Dynamic 1D Search and Processive Nucleosome Translocations by RSC and ISW2 Chromatin Remodelers

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Summary

Eukaryotic gene expression is linked to chromatin structure and nucleosome positioning by ATP-dependent chromatin remodelers that establish and maintain nucleosome-depleted regions (NDRs) near transcription start-sites. Conserved yeast RSC and ISW2 remodelers exert antagonistic effects on nucleosomes flanking NDRs, but the temporal dynamics of remodeler search, nucleosome engagement and mobilization for promoter accessibility are unknown. Using optical tweezers and 2-color single-particle imaging, we investigated the Brownian diffusion of RSC and ISW2 on free DNA and sparse nucleosome arrays. RSC and ISW2 rapidly scan DNA by one-dimensional hopping and sliding respectively, with dynamic collisions between remodelers followed by recoil or apparent co-diffusion. Static nucleosomes block remodeler diffusion resulting in remodeler recoil or sequestration. Remarkably, both RSC and ISW2 use ATP hydrolysis to translocate mono-nucleosomes processively at ~30 bp/sec for surprising distances on extended linear DNA. Processivity and opposing push-pull directionalities of nucleosome translocation shown by RSC and ISW2 shape the distinctive landscape of promoter chromatin.

Introduction

Eukaryotic chromatin structure is central to gene expression, with nucleosome positioning and composition being established and maintained by four families of ATP-dependent chromatin remodelers (SWI/SNF, CHD, ISWI, and INO80).[1-3] Active or poised gene promoters exhibit a defined chromatin architecture, the nucleosome-free or nucleosome-depleted region (NDR)[4,5] to which transcriptional machinery is recruited.[2] Genomic studies have shown that in budding yeast, RSC (SWI/SNF family) and ISW2 (ISWI family) remodelers have opposing directional effects on promoter nucleosome movements that widen or narrow the NDR with corresponding effects on transcription *in vivo*, giving rise to the concept of dynamic nucleosome pushing and pulling by remodelers to regulate promoter accessibility [6-9]. Remodelers essential for repositioning promoter nucleosomes and incorporating histone variant H2A.Z are enriched in vivo at the +1 and -1 nucleosomes and the intervening NDR DNA.[10,11]

Live-cell single particle tracking (SPT) studies have provided a window into the complexity of remodeler-chromatin interactions, revealing substantial binding frequencies, highly transient chromatin association, and stable residence times of only several seconds.[12,13] By integrating kinetic SPT findings with genomic and proteomic information, remodelers with opposing functions could be calculated to co-occupy the same promoter DNA at some frequency, suggesting a 'tug-of-war' competition between remodelers for the +1 and –1 nucleosomes flanking the NDR.[13] Moreover, chromatin-bound remodelers display several-fold higher diffusion coefficients than nucleosomal histones in living cells, indicating potential one-dimensional (1D) diffusion of bound-

state remodelers, i.e. local 1D search on nucleosome-depleted promoter chromatin[13] similar to 1D diffusion of bacterial DNA-binding proteins.[14,15] Consistent with this, yeast SWR1, an INO80 family remodeler that performs histone H2A.Z exchange, searches for nucleosome targets by 1D diffusion on free DNA constrained between reconstituted nucleosomes.[16] Although single-nucleosome fluorescence resonance energy transfer (FRET) studies have indicated that RSC, INO80, ACF, and Chd1, but not SWR1, can utilize ATP-hydrolysis to reposition nucleosome core histones on DNA at short length-scales[17-22] the processivity and extent of histone octamer movement thus catalyzed or 'nucleosome sliding' is unknown and dynamic remodeler engagement and directional nucleosome translocation have not been systematically studied.

Here, we investigate RSC and ISW2 remodeler 1D diffusion using naked DNA and sparsely reconstituted nucleosome array substrates stretched by optical tweezers and imaged by confocal microscopy to analyze remodeler-nucleosome interactions. Differences in remodeler diffusion on naked DNA under varying ionic strength and nucleotide conditions indicate that RSC scans DNA mainly by 1D hopping, while ISW2 undergoes 1D sliding. We also observed remodeler-remodeler collisions, and infrequent bypassing events. 1D interactions between a remodeler and a nucleosome are similarly dynamic, with many collisions resulting in confined diffusion or colocalization. In the presence of ATP, both RSC and ISW2 show striking processivity and nucleosome translocation in opposing directions on sparse nucleosome arrays. We discuss these findings in the context of target search and remodeler dynamics leading to NDR expansion or contraction.

Results

1D diffusion of RSC and ISW2 on DNA reveal distinct hopping and sliding modes

DNA helicases and the chromatin remodeler SWR1 have been shown to undergo 1D diffusion on dsDNA.[16,23] Given the enrichment of RSC and ISW2 at NDRs and flanking +1 and -1 nucleosomes[9,11], we hypothesized that both remodelers should be capable of free one-dimensional (1D) Brownian diffusion on dsDNA (**Fig. 1A**). Accordingly, we employed dual optical tweezers and scanning confocal microscopy at 72 nm (225 bp) 1D spatial resolution with laminar flow microfluidics (**Fig. S1A**) to directly visualize and quantify diffusion of RSC and ISW2 complexes on stretched lambda DNA (48.6 kbp) (**Fig. 1B, Materials and Methods**). We observed 1D dynamics of purified, functionally active, fluorescent RSC and ISW2 labeled at a HaloTag moiety fused to the catalytic ATPase (**Fig. S1B-C**; **Fig. S3A-B; Materials and Methods)**. In contrast to the stationary dCas9 control[24], both ISW2 and RSC display visible movements on DNA, as evidenced by the overlay of compiled kymographs exhibiting diffusion away from their initial binding sites (**Fig. 1C**). We use 20-frame (0.85 s) rolling windows along trajectory length to quantify instantaneous diffusion coefficients [short-range diffusion] (**Fig. 1D, Materials and Methods**), classifying trajectory segments into 'non-diffusive', low-diffusion, and high-diffusion groups for RSC (**Fig. 1D**) and ISW2 (**Fig. S1D**).

The 'non-diffusive' group, with diffusion coefficients less than 0.01 μm²/s, is indistinguishable from dCas9 and represents either no diffusion or very slow diffusion below the detection limit (**Fig. 1E**). Directional movements with speeds slower than 294 bp/s on linear DNA would not be detected using the rolling-window method but can be revealed when detection is extended over a longer duration [long-range diffusion, described later]. On average, one-third of time traces are classified as non-diffusive for the two remodelers (**Fig. S1D**). The high-diffusion group, with diffusion coefficients equal to or greater than 0.04 μm²/s, represents the upper-limit diffusion for both RSC and ISW2 (**Fig. 1E**). The data shows that remodelers undergo frequent transitions during 1D scanning between non-diffusion, low and high diffusion **(Fig.1D)**. These diffusive transitions could be due to differences in interaction energies with the underlying DNA sequence and intrinsic remodeler conformations that vary in DNA affinity along the lambda genome sequence.[25-27]

Under the same buffer conditions, RSC is more diffusive than ISW2, as evidenced by its larger diffusion coefficient (D_{coef}) (**Fig. 1E**) and increased duration in the high-diffusion category (**Fig. S1D**). Considering the larger size of RSC (~1 MDa) compared to ISW2 (~300 kDa), our observation is inconsistent with the predicted diffusion based on the Stokes-Einstein equation relating D_{coef} to the particle size, suggesting that the two remodelers utilize different diffusive mechanisms.[15,28-30] Of the two main types of 1D diffusion, hopping and sliding.[14,31,32] 1D hopping, but not 1D sliding, is sensitive to screening ions, and is not constrained to the DNA helical axis. In contrast, 1D sliding is constrained to follow the helical axis and is speed-limited by rotational drag.[33] We measured diffusion for each remodeler under increasing salt conditions and found that both RSC and ISW2 exhibit shorter bound lifetimes at higher salt, with ISW2 binding undetectable at 150 mM KCl (**Fig. 1F**). Importantly, however, RSC displays elevated high-diffusion coefficients (**Fig. 1G, Fig. S1E**) and longer duration in the high-diffusion category (**Fig. S1F)** while these parameters for ISW2 remains unaffected by elevated salt up to 100 mM KCl (**Fig. 1G, Fig. S1E-F**). These findings suggest that RSC largely utilizes a 1D hopping mode of diffusion, whereas ISW2 employs helical-coupled sliding (**Fig. 1H**). Additionally, we found that RSC displays a substantially higher diffusion with ATPγS, suggesting that conformational changes associated with nucleotide binding may induce a more diffusion-competent state (**Fig. S1G-H**).

