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Supplemental Information

A Polar and Nucleotide-Dependent Mechanism of Action for RAD51 Paralogs in RAD51 Filament Remodeling

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Supplemental Information: Taylor *et al*

Content:

Figure S1

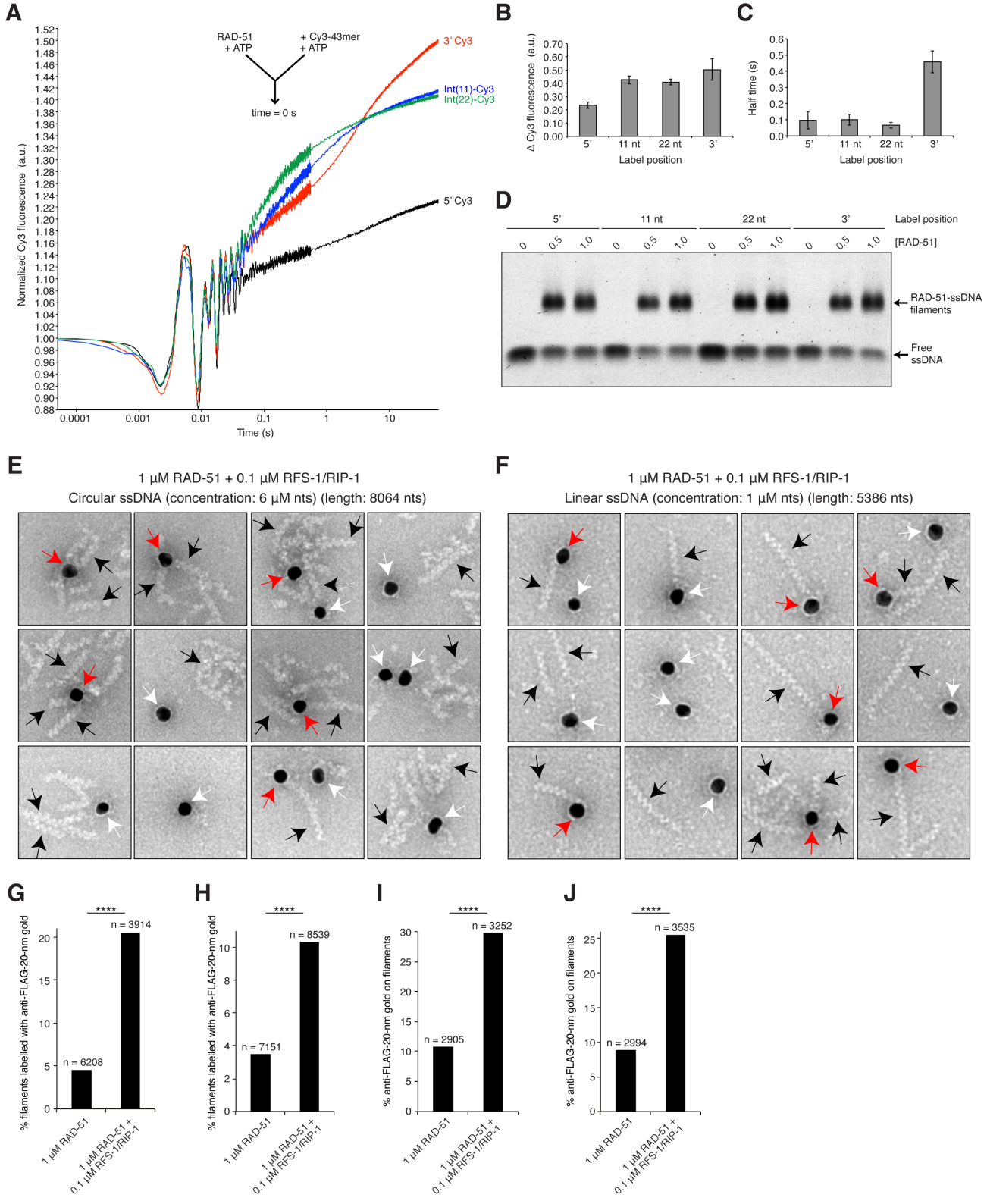
A
RAD-51 + ATP
+ Cy3-43mer + ATP
time = 0 s
3' Cy3
Int(11)-Cy3
Int(22)-Cy3
5' Cy3
Normalized Cy3 fluorescence (a.u.)
Time (s)
B
Δ Cy3 fluorescence (a.u.)
5'
11 nt
22 nt
3'
Label position
C
Half time (s)
Label position
D
Label position
[RAD-51]
RAD-51-ssDNA filaments
Free ssDNA
E
1 μM RAD-51 + 0.1 μM RFS-1/RIP-1
Circular ssDNA (concentration: 6 μM nts) (length: 8064 nts)
F
1 μM RAD-51 + 0.1 μM RFS-1/RIP-1
Linear ssDNA (concentration: 1 μM nts) (length: 5386 nts)
G
% filaments labelled with anti-FLAG-20-nm gold
n = 6208
n = 3914
****
1 μM RAD-51
1 μM RAD-51 + 0.1 μM RFS-1/RIP-1
H
% filaments labelled with anti-FLAG-20-nm gold
n = 7151
n = 8539
****
I
% anti-FLAG-20-nm gold on filaments
n = 2905
n = 3252
****
J
% anti-FLAG-20-nm gold on filaments
n = 2994
n = 3535
****

Figure S1: RAD-51 filament assembly on different oligonucleotides and RFS-1/RIP-1 binding to RAD-51-ssDNA filaments formed on circular ssDNA. Related to Figure 2.

