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Authors	Hsin-Yi Yeh, Guan-Chin Su, Kentaro Ito, Yumiko Kurokawa, Hiroshi Iwasaki, Hung-Wen Li, Chih-Hao Lu, Hsin-Yi Yeh, Guan-Chin Su
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Swi5-Sfr1 Stimulates Rad51 Recombinase Filament Assembly by Modulating Rad51 Dissociation

Chih-Hao Lu[1], Hsin-Yi Yeh[2], Guan-Chin Su[2], Kentaro Ito[3], Yumiko Kurokawa[3,¶], Hiroshi Iwasaki[3,*], Peter Chi[2,4,*] and Hung-Wen Li[1,*]

[1] Department of Chemistry, National Taiwan University, Taiwan

[2] Institute of Biochemical Sciences, National Taiwan University, Taiwan

[3] Institute of Innovative Research, Tokyo Institute of Technology, Japan

[4] Institute of Biological Chemistry, Academia Sinica, Taiwan

[*] To whom correspondence should be addressed:

Hiroshi Iwasaki, E-mail: hiwasaki@bio.titech.ac.jp

Peter Chi, E-mail: peterhchi@ntu.edu.tw

Hung-Wen Li, E-mail: hwli@ntu.edu.tw

[¶] current address: Center for Frontier Research, National Institute of Genetics, Japan

Author contributions: C.H.L., H.I., P.C. and H.W.L. designed research; C.H.L. performed all single-molecule experiments and analyzed data; H.Y.Y, G.C.S., K.I. and Y.K. purified proteins used in this article; C.H.L. H.I., P.C. and H.W.L. wrote the paper.

Key words:

homologous recombination/ single-molecule microscopy/ Rad51/ Swi5-Sfr1

ABSTRACT

Eukaryotic Rad51 protein is essential for homologous recombination repair of DNA double-strand breaks. Rad51 recombinases first assemble onto single-stranded DNA to form a nucleoprotein filament, required for function in homology pairing and strand exchange. This filament assembly is the first regulation step in homologous recombination. Rad51 nucleation is kinetically slow, and several accessory factors have been identified to regulate this step. Swi5-Sfr1 (S5S1) stimulates Rad51-mediated homologous recombination by stabilizing Rad51 nucleoprotein filaments, but the mechanism of stabilization is unclear. We used single-molecule tethered particle motion (TPM) experiments to show that mouse S5S1 (mS5S1) efficiently stimulates mouse RAD51 (mRAD51) nucleus formation, and inhibits mRAD51 dissociation from filaments. We also used single-molecule fluorescence resonance energy transfer (smFRET) experiments to show that mS5S1 promotes stable nucleus formation by specifically preventing mRAD51 dissociation. This leads to a reduction of nucleation size from three mRAD51 to two mRAD51 molecules in the presence of mS5S1. Compared to mRAD51, fission yeast Rad51 (SpRad51) exhibits fast nucleation but quickly dissociates from the filament. SpS5S1 specifically reduces SpRad51 disassembly to maintain a stable filament. These results clearly demonstrate the conserved function of S5S1 by primarily stabilizing Rad51 on DNA, allowing both the formation of the stable nucleus and the maintenance of filament length.

SIGNIFICANCE STATEMENT

In DNA homologous recombination, the recombinase-coated single-stranded DNA filament formation is the first committed step and is subject to tight regulation. Stabilization of nucleoprotein filament by accessory proteins can be achieved by enhancing filament formation, reducing filament disassembly or both. However, the mechanism of regulation is not understood by conventional biochemical methods. This is the first study of the mechanism of how accessory proteins stimulate filament assembly by applying single-molecule methods that allow us to monitor the binding of Rad51 on DNA in mouse and fission yeast. Our results show that Swi5-Sfr1 complex demonstrates the evolutionarily-conserved stimulation on Rad51 filament assembly by stabilizing Rad51 on DNA, allowing both the formation of the stable nucleus and the reduction of Rad51 dissociation.

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INTRODUCTION

Rad51 recombinases are essential for eukaryotic homologous recombination DNA repair(1, 2). As a replication fork encounters a lesion, collapsed forks lead to DNA double-strand breaks (DSBs). Homologous recombination is the major pathway to restart replication(3). To carry out homologous recombination repair, the broken end of DNA is resected to reveal a 3' protruded single-stranded DNA (ssDNA), which Rad51 binds to form a nucleoprotein filament(1, 2, 4-7). The resultant helical nucleoprotein filament is the active component responsible for homology searching and strand exchange and is essential for DSB repair and genomic maintenance.

Among the cascade of steps required for DSB repair, Rad51 nucleoprotein filament assembly is the committed step and is subject to tight regulation(1, 2, 8-10). Maintaining a stable but dynamic nucleoprotein filament is critical for recombinase function, as this filament is essential for both the homology search and strand exchange required for heteroduplex DNA formation. Filament assembly includes a rate-determining nucleation step, where several Rad51 molecules bind ssDNA to form a stable nucleus, followed by a faster extension step(11-14). The filament assembly involves monomer addition occurring both at 3′- and 5′- ends of the recombinase filament but with different rates, leading to the overall end preference. During strand exchange, this filament likely maintains a certain length through dissociating and rebinding of Rad51. Thus, the filament overall moves dynamically with polarity (15-18). Several proteins stimulate and regulate Rad51 filament assembly(19-26). Swi5 and Sfr1 were identified by genetic studies in the fission yeast *Schizosaccharomyces pombe (S. pombe)*(27, 28). Mutations in fission yeast *swi5* and *sfr1* decrease recombination rates and increase sensitivity to ionizing radiation and DNA damaging chemicals(27, 28). *In vitro* biochemical and biophysical studies showed that both fission yeast and mouse Swi5 and Sfr1 proteins form a heterodimeric complex that physically interacts with Rad51 to facilitate Rad51-mediated recombination(25, 26, 29-31). Both fission yeast and mouse Swi5-Sfr1 (S5S1) complexes stabilize the Rad51 presynaptic filament and increase the ssDNA-dependent ATPase activity of Rad51. Moreover, mouse S5S1 (mS5S1) has been reported to enhance the release of ADP from the mRAD51 presynaptic filaments(32).

How Rad51 nucleoprotein filaments are stabilized by these accessory factors is not clear. Stabilization could be achieved by increasing the Rad51 on-rate or decreasing the off-rate, or both. Conventional biochemical studies are based on averaged and equilibrium measurements, making it challenging to elucidate the molecular events responsible for these kinetic events. Here, we used single-molecule tethered particle motion (TPM) and fluorescence resonance energy transfer (smFRET)

experiments to characterize individual Rad51 filament assembly and disassembly kinetics as well as the nucleation process in real-time. Our single-molecule results support a model in which Swi5-Sfr1 stabilizes Rad51 on DNA by preventing its dissociation. This effect leads to both stable nucleus formation and longer lasting filament. Specifically, this stabilization decreases the nucleation size from 3 to 2 Rad51 in mouse and reduces filament disassembly nearly 3-fold in fission yeast. In spite of different kinetic properties of the mouse and fission yeast Rad51, Swi5-Sfr1 complex stimulates Rad51 process through a similar and conserved mechanism.

RESULTS

Mouse SWI5-SFR1 (mS5S1) stimulates mRAD51 nucleoprotein filament assembly. Among many accessory proteins regulating homologous recombination, the Swi5-Sfr1 complex stimulates various stages of the process, including filament assembly and strand exchange(25, 26, 28-35). Here, we took advantage of previously developed single-molecule tethered particle motion (TPM) experiments(36-38) to monitor Rad51 nucleoprotein filament assembly in real-time (Figure 1A). We designed a gapped DNA substrate containing a 135 nt secondary structure-free, poly dT ssDNA region ($(dT)_{135}$ gapped DNA, Figure 1A) immobilized on the surface of glass slide via a 5'-digoxigenin-anti-digoxigenin linkage. The substrate was annealed to a short oligo labeled with biotin at its 5' end, which was, in turn, attached to a streptavidin-coated polystyrene bead for visualization purposes. The 151 bp double-stranded handle in the gapped DNA was used to prevent potential Rad51-surface interaction during TPM experiments. Bead Brownian motion (BM) of DNA tether is constrained to a small region near the glass surface. The bead BM of bare $(dT)_{135}$ gapped DNA for assembly experiments and 301 bp duplex DNA used for control experiments are measured to be 21.3±5.78 nm and 35.2±3.77 nm (Figure S1A-S1B). In the presence of either ATP or AMPPNP, mRAD51 preferentially assembles onto the ssDNA region of the gapped substrate at 150 mM KCl(39) (Figure S1A & S1C-S1G). Assembly experiments were initiated by introducing a mRAD51-ATP mixture into the reaction chamber containing the surface-anchored gapped substrates. Increases in bead Brownian motion reveals nucleoprotein filament formation as mRAD51 assembling onto DNA increases ssDNA length and stiffness of the DNA tether. This results in a change in the spatial extent of bead BM(32, 40, 41). Analysis of individual single-molecule TPM time-courses reveals several kinetic parameters, including (i) the dwell time prior to bead BM increase (nucleation time, the time needed for RAD51 to form stable nuclei on ssDNA, Figure 1B-C), (ii) extension time (Figure 1D, the time required to add RAD51 monomers to ssDNA) and (iii) amount of BM increase (Figure 1E, reflecting the length of nucleoprotein

filament). Our results show that mRAD51 alone (0.8 μM) exhibits a nucleation time of 97.6±2.89 sec (Figure 1C). Interestingly, a shorter nucleation time (67.5±1.32 sec) was observed when mRAD51 was pre-incubated with more than 2-fold excess mS5S1 (Figure 1C). This observation demonstrates that mS5S1 stimulates the nucleation step of mRAD51 filament formation, which is rate-limiting. mS5S1 has neither dsDNA nor ssDNA affinity(25), so adding mS5S1 in the absence of mRAD51 did not change BM values (Figure S1H). Control experiments using a mS5S1 mutant that is defective for mRAD51 interaction, mSWI5$^{FL/AA}$-SFR1 (mS5^{FL}S1)(31), showed no stimulation of nucleation times (Figure 1C). Collectively, these findings suggest that the interaction of mRAD51-mS5S1 in solution stimulates mRAD51 nucleation. Within our experimental resolution, we did not observe any change in mRAD51 extension times in the presence of mS5S1 (Figure 1D & S2A). On the other hand, the mean BM increment increases in the presence of mS5S1 (Figure 1E), indicating that the mS5S1-RAD51 complex forms longer filaments than mRAD51 alone.

Stimulation of the mRAD51 nucleation depends on mS5S1 concentration. We next asked how much mS5S1 is required for maximum stimulation. We incubated various concentrations of mS5S1 (0-2.0 μM) with 0.8 μM mRAD51 in solution and observed filament assembly kinetics as in Figure 1A. Interestingly, a sigmoidal dependence of mS5S1/mRAD51 ratios is shown for nucleation rates (Figure 1F), with nucleation rates starting to increase at a ratio larger than 1.5 and reaching a constant maximum nucleation rate of ~0.015 s^{-1} when the ratio was larger than 2. This finding implies that two mS5S1 per one mRAD51 are required for the maximum nucleation stimulation. For the comparison purposes, we measured nucleation rates in the presence of the non-hydrolysable ATP analog, AMPPNP (black open square in Figure 1F). Under these conditions, the nucleation rate for mRAD51 alone is fast (~0.017 s^{-1}), suggesting that mRAD51 nucleating clusters are more stable in the absence of ATP hydrolysis, consistent with previous reports on bacterial RecA proteins(12, 42). It is possible that mS5S1 stimulates mRAD51 nucleation by stabilizing mRAD51 nucleating clusters.

Bead BM increment, indicative of the length of mRAD51 filament, on $(dT)_{135}$ gapped DNA without mS5S1 is 20.0±0.92 nm (Figure 1E and 1G), likely reflecting the equilibrium filament length of disassembly and assembly dynamics of the filament(14). The longer filament of 27.3±1.51 nm is seen in the presence of AMPPNP (black open square in Figure 1G), consistent with that mRAD51 disassembly requires ATP hydrolysis. Notably, in the presence of just 0.5-fold of mS5S1, mRAD51 forms longer, more stable filaments, which is consistent with previous biochemical studies (25, 31, 32). Control experiments confirm that longer filaments in the presence of either S5S1-ATP or AMPPNP do not result from

mRAD51 binding to the duplex handle of gapped DNA substrates (Figure S1A & S1C-S1G).

Molecular determinants of mSWI5-SFR1 stimulation on mRAD51 nucleation. In the rate-limiting nucleation step of the filament assembly, recombinases first form a stable nucleating cluster before extending into a longer functional filament. We used TPM experiments to measure how filament nucleation rates change with mRAD51 concentrations. The results are fitted to a power-law dependence, providing information about the mRAD51 nucleation unit of this rate-limited step. In the case of mRAD51 only, the fit returns n=2.43±0.46 (Figure 2A). As the fit renders the lower limit, our result suggests that three mRAD51 monomers are required for stable nucleating cluster formation. This is consistent with earlier work showing that human RAD51 (hRAD51) has a nucleation unit of three monomers(14). Adding a two-fold molar excess of $mS5^{FL}S1$ to mRAD51(green solid circle, Figure 2A) does not change the mRAD51 nucleation unit. However, adding more than a two-fold molar excess of mS5S1 to mRAD51 returned a power-law dependence of n=1.67±0.16 (Figure 2B). Thus, mS5S1-mRAD51 complex only needs two mRAD51 molecules to form a stable nucleation cluster. This reduction in the number of mRAD51 molecules required to form a stable nucleation cluster in the presence of mS5S1 provides a clear mechanism to increase the rate of the mRAD51 nucleoprotein assembly process.

During DNA double-stranded break (DSB) repair, the ends of the DNA breaks are resected to produce 3' ssDNA overhangs. Thus, the repair substrate possesses a double-strand/single-strand junction (ds/ss junction) and a 3' protruding ssDNA tail. Rad51 nucleation clusters could initiate filament assembly on ssDNA either near the ds/ssDNA junction or onto the free ssDNA sites along the tail. We compared the nucleation rates of mRAD51 in four gapped substrates containing one ds/ssDNA junction but different lengths of ssDNA tail (90, 135, 165 & 200 dT). Nucleation rates can be fitted linearly to ssDNA tail lengths, with the slope corresponding to the apparent nucleation rate on free ssDNA site (Figure 2C). For mRAD51 only, the dependence on ssDNA length was small, suggesting that mRAD51 alone prefers to nucleate near the junction. On the other hand, in the presence of a two-fold excess mS5S1, nucleation rates of mRAD51 showed a much stronger dependence on ssDNA lengths (filled squares, Figure 2C), with a ~6.5-fold increase in the slope ($(8.95\pm1.93)\times10^{-5}$ $s^{-1}nt^{-1}$ for mS5S1-mRAD51 and $(1.40\pm0.52)\times10^{-5}$ $s^{-1}nt^{-1}$ for mRAD51 alone, Figure 2C and Table S1). These data indicate that mS5S1 stimulates mRAD51 binding by increasing its ssDNA affinity. Previous studies showed that DSB ends are resected to lengths of up to several kilobases in cells(4, 5), therefore, by dramatically increasing the ssDNA affinity of mRAD51, mS5S1 can effectively stimulate mRAD51 filament assembly.

smFRET experiments reveal Rad51 binding and dissociation dynamics during nucleating events. In TPM assembly experiments, we monitored the filament assembly kinetics that led to the successful assembly of recombinase nucleoprotein filaments. Due to their limited spatiotemporal resolution, TPM experiments cannot detect dynamics during non-productive assembly events. For example, transient recombinase binding events are likely taking place but would be difficult to detect by TPM. To monitor transient binding events, we used single-molecule fluorescence resonance energy transfer (smFRET) to characterize nucleation dynamics of mRAD51 at high spatiotemporal resolution. The DNA substrates used in the smFRET experiments were short and composed of an 18 bp dsDNA handle and a short 3′ terminating dT overhangs (13 or 18 nt, Figure 3A & S3A-S3D). The fluorophore donor (Cy3) and acceptor (Cy5) dyes were positioned so that mRAD51 monomer binding and dissociation on the ssDNA region could be monitored. In the absence of mRAD51, ssDNA is flexible, and separation between the dye pairs is short, resulting in a high FRET efficiency (~ 0.85 for $(dT)_{13}$ and ~ 0.8 for $(dT)_{18}$, Figure S3A-S3D). When mRAD51 assembles onto ssDNA, the distance between the dye pair increased resulting in reduced FRET efficiency (Figure 3B-3C; S3C-S3D). Therefore, each high-to-low FRET transition represents one or more mRAD51 binding events. In the case of mRAD51 alone, using a shorter $(dT)_{13}$ substrate, the percentage of DNA molecules with FRET alternation observed within 3 minutes (binding fraction) was 18.5±3.62% (Figure S3E), with the time traces dominated by the protein-free, high-FRET state (Figure 3B, S3C & S4A). The low binding fraction and the transience of the low FRET states indicate that the $(dT)_{13}$ substrate is too short to form stable nucleating clusters of mRAD51. On the other hand, in the presence of mS5S1, the binding fraction of mRAD51 dramatically increases to 66.6±14.0% (Figure S3E) and the time traces are dominated by low-FRET states (Figure 3C, S3C & S4B), reflecting more RAD51 binding. This observation is consistent with our previous finding that mS5S1 reduces the nucleating unit from three mRAD51 molecules to two (Figure 2B-2C) and mS5S1 increases the ssDNA affinity of mRAD51 and stabilizes mRAD51 nucleating clusters on ssDNA (Figure 2C). Adding $mS5^{FL}S1$ leads to the similar consequences as mRAD51 alone case (Figure S3C, S3E, S4C & S5A), consistent with the inability of $mS5^{FL}S1$ to stimulate mRAD51 assembly. For the longer $(dT)_{18}$ ssDNA, mRAD51 alone results in stable binding with many more bound ssDNA molecules (58.0±7.51%) and the middle-to-low FRET signal is dominant (Figure S3D & S4C). We identified seven FRET states (Figure S5) and corresponding binding/dissociation rate constants (Figure S6) using the longer $(dT)_{18}$ substrates, reflecting the binding of up to six mRAD51 monomers.

The $(dT)_{13}$ substrates allowed at most four mRAD51 to bind the ssDNA but these binding events were not long enough to form stable filaments. We observed multiple FRET states, as well as alternations among these states, reflecting dynamics among multiple mRAD51 bound states. These intermediate FRET states are identified by the FRET histograms (Figure S3C-S3D). Previous work on budding yeast *S. cerevisiae* Rad51 (ScRad51) on the same $(dT)_{13}$ substrates resulted in five different FRET states, corresponding to between zero and four ScRad51 molecules bound(17). We used Bayesian analysis(43) to globally fit all of the FRET time-courses, and a total of four FRET states was best found in mRAD51-only experiments (Figure 3B). The four identified FRET states in mRAD51-only experiments match to zero, one, two and three Rad51-bound states seen in ScRad51 experiments(17), confirming the validity of our analysis. We did not observe the lowest FRET state seen in the ScRad51 studies (~0.1, the four Rad51-bound state, Figure 3B), reflecting that four mRAD51 oligomers in $(dT)_{13}$ substrates are not stable enough to be seen. On the other hand, in the mixture of mRAD51/mS5S1 complex, we identified five FRET states, even though the highest FRET state (protein-free state, state 0, Figure 3C) is less populated. To confirm these FRET state assignments, we analyzed the FRET time-courses to generate the transition density plots(18, 44) (TDP) (Figure 3D-3E) in both cases. The TDP analysis identifies the FRET states before and after each transition, and the heat maps allow the identifications of the distribution of multiple FRET states. For example, for the mRAD51-only case, a transition from FRET value of ~0.85 (state 0) to ~0.75 (state 1) will score in the "binding" section in the TDP, as it reflects the binding of the first mRAD51 onto the ssDNA. The mirror symmetry along the diagonal of TDP indicates the reversible changes between these FRET states.

The transitions seen in time-courses and TDP suggest that up to five FRET states observed represent the zero to four mRAD51 bound states in the $(dT)_{13}$ substrates. The intermediate FRET values identified for the mRAD51/S5S1 complex are different from the ones seen in ScRad51 and mRAD51-only cases. This difference likely reflects the steric effect of the large mRAD51-S5S1 complex (~ 80 kDa), driving separation between the donor and acceptor dye. The large mRAD51-S5S1 complex could also account for wider FRET values corresponding to zero, one and two mRAD51-bound states (Figure 3E). We also noted that total fluorescence signal increases upon mRAD51 binding (Figure 3B-C), suggesting a protein-induced fluorescence enhancement (PIFE) effect, consistent with the previous observation in ScRad51(16, 17, 45). This PIFE effect is more apparent in the presence of mS5S1 (Figure 3C & S7) or on the $(dT)_{18}$ substrates, potentially because mRAD51 is able to

fully extend towards the Cy3-tagged 3′ terminating end with mS5S1 or on the longer ssDNA.

Identification of these FRET states in time-courses allows us to determine the evolution of these FRET states and the dwell time associated with individual states. The FRET time-courses are best described by a consecutive and reversible kinetic model, where individual mRAD51 monomer can bind and dissociate during the nucleating cluster formation and the extension also occurs in monomer, with or without mS5S1 (Figure 3B-C). The kinetics of each binding ($k_{i \rightarrow i+1}$, i is a number of mRAD51 bound to ssDNA) and dissociation ($k_{i \rightarrow i-1}$) can be determined in both mRAD51-only and mRAD51-S5S1 mixtures (Figure 3F). Surprisingly, when comparing mRAD51-only (open bars, Figure 3F) and mRAD51-S5S1 mixture (filled bars, Figure 3F), mS5S1 significantly reduces the mRAD51 dissociation rates during the nucleating cluster formation. Although mS5S1 could possibly increase mRAD51 binding rates, the change is not significant in our experimental resolution. Therefore, the major stabilization effort of mS5S1 in mRAD51 nucleating cluster formation comes from the reduction in dissociation rates.