Remodeler-remodeler collisions during 1D search

Yeast gene promoters are bustling double-helical thoroughfares for transcription and chromatin regulators, the latter displaying macroscopic promoter occupancies of 10%-90% (despite rapid on-off kinetics) for any duration in live cells, compared to the ~5% occupancies of most general transcription factors[13,34]. This raises the possibility of encounters or collisions in 1D between two or more remodelers on the same length of accessible DNA. We used differentially labeled RSC and ISW2 to directly visualize heterotypic ISW2-RSC (**Fig. 2A**) and homotypic RSC-RSC (**Fig. 2B**) collisions, identifying three types of interactions (**Fig. 2C-E**) (i) short-lived colocalization (**Fig. 2C**) ($t_{1/2}$ 0.033 s for ISW2-RSC; $t_{1/2}$ 0.031 s for RSC-RSC) followed by recoil (**Fig. 2F, G**); (ii) longer-lived colocalization events, involving brief co-diffusion of two remodelers (**Fig. 3D**) ($t_{1/2}$ 0.68 s for ISW2-RSC; $t_{1/2}$ 0.70 s for RSC-RSC (**Fig. 2H, G**) (**Fig. S2A-D**) and (iii) rare bypass events (**Fig. 2E**), (0.2-0.3% of all colocalizations for both ISW2-RSC and RSC-RSC encounters (**Fig. 2E, J, K**). These findings suggest that remodelers can act not only as roadblocks but may also exhibit mutual affinity on encounter resulting in joint 1D diffusion.

Nucleosomes halt 1D diffusion and sequester RSC and ISW2

How do remodelers behave on encountering a nucleosome via 1D diffusion (**Fig. 3A**)? We investigated remodeler-nucleosome collisions using sparsely deposited nucleosome arrays reconstituted with site-specifically labeled histone octamers and imaged by pulsed (Cy3) or continuous (JFX554) laser excitation (**Fig. 3B-C**). We estimated the number of nucleosomes by counting force-induced unwrapping events at 15 pN or higher [25 nm length extension per nucleosome unwrapping](**Fig. 3D**)[35-37] and collected data on fibers containing ~10 nucleosomes/array (RSC and ISW2) and ~30 nucleosomes/array (RSC) (**Fig. 3E**). In the absence of ATP or with ATPγS, we documented collisions with recoil (**Fig. S3C, S3D**), stable colocalization (**Fig. S3E**), and nucleosome bypass on the stretched template (**Fig. S3F**); all event types sometimes observed within a single temporal trace (**Fig. 3F-G**). As noted earlier, in addition to high and low levels of diffusion, remodelers frequently transition into a 'non-diffusive' state [static sequestration or undetectable movements] of variable duration with a half-life on the order of 3-5 seconds on naked linear DNA (RSC; T=3.8 s, no ATP, T=3.3 s, ATPgS and ISW2; T=5.3 s, no ATP, T=3.5 s, ATPgS) (**Fig. 3H-K)**. However, on nucleosome encounter by either RSC or ISW2, non-diffusive remodeler dwell times increase substantially (~5 fold with no ATP; ~8 fold with ATPgS) (RSC; T=18 s, no ATP, T=28 s, ATPgS)(**Fig. 3H, L**)(ISW2; T=28 s, no ATP, T=28 s,

ATPgS)(**Fig. 3H-I, L-M**). We conclude that encounters in 1D between diffusing remodelers and static nucleosomes without ATP hydrolysis result in remodeler sequestration.

In the presence of ATP, we found little substantive change for RSC (T = 23 s, ATP), but the non-diffusive half-life for ISW2 decreased by ~2 fold (T=12 s, ATP) (**Fig. 3H-I, L-M**). While we cannot directly observe very short-range translocation events in our rolling window analysis, this decreased half-life for ISW2 is consistent with its known ATP-dependent dynamic interactions with the nucleosome substrate.[38]

ATP-dependent, processive and directional nucleosome translocation by RSC and ISW2

Because short-range analysis of 1D diffusion through MSD is unsuitable for directly visualizing the anticipated ATP-dependent nucleosome translocations by RSC and ISW2, we explored the potential for long-range nucleosome movements >300 bp (>5 s) over the entire period of fluorophore detection (up to several minutes). Both remodelers, acting on promoter nucleosomes, have competing effects on +1 nucleosome positioning *in vivo*.[11] RSC widens the NDR by pushing the +1 nucleosome further downstream, while ISW2 narrows the NDR by pulling it in the opposite direction.[11] Indeed, we observed numerous ATP-hydrolysis dependent nucleosome translocation events comprising 42% of RSC-nucleosome and 21% of ISW2-nucleosome encounters (**Fig. 4A-D; S4A-B**). Strikingly, nucleosome translocations are processive, as evidenced by the co-mobility of nucleosome and remodeler fluorescence in one direction for extended periods lasting up to minutes (**Fig. 4C-D**).

Interestingly, we observed a higher frequency of ATP-dependent translocating events at the start of imaging for RSC as compared to ISW2 **(Fig. 4G-H, blue**). Given that there is a lag before the start of imaging, this may be due to RSC encountering nucleosomes faster due to its higher 1D diffusivity. Alternatively, ISW2 may be often found searching at the start due to faster turnover after nucleosome encounter. Furthermore, translocation events often terminate in stable non-diffusive colocalization of remodeler with nucleosomes **(Fig. 4G-H, red**). Importantly, we document a small number of translocation termination events on visible encounter with a downstream nucleosome (N= 2 for RSC, N = 2 for ISW2) **(Fig. S4C-D**). While rare due to photobleaching of nucleosome fluorescence, the maximum translocation to termination distance is also close to the predicted average spacing between sparsely deposited nucleosomes on these arrays (~2000-5000 bp)(**Fig. 4I**). Altogether, the data suggests that downstream nucleosomes have a prominent role in blocking processive nucleosome translocation.

Notably, for the limited subset of RSC and ISW2 kymographs that display both 1D diffusion followed by nucleosome encounter and translocation, we observed a distinct bias in the direction of translocation. RSC shows a moderate preference for translocation in the same direction as approach to the nucleosome (6/9 events), while ISW2 exhibits a strong bias for translocation in the opposite direction to its approach (12/13 events) (**Fig. 4E-F; Figure S4E-F).** We also observed rare instances of directional switch during nucleosome translocation for RSC (9% of 65 traces), but none (out of 46 traces) for ISW2 (Fig. S4A, example #3). The observed bias provides direct visual evidence for opposing directions of nucleosome translocation that is a central feature of the push-pull model for expansion and contraction of the NDR.

We calculated the average translocation speeds both RSC and ISW2. Interestingly, of the translocating traces, 12% of RSC and 9% of ISW2 traces showed speed changes (**Fig. 4I**). To determine translocation speeds for both remodelers, we segmented time traces into single-speed constituents from all translocation events, measuring their duration and length (**Fig. 4J, Fig. S4G-H**). Of interest, ISW2 and RSC exhibited the same average translocation speed of 29 bp/s (RSC s.d. 9.8; ISW2 s.d. 14)(**Fig. 4K**). Uninterrupted, speed-constant RSC translocations show a duration of 33 seconds (+5 s/-6 s), and 940 bp (+160 s/-260 s) (**Fig. S4G**). ISW2-dependent

events were slightly shorter-lived, lasting 22 seconds (+24 s/-4 s) over 690 bp (+630 s/-160 s) (**Fig. S4H**).

In summary, RSC and ISW2 display surprising processivity in nucleosome translocation compared to demonstrated short-range nucleosome mobility characterized by single molecule FRET.[17-19,38-40] Remodelers search in 1D within the NDR to engage and reposition flanking nucleosomes, without dissociation-reassociation until blocked by distal nucleosomes (**Fig. 4L**), the direction of ensuing nucleosome translocation biased in a way that is highly relevant to the regulation of NDR size.

