# Supporting Information

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### SI Materials and Methods

**Reagents.** The DNA binding dyes, Sytox Orange, Hoechst 33258 (H33258), and YO-PRO-1, and the labeling dyes, Alexa Fluor 546 and Alexa Fluor 488 maleimide, were purchased from Invitrogen. YO-PRO-1, Sytox Orange, and the Alexa Fluor dyes were dissolved in dimethyl sulfoxide (DMSO) and kept at −20 °C. H33258 was dissolved in Nanopure water and stored at −20 °C.

**Proteins and DNA.** RecQ and SSB proteins were purified as described (7). The G26C SSB mutant was purified as described (40). The concentrations of RecQ and both wild-type and mutant SSB were determined by measuring the absorbance at 280 nm and using molar extinction coefficients of 14,800 $M^{-1}{\cdot}cm^{-1}$ and 27,880 $M^{-1}{\cdot}cm^{-1}$, respectively. Biotinylated BSA (Pierce) and streptavidin (Promega) were resuspended in 20 mM TrisOAc (pH 7.5) and stored at −20 °C. Phage λ DNA was purchased from New England Biolabs (NEB) and stored at 4 °C. Plasmid DNA (pUC19) was purified by alkaline lysis followed by equilibrium ultracentrifugation in a CsCl-ethidium bromide gradient (70). Purified pUC19 was linearized with HindIII (NEB). The DNA concentration was determined using a molar extinction coefficient of 6,600 $M^{-1}{\cdot}cm^{-1}$ at 260 nm.

**Labeling of the G26C SSB Mutant.** SSB with the glycine at position 26 mutated to a cysteine residue was labeled with the indicated Alexa Fluor maleimide dye as described (40). Briefly, mutant protein was dialyzed into labeling buffer [20 mM TrisOAc (pH 8.0), 0.5 M NaCl, 1 mM EDTA, and 20% (vol/vol) glycerol] to remove DTT from the storage buffer. The maleimide dye was added at a 10-fold molar excess relative to SSB monomer concentration, and the final volume of DMSO in the reaction was kept below 5% (vol/vol). Labeling was performed at 4 °C for 4 h. The reaction was then loaded onto a 20 mL, P-10 size exclusion column preequilibrated with labeling buffer (Bio-Rad). The labeled SSB, which eluted first, was collected and subsequently dialyzed into storage buffer [25 mM TrisOAc (pH 8.0), 0.5 M NaCl, 1 mM EDTA, 1 mM DTT, and 50% (vol/vol) glycerol]. Protein concentration was determined from the absorbance (A) at 280 nm after correcting for the contribution of the dye to the absorbance at 280 nm by using a correction factor ($C_f$) of 0.22 for both dyes as provided by the manufacturer: $A_{protein} = A_{measured} - 0.22 \times A_{dye@max}$. A molar extinction coefficient of 72,000 $M^{-1}{\cdot}cm^{-1}$ was used for the Alexa Fluor 488 dye to determine its concentration from the absorbance at 493 nm. A molar extinction coefficient of 112,000 $M^{-1}{\cdot}cm^{-1}$ was used for the Alexa Fluor 546 dye to determine its concentration from the absorbance at 556 nm. For both labeled SSB preparations, the degree of labeling indicated that one monomer of SSB was labeled with one dye molecule.

**Dye-Displacement Helicase Assays.** Ensemble helicase activity was measured using dye-displacement assays as previously described (16, 42), in a reaction solution containing 25 mM TrisOAc (pH 7.5), 1 mM $Mg(OAc)_2$, 1 mM ATP, 1 mM DTT, 1 mM phosphoenol pyruvate (PEP), dye, and 25 units/mL pyruvate kinase unless noted. Spectra were collected on a SLM fluorimeter controlled by Vinci Software (ISS). Slit widths were set to 8 mm for both excitation and emission. The reaction solution (350 μL) was added to a quartz cuvette (700 μL total volume; Starna Cells), and wild-type SSB was added at a final concentration of 1 μM of tetramer. Reactions were performed at 37 °C. Hoechst 33258 (300 nM) was excited at 355 nm and emission was monitored at 465 nm. To measure the displacement of YO-PRO-1 (50 nM), the solution was excited at 490 nm and emission was monitored at 520 nm; for ethidium bromide (1 μM), excitation was at 546 nm and emission was monitored at 595 nm. The fluorescence of free dye was observed before dsDNA was added at a final concentration of 1.0 μM (base pairs). The fluorescence of DNA-bound dye was then observed and recorded, followed by addition of RecQ (100 nM) to initiate the reaction unless noted. Helicase rates were determined as described (16). Briefly, the slope of the steady-state, linear portion of the unwinding curve was measured and divided by the difference in free and bound dye fluorescence ($\Delta F_{max}$) and then multiplied by the total DNA concentration. The extent of unwinding was determined by taking the fluorescence end point of the reaction, subtracting from the DNA-bound fluorescence, and dividing by the difference of the bound and free dye fluorescence. This fraction was assumed to reflect the total amount of DNA unwound because the fluorescence of the free dye is equal to that of Hoechst 33258 (H33258) or YO-PRO-1 in the presence of SSB-bound ssDNA.

