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1 **Swi5-Sfr1 Stimulates Rad51 Recombinase Filament Assembly by**  
2 **Modulating Rad51 Dissociation**

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

22

23 **Key words:**

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

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27 **ABSTRACT**

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

48

49 **SIGNIFICANCE STATEMENT**

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

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65 **INTRODUCTION**

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

74 Among the cascade of steps required for DSB repair, Rad51 nucleoprotein  
75 filament assembly is the committed step and is subject to tight regulation(1, 2, 8-10).  
76 Maintaining a stable but dynamic nucleoprotein filament is critical for recombinase  
77 function, as this filament is essential for both the homology search and strand  
78 exchange required for heteroduplex DNA formation. Filament assembly includes a  
79 rate-determining nucleation step, where several Rad51 molecules bind ssDNA to form  
80 a stable nucleus, followed by a faster extension step(11-14). The filament assembly  
81 involves monomer addition occurring both at 3'- and 5'- ends of the recombinase  
82 filament but with different rates, leading to the overall end preference. During strand  
83 exchange, this filament likely maintains a certain length through dissociating and  
84 rebinding of Rad51. Thus, the filament overall moves dynamically with polarity  
85 (15-18). Several proteins stimulate and regulate Rad51 filament assembly(19-26).  
86 Swi5 and Sfr1 were identified by genetic studies in the fission yeast  
87 *Schizosaccharomyces pombe* (*S. pombe*)(27, 28). Mutations in fission yeast *swi5* and  
88 *sfr1* decrease recombination rates and increase sensitivity to ionizing radiation and  
89 DNA damaging chemicals(27, 28). *In vitro* biochemical and biophysical studies  
90 showed that both fission yeast and mouse Swi5 and Sfr1 proteins form a  
91 heterodimeric complex that physically interacts with Rad51 to facilitate  
92 Rad51-mediated recombination(25, 26, 29-31). Both fission yeast and mouse  
93 Swi5-Sfr1 (S5S1) complexes stabilize the Rad51 presynaptic filament and increase  
94 the ssDNA-dependent ATPase activity of Rad51. Moreover, mouse S5S1 (mS5S1)  
95 has been reported to enhance the release of ADP from the mRAD51 presynaptic  
96 filaments(32).

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

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

111

## 112 **RESULTS**

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

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

155 **Stimulation of the mRAD51 nucleation depends on mS5S1 concentration.** We  
156 next asked how much mS5S1 is required for maximum stimulation. We incubated  
157 various concentrations of mS5S1 (0-2.0  $\mu$ M) with 0.8  $\mu$ M mRAD51 in solution and  
158 observed filament assembly kinetics as in Figure 1A. Interestingly, a sigmoidal  
159 dependence of mS5S1/mRAD51 ratios is shown for nucleation rates (Figure 1F), with  
160 nucleation rates starting to increase at a ratio larger than 1.5 and reaching a constant  
161 maximum nucleation rate of  $\sim 0.015$  s<sup>-1</sup> when the ratio was larger than 2. This finding  
162 implies that two mS5S1 per one mRAD51 are required for the maximum nucleation  
163 stimulation. For the comparison purposes, we measured nucleation rates in the  
164 presence of the non-hydrolysable ATP analog, AMPPNP (black open square in Figure  
165 1F). Under these conditions, the nucleation rate for mRAD51 alone is fast ( $\sim 0.017$  s<sup>-1</sup>),  
166 suggesting that mRAD51 nucleating clusters are more stable in the absence of ATP  
167 hydrolysis, consistent with previous reports on bacterial RecA proteins(12, 42). It is  
168 possible that mS5S1 stimulates mRAD51 nucleation by stabilizing mRAD51  
169 nucleating clusters.

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

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

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

198 During DNA double-stranded break (DSB) repair, the ends of the DNA breaks are  
199 resected to produce 3' ssDNA overhangs. Thus, the repair substrate possesses a  
200 double-strand/single-strand junction (ds/ss junction) and a 3' protruding ssDNA tail.  
201 Rad51 nucleation clusters could initiate filament assembly on ssDNA either near the  
202 ds/ssDNA junction or onto the free ssDNA sites along the tail. We compared the  
203 nucleation rates of mRAD51 in four gapped substrates containing one ds/ssDNA  
204 junction but different lengths of ssDNA tail (90, 135, 165 & 200 dT). Nucleation rates  
205 can be fitted linearly to ssDNA tail lengths, with the slope corresponding to the  
206 apparent nucleation rate on free ssDNA site (Figure 2C). For mRAD51 only, the  
207 dependence on ssDNA length was small, suggesting that mRAD51 alone prefers to  
208 nucleate near the junction. On the other hand, in the presence of a two-fold excess  
209 mS5S1, nucleation rates of mRAD51 showed a much stronger dependence on ssDNA  
210 lengths (filled squares, Figure 2C), with a ~6.5-fold increase in the slope  
211  $((8.95\pm 1.93)\times 10^{-5} \text{ s}^{-1}\text{nt}^{-1}$  for mS5S1-mRAD51 and  $(1.40\pm 0.52)\times 10^{-5} \text{ s}^{-1}\text{nt}^{-1}$  for  
212 mRAD51 alone, Figure 2C and Table S1). These data indicate that mS5S1 stimulates  
213 mRAD51 binding by increasing its ssDNA affinity. Previous studies showed that DSB  
214 ends are resected to lengths of up to several kilobases in cells(4, 5), therefore, by  
215 dramatically increasing the ssDNA affinity of mRAD51, mS5S1 can effectively  
216 stimulate mRAD51 filament assembly.

217 **smFRET experiments reveal Rad51 binding and dissociation dynamics during**  
218 **nucleating events.** In TPM assembly experiments, we monitored the filament  
219 assembly kinetics that led to the successful assembly of recombinase nucleoprotein  
220 filaments. Due to their limited spatiotemporal resolution, TPM experiments cannot  
221 detect dynamics during non-productive assembly events. For example, transient  
222 recombinase binding events are likely taking place but would be difficult to detect by  
223 TPM. To monitor transient binding events, we used single-molecule fluorescence  
224 resonance energy transfer (smFRET) to characterize nucleation dynamics of mRAD51  
225 at high spatiotemporal resolution. The DNA substrates used in the smFRET  
226 experiments were short and composed of an 18 bp dsDNA handle and a short 3'  
227 terminating dT overhangs (13 or 18 nt, Figure 3A & S3A-S3D). The fluorophore  
228 donor (Cy3) and acceptor (Cy5) dyes were positioned so that mRAD51 monomer  
229 binding and dissociation on the ssDNA region could be monitored. In the absence of  
230 mRAD51, ssDNA is flexible, and separation between the dye pairs is short, resulting  
231 in a high FRET efficiency ( $\sim 0.85$  for (dT)<sub>13</sub> and  $\sim 0.8$  for (dT)<sub>18</sub>, Figure S3A-S3D).  
232 When mRAD51 assembles onto ssDNA, the distance between the dye pair increased  
233 resulting in reduced FRET efficiency (Figure 3B-3C; S3C-S3D). Therefore, each  
234 high-to-low FRET transition represents one or more mRAD51 binding events. In the  
235 case of mRAD51 alone, using a shorter (dT)<sub>13</sub> substrate, the percentage of DNA  
236 molecules with FRET alternation observed within 3 minutes (binding fraction) was  
237  $18.5 \pm 3.62\%$  (Figure S3E), with the time traces dominated by the protein-free,  
238 high-FRET state (Figure 3B, S3C & S4A). The low binding fraction and the  
239 transience of the low FRET states indicate that the (dT)<sub>13</sub> substrate is too short to form  
240 stable nucleating clusters of mRAD51. On the other hand, in the presence of mS5S1,  
241 the binding fraction of mRAD51 dramatically increases to  $66.6 \pm 14.0\%$  (Figure S3E)  
242 and the time traces are dominated by low-FRET states (Figure 3C, S3C & S4B),  
243 reflecting more RAD51 binding. This observation is consistent with our previous  
244 finding that mS5S1 reduces the nucleating unit from three mRAD51 molecules to two  
245 (Figure 2B-2C) and mS5S1 increases the ssDNA affinity of mRAD51 and stabilizes  
246 mRAD51 nucleating clusters on ssDNA (Figure 2C). Adding mS5<sup>FL</sup>S1 leads to the  
247 similar consequences as mRAD51 alone case (Figure S3C, S3E, S4C & S5A),  
248 consistent with the inability of mS5<sup>FL</sup>S1 to stimulate mRAD51 assembly. For the  
249 longer (dT)<sub>18</sub> ssDNA, mRAD51 alone results in stable binding with many more bound  
250 ssDNA molecules ( $58.0 \pm 7.51\%$ ) and the middle-to-low FRET signal is dominant  
251 (Figure S3D & S4C). We identified seven FRET states (Figure S5) and corresponding  
252 binding/dissociation rate constants (Figure S6) using the longer (dT)<sub>18</sub> substrates,  
253 reflecting the binding of up to six mRAD51 monomers.

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

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

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

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

307 **Fission yeast Rad51 filament assembly is fast, with no apparent stimulation from**  
308 **S5S1.** Mouse mS5S1 stimulates mRAD51 activity by accelerating the nucleation step  
309 during the nucleoprotein filament assembly. Fission yeast Swi5-Sfr1 (SpS5S1) is also  
310 known to stimulate SpRad51 activity(26, 29, 30, 33, 35). To examine whether the  
311 activation mechanism by S5S1 is evolutionally conserved, we used the same TPM  
312 approach towards SpS5S1. Under the same reaction condition (150 mM KCl, pH=7.5),  
313 SpRad51 does not assemble on dsDNA in either ATP or AMPPNP (Figure S1I-S1J &  
314 S1L-S1M). At the same recombinase concentration (0.8  $\mu$ M), SpRad51 displayed  
315 much faster assembly kinetics than mouse mRAD51 (Figure 4A-4B, Figure 1B-1C).  
316 In the absence of S5S1, SpRad51 nucleation time is  $23.2 \pm 0.70$  s (Figure 4B), 4-fold  
317 faster than that observed for mRAD51 ( $97.6 \pm 2.89$  s) (Figure 1C). To see if SpS5S1  
318 complex further stimulates SpRad51 assembly, we used a reduced recombinase  
319 concentration (0.3  $\mu$ M) to allow nucleation rates to be determined accurately (Figure  
320 4E & S8). Interestingly, SpS5S1 did not stimulate SpRad51 nucleation at low  
321 concentration and even inhibited SpRad51 nucleation at higher concentrations (solid  
322 circles, Figure 4E & S8B). It is possible that SpRad51 nucleation is sufficiently fast  
323 that no additional stimulation is necessary, unlike the slow nucleation observed for  
324 mRAD51. As to the inhibition effect seen at higher SpS5S1 concentrations, it likely  
325 results from the ssDNA binding property of SpS5S1(26, 30). Even though SpS5S1  
326 has DNA affinity, its binding to DNA substrates does not alter bead BM (Figure  
327 S1K-S1N). The control experiments verify that a decrease in bead BM increment with  
328 increasing SpS5S1 concentration derives from reduced amounts of SpRad51 binding

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

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

350 **S5S1 prevents Rad51 filament disassembly.** In addition to accelerating the binding  
351 event, nucleoprotein filament stability can also be achieved by the prevention of  
352 filament dissociation. In the TPM-based disassembly experiments (Figure 5),  
353 surface-bound (dT)<sub>135</sub> gapped DNA substrates were first incubated with Rad51/ATP  
354 mixture, and then another mixture of Rad51, ATP and S5S1 was added to the  
355 microscope slide. This set of disassembly experiments was done for mouse (Figure  
356 5B) and fission yeast (Figure 5F) proteins, respectively. The two-stage incubation  
357 avoids potential ssDNA substrate competition between SpS5S1 and SpRad51.  
358 Extensive buffer wash containing no Rad51 but all other components removed free  
359 Rad51 from the reaction chamber. This was defined as time zero of the disassembly  
360 reaction. The bead Brownian motions are measured in real-time to monitor the  
361 filament length. Several kinetic parameters were determined, including (i) lifetime of  
362 the stable filament (dwell time prior to BM decrease), (ii) dissociation time, time for  
363 individual RAD51 monomer dissociation continuously and (iii) net BM decrease, a  
364 decrease in filament coverage. Our results showed that the mRAD51 filament is quite  
365 stable in ATP even without mS5S1, with minimum mean lifetime around  $350 \pm 39.3$   
366 sec (Figure 5C). In the presence of  $1.0 \mu\text{M}$  mS5S1, the lifetime of the mRAD51

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

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

405

## 406 **DISCUSSIONS**

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

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

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

458 Different amounts of S5S1 required to stimulate nucleation and reduce filament  
459 disassembly provides hints how S5S1 interacts with Rad51 molecules to achieve  
460 filament stabilization. For the mouse proteins, a two-fold excess of mS5S1 maximally  
461 stimulates mRAD51 nucleation (Figure 1F). However, stabilization in the disassembly  
462 experiments can be seen with the low coverage of mS5S1 complex (~17%, Figure 5C,  
463 0.05  $\mu$ M vs 0.3  $\mu$ M mRAD51 added). Previous structural studies suggested that S5S1  
464 is accommodated within the groove of the Rad51 filament(30). The different  
465 concentration requirements of S5S1 point to a model that S5S1 binding stabilizes  
466 adjacent Rad51 molecules through binding within the filament groove to prevent  
467 Rad51 dissociation (Figure 6 & S11). During the nucleus formation, more mS5S1 are  
468 required so most of Rad51 are stabilized by S5S1. As Rad51 dissociation is inhibited  
469 by S5S1, stable Rad51 nucleus can be formed more easily. On the other hand, as  
470 filament disassembly could take place at the filament end more frequently, filament  
471 stabilization can be achieved by those S5S1 binding to terminal Rad51. Therefore,  
472 low coverage S5S1 is sufficient. As S5S1 has also been shown to activate Rad51  
473 filaments(30, 32), S5S1 binding within Rad51 filament also contributes to the overall  
474 Rad51 activity stimulation. During the dynamic progression of strand exchange, low  
475 S5S1 coverage allows efficient budgeting of S5S1, so S5S1 can be available for  
476 stimulation at later stages of recombination progression.

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

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

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

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

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

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

530

## 531 **MATERIALS AND METHODS**

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

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

547 We used an inverted optical microscope (IX-71, Olympus) with a differential  
548 interference contrast (DIC) imaging mode to visualize tethers and measure bead  
549 Brownian motion (BM). Images of assembly experiments were acquired at 30 Hz  
550 using a Newvicon camera (Dage-MTI) and were analyzed using software written in  
551 Labview. The amplitude of tether Brownian motion is defined by the standard  
552 deviation of the bead centroid positions of 20 images using sliding windows. In  
553 addition to the DNA contour length change, polymer stiffness and camera exposure  
554 time alter the Brownian motion amplitudes in practice(42). For each independent  
555 TPM assembly experiments, images were first recorded for around 30 seconds (~  
556 1000 image frames) before the addition of Rad51 or Rad51-S5S1 mixture and then for

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

560 **Single-molecule fluorescence resonance energy transfer (smFRET) experiment**  
561 **and data analysis.** In smFRET experiments, PEGylated glass slides and coverslips  
562 were prepared as previously described(66). To perform smFRET experiments,  
563 reaction chambers were incubated with 20 µg/mL streptavidin for 5 min. Excess  
564 streptavidin was washed away with the buffer containing 20 mM Tris and 50 mM  
565 NaCl. 15 pM of 3'-biotinylated fluorophore-labeled hybrid DNA was then  
566 immobilized on the surface for 5 min. After 5 min incubation, free DNA was removed  
567 by flowing in mRAD51 imaging buffer containing 1 mM Trolox (Sigma-Aldrich), 2.6  
568 mM protocatechuic acid (PCA, Sigma-Aldrich), 0.21 units/mL protocatechuate  
569 3,4-dioxygenase (PCD, OYC Americas Inc.), 30 mM Tris, 2.5 mM magnesium  
570 chloride and 150 mM potassium chloride at pH 7.5. The reaction includes a mixture  
571 of 1 µM mRAD51 and 2 mM ATP in mRAD51 imaging buffer into reaction chambers.  
572 mS5S1 (or mS5FLS1)-containing experiments includes 1 µM mRAD51, 2 µM mS5S1  
573 (or mS5FLS1) and 2 mM ATP in mRAD51 imaging buffer. Under our imaging  
574 conditions, ~78 % of fluorophores in hybrid DNA survives for more than 200 sec.