Discussion

The historical framework for how DNA binding proteins find their targets at "faster-than-[3D] diffusion-controlled rates" demonstrated the importance of 1D diffusion on nonspecific DNA to facilitate target binding.[14] In this context we investigated the search processes of two chromatin remodelers RSC and ISW2, which target a subset of nucleosomes flanking the short stretches of nucleosome-depleted DNA regions (NDRs) at promoters genome-wide. We directly visualized 1D target search by RSC and ISW2 on extended naked DNA within a sparsely reconstituted nucleosome array as a surrogate for yeast NDRs. The two remodelers exhibit different modes of 1D scanning, as shown by their sensitivity to salt changes; ISW2 and RSC favor 1D sliding and 1D hopping during their scans of naked DNA, respectively.

Despite being vacant of nucleosomes, NDRs at promoter regions resemble congested marketplaces with active protein traffic from multiple transcription components and regulators. SPT studies from our lab have demonstrated a likelihood of two remodelers co-occupying the same promoter, raising the possibility of interference during target search process.[13] For the bacterial Lac operon, it has been previously theorized that molecular crowding limits diffusion to the "vacancies" between LacI and other proteins bound to DNA.[41] Supporting this concept, our data shows that RSC and ISW2, confine their diffusion. These collisions and colocalizations are highly transient on a sub-second timescale and 1D diffusion is also very fast, with RSC and ISW2 able to scan an NDR of 150 bp within 0.2 seconds. Thus, the search time after contact with the NDR constitutes only a small fraction of the overall *in vivo* remodeler lifetime bound to chromatin (several seconds).[13] These kinetics of remodeler 1D diffusion on NDRs are unlikely to be functionally rate-limiting, which underscores the importance of prior events involving 3D nucleoplasmic diffusion and recruitment to the NDR, and subsequent nucleosome engagement, remodeler sequestration and activation of the catalytic ATPase motor.

Notably, we directly captured long-range processive translocation of individual nucleosomes by both RSC and ISW2 over kbp-distances at speeds of 29 bp/s. Previous studies established remodeler-driven nucleosome translocations on short DNA fragments (~200 bp).[17-19,38-40] These studies revealed that RSC[18] and ISW2[39,40] generate unidirectional mononucleosome translocation events, whereas ISW1, CHD1, and the catalytic subunit of ISW2 (Isw2p) remodelers produce back and forth nucleosome translocations[17,22,40], at speeds of ~2 bp/s.[17] While, short-range ATP-dependent nucleosome translocations are not resolved by our instantaneous diffusion analysis limited to a 1D localization precision of 226±32 bp (72±10 nm), our long-range analysis demonstrates speeds >10-fold higher than previously reported for ACF.[17] This difference could be due to sub-saturating concentrations of ATP used in previous studies to resolve single base-pair stepping,[17-19,38-40] to our use of lambda DNA rather than the high-affinity 601-nucleosome positioning sequence, and to substrate tension (5 pN) from stretching arrays between two optical traps in the regime of unwrapping the outer turn of nucleosomal DNA.[36,42]

We also documented a few instances where processivity of nucleosome translocation was actually terminated by a second nucleosome in its path. This is fully consistent with the maximum translocation distance observed between sparsely reconstituted nucleosome arrays on lambda DNA. In the context of the native NDR, our findings suggest that once bound, remodelers rapidly

traverse the NDR by 1D sliding or hopping to engage with and processively translocate +1 and -1 nucleosomes until halted by upstream and downstream nucleosomes or other protein roadblocks.

It is of interest that histone H2A fluorescence is retained over long-lived nucleosome translocation events, indicating the absence of nucleosomal H2A dissociation or eviction. This is anticipated for ISW2, as the ISWI family repositions nucleosomes without histone eviction[43,44], but surprising for RSC which has been found to evict histone octamers.[45] However, our assays are conducted in the absence of histone chaperone Nap1 which is known to facilitate histone eviction.[46] The influence of histone chaperones on processive nucleosome translocation and histone eviction is a topic for future study.

RSC and ISW2 have opposing effects on positioning NDR-flanking nucleosomes *in vivo*, yet it has been unclear whether the two remodelers possess intrinsically opposed directionality during nucleosome translocation to either expand or contract NDR size.[47-49] Our findings for moderate bias for translocation by RSC in the same direction as approach to the nucleosome, and strong bias for translocation by ISW2 in the opposite direction provides direct evidence in support of the "push-pull" mechanism for controlling NDR width. Thus, 1D hopping diffusion by RSC on naked DNA between sparse nucleosome arrays, nucleosome engagement and translocation in the same direction as its approach expands the NDR (pushing from the NDR long-linker) while 1D sliding diffusion and nucleosome translocation by ISW2 in the opposite direction shrinks the NDR (pulling towards the NDR long-linker).

Indeed, the recent cryoEM structures of RSC[50,51] demonstrating asymmetric orientation of RSC DNA binding components on a long-linker nucleosome relative to ATPase contact at SHL-2 of the core particle provides the structural basis for movement of +1 and –1 nucleosomes away from the NDR. Although similar structural data for ISW2 is unavailable, a proposed model based on biochemical and footprinting analyses predict ISW2-driven nucleosome movement in the direction towards the NDR.[39] Additional mechanisms that may contribute to directional nucleosome movement include DNA sequence-binding preferences of RSC to GC-rich motifs and poly dA-dT stretches that may orient the remodeler on the NDR[52], the introduction of diffusion barriers in the NDR by stable, high-occupancy, pioneer-like transcription factors[49,53-55], and sensing of mechanically rigid DNA in the NDR as was shown for INO80.[56] The convergence of structural, biochemical, genomic, and single molecule imaging approaches offers deep insights and exciting opportunities for understanding the biogenesis and dynamics of chromatin accessibility that is fundamental to the regulation of genome-based activities.

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Declaration of Interests
Patents and patent applications describing improved fluorophores (with inventor LDL) are assigned to the Howard Hughes Medical Institute