Fission yeast Rad51 filament assembly is fast, with no apparent stimulation from S5S1. Mouse mS5S1 stimulates mRAD51 activity by accelerating the nucleation step during the nucleoprotein filament assembly. Fission yeast Swi5-Sfr1 (SpS5S1) is also known to stimulate SpRad51 activity(26, 29, 30, 33, 35). To examine whether the activation mechanism by S5S1 is evolutionally conserved, we used the same TPM approach towards SpS5S1. Under the same reaction condition (150 mM KCl, pH=7.5), SpRad51 does not assemble on dsDNA in either ATP or AMPPNP (Figure S1I-S1J & S1L-S1M). At the same recombinase concentration (0.8 μM), SpRad51 displayed much faster assembly kinetics than mouse mRAD51 (Figure 4A-4B, Figure 1B-1C). In the absence of S5S1, SpRad51 nucleation time is 23.2±0.70 s (Figure 4B), 4-fold faster than that observed for mRAD51 (97.6±2.89 s) (Figure 1C). To see if SpS5S1 complex further stimulates SpRad51 assembly, we used a reduced recombinase concentration (0.3 μM) to allow nucleation rates to be determined accurately (Figure 4E & S8). Interestingly, SpS5S1 did not stimulate SpRad51 nucleation at low concentration and even inhibited SpRad51 nucleation at higher concentrations (solid circles, Figure 4E & S8B). It is possible that SpRad51 nucleation is sufficiently fast that no additional stimulation is necessary, unlike the slow nucleation observed for mRAD51. As to the inhibition effect seen at higher SpS5S1 concentrations, it likely results from the ssDNA binding property of SpS5S1(26, 30). Even though SpS5S1 has DNA affinity, its binding to DNA substrates does not alter bead BM (Figure S1K-S1N). The control experiments verify that a decrease in bead BM increment with increasing SpS5S1 concentration derives from reduced amounts of SpRad51 binding

to the gapped ssDNA substrate (Gray solid circles in Figure 4F). To test whether or not ssDNA binding property of SpS5S1 inhibits SpRad51 filament assembly, we used a N-terminus truncation mutant of SpSfr1 in a complex with Swi5 (SpS5S1C). The N-terminal region of SpSfr1 possesses an initial interaction site with Rad51 to serve as an anchor and DNA binding site, both of which are overlapped with each other. Thus SpS5S1C is deficient in DNA binding but retaining SpRad51 filament stabilization(30). This DNA binding-deficient mutant showed neither inhibition nor stimulation of nucleoprotein filament formation even when two-fold excess amounts were included (open diamonds, Figure 4E). Therefore, SpS5S1 and SpRad51 compete for ssDNA binding and we conclude that SpS5S1 has no stimulatory effects on SpRad51 nucleation. In addition, the magnitude of BM increment (Figure 4F & S8D) drops with increasing amounts of wild-type SpS5S1, while the SpS5S1C mutant protein induced no apparent change. Thus, we confirmed that higher SpS5S1 concentrations can compete with SpRad51 for substrates.

With the fast nucleation rates observed, SpRad51 displays more apparent dependence on ssDNA length than mRAD51 (Figure S9A). A ~40-fold difference in slope observed and similar y-intercepts here suggest that SpRad51 has higher ssDNA binding affinity than mRAD51, and likely contributes to the faster nucleation rates. SpRad51 concentration-dependence of nucleation rates returns a power law fitting of 2.70±0.29, suggesting that three SpRad51 monomers are required for stable nucleation events (Figure S9B), similar to that of mRAD51 (Figure 2A).

S5S1 prevents Rad51 filament disassembly. In addition to accelerating the binding event, nucleoprotein filament stability can also be achieved by the prevention of filament dissociation. In the TPM-based disassembly experiments (Figure 5), surface-bound $(dT)_{135}$ gapped DNA substrates were first incubated with Rad51/ATP mixture, and then another mixture of Rad51, ATP and S5S1 was added to the microscope slide. This set of disassembly experiments was done for mouse (Figure 5B) and fission yeast (Figure 5F) proteins, respectively. The two-stage incubation avoids potential ssDNA substrate competition between SpS5S1 and SpRad51. Extensive buffer wash containing no Rad51 but all other components removed free Rad51 from the reaction chamber. This was defined as time zero of the disassembly reaction. The bead Brownian motions are measured in real-time to monitor the filament length. Several kinetic parameters were determined, including (i) lifetime of the stable filament (dwell time prior to BM decrease), (ii) dissociation time, time for individual RAD51 monomer dissociation continuously and (iii) net BM decrease, a decrease in filament coverage. Our results showed that the mRAD51 filament is quite stable in ATP even without mS5S1, with minimum mean lifetime around 350±39.3 sec (Figure 5C). In the presence of 1.0 μM mS5S1, the lifetime of the mRAD51

filament was further stabilized to 535±49.9 sec (~1.53-fold). Using low concentrations of mS5S1 (0.05 or 0.3 μM) also resulted in longer lifetimes (441±51.1 and 436±45.1 sec respectively, ~1.27-fold). This suggests that mS5S1 prevents mRAD51 filament disassembly. Control experiments using mS5^{FL}S1 mutant return no additional stabilization, confirming that mRAD51-S5S1 interaction is essential for mRAD51 nucleoprotein filament stabilization. We also noticed that the fraction of full-length mRAD51 filaments retained at the end of the 15-minute reaction, un-disassembled filament (Figure 5D), increases in the presence of mS5S1, correlating with the increased filament lifetime with mS5S1 (Figure 5C). However, once dissociation was initiated (as BM starts to decrease), the dissociation rate is similar either with or without mS5S1, within the resolution of our TPM measurements (Figure 5E). Compared to mRAD51, the SpRad51-alone filament is less stable (lifetime of 140±17.9 sec, Figure 5F-5G). Essentially all SpRad51 filaments were disassembled within 15 mins (Figure 5H). In the presence of SpS5S1, the lifetime of the SpRad51 filament is significantly increased, as was the fraction of filaments retained at the end of 15-minute observations. For example, 0.01 μM of SpS5S1 increases the lifetime to 382±32.6 sec (~2.73-fold). SpS5S1 increases the dissociation time as well, making the disassembly events slower (Figure 5I). SpS5S1C mutant also protects SpRad51 filament (empty bars in Figure 5G), but less effective than wild-type SpS5S1 (gray solid bars) especially at lower concentrations. This may be due to the lower affinity to SpRad51(35) because higher amount of SpS5S1C shows a similar ability to protect the filament disassembly. Therefore, we conclude that both S5S1 heterodimers act a stabilizer of Rad51 filament via direct interaction with the recombinase.

Comparing S5S1 from these two species, we found that 1.0 μM of mS5S1 only achieves ~1.53-fold increase in filament lifetime while SpS5S1 increases to ~2.73-fold. Considering the fast disassembly kinetics of SpRad51, SpS5S1 predominantly acts on this filament k_{off} step. In addition, comparing bead BM changes between the assembly and disassembly experiments (Figure S10), we found that not all mRAD51 dissociated from the gapped DNA substrates, reflecting the incomplete disassembly of mRAD51 filaments, as seen in human RAD51(14). On the contrary, SpRad51 was almost entirely released from DNA as filaments initiate disassembly even in the presence of SpS5S1, confirming that SpRad51 filament is more susceptible to disassembly. The reason why the apparent function of S5S1 seems to be different is due to the different stabilities of Rad51 filaments between yeast and mouse proteins. Thus, we conclude that the primary conserved function of S5S1 is to stabilize Rad51 on DNA, allowing both the formation of the stable nucleus and the maintenance of filament length.

DISCUSSIONS

Nucleoprotein filament assembly is the first committed step in homologous recombination and is targeted for regulation(1, 2, 23, 26, 30, 32, 46, 47). A nucleoprotein filament with sufficient length is advantageous for initial homology search. The filament is also expected to be dynamic during the directional exchange of different parts of duplex homologous DNA. Accessory proteins have been found to stimulate the recombination process by maintaining a stabilized and yet dynamic recombinase nucleoprotein filament. Stabilization of the nucleoprotein filament can be achieved by speeding up the filament assembly, reducing filament disassembly or both. However, these kinetic parameters are typically obscured in ensemble biochemical experiments. In this study, we used two different single-molecule tools to determine these kinetics parameters that allow us to characterize the mechanism of S5S1 regulation in mouse and fission yeast proteins. Several kinetic steps are involved in filament assembly: initial Rad51 binding and dissociation events leading to a stable nucleus, fast extension steps and dissociation events within growing filaments. S5S1 of both species stabilized Rad51 nucleoprotein filaments by preventing Rad51 dissociation from nucleation clusters and from the assembled filaments. Although the S5S1 complex of these species alters the kinetic steps differentially due to intrinsic characteristics of Rad51 recombinases in different species, they both prevent Rad51 dissociation to facilitate efficient recombination progression. Here, we used two complementary single-molecule tools to characterize the assembly. TPM experiments allow to characterize the formation and disassembly of individual stable filaments, and FRET experiments capture the dynamics of individual Rad51 binding and dissociation before a stable nucleus is formed. An apparent stimulation of the Rad51 filament nucleation by S5S1 seen in TPM experiments is attributed to the reduction of the dissociation rate in nucleus formation observed in FRET. A nearly 4-fold reduction of dissociation rate $(k_{1\to 0})$ of mRAD51 is seen in the present of mS5S1, but no apparent change in binding rates is seen (Figure 3F). This then leads to a reduction in nucleation size of three mRAD51 monomers to two mRAD51 monomers in the presence of mS5S1. Fission yeast Rad51 is fast in filament assembly, but is prone to disassembly.. SpS5S1 stabilizes the SpRad51 filaments by specifically preventing SpRad51 dissociation. Therefore, S5S1 of two species stabilize Rad51 filaments by using the same strategy of preventing Rad51 dissociation. Our single-molecule experiments speak specifically to the Rad51 state when it nucleates and extends on the DNA substrates, and our data are consistent with previously shown in the literature(14, 17). Previous works have suggested that recombinases from various species can exist in oligomers in solution(48-52). It is

possible that a structural transition is made in solution before or during the DNA binding, so smaller units of Rad51 oligomers are responsible for nucleation and individual Rad51 molecule is added during filament growth.

S5S1 association with Rad51 could either expose DNA binding domain of Rad51 or stabilize the oligomeric interface of Rad51. SpS5S1 has two Rad51 binding sites: a high-affinity one in N-terminus of Sfr1 and a low-affinity one in C-terminus(30). Mouse S5S1 has only one mRAD51 binding site, residing in the C-terminus(31). The difference in Rad51 binding affinity between mouse and fission yeast S5S1 is responsible for the observation that more S5S1 is required for the Rad51 stability in the cases of mS5S1 and SpS5S1C mutants (Figure 5C and 5G). Considering its large effect on both dissociation rates (FRET experiments) and disassembly rates (TPM disassembly experiments), we suggest that S5S1 acts by holding adjacent Rad51 molecules together, reducing dissociation. The interaction between S5S1 and RAD51 is thus essential for stimulation, confirmed by the abolished stimulation seen in $mS5^{FL}S1$ mutants (Figure 1C, 2A, 5C, and S3-S5).

Different amounts of S5S1 required to stimulate nucleation and reduce filament disassembly provides hints how S5S1 interacts with Rad51 molecules to achieve filament stabilization. For the mouse proteins, a two-fold excess of mS5S1 maximally stimulates mRAD51 nucleation (Figure 1F). However, stabilization in the disassembly experiments can be seen with the low coverage of mS5S1 complex (~17%, Figure 5C, 0.05 μM vs 0.3 μM mRAD51 added). Previous structural studies suggested that S5S1 is accommodated within the groove of the Rad51 filament(30). The different concentration requirements of S5S1 point to a model that S5S1 binding stabilizes adjacent Rad51 molecules through binding within the filament groove to prevent Rad51 dissociation (Figure 6 & S11). During the nucleus formation, more mS5S1 are required so most of Rad51 are stabilized by S5S1. As Rad51 dissociation is inhibited by S5S1, stable Rad51 nucleus can be formed more easily. On the other hand, as filament disassembly could take place at the filament end more frequently, filament stabilization can be achieved by those S5S1 binding to terminal Rad51. Therefore, low coverage S5S1 is sufficient. As S5S1 has also been shown to activate Rad51 filaments(30, 32), S5S1 binding within Rad51 filament also contributes to the overall Rad51 activity stimulation. During the dynamic progression of strand exchange, low S5S1 coverage allows efficient budgeting of S5S1, so S5S1 can be available for stimulation at later stages of recombination progression.

There exist several heterodimeric complexes regulating recombinase nucleoprotein filament stability(2-4, 53, 54). The elongated crescent-like structure of Hop2-Mnd1 shares a similar structural motif with S5S1. In both cases, the heterodimeric complex interacts with the groove of the Dmc1 and/or Rad51 filament

to stabilize the nucleoprotein filaments(1, 55-58). A Rad51 paralog complex in *C. elegans*, RFS-1/RIP-1 complex, also has been proposed to act similarly on Rad51 filament remodeling(54). *S. cerevisiae* Psy3-Csm2 dimer has been suggested to bind to the end of Rad51 filaments to achieve filament stabilization(53). Together with the structural evidence, it is possible that the mechanism proposed here likely serves as a general principle for these heterodimeric complexes involved in filament stabilization. For example, whether these accessory factors, such as BRCA2, PCSS complex and RAD51 paralogs(24, 53, 54), work synergistically on the filament stabilization. It is possible that these accessory proteins allow better modulation on filament dynamics for efficient strand exchange progression.

Differences in kinetics are seen between mouse RAD51 and SpRad51 recombinases. Mouse RAD51 is slower in nucleation, but SpRad51 is more prone to disassembly. We have found that mS5S1 acts effectively on the nucleation step while SpS5S1 acts primarily on the disassembly step to achieve nucleoprotein filament stabilization. However, DNA binding of SpS5S1 is shown to inhibit SpRad51 filament assembly (Figure 4E-4F) and strand-exchange(26, 33). It is likely that accessory proteins evolve to accommodate different kinetics characteristics of recombinases during speciation and play the different roles of in the progression of homologous recombination process. For example, SAXS and X-ray crystallographic studies showed that SpS5S1 fits into the helical groove of the SpRad51 filament and also extends onto ssDNA(29, 30, 35, 59). In addition to stabilizing SpRad51, SpS5S1 interacts with ssDNA provides addition safe latch on SpRad51. Budding yeast Mei5-Sae3 complex (ScM5S3), orthologs of Sfr1 and Swi5, has been shown to stabilize ScDmc1 filament and to stimulate ScDmc1-mediated strand exchange during meiosis (19, 60, 61). ScM5S3 possesses both ssDNA and dsDNA affinities, and it is possible that budding yeast M5S3 stabilizes ScDmc1 filament in a similar, general mechanism found in mouse and fission yeast.

ssDNA is likely bound by single-stranded DNA binding proteins, for example, replication protein A (RPA) to prevent nucleolytic cleavage. RPA binding to ssDNA is a physical barrier for Rad51 assembly. Previous findings demonstrate that fission yeast S5S1 works synergistically with Rad52 to stimulate the Rad51 assembly on RPA-coated ssDNA(28, 35). It would be interesting to see whether SpS5S1 also primarily acts on preventing SpRad51 disassembly in the presence of RPA.

Both mouse and fission yeast S5S1 have been shown to activate Rad51 filament by stimulating ssDNA-dependent ATPase activity of Rad51(30, 32). Moreover, mouse S5S1 enhances ATPase activities of mRAD51 by stimulating the release of ADP to maintain the filament in an active form(30, 32). E. coli RecA recombinases have also been shown to continuously hydrolyze ATP and bind new ATP molecules without

dissociating from DNA(62). It's possible that mS5S1 stabilizes interfaces between adjacent mRAD51 molecules within the filament during ATPase turnover events, and thus maintains active nucleoprotein filaments. It is important to note that co-factors like AMPPNP and Ca^{2+} increase the stability of Rad51 filaments by inhibiting ATP hydrolysis and maintaining the ATP-bound form of Rad51, and then these co-factors promote strand exchange activity(25, 33, 35, 63, 64). Therefore, in contrast to AMPPNP and Ca^{2+} effects, S5S1 stimulates Rad51 progression in a unique mechanism. Maintaining a dynamic nucleoprotein filament homeostasis requires Rad51 dissociation at appropriate rates. Swi5-Sfr1 and other accessory proteins serve the purpose to modulate dissociation rates of Rad51 to fine tune recombinase progression.

MATERIALS AND METHODS

Supporting Information. A detailed description of the DNA substrates preparations, proteins & buffer conditions and detailed experimental procedures of single-molecule tethered particle motion (TPM) assembly experiment are provided in Supporting Information.

Single-molecule TPM assembly experiment and data analysis. Streptavidin-labeled beads were prepared as previously described(65). In filament assembly experiments, glass slide was coated with 5 μg/mL anti-digoxigenin and blocked with 2 mg/mL bovine serum albumin (BSA) sequentially. 4 nM DNA substrates were incubated on the anti-digoxigenin-coated slide for 30 min followed by buffer washing to remove unbound DNA. 220 nm streptavidin-decorated polystyrene beads were then attached to the DNA substrates for microscopic visualization. All TPM reactions were performed in either mouse or fission yeast buffers supplemented with 1 mM DTT and 2 mM ATP. TPM assembly experiments were initiated by introducing a Rad51-ATP (or with S5S1) mixture into the reaction chamber containing the surface-anchored gapped substrates.

We used an inverted optical microscope (IX-71, Olympus) with a differential interference contrast (DIC) imaging mode to visualize tethers and measure bead Brownian motion (BM). Images of assembly experiments were acquired at 30 Hz using a Newvicon camera (Dage-MTI) and were analyzed using software written in Labview. The amplitude of tether Brownian motion is defined by the standard deviation of the bead centroid positions of 20 images using sliding windows. In addition to the DNA contour length change, polymer stiffness and camera exposure time alter the Brownian motion amplitudes in practice(42). For each independent TPM assembly experiments, images were first recorded for around 30 seconds (~ 1000 image frames) before the addition of Rad51 or Rad51-S5S1 mixture and then for

about 15 minutes (~ 30000 image frames) after the addition of the mixtures. For snapshot control experiments, 1000 image frames were recorded at 5-6 different field-of-views on coverslip after 5 min reaction (Figure S1).

Single-molecule fluorescence resonance energy transfer (smFRET) experiment and data analysis. In smFRET experiments, PEGylated glass slides and coverslips were prepared as previously described(66). To perform smFRET experiments, reaction chambers were incubated with 20 μg/mL streptavidin for 5 min. Excess streptavidin was washed away with the buffer containing 20 mM Tris and 50 mM NaCl. 15 pM of 3′-biotinylated fluorophore-labeled hybrid DNA was then immobilized on the surface for 5 min. After 5 min incubation, free DNA was removed by flowing in mRAD51 imaging buffer containing 1 mM Trolox (Sigma-Aldrich), 2.6 mM protocatechuic acid (PCA, Sigma-Aldrich), 0.21 units/mL protocatechuate 3,4-dioxygenase (PCD, OYC Americas Inc.), 30 mM Tris, 2.5 mM magnesium chloride and 150 mM potassium chloride at pH 7.5. The reaction includes a mixture of 1 μM mRAD51 and 2 mM ATP in mRAD51 imaging buffer into reaction chambers. mS5S1 (or mS5FLS1)-containing experiments includes 1 μM mRAD51, 2 μM mS5S1 (or mS5FLS1) and 2 mM ATP in mRAD51 imaging buffer. Under our imaging conditions, ~78 % of fluorophores in hybrid DNA survives for more than 200 sec.

We utilized objective-type total internal reflection fluorescence microscope (TIRFM, Olympus IX2) and 532 nm laser as excitation light source in smFRET experiments. Fluorescence intensity signals of both Cy3 and Cy5 were acquired with EMCCD (ProEM 512B, Princeton Instrument) at 20 Hz using a dual-view system. Emission movies of Cy3 and Cy5 fluorescence were recorded using a software program written in Labview 8.6. Colocalized Cy3 and Cy5 spots were analyzed using a mapping software program written in IDL. Fluorescence intensity time-traces of each individual mapped DNA molecule were analyzed using Matlab. Alternation in FRET values was analyzed using variational Bayesian analysis (vbFRET) to globally fit all time-courses(43).

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Figure Legends

Figure 1. Mouse SWI5-SFR1 (mS5S1) stimulates mRAD51 nucleoprotein filament assembly. (A) Schematic illustration of the RAD51 nucleoprotein assembly experiments. **(B)** Representative bead BM time-courses of mRAD51 (0.8 μM) assembly on $(dT)_{135}$ DNA substrates without mS5S1 (upper), with 1.6 μM mS5S1

(middle) or with 1.6 μM mS5^{FL}S1 mutant (lower). Gray bars correspond to the deadtime when recombinase mixtures with 2 mM ATP were introduced. Histograms of nucleation time **(C)**, mean extension time (second/RAD51) **(D)** and bead BM increment **(E)** of mRAD51 assembling. All experiments were carried out at 2 mM ATP. Error bar of nucleation rate was the standard deviation of the mean by bootstrapping 5000 times, and error bar of extension time is one standard error of the mean (SEM). **(F)** 0.8 μM mRAD51 was pre-incubated with various stoichiometric ratios of mS5S1 and the mixture was introduced into a reaction chamber containing surface-bound $(dT)_{135}$ gapped DNA. Nucleation rates are about constant (~0.010 s^{-1}) when the [mS5S1]/[mRAD51] ratio is less than 1.625. The nucleation rates of mRAD51 increase and achieve maximum value (~0.015 s^{-1}) when the ratio of [mS5S1]/[mRAD51] is larger than 2, suggesting that maximum nucleation stimulation occurs in the mixture of one mRAD51 and two mS5S1. Individual nucleation rates were obtained based on Maximum likelihood estimation (MLE). **(G)** Bead BM increment of mRAD51 assembly on $(dT)_{135}$ DNA substrates in the presence of indicated ratios of mS5S1 to mRAD51. Bead BM increment reflects the coverage of the RAD51 nucleoprotein filaments. mRAD51 forms longer and more stable filaments in the presence of mS5S1. All experiments were carried out at 2 mM ATP. Black open square represents the nucleation rate of mRAD51 in the presence of non-hydrolyzable ATP analog, AMPPNP, in the absence of mS5S1. Dash lines are the mean, and the shaded region span two standard deviations. Error bar of bead BM increment is one SEM.