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

585

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595

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598

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779

780 Figure Legends

781 **Figure 1. Mouse SWI5-SFR1 (mS5S1) stimulates mRAD51 nucleoprotein**  
782 **filament assembly. (A)** Schematic illustration of the RAD51 nucleoprotein assembly  
783 experiments. **(B)** Representative bead BM time-courses of mRAD51 (0.8  $\mu$ M)  
784 assembly on (dT)<sub>135</sub> DNA substrates without mS5S1 (upper), with 1.6  $\mu$ M mS5S1

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

807

808 **Figure 2. Mouse mS5S1 reduces nucleation unit of mRAD51 and increases the**  
809 **ssDNA affinity. (A-B)** RAD51 concentration dependence of filament nucleation  
810 obtained by TPM experiments. Power law fitting to the observed nucleation rates  
811 suggests the nucleation unit of RAD51:  $2.43 \pm 0.46$  for mRAD51 (A) and  $1.67 \pm 0.16$   
812 for mRAD51-S5S1 complex (B). Green open circles in (A) are nucleation rates of  
813 mRAD51 in the presence of excess mS5<sup>FL</sup>S1 mutants, defective in stimulating  
814 mRAD51. mS5S1 & mS5<sup>FL</sup>S1 are in two-fold excess in (A) & (B). (C) ssDNA length  
815 dependence of mRAD51 filament nucleation rate obtained by TPM experiments.  
816 Gapped DNA substrates contain only one 5' ds/ss junction but various lengths of  
817 ssDNA gaps (90-200 nt). As the gapped DNA substrate structure, overall nucleation  
818 rates are fitted to  $k_{ssDNA}^{app}(L_{ssDNA}) + k_{junction}^{app}$ , where  $k_{ssDNA}^{app}$  and  $k_{junction}^{app}$  are  
819 apparent ssDNA-dependent nucleation rate constant and apparent ds/ss  
820 junction-dependent nucleation rate constant. (Red) 0.8  $\mu\text{M}$  mRAD51 only and (blue)  
821 mixture of 0.8  $\mu\text{M}$  mRAD51 and 1.6  $\mu\text{M}$  mS5S1. All experiments were carried out at  
822 2 mM ATP.

823

824 **Figure 3. Single-molecule FRET experiments demonstrate that mS5S1 stabilizes**  
825 **mRAD51 nucleating clusters.** (A) Schematic illustration of single-molecule  
826 fluorescence resonance energy transfer (smFRET) experimental setup. mRAD51  
827 assembles onto (dT)<sub>13</sub> ssDNA results in the FRET decrease due to the increase of dye  
828 pair separation. (B–F) Single-molecule FRET observation of mRAD51 nucleating  
829 cluster dynamics. Exemplary FRET time trace of (B) mRAD51 and (C)  
830 mRAD51-S5S1 complex assembling on (dT)<sub>13</sub> ssDNA substrate. High FRET state  
831 (~0.8) corresponds to a DNA-only state, and low FRET state (0.0~0.6) corresponds to  
832 the mRAD51-bound state. (D–E) Transition density plots (TDP) clearly reflect 4 states  
833 (without mS5S1) and 5 states (with mS5S1) in mRAD51 nucleating cluster dynamics.  
834 (F) Rate constants of mRAD51 nucleating cluster dynamics in the absence (empty bar)  
835 and presence (solid bar) of mS5S1. Error bar of binding and dissociation rates were  
836 the standard deviation of the mean by bootstrapping 5000 times.

837

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

850

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

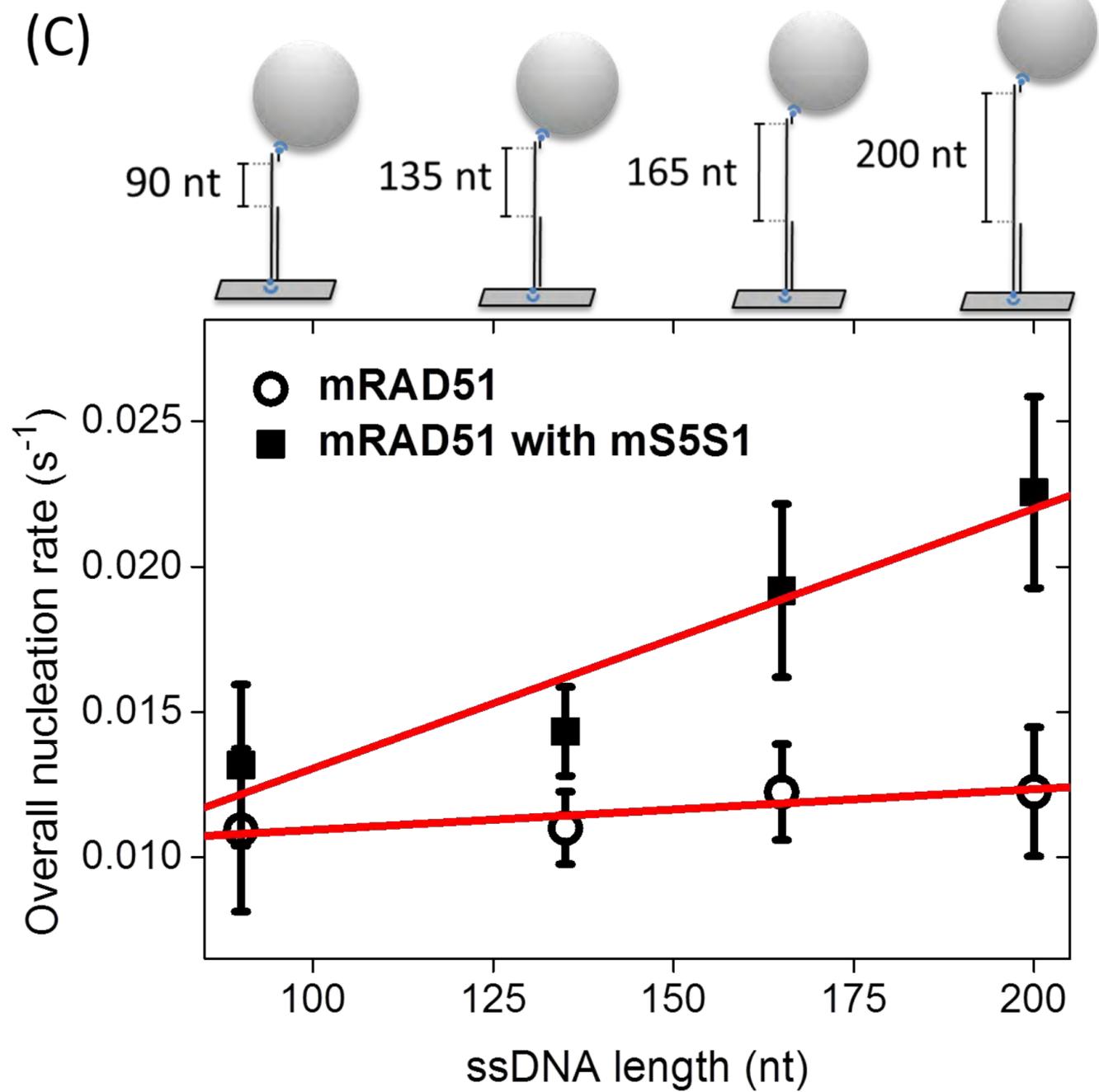
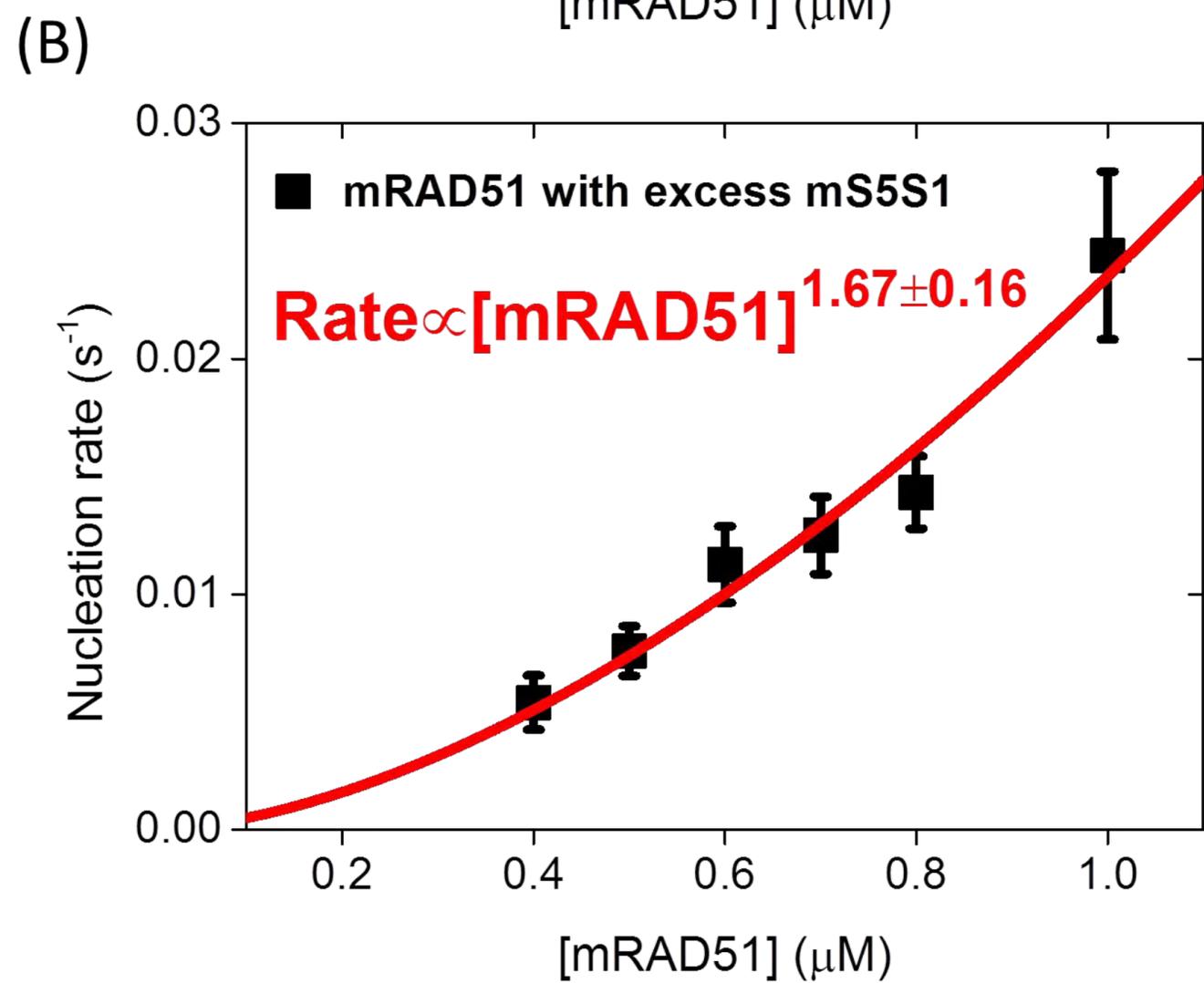
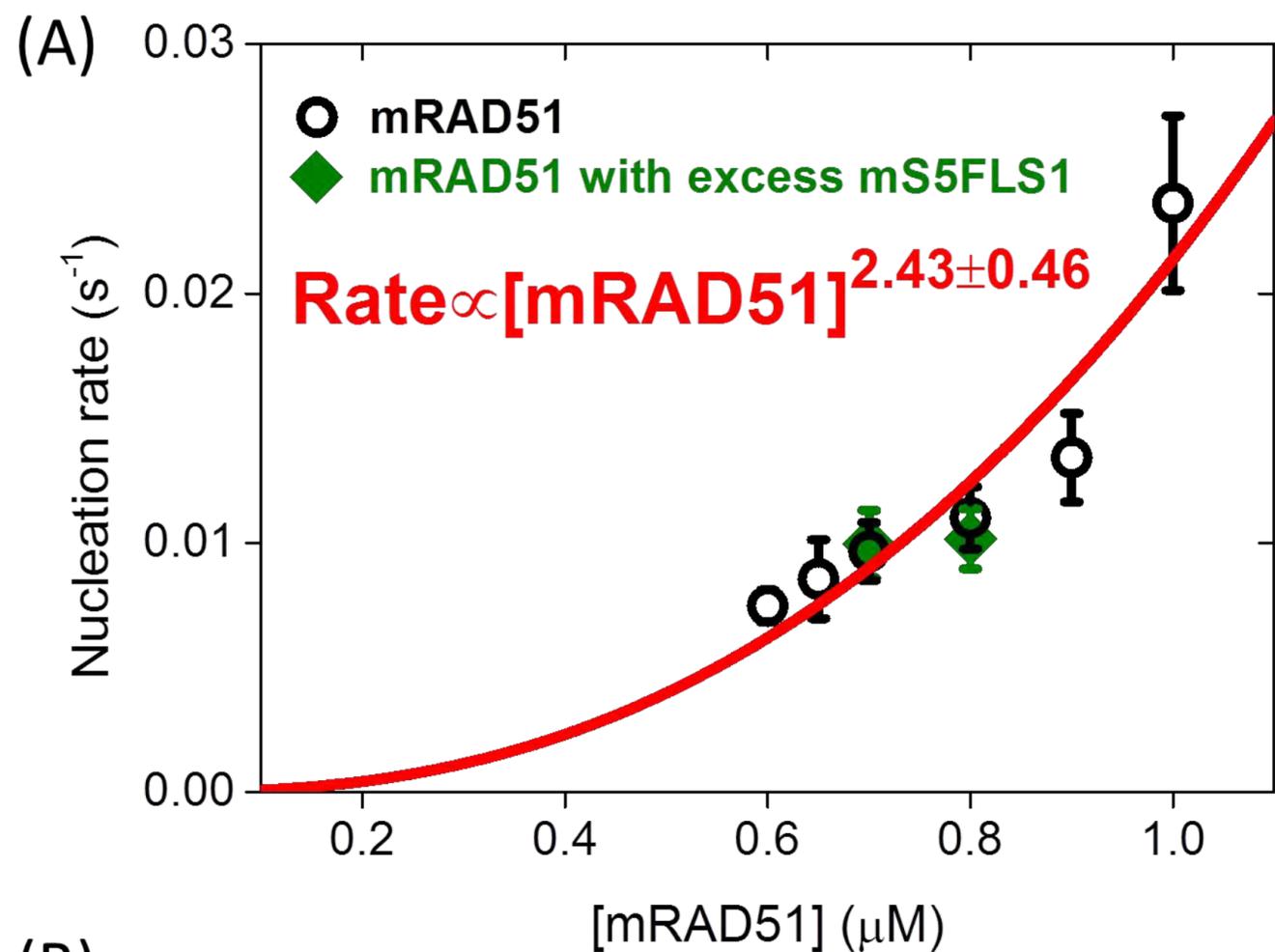
861 mRAD51 in the presence of various mS5S1 concentrations and nucleotide conditions.  
862 **(F)** Representative bead BM time-courses of SpRad51 disassembly without SpS5S1  
863 (top) and in the presence of 0.3  $\mu$ M SpS5S1 (bottom). **(G-I)** Kinetic parameters for  
864 fission yeast. The fraction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