Main Figures

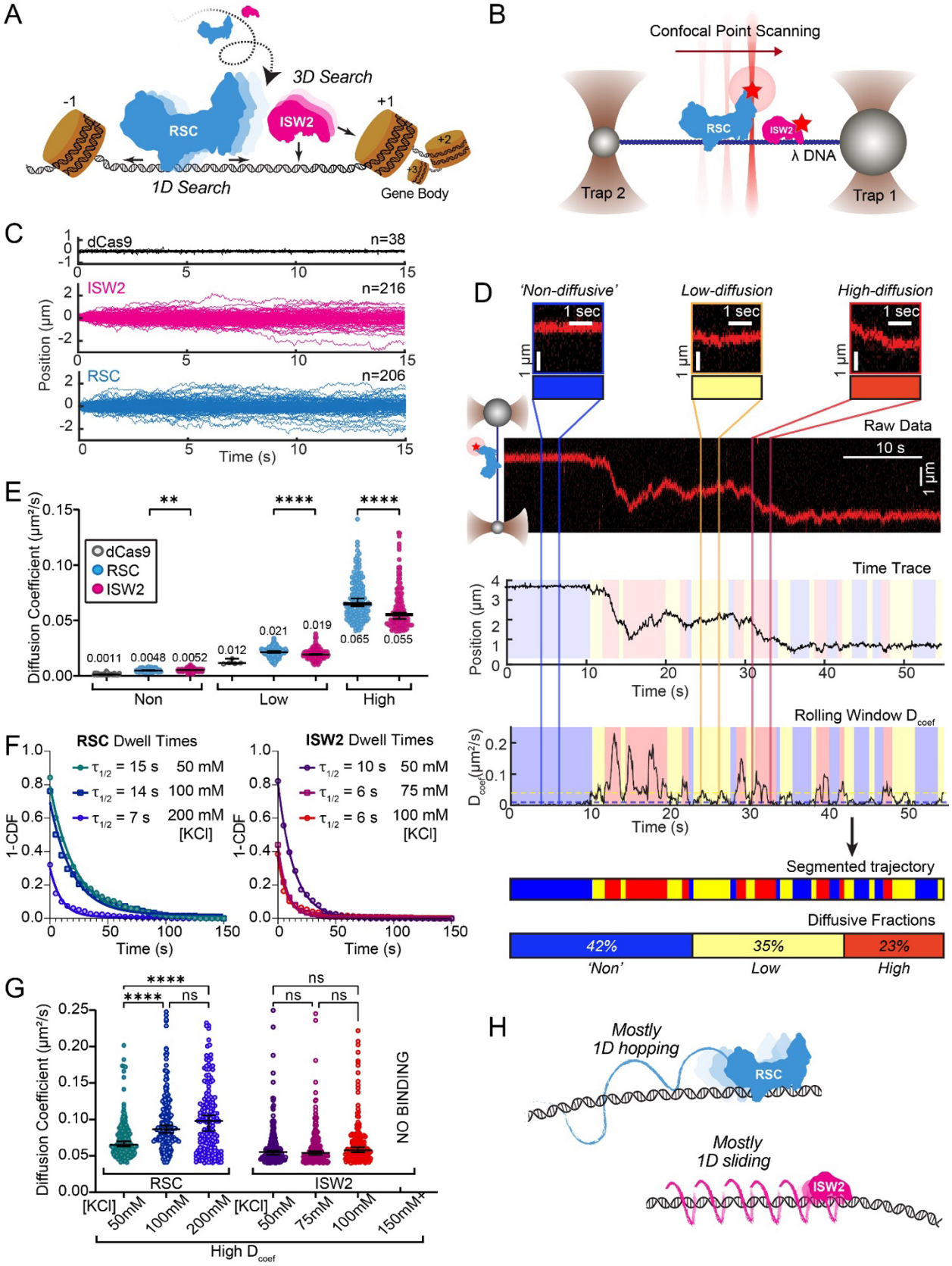

Figure 1. RSC and ISW2 Diffusion on Lambda DNA and Impacts of Ionic Strength. (A) Schematic: RSC and ISW2 bind nucleosome-free DNA at yeast promoters. (B) Optical tweezers and confocal scanning microscopy assay. (C) Aligned trajectories for RSC, ISW2, and dCas9. (D) Rolling window analysis assigns diffusion coefficients (Dcoef) and percentages for non-diffusive, low, and high diffusion. (E) Heterogeneous 1D diffusion of RSC and ISW2 on lambda DNA (50 mM KCl, no ATP). Scatter violin plots show mean Dcoef per diffusion type. Mann-Whitney tests compare RSC vs ISW2 distributions. Median Dcoef values with 95% confidence intervals are shown. (F) Dwell times for RSC and ISW2 at varying KCl concentrations; single-exponential decay fit and half-lives shown. (G) Ionic strength impact on high-mobility diffusion at different KCl concentrations. Scatter violin plots depict median values and 95% confidence intervals. (H) Model: RSC primarily undergoes 1D hopping, while ISW2 performs helically coupled 1D sliding.

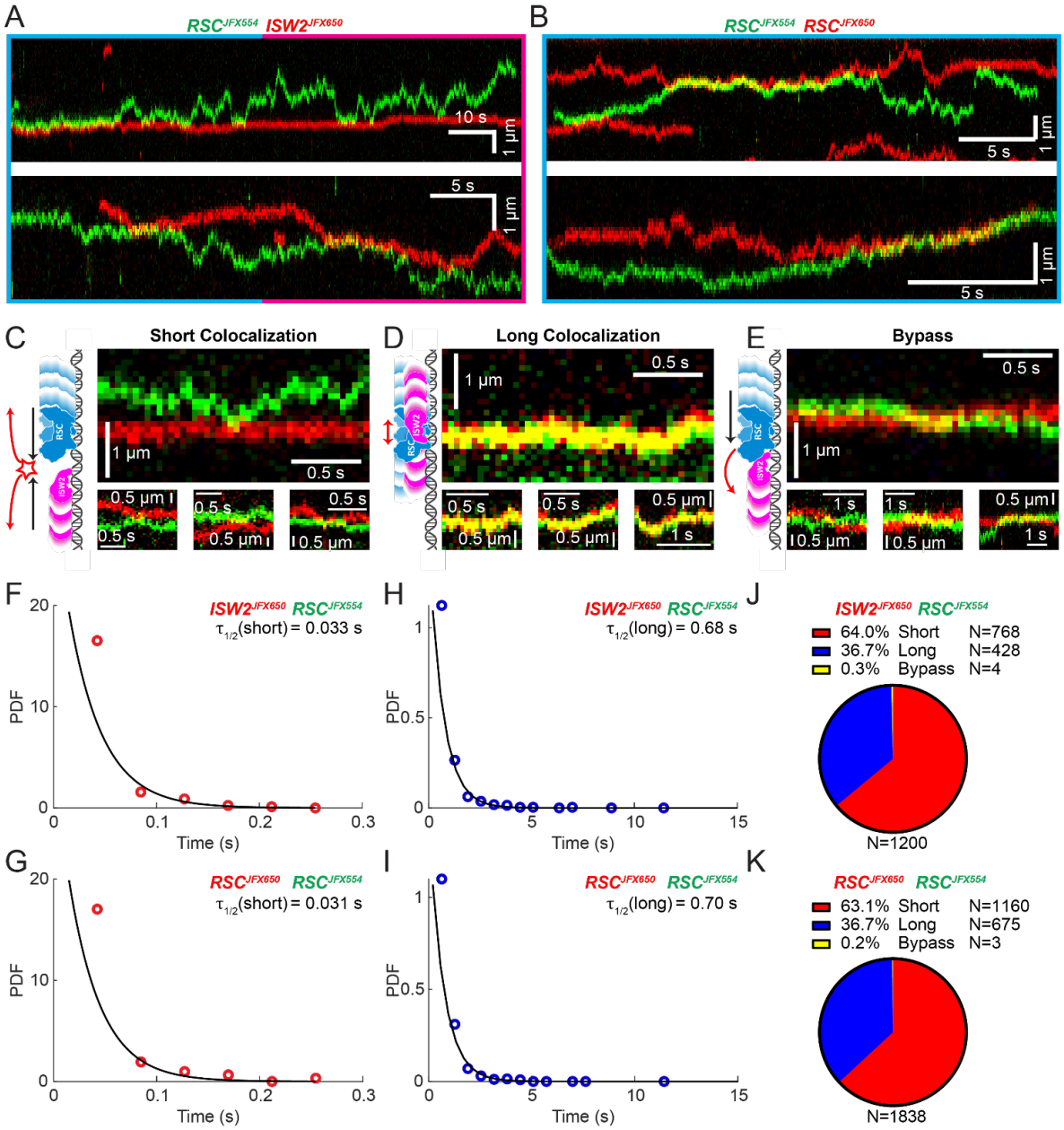

Figure 2. Bimolecular Remodeler-Remodeler Interactions During 1D Encounters on DNA. (A-B) Two-color kymographs of RSC-JFX554 and ISW2-JFX650 (A) or RSC-JFX554 and RSC-JFX650 (B) diffusing together on naked DNA. (C-E) Three types of encounter events are observed: short colocalizations (C), long colocalizations (D), and bypass events (E). Representative kymograph sections and corresponding cartoons illustrate each interaction. (F-G) Dwell times of short colocalization events for RSC-ISW2 (F) and RSC-RSC (G). (H-I) Dwell times of long colocalization events for RSC-ISW2 (H) and RSC-RSC (I). Interaction lifetimes are determined by measuring the time remodelers spend in close proximity. Interaction half-lives (τ) are calculated from single exponential fits to PDF plots of lifetimes. (J-K) Proportions of each event type quantified as pie charts for RSC-ISW2 (J) and RSC-RSC (K).

