

Pleomorphic effects of three small-molecule inhibitors on transcription elongation by *Mycobacterium tuberculosis* RNA polymerase

Omar Herrera-Asmat^{1,2,3,4,‡}, Alexander B. Tong^{1,4,5,‡}, Wenxia Lin^{4,6}, Tiantian Kong⁶, Juan R Del Valle⁷, Daniel G. Guerra³, Yon W. Ebright^{8,9}, Richard H. Ebright^{8,9}, Carlos Bustamante^{1,2,4,5,10-13}

1 Jason L. Choy Laboratory of Single-Molecule Biophysics, University of California, Berkeley, CA 94720.

2 Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

3 Laboratorio de Moléculas Individuales, Laboratorios de Investigación y Desarrollo, Facultad de Ciencias e Ingeniería, Universidad Peruana Cayetano Heredia, Av Honorio Delgado 430, San Martín de Porras, Lima 15102, Peru.

4 California Institute for Quantitative Biosciences, QB3, University of California, Berkeley, CA 94720.

5 Department of Chemistry, University of California, Berkeley, CA 94720.

6 Department of Biomedical Engineering, School of Medicine, Shenzhen University, Shenzhen, Guangdong 518000, China.

7 Department of Chemistry & Biochemistry, University of Notre Dame, IN 46556, USA.

8 Waksman Institute, Rutgers University, Piscataway, NJ 08854.

9 Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, NJ 08854.

10 Department of Physics, University of California, Berkeley, CA 94720.

11 Biophysics Graduate Group, University of California, Berkeley, CA 94720.

12 Kavli Energy Nanoscience Institute, University of California, Berkeley, CA 94720.

13 Howard Hughes Medical Institute, University of California, Berkeley, CA 94720.

‡ These authors contributed equally to this work.

Abstract

The *Mycobacterium tuberculosis* RNA polymerase (MtbRNAP) is the target of the first-line anti-tuberculosis inhibitor rifampin, however, the emergence of rifampin resistance necessitates the development of new antibiotics. Here, we communicate the first single-molecule characterization of MtbRNAP elongation and its inhibition by three diverse small-molecule inhibitors: N(α)-aroyl-N-aryl-phenylalaninamide (D-IX216), streptolydigin (Stl), and pseudouridimycin (PUM) using high-resolution optical tweezers. Compared to *Escherichia coli* RNA polymerase (EcoRNAP), MtbRNAP transcribes more slowly, has similar mechanical robustness, and only weakly recognizes *E. coli* pause sequences. The three small-molecule inhibitors of MtbRNAP exhibit strikingly different effects on transcription elongation. In the presence of D-IX216, which inhibits RNAP active-center bridge-helix motions required for nucleotide addition, the enzyme exhibits transitions between slowly and super-slowly elongating inhibited states. Stl, which inhibits the RNAP trigger-loop motions also required for nucleotide addition, inhibits RNAP primarily by inducing pausing and backtracking. PUM, a nucleoside analog of UTP, in addition to acting as a competitive inhibitor, induces the formation of slowly elongating RNAP inhibited states. Our results indicate that the three classes of small-molecule inhibitors affect the enzyme in distinct ways and show that the combination of Stl and D-IX216, which both target the RNAP bridge helix, has a strong synergistic effect on the enzyme.

Introduction

Tuberculosis (TB) is the second most prevalent infectious disease causing morbidity and mortality worldwide (after COVID-19). There are 10 million new cases and 1.5 million deaths yearly, which incur \$12 billion in medical and economic costs¹. Rifampin is a small-molecule inhibitor of *Mycobacterium tuberculosis* RNA polymerase (MtbRNAP) and is a first-line treatment for TB^{2,3}. The emergence and proliferation of rifampin resistance in *M. tuberculosis* have led to an effort to identify and develop new small-molecule inhibitors that effectively inhibit rifampin-resistant MtbRNAP⁴⁻⁷. A thorough understanding of the activity of MtbRNAP and its interactions with these new inhibitors is critical for informing and facilitating inhibitor development against TB.

In the last three decades, single-molecule optical tweezers studies of *E. coli* RNAP (EcoRNAP) and eukaryotic RNAP II have provided key insights on transcription elongation^{8,9}, including the direct, real-time observation of single-base-pair stepping^{10,11}. In addition, these studies have revealed details about sequence-dependent pausing, factor-dependent pausing^{12,13}, and backtracking¹³⁻¹⁶. However, single-molecule studies of MtbRNAP and its inhibition by small-molecule inhibitors have been scarce. Current studies have primarily been structural and bulk assays¹⁷⁻²³, with only one study by single-molecule methods addressing initiation²⁴.

Here, we present a study of transcription elongation by MtbRNAP and its inhibition by small-molecule inhibitors using high-resolution single-molecule optical tweezers. First, we characterize *in singulo* the transcription elongation of MtbRNAP alone, including its pause-free velocity and mechanical robustness. Next, we assess three small-molecule inhibitors that target the transcription elongation of MtbRNAP. These inhibitors bind to sites distinct from rifampin and remain effective despite polymerase mutations that confer resistance to rifampin. These inhibitors are D-IX216 (an N(α -aroyl-N-aryl-phenylalaninamide)^{25,26}, streptolydigin (Stl)²⁷⁻³², and pseudouridimycin (PUM)³³⁻³⁵.

N(α -aroyl-N-aryl-phenylalaninamides selectively inhibit Mycobacterial RNAPs²⁶ by binding to a site at the N-terminal end of the bridge helix. This site is conserved in Mycobacterial polymerases but not in other bacterial polymerases. The bound inhibitor interferes with the conformational dynamics of the bridge helix that are necessary for nucleotide addition^{25,26,36,37}. In this study we used D-IX216, we find that addition of this inhibitor results in the enzyme transitioning to different inhibition states that add nucleotides much more slowly. Stl acts as a broad-spectrum inhibitor of bacterial RNAPs^{27-29,31,32}. Stl binds to the region between the bridge helix and trigger loop, interfering with the closing of the trigger loop, which is required for nucleotide addition^{29,32}. We find that Stl stabilizes pausing by the enzyme. PUM is another broad-spectrum inhibitor of bacterial RNAPs³³ and is a nucleoside analog of UTP^{33-35,38,39}, competitively inhibiting the binding of UTP. Additionally, PUM also appears to have an additional inhibitory effect on the enzyme³⁵. Finally, when combined, D-IX216 and Stl exhibit synergistic inhibition of the enzyme.

Results

Biophysical and mechanochemical characterization of MtbRNAP

We studied the elongation of MtbRNAP using a high-resolution optical tweezers assay, where the RNAP and one end of the DNA template were tethered to polystyrene microspheres held in optical traps (Figure 1a, left panel). We collected single-molecule trajectories of MtbRNAP transcription across a ~3kb template with a sequence derived from *M. tuberculosis*.

Molecular trajectories were first obtained in *assisting constant force* mode (Figure 1b), where the upstream DNA is tethered, causing the force applied by the traps to aid the movement of the RNAP. We also obtained trajectories in *opposing constant force* mode (Figure 1c), where the downstream DNA is tethered, and the force hinders the enzyme's movement. In both cases, as the RNAP moves, the length of the tether changes, and the position of the movable trap is adjusted via force feedback to maintain a constant tension on the tether.

Transcription by MtbRNAP under an assisting constant force of 18 pN comprises periods of continuous activity at a consistent velocity interspersed with long pauses, a behavior also described for EcoRNAP^{40–42} (Figure 1a, right panel). Plots of the velocity distribution of all molecular trajectories for both enzymes show a population at zero velocity corresponding to transcriptional pauses, and another corresponding to the pause-free velocity of the enzyme (Figure 1d, right panel). The pause-free velocity of MtbRNAP is measured by this method is 23.1 ± 5.1 nt/s, approximately half that of EcoRNAP, which is 42.7 ± 8.6 nt/s (Figure 1d, right panel).

To get a more precise characterization of the dynamics of RNAP elongation, we obtained the dwell time at each nucleotide by fitting the molecular traces to a monotonic one base-pair (bp) staircase function. We then modeled the resulting dwell time

distribution (DTD) as a sum of exponentials $(\sum_{i=1}^n a_i e^{-k_i t})$ ^{43,44}. We interpret these different exponentials as distinct states of the enzyme: a_i represents the state probability ($\sum_{i=1}^n a_i = 1$), while k_i indicates the kinetic rate of the i th state, such that $k_1 > k_2 > \dots > k_n$. The fastest state typically corresponds to the pause-free state, whereas the slower states reflect various dwell times associated with the paused states of the enzyme, including the relatively short elemental pauses and longer backtracking pauses.

For EcoRNAP, three exponential components effectively describe the DTD. The majority of the steps ($92.4\% \pm 1.3\%$, 95% CI), belong to the fastest state (state 1), with a rate of 38.3 ± 1.0 nt/s ($k_{\text{pause-free}}$ [k_{pf}], 95% CI), which corresponds to the average rate of completing a pause-free nucleotide addition cycle⁴³. State 2, comprising $7.2\% \pm 1.3\%$ (95% CI) of steps, corresponded to a slower rate of 6.2 ± 1.0 nt/s ($k_{\text{elemental-pause}}$ [k_{ep}], 95% CI), which we associate with the rate of escape by RNAP from elemental pauses⁴⁵. Finally, the remaining $0.31\% \pm 0.16\%$ (95% CI) of steps are state 3, which corresponds to an even slower rate of 0.34 ± 0.15 nt/s (95% CI), reflecting long pauses (k_{lp}).

For MtbRNAP, we also identified three populations with a k_{pf} of 20.7 ± 0.4 nt/s, a k_{ep} 5.2 ± 0.6 s⁻¹, and a k_{lp} of 0.52 s⁻¹ with similar population percentages to EcoRNAP (Figure 1e). The pause-free velocity values obtained from the velocity distribution

(Figure 1d, right panel) and DTD (Figure 1e) coincide well. We repeated this characterization for MtbRNAP under various magnitudes of assisting constant forces, ranging from 8 pN to 18 pN, and observed that k_{pf} slightly increases with greater force, while k_{el} was mostly unchanged. Conversely, when conducting the experiment under opposing constant forces from 4 to 18 pN, there are no dramatic effects on k_{pf} or k_{el} (Figure 2a).

Next, we performed experiments in *opposing passive mode*, where the downstream DNA is tethered, as in opposing constant force mode, but the traps are not moved, leading to an increase in force as transcription progresses (Figure 2b). Opposing passive mode is used to determine the stalling behavior and stall force of the polymerase. At low forces, MtbRNAP transcription velocity exhibits minimal response to the opposing force until a maximum force (F_{stall}) is reached, beyond which the enzyme drastically slows, stalls, and backtracks (Figure 2b)⁴⁵. On average, MtbRNAP stalled at 19.3 ± 1.2 pN (s.e.m.)⁴⁶. This force-induced stalling is often accompanied by backtracking (Figure 2c). By comparison, EcoRNAP stalls at 17.1 ± 1.1 pN (s.e.m.) and is also accompanied by backtracking, consistent with previous studies^{9,16,49}. The force-velocity curves can be fit to an unrestrained Boltzmann relation $v(f)=(1+A)/(1+A\exp(f\delta/kT))$, where the process is split into biochemical (force-independent) and mechanical (force-dependent) terms. The ratio of these rates at zero force is the parameter A, and the strength of force dependence is δ (Figure 2d, see Methods)¹¹. For both Eco and Mtb RNAPs, we find that $A \ll 1$, indicating that biochemical transitions are rate-limiting at zero force in these instances, in line with prior findings for EcoRNAP⁹. The value of δ is the distance to the transition state of polymerase stalling via backtracking, so we interpret this as the backtracking distance required by the RNAP to stall⁴⁶. For MtbRNAP, we obtain a value of 14 ± 1 nt (95% CI) (Figure 2d), comparable to the 13 ± 1 nt observed for EcoRNAP and findings from earlier studies on EcoRNAP⁹.

MtbRNAP weakly recognizes pause sequences of EcoRNAP.