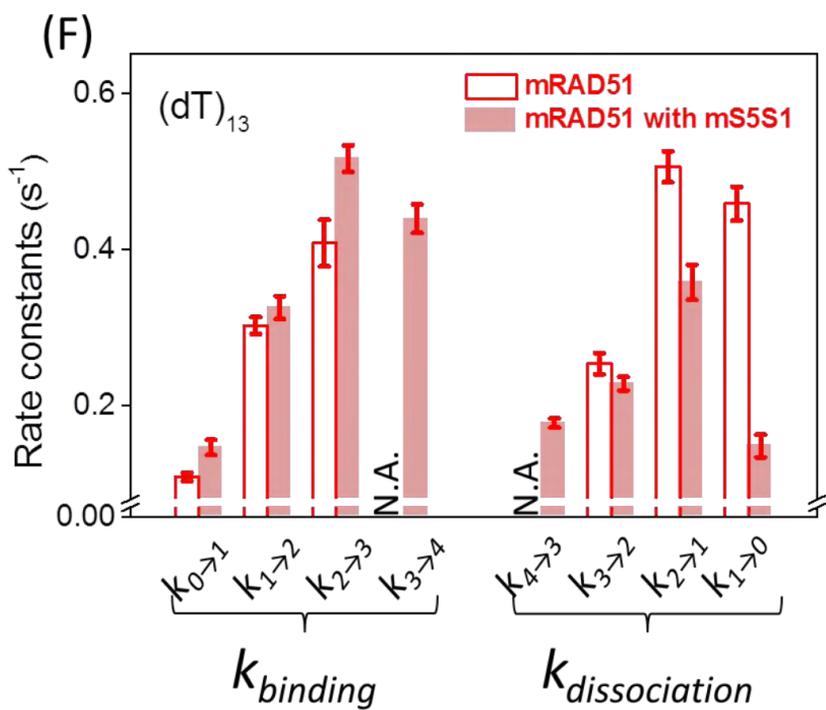
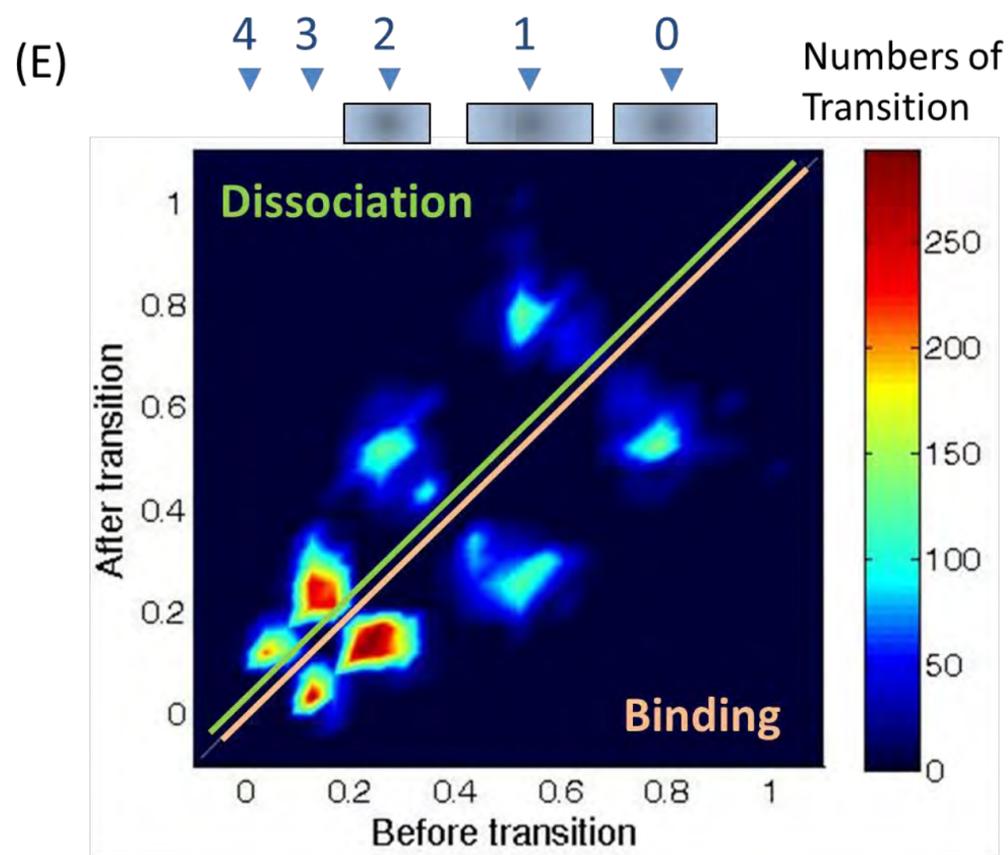
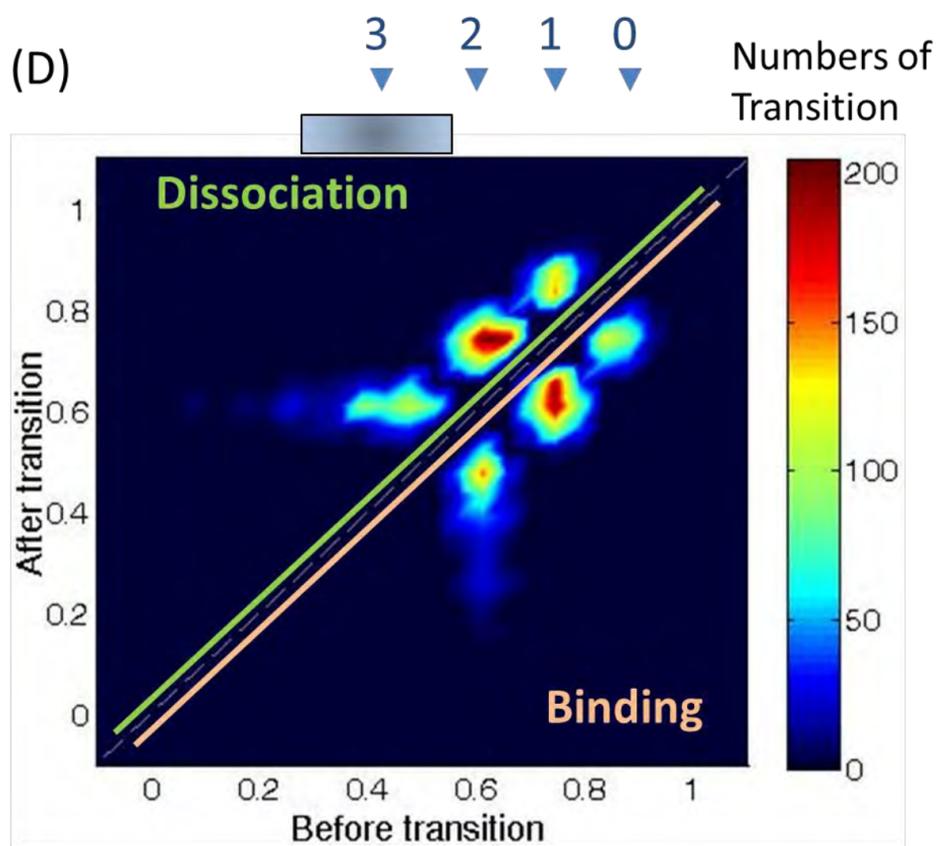
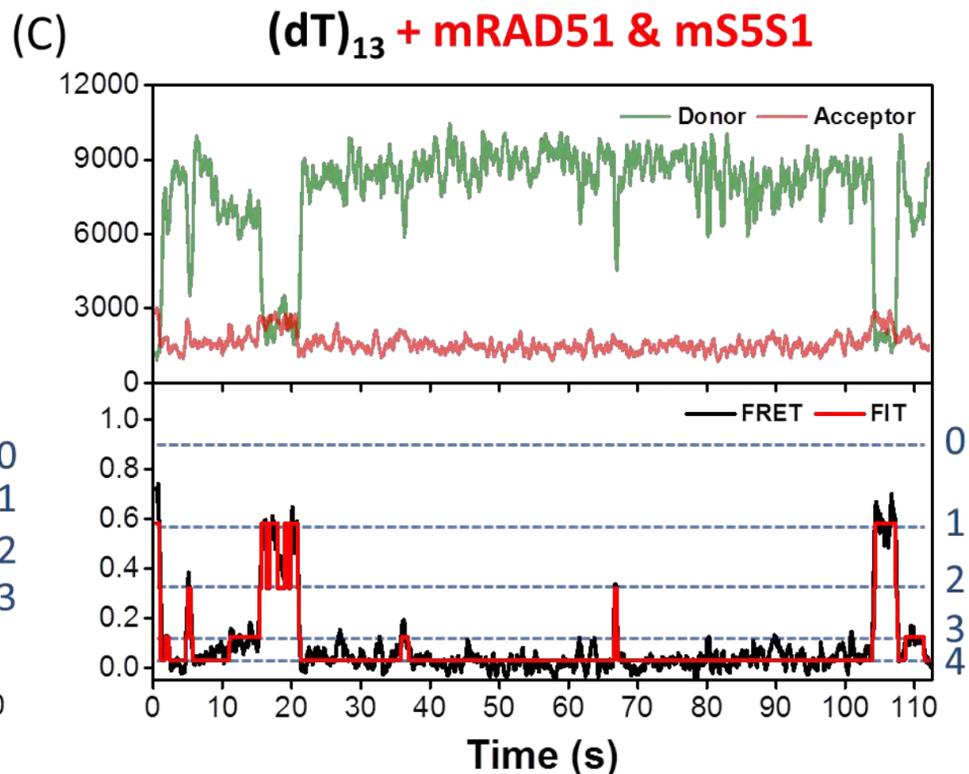
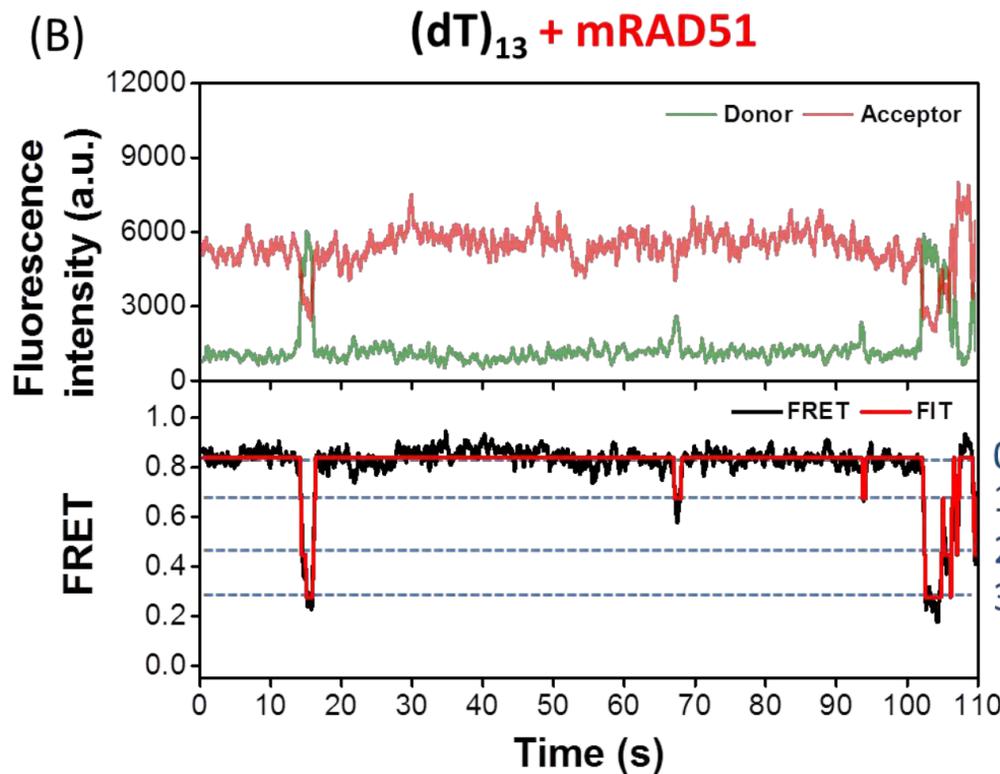
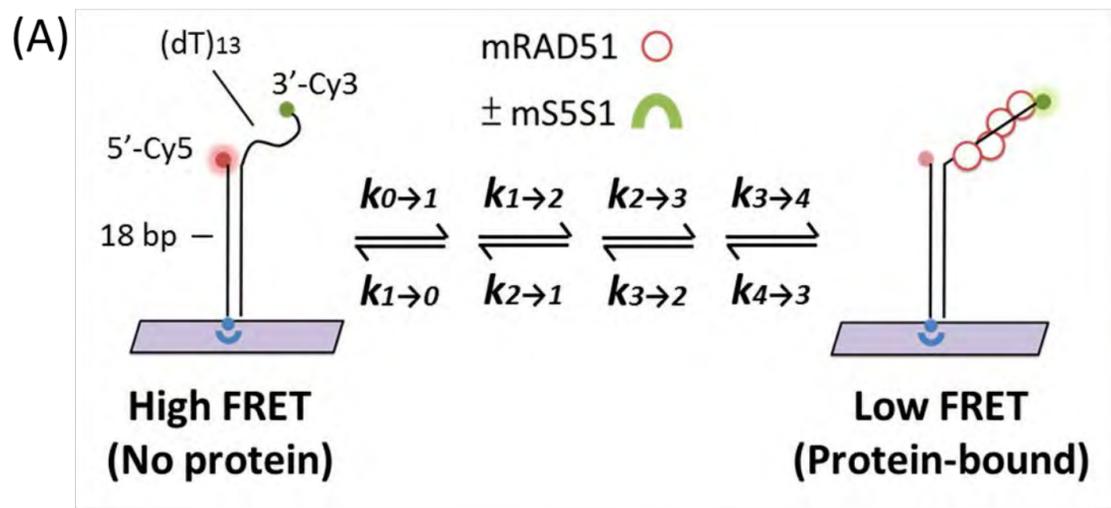
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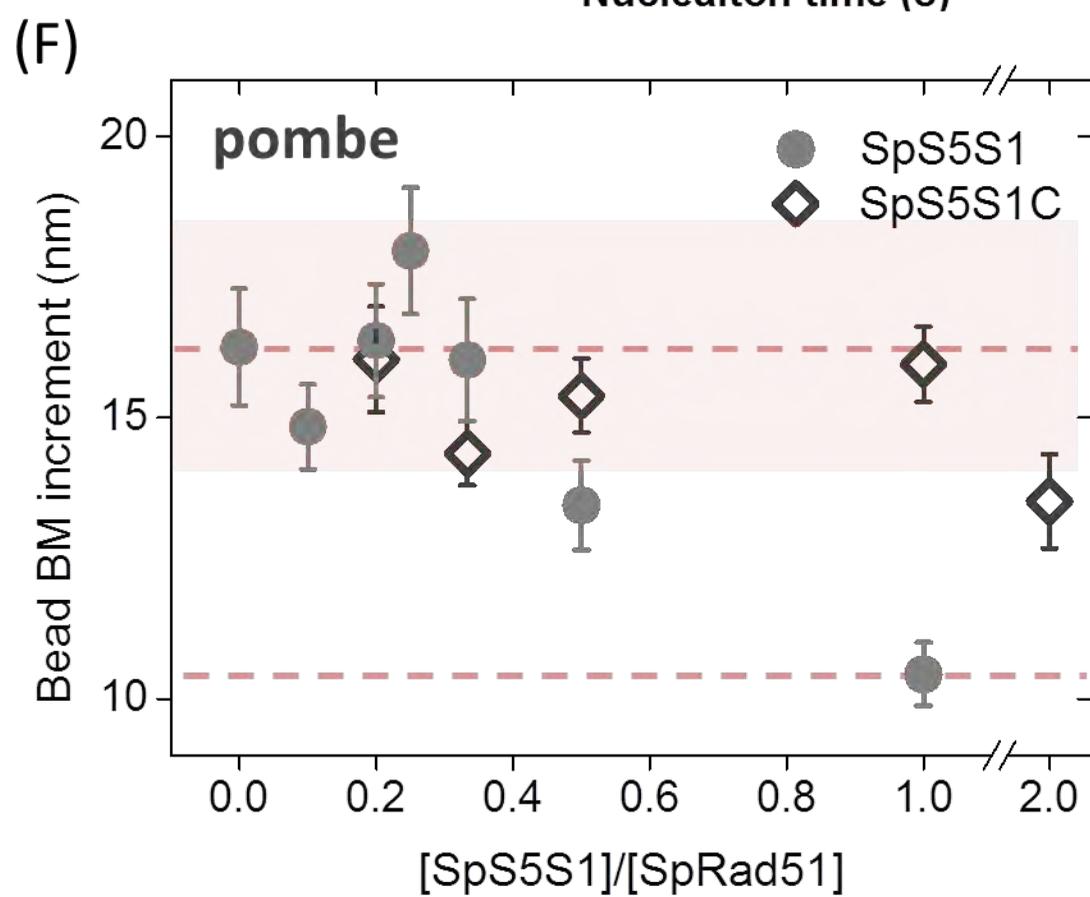
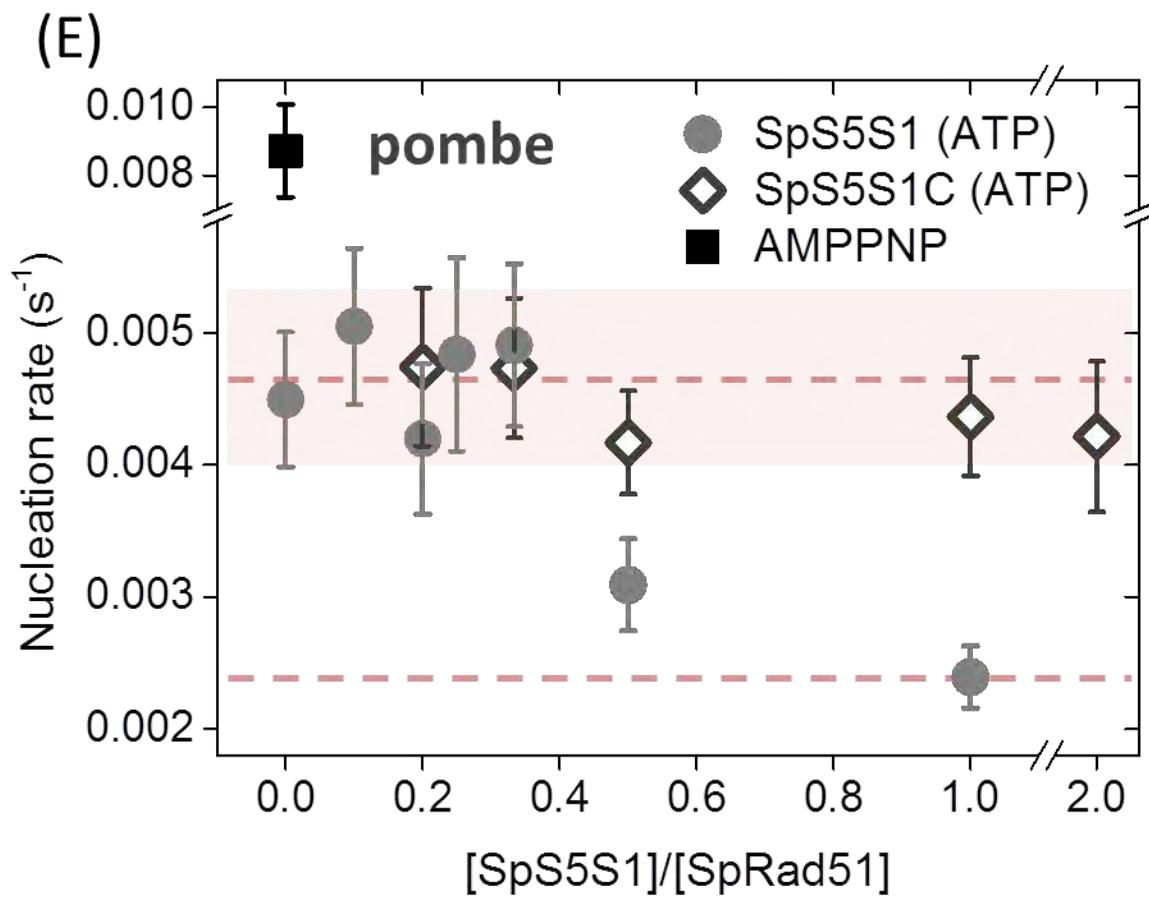
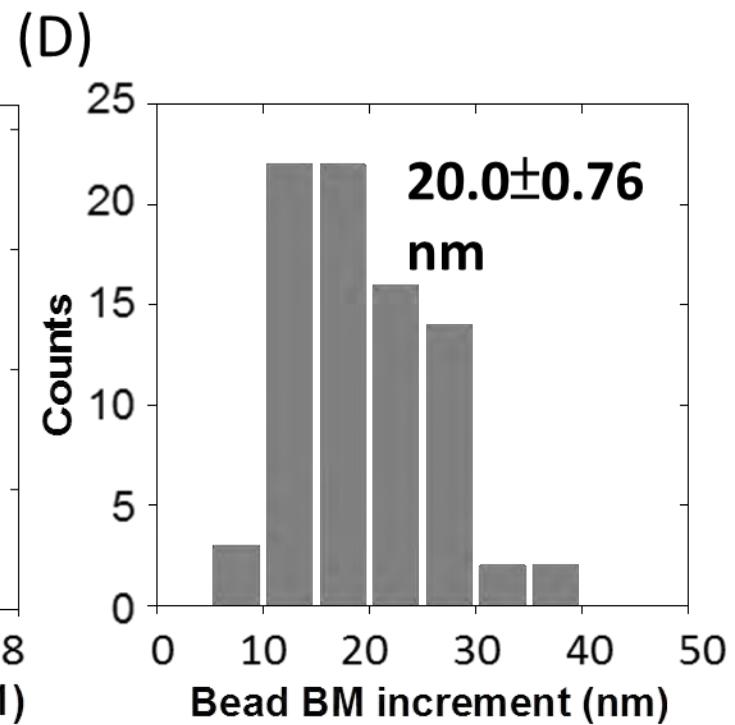
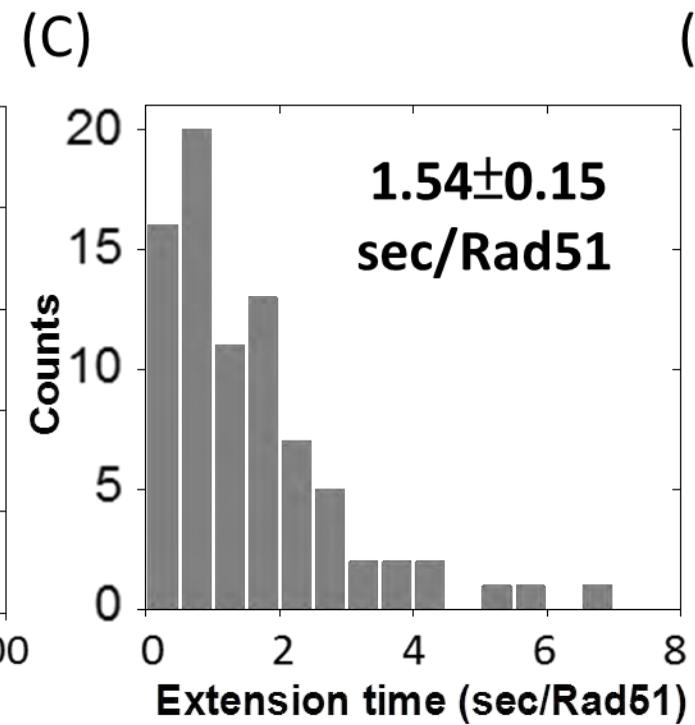
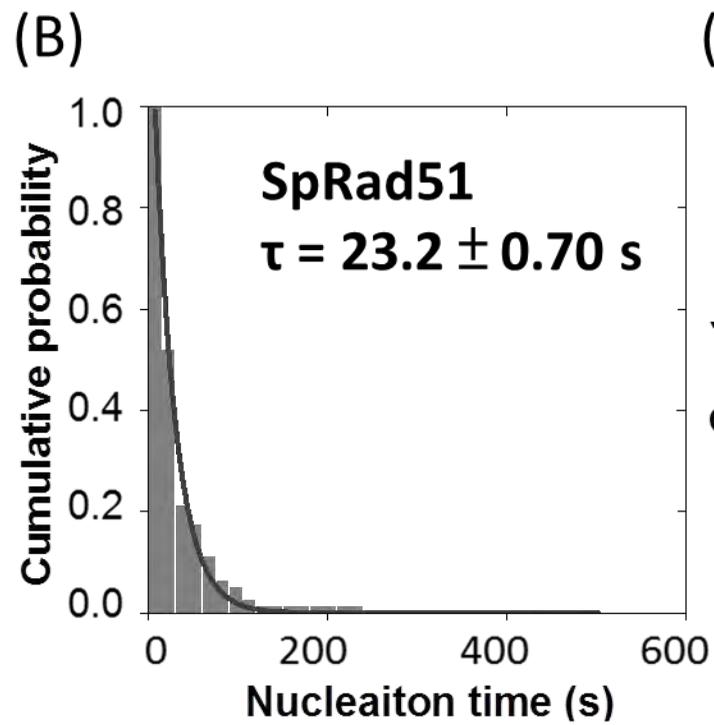
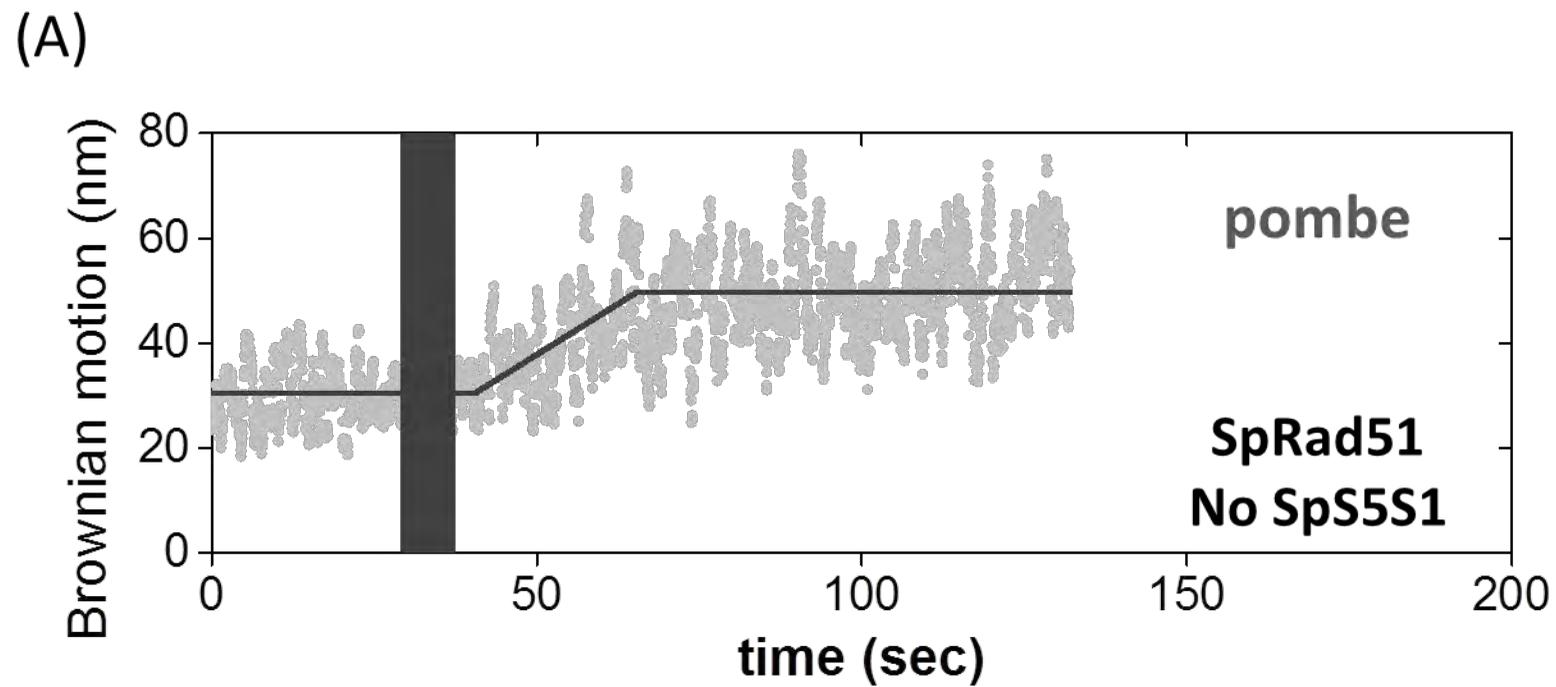
869 **Figure 6. Proposed models for regulating Rad51 nucleoprotein filament**  
870 **formation by the S5S1 complex.** Swi5-Sfr1 stabilizes Rad51 on ssDNA primarily by  
871 preventing its dissociation. This stabilization effect leads to a stable nucleating cluster  
872 formation and a reduction in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