**Flow Cell Construction and Surface Modification of Glass Coverslips.** Glass coverslips were cleaned by submerging them in a solution of 95% ethanol, saturated with KOH, for 1 h, followed by sequential rinsing with Nanopure water and methanol. Channels and holes were etched into glass slides using a 30-W Epilog mini laser engraver (37, 38, 71). A coverslip spanning the etched surface on the glass was glued on using UV Optical adhesive no. 74 (Norland Products). The glue was cured by placing the flow cell a distance of 30 cm from a 100-Watt high-pressure mercury plasma arc-discharge lamp (Zeiss 100 W HBO lamp) for 20 min. Each flow channel measured 3 × 13 mm in width and length, respectively, and 100 μm deep. PEEK tubing with a 0.5-mm inner diameter (Upchurch Scientific) was attached to each of the etched holes using a 5-min epoxy (Devcon) to create ports. Before use, each channel was cleaned with 1 M NaOH for 30 min, rinsed with water, and finally filled with SM buffer, which consisted of 20 mM TrisOAc (pH 7.5), 20% (wt/vol) sucrose, and 50 mM DTT. Next, a solution of 1 mg/mL biotin–BSA in SM buffer was flowed in and incubated in each channel for 10 min at room temperature. Free biotin–BSA was washed with SM buffer, and then the channel was filled with 0.1 mg/mL streptavidin in SM buffer and incubated for 10 min at room temperature. Free streptavidin was removed by rinsing the flow cells with SM buffer. The flow cell was then filled with a solution of 1 mg/mL Roche Blocking Reagent in SM buffer. The flow cell was connected to a syringe pump and washed with SM buffer before conducting the helicase reactions.

**Incorporating Biotin Groups into λ Phage DNA.** The *cos* sites of λ phage DNA were filled in using a reaction (30 μL) containing 5 units Klenow fragment (3′ → 5′ exo⁻; NEB), λ phage DNA (80 ng/μL), and 33 μM each of dATP, dCTP, dTTP, and 33 μM biotin-11-dGTP (Perkin-Elmer) (38, 71). The reaction solution was incubated at 37 °C for 15 min before EDTA was added at a final concentration of 10 mM to stop the reaction. The polymerase was inactivated by incubation at 75 °C for 20 min. The reaction was diluted to a final volume of 100 μL by adding Nanopure water. Free nucleotides were removed from the solution using a MicroSpin S-200 HR column (GE Healthcare), equilibrated in buffer containing 10 mM Tris HCl (pH 8.0), and 1 mM EDTA. The DNA concentration was determined by measuring the absorbance at 260 nm using a molar extinction coefficient of 6,600 $M^{-1}{\cdot}cm^{-1}$ (70).

**Single-Molecule TIRF Measurements.** Unwinding of λ DNA molecules was observed on an Eclipse TE2000-U inverted microscope

with a TIRF attachment (Nikon), using a CFI Plan Apo TIRF 100×, 1.49 N.A., oil-immersion objective (37). Excitation was by 488-nm (Picarro) or 561-nm (Cobolt) lasers. Fluorescence emission was detected and separated into 515 nm (30-nm bandpass) and 600 nm (40-nm bandpass) components (Optical Insights), imaged on an iXon CCD camera (Andor), and processed using Andor imaging software. Images were collected by averaging four 50-ms exposures, every 2 s.