Figure 2. Mouse mS5S1 reduces nucleation unit of mRAD51 and increases the ssDNA affinity. (A-B) RAD51 concentration dependence of filament nucleation obtained by TPM experiments. Power law fitting to the observed nucleation rates suggests the nucleation unit of RAD51: 2.43±0.46 for mRAD51 (A) and 1.67±0.16 for mRAD51-S5S1 complex (B). Green open circles in (A) are nucleation rates of mRAD51 in the presence of excess mS5^{FL}S1 mutants, defective in stimulating mRAD51. mS5S1 & mS5^{FL}S1 are in two-fold excess in (A) & (B). **(C)** ssDNA length dependence of mRAD51 filament nucleation rate obtained by TPM experiments. Gapped DNA substrates contain only one 5' ds/ss junction but various lengths of ssDNA gaps (90-200 nt). As the gapped DNA substrate structure, overall nucleation rates are fitted to $k_{ssDNA}^{app}(L_{ssDNA})+k_{junction}^{app}$, where k_{ssDNA}^{app} and $k_{junction}^{app}$ are apparent ssDNA-dependent nucleation rate constant and apparent ds/ss junction-dependent nucleation rate constant. (Red) 0.8 μM mRAD51 only and (blue) mixture of 0.8 μM mRAD51 and 1.6 μM mS5S1. All experiments were carried out at 2 mM ATP.

Figure 3. Single-molecule FRET experiments demonstrate that mS5S1 stabilizes mRAD51 nucleating clusters. (A) Schematic illustration of single-molecule fluorescence resonance energy transfer (smFRET) experimental setup. mRAD51 assembles onto $(dT)_{13}$ ssDNA results in the FRET decrease due to the increase of dye pair separation. **(B–F)** Single-molecule FRET observation of mRAD51 nucleating cluster dynamics. Exemplary FRET time trace of **(B)** mRAD51 and **(C)** mRAD51-S5S1 complex assembling on $(dT)_{13}$ ssDNA substrate. High FRET state (~0.8) corresponds to a DNA-only state, and low FRET state (0.0~0.6) corresponds to the mRAD51-bound state. **(D-E)** Transition density plots (TDP) clearly reflect 4 states (without mS5S1) and 5 states (with mS5S1) in mRAD51 nucleating cluster dynamics. **(F)** Rate constants of mRAD51 nucleating cluster dynamics in the absence (empty bar) and presence (solid bar) of mS5S1. Error bar of binding and dissociation rates were the standard deviation of the mean by bootstrapping 5000 times.

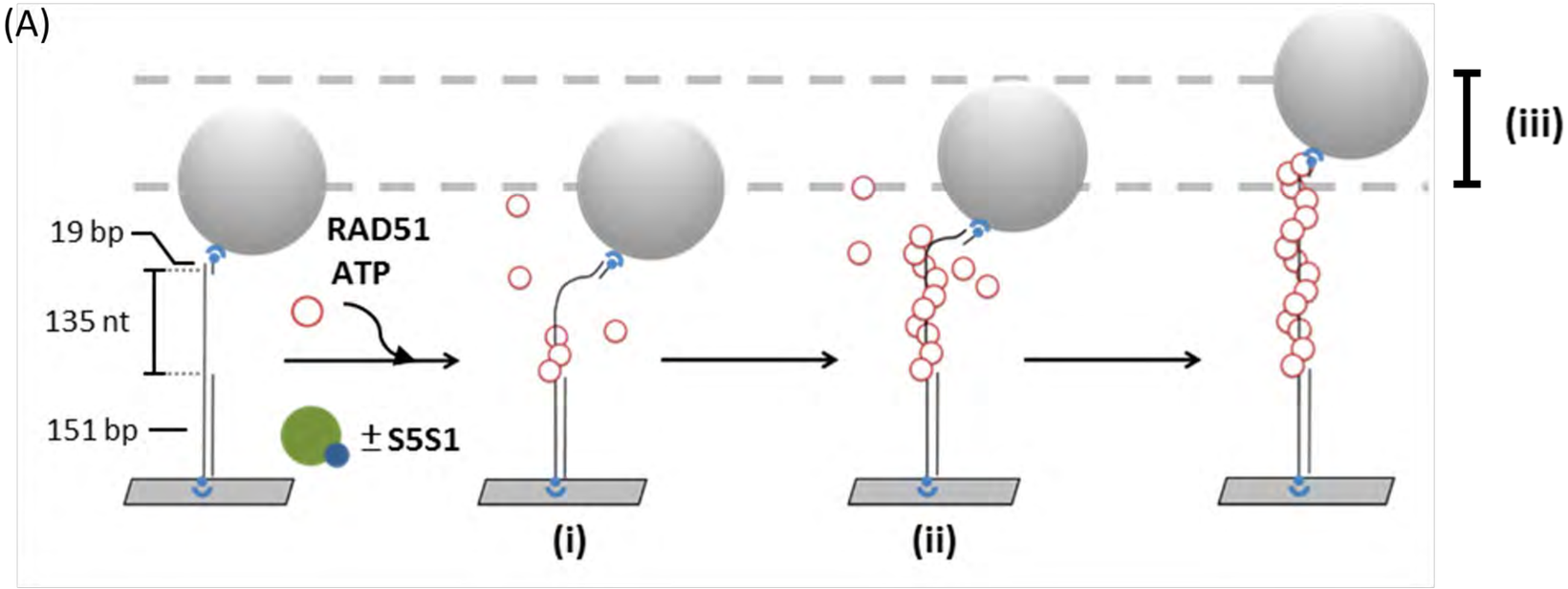
Figure 4. Fission yeast SpS5S1 does not stimulate SpRad51 filament assembly. (A) Representative bead BM time-courses of fission yeast Rad51 (0.8 μM) assembly on the $(dT)_{135}$ DNA substrates without S5S1. **(B)** Nucleation time, **(C)** mean extension time (second/Rad51) and **(D)** bead BM increment analyzed from individual assembly time-courses. Nucleation time histograms are fitted by single exponential decay. **(E)** Nucleation rates of various concentrations of SpS5S1 at constant 0.3 μM of SpRad51. **(F)** Bead BM increments of SpRad51 assembly at various ratios of SpS5S1 and SpRad51 mixtures also decreased at higher SpS5S1 concentrations. All experiments were carried out at 2 mM ATP and 0.3 μM SpRad51. Gray solid circles are from wild-type SpS5S1 experiments. Black diamonds are from the N-terminus truncation mutant of SpS5S1 (SpS5S1C) deficient in ssDNA binding. Dash lines are the mean, and the shaded region span two standard deviations.

Figure 5. Nucleoprotein filament disassembly experiments showed that S5S1 prevent Rad51 filament disassembly. (A) Schematic illustration of nucleoprotein filament disassembly experiments using the TPM setup. **(B)** Representative bead BM time-courses of mouse mRAD51 disassembly on $(dT)_{135}$ DNA substrates without mS5S1 (top) and in the presence of 1.0 μM mS5S1 (bottom). mRAD51 filaments were pre-assembled in the presence of ATP. Dark grey bars stand for void time for extensive buffer wash to remove free mRAD51. A lifetime of the pre-assembled filament before the BM decrease dictates the mRAD51 disassembly kinetics. **(C)** Mean lifetime of mouse mRAD51 nucleoprotein filament, **(D)** fraction of the un-disassembled filament within 15 minutes and **(E)** mean dissociation time per

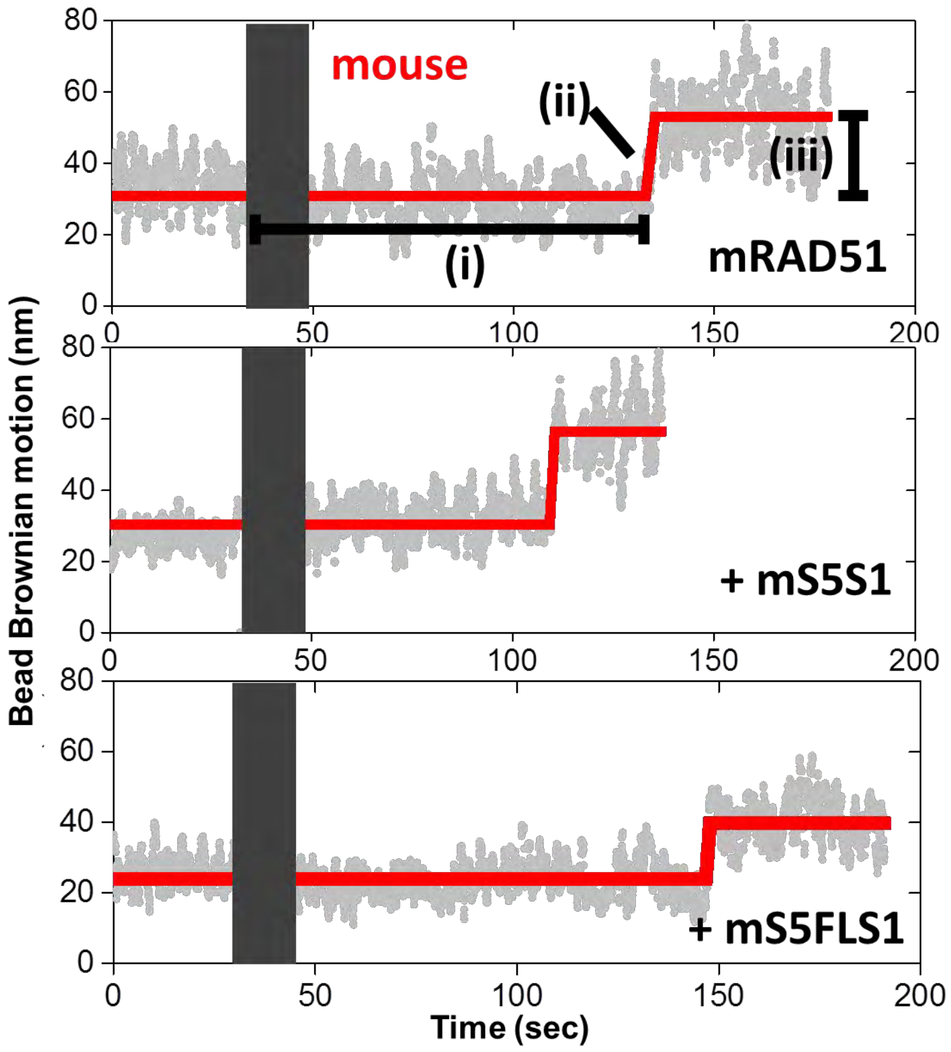
mRAD51 in the presence of various mS5S1 concentrations and nucleotide conditions. **(F)** Representative bead BM time-courses of SpRad51 disassembly without SpS5S1 (top) and in the presence of 0.3 μM SpS5S1 (bottom). **(G-I)** Kinetic parameters for fission yeast. The fraction of un-disassembled tethers is correlated with the mean lifetime of the SpRad51 filament in both species. In (G-I), the N-terminus truncation S5S1C mutants (open bars) are deficient in DNA binding. All experiments were carried out at 2 mM ATP. Error bar is one standard error of the mean.

Figure 6. Proposed models for regulating Rad51 nucleoprotein filament formation by the S5S1 complex. Swi5-Sfr1 stabilizes Rad51 on ssDNA primarily by preventing its dissociation. This stabilization effect leads to a stable nucleating cluster formation and a reduction in filament disassembly. In spite of different kinetic properties of mouse and fission yeast Rad51, Swi5-Sfr1 complex stimulates Rad51 process through a general, evolutionally-conserved mechanism. Red half-arrows indicate the kinetic steps affected by S5S1.

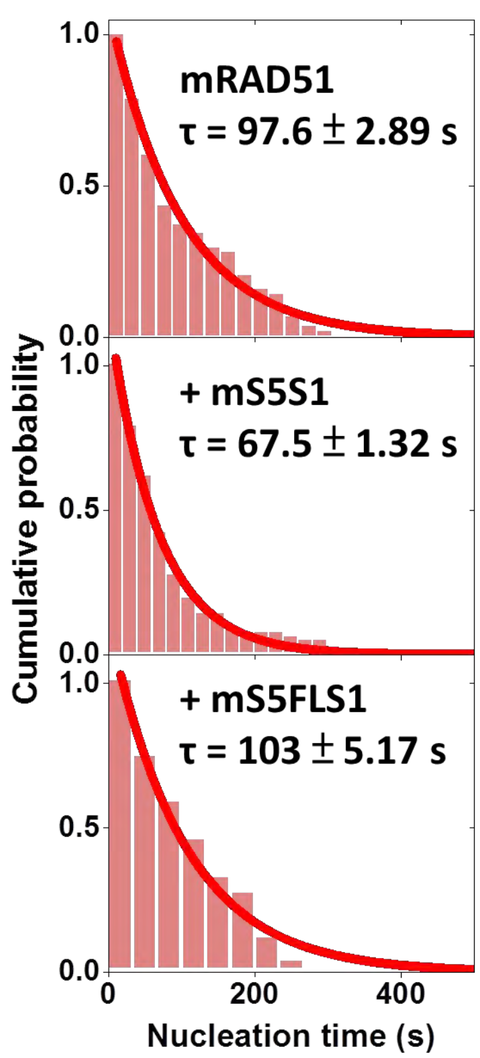
(A)


19 bp
135 nt
151 bp
RAD51
ATP
±S5S1
(i)
(ii)
(iii)

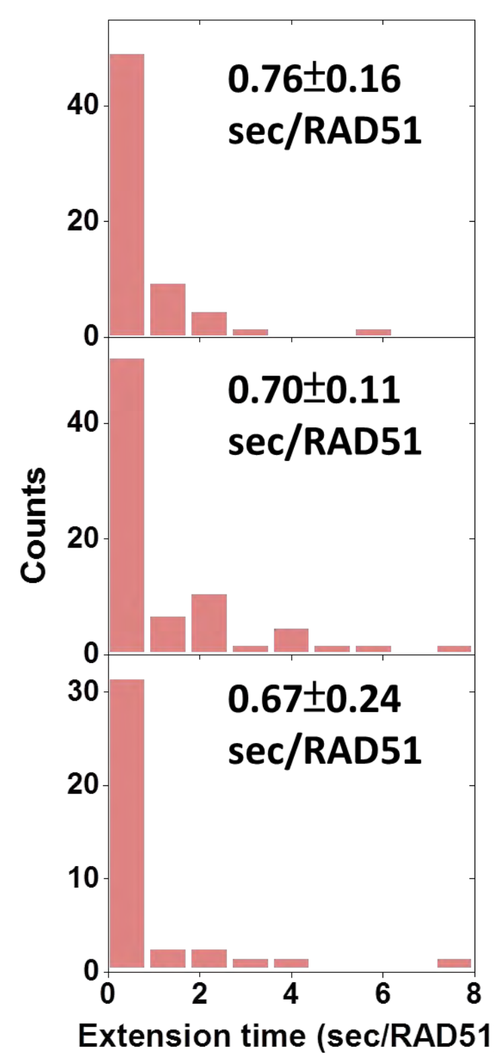
(B)


mouse
(ii)
(iii)
(i)
mRAD51
+ mS5S1
+ mS5FLS1
Bead Brownian motion (nm)
Time (sec)

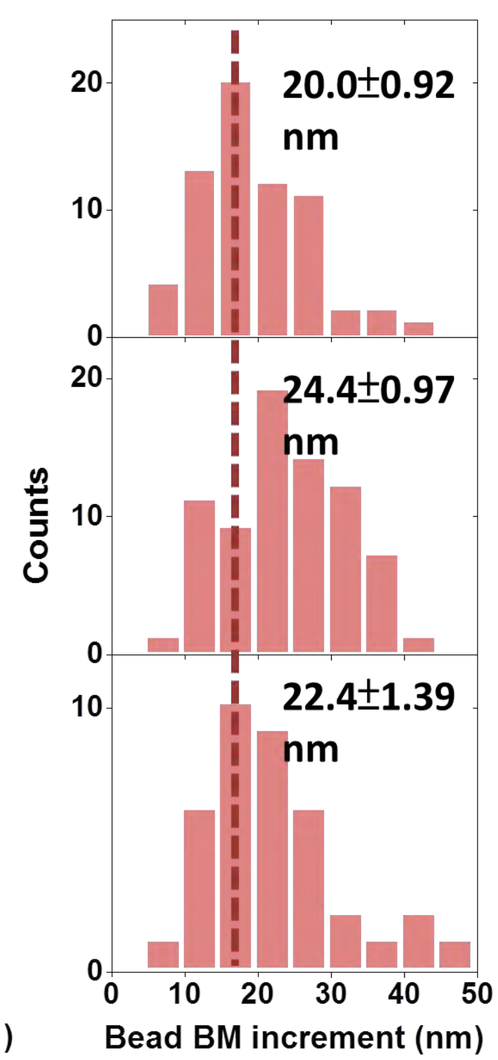
(C)


mRAD51
τ = 97.6 ± 2.89 s
+ mS5S1
τ = 67.5 ± 1.32 s
+ mS5FLS1
τ = 103 ± 5.17 s
Cumulative probability
Nucleation time (s)

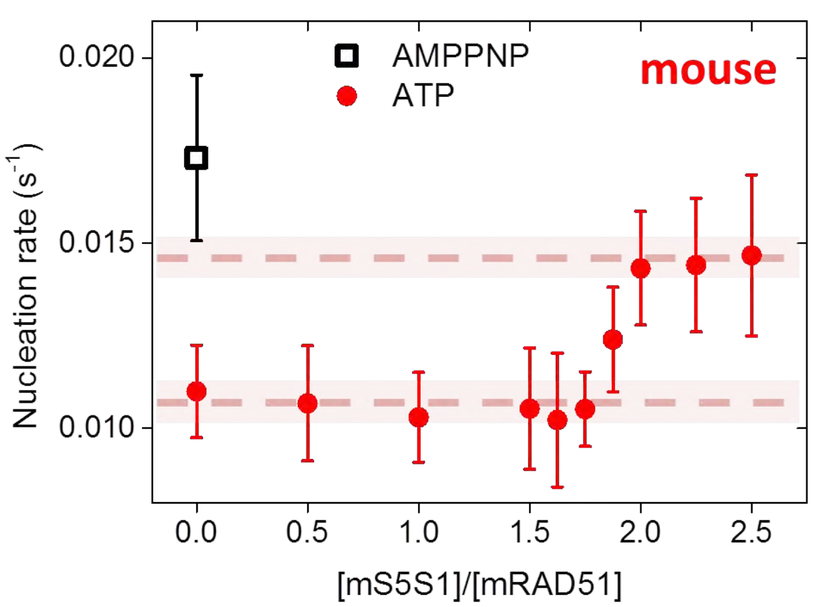
(D)


0.76±0.16
sec/RAD51
0.70±0.11
sec/RAD51
0.67±0.24
sec/RAD51
Counts
Extension time (sec/RAD51)

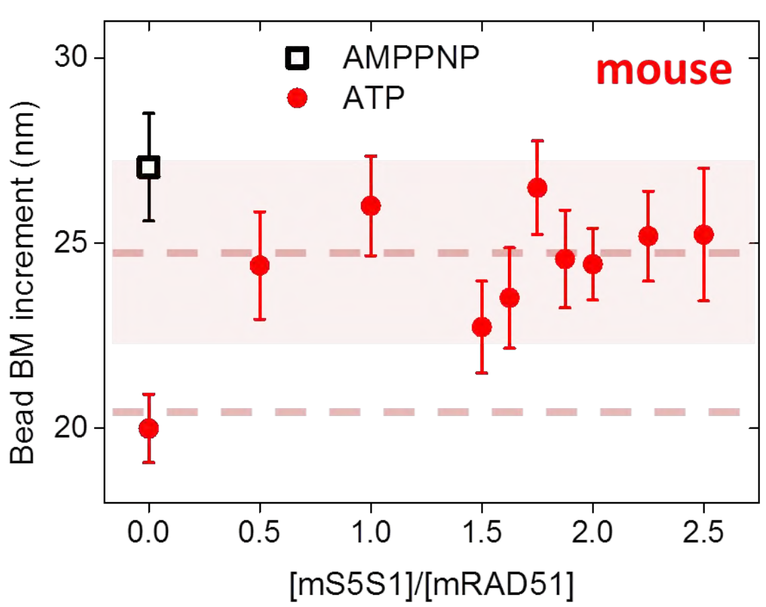
(E)


20.0±0.92
nm
24.4±0.97
nm
22.4±1.39
nm
Counts
Bead BM increment (nm)