We have observed that MtbRNAP tends to pause less frequently than EcoRNAP under an assisting constant force on both the Eco and Mtb templates (Figure 1e). To gain a clearer understanding of the pausing dynamics, we utilized a *molecular ruler* template to compare the pausing behavior in a sequence-dependent manner. A molecular ruler consists of a tandem repeat of a sequence harboring one or more strong pauses (Figure 3a)⁴⁸. Because the trajectories of the different traces obtained on our high-resolution optical tweezers instrument all display the repeating pattern of pauses, it is possible to align them and determine the precise position of the RNAP on the template with nearly single base pair resolution^{13,49}. Without a ruler, uncertainties in position caused by bead size variations or calibration errors cause the location of RNAP on the template to be not precisely known. We previously developed a ruler for EcoRNAP featuring five pauses designated as *a*, *b*, *c*, *d*, and *his*. However, we discovered that this ruler was ineffective for MtbRNAP, which does not pause strongly enough at those locations, as seen in the heights of the peaks of the residence time histogram (RTH) of the repeat (Figure 3b, c). We confirmed this behavior using a gel

assay, which shows different and often weaker pausing by MtbRNAP at these locations (Figure 3d). Notably, MtbRNAP demonstrates reduced recognition of the elemental pauses *a*, *b*, and *d*, and surprisingly did not strongly recognize the strong hairpin pause sequence *his* from *E. coli*.

Since MtbRNAP recognized pause *c* the strongest (Figure 3d), we designed a new molecular ruler based on this sequence. The *Mtb* molecular ruler consists of eight repeats of the elemental pause *c*, inserted within a fragment of the *M. tuberculosis rpoB* gene. MtbRNAP pauses strongly at the expected location of 50 nt into the 68 nt repeat that makes up the ruler, with minor pauses at position 5 and 30 nt in the repeat region (Figure 3 – figure supplement 1). Using this tool, we proceeded to study the effects of the inhibitors in a sequence-dependent manner.

D-IX216 inhibits elongation by MtbRNAP by switching it into interchangeable slow and super-slow inhibited states of transcription elongation

We investigated the effects of D-IX216 on the elongation activity of MtbRNAP. D-IX216 is an analog of D-AAP1²⁶, having a 2-thiophenyl group in place of a phenyl group as ring A and a 5-fluoro-2-piperazin-1-yl-phenyl instead of 2-methyl-phenyl as ring C (Figure 4 – figure supplement 1 a). D-IX216 is ten times more potent (IC₅₀ of 100 nM determined via bulk transcription methods) against MtbRNAP compared to D-AAP1 (IC₅₀ of 1 μM)^{25,26}, and is selective for MtbRNAP, not affecting EcoRNAP at these concentrations (Figure 4—figure supplement 1 b-d).

Upon adding 70-560 nM D-IX216 to a transcribing MtbRNAP (Figure 4a) under 18pN of assisting constant force, the elongation trajectories exhibited periods of transcription with three distinct global (i.e., including pauses) transcription velocities: one with *fast*, one with *slow*, and one with *super-slow* activity (Figure 4b), each lasting for at least tens of seconds (Figure 4—figure supplement 1e-f, and Figure 4—figure supplement 2 a). The slow and super-slow were about 5 and 50 times slower than the fast, respectively. Additionally, the super-slow exhibited less than half the processivity (both in terms of the number of base pairs transcribed before tether break/detachment and in terms of the number of base pairs transcribed before recovery from inhibition) compared to the slow activity (Figure 4—figure supplement 2 b). Interestingly, changing the direction of the applied force did not greatly alter the interconversion rate between these activities (Figure 4—figure supplement 2 b,c).

The fast activity resembled the enzyme's behavior in the absence of D-IX216. DTD analysis of this activity yields values similar to D-IX216-free MtbRNAP, further suggesting that the fast activity corresponded to a D-IX216-free enzyme (Figure 4—figure supplement 3). Hence, we identify the fast DTD states as the pause-free, elemental pause, and the long pause states. In contrast, the slow and super-slow activities were exclusively observed with D-IX216 present, indicating that they arise from its binding to the RNAP. We shall refer to the slow and super-slow as *inhibited states* of the enzyme, to differentiate them from the nucleotide addition states obtained from DTD analysis mentioned earlier. When bound to D-IX216, MtbRNAP displayed a consistent slow nucleotide addition rate lasting tens of seconds (Figure 4a, b). The inhibited states persist for several steps and can interconvert with each other (Figure

4—figure supplement 2 b,c). DTD analysis of these inhibited states revealed that the slow and super-slow inhibited states consist of multiple (nucleotide addition) states, each exhibiting distinct nucleotide addition rates (Fig 4 c, d). We emphasize here that inhibited states refer to a persistent activity of the enzyme lasting for multiple incorporation cycles, while nucleotide addition states may change between every step. The rates of the various nucleotide addition states found for the slow and super-slow inhibited states do not match those of the pause-free, elemental pause, or long pause states (Fig 4d), so we conclude that these are states distinct from the normal ones visited by the polymerase.

When the concentration of D-IX216 was increased from 70 nM to 560 nM, the kinetic parameters extracted from the DTD analysis did not greatly change (Figure 4—figure supplement 4 a), suggesting that the slow and super-slow inhibited states occur during the prolonged binding of a single D-IX216 molecule. This inference was supported by our washing experiments, which revealed that removing D-IX216 from the transcription medium of the affected polymerase did not immediately restore normal enzyme activity (Figure 4—figure supplement 1f). If this interpretation is correct, the conversion to the slow activity results from a binding event, while reversion to the fast activity is due to unbinding. Using the lifetimes of the fast and slow activities in the 280 nM case, we calculated a k_{on} , k_{off} , and K_D for D-IX216, obtaining $0.133 \pm 0.006 \mu\text{M}^{-1}\text{s}^{-1}$, $0.0032 \pm 0.0014 \text{s}^{-1}$, and $24.1 \pm 10.6 \text{nM}$, respectively (Figure 4—figure supplement 4 b). The K_D so attained is consistent with the IC_{50} of $\sim 100 \text{nM}$. We have no explanation for what process is involved in the interconversion between the slow and the super-slow inhibited states, but we speculate that it may correspond to two distinct binding modes of the inhibitor to the enzyme, affecting its activity differently.

We also examined whether the induced slowing by D-IX216 could be due to reduced rNTP affinity by decreasing the rNTP concentration from $250 \mu\text{M}$ to $25 \mu\text{M}$ ($\sim 5\times K_M$ to $\sim 0.5\times K_M$ for the uninhibited polymerase) in the presence of D-IX216. However, the DTD behavior of the slow and super-slow inhibited states remained unchanged (Figure 4—figure supplement 4 c), suggesting that rNTP binding is not rate-limiting in these inhibited states, unlike what is expected at these concentrations in the absence of the inhibitor.

Lastly, we investigated the effects of D-IX216 on transcription across the Mtb molecular ruler to determine if these effects were sequence-dependent. Our findings revealed that slow or super-slow events occurred randomly along the DNA template, consistent with our observations using the original sequence of Mtb DNA (Figure 4a). When MtbRNAP is in the slow inhibited state while crossing the repeats region, we observed that D-IX216 increases the residence time at every position (Figure 4e). We normalized the RTH values to the median residence time of each inhibited state to account for the different incorporation times associated with the fast, slow, and super-slow inhibited states. (Figure 4e, middle). This normalization demonstrated that the relative strength of the designated pause increased, as indicated by the heightened RTH peak (Figure 4e, right). If pause c is an off-pathway pause, a kinetic competition should exist between forward translocation and pausing. Therefore, we attribute the increased strength of this pause to a decrease in the transcription rate caused by the

inhibitor. In conclusion, we found that D-IX216 globally slows MtbRNAP translocation in a sequence-independent manner.

Streptolydigin induces backtracking in MtbRNAP

Using the Mtb molecular ruler, we added 4.5-45 μM Stl to a transcribing MtbRNAP under 18 pN of assisting constant force. We observed that Stl induced long pauses both inside and outside the repeat region (Figure 5a). The probability of entering these long pauses (lasting over 1 second) increased from 0.42% without the inhibitor to 0.76% with Stl. Of the enzymes that recovered, the long pause consists of two components; first, the RNAP pauses with an average duration of 10 ± 1.3 s (95% CI); then, it backtracks by 7 ± 1 nt (s.e.m.) and spends an additional 32 ± 4 s (95% CI) paused before transcription resumes (Figure 5b). In contrast, previous studies have shown that Stl does not affect the backtracking dynamics of EcoRNAP³⁰⁻³².

We also conducted a real-time bulk fluorescence assay where a fluorescent adenosine analog 2-aminopurine (2AP) in the template strand changes its brightness depending on whether it is basepaired or not. In this assay, we assemble the RNAP bubble to be just in front of the 2AP, whereby translocation by the RNAP causes the 2AP to un-basepair and fluoresce more strongly. The cognate rNTP is not present in solution, so the fluorescence persists after translocation. This allows us to distinguish between the post-translocated and the other states of the enzyme (pre-translocated or otherwise, see Figure 5—figure supplement 1 a). We used this assay to monitor the translocation state of RNAPs without cognate nucleotide and in the presence of 7.5 μM Stl^{51,52}. These experiments were conducted without pyrophosphate to prevent the inherent editing of the enzyme in the backtracked state. In the absence of Stl, the MtbRNAP is predominantly found in the post-translocated state, while in the presence of Stl, only ~20% of RNAPs remained in the post-translocated state (Figure 5—figure supplement 1 b). This reduction in the amount of post-translocated MtbRNAPs with Stl is consistent with the increase in backtracking observed in the single molecule traces (Fig 5b).

The recovery from the Stl-induced pauses before and after backtracking both exhibited single-exponential distributions (Fig 5c). A single-exponential distribution of backtrack durations is associated with *deep backtracking* and recovery via endonucleolysis, whereas a power law distribution ($t^{-3/2}$) corresponds to diffusive recovery from more shallow backtracking (Figure 5d).

Next, we compared the effect of Stl on the DTD under assisting and opposing constant forces. We found that Stl did not affect the MtbRNAP pause-free velocity under assisting constant force (Figure 5e). However, under opposing constant force, which favors the pre-translocated state of the polymerase, Stl slightly reduced the polymerase velocity and greatly increased the occurrence and duration of long pauses compared to the enzyme without the inhibitor (Figure 5e), consistent with reports regarding Stl's effect on EcoRNAP³⁰.

Our examination of the RTHs across the molecular ruler by MtbRNAP under assisting constant force with and without Stl, revealed that this inhibitor increased the duration of the designed pause in the tandem repeat region by 30%, but did not

introduce additional sequence-dependent pause sites (Figure 5f). This observation suggests that Stl binding is more favorable when the enzyme is paused²⁹. This inference is supported by the fact that we did not observe an increased residence time background in the presence of Stl (outside of repeat pauses).

A recent article noted that factors GreA and GreB enhanced Stl's pausing effects on EcoRNAP under opposing force³⁰. However, when we assayed MtbRNAP elongation under comparable conditions using GreA (as GreB has not been found in Mtb), we observed no obvious change in long pause duration (Figure 5g). These differing results may reflect differences in the mechanism of Stl inhibition between EcoRNAP and MtbRNAP.

Pseudouridimycin converts MtbRNAP into two slowly elongating inhibited states and increases the density of pauses in EcoRNAP

When 1 μ M PUM was added to a transcribing EcoRNAP, pauses lasting 5.9 ± 1.8 s on average were observed (pause escape rate of 0.17 ± 0.04 s⁻¹), representing 2% of the enzyme's steps (Figure 6a). Occasionally, we noted longer pauses lasting several hundreds of seconds. Additionally, we rarely (in less than 6% of traces) observed sections where EcoRNAPs converted to one of two states of slower nucleotide addition (slow and super-slow). These states resemble those caused by D-IX216 on MtbRNAP, so we shall also refer to them as inhibited states; however the enzyme was not observed to recover (Figure 6b, c).

Using the molecular ruler method with EcoRNAP, our experiments showed that PUM increased the RTH peaks of the *E. coli* native pauses *his* and *d*, reduced the RTH peak of native pause *c*, and sporadically introduced more sequence-dependent pauses along the template, and in a concentration-dependent manner (Figure 6d). As PUM is an rUTP analog, it is reasonable to conclude that these additional sequence-dependent pauses occur at U incorporation sites, as it seems to be the case for the additional pausing around the *his* and *d* sites (Figure 6d). However, due to the ± 3 bp resolution of our technique, we cannot confidently assert that all of these new peaks correspond to U incorporation sites¹⁵.