(**A**) Average normalized Cy3-43mer fluorescence profiles plotted as a function of time. The arrow indicates the components of the two syringes rapidly mixed at the 0 s time point in a stopped-flow instrument. 1 μM RAD-51 pre-incubated with ATP was mixed with 15 nM Cy3-43mer. 5'-, Int(11)-, Int(22)- and 3'-Cy3 constructs were analysed (n = 9).

(**B**) Graph of average Δ Cy3 fluorescence for the data presented in **A** (errors: s.d.).

(**C**) Graph of average half-time for the fluorescence change for the data presented in **A** (errors: s.d.).

(**D**) Protein-DNA complexes formed by RAD-51 on the 5'-, Int(11)-, Int(22)- and 3'-Cy3-43mer constructs with ATP resolved in native agarose gels.

(**E** and **F**) Example images of anti-FLAG-20-nm gold particles bound (red arrows) or unbound (white arrows) to RAD-51-ssDNA filaments (black arrows) formed in the presence of RFS-1/RIP-1 on either (**E**) circular p8064 ssDNA or (**F**) linearized PhiX ssDNA.

(**G-H**) Quantification of % RAD-51-ssDNA filaments (including aggregated filaments) formed on circular ssDNA labelled with anti-FLAG-20-nm gold particles ± RFS-1/RIP-1 from two independent experiments. **** Two-tailed Chi-square test of independence, $p < 0.0001$.

(**I-J**) Quantification of % anti-FLAG-20-nm gold particle binding to RAD-51-ssDNA filaments (including aggregated filaments) formed on circular ssDNA ± RFS-1/RIP-1 from 2 independent experiments. **** Two-tailed Chi-square test of independence, $p < 0.0001$.

Figure S2

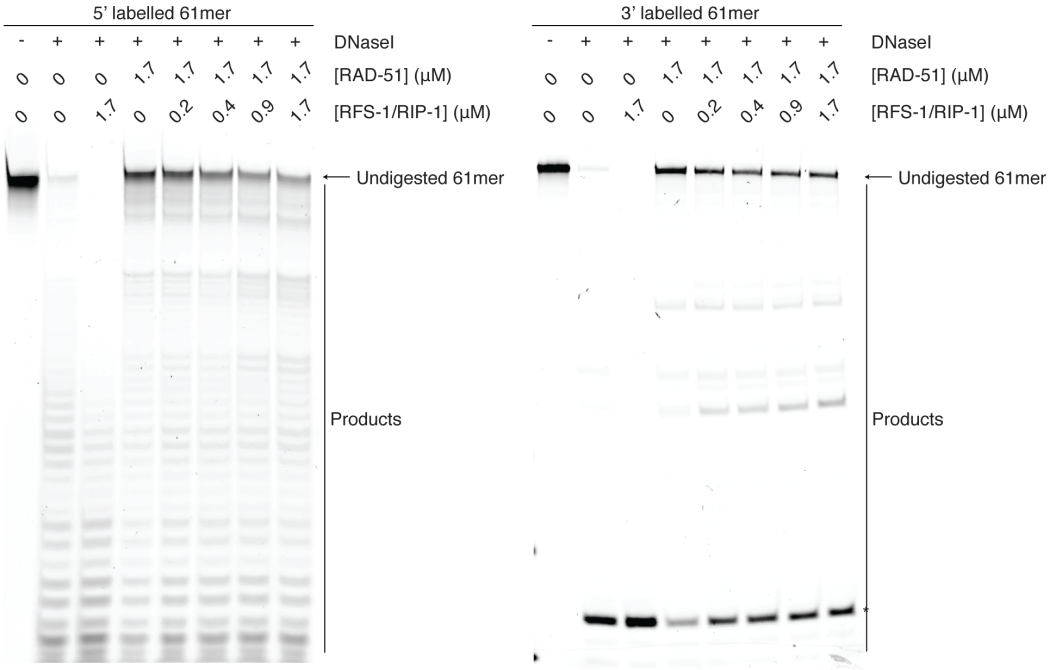
5' labelled 61mer
3' labelled 61mer
DNaseI
[RAD-51] (μM)
[RFS-1/RIP-1] (μM)
Undigested 61mer
Products

Figure S2: RFS-1/RIP-1 propagates filament DNaseI sensitization beyond immediate proximity to the 5' filament end. Related to Figure 3.
DNaseI protection assay on protein-DNA complexes formed by RAD-51 and RFS-1/RIP-1 on 5' or 3' fluorescently-labelled 61mer ssDNA. Products were resolved by denaturing PAGE.