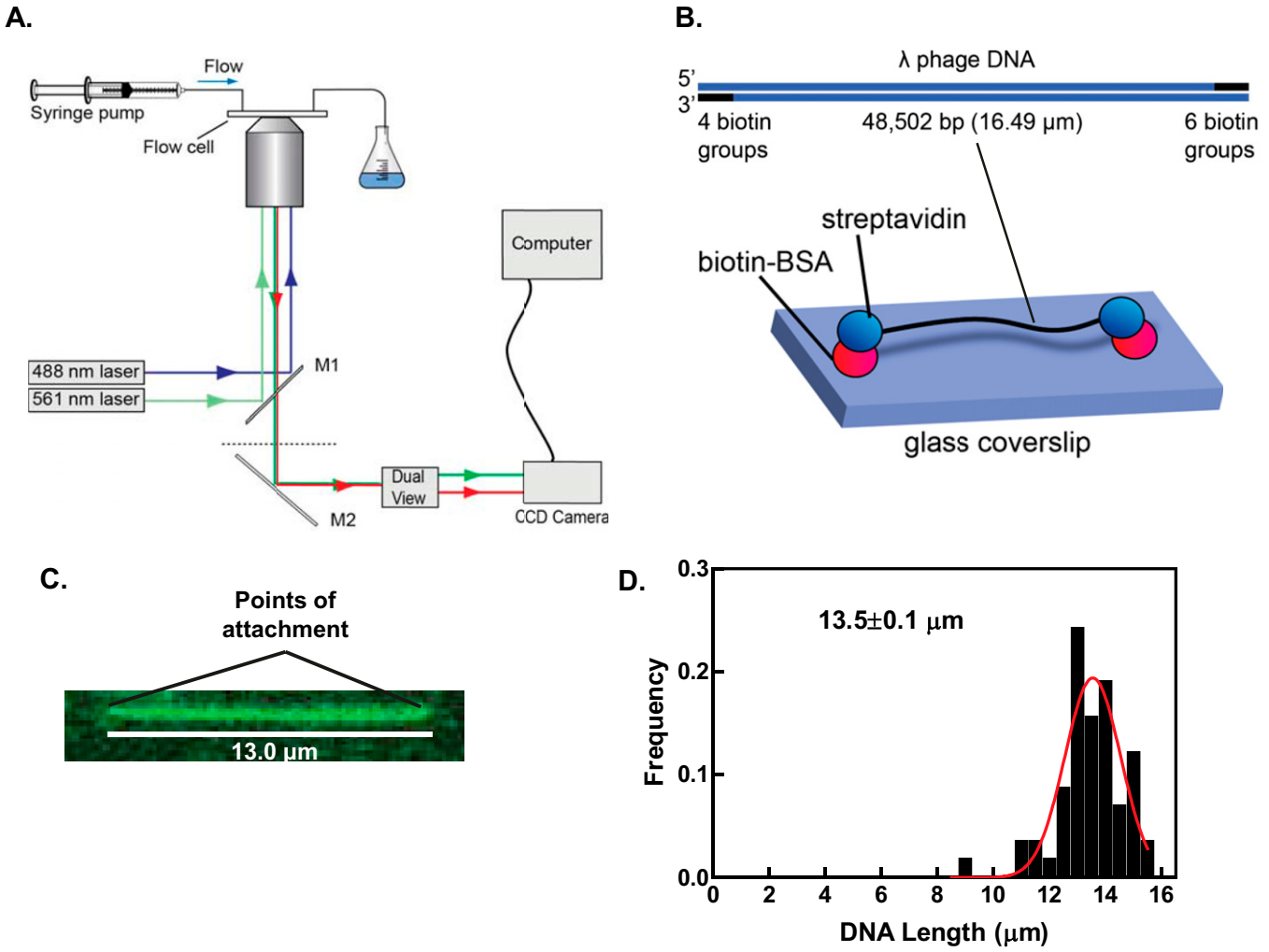



**Fig. S1.** TIRF microscopy is used to visualize unwinding of surface-tethered DNA molecules. (*A*) Schematic of the TIRF microscope system used to visualize DNA unwinding by RecQ. Flow cells were mounted on an oil-immersion 100x TIRF objective. Two lasers, 488 nm and 561 nm, were guided into the microscope objective using dichroic mirrors (M1 and M2). The fluorescence emission was filtered through the dual-view apparatus, which separates the red and green components of the emitted fluorescence. A CCD camera controlled by a computer was used to collect the fluorescence images. Laser shutters were coordinated with the iXon camera to illuminate the sample only during exposure times and reduce photobleaching of the fluorophores. (*B*, *Top*) Diagram of the phage λ DNA molecule with the indicated number of biotin groups incorporated within the 12-nt *cos* overhangs. (*Bottom*) Illustration of biotinylated λ DNA attached at both ends via biotin–streptavidin linkage. (*C*) Image of a λ DNA molecule attached to the glass surface, stained with YO-PRO-1 (100 nM), and illuminated with the 488-nm laser. The image is false colored in green and the attachment points to the glass surface are indicated. (*D*) Histogram of the lengths of 58 molecules of λ phage DNA doubly tethered to the glass surface. We fit the data to a Gaussian function and calculated a mean length of 13.5 ± 0.1 μm. This value is consistent with the observed relative extension of singly tethered λ phage DNA molecules under shear flow (72), indicating that for our doubly tethered DNA molecules, the flow-induced shear force, and not the density of streptavidin, limits dsDNA extension.

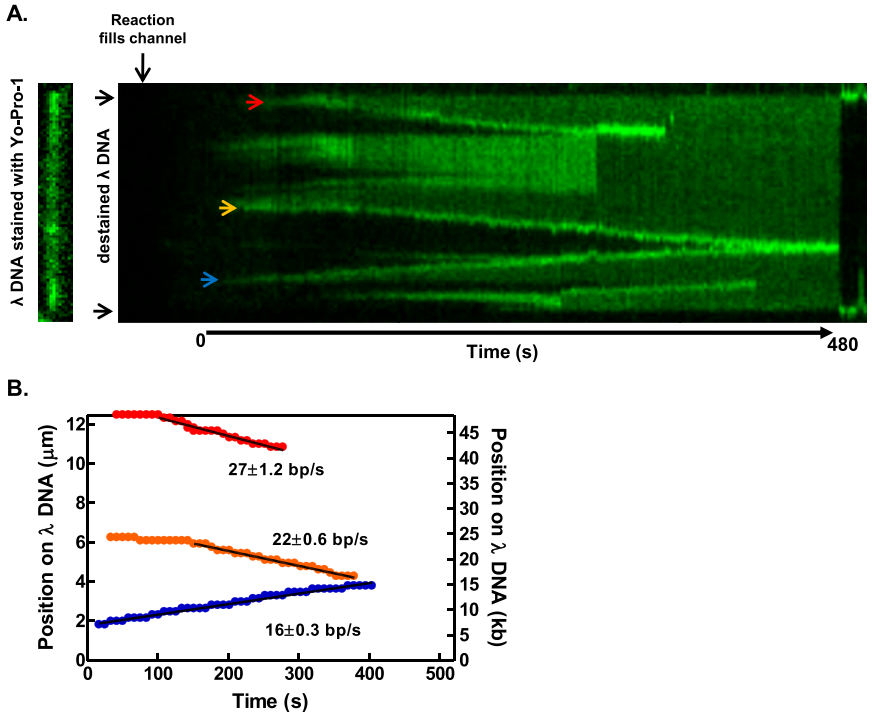



**Fig. S2.** Converging unwinding forks show no change in rate. (*A*) Kymograph of the DNA molecule in Fig. 1 and Movie S1 being unwound in a reaction containing RecQ (80 nM), ATP (1 mM), and $^{AF488}$SSB (60 nM). Three unwinding forks are observed (red, orange, and blue arrows). The orange and blue forks converge toward the end of the experiment, resulting in breakage of the molecule. (*B*) Plot of the position of each unwinding fork in *A* as a function of time (colored circles). The rate of each fork is determined by a fit to the linear portion of the position (black lines). The values of these rates are shown for each plot. At 380 s, the orange and blue forks merge and their positions are indistinguishable, given the microscope resolution. The progression of these two forks appears unchanged and linear as they converge.