Interestingly, when 1 μ M PUM was added to MtbRNAP, a higher percentage of RNAPs (16%) exhibited conversions to slow and super-slow inhibited states reminiscent of D-IX216 binding (Figure 7a). However, unlike with D-IX216, we did not observe any interconversions between the slow and super-slow inhibited states with PUM. The DTD analysis indicated that the slow inhibited states induced by PUM were composed of nucleotide addition states that were slower than wild-type (Fig 7b). Comparing the addition states from the slow inhibited states induced by D-IX216 and PUM, we found differences in both global velocity and addition states, similarly so for the super-slow inhibited states (Figure 4d and Figure 7—figure supplement 1 a,b). Furthermore, in the presence of PUM, the polymerase displayed erratic behaviors, including the enzyme "hopping" back and forth in 10–20 bp increments, moving steadily backward (which differs from traditional backtracking), and pausing for extended periods, sometimes for hundreds of seconds (Figure 7—figure supplement 1 c,d). PUM had minimal effect on the RTH of the repeats, exhibiting only a slight increase in the strength of pause *c*,

which we attribute to the inhibitor's preference for binding to already paused polymerases (Figure 7c).

Mixing of Stl and D-IX216 shows a synergistic effect on transcription arrest

Treatment of tuberculosis usually involves a combination of antibiotics⁵³. Accordingly, we monitored transcription by MtbRNAP in the presence of both Stl and D-IX216. Notably, Stl and D-IX216 both bind to the bridge helix of the polymerase, in distinct pockets^{26,29,32,36}, which suggests that they may affect each other's binding.

When 560 nM of D-IX216 and 15 μ M of Stl were added to a transcribing MtbRNAP, the enzyme displayed either long pauses or a terminal arrest following a period of slow transcription (Fig 8a, b). The average duration of these long pauses, defined as those lasting over 5 s, was 25.2 ± 5.1 s (s.e.m.) in the presence of both inhibitors. This duration is consistent with the distribution of pause durations of MtbRNAP with 15 μ M Stl alone (27.5 ± 6.7 s, s.e.m.). This suggests that these long pauses may be attributed to Stl.

Interestingly, the arrest events following periods of slow transcription were not observed with either Stl or D-IX216 alone. The velocities during these slow sections appear to correspond to the slow and super-slow inhibited states induced by D-IX216 alone (Fig 8b). These studies appear consistent with the hypothesis that these slow transcription periods reflect the binding of D-IX216, which transitions the RNAP into the slow or super-slow inhibited state). Subsequently, the binding of Stl leads to a permanent pause (arrest).

Interestingly, while on average it takes 60 s for an Stl molecule to bind to an actively transcribing MtbRNAP at the concentration of the inhibitor used, in the presence of D-IX216, pauses arise within ~ 10 s after transcription slows down. This suggests that Stl binds more readily to D-IX216-bound MtbRNAP (indicating an increased k_{on}) than to the transcribing enzyme alone. This observation aligns with previous findings that Stl prefers to bind to already paused polymerases²⁹.

In conclusion, our evidence indicates that the binding of D-IX216 can increase the k_{on} of Stl, leading to the arrest of the enzyme. This suggests a synergistic effect between these two inhibitors, converting the lethargic nucleotide addition activity observed with D-IX216 alone into complete transcription arrests.

Discussion and Conclusion

Our findings indicate that the global transcription velocity of MtbRNAP is slower than that of EcoRNAP, which mirrors the trend in total transcription rates observed *in vivo*⁵⁴. In addition, whereas MtbRNAP has comparable mechanical robustness to EcoRNAP, it generally pauses more weakly or less. Our molecular ruler and bulk data suggest that the consensus pause sequences of the *E. coli* enzyme are also recognized by MtbRNAP, but again with less strength.

Our single-molecule studies demonstrate that three small-molecule inhibitors of MtbRNAP: D-IX216, Stl, and PUM, do not act as chain terminators, but instead slow transcription elongation in distinct ways.

D-IX216 inhibits transcription by inducing two distinct inhibited states that incorporate nucleotides more slowly. Our findings show that this inhibitor does not completely stop nucleotide incorporation, suggesting that it is engaged during elongation and exerts an inhibitory effect that differs from other inhibitors that target the bridge helix of the RNAP^{26,32,36,37,51,55–58}. The periods of recovery from transcription elongation inhibition observed in our molecular trajectories likely correspond to inhibitor dissociation. The different states (obtained from DTD), associated with different transcription rates, may reflect stochastic differences in the functional interactions between the catalytic elements of the RNAP and the inhibitor. Future research should investigate the causes of the nucleotide addition states and inhibited states.

Previous single-molecule studies have shown that Stl lengthens existing pauses in EcoRNAP, which is consistent with our observation that pause c is extended with Stl in MtbRNAP. However, in those studies, EcoRNAP was not found to backtrack after inhibition by Stl, which is an interesting difference in inhibition mechanism between these two polymerases.³⁰

PUM increases the pause density in both EcoRNAP and MtbRNAP by competing with UTP incorporation³⁵. In addition, PUM can transform the active polymerase into two distinct slowly-elongating inhibited states that do not appear to interconvert. We find this result surprising, as many nucleotide analog inhibitors of polymerases just cause pausing or termination, as is the case with several inhibitors being studied for use against SARS-CoV-2^{71–73}. It is possible that the inhibited states induced by D-IX216 and PUM share similar underlying mechanisms of inhibition, but if this is the case, it is not reflected in the different effects that these inhibitors have on the rate of elongation and DTD.

Both Stl and D-IX216 bind to the bridge helix of MtbRNAP. When administered together, D-IX216 binding seems to promote the binding of Stl, resulting in more potent inhibition (or arrest) of the enzyme. Since the standard of care for the treatment of tuberculosis infection involves a combination of antibiotics, identifying inhibitors that work together effectively is particularly valuable, and is not routinely the case⁵⁹. The results presented here with the combination of Stl and D-IX216 provide a basis for their possible use in combination therapy.

Single-molecule studies can provide details in the mechanism of action of inhibitors^{71–74}. For instance, this study shows that PUM, ostensibly an rUTP analog, surprisingly also can induce the RNAP into states with slow activity. In addition, we see that Stl induces the RNAP to backtrack, which is a state often difficult to detect with bulk assays⁷¹. Having these additional mechanistic insights will allow medicinal chemists to make more informed choices in developing ideal combinations of drugs against multi-drug resistant diseases such as tuberculosis.

Methods

A list of oligos, plasmids, and cells used are available in Supplementary File 1. A list of conditions studied in the optical tweezers assay, including numbers of traces for each, is included in Supplementary File 2.

Plasmid preparation

To make the vectors for MtbRNAP biotinylation, we performed round-the-horn site-directed mutagenesis of plasmid pET-DUET-BC⁶⁰ using long PCR to introduce an avitag or a sortase tag (sortag) sequence at the c-terminus of the rpoC gene¹³. The resulting plasmids were named pET-DUET-BC-avitag or pET-DUET-BC-sortag, respectively. As for the vectors for expressing Mtb transcription factors, we amplified the corresponding gene from the Mtb genome and inserted it into the plasmid pET His6 TEV LIC (Addgene, # 29653) using ligation-independent cloning⁶¹.

To make plasmids with the DNA templates for the single-molecule assays, we took pUC19 and cloned the promoter AC50, the 10-bp stalling cassette, and the transcribable region, which derived from a ~ 2.7 kb region of genes rpoB and C from the *M. tuberculosis* Erdman genome (a gift from Professor Sara Stanley). We used an additional round-the-horn site mutagenesis to remove the lac promoter. We named this plasmid pUC19-del-Lac-AC50-MtbrpoBC. We used this plasmid to create another plasmid containing a molecular ruler. First, we removed one side of the BsaI in the rpoC gene by inserting a 255 bp DNA fragment of terminator WhiB1. Second, we replaced the stalling sequence with a 20-bp CU-less cassette using round-the-horn mutagenesis. Third, we used NEBuilder® HiFi DNA Assembly Master Mix (NEB E2621S) to insert a synthetic DNA fragment, an 8x repeat of a 63 bp sequence of pause c (Genescript), into the rpoB gene. The resulting plasmid was named pSGUML1T1.

DNA template preparation

We made a single-end digoxigenin-labeled DNA template (without a ruler) using PCR by amplifying approximately 4.2 kb or 2.8 kb from plasmid pUC19-del-Lac-AC50-MtbrpoBC, depending on whether the assay is for opposing or assisting force, and utilized a dig-labeled primer.

To make the DNA template containing the molecular ruler, we linearized the plasmid pSGUML1T1 with BsaI and filled the sticky ends by using Klenow Fragment in the presence of either ddATP or ddCTP to produce the opposing or assisting force template, respectively¹³.

In addition, DNA handles were created using PCR with a biotinylated primer along with another primer, either double-dig labeled or encoding a restriction enzyme site for generating sticky ends for later ligation.

Production of biotinylated and non-biotinylated MtbRNAP

We produced the holoenzyme of MtbRNAP with sigma factor A by co-expressing its subunits in *E. coli*⁶⁰. We biotinylated the avitag-MtbRNAP by co-expressing the plasmids pACYD-DUET-AOS and pDUET-BC-avitag with the plasmid pCDF-BirA (a gift from Professor Nicola Burgess-Brown). This protein was used for the assay under opposing force with Stl and GreA. Alternatively, we biotinylated the sortag-MtbRNAP (LPETG at the c-terminal of β') after purification using an *in vitro* reaction of the polymerase, the enzyme sortase A⁶² and GGGL-biotin (Genescript)⁶³.

Production of transcription factors MtbCarD and MtbGreA

We purified the transcription factor CarD as before⁶⁰. To obtain MtbGreA, we expressed it as a his-tagged protein in *E. coli* BL21(DE). We captured this protein with HisTrap® (Cytiva) and used a TEV protease cleavage reaction⁶⁴ to cleave its histidine tag. To remove the cleaved tag, we used reverse nickel chromatography. The final purification step was done with anion exchange chromatography (HiTrap Q, Cytiva) in a Tris-saline buffer.

***In vitro* transcription bulk assay**

We used an artificial bubble-based transcription to assess the recognition of the elemental pause sequences by MtbRNAP using a FAM-labeled RNA⁶⁰ and assessed transcript size via gel. Raw gels are available in the Source Data Files related to the relevant figures.

Ternary elongation complex formation

We stalled the biotinylated MtbRNAP on a cassette lacking one or two nucleotides to form the ternary elongation complex (TEC) to study elongation, as previously described^{65,66}.

Sample preparation for single molecule optical tweezers transcription elongation assay

To create the promoter-initiated stalled MtbRNAP elongation complexes, we utilized MtbCarD. First, we pre-incubated approximately 1 μM of biotinylated-MtbRNAP with around 10 μM of MtbCarD at 37 °C for 5 minutes in a final volume of 2 μL. Subsequently, we diluted the biotinylated MtbRNAP in buffer TB40 (20 mM Tris-HCl pH 7.9, 40 mM KCl, 5 mM MgCl₂, and 0.5 mM TCEP) to a concentration of 10 nM. We then stalled 5 nM of polymerases by incubating them with 150 μM GpG, 2 nM Mtb DNA template in stall Buffer (20 mM Tris-HCl pH 7.9, 3 μM rATP, 3 μM rGTP, and 3 μM rCTP) at 37 °C for 20 minutes. If we were to use the Eco DNA repeat, we would substitute GpG with ApU⁶⁷.

To attach the DNA handles or stall complexes to the microbead surfaces, we used a few different methods. In the first method, we incubated a 0.1 nM stalled complex labeled with double-digoxigenin with 0.05-0.1% passivated anti-dig beads in TB40 buffer for 10 minutes at 22 °C. Then, we added 0.05 μg/μL heparin at 22 °C for 5 minutes to remove non-competent polymerases. In the second method, we incubated 0.2-0.5 nM digoxigenin-labeled DNA handle with 300 nM neutravidin and the same amount of anti-digoxigenin beads at 22 °C for 10 minutes, followed by the addition of heparin. In the third method, we conducted ligation-based deposition by incubating a 0.1 nM stall complex with 0.1-0.05% passivated oligo beads, 0.1 mM rATP, and 4 U/μL T4 DNA ligase in TB40 buffer at 22 °C for 45 minutes to facilitate the ligation reaction. This was followed by a 5-minute treatment with heparin at 22 °C. In the fourth method, we attached 0.2-0.5 nM DNA handle by incubating it with passivated complementary oligo beads, 300 nM neutravidin, 0.1 mM rATP, and 4 U/μL of T4 DNA ligase in TB40 buffer for an identical prolonged incubation, followed by heparin treatment. The bead

depositions were either kept on ice for immediate use, or preserved by flash freezing and stored at -80°C.