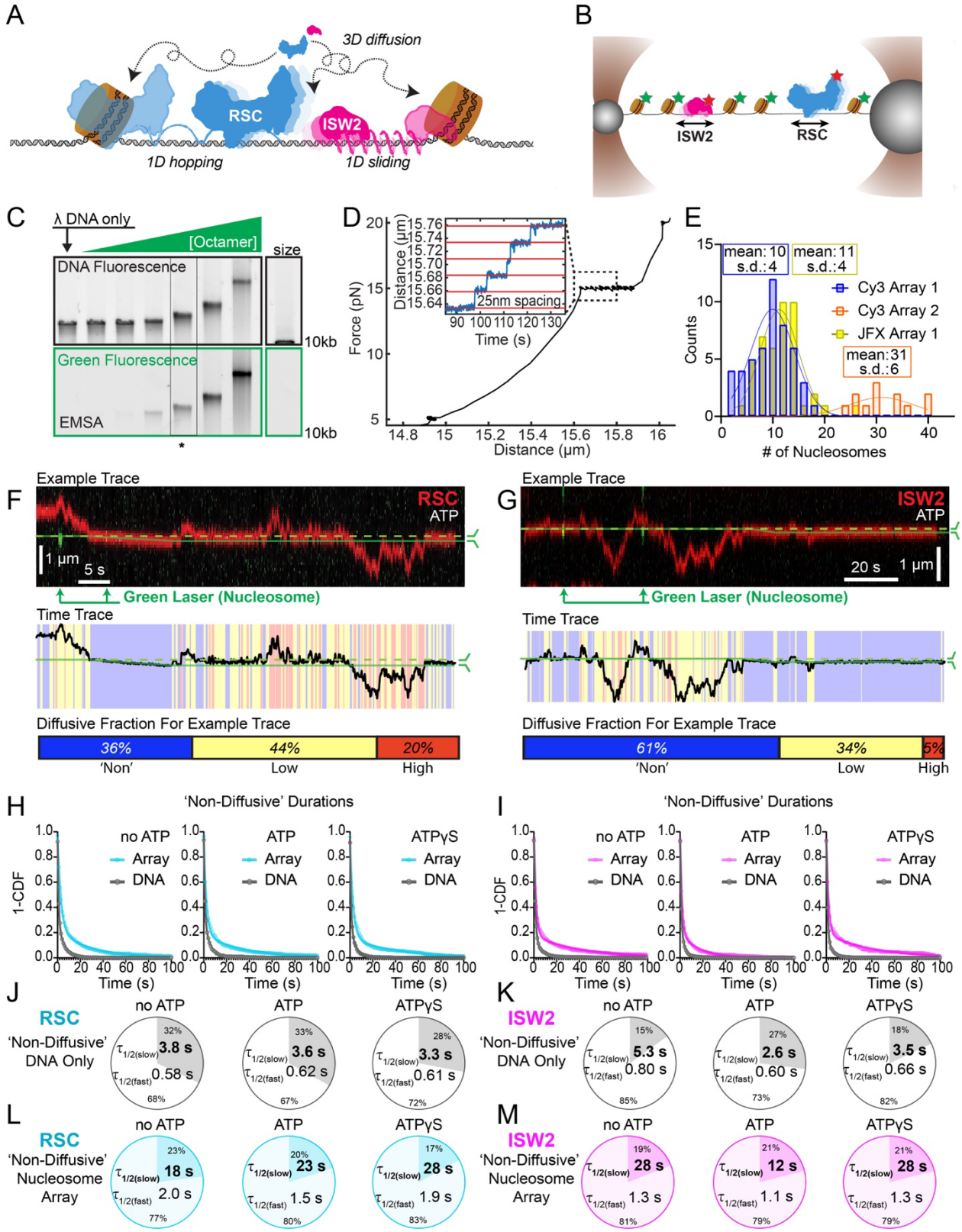

Figure 3. Nucleosomes Constrain 1D Diffusion of RSC and ISW2. (A) Schematic: RSC and ISW2 interact with promoters and flanking nucleosomes through 3D and 1D search processes. (B) Optical tweezers and confocal microscopy assay for measuring remodeler diffusion on lambda

DNA nucleosome arrays. (C) Electrophoretic mobility shift assay (EMSA) of lambda nucleosome arrays with increasing octamer concentration. The asterisk indicates a condition that produced ~10 nucleosomes per array. (D) Force distance plots are used to assess the number of nucleosomes on arrays, with ~25 nm lengthening per nucleosome at 15 pN (inset). (E) Histograms show nucleosome numbers per array for three arrays used, with mean values and standard deviations. (F-G) Kymograph segments of RSC (F) and ISW2 (G) encountering a nucleosome on the array with 1 mM ATP. Diffusion levels are binned on time trace plots. (H-I) Survival plots of dwell times in the 'non-diffusive' state for RSC (H) and ISW2 (I) on naked DNA or nucleosome array. (J-M) Half-lives are determined using double exponential fits and are indicated for the slow and fast percentages (pie charts). Values are given for various ATP conditions and for diffusion on naked DNA (RSC; J-ISW2; K) and nucleosome array (RSC; L-ISW2; M).

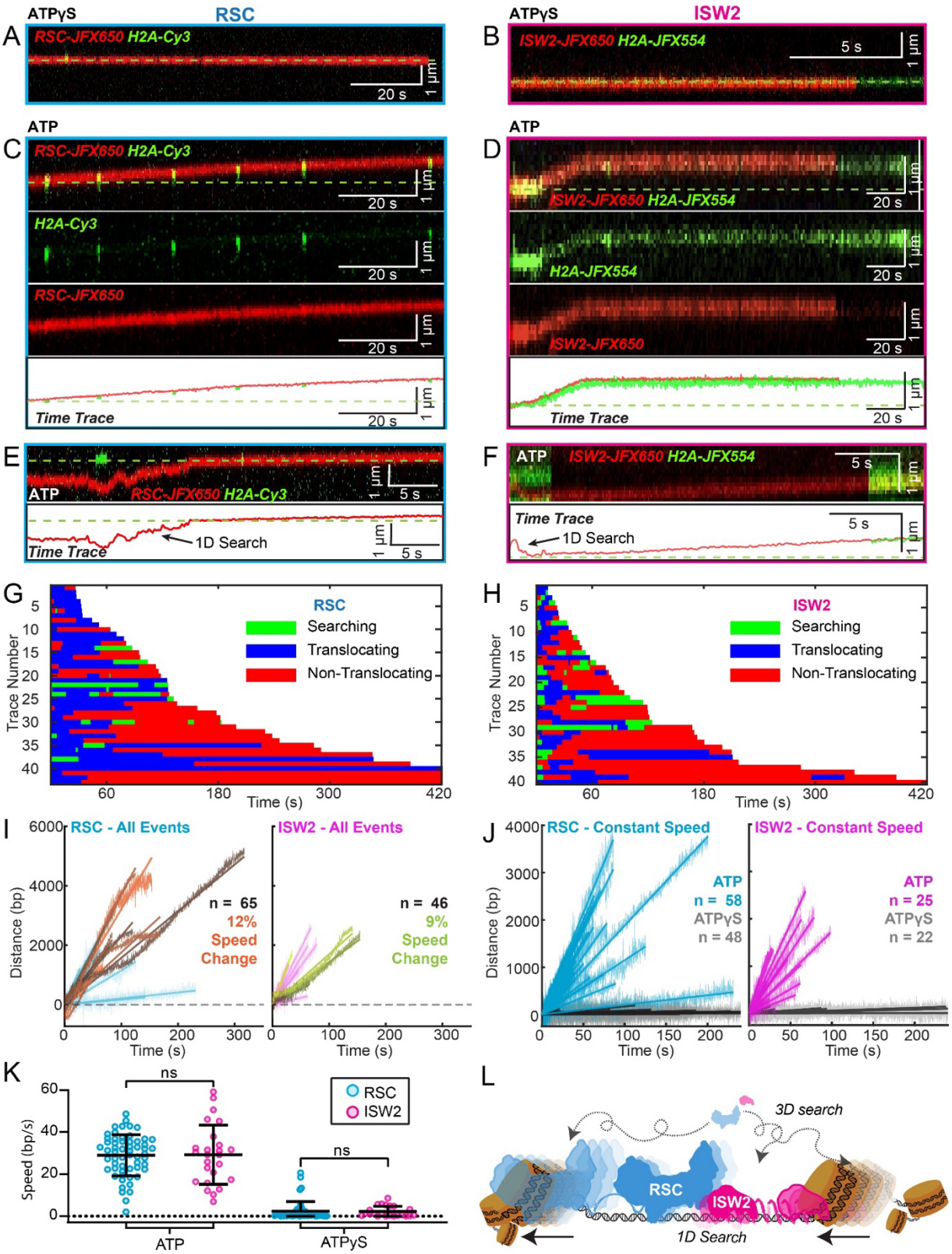

Figure 4. ATP-Dependent Processive Nucleosome Translocation by RSC and ISW2. (A-B) Kymographs of RSC (A) and ISW2 (B) showing static colocalization in 1 mM ATPγS. (C-D) Kymographs of RSC (C) and ISW2 (D) moving directionally with nucleosome signals in 1 mM ATP.

