1 had no effect on RecA-mediated ATP hydrolysis in the presence of 80-mer ssDNA. The asterisk denotes the ^{32}P label. Error bars represent the standard deviation ($\pm\text{SD}$) calculated based on at least three independent experiments. Symbol: S5S1, SWI5-SFR1.

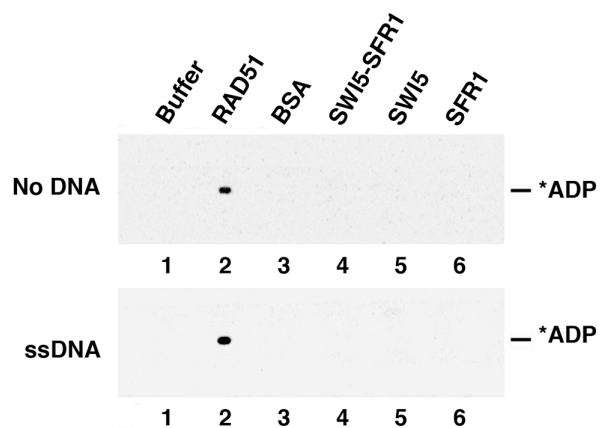
A



B

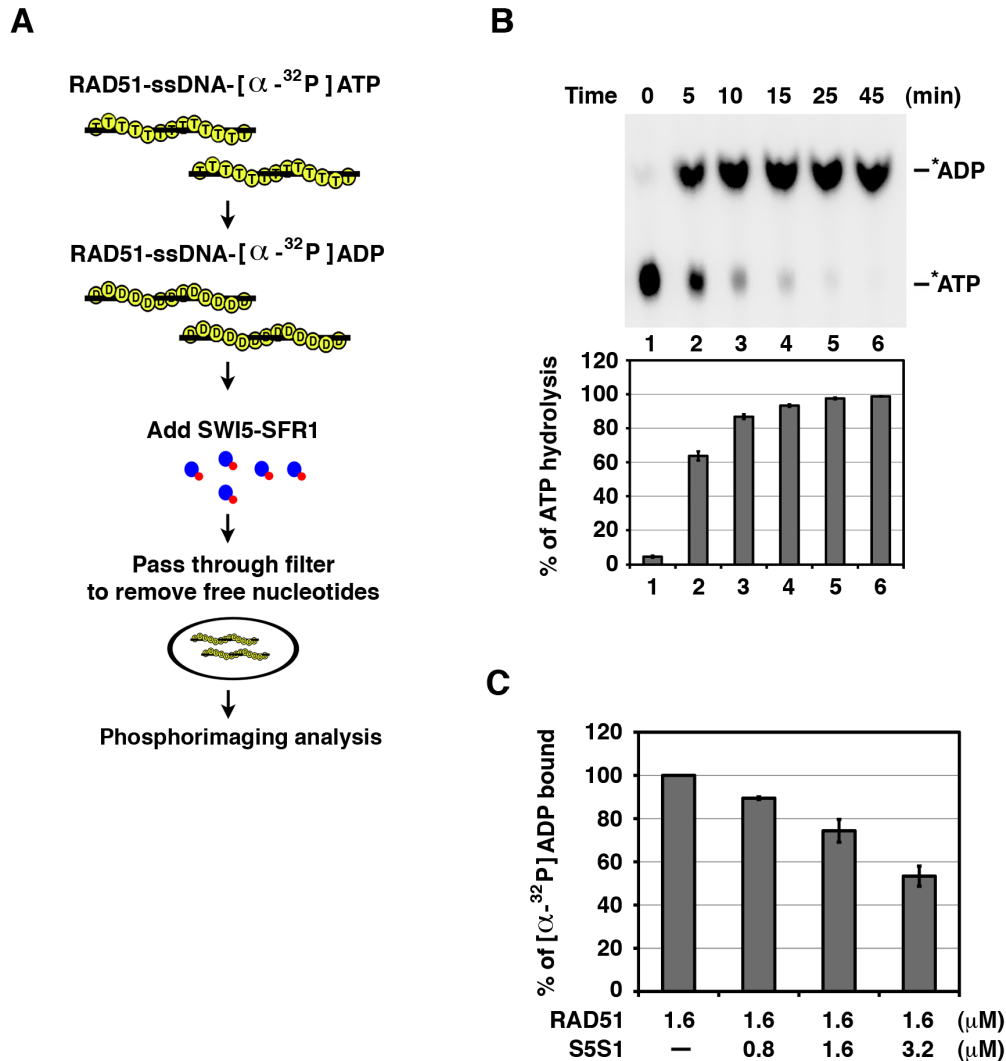


C



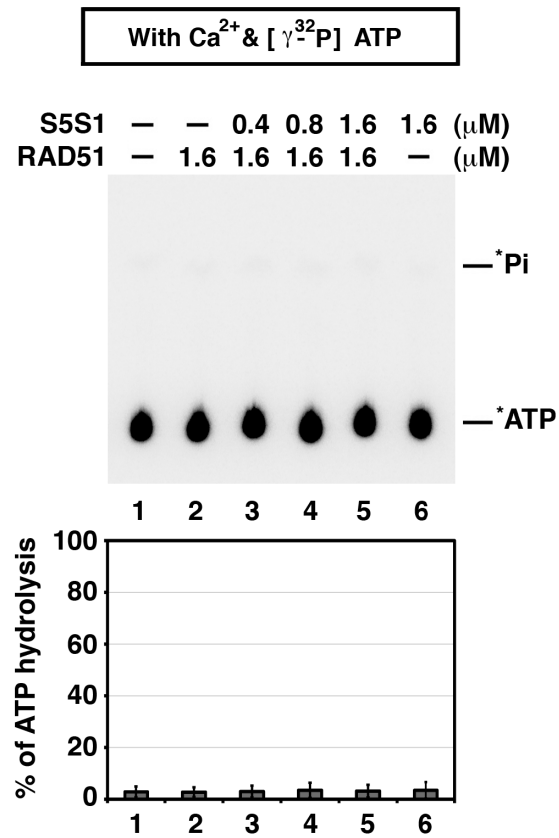
Supplementary Figure S3. SWI5, SFR1, or SWI5-SFR1 complex are devoid of nucleotide binding activity

(A) Shown are results from filter-binding experiments to demonstrate that SWI5, SFR1, and SWI5-SFR1 are devoid of the ability to bind [γ - 32 P] ATP (panel I) or [35 S] ATP- γ -S (panel II). Note that Ca^{2+} was added to prevent hydrolysis of the radiolabeled ATP. RAD51 served as a positive control. (B) UV crosslinking was used to demonstrate that SWI5-SFR1 lacks the ability to bind [α - 32 P] ATP. (C) Results from filter-binding experiments showing that SWI5, SFR1, and SWI5-SFR1 are devoid of ADP binding activity. (A and C) The radiolabeled nucleotides are highlighted by an asterisk.