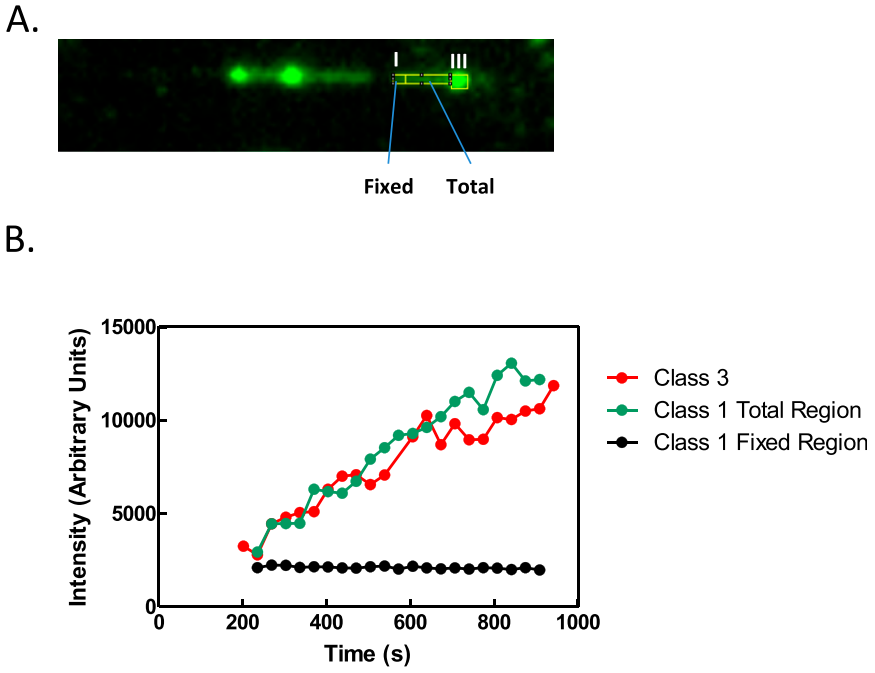



**Fig. S3.** Class III region intensity increases with unwinding fork progression and correlates with the intensity of a class I region. (*A*) Image of a DNA molecule being unwound by RecQ (100 nM) from Fig. 2. The frame represents 538 seconds of elapsed time. The outlined regions show the selected area in which the integrated intensity was measured for class I and class III regions. The measured area of the class III region was increased as the region grew in size, while the class I region area was both measured in one region (Fixed) or as it grew in length along the molecule (Total). The frame image was scaled using interpolation for clarity. (*B*) Graph of the intensity measurements from class I and class III regions during unwinding fork progression. The integrated intensity of each class type was measured every 4 frames from the corresponding movie using increasing outlined regions and is shown as a function of elapsed time.

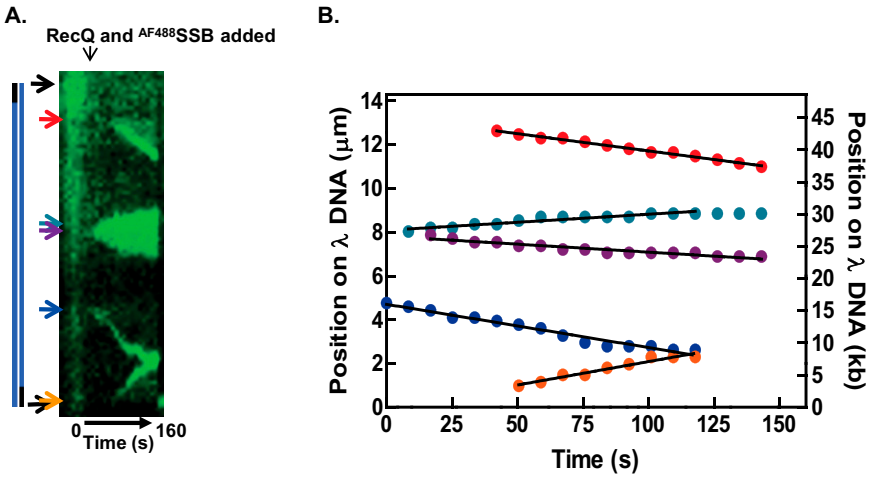



**Fig. S4.** Unwinding forks can proceed in a bidirectional fashion. (*A*) Kymograph of a molecule of DNA being unwound in a reaction containing RecQ (40 nM), ATP (1 mM), and $^{AF488}$SSB (60 nM) at 37 °C. Three unwinding forks are observed to be class I or III (red, blue, and orange arrows), whereas another unwinding fork, class II, initiates in the DNA molecule and grows in a bidirectional fashion (teal and purple arrows). (*B*) Plot of the position of each unwinding fork in *A* as a function of time. At 125 s, the orange and blue forks are too close, given our resolution, to distinguish the position of either one. The pause in the blue fork appears to be more distinctive because a scaling function was used in ImageJ to smooth the visual display of the magnified image. However, the data points in *B* were taken directly from individual frames that were not subjected to smoothing and are, thus, experimentally accurate (Movie S3).