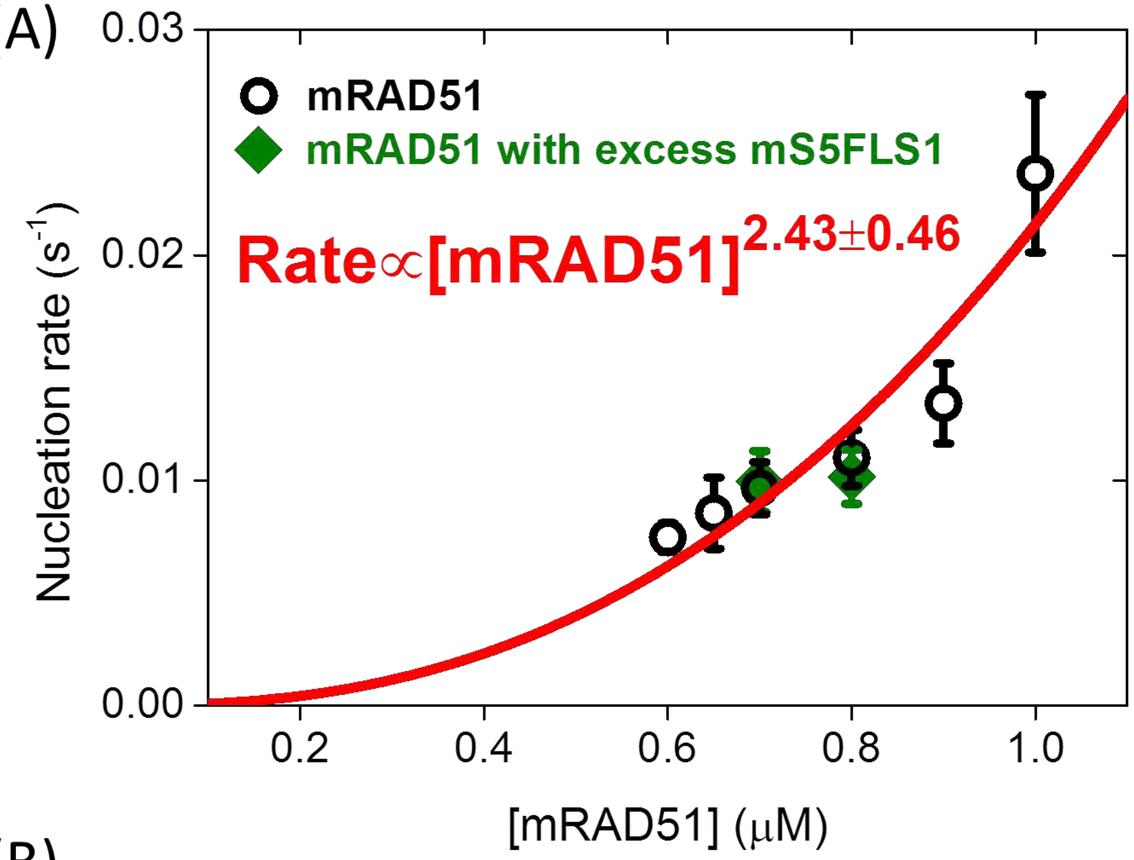
(F)


AMPPNP
ATP
mouse
Nucleation rate (s⁻¹)
[mS5S1]/[mRAD51]

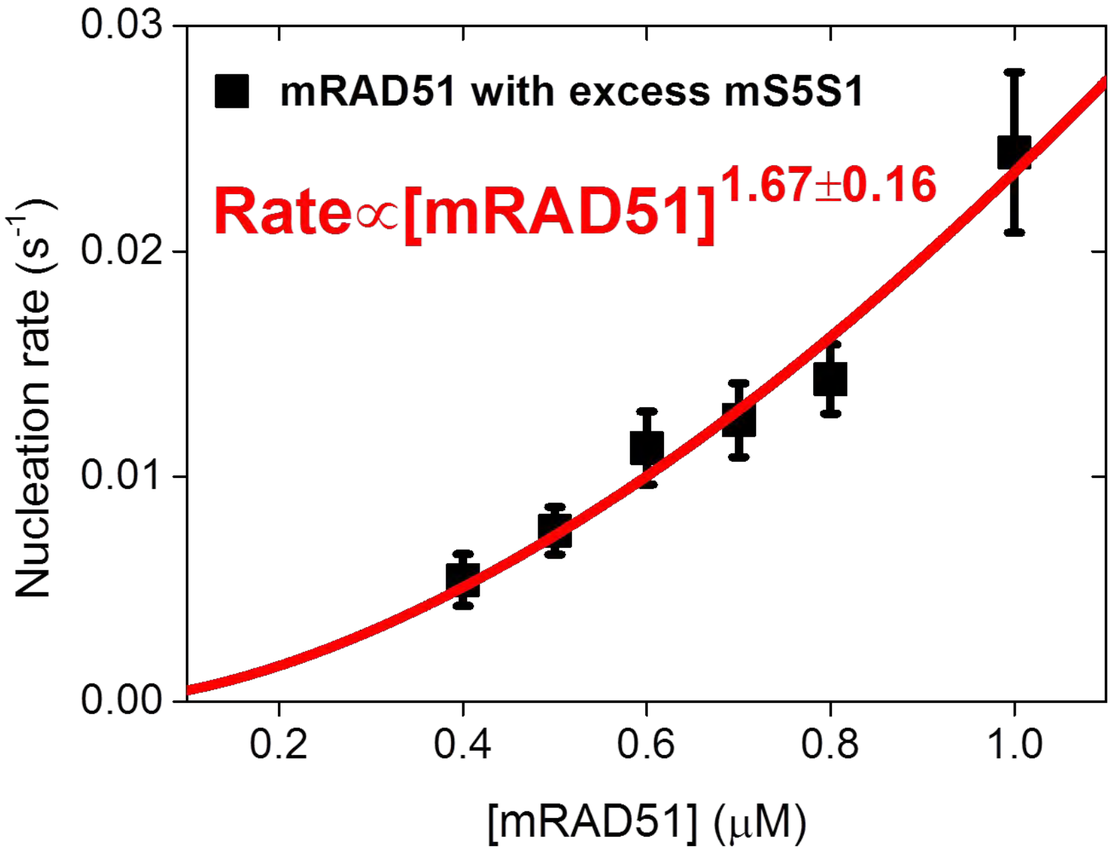
(G)


AMPPNP
ATP
mouse
Bead BM increment (nm)
[mS5S1]/[mRAD51]

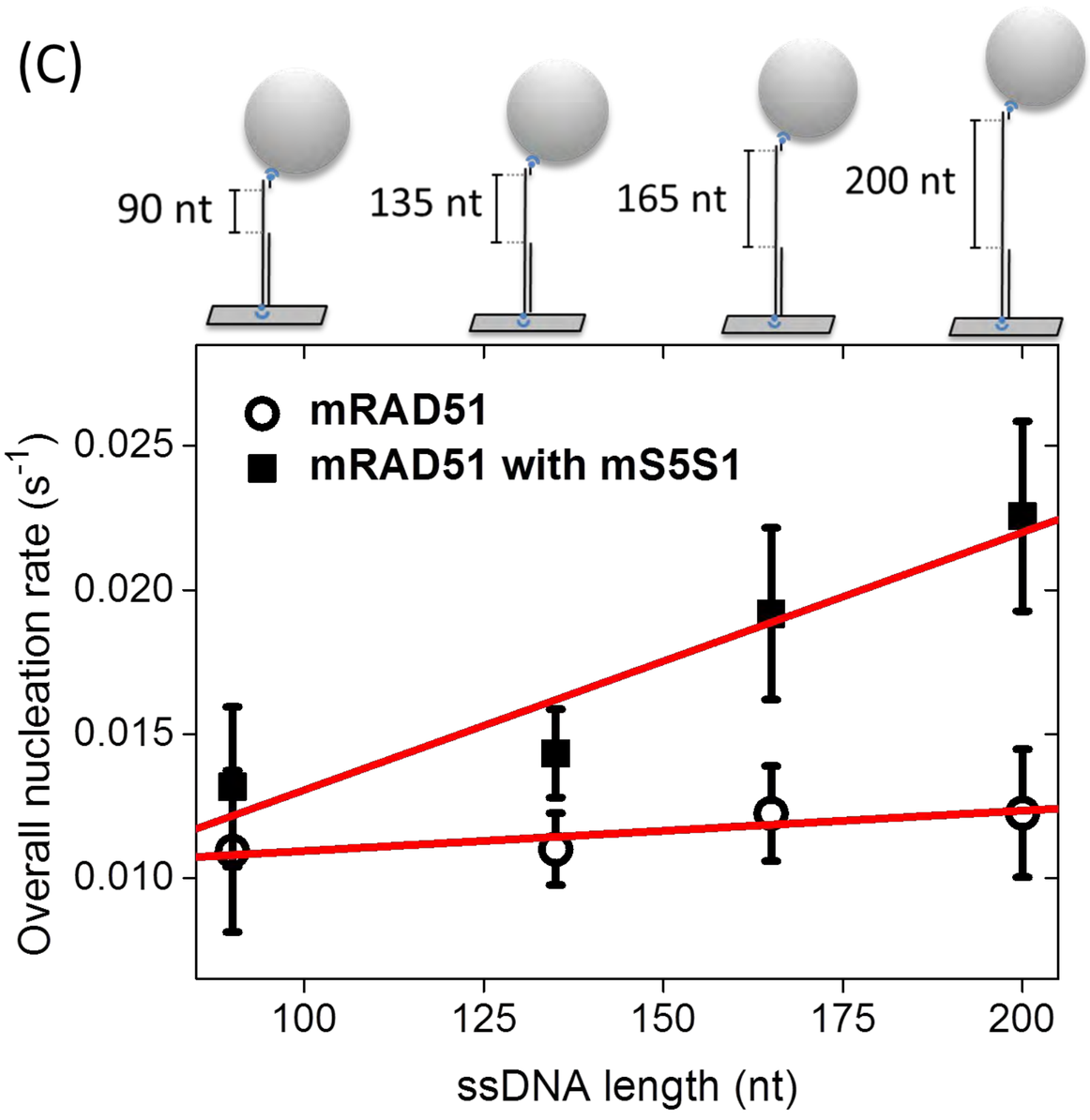
(A)


mRAD51
mRAD51 with excess mS5FLS1
Rate∝[mRAD51]2.43±0.46
Nucleation rate (s-1)
0.03
0.02
0.01
0.00
0.2
0.4
0.6
0.8
1.0
[mRAD51] (μM)

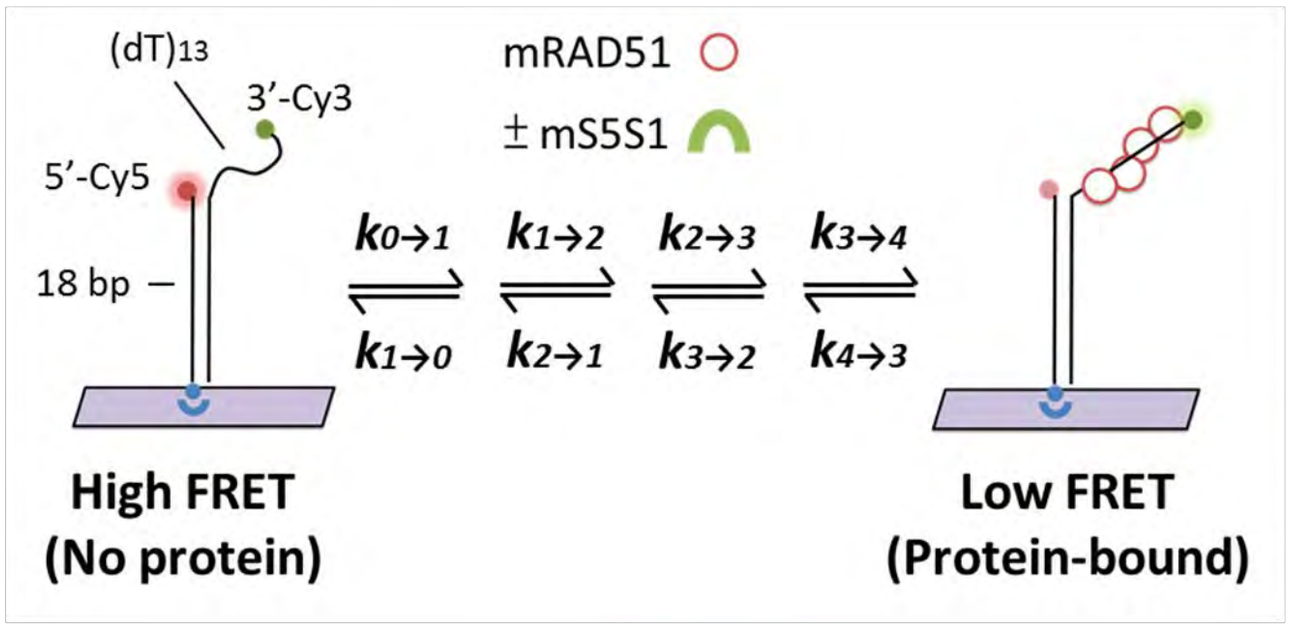
(B)


mRAD51 with excess mS5S1
Rate∝[mRAD51]1.67±0.16
Nucleation rate (s-1)
0.03
0.02
0.01
0.00
0.2
0.4
0.6
0.8
1.0
[mRAD51] (μM)

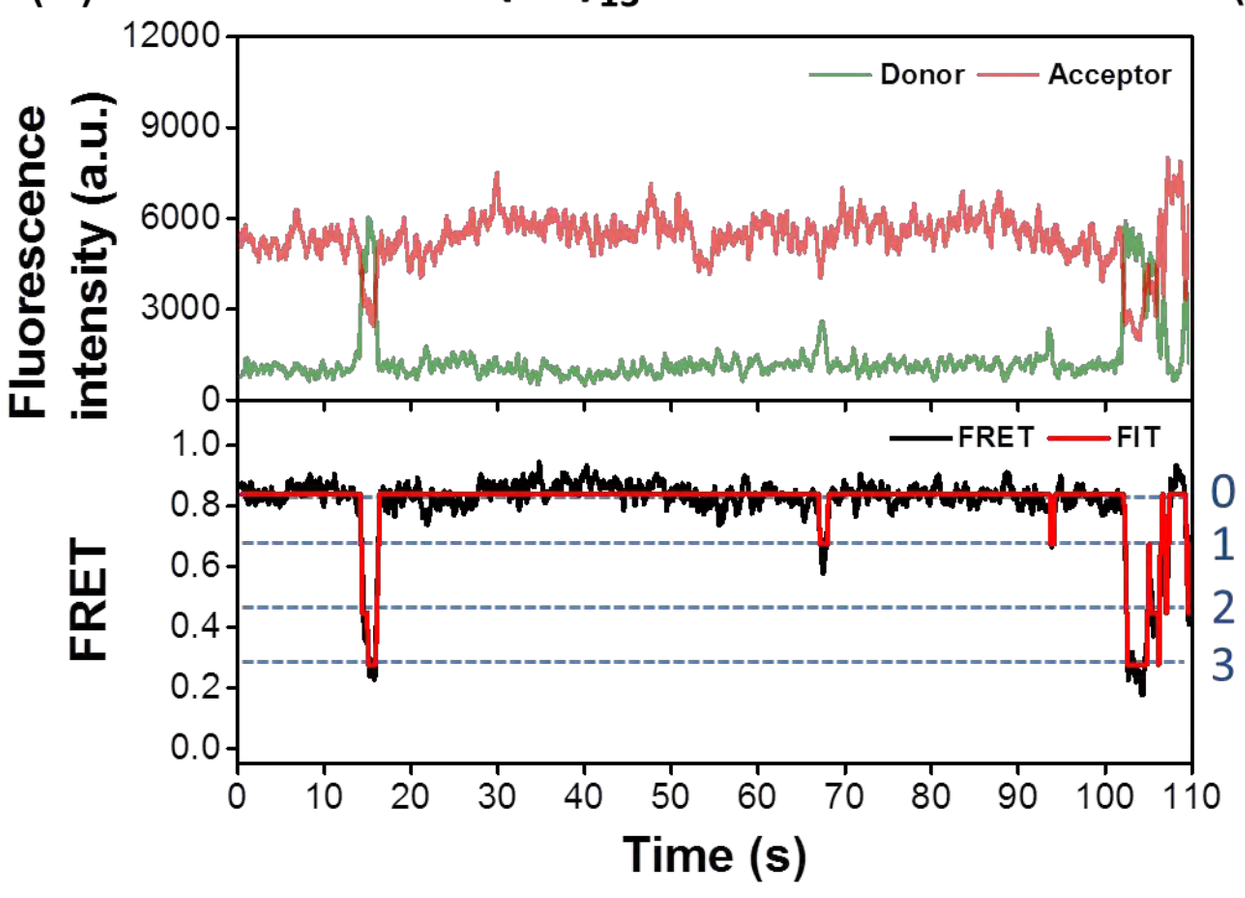
(C)


90 nt
135 nt
165 nt
200 nt
mRAD51
mRAD51 with mS5S1
Overall nucleation rate (s-1)
0.025
0.020
0.015
0.010
100
125
150
175
200
ssDNA length (nt)

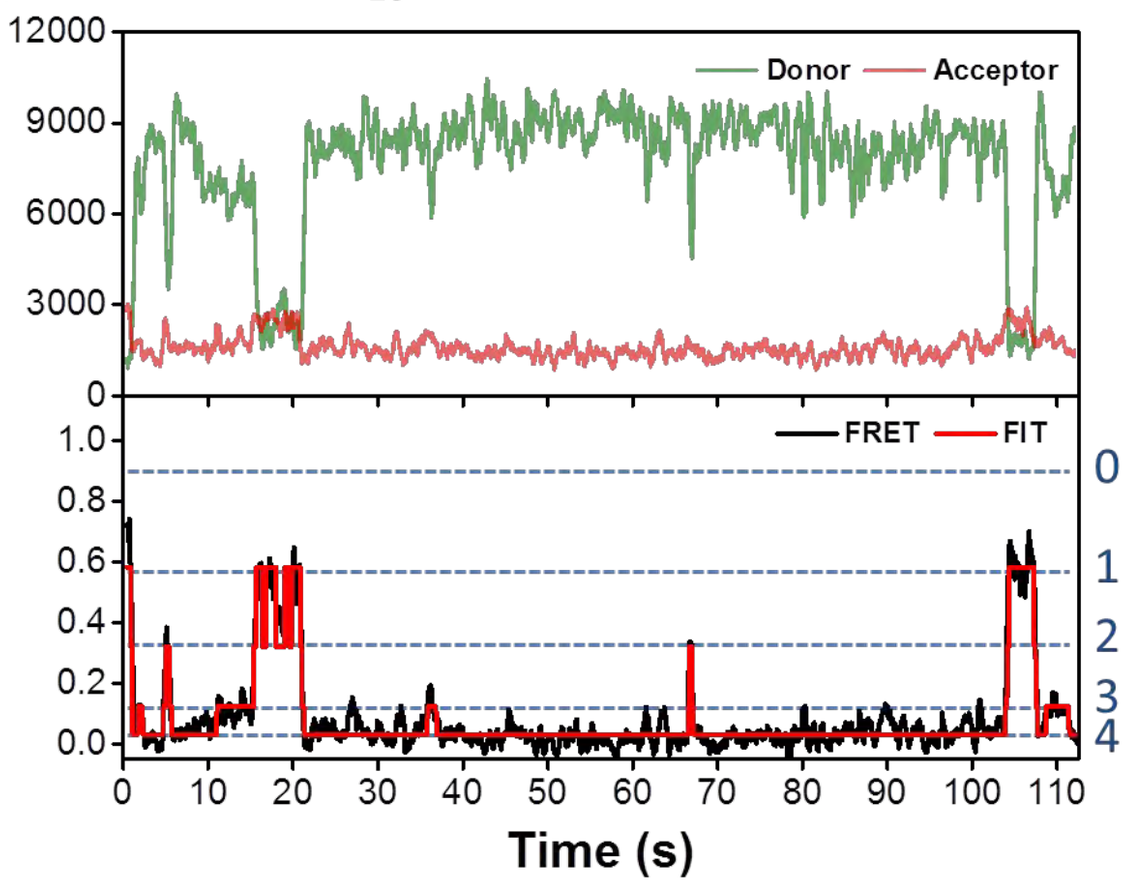
(A)


(dT)13
3'-Cy3
5'-Cy5
18 bp
mRAD51
± mS5S1
k0→1
k1→2
k2→3
k3→4
k1→0
k2→1
k3→2
k4→3
High FRET
(No protein)
Low FRET
(Protein-bound)

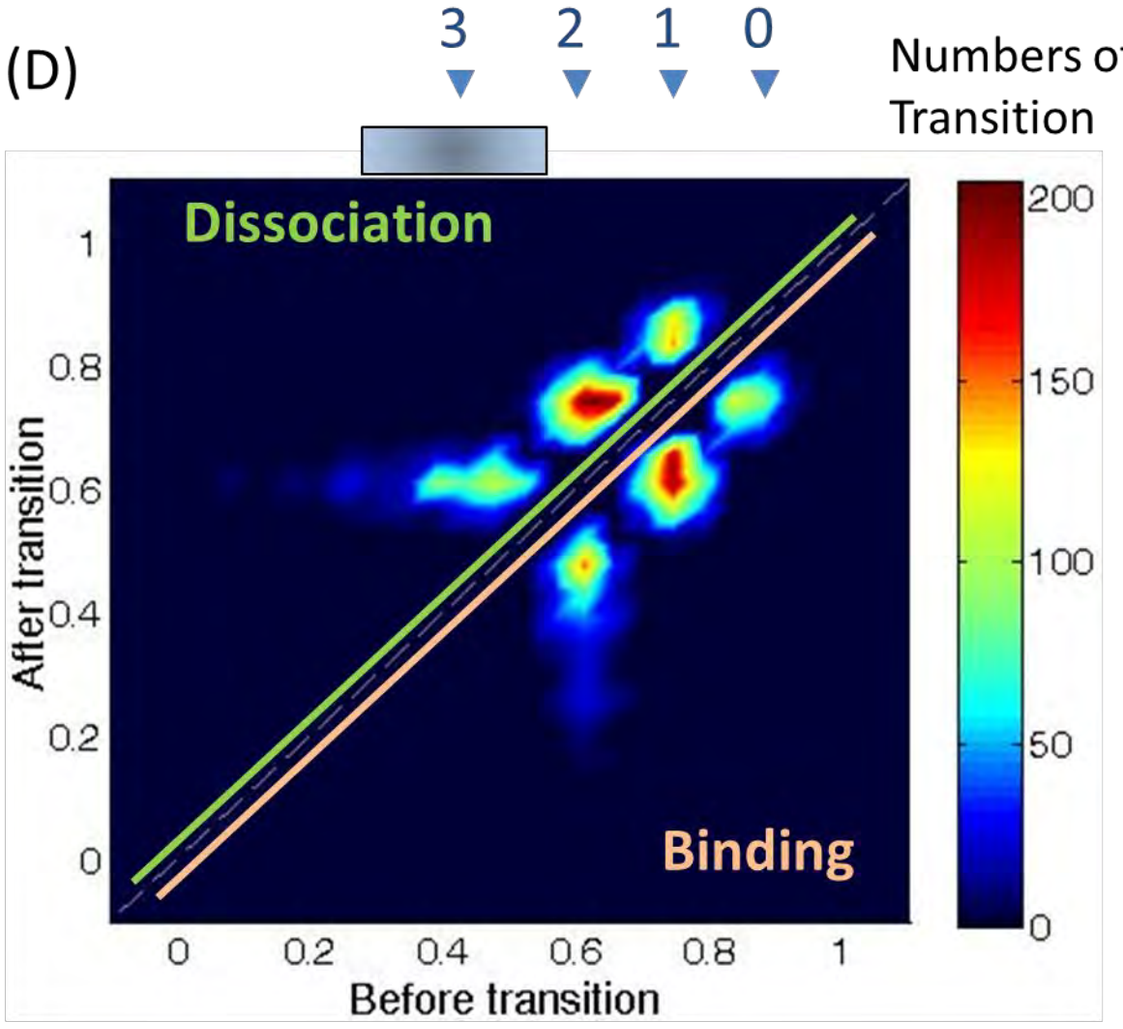
(B)