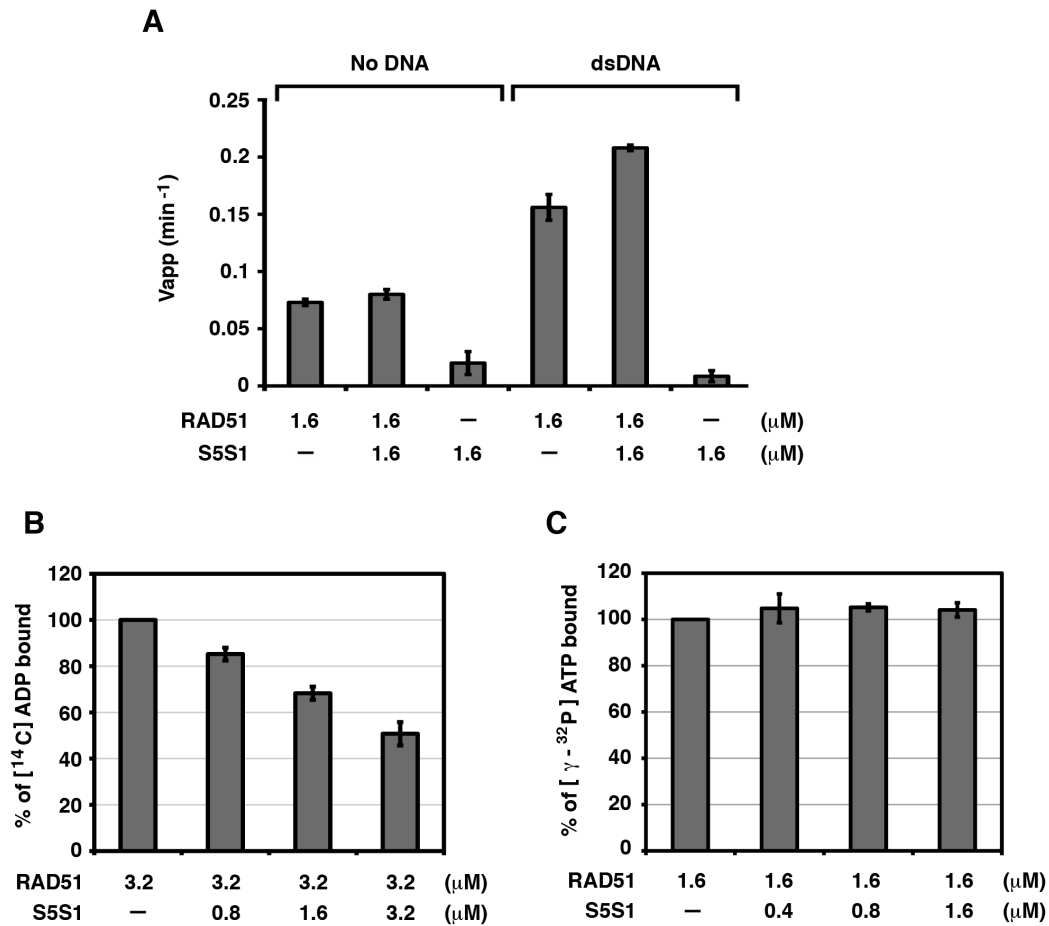
Supplementary Figure S4. SWI5-SFR1 facilitates ADP release from the RAD51 filament

(A) Schematic of the filter-binding assay to monitor ADP release from the RAD51 presynaptic filament that has been converted from the ATP to ADP bound form. (B) Thin layer chromatography to measure the hydrolysis of [α - 32 P] ATP by RAD51. The asterisk denotes the 32 P label. (C) SWI5-SFR1 enhances ADP release from the RAD51 presynaptic filament in a protein concentration-dependent manner. (B and C) Error bars represent the standard deviation (\pm SD) calculated based on at least three independent experiments. Symbols: S5S1, SWI5-SFR1