For every 10 µL of deposited beads, we added 1,000-1,500 µL of degassed TB130 Buffer (20 mM Tris-HCl pH 7.9, 130 mM KCl, 10 mM MgCl₂, 1 mM TCEP and 5 mM NaN₃) before injecting into the fluidics chamber.

In the experiments with MtbCarD and MtbGreA, the final amount of initiating CarD contributed to the amount free in solution in elongation was as low as ~13 pM (diluted from 100-120 nM MtbCarD), and neither causes any effects on pausing or velocity.

High-resolution optical tweezer transcription elongation assay

In Figure 1a there is a representation of the optical tweezers setup, and in Figure 1b, 1c, and 2b there are representations of assisting constant force, opposing constant force, and opposing passive mode experiments. We used a custom home-built timeshared dual-trap optical tweezers system^{67,68}. In short, the single molecule assay to measure transcription elongation involves two beads, one coated with RNAP stalled complexes, and one coated with DNA handles with neutravidin on the free end. The two beads are caught in separate traps, and when the beads are brought in close proximity, the neutravidin on the end of the DNA handle binds to the biotin attached to the RNAP and a tether is formed. The beads are brought apart, and the DNA handle separates the RNAP from the surface of the bead to reduce photodamage. We also used an oxygen-scavenger system based on sodium azide to increase the robustness of collecting processive activity. For both EcoRNAP and MtbRNAP, the attachment to biotin is done via its β' subunit.

We injected the microbeads into a microfluidics chamber through different channels to diffuse them to a central main channel, trapped them by optical tweezers and brought them close to each other from the tether. To resume the elongation activity, we flowed a solution containing rNTPs, transcription factors, and/or inhibitors through a shunt positioned very close to the tethered complex. We used fluidic valves to control the flow via gravity. When transcribing the Eco repeat sequence, we used a nucleotide cocktail of 1 mM rUTP, 1 mM rGTP, 0.5 mM rATP, and 0.25 mM rCTP¹³ to match previous studies.

The instrument records the trap separation and monitors the displacement of the beads from the centers of the traps, and handles force feedback for the constant force experiments⁶³. Calibration from raw voltages from the instrument to distance (bead separation) and applied force is done via power spectrum calibration to the expected Brownian motion of a bead held in a trap⁶⁷.

Analysis of single-molecule complete trajectories of transcription elongation

All analysis, including the processing of the raw instrument data to forces and bead-to-bead distances and analysis procedures is done via custom code written in MATLAB, and is available at <https://doi.org/10.5281/zenodo.15232932>.

The collected traces are first selected for their length and undergo an initial sorting step where traces with unusually high instrumental noise or those that are unfit for

analysis (two RNAPs tethered at the same time, too short transcription, etc.) are removed.

For calculating velocity distributions, a Savitzky-Golay differentiating filter of degree 1, width 1s is used to extract the velocity of the trace at every point. These velocities are binned and least-squares fit to the sum of two Gaussians, one with mean 0 bp/s.

To get the dwells of the polymerase, we fit the traces as a staircase with a 1 bp step size. The complementary cumulative distribution function (CCDF) of the dwell time

distribution (DTD) is fit to a sum of exponentials $\sum_{i=1}^n a_i e^{-k_i t}$, where i represents the i -th

nucleotide addition state of the RNA polymerase, a_i is the state probability ($\sum_i a_i = 1$), and k_i is the kinetic rate such that $k_1 > k_2 > \dots > k_n$. The CCDF is fit to the sum of up to 6 exponentials, and the best is chosen via the Akaike information criterion.

We calculated the residence time histogram (RTH) of the polymerase transcribing the pauses repeat according to a previous publication¹³. Briefly, the repeating pauses lets us determine a scale factor and offset factor to each transcription trace based on the location of the pauses. The scale is chosen to space the pauses 68 bp apart, and the offset is chosen to place the pause at position 50 bp into the repeat. This is done by taking the RTH of the repeat, averaged across the 8 repetitions, for a range of scale factors. The one that aligns the pauses best is the one with the most peaked RTH (a poor scale factor does not align the pauses, so makes the RTH flat, a good scale factor aligns the pauses and thus it will show up as a peak). The offset factor is then chosen to place the location of the strongest peak in the best RTH at 50 bp.

For the analysis of passive mode data, individual stalling force-velocity profiles are generated and the force F and velocity V is transformed into normalized units v and f , with the velocity normalized by dividing by the maximum force V_{max} and the force normalized by dividing by the stall force, which is the force at which the velocity is half of V_{max} . These individual force-velocity profiles are averaged together and fit to the function $v(f) = (1+A)/(1+A \exp(f\delta/kT))$. For a short derivation, let the nucleotide addition cycle be made up of two steps that occur sequentially, one force-independent (comprising the biochemical transitions) and force-dependent (comprising the mechanical transitions). Let the rates of these terms be $k_{biochemical} = k_b$ and $k_{mechanical}(F) = k_m \exp(-Fd/kT)$. For the mechanical term, the system must move some distance d against the optical tweezers' force F to reach the transition state, so the transition state energy is raised by Fd and the rate is affected via a Boltzmann factor. The transcription velocity is then the inverse of the sum of the inverses of these two rates: $V(F) = (k_b^{-1} + k_m^{-1} \exp(Fd/kT))^{-1}$. Let $V_{max} = V(0) = 1/(k_b^{-1} + k_m^{-1})$, so the normalized velocity $v(F) = V(F)/V_{max} = k_b^{-1} + k_m^{-1} / (k_b^{-1} + k_m^{-1} \exp(Fd/kT))$. Let $A = k_b/k_m$ and let $Fd = f\delta$ (to transform Fd (unit pN * nm) into $f\delta$ (unit normalized force * 'normalized distance')) to get the stated relation, $v(f) = (1+A)/(1+A \exp(f\delta/kT))$. A is a parameter that dictates the ratio between the biochemical (force-independent) and mechanical (force-dependent) rates, and δ is the force dependence of the mechanical term. We divide 'normalized distance' by F_{stall} to get the distance to transition state in length units since $Fd = f\delta$ and $f = F/F_{stall}$.

609
610 **Materials, Data, and Code Availability Statement**

611 All oligos/plasmids used in this study are available upon reasonable request. See
612 Supplementary File 1 for a list of materials. Transcription traces are available from
613 Dryad at <https://doi.org/10.5061/dryad.2fqz6130m>. Matlab code used for analysis is
614 available on Github at <https://doi.org/10.5281/zenodo.15232932>.
615

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624

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Figure Legends

Figure 1: Single-molecule transcription elongation by *M. tuberculosis* RNA polymerase under high-resolution optical tweezers

- a) (left) The experimental optical tweezer setup is shown, which tethers and isolates the RNAP from the laser beams through a DNA handle and template. (right) A few single-molecule transcription traces are shown, with the pauses (red) and the pause-free periods (green) highlighted.
- b) A diagram of the assisting constant force mode is shown. The traps are moved apart as the RNAP transcribes to keep the force constant.
- c) A diagram of the opposing constant force mode is shown. The traps are moved closer as the RNAP transcribes to keep the force constant.
- d) (left) Representative trajectories of transcription by EcoRNAP (black) and MtbRNAP (blue) under ~18 pN assisting constant force and saturating concentration of rNTPs (~1 mM) are shown. The DNA template for MtbRNAP derives from *M. tuberculosis* genes *rpoB* and *rpoC*. (right) Velocity distributions of these trajectories are shown, with a fit to a sum of two Gaussians, one with mean zero (pauses) and one with positive mean (pause-free velocity).
- e) The populations and rates obtained via DTD analysis are shown for EcoRNAP and MtbRNAP on Eco and Mtb templates. The traces were collected under saturating concentrations of nucleotides and 15-20 pN of assisting constant force for EcoRNAP or 18 pN for MtbRNAP.

Figure 2: Mechanochemical characterization of the elongation by *M. tuberculosis* RNA polymerase under high-resolution optical tweezers

- a) The effect of magnitude and direction of applied force on the kinetic parameters obtained from DTD analysis is shown. Positive forces are assisting constant forces, negative are opposing constant forces.
- b) A diagram of opposing passive mode is shown. The traps are held stationary as the RNAP transcribes, leading to an increase in force.
- c) Example opposing passive mode traces are shown. The MtbRNAP transcribes at near constant velocity before suddenly stalling and backtracking.
- d) Force-velocity relationships for MtbRNAP and EcoRNAP obtained from passive mode experiments are shown and fit to a generalized Boltzmann relation (right). Both polymerases' velocities remain insensitive to force until they reach similar stall forces, on average $19.3 \text{ pN} \pm 1.2 \text{ pN}$ for MtbRNAP, and $17.1 \pm 1.1 \text{ pN}$ for EcoRNAP.

Figure 3: MtbRNAP pauses less efficiently than EcoRNAP on Eco pauses

- a) Representation of the template containing a molecular ruler to study EcoRNAP pauses. The DNA template consists of 8 repeats of five Eco elementary pauses, *a*, *b*, *c*, *d*, and *his*.
- b) Example traces of single Eco and Mtb RNA polymerases transcribing the *E. coli* molecular ruler are shown. The eight repeats are denoted as vertical arrows, and the location of the pauses within each repeat are shown as horizontal colored lines. We collected the transcription activities using a cocktail of rNTPs (1 mM rUTP, 1 mM rGTP, 0.5 mM rATP, and 0.25 mM rCTP) at 15-20 pN for EcoRNAP and 18 pN for MtbRNAP. The zoom on the right shows the alignment of the trace pauses with the expected pause locations.
- c) Average residence time histograms of EcoRNAP and MtbRNAP display distinctive patterns of pause strength (peak height) and location. MtbRNAP more weakly recognizes the Eco pauses.
- d) Comparison of the transcription gel bands at the elemental pause sites between MtbRNAP and EcoRNAP.

Figure 3—Source Data 1: Original raw gels for Figure 3d

Figure 3—Source Data 2: Full gel images for Figure 3d, with labels.

Figure 4: The inhibition of MtbRNAP by D-IX216 involves the conversion to one of two slowly elongating inhibited states

- a) Example traces of MtbRNAP transcribing in the absence (grey) and presence (red) of 140 nM D-IX216 are shown.
- b) Traces exhibiting the three states, fast, slow, and super-slow, are shown and the regions are colored blue, orange, and red, respectively. Transcription in the absence of D-IX216 is shown in grey. The traces plotted in this figure were collected at 18 pN of assisting constant force, 1 mM rNTPs, and 140 nM D-IX216.
- c) Comparison of the complementary cumulative distribution function (CCDF) of the dwell time distributions (DTD) of the fast, slow and super-slow states.
- d) DTD analysis of the fast, slow, and super-slow inhibited states are shown. Pf: pause-free, ep: elemental pause, lp: long pause.
- e) (left) MtbRNAP traces crossing the Mtb molecular ruler are shown in varying states of inhibition. (right) The residence time histograms of these inhibited states are shown both in seconds or normalized to the median residence time.

Figure 5: Streptolydigin inhibits MtbRNAP by enhancing pausing and inducing backtracking during pausing

- a) Example traces of MtbRNAP transcription in the absence (grey) and presence (green) of 15 μ M Stl, with 18 pN of assisting constant force and saturating rNTP concentrations.
- b) Details of a backtracking event induced by Stl are shown, exhibiting a process where there is both an initial pre-backtrack pause, a backtrack, and then recovery from that backtrack.
- c) Fittings for the CCDFs of the pre-backtrack and recovery times of Stl-induced pauses to single exponentials are shown.
- d) The comparison between the fitting of the backtrack recovery between two models is shown: a single-exponential distribution corresponding to deep backtracking and a power law distribution corresponding to shallow backtracking.
- e) The kinetic parameters obtained from DTD analysis in the presence of Stl are shown, under a constant 18 pN assisting or 4 pN opposing constant forces.
- f) Average residence time histograms of MtbRNAP transcribing the repeat of the molecular ruler in the absence and presence of Stl is shown.
- g) Kinetic parameters extracted from DTD analysis of MtbRNAP traces under constant 5 pN of opposing force and 15 μ M Stl in the absence or presence of 0.5 μ M MtbGreA are shown.