Figure S3

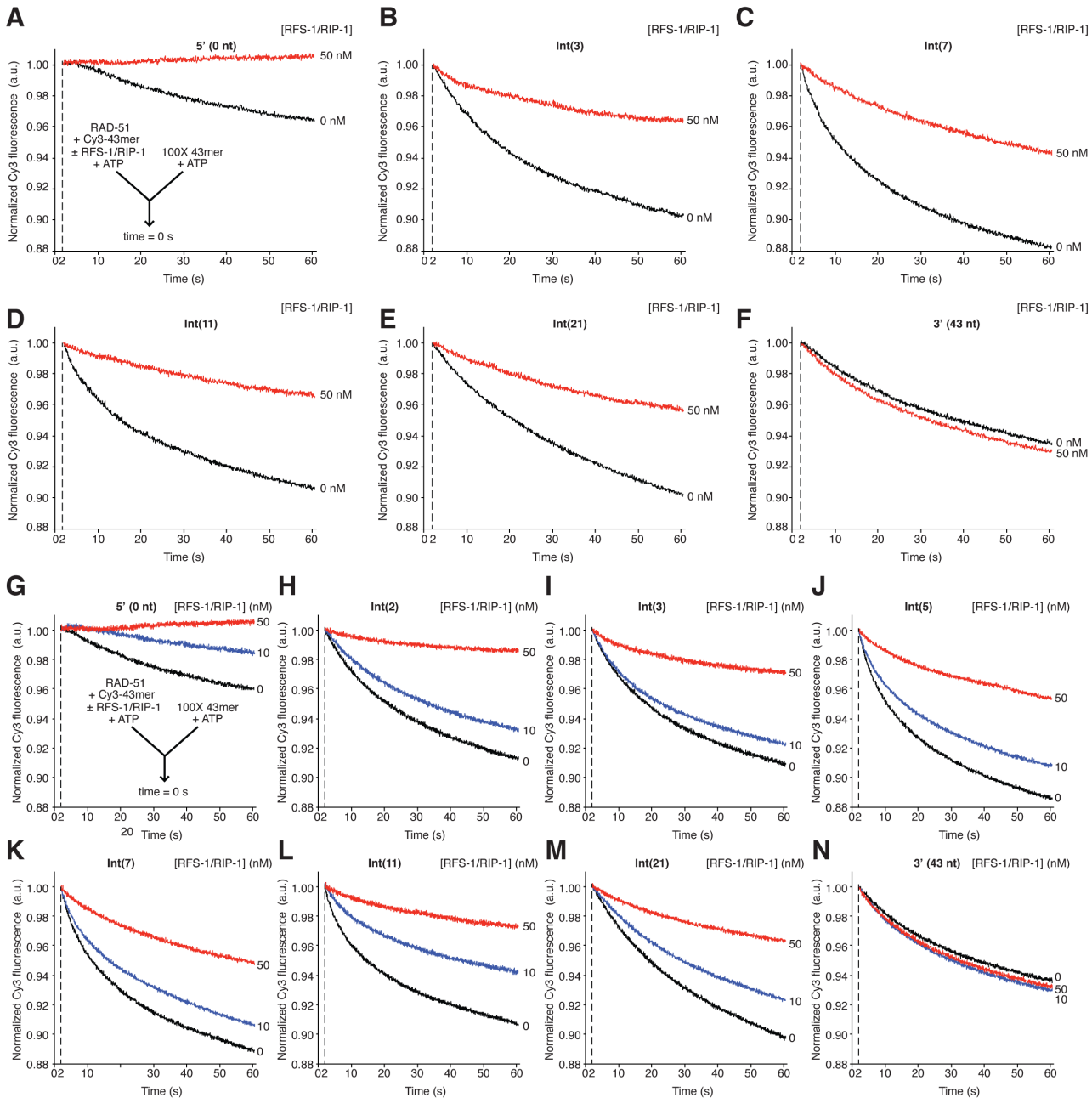
A
[RFS-1/RIP-1]
5' (0 nt)
50 nM
0 nM
RAD-51
+ Cy3-43mer
± RFS-1/RIP-1
+ ATP
100X 43mer
+ ATP
time = 0 s
Normalized Cy3 fluorescence (a.u.)
Time (s)
B
[RFS-1/RIP-1]
Int(3)
50 nM
0 nM
C
[RFS-1/RIP-1]
Int(7)
50 nM
0 nM
D
[RFS-1/RIP-1]
Int(11)
50 nM
0 nM
E
[RFS-1/RIP-1]
Int(21)
50 nM
0 nM
F
[RFS-1/RIP-1]
3' (43 nt)
0 nM
50 nM
G
5' (0 nt)
[RFS-1/RIP-1] (nM)
50
10
0
RAD-51
+ Cy3-43mer
± RFS-1/RIP-1
+ ATP
100X 43mer
+ ATP
time = 0 s
H
Int(2)
[RFS-1/RIP-1] (nM)
I
Int(3)
[RFS-1/RIP-1] (nM)
J
Int(5)
[RFS-1/RIP-1] (nM)
K
Int(7)
[RFS-1/RIP-1] (nM)
L
Int(11)
[RFS-1/RIP-1] (nM)
M
Int(21)
[RFS-1/RIP-1] (nM)
N
3' (43 nt)
[RFS-1/RIP-1] (nM)

Figure S3: RFS-1/RIP-1 propagates filament stabilization beyond immediate proximity to the 5' filament end. Related to Figure 3.

(**A-F**) Average normalized Cy3-43mer fluorescence profiles plotted as a function of time for the data in Figure 3F. The arrow indicates the components of the two syringes rapidly mixed at the 0 s time point in a stopped-flow instrument. RAD-51-ssDNA filaments pre-formed with 1 μM RAD-51 + 15 nM Cy3-43mer ± 50 nM RFS-1/RIP-1 for 10 min were mixed with 100-fold excess unlabelled 43mer. Label position is indicated top centre of each profile (n = 6-8).