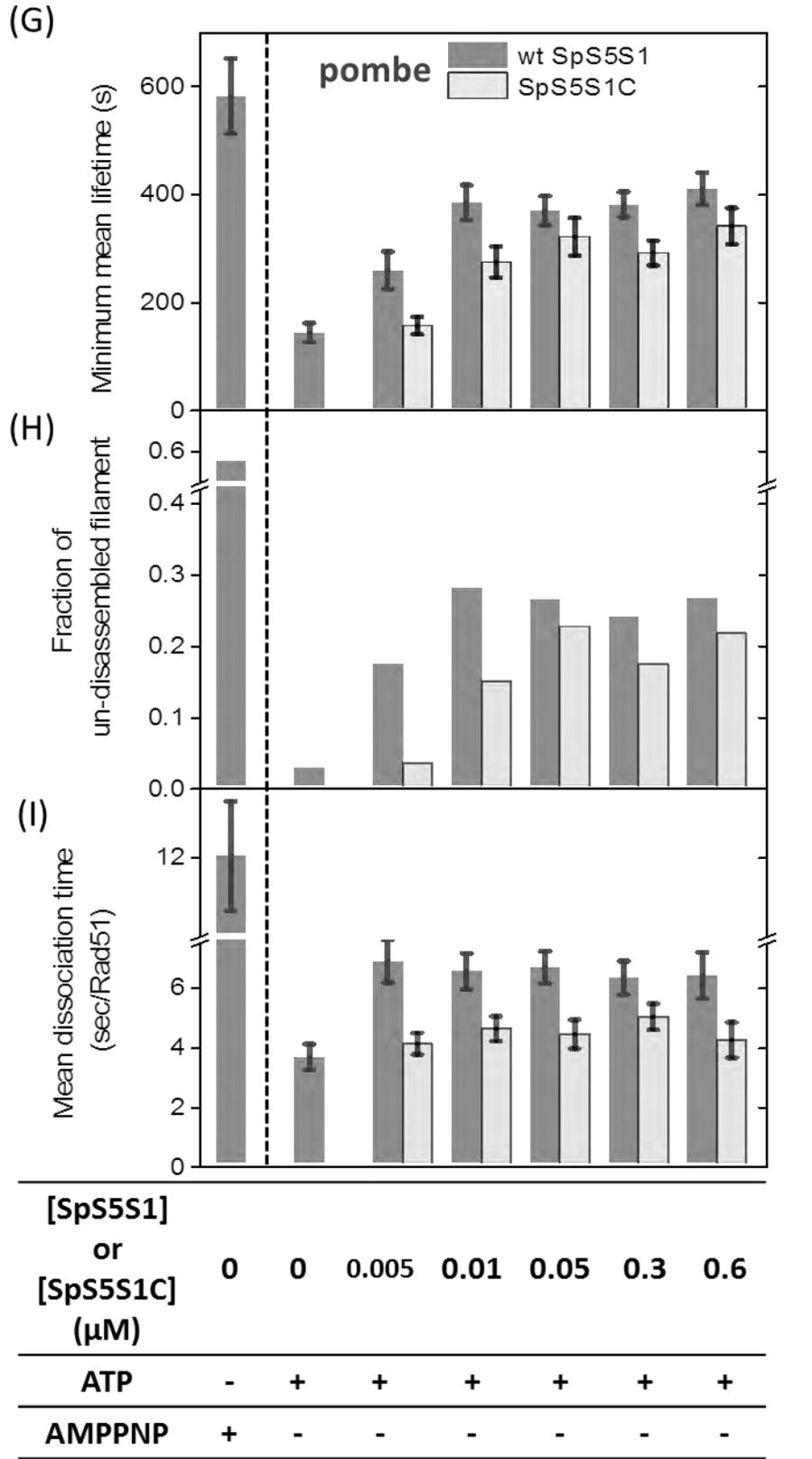
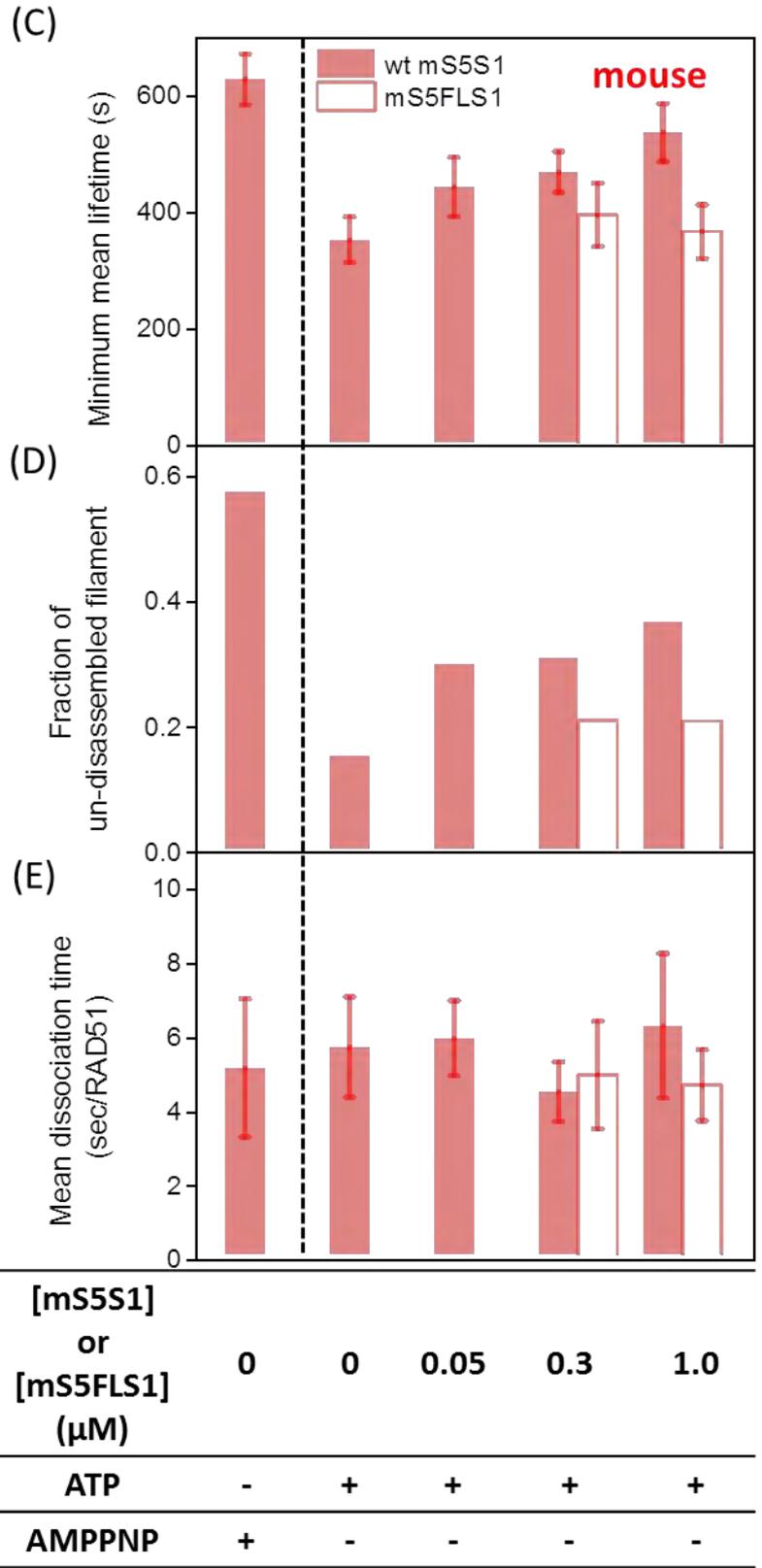
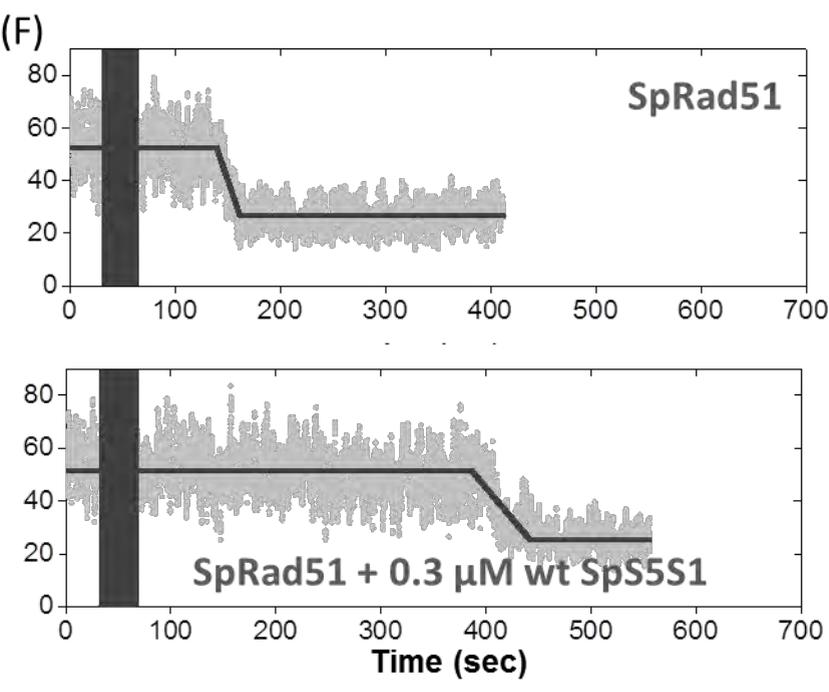
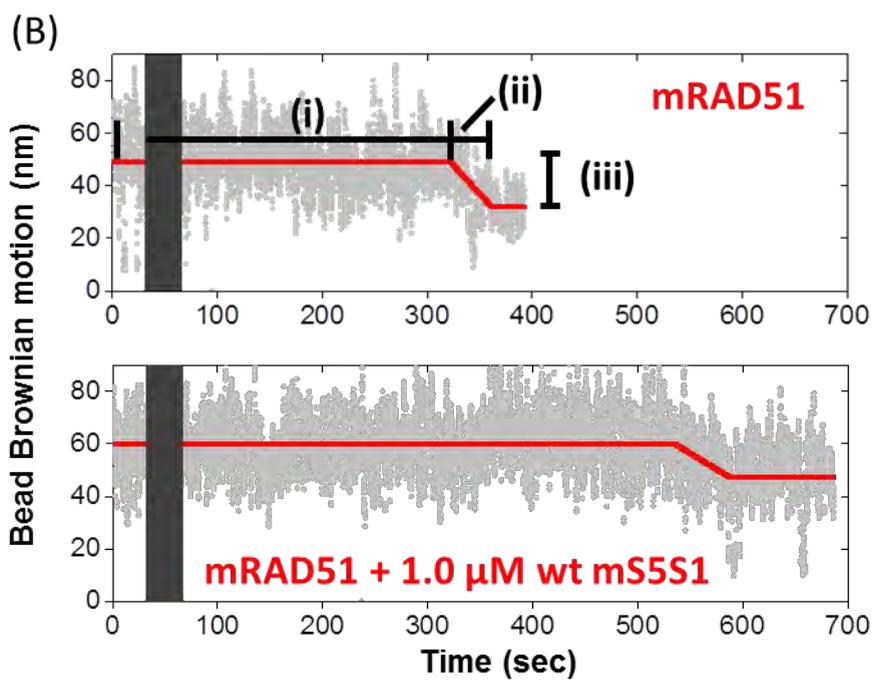
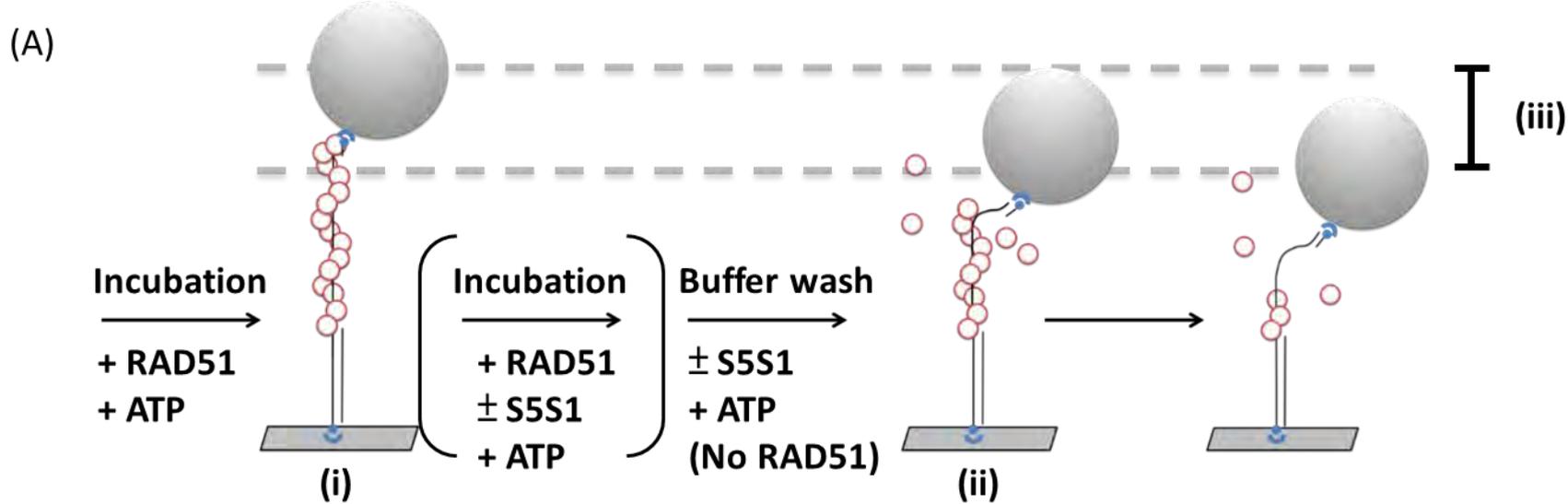
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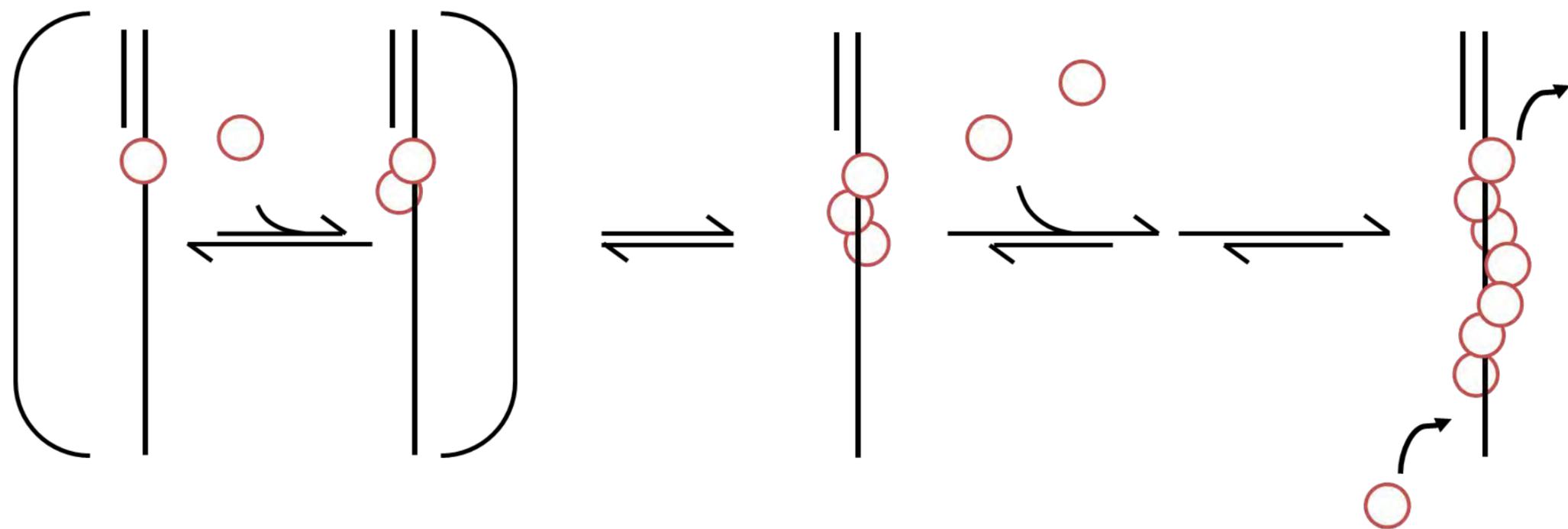