Figure 6: PUM inhibits EcoRNAP by enhancing pausing and infrequently inducing slowly elongating inhibited states

- a) Single-molecule traces of EcoRNAP transcription in the absence (black) and presence (purple) of 1 μ M PUM are shown. Traces were collected at 10 pN constant assisting force and 1 mM rNTPs.
- b) A comparison of the fast state and the infrequent slow and super-slow inhibited states with PUM are shown.
- c) Kinetics parameters attained from DTD analysis of the three regions of EcoRNAP with PUM are shown. Pf: pause-free, ep: elemental pause, lp: long pause.
- d) (left) Example traces of EcoRNAP crossing the molecular ruler in the presence of PUM are shown. (right) The average residence time histogram of EcoRNAP crossing one repeat is shown at varying ratios of PUM to 1 mM rUTP.

Figure 7: Inhibition of MtbRNAP by PUM involves induction of slowly elongating inhibited states

- a) (left) Single-molecule MtbRNAP transcription in the absence (grey) and presence (purple) of 1 μ M PUM are shown. (right) Examples of transcription in the slow inhibited state, the super-slow inhibited state, and a dynamic pause are shown. Transcription was carried out under 18pN of assisting constant force and 1 mM rNTPs.
- b) Kinetic parameters attained from DTD analysis of the three regions of MtbRNAP with PUM are shown. Pf: pause-free, ep: elemental pause, lp: long pause.
- c) The average residence time histogram of a repeat of MtbRNAP transcribing the Mtb molecular ruler in the presence and absence of 1 μ M PUM is shown

Figure 8: Inhibition of MtbRNAP by a combination of D-IX216 and Stl

- a) Comparison of transcription elongation traces of single-molecules of MtbRNAP obtained with D-IX216, Stl, or both are shown.
- b) A zoom of the traces with D-IX216 and Stl just before arrest are shown. The blue dotted guide lines match the average velocity of MtbRNAP in the fast, slow, and super-slow inhibited states from left to right.

Figure Supplement Legends

Figure 3 – figure supplement 1: The design of a molecular ruler to study pausing in MtbRNAP with high-resolution optical tweezers

- a) An artificial transcription bubble scheme with a 5'-FAM labeled RNA was used for a bulk elongation assay. The stalled ternary elongation complex at position G15 (TEC_{G15}) resumes RNA polymerization activity upon adding 250 μ M rNTPs, and pauses at pause *c* (TEC_{A26}).
- b) MtbRNAP efficiently recognizes sequence derived from *E. coli* elemental pause *c*. (left) A urea PAGE gel that shows the clearance of the pause site at TEG_{A26} with a mean duration of ~ 27 sec (inverse of the pause escape rate). (right) The quantification of the bands and their fitting to a bi-exponential decay. The extracted parameters indicate that the pause escape rate is similar in duration to the *his* pause of EcoRNAP⁷⁰.
- c) The *Mtb* molecular ruler enables the analysis of sequence dependent pausing by MtbRNAP at the single-molecule level. Example traces are shown on the left, collected at 1 mM rNTPs and 18 pN assisting constant force. Below is a zoom around the designed pause. The RTH on the right shows the strong pausing at 50 bp, and how the strength changes with the direction of the applied force (18 pN assisting constant force vs. 9 pN opposing constant force)

Figure 3 – figure supplement 1—Source Data 1: Original raw gels for Figure 3-figure supplement 1 b

Figure 3 – figure supplement 1—Source Data 2: Full gel images for Figure 3-figure supplement 1 b, with labels

Figure 4 – figure supplement 1: D-IX216 specifically slows down the MtbRNAP transcription

- a) The chemical representations of the N α -aroyl-N-aryl-phenylalaninamides D-AAP1 and D-IX216 are shown. Rings A and C are modified to increase the affinity of D-IX216 for MtbRNAP²⁵.
- b) D-IX216 does not alter EcoRNAP activity. An example trace of a two-shunt experiment shows that 280 nM D-IX216 does not affect EcoRNAP activity. The dotted boxes denote what solution is flowing out of the shunt at what time.
- c) The kinetic parameters obtained from DTD analysis of EcoRNAP in the absence and presence of D-IX216 is shown. In this dataset, there were too few events to fit a third exponential (long pauses).
- d) This buffer exchange control experiment shows that the RNAP quickly responds to buffer changes. When nucleotides are removed, MtbRNAP immediately pauses until the nucleotides are reintroduced.
- e) D-IX216 slows down MtbRNAP rather than pausing or halting it. The trajectory of a single MtbRNAP elongating at constant 18 pN assisting constant force with 280 nM D-IX216 and saturating nucleotides (~1 mM) shows a slow state that eventually instantly recovers its velocity (~ 20 bp/s). Depending on the condition, up to ~ 41% of polymerases can recover their initial velocity. The dotted boxes show the start and duration of the shunt opening to supplement the inhibitor and nucleotides. The apparent association time of D-IX216 binding corresponds to the time between the shunt opening to introduce D-IX216 and the switch's appearance. On the other hand, the apparent D-IX216 dissociation time corresponds to the slow region's lifetime.
- f) D-IX216 is still engaged during MtbRNAP elongation. We performed a buffer exchange using two shunts to remove the inhibitor from the affected polymerase. However, this did not immediately restore its global velocity. Towards the end of the trace, the polymerase might detach from the DNA template, leading to tether break events or stalls in 45% and 13% of the polymerases, respectively.

Figure 4 – figure supplement 2: Interconversion between the slow and super-slow inhibited states

- a) The CCDFs of lifetimes of the slow and super-slow inhibited states are shown with a fitting to a single exponential decay. The lifetime was measured as the time between switching into that state, until the motor either recovered (fast) or switched into the other state (e.g. a switch from slow to super-slow). Tether-break events were not included.
- b) Comparison of the processivity (in terms of kb extended by the elongating polymerase before tether break (template dissociation), or in terms of kb before a switch event of the super-slow and slow inhibited states is shown. Processivity is lower in the super-slow inhibited state than in the slow inhibited state by both definitions. The data for the bar plots were obtained from combined data of assisting and opposing constant force experiments.
- c) Transition maps under assisting and opposing constant force of the different states (fast, slow, and super-slow) are shown.

Figure 4 – figure supplement 3: The fast elongating state of MtbRNAP observed in the presence of D-IX216 corresponds to the inhibitor-free state

- a) Example fittings of the dwell times for the inhibitor-free and the various inhibited states of MtbRNAP are shown.
- b) The CCDFs of the DTDs for fast elongating polymerases observed with (black and dark blue) and without D-IX216 (cyan).
- c) The kinetic parameters from DTD analysis for the enzyme without D-IX216 and the enzyme in the fast state with D-IX216 are shown. No notable differences are found between the inhibitor-free and the fast state with D-IX216.

- Figure 4 – figure supplement 4: Effect of different D-IX216 and nucleotide concentrations on the slow and super-slow inhibited states of MtbRNAP**
- a) The concentration of D-IX216 did not impact the kinetic parameters obtained by DTD analysis of MtbRNAP in the slow inhibited states. This suggests that a single molecule of D-IX216 causes the conversion into the slow state.
 - b) Measurement of the dissociation constant (K_D) of D-IX216 for the elongating MtbRNAP. The association and dissociation times obtained from the duration of the fast, slow, and super-slow inhibited states in the single-molecule traces are fit to single-exponentials to obtain k_{on} and k_{off} . Their ratio is K_D .
 - c) The concentration of rNTPs did not affect the kinetic parameters in the slow inhibited state of MtbRNAP induced by D-IX216. This indicates that the slowing by D-IX216 is not due to a reduction in nucleotide affinity. These experiments were done in semipassive assisting force mode, where the optical traps are moved stepwise to keep the force within a set range. In other words, the traps are held at constant position, causing the force to fall as transcription progresses, until it reaches some lower force limit (in this case, 10 pN) after which the traps move apart to raise the force to some upper force limit (in this case, 18 pN).

Figure 5 – figure supplement 1: Streptolydigin (Stl) induces reduced post-translocated states in MtbRNAP

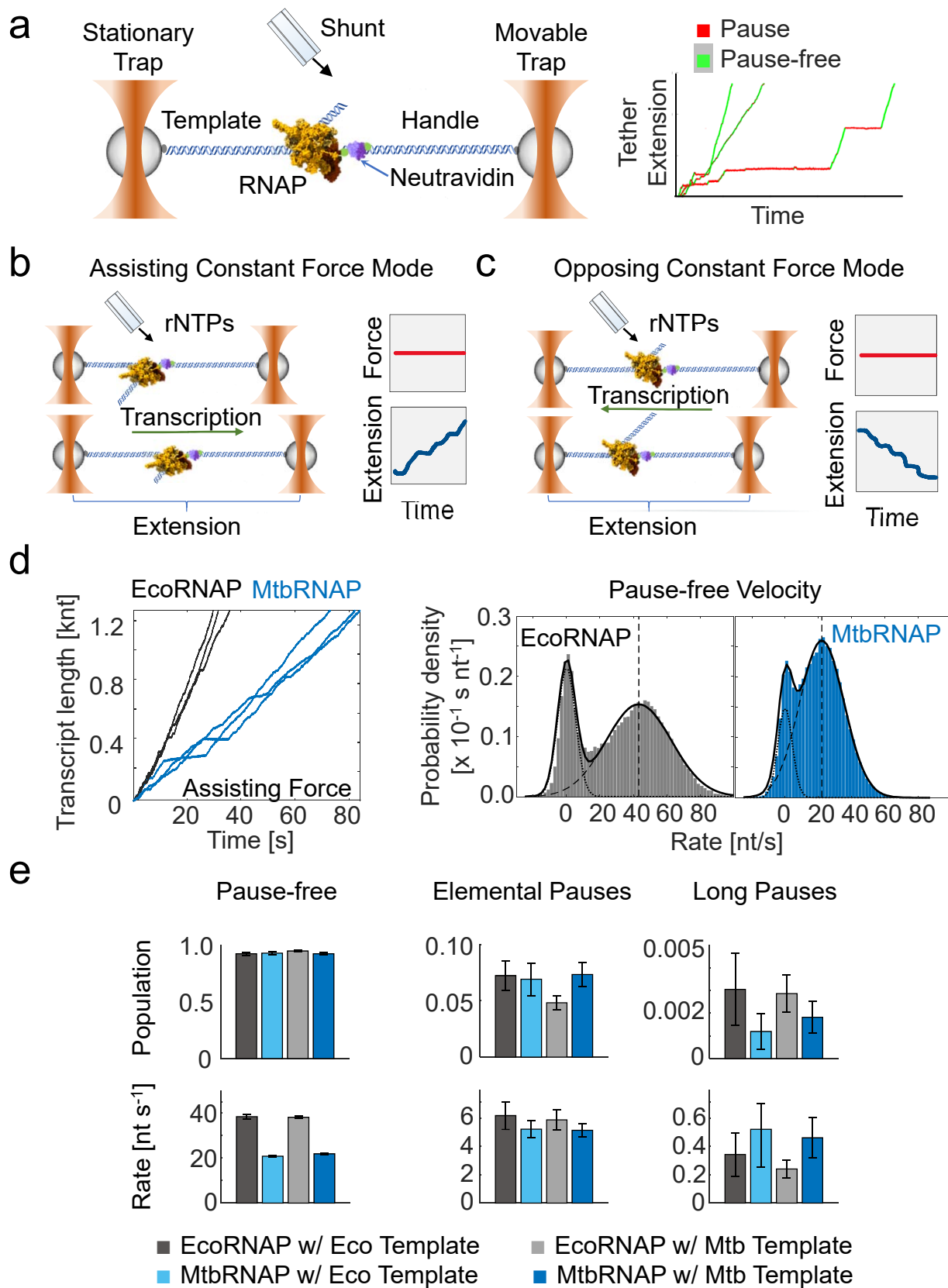
- a) A bulk assay was performed to measure the translocation of RNAP by one nucleotide using the Mtb ternary elongation complex. The technique involved real-time measurement of the fluorescent enhancement at 375 nm of a nitrogenous base analog, 2-aminopurine⁵². This analog was positioned on the template strand of the DNA-RNA hybrid, initially at position +2, and then moved towards position +1 due to the RNAP translocation process. The workflow included the use of a fluorescent artificial bubble to measure the effect of Stl on the translocation state of MtbRNAP. After the RNAP incorporated one rATP, the translocation state was measured by the dye's fluorescence in the following base in the template DNA. When the base was paired, fluorescence was low due to quenching; when the base was unpaired, such as in the post-translocated state, the fluorescence was high. The assay conditions consisted of transcription buffer and 100 μ M rATP, along with either 100 μ M dATP, 0.25 μ M MtbGreA, 7.5 μ M Stl, or 0.25 μ M MtbGreA / 7.5 μ M Stl.
- b) The comparison of different conditions of the translocation trace of the MtbRNAP in the presence of cognate nucleotide (rATP) and Stl shows that the inhibitor allows only ~ 20% of the MtbTEC to attain the post-translocated state (green dots). The addition of MtbGreA was used as a positive control for post-translocation.