(**G-N**) Average normalized Cy3-43mer fluorescence profiles plotted as a function of time for the data in Figure 3G. RAD-51-ssDNA filaments pre-formed with 1 μM RAD-51 + 15 nM Cy3-43mer and the indicated concentration of RFS-1/RIP-1 for 10 min were mixed with 100-fold excess unlabelled 43mer. Label position is indicated top centre of each profile (n = 5-8).

Figure S4

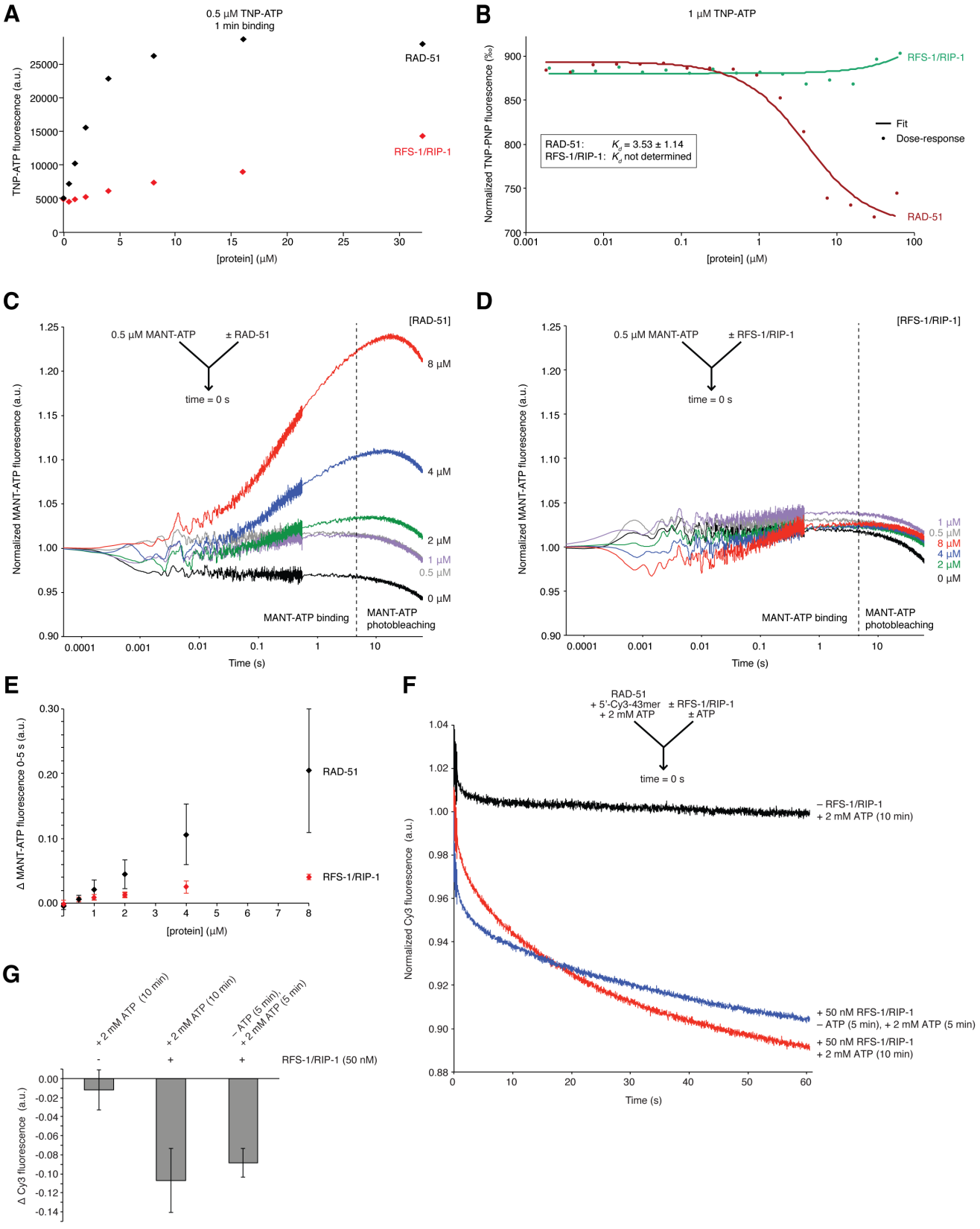
A
0.5 μM TNP-ATP
1 min binding
TNP-ATP fluorescence (a.u.)
[protein] (μM)
RAD-51
RFS-1/RIP-1
B
1 μM TNP-ATP
Normalized TNP-PNP fluorescence (‰)
[protein] (μM)
RFS-1/RIP-1
Fit
Dose-response
RAD-51:
RFS-1/RIP-1:
RAD-51
C
[RAD-51]
0.5 μM MANT-ATP
± RAD-51
time = 0 s
Normalized MANT-ATP fluorescence (a.u.)
Time (s)
8 μM
4 μM
2 μM
1 μM
0.5 μM
0 μM
MANT-ATP binding
MANT-ATP photobleaching
D
[RFS-1/RIP-1]
0.5 μM MANT-ATP
± RFS-1/RIP-1
time = 0 s
Normalized MANT-ATP fluorescence (a.u.)
Time (s)
1 μM
0.5 μM
8 μM
4 μM
2 μM
0 μM
MANT-ATP binding
MANT-ATP photobleaching
E
Δ MANT-ATP fluorescence 0-5 s (a.u.)
[protein] (μM)
RAD-51
RFS-1/RIP-1
F
RAD-51
+ 5'-Cy3-43mer
+ 2 mM ATP
± RFS-1/RIP-1
± ATP
time = 0 s
Normalized Cy3 fluorescence (a.u.)
Time (s)
– RFS-1/RIP-1
+ 2 mM ATP (10 min)
+ 50 nM RFS-1/RIP-1
– ATP (5 min), + 2 mM ATP (5 min)
+ 50 nM RFS-1/RIP-1
+ 2 mM ATP (10 min)
G
+ 2 mM ATP (10 min)
+ 2 mM ATP (10 min)
– ATP (5 min),
+ 2 mM ATP (5 min)
RFS-1/RIP-1 (50 nM)
Δ Cy3 fluorescence (a.u.)