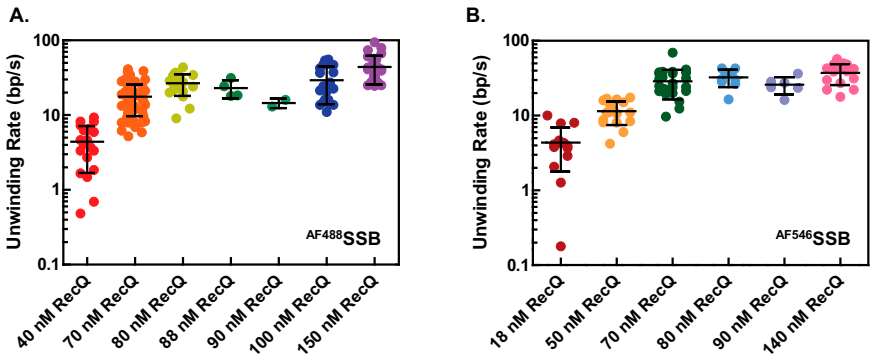



**Fig. S5.** Increasing RecQ concentration results in a distribution of increasing unwinding rates. (*A*) Distribution of the rates of unwinding by RecQ tracked by $^{AF488}$SSB and (*B*) $^{AF546}$SSB. Each circle represents a measured rate from an unwinding fork. The mean and SD at each concentration is shown by a horizontal line and error bar.

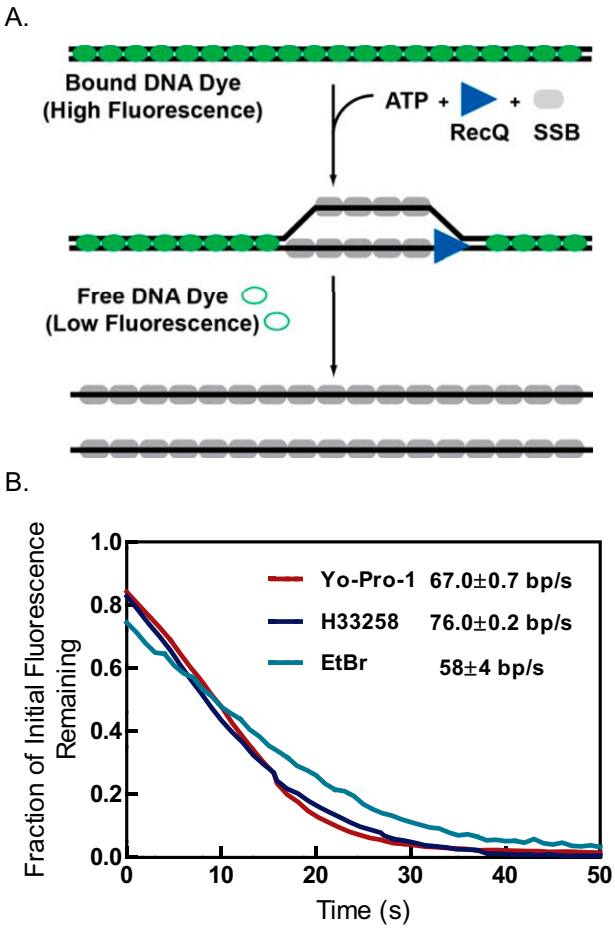



**Fig. S6.** DNA unwinding by RecQ is not substantially affected by YO-PRO-1. (*A*) Illustration of the dye-displacement helicase assay. This assay uses the change in fluorescence of a DNA-binding dye to measure DNA unwinding (16, 42). For certain DNA-binding dyes, the quantum yield of the dye increases upon binding to dsDNA, resulting in higher fluorescence relative to free dye in solution or to dye bound to ssDNA. Upon unwinding dsDNA to ssDNA, the dye is displaced, resulting in a decrease in fluorescence. (*B*) Traces from the dye-displacement assay measuring RecQ (100 nM) mediated unwinding of plasmid DNA (1 μM, in base pairs) as a function of time. Red, blue, and green traces depict displacement of YO-PRO-1, H33258, and ethidium bromide (EtBr), respectively. The unwinding rates reported are from the steady-state linear phase.