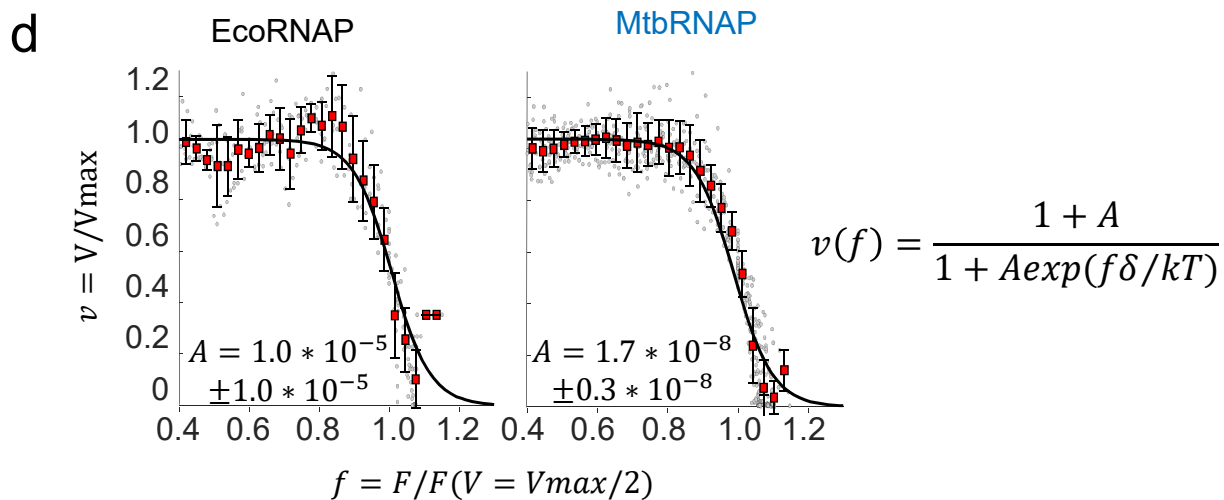
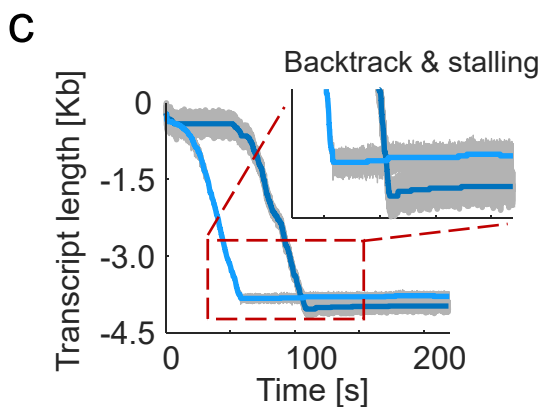
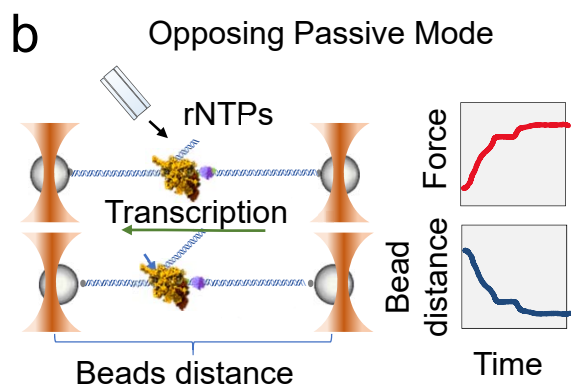
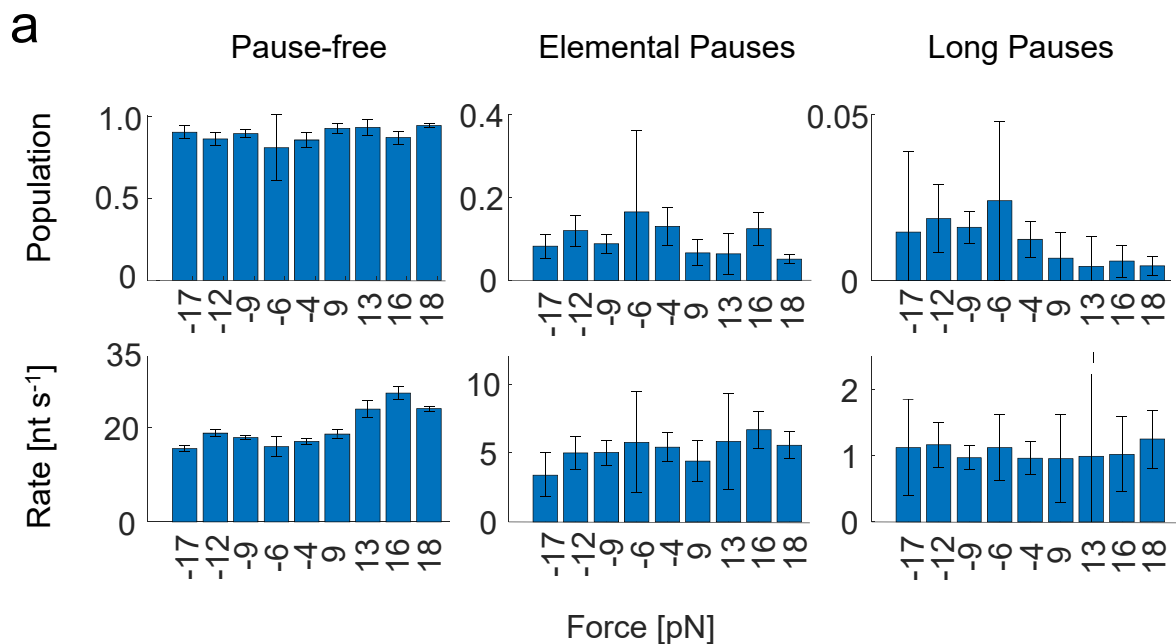
Figure 7 – figure supplement 1: Comparison of events found in transcription with antibiotics

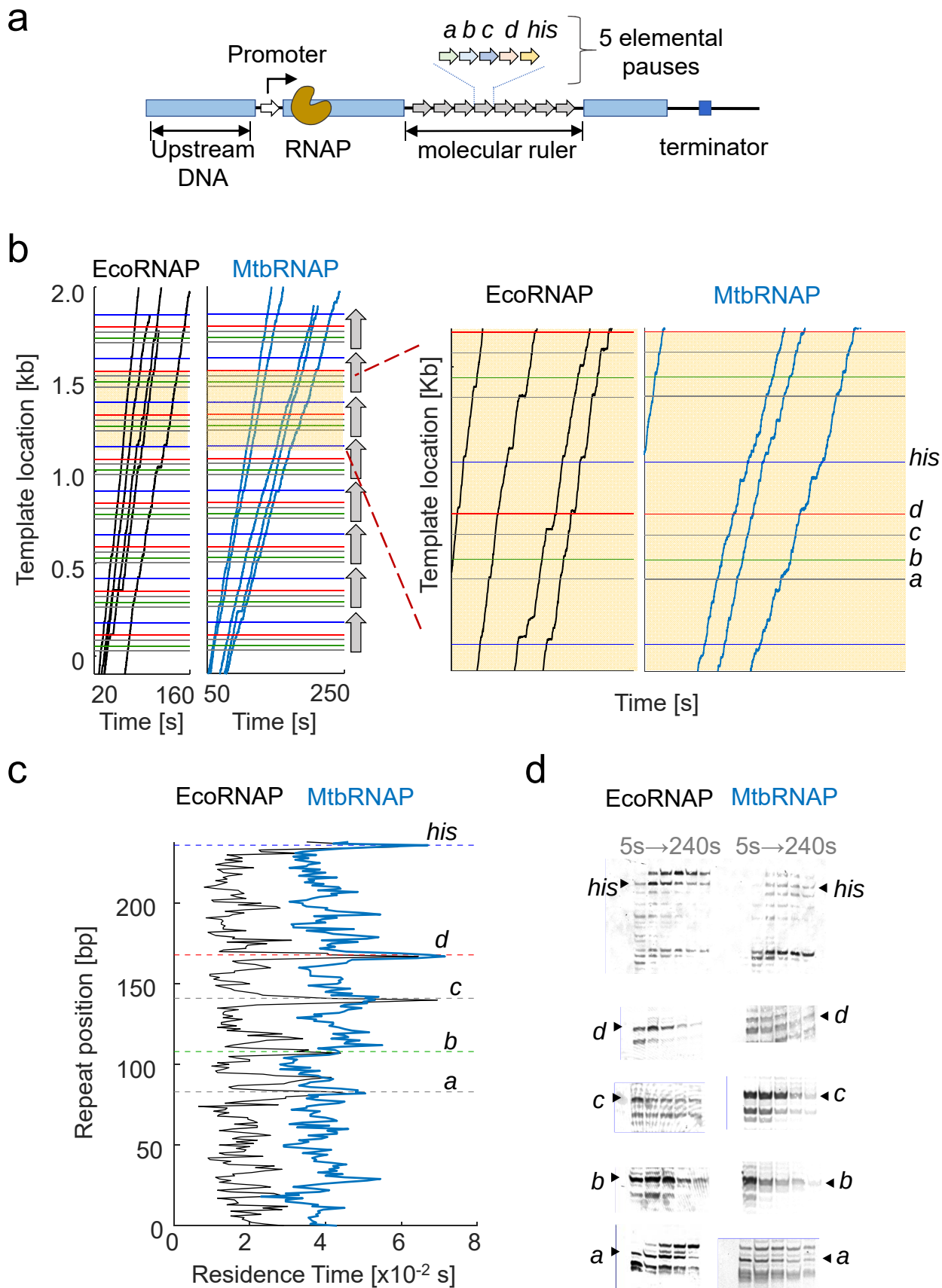
- a) Example traces in the slow inhibited state caused by PUM and D-IX216 are shown.
- b) Example traces in the super-slow inhibited state caused by PUM and D-IX216 are shown.
- c) Comparison of a common pause obtained in the absence of inhibitor versus a dynamic pause caused by PUM is shown.
- d) Comparison of a backtrack event observed in the presence of Stl compared to a rare non-traditional backtrack event induced by PUM is shown.