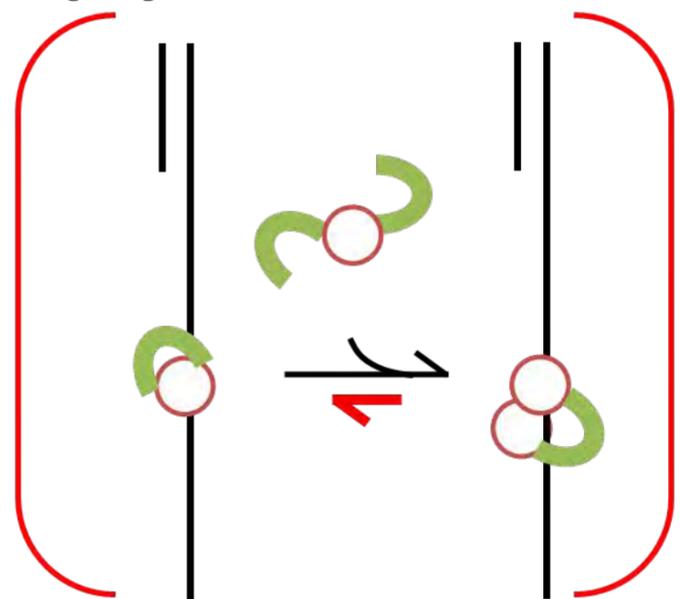
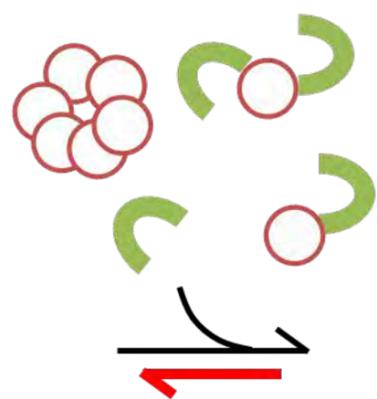
○ Rad51

⤿ 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**

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### **This PDF file includes:**

Supplementary text

SI Materials and Methods

Figures S1 to S11

Tables S1 to S10

References for SI reference citations

**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

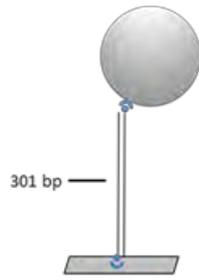
**DNA substrates.** The (dT)<sub>135</sub> gapped DNA substrate for TPM assembly and disassembly experiments contains 135 nt poly dT sandwiched by a 151 bp, 5'-digoxigenin-labeled dsDNA handle and a 5'-biotin-labeled 19 bp handle. To prepare the (dT)<sub>135</sub> gapped DNA, we first used auto-sticky 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'- ATCGGTTCGACGCTCTCCC TT/idSp/TGCGACTCCTGCATTAGGAA) using pBR322 as template. Oligos containing 135 thymidylates (5'-AAGGGAGAGCGTCGACCGAT(T)<sub>135</sub>CTTACTGTCATGCCATCCG) 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)<sub>135</sub> gapped DNA. (dT)<sub>90</sub>, (dT)<sub>100</sub>, (dT)<sub>165</sub> and (dT)<sub>200</sub> gapped DNA substrates were made with the same procedure as (dT)<sub>135</sub> DNA preparation, but using oligos with various lengths of (dT)<sub>n</sub> (n=90, 100, 165 and 200) (Figure S9). Oligos were purchased from Gene Link (oligos containing (dT)<sub>165</sub> and (dT)<sub>200</sub>), Integrated DNA Technologies (the primer with one abasic site and oligos containing (dT)<sub>90</sub>, (dT)<sub>100</sub> and (dT)<sub>135</sub>) 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)<sub>n</sub>/Cy3-3') in the buffer containing 20 mM Tris and 0.5 M NaCl at pH = 8.

**Proteins and buffers.** mRAD51, mS5S1, mS5<sup>FL/AA</sup>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.

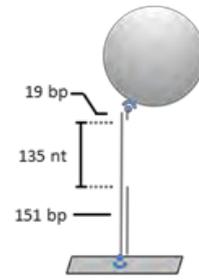
**Detailed experimental procedures of single-molecule tethered particle motion (TPM) assembly experiment.** For S5S1 titration experiments (Figure 1 and 4), 0.8 μM mouse RAD51 (or 0.3 μM fission yeast Rad51) was pre-incubated with the indicated concentrations of mouse S5S1 (or fission yeast S5S1) in 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  $\mu\text{M}$  mRAD51 plus 1.6 or 2.0  $\mu\text{M}$  (2-fold excess) mS5S1 (Figure 2B) were pre-incubated in corresponding buffers for 10-15 min at 37°C before experiments. For binding preference determination (Figure 2C & S9B), 0.8  $\mu\text{M}$  mRAD51, 0.8  $\mu\text{M}$  mRAD51 plus 1.6  $\mu\text{M}$  mS5S1 or 0.5  $\mu\text{M}$  SpRad51 in corresponding reaction buffers were incubated for 10-15 min at 37°C.