(dT)13 + mRAD51
Donor
Acceptor
Fluorescence intensity (a.u.)
FRET
FIT
0
1
2
3
Time (s)

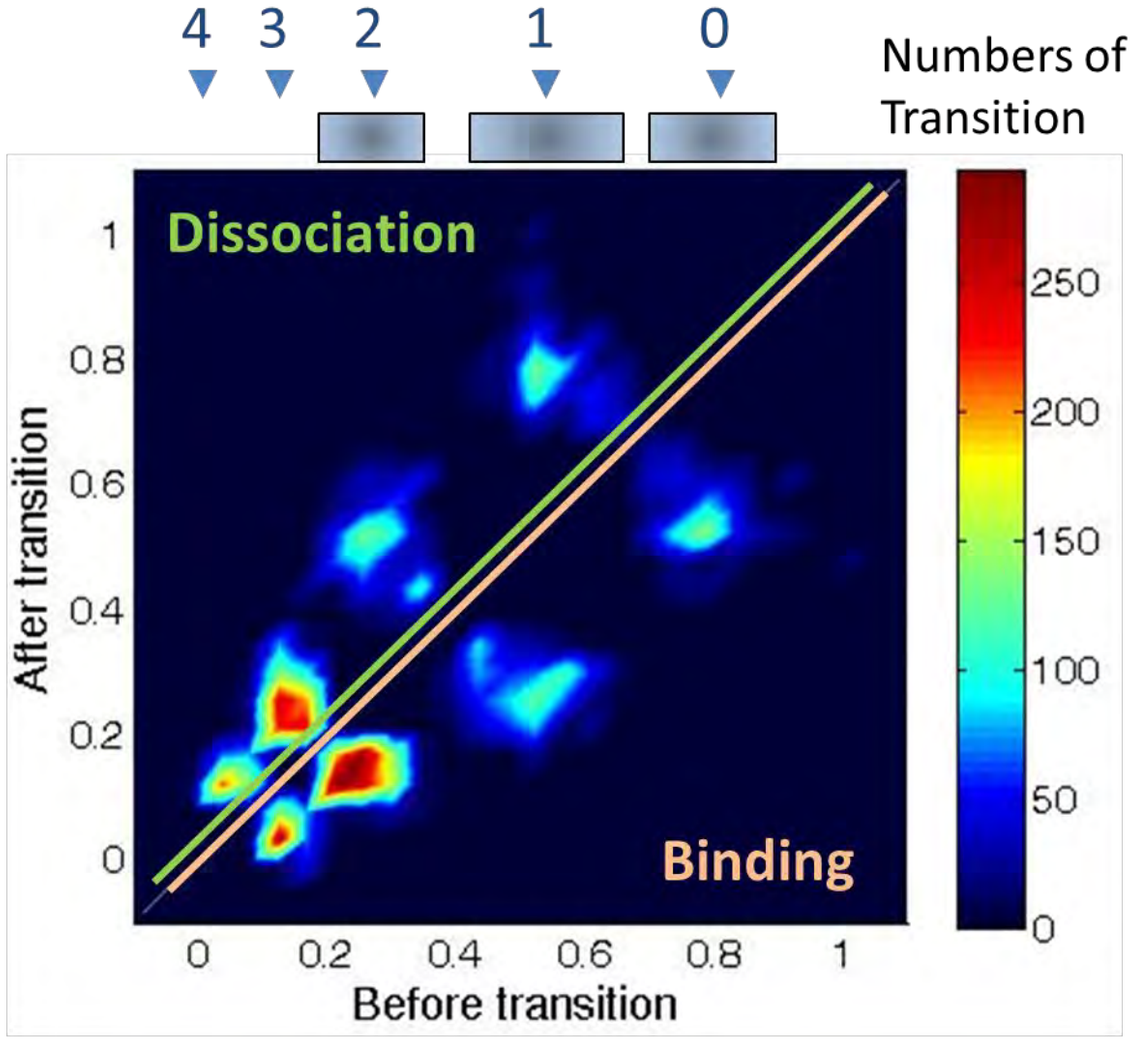
(C)


(dT)13 + mRAD51 & mS5S1
Donor
Acceptor
FRET
FIT
0
1
2
3
4
Time (s)

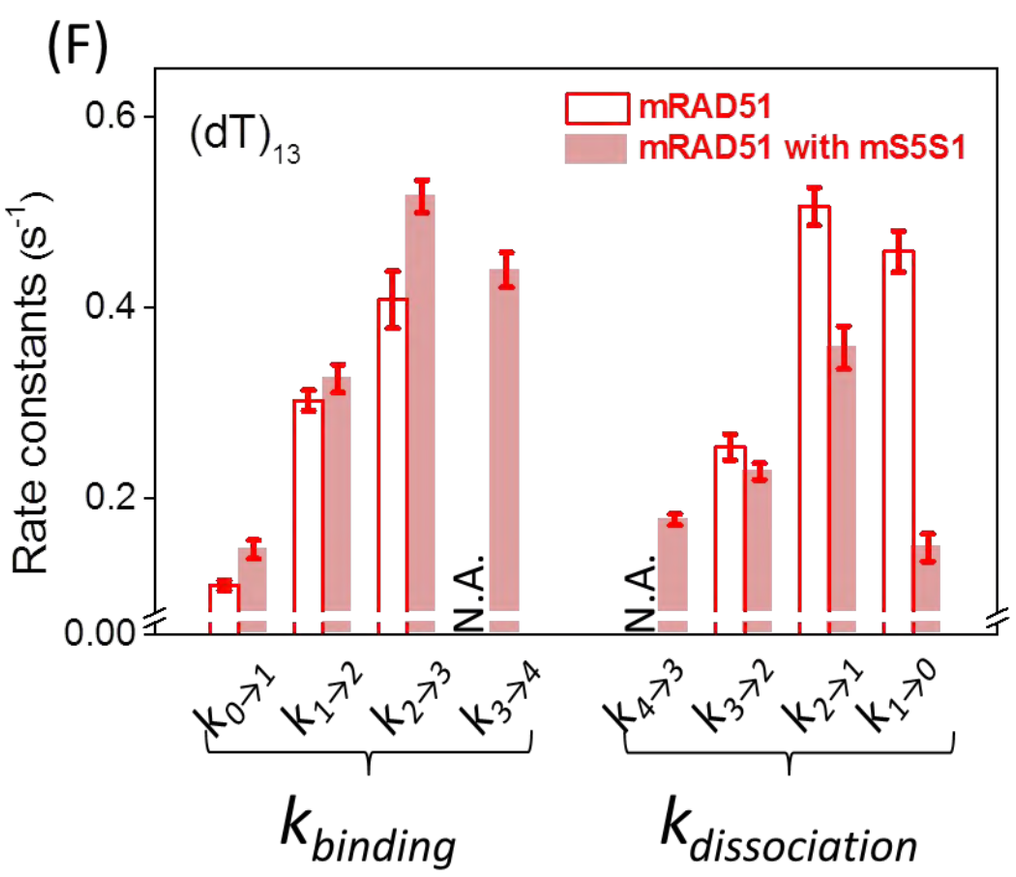
(D)

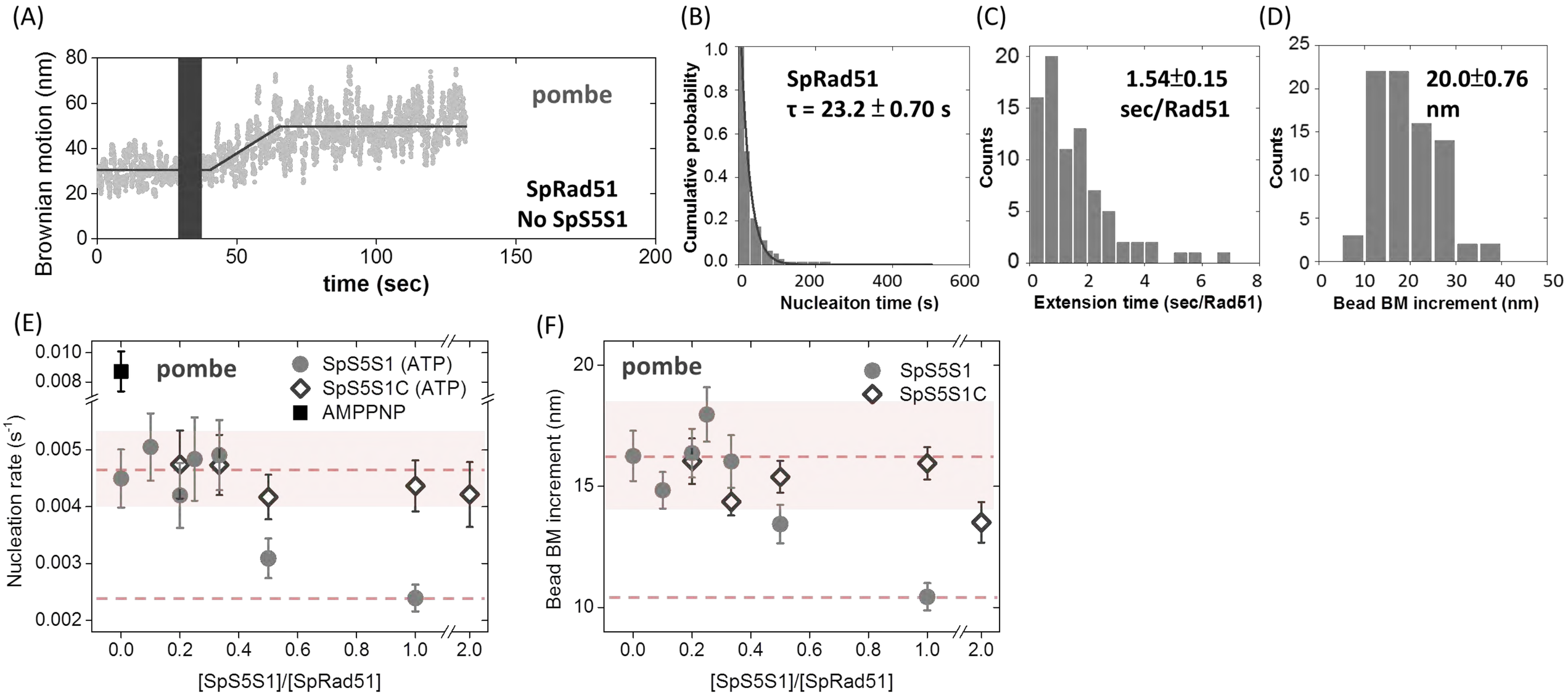

3
2
1
0
Numbers of Transition
Dissociation
Binding
After transition
Before transition

(E)

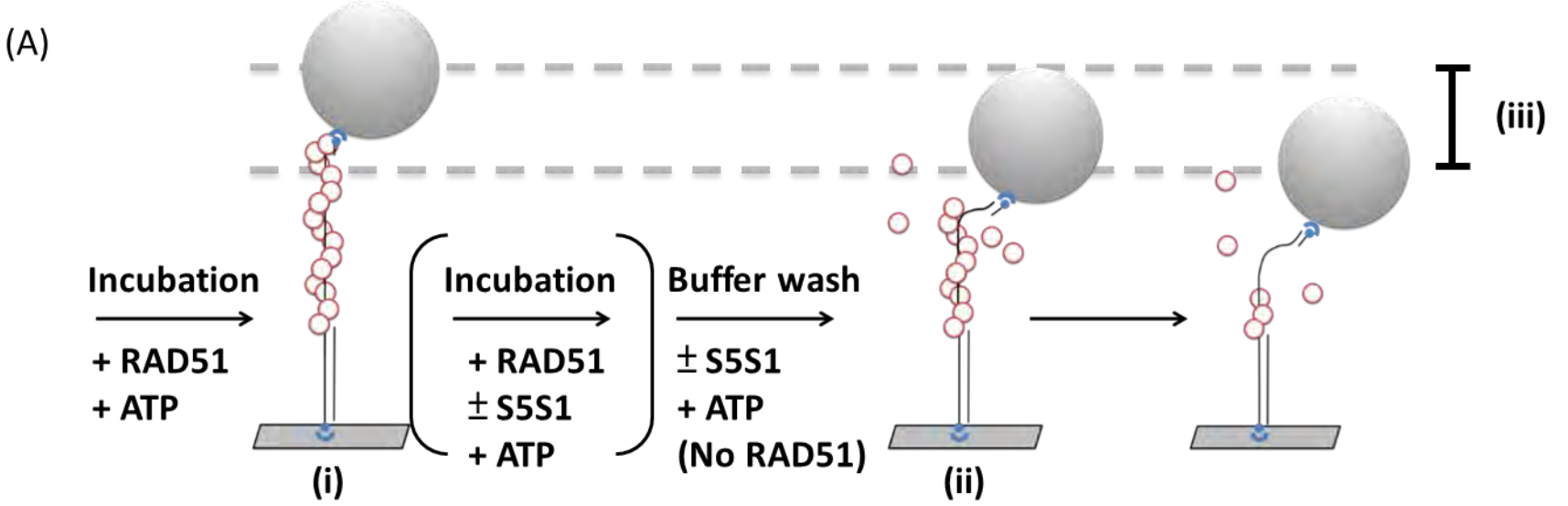

4
3
2
1
0
Numbers of Transition
Dissociation
Binding
After transition
Before transition

(F)

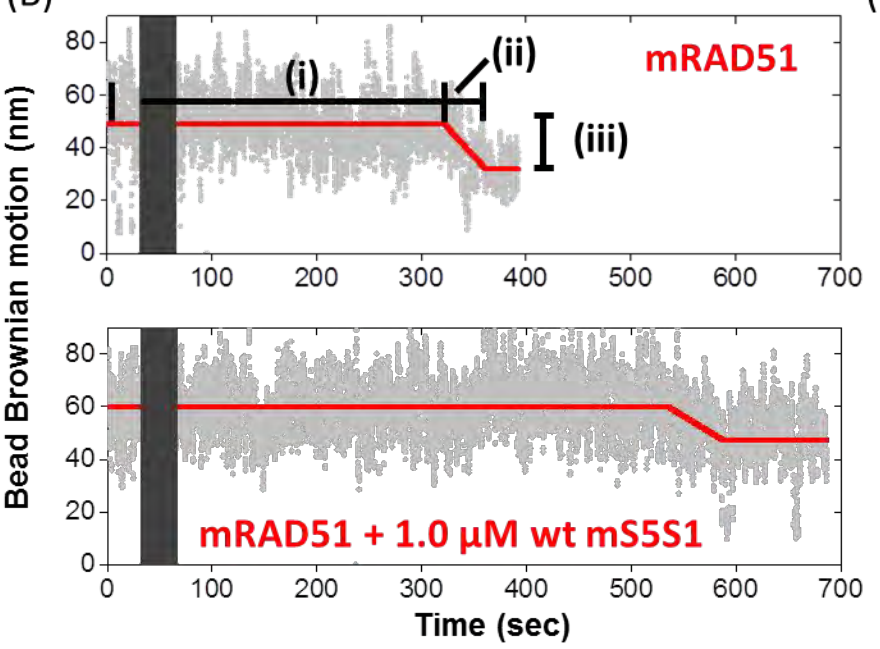

(dT)13
mRAD51
mRAD51 with mS5S1
Rate constants (s-1)
N.A.
N.A.
kbinding
kdissociation


(A)
Brownian motion (nm)
time (sec)
pombe
SpRad51
No SpS5S1
(B)
Cumulative probability
Nucleaiton time (s)
SpRad51
τ = 23.2 ± 0.70 s
(C)
Counts
Extension time (sec/Rad51)
1.54±0.15
sec/Rad51
(D)
Counts
Bead BM increment (nm)
20.0±0.76
nm
(E)
pombe
SpS5S1 (ATP)
SpS5S1C (ATP)
AMPPNP
Nucleation rate (s-1)
[SpS5S1]/[SpRad51]
(F)
pombe
SpS5S1
SpS5S1C
Bead BM increment (nm)
[SpS5S1]/[SpRad51]

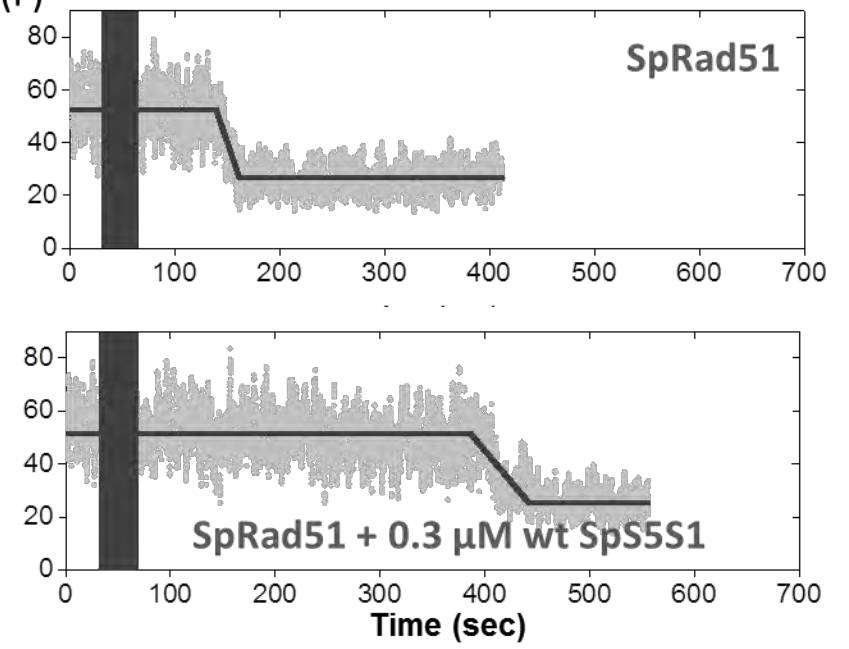
(A)



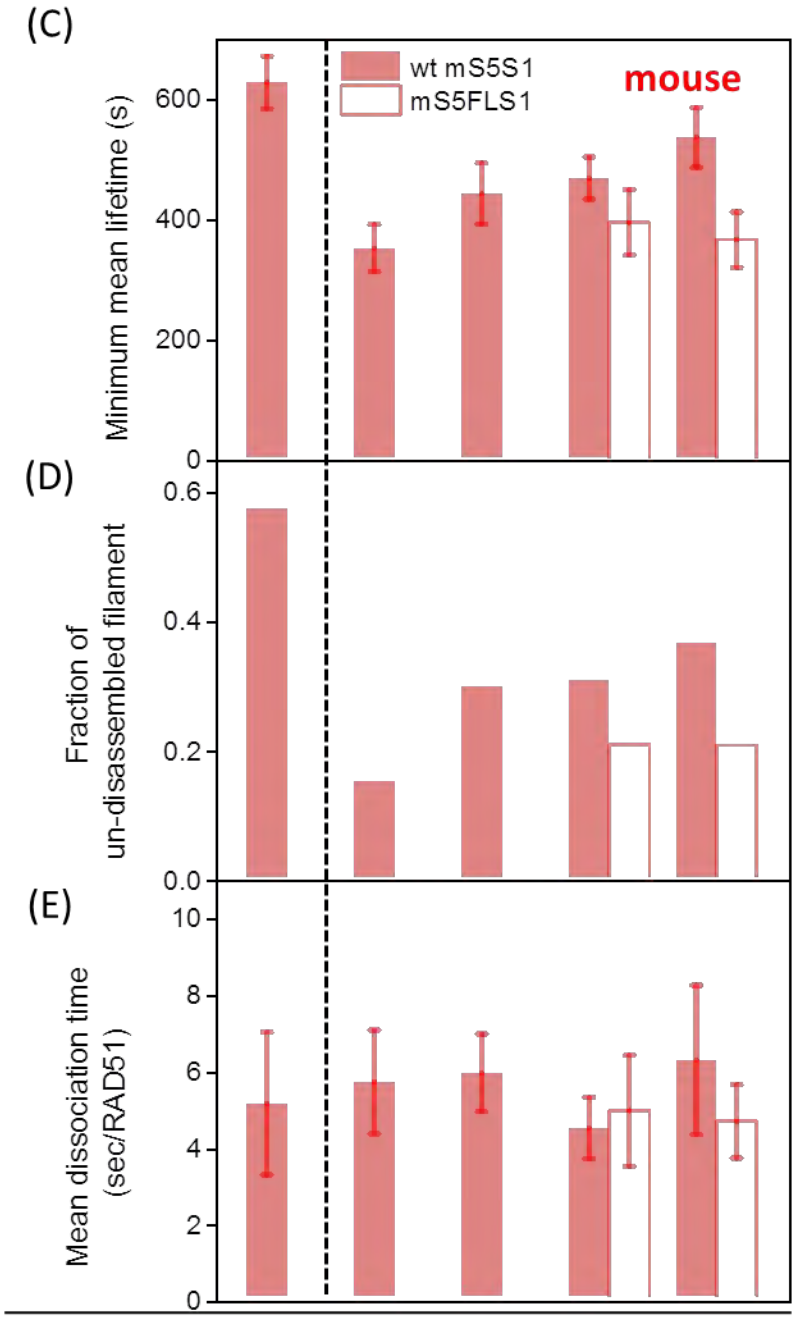
(B)