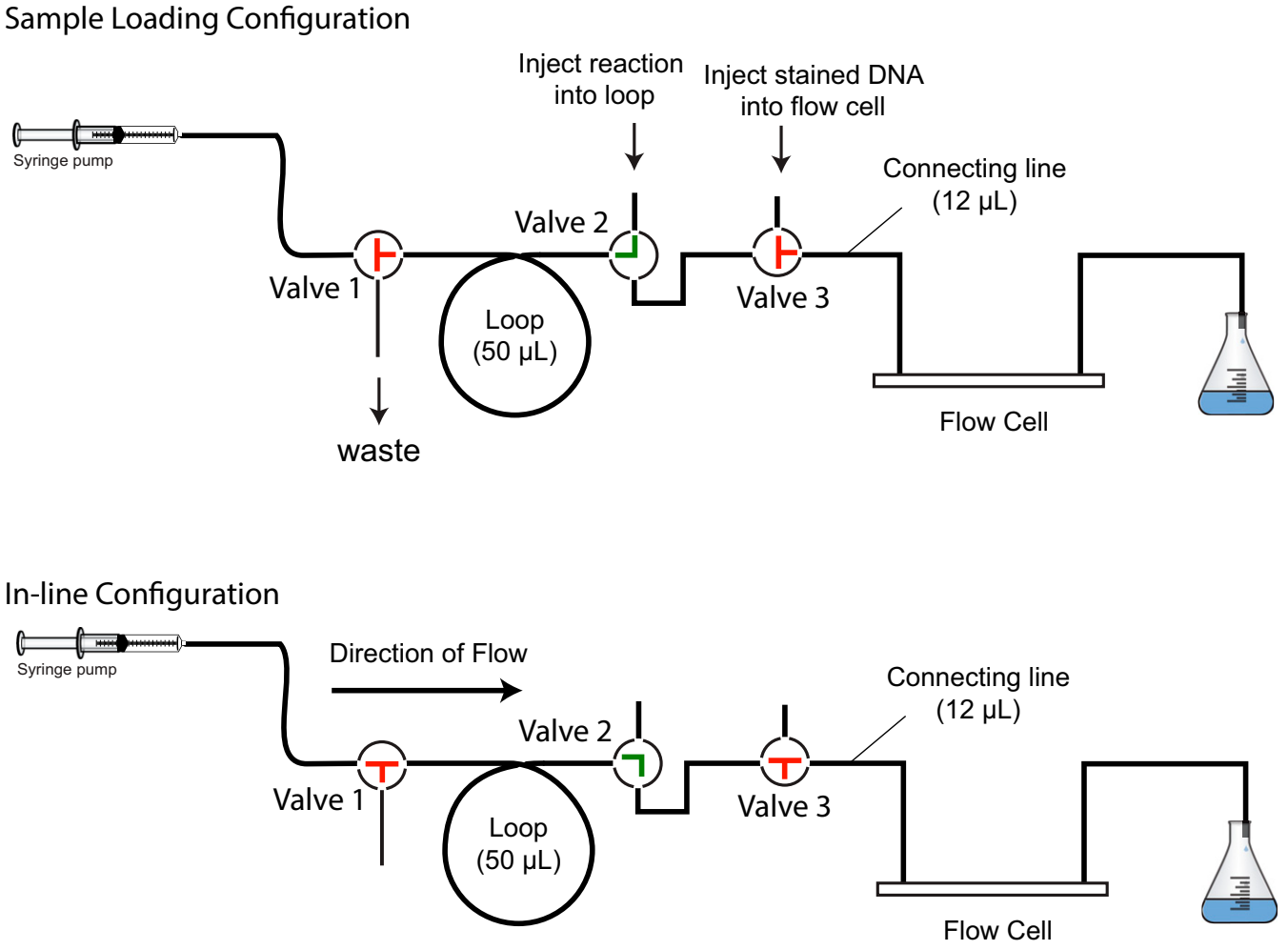



**Fig. S7.** A microfluidic injection system to rapidly exchange components in a single-channel TIRF flow cell. A schematic of the system of PEEK tubing connected by PEEK switching valves that was used to remove free RecQ from the flow channel is shown. This system is connected upstream of the flow cell and downstream of the syringe pump. In this system PEEK tubing connects two 3-port "T" valves (valve 1 and valve 3), a 3-port "90°" valve (valve 2), and a 50-µL loop. The syringe is initially filled with SM buffer with 75 nM Sytox Orange. Using the sample loading configuration (*Top*), DNA stained with Sytox Orange is injected into the flow cell with valve 3 in the indicated position, allowing DNA to attach to the surface of the flow cell. The system is then set to the in-line configuration (*Bottom*), and the syringe pump is turned on. The resulting flow of SM buffer into the flow cell extends the attached DNA and allows attachment of the other end. After locating doubly tethered DNA, the system is put back into the sample loading configuration. A reaction solution containing RecQ, ATP, Sytox Orange, and $^{AF488}$SSB is injected into the 50-µL loop, whereas the syringe is filled with a solution of ATP, Sytox Orange, and $^{AF488}$SSB that lacks RecQ. The valves are set to the in line configuration and the syringe pump is turned on to push the 50-µL reaction solution within the loop into the flow cell to initiate unwinding by RecQ. Another 50 µL of the solution lacking free RecQ flushes the flow cell, thereby removing free RecQ protein.