Figure S4: RFS-1/RIP-1 binds nucleotides weakly and slowly relative to RAD-51. Related to Figure 5.
(**A**) TNP-ATP fluorescence measured in a microplate reader following 1 minute binding to different concentrations of RAD-51 or RFS-1/RIP-1.
(**B**) Microscale thermophoresis measurements of normalized TNP-ATP fluorescence upon binding to different concentrations of RAD-51 or RFS-1/RIP-1. Experimentally determined dose-response values and attempted fit results are indicated.
(**C-D**) Average normalized MANT-ATP fluorescence profiles plotted as a function of time. The arrow indicates the components of the two syringes rapidly mixed at the 0 s time point in a stopped-flow instrument. The indicated concentrations of protein were mixed with 0.5 μM MANT-ATP. (**C**) RAD-51 (n = 5-7). (**D**) RFS-1/RIP-1 (n = 7).
(**E**) Graph of protein concentration-dependence of Δ MANT-ATP fluorescence for MANT-ATP binding to RAD-51 and RFS-1/RIP-1 for the data presented in **C-D** (errors: s.d.).
(**F**) Average normalized Cy3-43mer fluorescence profiles plotted as a function of time. RAD-51-ssDNA filaments pre-formed with 1 μM RAD-51 + 15 nM Cy3-43mer + 2 mM ATP for 10 min were mixed with buffer or 50 nM RFS-1/RIP-1 either pre-incubated for 10 min (red and black traces) or with buffer + 50 nM RFS-1/RIP-1 pre-incubated without nucleotide for 5 min before addition of 2 mM ATP for a further 5 min (blue trace) (n = 6-9).
(**G**) Graphs of average Δ Cy3 fluorescence for the data presented in **F** (errors: s.d.).

Supplemental Experimental Procedures:

Protein expression and purification

RAD-51 and RFS-1/RIP-1 were purified as described previously (Taylor et al., 2015). RAD-51 was expressed using the Champion pET-SUMO system (Life Technologies) in BL21(DE3) One Shot *E. coli*. The culture was grown in LB supplemented with 50 µg/ml kanamycin at 37 °C to OD_{600} of 0.6-0.8, before induction for 4 h with 1 mM IPTG at 30 °C. Pellets were resuspended in 400 ml ice cold Lysis Buffer (50 mM potassium phosphate (pH 7.8), 1 M KCl, 10% glycerol) supplemented with cOmplete, EDTA-free protease inhibitor cocktail tablets (Roche) (1 tablet per 25 ml buffer), and mixed well with a magnetic stirrer at 4 °C until the mixture was homogenous. All subsequent steps were carried out at 4 °C. The cells were lysed by addition of Triton X-100 to 0.1%. The lysate was sonicated and cleared using a Ti45 rotor (Beckman Coulter) at 40,000 rpm for 60 min. Imidazole was added to the supernatant to a final concentration of 25 mM and applied to 12 ml bed volume of Ni-NTA agarose affinity gel (Qiagen 30210) which had been pre-washed with Binding Buffer (50 mM potassium phosphate (pH 7.8), 1 M KCl, 10% glycerol, 25 mM imidazole (pH 7.5)). The protein was bound to the beads by rotating for 2 h then applied to 6 x 25 ml batch purification columns. The flowthrough was discarded and the beads washed with 300 ml Binding Buffer and 300 ml Binding Buffer containing 50 mM imidazole (50 ml per column). The protein was eluted with Binding Buffer containing 200 mM imidazole by passing 8 ml twice over each column then washing with a further 4 ml of this buffer per column (total eluate volume 72 ml), and dialyzed against 4 L Dialysis Buffer (20 mM Tris-HCl (pH 8.0), 300 mM KCl, 10% glycerol) overnight using 10 kDa MWCO SnakeSkin dialysis tubing (Thermo Scientific). Some precipitate was observed the following day, but most protein remained in solution. The His-SUMO tag was cleaved to yield native RAD-51 by addition of His-tagged Ulp1 SUMO protease for 45 min. The protein was centrifuged and the soluble fraction collected and bound to the same batch of NiNTA agarose affinity gel used for purification after regeneration according to the manufacturer's instructions to remove the SUMO protease and His-SUMO tag. The flowthrough containing native RAD-51 was collected and the resin washed with an additional 18 ml (3 ml per column) of Dialysis Buffer. These were pooled (total 90 ml) and mixed at 1:1 ratio with Dilution Buffer (20 mM Tris-HCl (pH 8.0), 10% glycerol, 2 mM EDTA, 1 mM DTT) (total 180 ml) to reduce salt concentration to 150 mM KCl. The protein was bound to a 1 ml Mono Q 5/50 GL column (GE Healthcare) at 0.5 ml / min using an Äkta Explorer HPLC system and washed with 30 ml R buffer (20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM EDTA, 0.5 mM DTT) supplemented with 100 mM KCl then 20 ml R buffer supplemented with 150 mM KCl. The protein was eluted with a 30 ml gradient 150-640 mM KCl in R buffer and 0.5 ml fractions collected. The peak fractions were pooled and either dialysed against Storage Buffer for 1 h, frozen in small aliquots in liquid nitrogen and stored at –80 °C, or concentrated and frozen directly in the elution buffer. This method typically yielded 1.5-2.5 mg/ml recombinant RAD-51 (40-65 µM) which could be concentrated as high as 640 µM without precipitation.