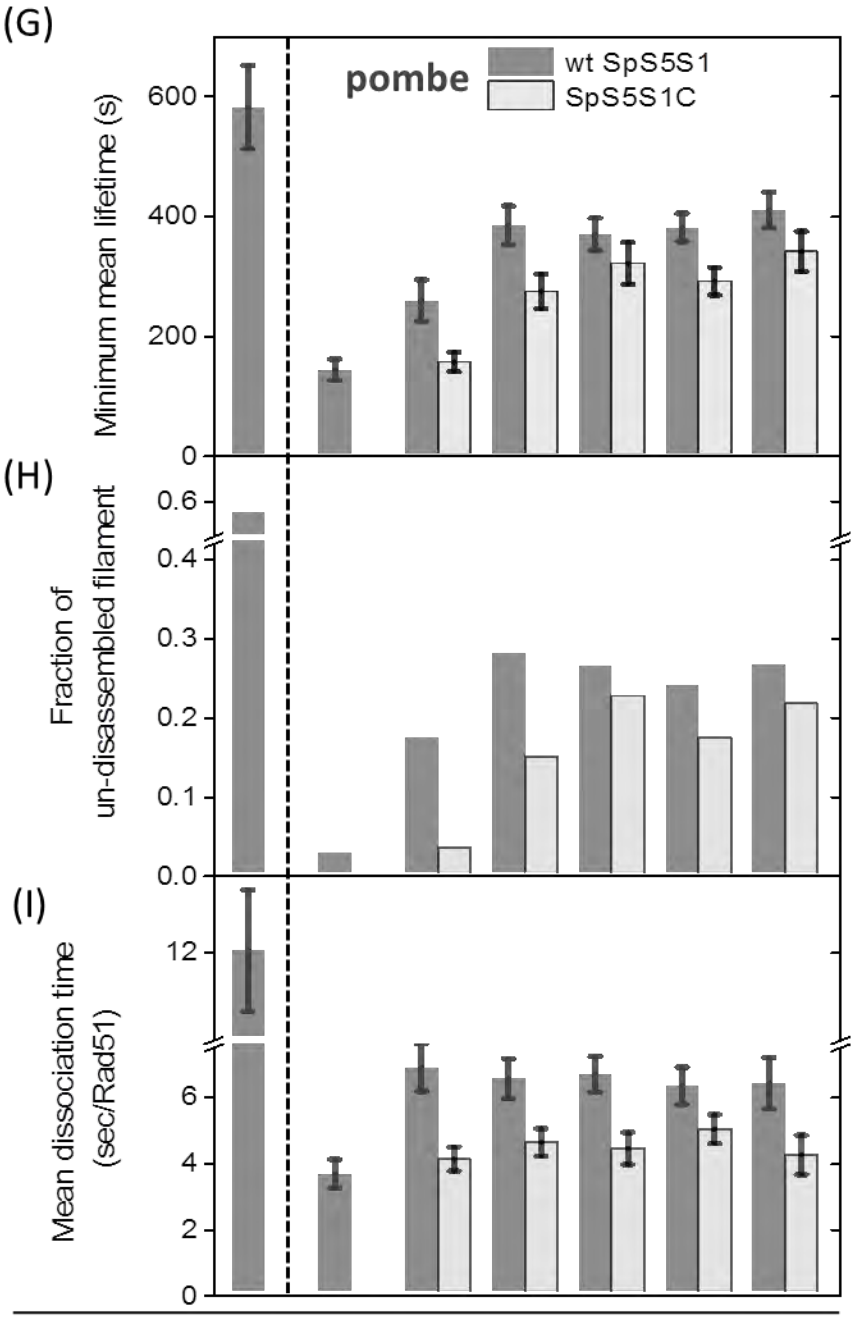
(F)



(C)



(G)



[mS5S1] or [mS5FLS1] (µM)	0	0	0.05	0.3	1.0
ATP	-	+	+	+	+
AMPPNP	+	-	-	-	-

[SpS5S1] or [SpS5S1C] (µM)	0	0	0.005	0.01	0.05	0.3	0.6
ATP	-	+	+	+	+	+	+
AMPPNP	+	-	-	-	-	-	-

Rad51

Swi5-Sfr1

(A) - Swi5-Sfr1

(B) + Swi5-Sfr1

Nucleating cluster stabilization

Filament stabilization

Supplementary Information for

Swi5-Sfr1 Stimulates Rad51 Recombinase Filament Assembly by Modulating Rad51 Dissociation

Chih-Hao Lu[1], Hsin-Yi Yeh[2], Guan-Chin Su[2], Kentaro Ito[3], Yumiko Kurokawa[3,¶], Hiroshi Iwasaki[3,*], Peter Chi[2,4,*] and Hung-Wen Li[1,*]

[1] Department of Chemistry, National Taiwan University, Taiwan
[2] Institute of Biochemical Sciences, National Taiwan University, Taiwan
[3] Institute of Innovative Research, Tokyo Institute of Technology, Japan
[4] Institute of Biological Chemistry, Academia Sinica, Taiwan

[*] To whom correspondence should be addressed:
Hiroshi Iwasaki, E-mail: hiwasaki@bio.titech.ac.jp
Peter Chi, E-mail: peterhchi@ntu.edu.tw
Hung-Wen Li, E-mail: hwli@ntu.edu.tw
[¶] current address: Center for Frontier Research, National Institute of Genetics, Japan

This PDF file includes:

Author contributions: C.H.L., H.I., P.C. and H.W.L. designed research; C.H.L. performed all single-molecule experiments and analyzed data; H.Y.Y, G.C.S., K.I. and Y.K. purified proteins used in this article; C.H.L. H.I., P.C. and H.W.L. wrote the paper.

SI Materials and Methods

DNA substrates. The $(dT)_{135}$ gapped DNA substrate for TPM assembly and disassembly experiments contains 135 nt poly dT sandwiched by a 151 bp, 5′-digoxigenin-labeled dsDNA handle and a 5′-biotin-labeled 19 bp handle. To prepare the $(dT)_{135}$ gapped DNA, we first used auto-sticky polymerase chain reaction (PCR)(1) to prepare a 131/151 hybrid DNA with a 20 nt 5′-overhang using a digoxigenin-labeled primer (5′-dig/CGTGGGTATGGTGGCAGG), and a primer containing an abasic nucleotide at the 21th position (5′- ATCGGTCGACGCTCTCCC TT/idSp/TGCGACTCCTGCATTAGGAA) using pBR322 as template. Oligos containing 135 thymidylates (5′-AAGGGAGAGCGTCGACCGAT$(T)_{135}$CTTACTG TCATGCCATCCG) was first phosphorylated by T4 PNK (NEB) and then ligated with the 131/151 hybrid in the presence of T4 DNA ligase (NEB) to generate a 151/305 hybrid DNA. After gel purification, the 151/305 hybrid DNA was then annealed with a biotin-tagged primer (5′-biotin-CGGATGGCATGACAGTAAG) to create the final $(dT)_{135}$ gapped DNA. $(dT)_{90}$, $(dT)_{100}$, $(dT)_{165}$ and $(dT)_{200}$ gapped DNA substrates were made with the same procedure as $(dT)_{135}$ DNA preparation, but using oligos with various lengths of $(dT)_n$ (n=90, 100, 165 and 200) (Figure S9). Oligos were purchased from Gene Link (oligos containing $(dT)_{165}$ and $(dT)_{200}$), Integrated DNA Technologies (the primer with one abasic site and oligos containing $(dT)_{90}$, $(dT)_{100}$ and $(dT)_{135}$) and Bio Basic Inc. (digoxigenin-labeled primers). For smFRET experiments, the surface-anchored hybrid DNA substrates were prepared by annealing a 5′-Cy5 and 3′-biotin (5′-Cy5/GCCTCGCTGCCGTCGCCA/bio-3′) double-labeled oligo and a 3′-Cy3-labeled oligo containing various numbers of thymidylate at 3′ overhang (5′-TGGCGACGGCaGCGAGGC$(dT)_n$/Cy3-3′) in the buffer containing 20 mM Tris and 0.5 M NaCl at pH = 8.

Proteins and buffers. mRAD51, mS5S1, $mS5^{FL/AA}S1$, SpRad51, SpS5S1, and SpS5S1C were purified as previously described(2-5). All mouse experiments were carried out with buffer containing 30 mM Tris, 2.5 mM magnesium chloride and 150 mM potassium chloride at pH=7.5. Fission yeast reactions were performed with buffer containing 25 mM Tris, 3 mM magnesium acetate and 150 mM potassium chloride at pH=7.5. ATP and AMPPNP were purchased from Sigma-Aldrich.

Detailed experimental procedures of single-molecule tethered particle motion (TPM) assembly experiment. For S5S1 titration experiments (Figure 1 and 4), 0.8 μM mouse RAD51 (or 0.3 μM fission yeast Rad51) was pre-incubated with the indicated concentrations of mouse S5S1 (or fission yeast S5S1) in corresponding buffers to form complexes at 37°C for 10-15 min. The RAD51-S5S1-ATP mixture

was cooled down to the room temperature and flowed into the reaction chamber containing bead-tagged DNA substrates. For nucleation unit determination, mRAD51 (or SpRad51) at different concentrations (Figure 2A & S9B) or 0.4-1.0 μM mRAD51 plus 1.6 or 2.0 μM (2-fold excess) mS5S1 (Figure 2B) were pre-incubated in corresponding buffers for 10-15 min at 37^{o}C before experiments. For binding preference determination (Figure 2C & S9B), 0.8 μM mRAD51, 0.8 μM mRAD51 plus 1.6 μM mS5S1 or 0.5 μM SpRad51 in corresponding reaction buffers were incubated for 10-15 min at 37^{o}C.

Single-molecule TPM disassembly experiment and data analysis. In disassembly experiments, we used silanized glass slide to avoid extensive surface-protein interaction. To prepare the silanized surface, the glass slides were sequentially sonicated in 2 M KOH for 5 min, 99 % ethanol for 15-20 min and ddH_2O for 15-20 min. After these sonication steps, slides were rinsed with ddH_2O and dried with N_2 gas. Glass slides were then functionalized in a solution containing 1,7-dichloro-octamethytetrasiloxane (Sigma-Aldrich) in 99 % ethanol in the dark overnight at room temperature. Slides were then rinsed with 99 % ethanol and ddH_2O alternatively and dried with N_2. Surface-bound $(dT)_{135}$ gapped DNA substrates were pre-incubated with mixtures of either 0.8 μM mRAD51-2 mM ATP or 0.8 μM SpRad51-2 mM ATP to form nucleoprotein filaments. After 5-10 min incubation, free mRAD51 or SpRad51 were removed with reaction buffer containing no mRAD51/SpRad51. The extensive wash used in our experiments didn't lead to disruption of protein filaments. For disassembly experiments involving S5S1, we added pre-incubated mixtures including 0.3 μM mRAD51 (or SpRad51), indicated amounts of mS5S1 (or SpS5S1), 2 mM ATP and ATP regeneration system (1 mM phosphoenolpyruvate and 4 units/ml pyruvate kinase) into reaction chambers and incubated for 10-15 min to form S5S1-coated Rad51-ssDNA filaments. After 10-15 min incubation, free Rad51 were removed by reaction buffer containing S5S1, ATP, and ATP regeneration system but without mRAD51/SpRad51. Tethers with BM ranging from 35 to 80 nm were scored as Rad51 nucleoprotein filaments (Figure S1).

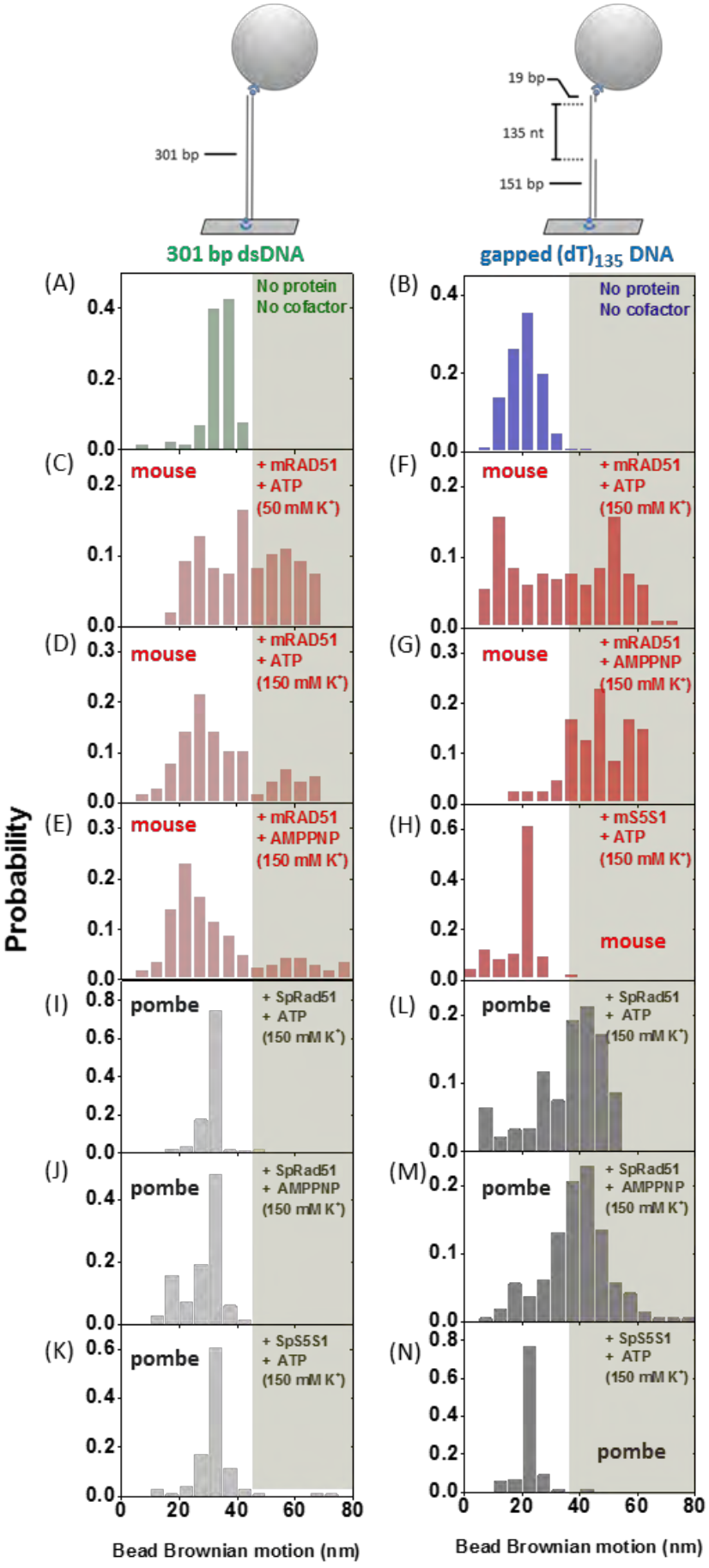

301 bp
19 bp
135 nt
151 bp
301 bp dsDNA
gapped (dT)135 DNA
(A)
No protein
No cofactor
(B)
No protein
No cofactor
(C)
mouse
+ mRAD51
+ ATP
(50 mM K+)
(F)
mouse
+ mRAD51
+ ATP
(150 mM K+)
(D)
mouse
+ mRAD51
+ ATP
(150 mM K+)
(G)
mouse
+ mRAD51
+ AMPPNP
(150 mM K+)
(E)
mouse
+ mRAD51
+ AMPPNP
(150 mM K+)
(H)
+ mS5S1
+ ATP
(150 mM K+)
mouse
(I)
pombe
+ SpRad51
+ ATP
(150 mM K+)
(L)
pombe
+ SpRad51
+ ATP
(150 mM K+)
(J)
pombe
+ SpRad51
+ AMPPNP
(150 mM K+)
(M)
pombe
+ SpRad51
+ AMPPNP
(150 mM K+)
(K)
pombe
+ SpS5S1
+ ATP
(150 mM K+)
(N)
+ SpS5S1
+ ATP
(150 mM K+)
pombe
Probability
Bead Brownian motion (nm)
Bead Brownian motion (nm)

Figure S1. Bead BM histograms of RAD51/S5S1 assembly on either 301 bp dsDNA or $(dT)_{135}$ gapped DNA. Brownian motion (BM) amplitudes of **(A)** bare 301 bp dsDNA and **(B)** bare $(dT)_{135}$ gap DNA substrates are about 35.2±3.77 and 21.3± 5.78 nm, respectively. BM higher than 35 nm (for $(dT)_{135}$ gapped DNA experiments) or 45 nm (for 301 bp dsDNA experiments) is considered to be extended by recombinases (yellow shaded region). Mouse mRAD51 binds to duplex DNA substrates at low salt **(C)**, but shows a reduced dsDNA affinity at higher (150 mM) KCl concentration in the presence of either **(D)** ATP **or (E) AMPPNP** . In contrast, mouse mRAD51 assembles efficiently on the $(dT)_{135}$ gapped DNA under ATP **(F)**, and it assembles much faster under AMPPNP **(G)**. mS5S1 didn't alter BM **(H)**, consistent with the biochemical characterization that mS5S1 has no ssDNA affinity. For pombe, SpRad51 has negligible dsDNA affinity and caused no increase in bead BM in either ATP **(I) or AMPPNP (J)**. **(L-M)** In contrast, SpRad51 preferentially assembled onto ssDNA region of $(dT)_{135}$ gapped DNA at higher KCl concentration in the presence of two cofactors, resulting in the increase of bead BM. Even though SpS5S1 can bind dsDNA and ssDNA, its binding does not change the BM of both substrates **(K and N)**. All assembly experiments were performed at 2 mM ATP or AMPPNP and collected after 5 minutes of protein introduction.

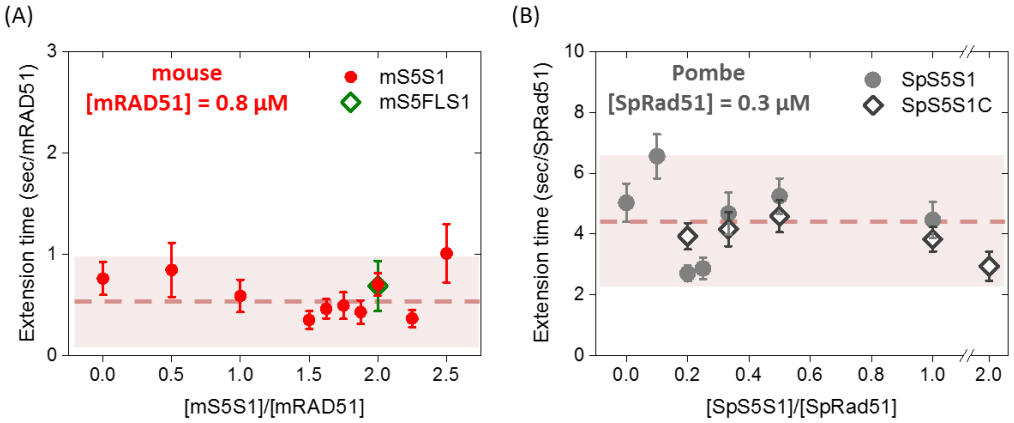
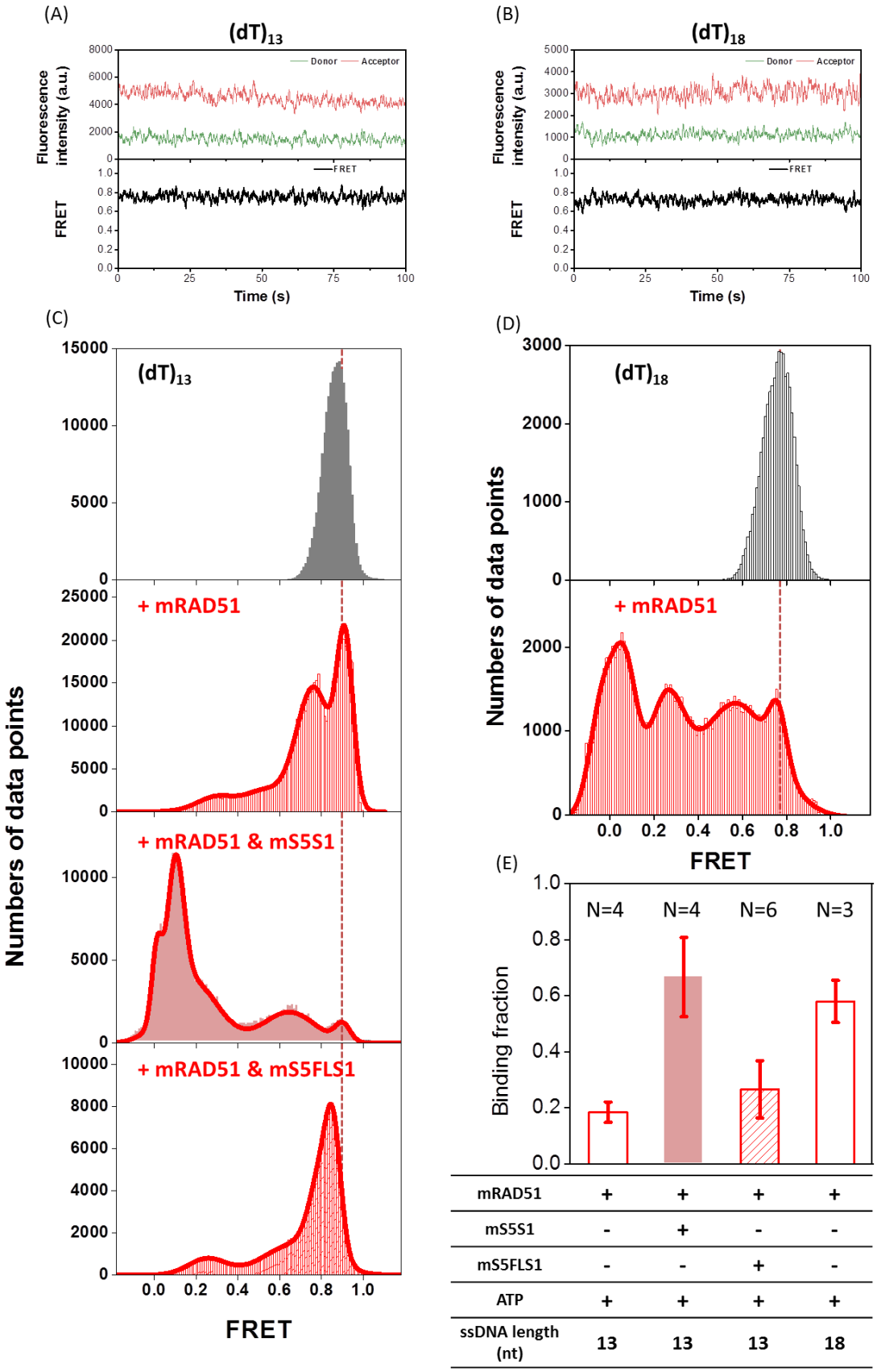


Figure S2. S5S1 shows negligible effects on Rad51 extension time in both two species. At fixed Rad51 concentrations (0.8 µM for mRAD51 and 0.3 µM for SpRad51), Rad51 extension times of both **(A)** mouse and **(B)** pombe display no apparent dependence on S5S1 concentration. Dash line is the mean of all measurements, and the shaded region span two standard deviations. Error bar is one standard error of the mean. Extension times of each concentration ratios are determined as the mean from at least 5 independent experiments.



mRAD51	+	+	+	+
mS5S1	-	+	-	-
mS5FLS1	-	-	+	-
ATP	+	+	+	+
ssDNA length (nt)	13	13	13	18

Figure S3. FRET histograms and binding fraction of mRAD51 assembling under different conditions. (A) & **(B)** In the absence of proteins, DNA molecules exist in

the high FRET state owing to the flexibility of ssDNA region. Both bare DNA substrates of different ssDNA lengths exhibit a high FRET state (E ~ 0.7-0.8). **(C)** FRET histograms of $(dT)_{13}$ DNA substrate in the absence of mRAD51 (top panel); in the presence of mRAD51 (second panel from top); in the presence of mRAD51 and mS5S1 mixture (third panel from top) or in the presence of mRAD51 and $mS5^{FL}S1$ mixture (bottom panel). **(D)** FRET histograms of $(dT)_{18}$ DNA substrate in the absence (upper) or in the presence (lower) of mRAD51. **(E)** Binding fraction of mRAD51 assembling on (dT)13 DNA substrate increases in the presence of mS5S1**.** At this $(dT)_{13}$ substrate, binding fraction of mRAD51-only is 18.5±3.62%, and that of mRAD51 and mS5S1 mixture is 66.6±14.0%. mRAD51 and $mS5^{FL}S1$ mixture gives a binding fraction of 26.6±10.2%, which is similar to that of mRAD51-only case. mRAD51 concentration is 1.0 μM, mS5S1 and $mS5^{FL}S1$ concentration are 2.0 μM. At longer $(dT)_{18}$ DNA substrates, the binding fraction is 58.0±7.51%. *N* indicates the number of independent experiments. Dash lines were drawn for guidance purpose.