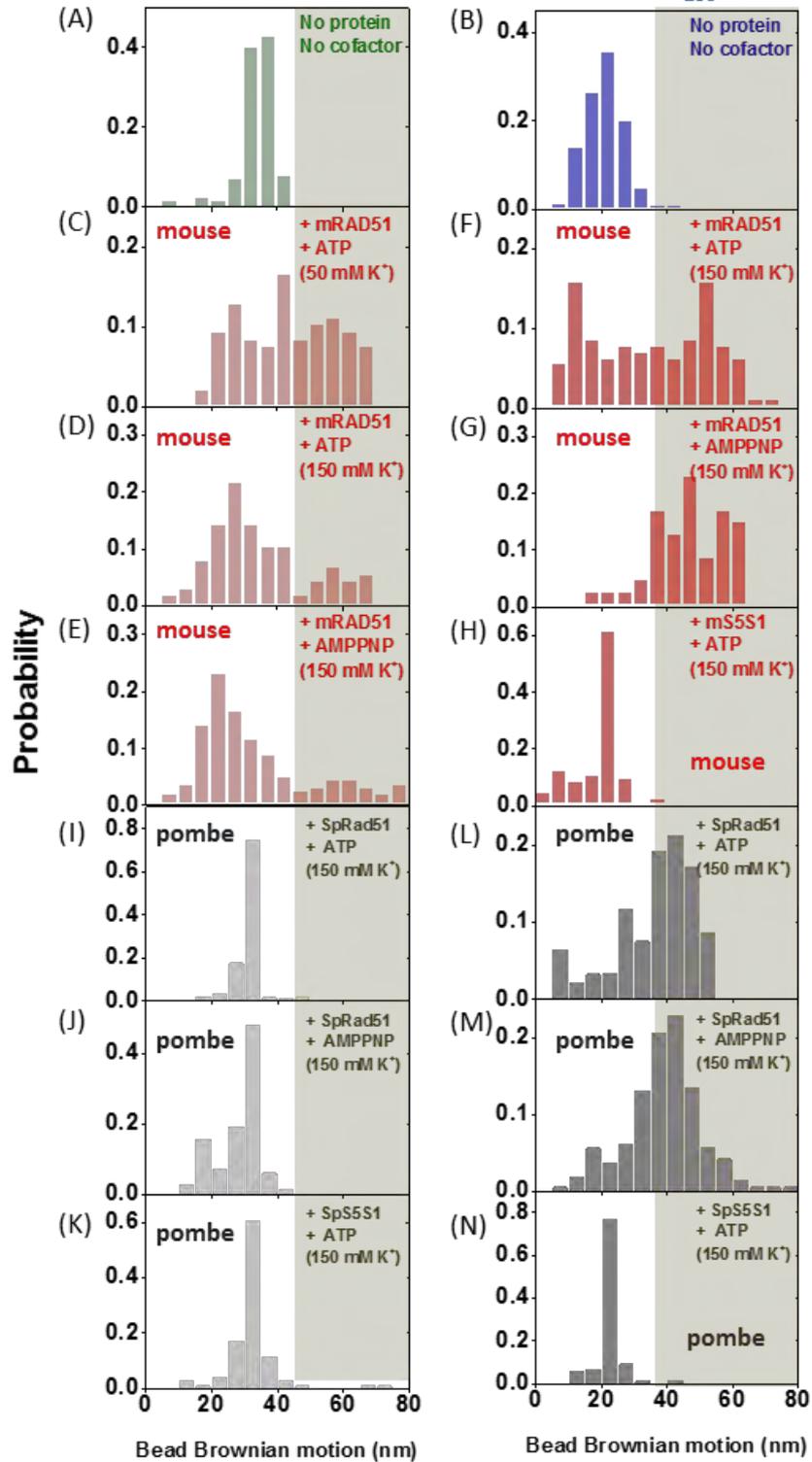
**Single-molecule TPM disassembly experiment and data analysis.** In disassembly experiments, we used silanized glass slide to avoid extensive surface-protein interaction. To prepare the silanized surface, the glass slides were sequentially sonicated in 2 M KOH for 5 min, 99 % ethanol for 15-20 min and ddH<sub>2</sub>O for 15-20 min. After these sonication steps, slides were rinsed with ddH<sub>2</sub>O and dried with N<sub>2</sub> gas. Glass slides were then functionalized in a solution containing 1,7-dichloro-octamethyltetrasiloxane (Sigma-Aldrich) in 99 % ethanol in the dark overnight at room temperature. Slides were then rinsed with 99 % ethanol and ddH<sub>2</sub>O alternatively and dried with N<sub>2</sub>. Surface-bound (dT)<sub>135</sub> gapped DNA substrates were pre-incubated with mixtures of either 0.8  $\mu\text{M}$  mRAD51-2 mM ATP or 0.8  $\mu\text{M}$  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  $\mu\text{M}$  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).



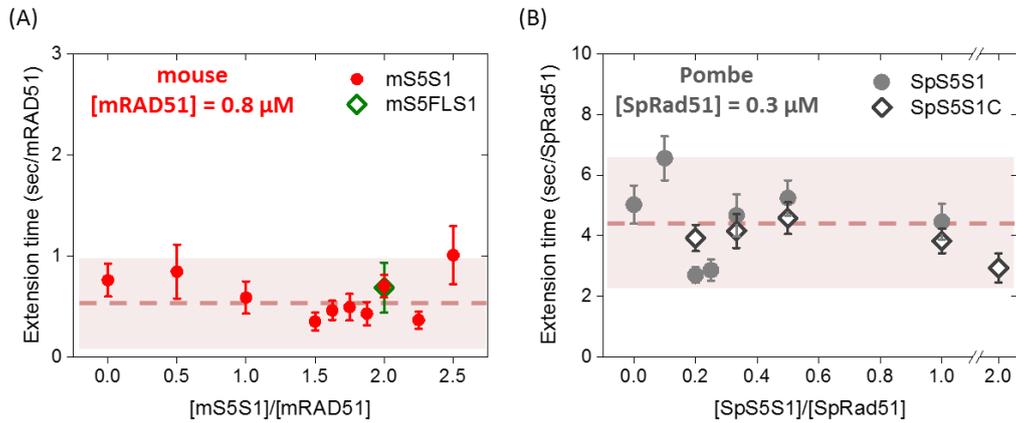
301 bp dsDNA



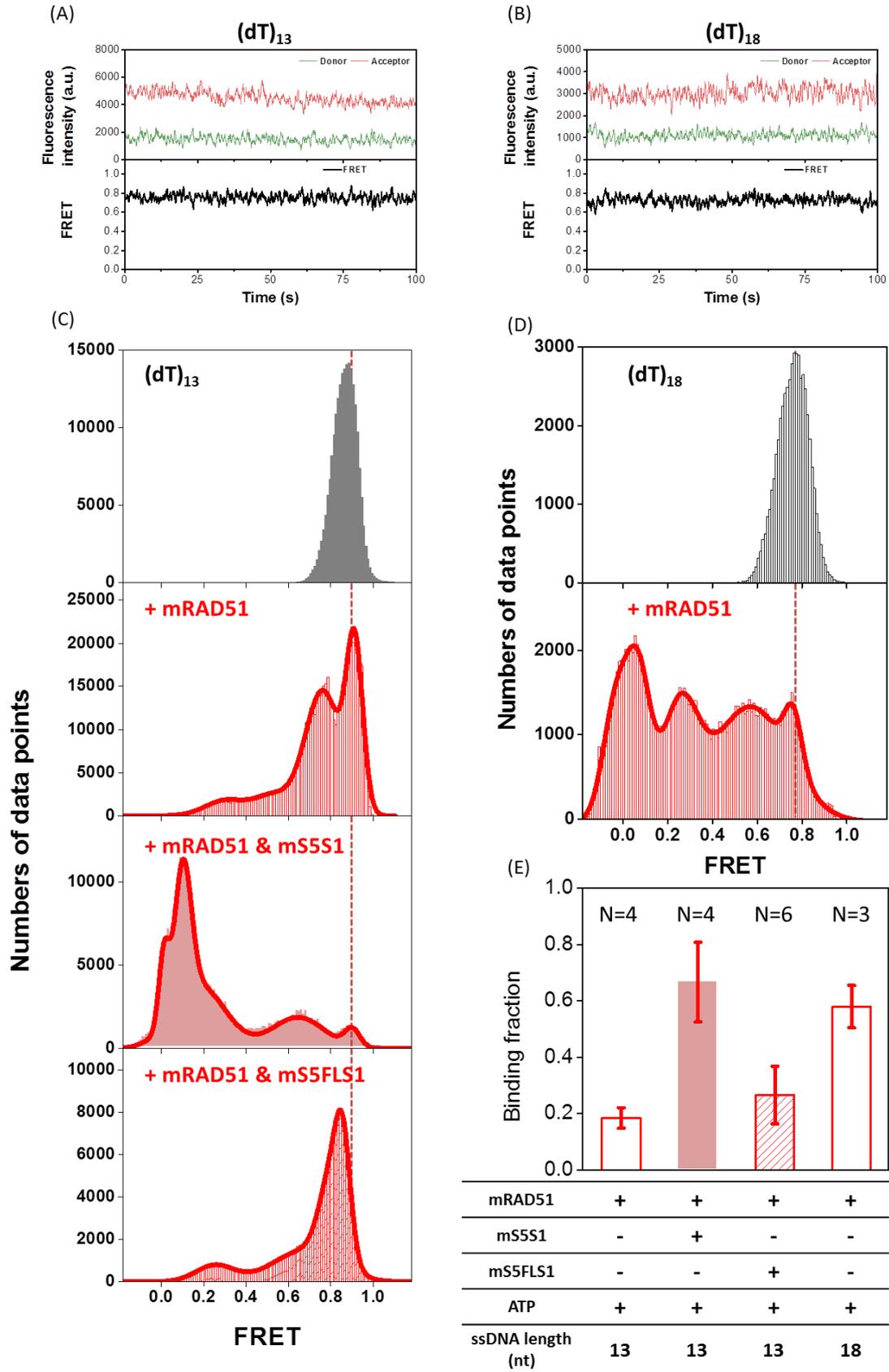
gapped (dT)<sub>135</sub> DNA



**Figure S1. Bead BM histograms of RAD51/S5S1 assembly on either 301 bp dsDNA or (dT)<sub>135</sub> gapped DNA.** Brownian motion (BM) amplitudes of **(A)** bare 301 bp dsDNA and **(B)** bare (dT)<sub>135</sub> gap DNA substrates are about 35.2±3.77 and 21.3±5.78 nm, respectively. BM higher than 35 nm (for (dT)<sub>135</sub> 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)<sub>135</sub> 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)<sub>135</sub> 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.

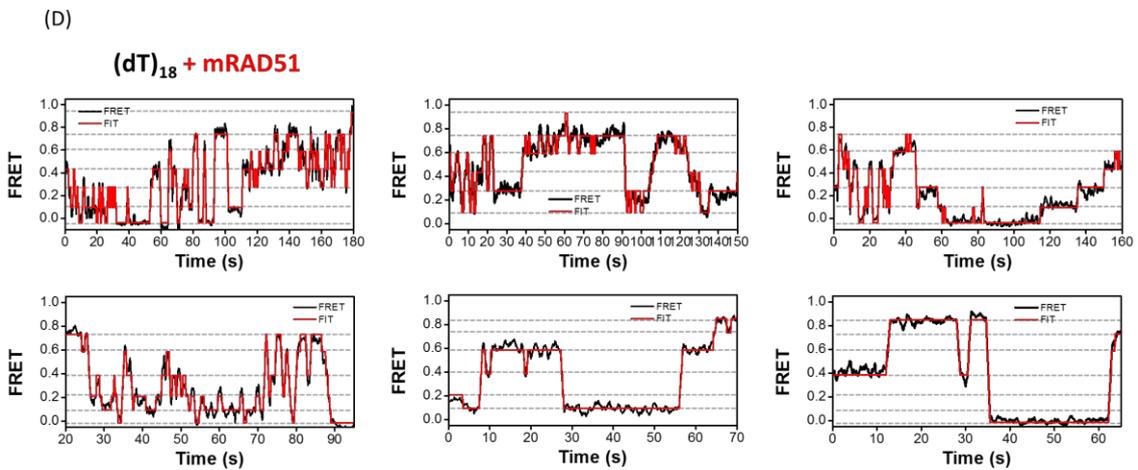
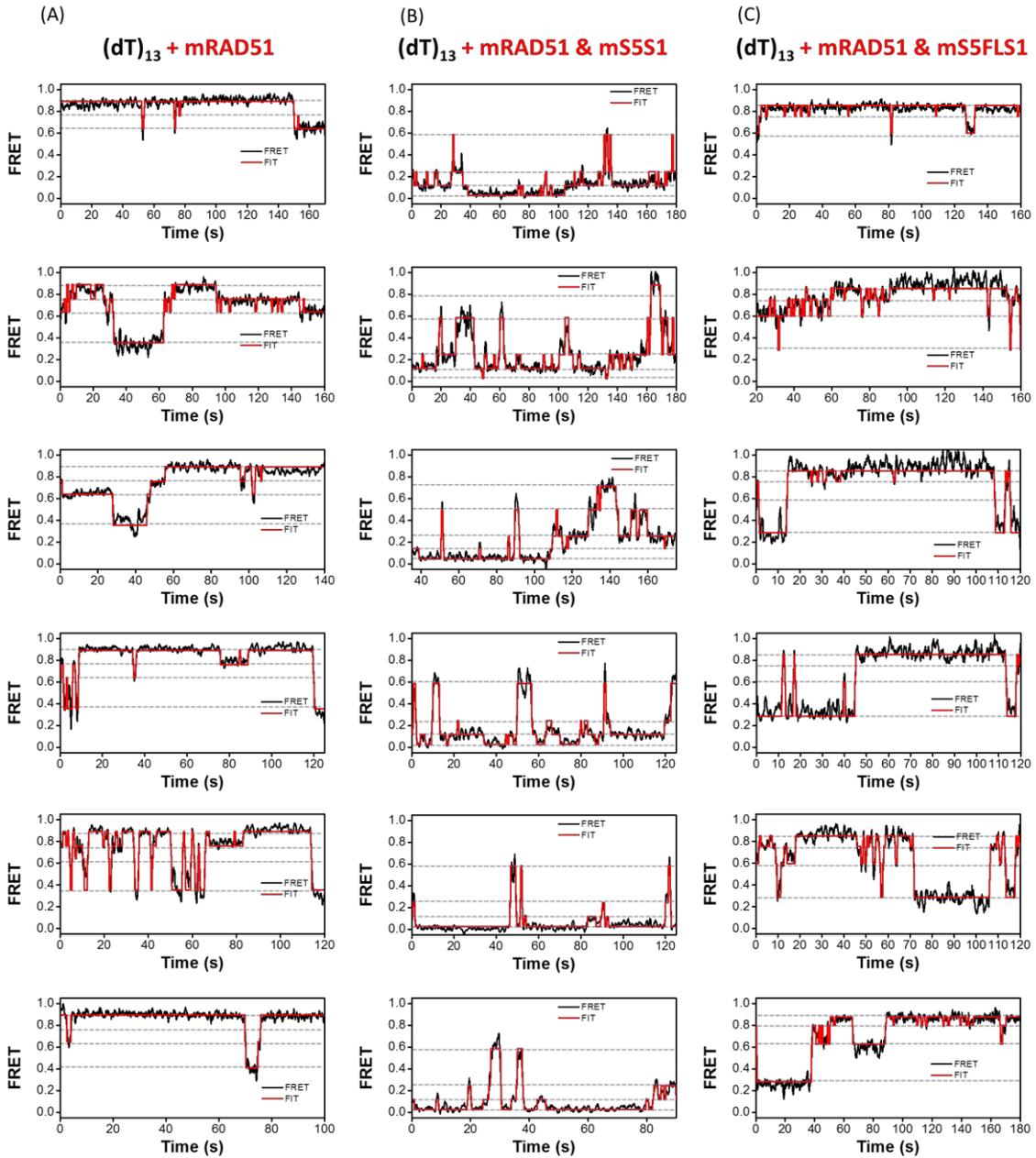


**Figure S2. S5S1 shows negligible effects on Rad51 extension time in both two species.** At fixed Rad51 concentrations (0.8  $\mu\text{M}$  for mRAD51 and 0.3  $\mu\text{M}$  for SpRad51), Rad51 extension times of both (A) mouse and (B) pombe display no apparent dependence on S5S1 concentration. Dash 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.