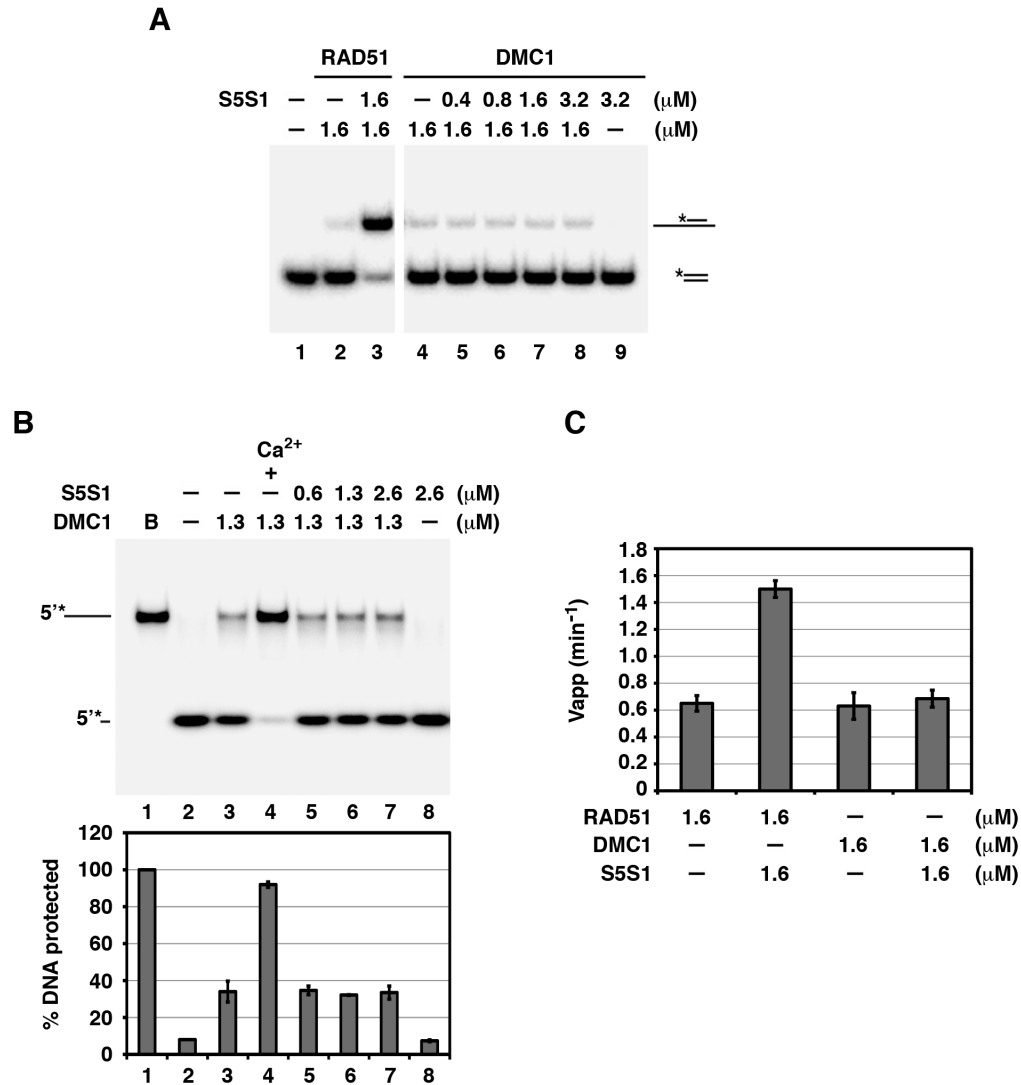
Supplementary Figure S5. Inhibition of RAD51-mediated ATP hydrolysis by Ca²⁺

Thin layer chromatography was used to measure the rate of RAD51-mediated ATP hydrolysis with 10 mM Ca²⁺ present. The results were graphed. The asterisk denotes the ³²P label. Error bars represent the standard deviation (±SD) calculated based on at least three independent experiments. Symbol: S5S1, SWI5-SFR1.



Supplementary Figure S6. Effects of SWI5-SFR1 on the functional attributes of the RAD51-dsDNA filament

(A) SWI5-SFR1 stimulates ATP hydrolysis by the RAD51-dsDNA filament. (B) SWI5-SFR1 enhances the release of ADP from the RAD51-dsDNA filament. (C) SWI5-SFR1 does not alter ATP binding affinity of the RAD51-dsDNA filament. (A-C) Error bars represent the standard deviation ($\pm\text{SD}$) calculated based on at least three independent experiments. Symbols: S5S1, SWI5-SFR1



Supplementary Figure S7. SWI5-SFR1 has no effect on the functional attributes mouse DMC1 recombinase

(A) SWI5-SFR1 has no effect on DMC1-mediated homologous DNA pairing. The asterisk denotes ³²P label in the DNA strand. (B) The DMC1 presynaptic filament was challenged with exonuclease I in the absence or presence of the indicated concentrations of SWI5-SFR1. Note that Ca²⁺ was included as a positive control (lane 4). The results were graphed. (C) SWI5-SFR1 has no effect on the ATPase activity of the DMC1 presynaptic filament. (B-C) Error bars represent the standard deviation (\pm SD) calculated based on at least three independent experiments. Symbols: S5S1, SWI5-SFR1

Supplementary References

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