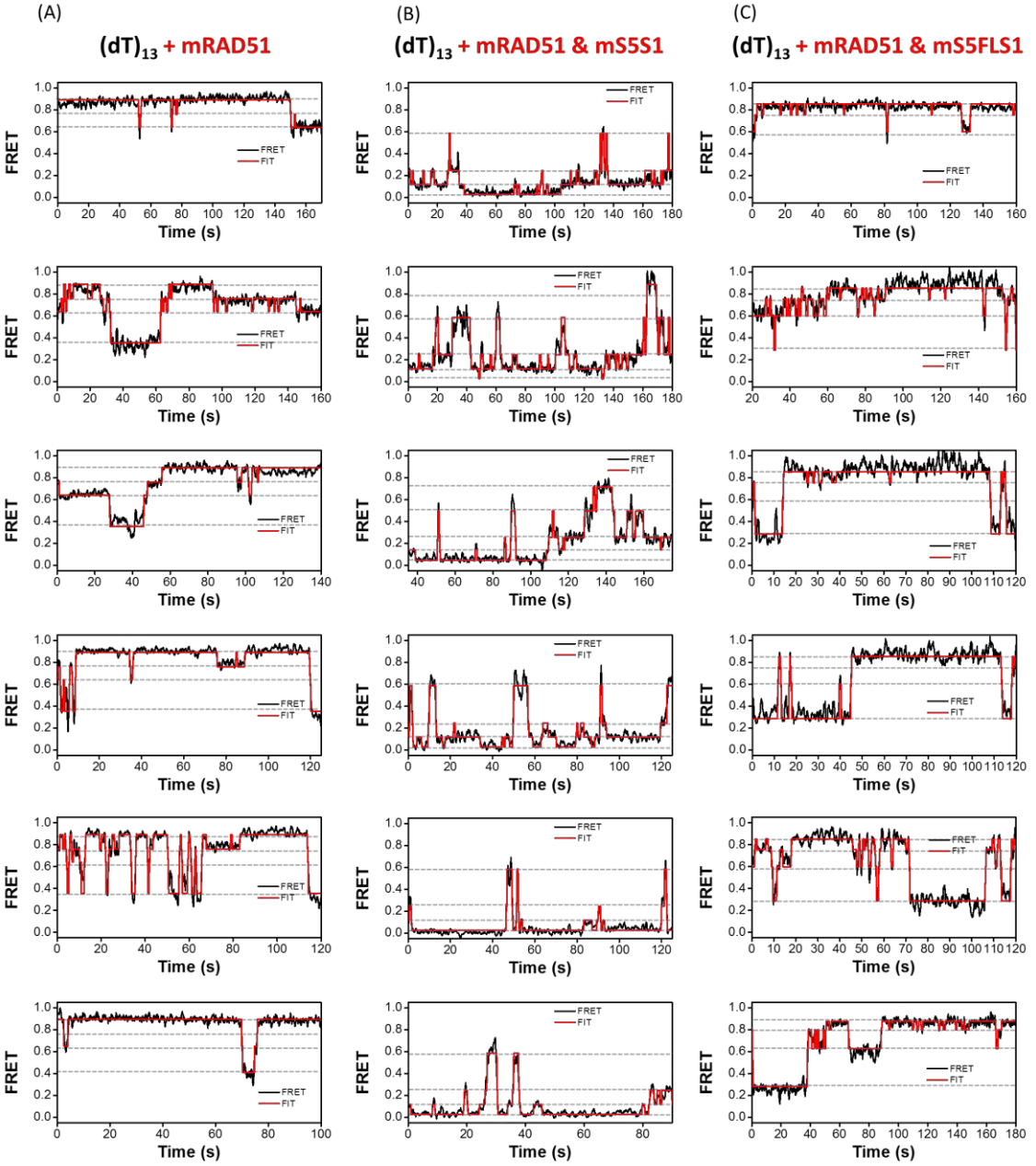

(A)
(dT)13 + mRAD51
(B)
(dT)13 + mRAD51 & mS5S1
(C)
(dT)13 + mRAD51 & mS5FLS1
(D)
(dT)18 + mRAD51
FRET
FIT
Time (s)

Figure S4. Representative FRET time traces of mRAD51 assembling under three different conditions: (A) on $(dT)_{13}$ substrate; **(B)** on $(dT)_{13}$ substrate in the presence of 2 μM mS5S1; **(C)** on $(dT)_{13}$ substrate in the presence of 2 μM $mS5^{FL}S1$; **(D)** on $(dT)_{18}$ substrate.

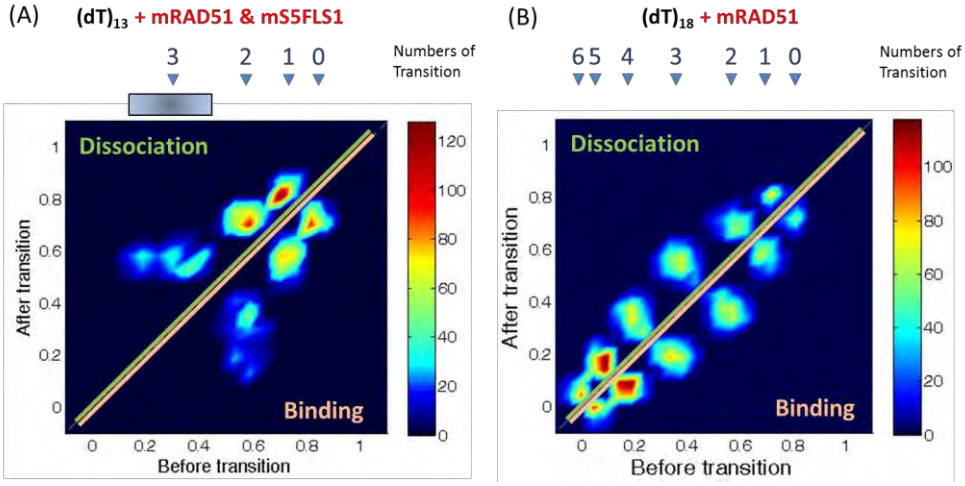


Figure S5. Transition density plot of mRAD51 assembly on $(dT)_{13}$ substrate in the presence of $mS5^{FL}S1$ and $(dT)_{18}$ substrate in the absence of mS5S1. (A) Adding $mS5^{FL}S1$ mutant gave similar transition with RAD51-only case. **(B)** TDP of mRAD51 assembly on $(dT)_{18}$ substrate clearly identifies 7 states, corresponding to zero, one to six mRAD51 monomer(s) binding.

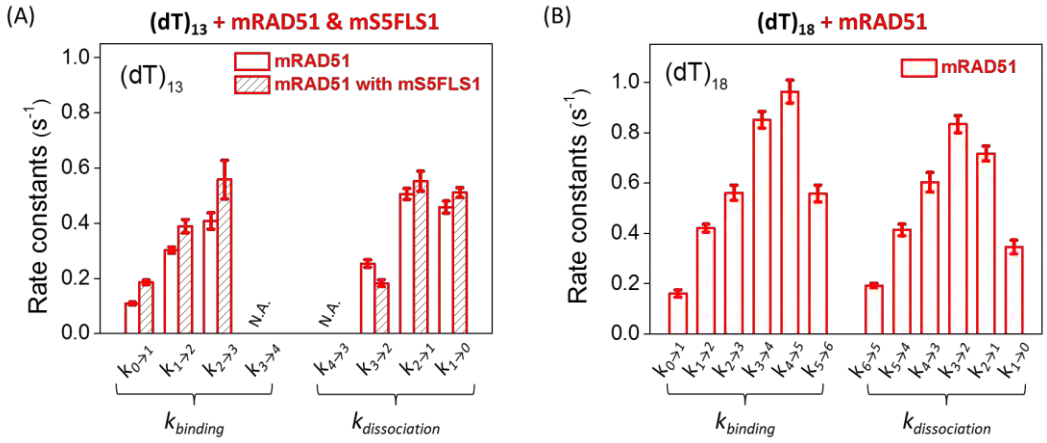


Figure S6. Rate constants of mRAD51 assembling on (A) on $(dT)_{13}$ substrate in the presence of $mS5^{FL}S1$ and (B) $(dT)_{18}$ DNA substrate in the absence of mS5S1.

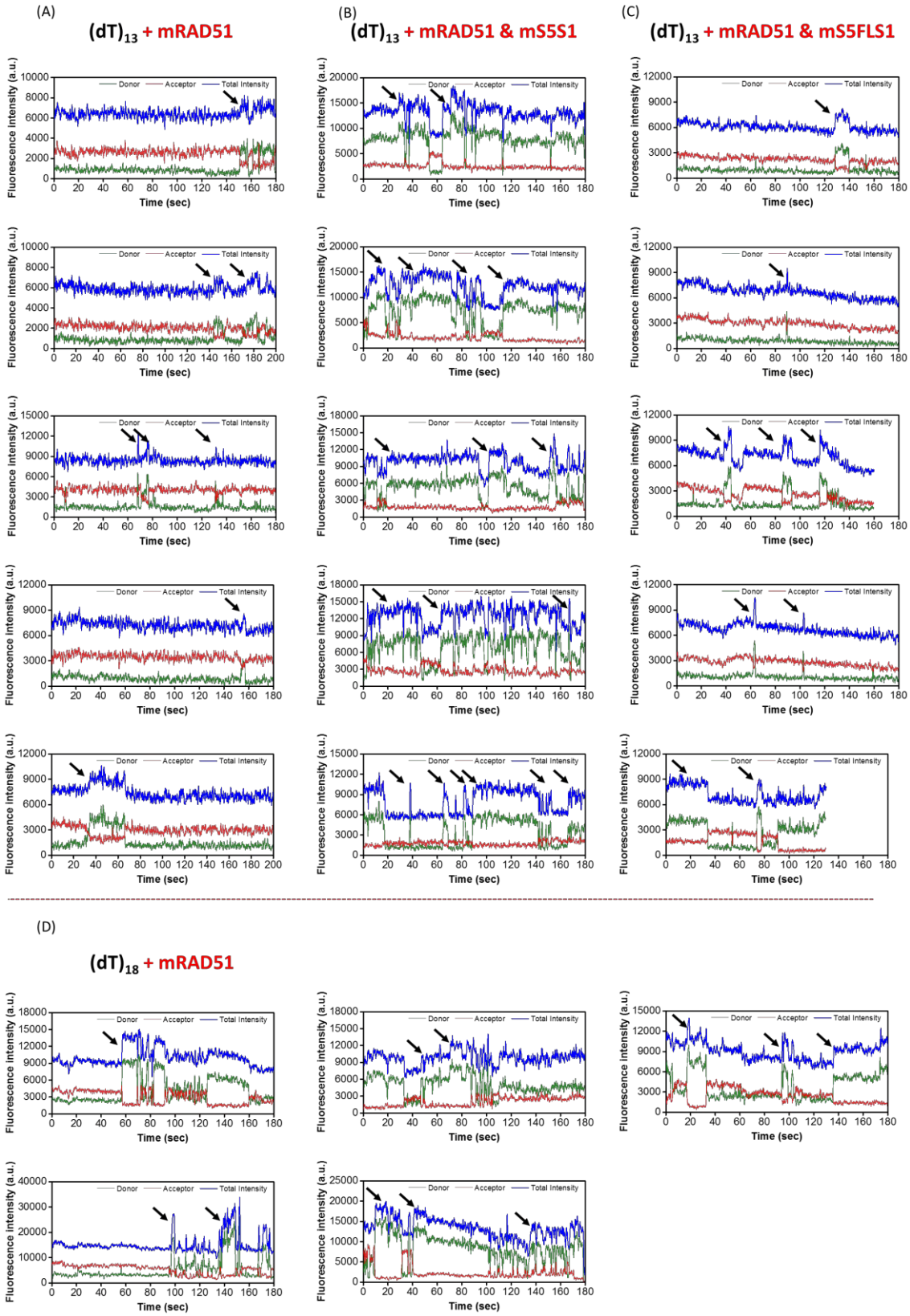


Figure S7. Protein-induced fluorescence enhancement (PIFE) effects are more apparent as mRAD51 assembles on $(dT)_{13}$ substrate in the presence of mS5S1 or on longer $(dT)_{18}$ substrate. Representative fluorescence intensity time traces of

mRAD51 assembling under three different conditions: **(A)** on $(dT)_{13}$ substrate; **(B)** on $(dT)_{13}$ substrate in the presence of 2 μM mS5S1; **(C)** on $(dT)_{13}$ substrate in the presence of 2 μM mS5^{FL}S1; **(D)** on $(dT)_{18}$ substrate. Black arrows indicate the increase in total fluorescence intensity owing to the Cy3 PIFE effect.

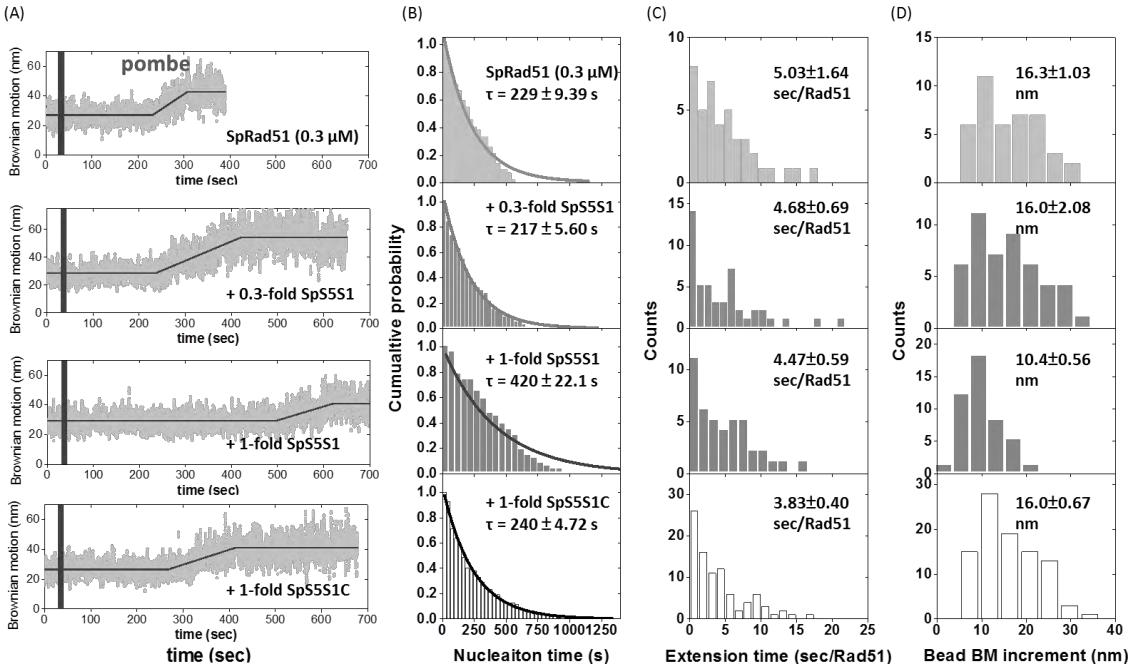


Figure S8. SpRad51 nucleoprotein filament assembly experiments. (A) Representative bead BM time-courses of SpRad51 (0.3 μM) assembly on $(dT)_{135}$ DNA substrates without SpS5S1 (top), with 0.3-fold (0.1 μM) SpS5S1 (middle top), with 0.3 μM SpS5S1 (middle bottom) or with 0.3 μM SpS5S1C mutant (bottom). Gray bars correspond to the deadtime when recombinase mixtures with 2 mM ATP were introduced. Histograms of nucleation time **(B)**, mean extension time (second/Rad51) **(D)** and bead BM increment **(E)** of SpRad51 assembling. All experiments were carried out at 2 mM ATP. Error bar of nucleation rate was the standard deviation of the mean by bootstrapping 5000 times, and error bar of extension time and bean BM increment is one SEM.

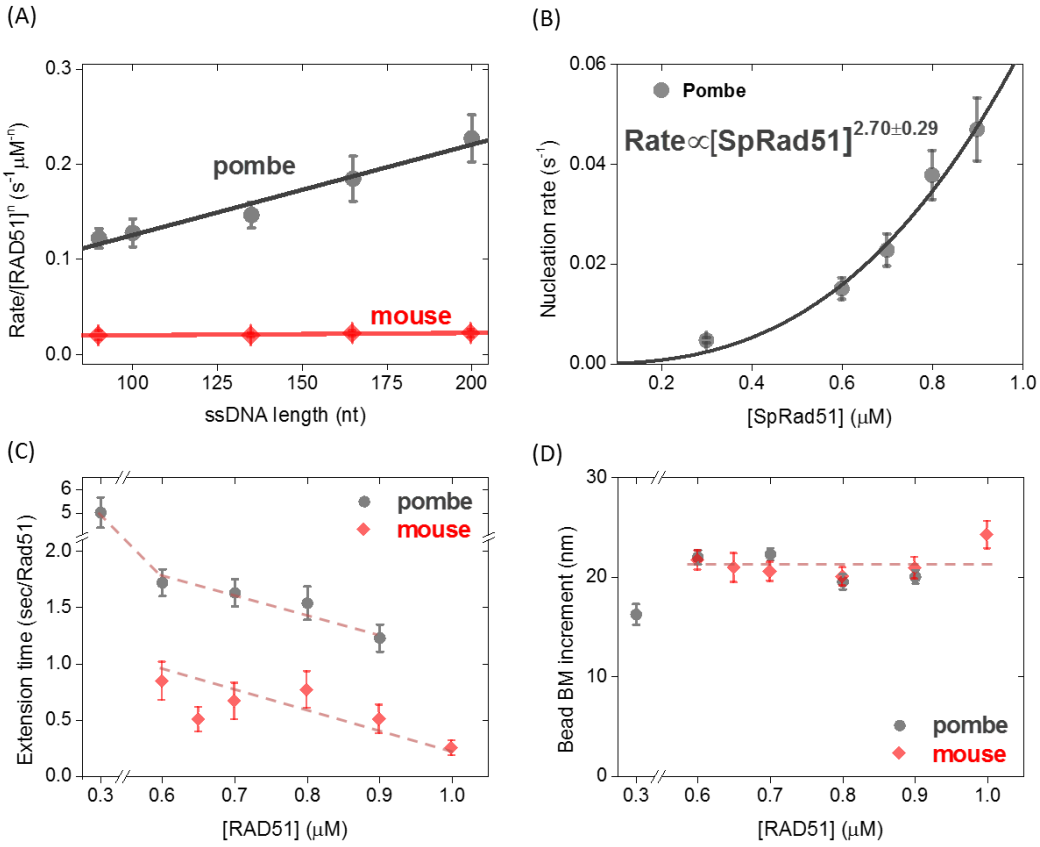


Figure S9. Kinetic parameters of SpRad51 nucleoprotein filament assembly and comparison with mRAD51. (A) ssDNA length dependence of pombe and mouse Rad51 obtained from TPM experiments. Nucleation rates are fitted by a $Rate/[RAD51]^n = k_{ssDNA}*L_{ssDNA}+k_{junction}$. Compared to mRAD51, SpRad51 shows a strong tendency to form nuclei on the ssDNA tail of the resected DNA. **(B)** SpRad51 concentration dependence of filament nucleation obtained by TPM experiments. Power-law fitting returns the nucleation unit of n=2.70±0.29 for SpRad51. **(C)** Extension time obtained from the slope of the BM time-courses and was expressed by the time required to add one Rad51 onto the filament. SpRad51 extends slower than mRAD51. **(D)** Similar bead BM increments between mRAD51 and SpRad51 assembly indicate the similar nucleoprotein filament structure of these two recombinases. At low SpRad51 concentration (0.3 μM), the equilibrium filament length is shorter. Dashed lines are drawn for guidance purpose. Error bar is one standard error of the mean.