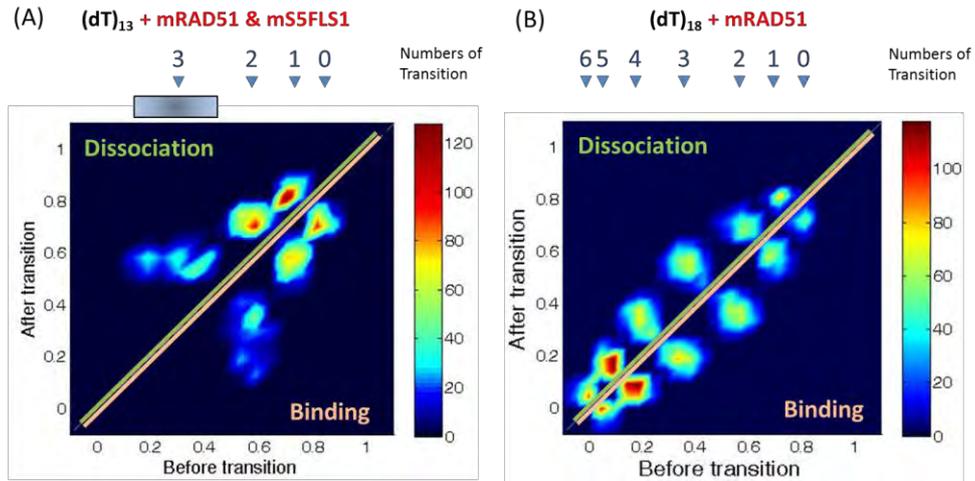


**Figure S3. FRET histograms and binding fraction of mRAD51 assembling under different conditions. (A) & (B)** In the absence of proteins, DNA molecules exist in

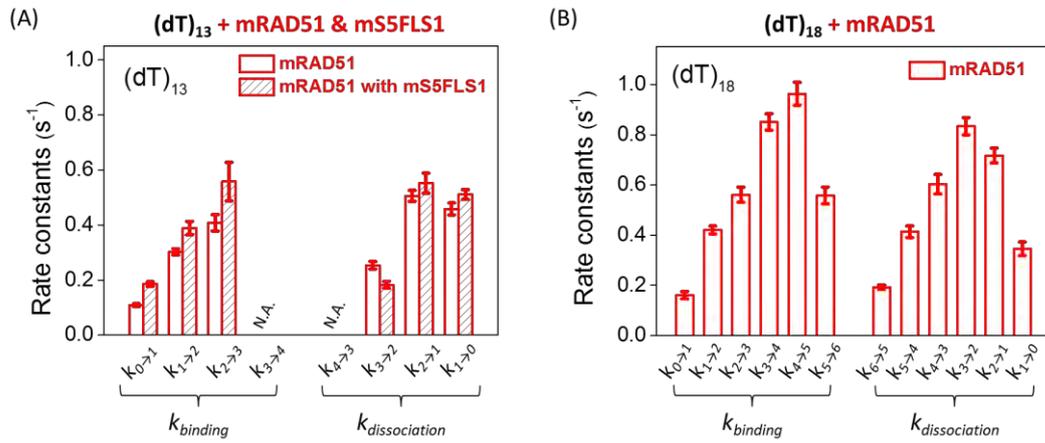
the high FRET state owing to the flexibility of ssDNA region. Both bare DNA substrates of different ssDNA lengths exhibit a high FRET state ( $E \sim 0.7-0.8$ ). **(C)** FRET histograms of  $(dT)_{13}$  DNA substrate in the absence of mRAD51 (top panel); in the presence of mRAD51 (second panel from top); in the presence of mRAD51 and mS5S1 mixture (third panel from top) or in the presence of mRAD51 and mS5<sup>FL</sup>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  $18.5 \pm 3.62\%$ , and that of mRAD51 and mS5S1 mixture is  $66.6 \pm 14.0\%$ . mRAD51 and mS5<sup>FL</sup>S1 mixture gives a binding fraction of  $26.6 \pm 10.2\%$ , which is similar to that of mRAD51-only case. mRAD51 concentration is  $1.0 \mu\text{M}$ , mS5S1 and mS5<sup>FL</sup>S1 concentration are  $2.0 \mu\text{M}$ . At longer  $(dT)_{18}$  DNA substrates, the binding fraction is  $58.0 \pm 7.51\%$ .  $N$  indicates the number of independent experiments. Dash lines were drawn for guidance purpose.



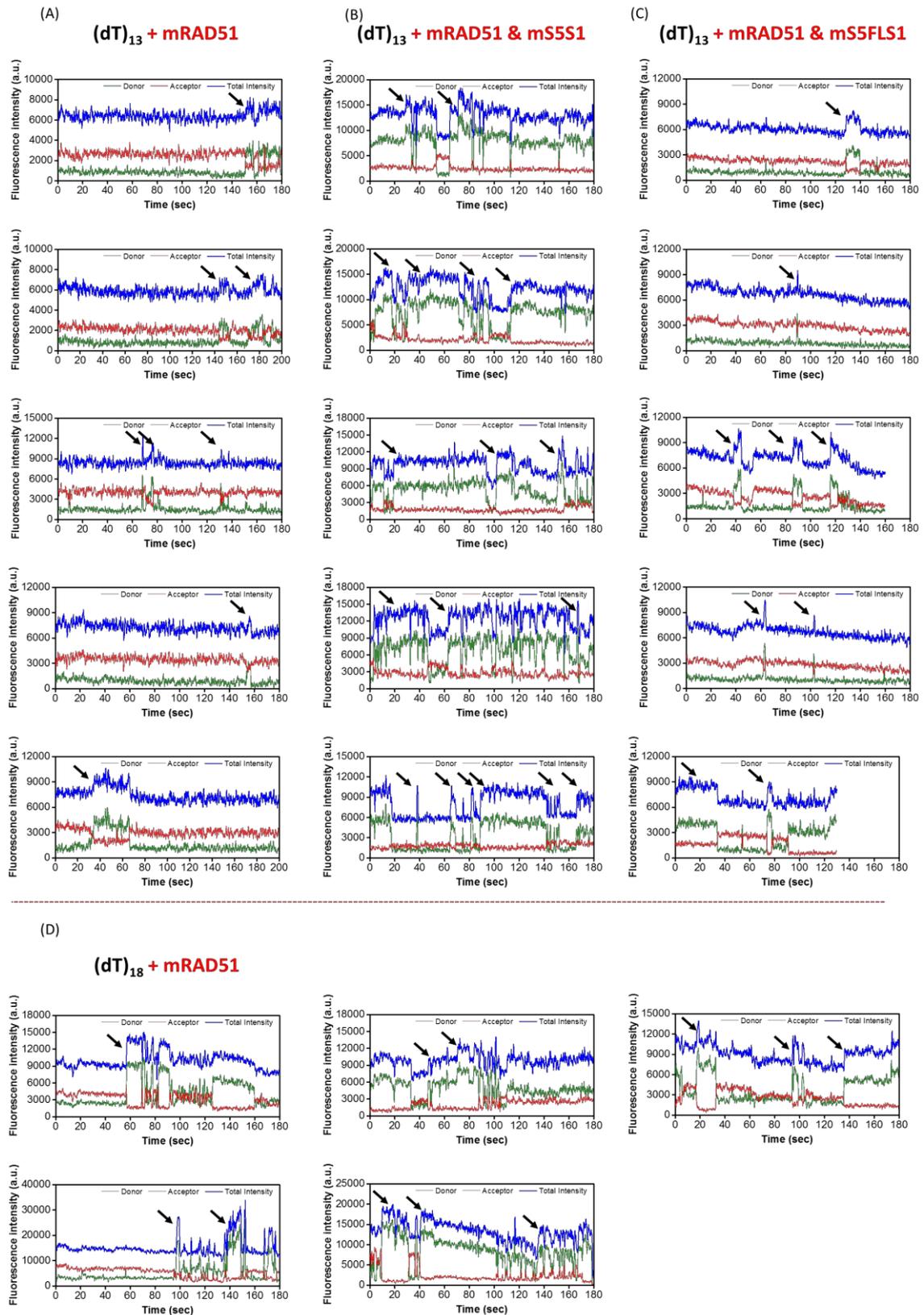
**Figure S4. Representative FRET time traces of mRAD51 assembling under three different conditions: (A)** on (dT)<sub>13</sub> substrate; **(B)** on (dT)<sub>13</sub> substrate in the presence of 2 μM mS5S1; **(C)** on (dT)<sub>13</sub> substrate in the presence of 2 μM mS5<sup>FL</sup>S1; **(D)** on (dT)<sub>18</sub> substrate.



**Figure S5. Transition density plot of mRAD51 assembly on  $(dT)_{13}$  substrate in the presence of  $mS5^{FLS1}$  and  $(dT)_{18}$  substrate in the absence of  $mS5S1$ . (A) Adding  $mS5^{FLS1}$  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.**

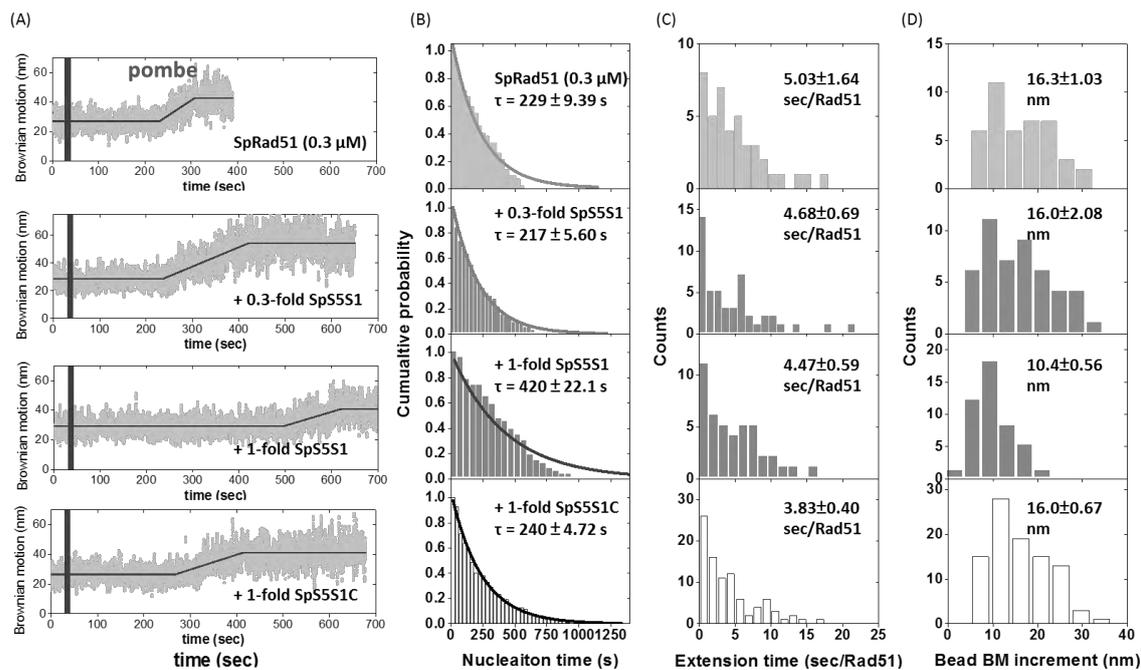


**Figure S6. Rate constants of mRAD51 assembling on (A) on (dT)<sub>13</sub> substrate in the presence of mS5<sup>FL</sup>S1 and (B) (dT)<sub>18</sub> DNA substrate in the absence of mS5S1.**

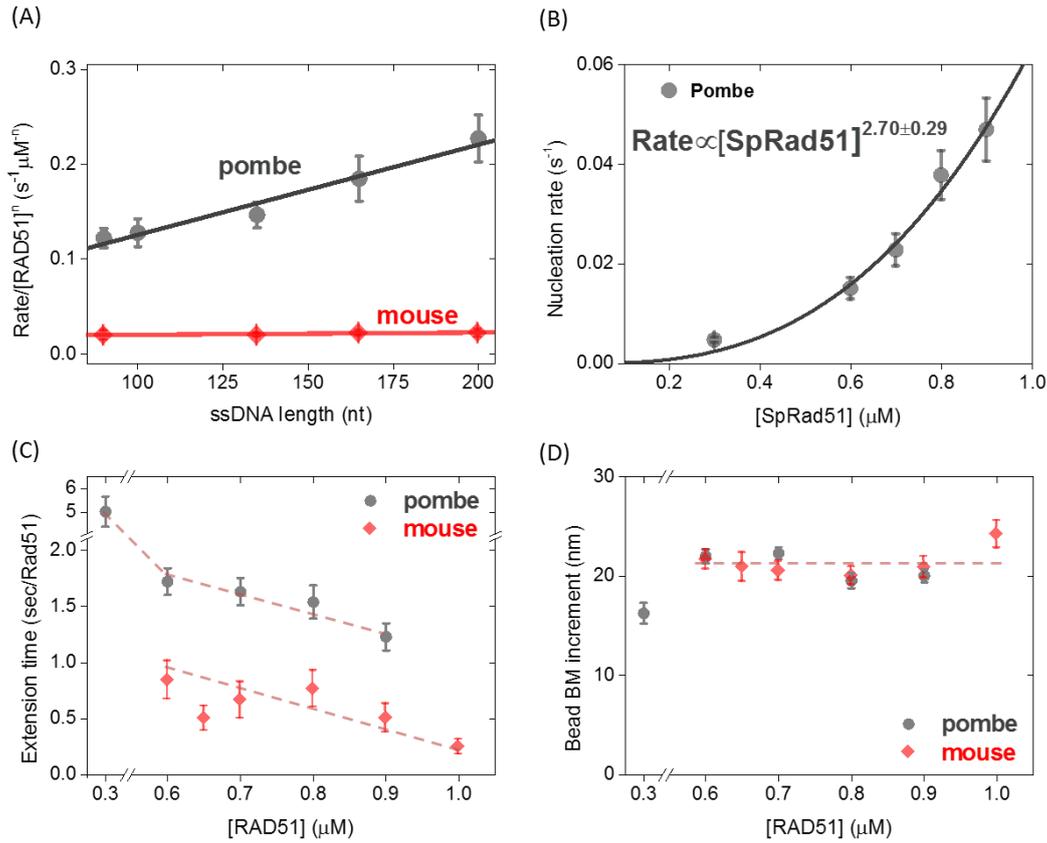


**Figure S7. Protein-induced fluorescence enhancement (PIFE) effects are more apparent as mRAD51 assembles on (dT)<sub>13</sub> substrate in the presence of mS5S1 or on longer (dT)<sub>18</sub> substrate. Representative fluorescence intensity time traces of**

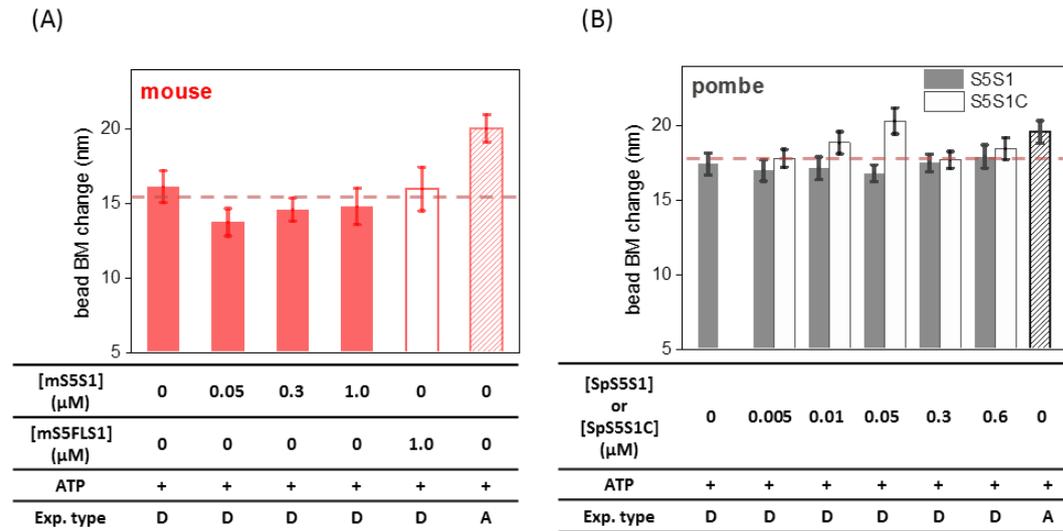
mRAD51 assembling under three different conditions: **(A)** on (dT)<sub>13</sub> substrate; **(B)** on (dT)<sub>13</sub> substrate in the presence of 2 μM mS5S1; **(C)** on (dT)<sub>13</sub> substrate in the presence of 2 μM mS<sup>FL</sup>S1; **(D)** on (dT)<sub>18</sub> substrate. Black arrows indicate the increase in total fluorescence intensity owing to the Cy3 PIFE effect.