RFS-1/RIP-1 was expressed in budding yeast cells by growing a 100 L culture in a 120 L BIOFLO 5000 fermenter (New Brunswick Scientific) in YP media + 2% raffinose at 30°C, pH 5.8, agitation 200 rpm and airflow 20 L/min until OD_{600} of 0.7-0.8, before induction for 5 h with 2% galactose. The cells were harvested and washed twice with Yeast Wash Buffer (25 mM HEPES-KOH (pH 7.5), 1 M sorbitol) then once with Buffer K (45 mM HEPES-KOH, 10% glycerol, 0.1 M potassium glutamate, 5 mM magnesium acetate) supplemented with 0.02% NP-40. The cells were then resuspended in 70 ml buffer K + 0.02% NP-40 supplemented with cOmplete, EDTA-free protease inhibitor cocktail tablets (Roche) (1 tablet per 25 ml buffer) and frozen dropwise in liquid nitrogen. Cells were lysed by crushing in a freezer mill (SPEX CertiPrep 6850) for 6 x 2 min cycles at rate 15 under liquid nitrogen and the frozen cell powder stored at –80°C. For purification, the cell powder was thawed to room temperature in a water bath, diluted with Buffer K and the potassium glutamate concentration adjusted to 0.5 M and mixed well with a magnetic stirrer at 4°C until the lysate was homogenous. All subsequent steps were carried out at 4 °C. The lysate was cleared in an Optima LE-80K Ultracentrifuge (Beckman Coulter) using a Ti45 rotor at 40,000 rpm for 90 min and the supernatant was applied to 10 ml bed volume of anti-FLAG M2 affinity gel (Sigma A2220). The protein was bound to the beads by rotating overnight then applied to 5 x 25 ml batch purification columns. The flow-through was discarded and the beads washed with 750 ml Buffer K (150 ml per column). The protein was eluted with 1 mg/ml 3xFLAG peptide in Buffer K by passing 2 ml twice over each column then washing with 4 ml Buffer K per column (total eluate volume 30 ml), and dialyzed against 4 L Storage Buffer (20 mM Tris-acetate (pH 8.0), 100 mM potassium acetate, 10% glycerol, 1 mM EDTA, 0.5 mM DTT) overnight using 10 kDa MWCO SnakeSkin dialysis tubing (Thermo Scientific). The protein was then concentrated on a 30 kDa MWCO Amicon Ultra-15 Centrifugal Filter Unit pre-washed with water and Storage Buffer to a final volume of 1 ml, frozen in small aliquots in liquid nitrogen and stored at –80 °C. This method typically yielded 3-4.4 mg/ml recombinant RFS-1/RIP-1 (55-80 µM complex) devoid of detectable nuclease activity. For electron microscopy studies, 100 µl concentrated RFS-1/RIP-1 was subsequently applied to a Superdex S200 5/150 GL column (GE Healthcare) in Storage Buffer and the peak fractions collected, pooled and stored in 1.45 µM aliquots.

EMSA

RAD-51 was mixed with a master mix containing 645 nM (nucleotides) of the indicated Cy3-labelled $(dT)_{43}$ oligonucleotide, 20 mM Tris-HCl (pH 7.5), 8% glycerol, 1 mM DTT, 50 mM sodium acetate, 2 mM $MgCl_2$ and 2 mM ATP, in 10 µl reaction volume at 25°C. Protien-ssDNA complexes were crosslinked with 0.25% glutaraldehyde for 10 min at 25 °C. Reactions were resolved on 1% agarose gels in 1X TBE (70 V, 2 h 20 min) at 4 °C. Gels were imaged on a FLA-9000 scanner (Fujifilm).

Nuclease protection assays

Proteins were diluted from concentrated stocks into T Buffer (25 mM Tris-HCl (pH 7.5), 10% glycerol, 0.5 mM EDTA (pH 8.0), 50 mM KCl), which was also used in no protein controls. Proteins were pre-incubated for 5 min on ice then mixed with 7310 nM (nucleotides) 5'- or 3'-fluorescein-labelled 61mer oligonucleotide (GACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCC) in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 1 mM ATP, 1 mM $MgCl_2$, 1 mM $CaCl_2$, in 10 µl reaction volume at 25 °C for 10 min. 1 µl (2 U) bovine pancreatic DNaseI (New England Biolabs) was then added for 20 min at 25 °C. The samples were deproteinized with 0.125% SDS and 12.5 µg proteinase K for 10 min at 37 °C and resolved in 10% native polyacrylamide gels in 1X TBE (110 V, 50 min) or in 20% denaturing polyacrylamide urea gels in 1X TBE (15 W, 90 min). Gels were imaged on a FLA-9000 scanner (Fujifilm) and quantified with Multi Gauge V3.2 (Fujifilm). After adjusting for background, the % protection was determined for all samples then normalized to the value for RAD-51 only. Average relative protection values from at least 5 experiements were then determined.