Supplementary File 1 Tables of Oligos Plasmids and Cells.docx

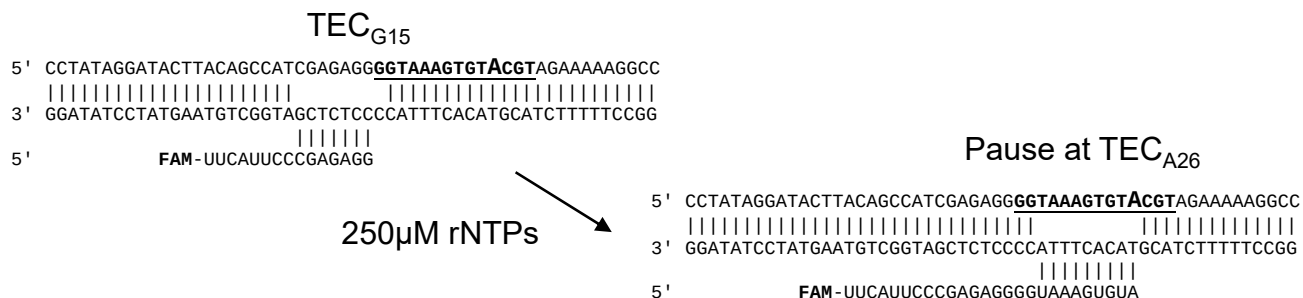
Supplementary File 2 Summary of Traces



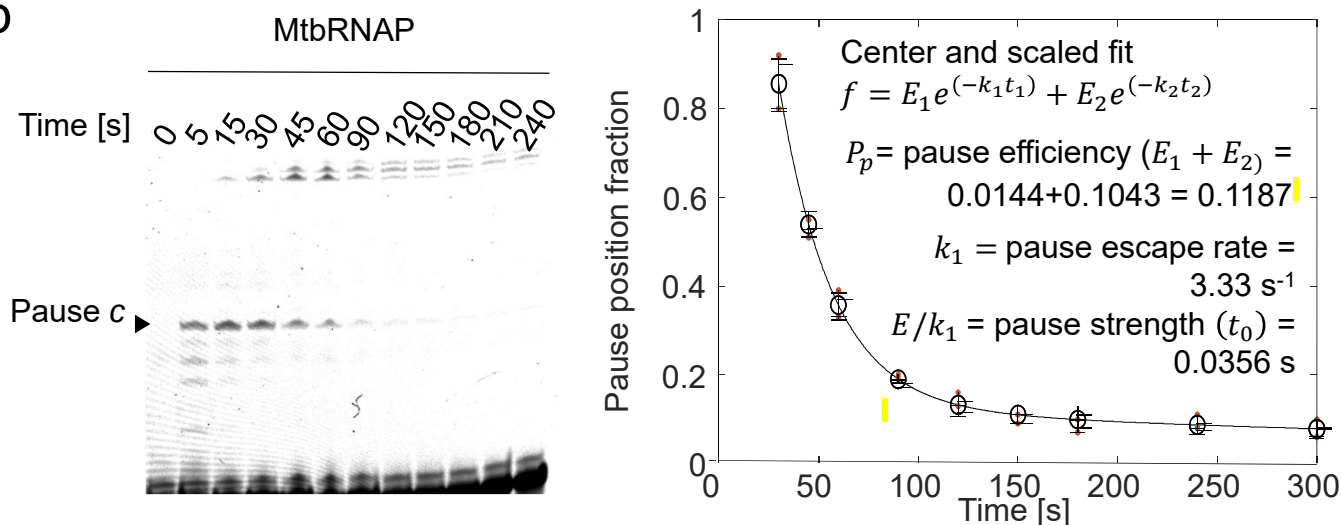




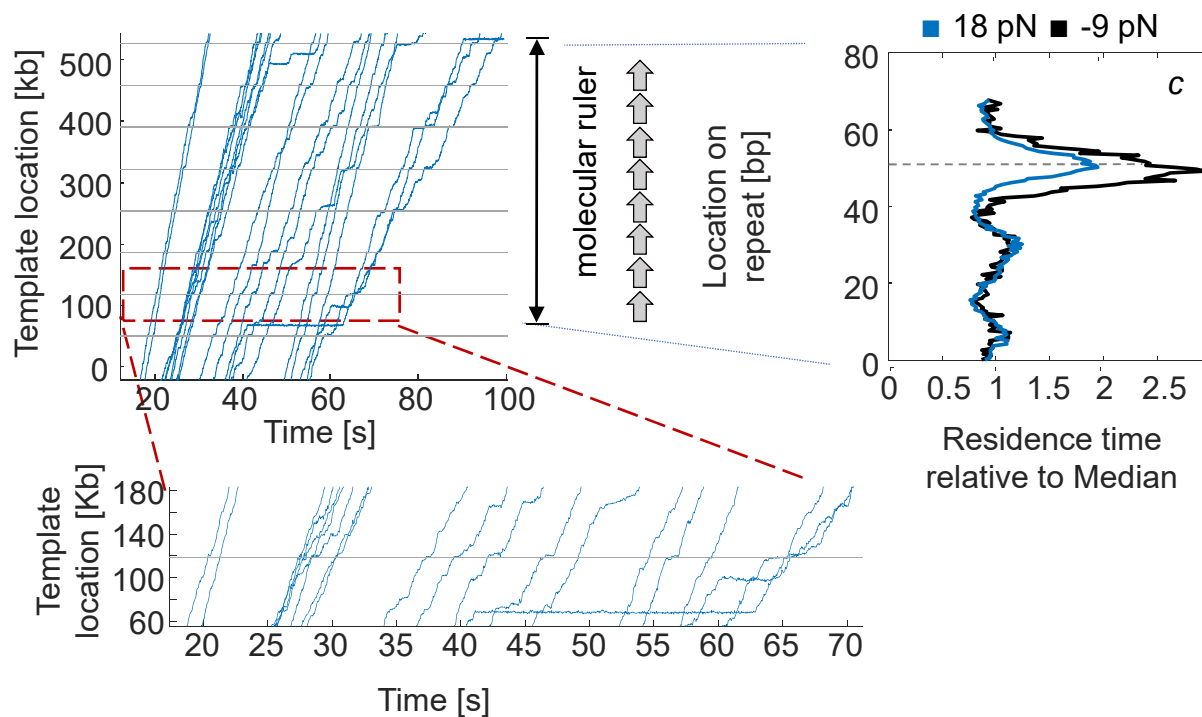
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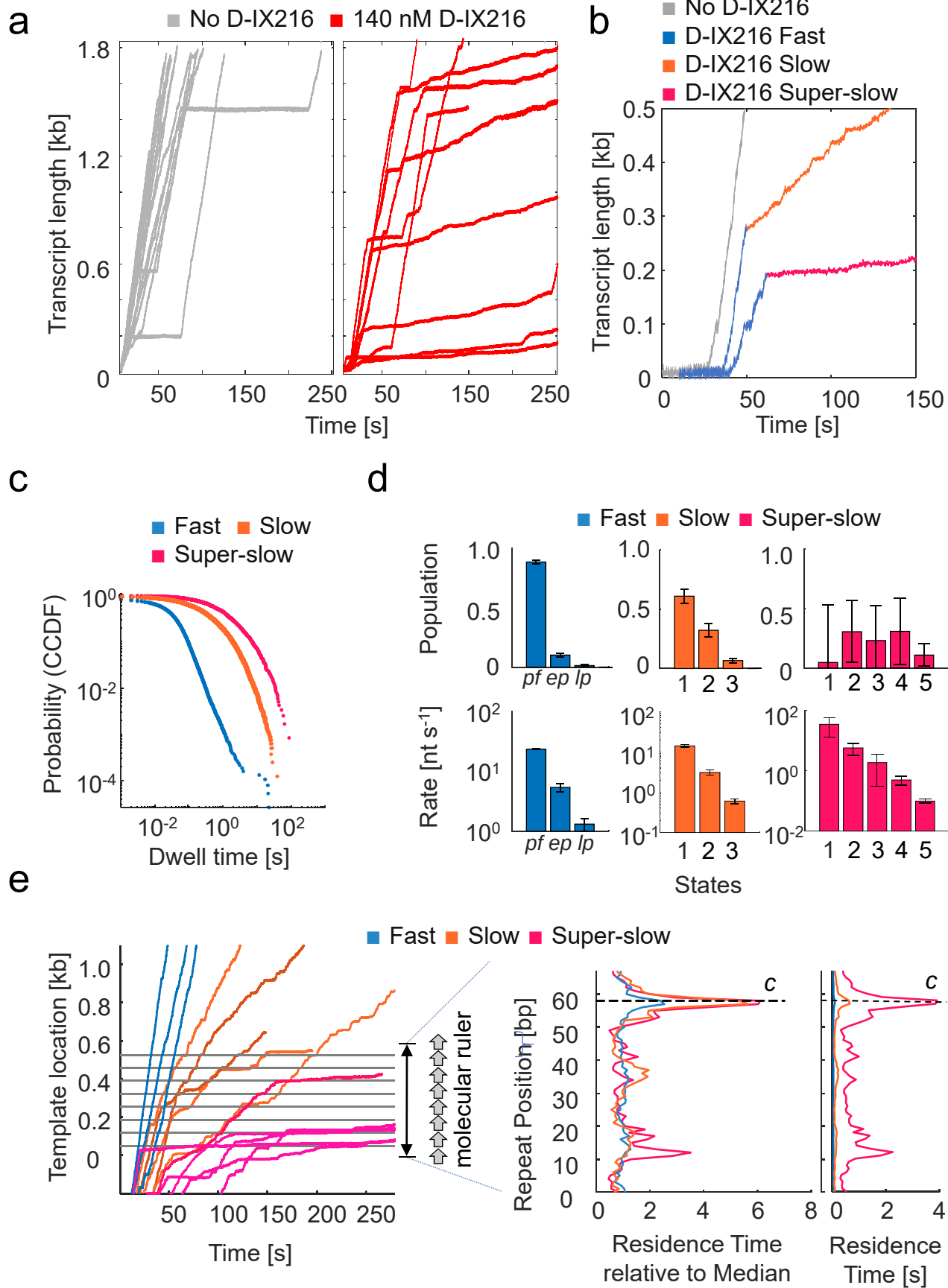