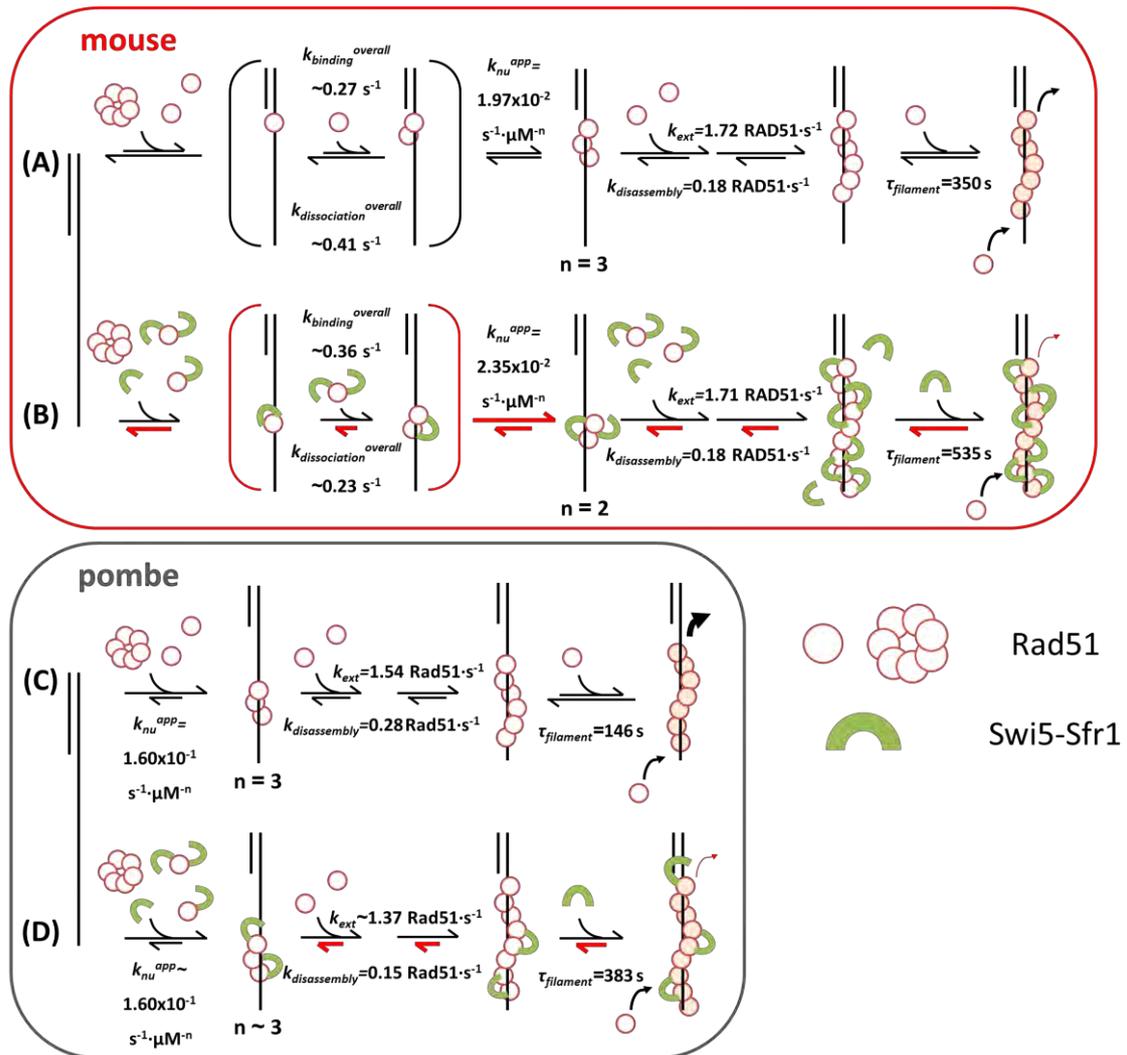
**Figure S8. SpRad51 nucleoprotein filament assembly experiments.** (A) Representative bead BM time-courses of SpRad51 (0.3 μM) assembly on (dT)<sub>135</sub> DNA substrates without SpS5S1 (top), with 0.3-fold (0.1 μM) SpS5S1 (middle top), with 0.3 μM SpS5S1 (middle bottom) or with 0.3 μM SpS5S1C mutant (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) (C) and bead BM increment (D) 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 bead 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  $\text{Rate}/[\text{RAD51}]^n = k_{ssDNA} * L_{ssDNA} + k_{junction}$ . 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  $n=2.70 \pm 0.29$  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  $\mu\text{M}$ ), 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<sup>FL</sup>S1 are all smaller than BM increment in mRAD51 assembly experiment (the last column in dashed bar) as (dT)<sub>135</sub> 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  $\mu\text{M}$  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] ( $\mu\text{M}$ )	[mS5S1]/[mRAD51]	SWI5-SFR1 type	Cofactor type	Nucleation rate ( $\text{s}^{-1}$ )	Extension time (sec/RAD51)	Bead BM increment (nm)	<i>n</i> (Numbers of molecules)
0.8	0	--	ATP	$(1.10 \pm 0.12) \cdot 10^{-2}$	$0.76 \pm 0.16$	$20.0 \pm 0.92$	67
	0.5	Wild-type mS5S1		$(1.07 \pm 0.16) \cdot 10^{-2}$	$0.85 \pm 0.27$	$24.4 \pm 1.45$	43
	1			$(1.03 \pm 0.12) \cdot 10^{-2}$	$0.59 \pm 0.16$	$26.0 \pm 1.35$	52
	1.5			$(1.05 \pm 0.16) \cdot 10^{-2}$	$0.35 \pm 0.09$	$22.7 \pm 1.24$	44
	1.625			$(1.02 \pm 0.18) \cdot 10^{-2}$	$0.46 \pm 0.09$	$23.5 \pm 1.36$	48
	1.75			$(1.05 \pm 0.10) \cdot 10^{-2}$	$0.49 \pm 0.13$	$26.5 \pm 2.26$	51
	1.875			$(1.24 \pm 0.14) \cdot 10^{-2}$	$0.43 \pm 0.11$	$24.6 \pm 1.32$	59
	2			$(1.43 \pm 0.15) \cdot 10^{-2}$	$0.70 \pm 0.11$	$24.4 \pm 0.97$	76
	2.25			$(1.44 \pm 0.18) \cdot 10^{-2}$	$0.37 \pm 0.08$	$25.2 \pm 1.21$	54
	2.5			$(1.49 \pm 0.24) \cdot 10^{-2}$	$1.01 \pm 0.29$	$25.2 \pm 1.79$	30
	2			mS5 <sup>FL/AA</sup> S1 mutant	$(1.02 \pm 0.12) \cdot 10^{-2}$	$0.67 \pm 0.24$	$22.5 \pm 1.39$
	0	--	AMPPNP	$(1.73 \pm 0.22) \cdot 10^{-2}$	$0.37 \pm 0.07$	$27.1 \pm 1.46$	45

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

Initial [mRAD51] ( $\mu\text{M}$ )	Initial [mS5S1] ( $\mu\text{M}$ )	SWI5-SFR1 type	Cofactor type	Nucleation rate ( $\text{s}^{-1}$ )	Extension time (sec/RAD51)	Bead BM increment (nm)	<i>n</i> (Numbers of molecules)
No mS5S1							
0.6	0	--	ATP	$(7.47 \pm 0.70) \cdot 10^{-3}$	$0.80 \pm 0.20$	$21.7 \pm 0.97$	92
0.65				$(8.54 \pm 1.56) \cdot 10^{-3}$	$0.50 \pm 0.11$	$20.9 \pm 1.45$	44
0.7				$(9.65 \pm 1.16) \cdot 10^{-3}$	$0.66 \pm 0.16$	$20.5 \pm 0.97$	60
0.8				$(1.10 \pm 0.12) \cdot 10^{-2}$	$0.76 \pm 0.16$	$20.0 \pm 0.92$	67
0.9				$(1.34 \pm 0.19) \cdot 10^{-2}$	$0.50 \pm 0.13$	$20.9 \pm 1.08$	41
1.0				$(2.36 \pm 0.35) \cdot 10^{-2}$	$0.24 \pm 0.07$	$24.2 \pm 1.38$	39
With mS5S1							
0.4	1.6	Wild-type mS5S1	ATP	$(5.34 \pm 1.15) \cdot 10^{-3}$	$0.80 \pm 0.20$	$21.1 \pm 1.57$	29
0.5				$(7.59 \pm 1.05) \cdot 10^{-3}$	$0.49 \pm 0.12$	$25.7 \pm 1.79$	37
0.6				$(1.13 \pm 0.15) \cdot 10^{-2}$	$0.62 \pm 0.17$	$24.7 \pm 1.63$	33
0.7				$(1.25 \pm 0.16) \cdot 10^{-2}$	$0.34 \pm 0.11$	$27.6 \pm 1.92$	31
0.8				$(1.43 \pm 0.15) \cdot 10^{-2}$	$0.70 \pm 0.11$	$24.4 \pm 0.97$	76
1.0	2.0			$(2.44 \pm 0.36) \cdot 10^{-2}$	$0.69 \pm 0.15$	$25.7 \pm 1.74$	37
0.7	1.6	mS5 <sup>FL/AA</sup> S1 mutant	ATP	$(9.95 \pm 1.35) \cdot 10^{-3}$	$0.82 \pm 0.20$	$21.0 \pm 1.17$	52
0.8				$(1.02 \pm 0.12) \cdot 10^{-2}$	$0.67 \pm 0.24$	$22.5 \pm 1.39$	39

**Table S5.** Summary of data in Figure 2C.

<b>Initial [mRAD51] (<math>\mu\text{M}</math>)</b>	<b>Initial [mS5S1] (<math>\mu\text{M}</math>)</b>	<b>SWI5-SFR1 type</b>	<b>Cofactor type</b>	<b>ssDNA length (nt)</b>	<b>Nucleation rate (<math>\text{s}^{-1}</math>)</b>	<b><i>n</i> (Numbers of molecules)</b>
No mS5S1						
0.8	0	--	ATP	90	$(1.09 \pm 0.28) * 10^{-2}$	31
				135	$(1.10 \pm 0.12) * 10^{-2}$	67
				165	$(1.22 \pm 0.17) * 10^{-2}$	33
				200	$(1.23 \pm 0.22) * 10^{-2}$	42
With mS5S1						
0.8	1.6	Wild-type mS5S1	ATP	90	$(1.32 \pm 0.28) * 10^{-2}$	32
				135	$(1.43 \pm 0.15) * 10^{-2}$	76
				165	$(1.94 \pm 0.42) * 10^{-2}$	35
				200	$(2.26 \pm 0.22) * 10^{-2}$	36

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

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

(dT) <sub>18</sub>									
1.0	0	--	ATP	$k_{0 \rightarrow 1}$	0.16±0.01	$k_{1 \rightarrow 0}$	0.35±0.03	58.0±7.51	467
				$k_{1 \rightarrow 2}$	0.42±0.02	$k_{2 \rightarrow 1}$	0.72±0.03		
				$k_{2 \rightarrow 3}$	0.56±0.03	$k_{3 \rightarrow 2}$	0.83±0.03		
				$k_{3 \rightarrow 4}$	0.85±0.03	$k_{4 \rightarrow 3}$	0.60±0.04		
				$k_{4 \rightarrow 5}$	0.96±0.06	$k_{5 \rightarrow 4}$	0.41±0.02		
				$k_{5 \rightarrow 6}$	0.56±0.03	$k_{6 \rightarrow 5}$	0.19±0.01		

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

<b>Initial [SpRad51] (<math>\mu\text{M}</math>)</b>	<b>[SpS5S1]/[SpRad51]</b>	<b>Swi5-Sfr1 type</b>	<b>Cofactor type</b>	<b>Nucleation rate (<math>\text{s}^{-1}</math>)</b>	<b>Extension time (sec/Rad51)</b>	<b>Bead BM increment (nm)</b>	<b><i>n</i> (Numbers of molecules)</b>
0.3	0	--	ATP	$(4.50 \pm 0.51) * 10^{-3}$	$5.03 \pm 0.64$	$16.3 \pm 1.04$	42
	0.1	Wild-type SpS5S1		$(5.05 \pm 0.59) * 10^{-3}$	$6.56 \pm 0.73$	$14.8 \pm 0.76$	68
	0.2			$(4.20 \pm 0.57) * 10^{-3}$	$2.70 \pm 0.26$	$16.4 \pm 1.01$	38
	0.25			$(4.84 \pm 0.73) * 10^{-3}$	$2.87 \pm 0.34$	$18.0 \pm 1.12$	41
	0.33			$(4.91 \pm 0.62) * 10^{-3}$	$4.68 \pm 0.69$	$16.0 \pm 1.08$	48
	0.5			$(3.09 \pm 0.35) * 10^{-3}$	$5.25 \pm 0.58$	$13.4 \pm 0.80$	52
	1			$(2.39 \pm 0.24) * 10^{-3}$	$4.47 \pm 0.59$	$10.4 \pm 0.56$	45
	0.2			SpS5S1C mutant	$(4.74 \pm 0.60) * 10^{-3}$	$3.93 \pm 0.44$	$16.0 \pm 0.94$
	0.33	$(4.73 \pm 0.53) * 10^{-3}$			$4.16 \pm 0.56$	$14.4 \pm 0.57$	73
	0.5	$(4.17 \pm 0.39) * 10^{-3}$			$4.58 \pm 0.52$	$15.4 \pm 0.66$	82
	1	$(4.37 \pm 0.45) * 10^{-3}$			$3.83 \pm 0.40$	$15.9 \pm 0.67$	94
	2	$(4.21 \pm 0.57) * 10^{-3}$			$2.94 \pm 0.48$	$13.5 \pm 0.83$	35
	0	--			AMPPNP	$(8.72 \pm 1.36) * 10^{-3}$	$2.89 \pm 0.70$

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

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

**Table S9.** Summary of data in Figure S9A.

Species	Initial [Rad51] ( $\mu\text{M}$ )	Initial [S5S1] ( $\mu\text{M}$ )	Swi5-Sfr1 type	Cofactor type	ssDNA length (nt)	Nucleation rate constant ( $\text{s}^{-1}\cdot\mu\text{M}^{-n}$ )	<i>n</i> (Numbers of molecules)
Mouse	0.8	0	--	ATP	90	$(1.88\pm 0.48)\cdot 10^{-2}$	31
					135	$(1.89\pm 0.21)\cdot 10^{-2}$	67
					165	$(2.10\pm 0.28)\cdot 10^{-2}$	33
					200	$(2.11\pm 0.38)\cdot 10^{-2}$	42
fission yeast	0.8	0	--	ATP	90	$(1.22\pm 0.10)\cdot 10^{-1}$	145
					100	$(1.28\pm 0.15)\cdot 10^{-1}$	49
					135	$(1.47\pm 0.14)\cdot 10^{-1}$	131
					165	$(1.85\pm 0.24)\cdot 10^{-1}$	77
					200	$(2.27\pm 0.25)\cdot 10^{-1}$	81

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

Initial [SpRad51] ( $\mu\text{M}$ )	Initial [SpS5S1] ( $\mu\text{M}$ )	Swi5-Sfr1 type	Cofactor type	Nucleation rate ( $\text{s}^{-1}$ )	Extension time (sec/Rad51)	Bead BM increment (nm)	<i>n</i> (Numbers of molecules)
0.3	0	--	ATP	$(4.50\pm 0.51)\cdot 10^{-3}$	$5.03\pm 0.64$	$16.3\pm 1.04$	42
0.6				$(1.49\pm 0.22)\cdot 10^{-2}$	$1.72\pm 0.12$	$22.0\pm 0.70$	81
0.7				$(2.26\pm 0.32)\cdot 10^{-2}$	$1.63\pm 0.12$	$22.3\pm 0.61$	104
0.8				$(3.77\pm 0.49)\cdot 10^{-2}$	$1.54\pm 0.15$	$19.5\pm 0.76$	81
0.9				$(4.68\pm 0.63)\cdot 10^{-2}$	$1.22\pm 0.12$	$20.0\pm 0.68$	104

## SI References

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