The green laser is either blinked or continuous, and the initial position of the nucleosome is marked with a dashed green line. (E-F) Examples of translocation events following 1D encounter for RSC (E) and ISW2 (F). (G-H) Trace fragmentation plots illustrate translocating (blue), non-translocating (red), and 1D-search (green) segments for RSC (G) and ISW2 (H). (I) Linear fit to all translocation events, highlighting speed changes. (J) Linear fits to constant-speed segments (>300 bp, >5 s, r2 >0.5), with ATPγS static colocalization controls in gray. (K) Scatter plots of translocation speeds for RSC (cyan) and ISW2 (magenta), including mean values and standard deviation error bars; ATPγS control shown in gray. (L) Summary schematic.

Materials and Methods

Dual Optical Tweezers and Confocal Microscopy

Imaging was performed using a commercial optical tweezer combined with fluorescence microscope, C-Trap (LUMICKS, Amsterdam). A laminar flow-based microfluidics chamber was used for data collection, and reproducible measurements of remodeler diffusion were made in the absence of flow in the channel 4 protein reservoir (**Fig. S1A**). The microfluidics system was passivated before imaging: BSA (0.1% w/v in PBS) and Pluoronics F128 (0.5% w/v in PBS) were each flowed for 30 min, followed by a 30-min flush with PBS. Bacteriophage λ DNA, dual-labeled with 3x-Biotin at one end and 3x-Digoxigenin at the other, was tethered between 4.38-µm SPHERO Streptavidin Coated polystyrene beads (Spherotech, Cat. No. SVP-40-5) in Trap1 and 2.12-µm SPHERO Anti-Dig polystyrene beads (Spherotech, Cat. No. DIGP-20-2) in Trap2 (**Fig. 1B**). The trapping laser was set to 100% and overall power to 30%, with a Trap 1 split power of 60%. The tethered λ DNA was stretched to 5 pN force, and trap positions were fixed during kymograph acquisition.

Either 500 pM or 1 nM $RSC^{Sth1\text{-}3xFlag\text{-}Halo}$, or 136.6 pM $ISW2^{Isw2\text{-}3xFlag\text{-}Halo}$ in imaging buffer, were flowed at low pressure. Imaging buffer consisted of saturated Trolox solution and RSC reaction buffer (10 mM Tris, pH 7.4, 50 mM KCl, 3 mM MgCl2, 0.1 mg/ml BSA). The same imaging buffer was used for both RSC and ISW2. For confocal microscopy, a 42.4 ms line time, 6.5% red laser power, and 15% green laser power were utilized. Emission filters for blue (500/525 nm), green (545/620 nm), and red (650/750 nm) lasers were employed.

Purification and Labeling of Chromatin Remodeling Complexes

Catalytic subunit genes Sth1 and Isw2 were C-terminally tagged with 3xFlag-HaloTag at their endogenous loci in S. cerevisiae for native remodeler complex purification (**Fig. S1B**). Yeast cultures (4 L) were grown in YPAD medium (1% Bacto yeast extract, 2% Bacto peptone, 3% dextrose, 0.004% Adenine hemisulfate) to an optical density (OD600) of 3.5-4.0. Cells were harvested by centrifugation, washed twice in water, once in resuspension buffer (200 mM HEPES, 1 mM EDTA, 40% glycerol, 100 mM KOAc, 0.284 µg ml–1 leupeptin, 1.37 µg ml–1 pepstatin A, 0.17 mg ml–1 PMSF, 0.33 mg ml–1 benzamidine). The cell pellet was flash-frozen in liquid nitrogen. Lysis was achieved by cryo-milling (Spex Freezer/Mill 6870) for 15 cycles with alternating 1-min "on" and 1-min rest periods. The resulting powder was resuspended in approximately half of the powder volume of lysis buffer (150 mM HEPES pH 7.6, 1 mM EDTA, 2 mM MgCl2, 20% glycerol, 100 mM KOAc, 5 mM β-mercaptoethanol, 0.284 µg ml–1 leupeptin, 1.37 µg ml–1 pepstatin A, 0.17 mg ml–1 PMSF, 0.33 mg ml–1 benzamidine, 0.5 mM NaF, 5 mM β-Glycerophosphate). Protein extraction was carried out with the addition of 0.3 M KCl and incubated at 4°C for 30 min. The extract was cleared by centrifugation at 25,000 rpm at 4°C for 2 h, and the supernatant was incubated with pre-equilibrated 1 mL anti-FLAG M2 agarose (Sigma-Aldrich) for 4 h at 4°C with gentle rotation.

The agarose resin was washed five times with high salt wash buffer (20 mM HEPES pH 7.6, 0.2 mM EDTA, 10% glycerol, 500 mM KOAc, 0.01% IGEPAL CA-630, 0.284 µg ml–1

leupeptin, 1.37 µg ml–1 pepstatin A, 0.17 mg ml–1 PMSF, 0.33 mg ml–1 benzamidine) and three times with regular salt wash buffer (20 mM HEPES pH 7.6, 0.2 mM EDTA, 10% glycerol, 300 mM KOAc, 0.01% IGEPAL CA-630, 0.284 µg ml–1 leupeptin, 1.37 µg ml–1 pepstatin A, 0.17 mg ml–1 PMSF, 0.33 mg ml–1 benzamidine). The bead-bound remodeler complex was eluted twice using 0.5 mg/mL 3x Flag peptide (APExBIO), with the first elution incubated for at least 2 h. The eluate was concentrated using a 100 kDa MWCO centricon column (EMD Millipore), flash-frozen, and stored at -80°C until further processing.

For fluorescent labeling, the eluate was thawed on ice, incubated with 2 µM JFX650 or JFX554 (sourced from Luke Lavis) for 2 h at 4°C with gentle shaking. The eluate was then applied to a 20-60% glycerol gradient in gradient buffer (25 mM HEPES-KOH pH 7.6, 1 mM EDTA, 2 mM $MgCl_2$, 0.01% NP-40, 300 mM KOAc) for velocity sedimentation, allowing further purification of complexes and separation from unbound free dyes. Centrifugation was performed at 45,000 rpm for 20 h at 4°C in an SW 60-T rotor. Peak fractions were analyzed by SDS-PAGE and Flamingo Fluorescent Stain (Bio-Rad) (**Fig. S1C**). Protein concentration was determined by comparing to a serially diluted BSA standard in SDS-PAGE.

Gel-Based Nucleosome Sliding Assay

RSC nucleosome sliding reactions were performed using 20 nM nucleosome [43N43, Cy5DNA], 10 nM $RSC^{Sth1\text{-}3F\text{-}Halo}$, and 1 mM ATP in a buffer containing 10 mM Tris (pH 7.4), 50 mM KCl, 3 mM MgCl2, and 0.1 mg/mL BSA[57]. ISW2 nucleosome sliding reactions were conducted with 33.3 nM nucleosome [80N3, Cy5-DNA, Cy3-H2A], 6.15 nM $ISW2^{Isw2\text{-}3F\text{-}Halo}$, and 1 mM ATP in a buffer composed of 25 mM HEPES-KOH (pH 7.6), 50 mM KCl, 5 mM MgCl2, 0.1 mg/mL BSA, and 5% glycerol. All reactions had a 10 µL total volume and were incubated at 30°C with gentle mixing. At designated time points, reactions were quenched by adding 3 µg of single-stranded DNA (SS-DNA) and 5 mM EDTA. Samples were subsequently loaded onto 4.5% or 6% native polyacrylamide gels, and the gels were imaged using Cy5 excitation on a Typhoon Imager System (**Fig. S3A-B**).