Immuno-gold electron microscopy

RFS-1/RIP-1 was purified via a gel filtration column prior to use as described above. Anti-FLAG antibody (Sigma F3165) was conjugated to 20-nm gold particles using the InnovaCoat Gold Conjugation Mini kit (Innova Biosciences). 1 µM RAD-51 ± 0.1 µM RFS-1/RIP-1 and 1 µM (nucleotides) of linearized PhiX ssDNA (length: 5386 nucleotides) (gift of Michael Mcilwraith and Stephen West) or 6 µM (nucleotides) of p8064 circular ssDNA (length: 8064 nucleotides) (Tilibit Nanosystems) were co-incubated in 20 mM Triethanolamine-HCl (pH 7.5), 8% glycerol, 1 mM DTT, 50 mM sodium acetate, 2 mM $MgCl_2$, 2 mM ATP for 10 min at 25 °C, before incubation with anti-FLAG-20-nm gold for a further 5 min at 25 °C. Samples were applied to glow-discharged continuous carbon-coated grids, negatively stained by 2% uranyl acetate, and by negative stain as described previously (Taylor et al., 2015).

For quantification of anti-FLAG-20-nm gold binding to filaments in Figure 2E-G, images (2120 nm x 1511 nm) were acquired at nominal magnification 67,000X from between 70 and 125 fields of view per condition, and scored for number of anti-FLAG-20-nm gold particles unbound to filaments or bound to filaments >50 nm in length. Only gold particles directly on filaments and not those juxtaposed were counted. Clusters of >3 gold particles were not counted. Filament clumps of >4 filaments were not counted.

For quantification of filaments associated with anti-FLAG-20-nm gold in Figure S1G-H, the same images were scored for number of filaments bound or unbound to anti-FLAG-20-nm gold. Clumps of filaments indistinguishable as individual filaments for counting were scored as one. Clusters of >3 gold particles were not counted. Anti-FLAG-20-nm gold particles were also re-scored as unbound to filaments or bound to filaments by these alternative criteria (Figure S1I-J), and showed a similar fold enrichment in the presence of RFS-1/RIP-1 to Figure 2E-F.

Statistical analysis was performed in GraphPad Prism 6 (GraphPad Software). All images were scored by a researcher blinded to the identity of the samples.

Stopped-flow assays and data analysis

Stopped-flow experiments were performed using an SFM-300 stopped-flow machine (Bio-Logic) fitted with a MOS-200 monochromator spectrometer (Bio-Logic) with excitation wavelength set at 545 nm. Fluorescence measurements were collected with a 550 nm long pass emission filter. The machine temperature was maintained at 25 °C with a circulating water bath.

For all experimental setups, a master mix containing all common reaction components for each of the two syringes was prepared to which variable components were added to generate the mixtures for individual syringes for different experimental conditions. These individual syringe mixtures are indicated in the mixing schemes. Since equal volumes were injected into the mixing chamber from each syringe, the two solutions became mutually diluted. Therefore, all reaction components common to each syringe were prepared at the final concentration, whereas reaction components present in only one syringe were added at twice the desired final concentration. All concentrations quoted represent final concentrations after mixing. Components of each syringe were pre-incubated for 10 min before the start of experiments to allow the contents to reach equilibrium.

All reactions were performed in Stopped Flow Buffer (50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, 2 mM ATP) except where indicated to determine the nucleotide-dependency of RFS-1/RIP-1 activity in Figure 5. All reactions contained 15 nM (moles) Cy3 fluorescently labelled $(dT)_{43}$ or $(dT)_{23}$ oligonucleotide (Cy3-43mer or Cy3-23mer), with the fluorophore either conjugated to the 5' or 3' end or integrated into the DNA backbone after the indicated nucleotide

position. Proteins were added directly from concentrated stocks. Unlabelled $(dT)_{43}$ or $(dT)_{23}$ oligonucleotide was used at 1500 nM (moles) (100-fold excess) in competition experiments. For all experiments, controls were also performed for buffer alone with and without DNA to confirm fluorescence signal stability over the time course of the experiments (data not shown). For experiments on pre-formed RAD-51 filaments we employed a saturating concentration of RAD-51 (1 μM on 43mer, 750 nM on 23mer), to ensure all ssDNA present was coated by RAD-51 filaments.

Fluorescence measurements for most experiments were collected according to the following protocol: (1) every 0.00005 s from 0-0.05 s; (2) every 0.0005 s from 0.05-0.56 s; (3) every 0.02 s from 0.56-60.54 s.

For each condition analysed, traces were collected from between four and twenty-two independent reactions (n = 4-22, see individual figure legends for details). In Figure 4, these were pooled from two or three independent experiments. For presentation, average traces for each experiment were generated. Absolute fluorescence values were converted to arbitrary units by a normalization procedure to facilitate comparison. For all experiments the raw data were normalized to the same fluorescence value for the 0 s time point, except for the unlabelled DNA competition experiments (Figure 3, 4, S2) where raw data were normalized to the same value for Cy3 fluorescence at the 2.01998 s time point and truncated before this, as previously (Taylor et al., 2015).