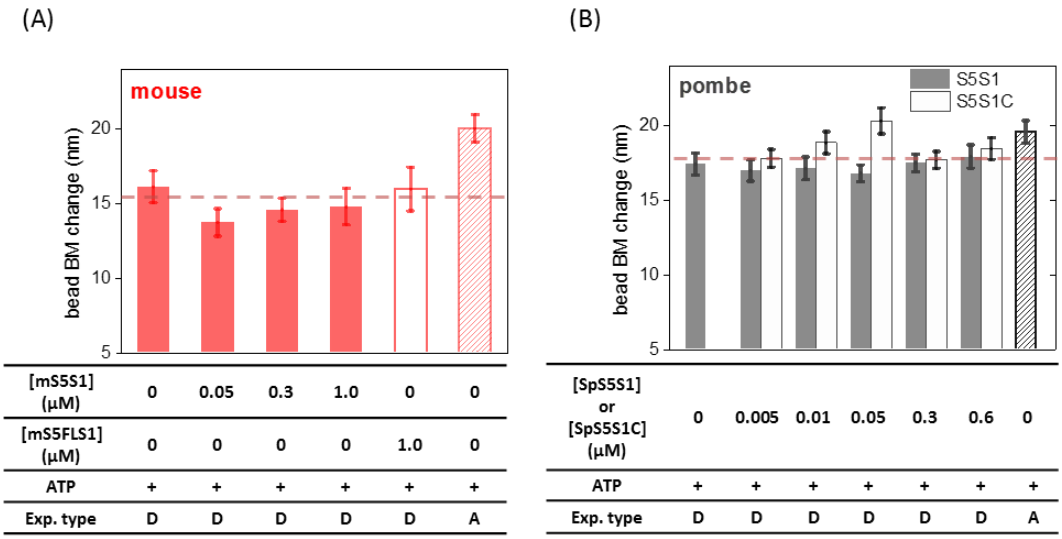


Figure S10. Filament disassembly of mRAD51 and SpRad51 filaments. (A) Bead BM decreases in TPM disassembly experiments of mRAD51 with either S5S1 or $S5^{FL}S1$ are all smaller than BM increment in mRAD51 assembly experiment (the last column in dashed bar) as $(dT)_{135}$ gapped DNA substrate was used in both assembly and disassembly experiments, implicating that not all mRAD51 dissociated from the gapped DNA substrates. **(B)** Compared to mRAD51 disassembly, SpRad51 most entirely dissociated from DNA. Both wild-type SpS5S1 (gray bars) and SpS5S1C mutants (light bars) showed a similar pattern. 0.8 μM of mRAD51 and SpRad51, a specified amount of S5S1 and ATP were used in the preparation of the filament before the disassembly was initiated. Dashed lines are drawn for guidance purpose (A: assembly experiment; D: disassembly experiment). Error bar is one standard error of the mean.

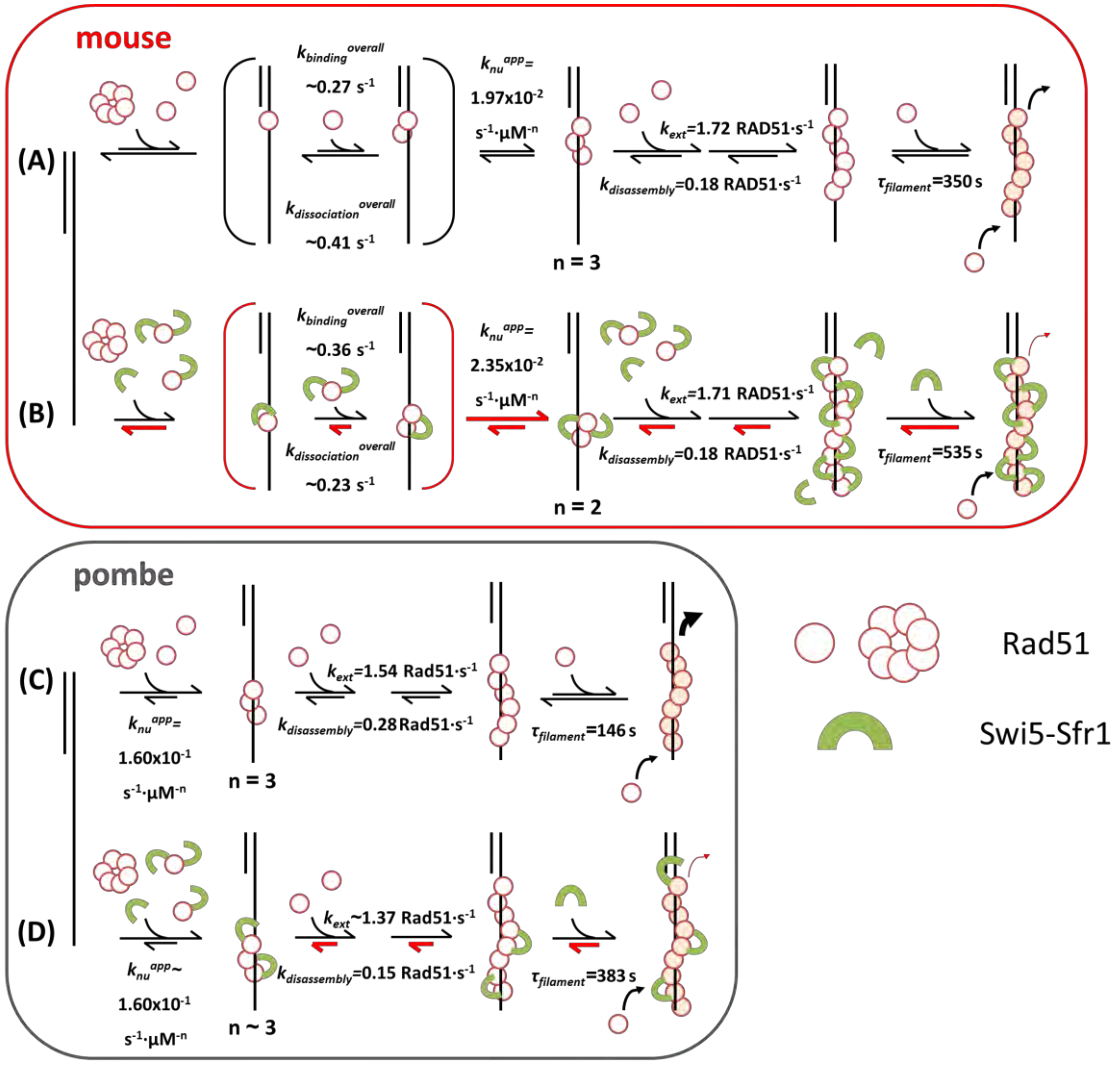


Figure S11. Kinetic parameters for S5S1-regulated Rad51 presynaptic filament formation obtained collectively from smFRET and TPM experiments. (A) Mouse RAD51 forms a stable nucleating cluster made of 3 monomers and extends on ssDNA as a monomer. **(B)** In the presence of mS5S1, mRAD51 interacts with S5S1 to form complex in solution. mS5S1 stimulates mRAD51 nucleation step by reducing mRAD51 nucleation unit from 3 to 2 monomers, stabilizing mRAD51 nucleating clusters and increasing mRAD51 ssDNA affinity. Also, mS5S1 prevents mRAD51 nucleoprotein filament disassembly. **(C & D)** Fission yeast SpS5S1 showed no stimulation on SpRad51 nucleation. Compared to mRAD51, SpRad51 displays a much higher nucleation rate owing to greater k_{ssDNA}. 3 SpRad51 monomers are required for stable nucleating cluster formation. SpRad51 filament is prone to disassembly compared to mRAD51. SpS5S1 efficiently prevents the disassembly of SpRad51 filament, and only small amounts of SpS5S1 is sufficient for stabilization. Different regulation strategies among species allow S5S1 to stabilize Rad51 filament efficiently. Red half-arrows indicate the kinetic steps affected by the S5S1.

Table S1. Summary of *apparent* nucleation rate constants of mRAD51 and mRAD51-S5S1 assembly on various gapped DNA substrates from Figure 2C. Error bar is the standard error of the fits.

	mRAD51	mRAD51-S5S1
k_{ssDNA}^{app} ($s^{-1} \cdot nt^{-1}$)	$(1.40 \pm 0.52) \times 10^{-5}$	$(8.95 \pm 1.93) \times 10^{-5}$
$k_{junction}^{app}$ (s^{-1})	$(9.54 \pm 0.70) \times 10^{-3}$	$(4.11 \pm 2.96) \times 10^{-3}$

Table S2. Summary of nucleation rate constants of mRAD51 and SpRad51 assembly on various gapped DNA substrates in Figure S9A. Error bar is the standard error of the fits.

	mRAD51	SpRad51
k_{ssDNA} ($s^{-1} \cdot nt^{-1} \cdot \mu M^{-n}$)	$(2.41 \pm 0.90) \times 10^{-5}$	$(9.50 \pm 0.99) \times 10^{-4}$
$k_{junction}$ ($s^{-1} \cdot \mu M^{-n}$)	$(1.64 \pm 0.14) \times 10^{-2}$	$(3.07 \pm 1.43) \times 10^{-2}$

Table S3. Summary of data in Figure 1 & S2A.

Initial [mRAD51] (μM)	**[mS5S1]/ [mRAD51]**	**SWI5-SFR1 type**	**Cofactor type**	**Nucleation rate (s^{-1})**	**Extension time (sec/RAD51)**	**Bead BM increment (nm)**	***n*** **(Numbers of molecules)**
0.8	0	--	ATP	$(1.10\pm0.12)*10^{-2}$	0.76±0.16	20.0±0.92	67
	0.5	Wild-type mS5S1		$(1.07\pm0.16)*10^{-2}$	0.85±0.27	24.4±1.45	43
	1			$(1.03\pm0.12)*10^{-2}$	0.59±0.16	26.0±1.35	52
	1.5			$(1.05\pm0.16)*10^{-2}$	0.35±0.09	22.7±1.24	44
	1.625			$(1.02\pm0.18)*10^{-2}$	0.46±0.09	23.5±1.36	48
	1.75			$(1.05\pm0.10)*10^{-2}$	0.49±0.13	26.5±2.26	51
	1.875			$(1.24\pm0.14)*10^{-2}$	0.43±0.11	24.6±1.32	59
	2			$(1.43\pm0.15)*10^{-2}$	0.70±0.11	24.4±0.97	76
	2.25			$(1.44\pm0.18)*10^{-2}$	0.37±0.08	25.2±1.21	54
	2.5			$(1.49\pm0.24)*10^{-2}$	1.01±0.29	25.2±1.79	30
	2	$mS5^{FL/AA}S1$ mutant		$(1.02\pm0.12)*10^{-2}$	0.67±0.24	22.5±1.39	39
	0	--	AMPPNP	$(1.73\pm0.22)*10^{-2}$	0.37±0.07	27.1±1.46	45

Table S4. Summary of data in Figure 2A, 2B, S9C & S9D.

Initial [mRAD51] (μM)	**Initial [mS5S1] (μM)**	**SWI5-SFR1 type**	**Cofactor type**	**Nucleation rate (s^{-1})**	**Extension time (sec/RAD51)**	**Bead BM increment (nm)**	***n*** **(Numbers of molecules)**
No mS5S1							
0.6	0	--	ATP	$(7.47\pm0.70)*10^{-3}$	0.80±0.20	21.7±0.97	92
0.65				$(8.54\pm1.56)*10^{-3}$	0.50±0.11	20.9±1.45	44
0.7				$(9.65\pm1.16)*10^{-3}$	0.66±0.16	20.5±0.97	60
0.8				$(1.10\pm0.12)*10^{-2}$	0.76±0.16	20.0±0.92	67
0.9				$(1.34\pm0.19)*10^{-2}$	0.50±0.13	20.9±1.08	41
1.0				$(2.36\pm0.35)*10^{-2}$	0.24±0.07	24.2±1.38	39
With mS5S1							
0.4	1.6	Wild-type mS5S1	ATP	$(5.34\pm1.15)*10^{-3}$	0.80±0.20	21.1±1.57	29
0.5				$(7.59\pm1.05)*10^{-3}$	0.49±0.12	25.7±1.79	37
0.6				$(1.13\pm0.15)*10^{-2}$	0.62±0.17	24.7±1.63	33
0.7				$(1.25\pm0.16)*10^{-2}$	0.34±0.11	27.6±1.92	31
0.8				$(1.43\pm0.15)*10^{-2}$	0.70±0.11	24.4±0.97	76
1.0	2.0			$(2.44\pm0.36)*10^{-2}$	0.69±0.15	25.7±1.74	37
0.7	1.6	$mS5^{FL/AA}S1$ mutant		$(9.95\pm1.35)*10^{-3}$	0.82±0.20	21.0±1.17	52
0.8				$(1.02\pm0.12)*10^{-2}$	0.67±0.24	22.5±1.39	39

Table S5. Summary of data in Figure 2C.

Initial [mRAD51] (μM)	**Initial [mS5S1] (μM)**	**SWI5-SFR1 type**	**Cofactor type**	**ssDNA length (nt)**	**Nucleation rate (s^{-1})**	***n*** **(Numbers of molecules)**
No mS5S1						
0.8	0	--	ATP	90	$(1.09\pm0.28)*10^{-2}$	31
				135	$(1.10\pm0.12)*10^{-2}$	67
				165	$(1.22\pm0.17)*10^{-2}$	33
				200	$(1.23\pm0.22)*10^{-2}$	42
With mS5S1						
0.8	1.6	Wild-type mS5S1	ATP	90	$(1.32\pm0.28)*10^{-2}$	32
				135	$(1.43\pm0.15)*10^{-2}$	76
				165	$(1.94\pm0.42)*10^{-2}$	35
				200	$(2.26\pm0.22)*10^{-2}$	36

Table S6. Summary of data in Figure 3F, S3E & S6.

Initial [mRAD51] (μM)	Initial [mS5S1] (μM)	SWI5-SFR1 type	Cofactor type	Binding rate (s^{-1})		Dissociation rate (s^{-1})		Binding fraction (%)	n (Numbers of molecules)
$(dT)_{13}$									
1.0	0	--	ATP	$k_{0\to1}$	0.11±0.01	$k_{1\to0}$	0.46±0.02	18.5±3.62	315
				$k_{1\to2}$	0.30±0.01	$k_{2\to1}$	0.51±0.02		
				$k_{2\to3}$	0.41±0.03	$k_{3\to2}$	0.25±0.01		
				$k_{3\to4}$	--	$k_{4\to3}$	--		
	2.0	Wild-type mS5S1		$k_{0\to1}$	0.15±0.01	$k_{1\to0}$	0.15±0.01	66.6±14.0	472
				$k_{1\to2}$	0.33±0.01	$k_{2\to1}$	0.36±0.02		
				$k_{2\to3}$	0.52±0.02	$k_{3\to2}$	0.23±0.01		
				$k_{3\to4}$	0.44±0.02	$k_{4\to3}$	0.18±0.01		
	2.0	$mS5^{FL/AA}S1$ mutant		$k_{0\to1}$	0.17±0.01	$k_{1\to0}$	0.48±0.02	25.0±8.51	120
				$k_{1\to2}$	0.38±0.02	$k_{2\to1}$	0.50±0.03		
				$k_{2\to3}$	0.45±0.06	$k_{3\to2}$	0.17±0.01		
				$k_{3\to4}$	--	$k_{4\to3}$	--		

$(dT)_{18}$									
1.0	0	--	ATP	$k_{0\rightarrow1}$	0.16±0.01	$k_{1\rightarrow0}$	0.35±0.03	58.0±7.51	467
				$k_{1\rightarrow2}$	0.42±0.02	$k_{2\rightarrow1}$	0.72±0.03		
				$k_{2\rightarrow3}$	0.56±0.03	$k_{3\rightarrow2}$	0.83±0.03		
				$k_{3\rightarrow4}$	0.85±0.03	$k_{4\rightarrow3}$	0.60±0.04		
				$k_{4\rightarrow5}$	0.96±0.06	$k_{5\rightarrow4}$	0.41±0.02		
				$k_{5\rightarrow6}$	0.56±0.03	$k_{6\rightarrow5}$	0.19±0.01		

Table S7. Summary of data in Figure 4E, 4F, S2B & S8.

Initial [SpRad51] (μM)	[SpS5S1]/ [SpRad51]	Swi5-Sfr1 type	Cofactor type	Nucleation rate (s^{-1})	Extension time (sec/Rad51)	Bead BM increment (nm)	*n* (Numbers of molecules)
0.3	0	--	ATP	$(4.50\pm0.51)*10^{-3}$	5.03±0.64	16.3±1.04	42
	0.1	Wild-type SpS5S1		$(5.05\pm0.59)*10^{-3}$	6.56±0.73	14.8±0.76	68
	0.2			$(4.20\pm0.57)*10^{-3}$	2.70±0.26	16.4±1.01	38
	0.25			$(4.84\pm0.73)*10^{-3}$	2.87±0.34	18.0±1.12	41
	0.33			$(4.91\pm0.62)*10^{-3}$	4.68±0.69	16.0±1.08	48
	0.5			$(3.09\pm0.35)*10^{-3}$	5.25±0.58	13.4±0.80	52
	1			$(2.39\pm0.24)*10^{-3}$	4.47±0.59	10.4±0.56	45
	0.2	SpS5S1C mutant		$(4.74\pm0.60)*10^{-3}$	3.93±0.44	16.0±0.94	50
	0.33			$(4.73\pm0.53)*10^{-3}$	4.16±0.56	14.4±0.57	73
	0.5			$(4.17\pm0.39)*10^{-3}$	4.58±0.52	15.4±0.66	82
	1			$(4.37\pm0.45)*10^{-3}$	3.83±0.40	15.9±0.67	94
	2			$(4.21\pm0.57)*10^{-3}$	2.94±0.48	13.5±0.83	35
	0	--	AMPPNP	$(8.72\pm1.36)*10^{-3}$	2.89±0.70	16.6±0.92	30

Table S8. Summary of data in Figure 5 & S10.

Species	Final [S5S1] (μM)	Swi5-Sfr1 type	Cofactor type	Minimum mean lifetime (sec)	Fraction of un-disassembled filament (%)	Disassembly time (sec/Rad51)	Bead BM decrease (nm)	*n* (Numbers of molecules)
Mouse	0	--	ATP	350±39.3	17.0	5.62±1.35	16.1±1.08	47
	0.05	Wild-type mS5S1		441±51.1	29.5	5.86±1.02	13.7±0.93	44
	0.3			467±35.2	30.5	4.41±0.80	14.5±0.79	83
	1.0			535±49.9	34.1	6.20±1.96	14.7±1.22	44
	0.3	$mS5^{FL/AA}S1$ mutant		393±54.9	20.5	4.86±1.46	18.0±1.51	39
	1.0			364±46.4	22.7	4.59±0.97	15.9±1.48	44
	0	--	AMPPNP	627±43.9	57.9	5.06±1.88	15.6±1.61	56
Fission yeast	0	--	ATP	140±17.9	2.47	3.55±0.44	17.4±0.74	81
	0.005	Wild-type SpS5S1		256±34.6	17.1	6.77±0.72	16.9±0.71	82
	0.01			382±32.6	27.9	6.43±0.61	17.1±0.68	122
	0.05			367±27.1	26.2	6.57±0.54	16.8±0.56	162
	0.3			378±23.4	23.8	6.21±0.57	17.5±0.59	210
	0.6			407±30.1	25.4	6.29±0.78	17.9±0.78	118
	0.005	SpS5S1C mutant		153±16.4	2.91	4.00±0.37	17.8±0.60	95
	0.01			271±28.8	14.7	4.51±0.41	18.8±0.74	75
	0.05			318±35.1	19.8	4.32±0.49	20.3±0.88	75
	0.3			288±23.0	17.1	4.91±0.44	17.7±0.56	123
	0.6			338±33.8	20.0	4.12±0.59	18.4±0.73	70
	0	--	AMPPNP	579±69.8	48.1	11.9±2.79	11.8±1.12	27

Table S9. Summary of data in Figure S9A.

Species	Initial [Rad51] (μM)	Initial [S5S1] (μM)	Swi5-Sfr1 type	Cofactor type	ssDNA length (nt)	Nucleation rate constant ($s^{-1}\cdot\mu M^{-n}$)	*n* (Numbers of molecules)
Mouse	0.8	0	--	ATP	90	$(1.88\pm0.48)*10^{-2}$	31
					135	$(1.89\pm0.21)*10^{-2}$	67
					165	$(2.10\pm0.28)*10^{-2}$	33
					200	$(2.11\pm0.38)*10^{-2}$	42
fission yeast	0.8	0	--	ATP	90	$(1.22\pm0.10)*10^{-1}$	145
					100	$(1.28\pm0.15)*10^{-1}$	49
					135	$(1.47\pm0.14)*10^{-1}$	131
					165	$(1.85\pm0.24)*10^{-1}$	77
					200	$(2.27\pm0.25)*10^{-1}$	81

Table S10. Summary of data in Figure S9B-S9D.

Initial [SpRad51] (μM)	Initial [SpS5S1] (μM)	Swi5-Sfr1 type	Cofactor type	Nucleation rate (s^{-1})	Extension time (sec/Rad51)	Bead BM increment (nm)	*n* (Numbers of molecules)
0.3	0	--	ATP	$(4.50\pm0.51)*10^{-3}$	5.03±0.64	16.3±1.04	42
0.6				$(1.49\pm0.22)*10^{-2}$	1.72±0.12	22.0±0.70	81
0.7				$(2.26\pm0.32)*10^{-2}$	1.63±0.12	22.3±0.61	104
0.8				$(3.77\pm0.49)*10^{-2}$	1.54±0.15	19.5±0.76	81
0.9				$(4.68\pm0.63)*10^{-2}$	1.22±0.12	20.0±0.68	104

SI References

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