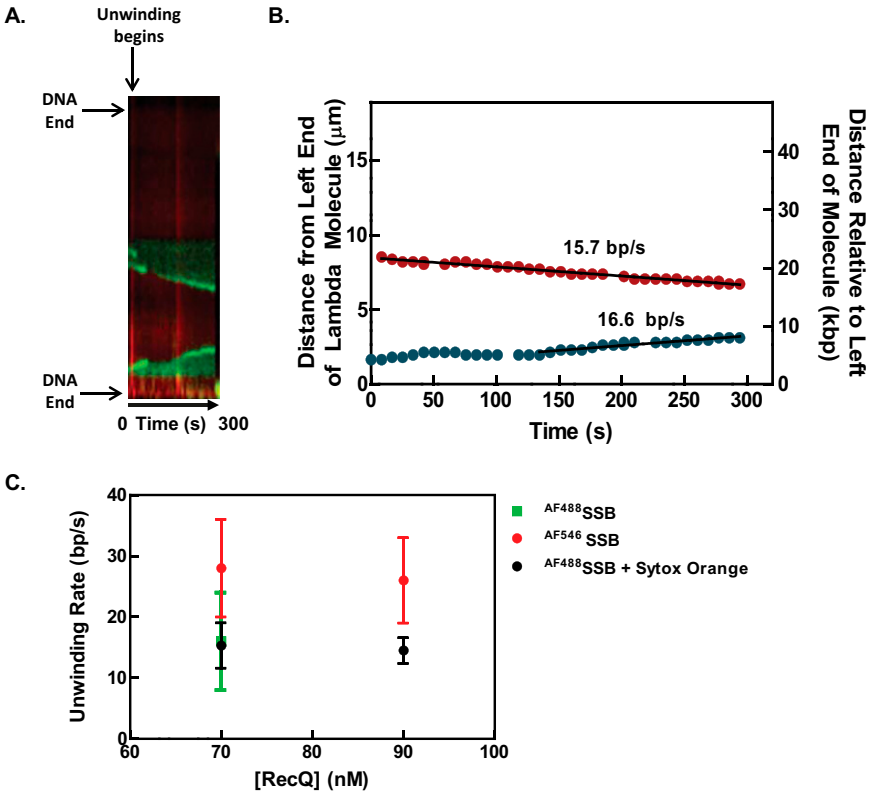



**Fig. S8.** Sytox Orange can be used to track dsDNA during unwinding. (*A*) Kymograph of a DNA molecule stained with Sytox Orange while being unwound by RecQ (90 nM) in the presence of $^{AF488}$SSB. (*B*) The unwinding rate was measured by tracking the fluorescence of $^{AF488}$SSB and fitting the linear portion of the traces. (*C*) The rates of the unwinding forks in the presence of Sytox Orange are within the range of rates measured using $^{AF488}$SSB or $^{AF546}$SSB.

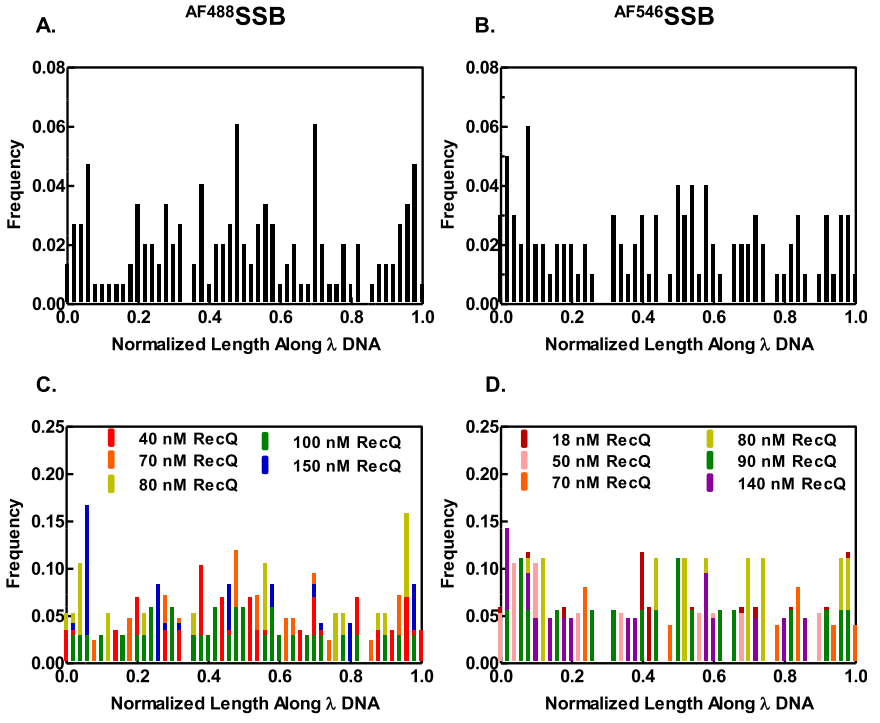
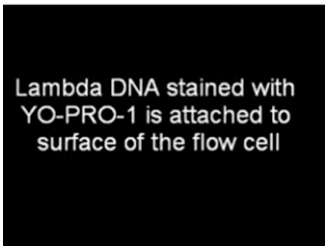
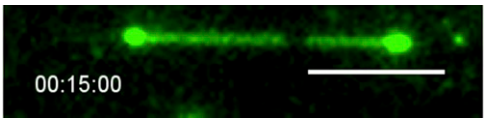



**Fig. S9.** RecQ initiates unwinding randomly along dsDNA. (*A*) Cumulative distribution of the unwinding initiation positions along λ DNA plotted as a histogram for experiments using $^{AF488}$SSB ($n$ = 148) and (*B*) $^{AF546}$SSB ($n$ = 109). The position of each event was measured relative to one end of the DNA and was normalized to the end-to-end length of each molecule for comparison between molecules. (*C*) Distribution in *A* shown for each RecQ concentration (40 nM RecQ, $n$ = 29; 70 nM RecQ, $n$ = 42; 80 nM RecQ, $n$ = 19; 100 nM RecQ, $n$ = 34; and 150 nM RecQ, $n$ = 24). (*D*) Distribution in *B* shown for each RecQ concentration (18 nM RecQ, $n$ = 17; 50 nM RecQ, $n$ = 19; 70 nM RecQ, $n$ = 25; 80 nM RecQ, $n$ = 9; 90 nM RecQ, $n$ = 18; and 140 nM, $n$ = 21).