For analysis, for all experiments in Figures 2, 5, S1 and S4F, ten-point moving averages were calculated on each individual normalized trace, which were used to define initial (0 s) and final (60.54 s) fluorescence values and Δ Cy3 fluorescence values for each experiment. Half-times in Figure S1 were measured as the time points where the fluorescence from moving averages was closest to the value calculated for the Δ Cy3 fluorescence midpoint. Average values and associated standard deviations were then calculated. For competition experiments (Figure 3, 4, S2), the relatively small changes in fluorescence observed meant the data was too noisy for analysis from moving averages for Δ Cy3 fluorescence in this way. For these experiments, Δ Cy3 fluorescence values were calculated on each individual normalized trace as the difference between the mean fluorescence values measured across all time points from 1.01998 to 2.99998 s (start value, on either side of the adjusted start at 2 s) and 58.54 to 60.54 s (end value). Average values and associated standard deviations were then calculated. Positive and negative Δ Cy3 fluorescence values represent increases and decreases in fluorescence respectively. To determine the % of Cy3 fluorescence reduction observed for RAD-51 only in Figures 3F, G and 4G, J, the Δ Cy3 fluorescence value for each trace calculated in the presence of RFS-1/RIP-1 was divided by the average Δ Cy3 fluorescence value for RAD-51 alone and multiplied by 100. Average values and associated standard deviations or standard errors of the mean were then calculated.

For experiments testing protein binding to MANT-ATP (ThermoFisher Scientific M12417), reactions were performed in Stopped Flow Buffer without ATP. MANT-ATP (final concentration 0.5 μM) was mixed with increasing amounts of either RAD-51 or RFS-1/RIP-1. Excitation wavelength was set at 365 nm and fluorescence measurements were collected with a 395 nm long pass emission filter. For analysis, Δ MANT-ATP fluorescence values for the binding phase (before photobleaching) were calculated on each individual normalized trace as the difference between the mean fluorescence values measured across all time points from 0 to 0.01 s (start value) and 4.51998 to 5.49998 s (end value, on either side of 5 s). Average values and associated standard deviations were then calculated.

TNP-ATP fluorescence measurements and microscale thermophoresis

Fluorescence intensity of TNP-ATP (Sigma-Aldrich SML0740) stained protein solutions was measured using Infinity F500 microplate reader (Tecan Group Ltd.) in 96 well plates (25 °C). The excitation and emission wavelengths were chosen at 485 (20) nm and 535 (25) nm, respectively. Increasing amounts of either RAD-51 or RFS-1/RIP-1 were mixed with TNP-ATP (final concentration 0.5 μM) and samples measured after 1 minute.

Binding affinity quantifications via microscale thermophoresis were performed using the Monolith NT.115 (Nanotemper Technologies). Affinity measurements were performed by using MST buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$), supplemented with 0.05% Tween-20. Samples were loaded into NT.115 standard capillaries (NanoTemper Technologies). Measurements were performed at 25 °C, 100% LED, 40% IR laser power and constant concentration of 1 μM TNP-ATP and increasing concentration of purified proteins. Data were analysed by the MO.Affinity Analysis software (NanoTemper Technologies).

ssDNA curtains assays

Flowcells were prepared as previously described (Gibb et al., 2012; Gorman et al., 2010). The single-stranded DNA substrates were prepared by rolling circle replication using an M13mp18 single strand plasmid annealed to a biotinylated primer, as described (Gibb et al., 2012; Gibb et al., 2014). For the extension and visualization of the ssDNA-ScRPA-eGFP curtain, *Saccharomyces cerevisiae* RPA-eGFP (1 nM) was flown in at 0.8 ml/min with BSA buffer (40 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM DTT, and 0.2 mg/ml BSA). After 2 minutes, 150 μl of 7 M urea was injected to remove protein aggregates and unresolved secondary structures followed by 15 minutes of continuous flow of 1 nM ScRPA-eGFP in BSA buffer.

RAD-51 filaments were assembled using CeH buffer (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.2 mg/ml BSA) unless otherwise indicated. Excess ScRPA-eGFP was flushed out with CeH buffer at 1 ml/min for a minimum of 2 minutes before injecting the indicated concentration of RAD-51. For sample injections of

RFS-1/RIP-1, the flowcell chamber is first equilibrated to the appropriate buffer (CeH buffer with the nucleotide RFS-1/RIP-1 is incubated in and/or ScRPA-eGFP) with a one minute buffer flow at 1 ml/min. RFS-1/RIP-1 was pre-incubated with 2 mM ATP, ADP or ATPγS as indicated for at least 10 minutes prior to introduction to the flow cell.

For visualization, a 488 nm laser is used to illuminate the eGFP molecules. 100 msec exposure images are captured at 20 second intervals unless indicated otherwise. For analysis, kymograms of individual ssDNA molecules were generated using Fiji (ImageJ 1.48b, Wayne Rasband, National Institutes of Health, USA). Fluorescence intensity of the kymograms are adjusted based on the intensity profile of the background kymograms. The average pixel intensity for each time point is normalized so that the first frame has an intensity of 1.

Supplemental References:

Gibb, B., Silverstein, T.D., Finkelstein, I.J., and Greene, E.C. (2012). Single-Stranded DNA Curtains for Real-Time Single-Molecule Visualization of Protein-Nucleic Acid Interactions. Analytical Chemistry *84*, 7607-7612.

Gorman, J., Fazio, T., Wang, F., Wind, S., and Greene, E.C. (2010). Nanofabricated Racks of Aligned and Anchored DNA Substrates for Single-Molecule Imaging. Langmuir *26*, 1372-1379.