Single Molecule Tracking and Analysis

Kymographs were analyzed using the Pylake KymoTracker widget (Lumicks.pylake, ver. 0.10.0) in Python. The following parameters were applied for particle tracking: line width: 0.4 um, minimum length: 8 pixels, pixel threshold (minimum pixel intensity): 3, window (maximum frames of gap allowed to connect two lines as one track): 8, sigma (fluctuation in the molecule's position over time): 0.14, velocity: 0.00, Refine lines: yes. For tracks with gaps that could not be connected by the default parameters (more commonly observed for long-lived tracks), the 'Connect line' function was used to manually connect two lines. Notably, all movies were acquired without laminar flow, resulting in a low rate of new protein binding during the movie. Sigma and/or velocity parameters were increased to track highly diffusive molecules.

Rolling Window Diffusion Analysis Pipeline

To capture transitions in diffusive states, 20-frame window sub-trajectories were made from the beginning to the end of each trajectory. The 20-frame window size was determined by trial-and-error, with shorter windows (e.g., 5, 10 frames) resulting in noisy detection of spurious diffusion peaks and longer windows (e.g., 35, 50 frames) causing averaging of diffusion peaks. Diffusion coefficients (D) were calculated for each window using a MATLAB class called msdanalyzer[58], where D was estimated from the linear regression fitting of the mean squared displacement (MSD) plots of rolling windows using the first five time points. D is calculated as:

$$D = \frac{1}{2d} \times \frac{MSD(dt)}{dt}$$

where d is the number of dimensions (1 in this case).

Each window was then classified as non-diffusive, low-diffusive, or high-diffusive states. Based on the dCas9 profile as a control for truly immobile particles, we set < 0.01 μm²/s as the threshold for the immobile state. The thresholds for low-diffusive (D < 0.04 μm²/s) and high-diffusive (D > 0.04 μm²/s) states were set at 0.04 μm²/s. The mean of all D values associated with each diffusive state was calculated to obtain D values for the three states per trajectory (**Fig. 1D**).

Four crRNAs specific to lambda DNA were used (**Table 1**) to immobilize dCas9 bound to lambda DNA in standard Cas9 cleavage buffer. To quantify state durations and relative percentages of the three diffusive states per trajectory, two additional filtering steps were applied to reduce the detection of spurious diffusive transitions, which mainly arise from tracking errors and noise in raw kymographs. First, the "smooth()" MATLAB function (default parameter) was used to smooth the position vector using a moving average filtering method. The smoothed data was then used to classify each window into three diffusive states. The "bwconncomp.m" MATLAB function was used to connect neighboring windows with the same diffusive states into segments of non-diffusive, low-diffusive, and high-diffusive states. A second filtering step removed segments shorter than 10 consecutive windows as spurious detections. Finally, the lengths of each segment were used to compute state durations, and the net lengths of each of the three diffusive segments over the total trajectory length were used to determine the relative percentages of the three diffusive states per trajectory.

Bimolecular Remodeler-Remodeler Interaction Analysis

To define colocalizations, we performed the following steps. Firstly, we calculated the diffusion coefficient for each molecule over time using time windows, specifically utilizing 20 exposure time intervals in our study. Next, we determined the mean diffusion coefficient for each molecule. Based on the mean diffusion coefficients, we determined the maximum displacements that can occur between two molecules assuming independent movement, employing a simple Brownian motion model. The maximum distance between two molecules was calculated using:

$$x_1 = \sqrt{2\mu_{D1}\Delta t}$$
$$x_2 = \sqrt{2\mu_{D2}\Delta t}$$

where μ_1 and μ_2 represent the mean diffusion coefficients of the first and second molecules, respectively, and Δt denotes the camera exposure time. Consequently, the maximum distance between two molecules is given by:

$$d = x_1 + x_2$$

For RSC-RSC interactions, the average threshold was found to be 0.31 μm, and for RSC-ISW2 interactions, it was 0.29 μm.

To reduce noise arising from shot noise, we applied a Gaussian filter to smooth the trajectories, using a time window of 5 exposure time points. Subsequently, we identified time points at which the distance between molecules, based on the smoothed trajectories, fell below the threshold, enabling the determination of colocalized time points. These colocalized time points were then categorized into short and long colocalizations. Specifically, we set a threshold of 5 exposure times (the same time window used for trajectory smoothing). If the duration of colocalization exceeded this threshold, it was automatically classified as a long colocalization. For colocalizations below the threshold, we assessed the duration of colocalization and the local diffusion coefficients. If the displacement of the molecular distance exceeded the calculated average displacement, it was categorized as a long colocalization; otherwise, it was considered a short colocalization.

To validate the short colocalizations, we examined the diffusion coefficient for this part of the trajectories, ensuring that the molecular distance remained within the threshold and subsequently exited it. If the calculated diffusion coefficient for this segment was greater than

the average diffusion coefficient, we classified the colocalization as a short colocalization; otherwise, it was considered a long colocalization. In summary, our colocalization analysis involved calculating diffusion coefficients, determining thresholds, smoothing trajectories, identifying colocalized time points, categorizing colocalizations into short and long durations, and verifying short colocalizations based on the diffusion coefficient. These steps allowed us to define colocalizations and evaluate their durations in our study (**Fig. S2A-F**).

Dwell time analysis was performed using the Akaike Information Criterion (AIC) to determine the most probable number of lifetime models. In this case, a single exponential model was identified. Subsequently, lifetime estimation was carried out using the Maximum Likelihood Estimation (MLE) method with the XYZ package in MATLAB. The likelihood is expressed as

$$P(\boldsymbol{t}|\tau) = \prod_{i=1}^{I} Exp(t_i\,;\,\tau) = \prod_{i=1}^{I} \frac{1}{\tau} e^{-\frac{t_i}{\tau}} = \frac{1}{\tau^I} \exp\left(-\frac{1}{\tau}\sum_{i=1}^{I} t_i\right).$$

Lambda DNA Preparation with Biotin and Digoxigenin Labeling

Lambda DNA was prepared with three biotins on one end and three digoxigenins on the other end using the following protocol. Custom oligos (**Table S1**) were sourced from IDT, and lambda DNA was acquired from NEB (cat# N3011S). Oligo 1 was annealed to lambda DNA by adding a 25-fold molar excess to the DNA in an annealing buffer containing 30 mM HEPES (pH 7.5) and 100 mM KCl. The mixture was heated at 70°C for 10 minutes and cooled gradually to room temperature. Subsequently, 2 µL of NEB T4 DNA ligase (400U, cat# M0202S) and its corresponding buffer with ATP were added, followed by a 30-minute incubation at room temperature. A 50-fold molar excess of oligo 2 was then introduced, along with an additional 1 µL of T4 DNA ligase and its buffer containing ATP, with volume adjustments. The mixture was incubated at room temperature for another 30 minutes and heat-inactivated at 65°C for 10 minutes. The end-labeled lambda DNA was purified using the Qiaex II gel-extraction DNA clean-up kit, as per the manufacturer's instructions (Qiagen, cat# 20021).

Lambda DNA Preparation with Biotin Labeling on Both Ends

Lambda DNA was also prepared with three biotins on each end using an adapter oligo (**Table 1**) to recycle the 3x-Biotin Cos1 Oligo. The protocol mentioned above was followed, with some modifications. An equimolar mixture of adapter oligo and the 3x-Biotin Cos1 Oligo were annealed by heating and gradually cooling using IDT protocols and buffers. This adapted oligo was then used instead of oligo 2 in the aforementioned protocol.