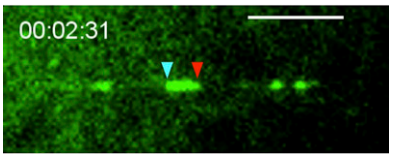

**Movie S1.** Destaining of a YO-PRO-1–stained λ DNA molecule, followed by RecQ-dependent unwinding. Initially, the DNA is fluorescent due to intercalation of the dye molecules. As a solution containing 200 mM NaCl is flowed into the flow cell, the dye dissociates and fluorescence disappears. Subsequently, a solution containing of 80 nM RecQ and $^{AF488}$SSB is flowed into the flow cell to initiate unwinding; reaction is the same as shown in Fig. 1 *B–F*. The solution fills the channel 24 s into the movie. The flow, when on, goes from left to right in the movie. The elapsed time is indicated in hours:minutes:seconds. (Scale bar, 5 μm.)

Movie S1



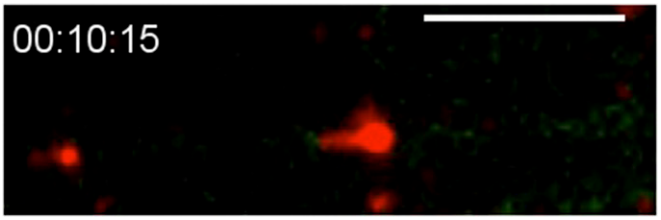

**Movie S2.** Unwinding of a λ DNA molecule in a solution containing 100 nM RecQ and $^{AF488}$SSB. Initially, the DNA is not seen due to the absence of fluorescent dye. The flow, when on, is from left to right, and the RecQ and $^{AF488}$SSB fill the channel 24 s into the movie. The elapsed time is indicated in hours:minutes:seconds. (Scale bar, 5 μm.)

Movie S2



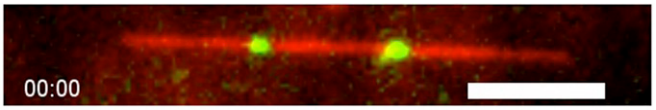

**Movie S3.** Unwinding of a λ DNA molecule in a solution of 40 nM RecQ and $^{AF488}$SSB at 37 °C. Initially, the DNA is not seen due to the absence of fluorescent dye. A white arrow indicates initiation of a class 2 unwinding fork that then moves in a bidirectional fashion as indicated by the cyan (left end) and red (right end) arrows. The flow, when on, is from left to right in the movie and the RecQ and $^{AF488}$SSB fill the channel 33 s into the movie. The elapsed time is indicated in hours:minutes:seconds. (Scale bar, 5 μm.)

Movie S3



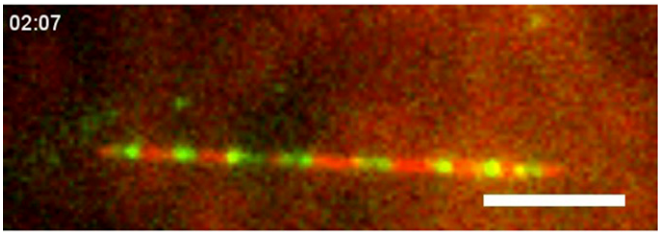

**Movie S4.** Unwinding of a λ DNA molecule in a solution of 50 nM RecQ and $^{AF546}$SSB. Initially, the DNA is apparent due to the binding of the fluorescent YO-PRO-1 dye. The flow, when on, is from left to right in the movie, and RecQ and $^{AF546}$SSB fill the channel 58 s into the movie. The elapsed time is indicated in hours:minutes:seconds. (Scale bar, 5 μm.)

Movie S4




**Movie S5.** Reinitiation of DNA unwinding by RecQ. A two-color image of a λ DNA molecule containing two unwound regions is shown. The DNA molecule was only partially unwound in a previous experiment and unwinding had apparently halted after several kilobase pairs of unwinding. The dsDNA is stained with Sytox Orange and the two unwound regions are marked with $^{AF488}$SSB. At ~15 s into the movie, a solution containing 70 nM RecQ, 60 nM $^{AF488}$SSB, 1 mM ATP, 1 mM $Mg(OAc)_2$, and 75 nM Sytox Orange is introduced into the flow cell, using the microfluidic injection system shown in Fig. S7. The flowcell is finished filling at ~47 s, at which point the Sytox Orange in the flow cell starts photobleaching, resulting in decreased background fluorescence. Subsequent continued unwinding (as class I/III events) from the preexisting unwound regions is now evident, until the DNA molecule breaks. The time is indicated in minutes:seconds.

Movie S5




**Movie S6.** Unwinding of DNA by stable RecQ oligomers. The elapsed time is indicated in hours:minutes:seconds. DNA unwinding was initiated by using the microfluidic injection system (Fig. S7) with the loop containing 70 nM RecQ, 60 nM $^{AF488}$SSB, 1 mM ATP, 1 mM $Mg(OAc)_2$, and 75 nM Sytox Orange in SM buffer. This solution is pumped into and fills the flow cell in Fig. 4*A* at 39 s into the movie. Subsequently, 50 μL of a solution containing 60 nM $^{AF488}$SSB, 1 mM ATP, 1 mM $Mg(OAc)_2$, and 75 nM Sytox Orange is pumped into the flow cell to remove the free RecQ, starting at 1:03 into the movie and ending at 1:27. The forks are observed to continue to unwind after removing free RecQ.

Movie S6