Lambda Nucleosome Array Preparation

A salt gradient dialysis method was employed to reconstitute nucleosomes onto lambda DNA, using optimized laboratory procedures based on established protocols[59,60]. The buffers used in this reconstitution included high salt buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 2 M NaCl, 5 mM 2-Mercaptoethanol (BME)) and low salt buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 50 mM NaCl, 5 mM BME). Cy3-labeled H2A-containing octamer, prepared as formerly described[61], was titrated onto lambda DNA (either 0.5 µg or 1 µg DNA) at molar ratios of 40:1, 20:1, 10:1, 5:1, and 2.5:1. Reconstitution reactions were carried out in 10 mM Tris pH 7.5, 1 mM EDTA pH 8.0, 0.1 mg/mL BSA Roche (cat# 10711454001), and 1 mM BME. A 16-hour dialysis was set up using a 7 kDa MWCO Slide-A-Lyzer MINI Dialysis Device (cat# 69560), placed in a flotation device in high-salt buffer. Low-salt buffer was gradually added to the high-salt buffer throughout the dialysis, with constant stirring. After the dialysis period, the solution was replaced with 100% low-salt buffer and allowed to dialyze for an additional hour. The reconstitution efficiency was assessed using an electrophoretic mobility shift assay (EMSA) by running lambda nucleosome arrays on a 0.5% agarose gel in 0.5x TBE (**Fig. 3C**).

Histone Labeling, Octamer Reconstitution, and Purification

H2A(K120C)/H2B dimers, synthesized in-house, were labeled with maleimide-JFX554 (gift of Luke D. Lavis) using a standard protein maleimide labeling protocol with a 50-fold molar excess of dye to protein. The reaction was allowed to proceed for 3 hours at room temperature before quenching with BME. A small aliquot was reserved to assess labeling efficiency, and excess free dye was removed through three successive buffer exchanges using a 10K MWCO Amicon spin filter. Labeling efficiency was estimated at 61% based on nanodrop spectral absorption readings at 554 nm and 280 nm, and the extinction coefficients of the dye and protein. The labeled H2A/H2B dimer was then denatured overnight in denaturing buffer (7 M guanidinium chloride, 10 mM DTT, 20 mM Tris pH 7.5). The following day, a 1.5-fold molar excess of denatured H2A/H2B was mixed with H3 and H4, both solubilized in denaturing buffer, and incubated for 1 hour at room temperature. 0.5 mg of histones H2A and H2B were used in this step.

Octamer refolding was conducted by successively dialyzing the mixture into 1L of refolding buffer (2 M NaCl, 5 mM BME, 1 mM EDTA, 10 mM Tris HCl pH 7.5) four times, with each dialysis separated by approximately 12 hours. The samples were then size-excluded on a Superdex 200 column equilibrated with refolding buffer using an AKTA Fast Protein Liquid Chromatograph (FPLC). Octamer fractions were collected, and SDS-PAGE was employed to determine which peak fractions to pool. A second estimation of labeling efficiency at 68% was obtained after octamer purification. Octamer samples were stored at 1 mg/mL concentrations in 2 M NaCl and 50% glycerol at -20°C until further use.

Lambda Nucleosome Array Imaging and Validation

Lambda nucleosome arrays (biotin labeled on both ends) were captured by oscillating the distance between two trap centers (traps 1 and 2 with a ~4.38 μm diameter streptavidin-coated polystyrene bead under buffer flow (<0.2 bar) containing approximately 63 pg/μL lambda nucleosome array. When the force measured on trap 2 exceeded 5 pN, the oscillation and buffer flow were stopped. The distance between the beads was adjusted to achieve a force of ~0 pN on trap 2, and the traps were moved into the protein channel. Buffer flow was maintained at <0.1 bar for a maximum of 30 seconds to refresh protein in the channel and promote remodeler binding. This flow did not disrupt the nucleosome array stability. The DNA was then pulled to a tension of 5pN, at which point the distance between the two traps was fixed and imaging was started. For imaging Cy3-labeled nucleosomes, a time-lapse scheme in the green channel was used to preserve fluorescence signal lifetime, despite poorer photostability compared to JFX-labeled remodelers. Green excitation (15% of maximum) was intermittently applied to visualize nucleosome positions on the array, with approximately 1 second of green excitation used at various time points. Red excitation (6.5% of maximum) was applied continuously. After JFX-650 labeled remodelers bleached, green excitation continued until the remaining green signal photobleached. Lambda nucleosome arrays contained a variable number of nucleosomes. To determine the mean number of nucleosomes per array, the following procedure was employed. After imaging, the lambda nucleosome array was moved to a buffer-only channel, and nucleosomes were forcibly unwrapped. A force-clamp between 15-20 pN was applied to visualize individual unwrapping events (**Fig. 3D-E**). Once unwrapping events ceased, the force was increased to 40 pN and then 60 pN+ to denature the DNA. If the DNA remained intact, a second force-distance curve was collected to visualize DNA free of nucleosomes.

Remodeler-Nucleosome Colocalization Analysis Pipeline

To colocalize remodeler signals with nucleosome signals, we first processed nucleosome signals by performing linking analysis and marking signals to be excluded from colocalization based on specific criteria. As mentioned previously, the green laser was pulsed to extend the fluorescence lifetime of Cy3-labeled nucleosomes, which constituted 2/3 of the data

collected in this study. A JFX-nucleosome was created to extend the fluorescence lifetime of the nucleosome, achieving a duration comparable to that of the remodeler. We tested both pulsing and constant exposure strategies, ultimately opting for constant exposure due to the similar fluorescence lifetimes of the JFX-nucleosome and remodeler. The JFX-nucleosome was brighter, allowing for lower green excitation power (10%), resulting in longer fluorescence lifetimes. However, one disadvantage of using the JFX-nucleosome was signal dimming and blinking, as observed in **Fig. 4D**. For data involving pulsed green lasers, we extended nucleosome positional information by using the last visible fluorescence signal before laser pulsing or photobleaching. This approach enabled generation of positional information for all nucleosome datasets, regardless of fluorophore used, facilitating remodeler-nucleosome colocalization analysis.

Nucleosome fluorescent signals were omitted from consideration if unstable, likely due to non-specific adsorption of fluorescently labeled histones remaining after reconstitution of lambda nucleosome arrays. These signals were also considered in linking analysis, and stable signals that later linked to an unstable signal were removed from colocalization analysis. Remodelers colocalizing within 0.17 pixels (~500 bps) of the nucleosome signal were considered colocalized, and their molecular identifiers were recorded for immobility analysis.

Translocation Analysis Pipeline

Kymographs obtained in the presence of 1 mM ATP were evaluated for translocation based on directional motion exhibited by either remodeler or nucleosome signals, or their colocalization. Traces were categorized into segments displaying 1D search, stable non-translocating engagement, or translocation. Stable nucleosome colocalized signals in kymographs with 1 mM ATPγS were used as a control condition, as translocation should not occur due to its ATP dependence. Time trace information for translocating segments was fitted to a linear regression model. In cases of poor fits, traces were re-evaluated for speed changes. Translocation events with speed changes were fitted separately using linear regressions for each segment of uniform speed. Translocation events lasting less than 5 seconds, spanning less than 300 bps, or exhibiting an R^2 value below 0.5 were excluded from reporting, as they could not be distinguished from fits in the ATPγS control condition.

IDENTITY	SEQUENCE
Cas9 crRNA sequence “lambda 1”	5'- / AltR 1 /rGrUrG rArUrA rArGrU rGrGrA rArUrG rCrCrA rUrGrG rUrUrU rUrArG rArGrC rUrArU rGrCrU / AltR2/ -3'
Cas9 crRNA sequence “lambda 2”	5'- / AltR 1 /rCrUrG rGrUrG rArArC rUrUrC rCrGrA rUrArG rUrGrG rUrUrU rUrArG rArGrC rUrArU rGrCrU / AltR2/ -3'
Cas9 crRNA sequence “lambda 3”	5'- /AltRl /rCrArG rArUrA rUrArG rCrCrU rGrGrU rGrGrU rUrCrG rUrUrU rUrArG rArGrC rUrArU rGrCrU / AltR2/ -3'
3x-biotin-cos1 oligo	5' - /5Phos/ AGG TCG CCG CCC TT/iBiodT/TT/iBiodT/TT/3BiodT/-3'
3x-digoxigenin-cos2 oligo	5'- /5Phos/ GGG CGG CGA CCT TT/iDigN/TT/iDigN/TT/3DigN/-3'
Adapter oligo for lambda DNA dual end biotin labeling	5'- /5Phos/ GGG CGG CGA CCT TGC A-3'

Table 1. crRNA sequences for dCas9 binding oligonucleotide sequences used for lambda DNA preparation

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