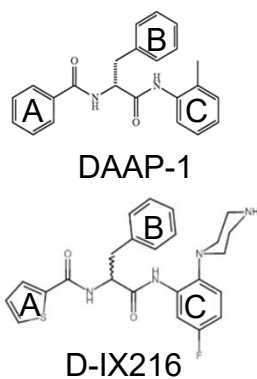
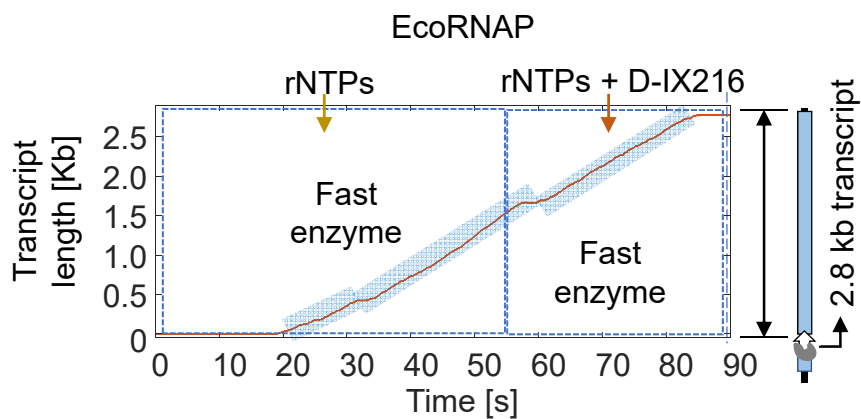
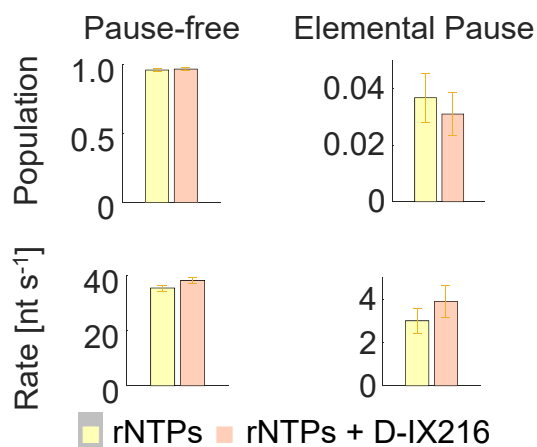
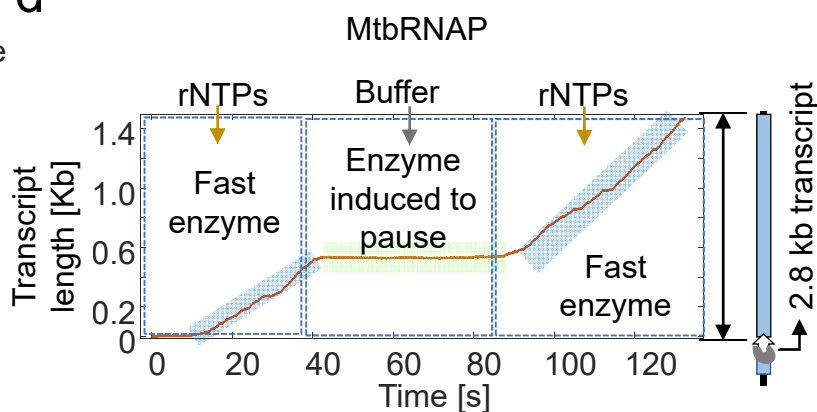
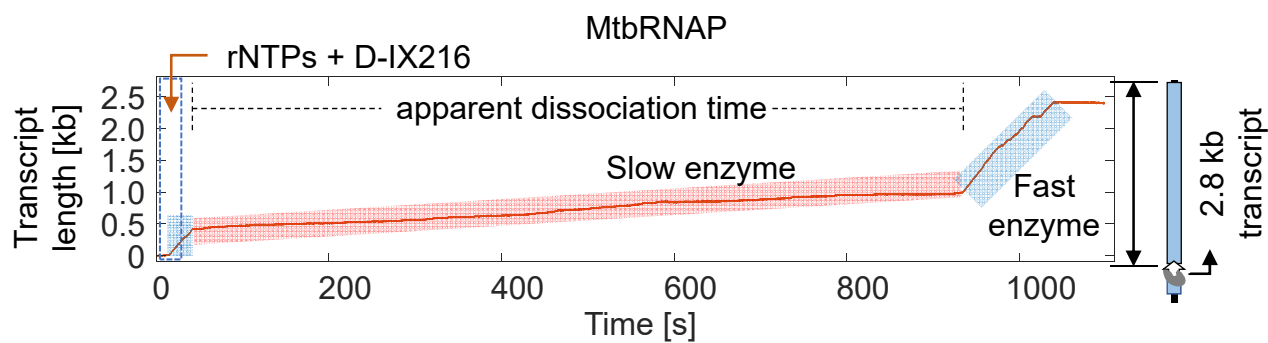
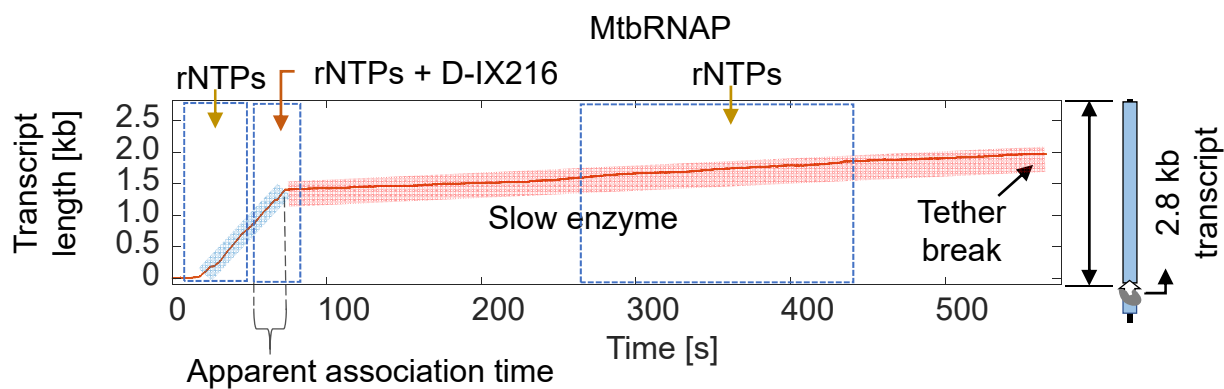
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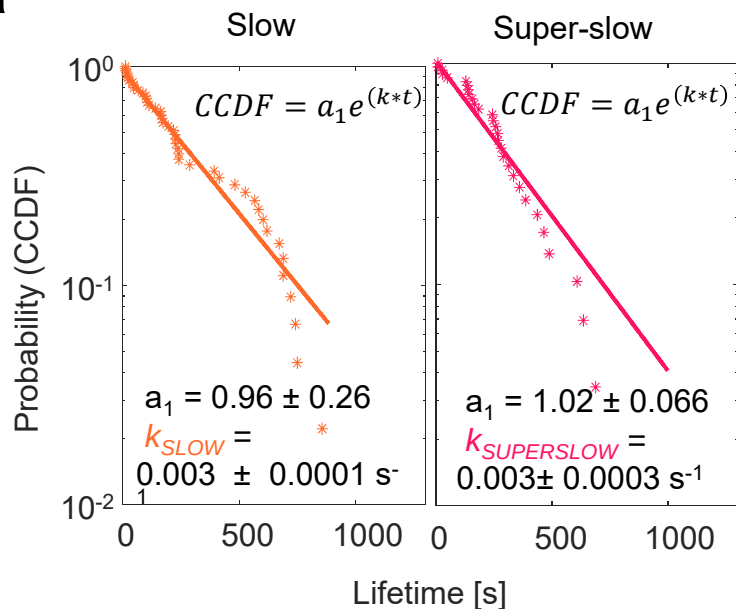
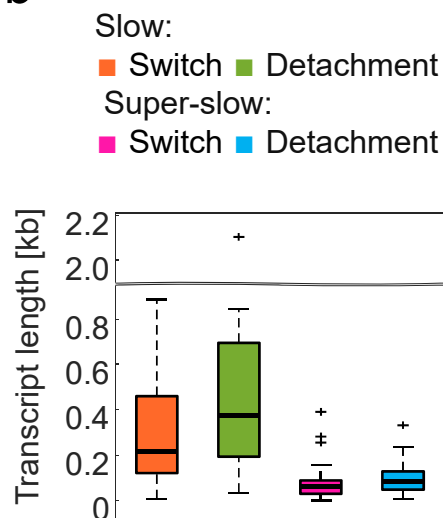
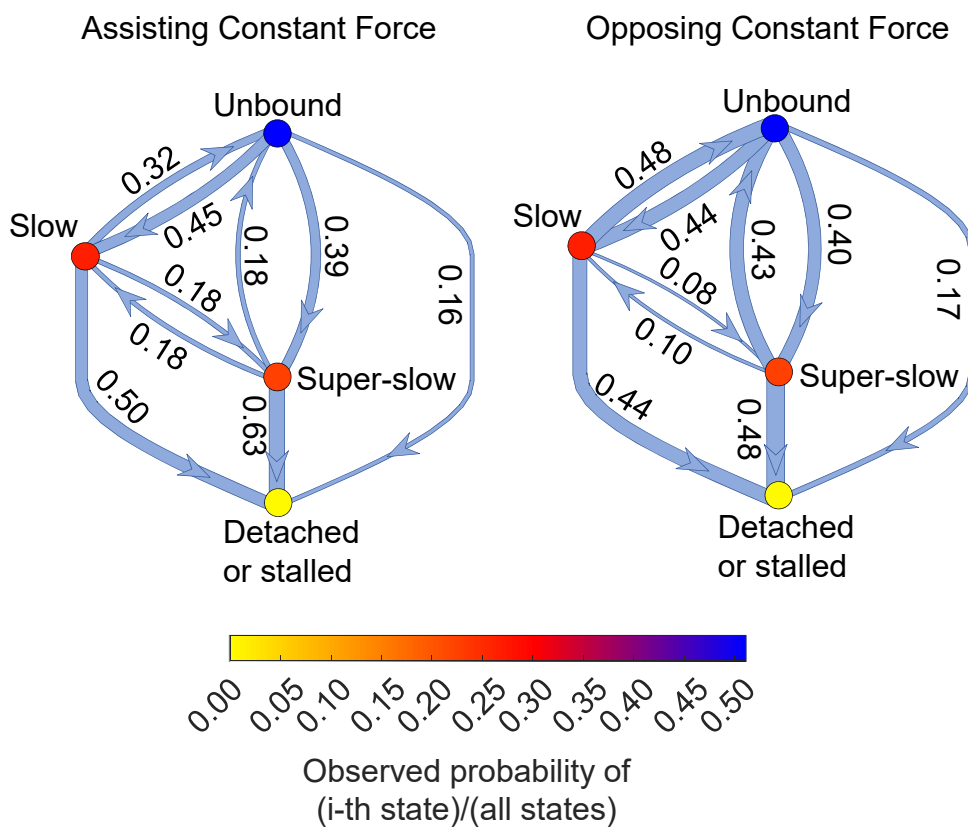


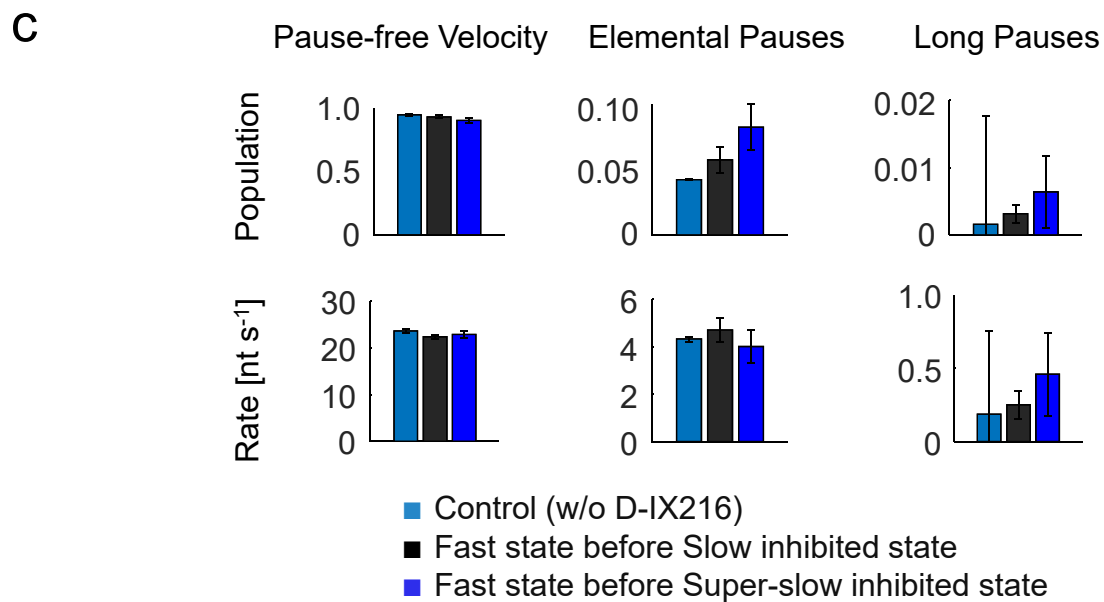
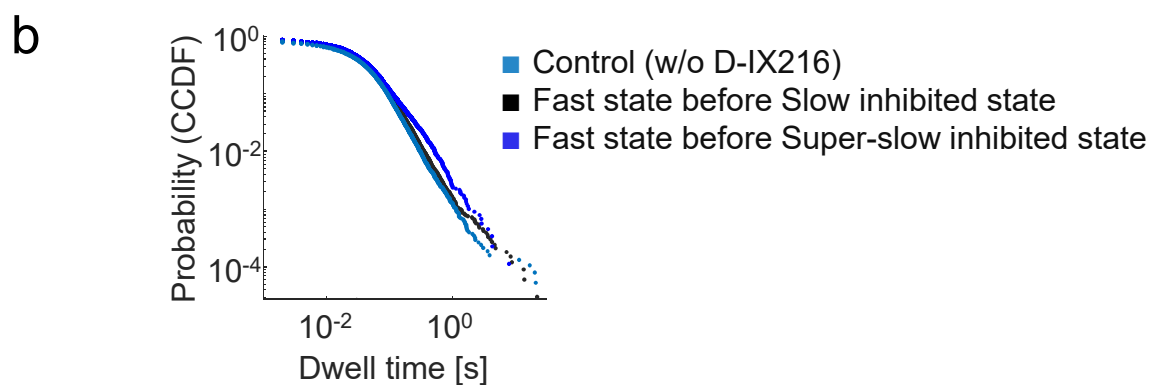
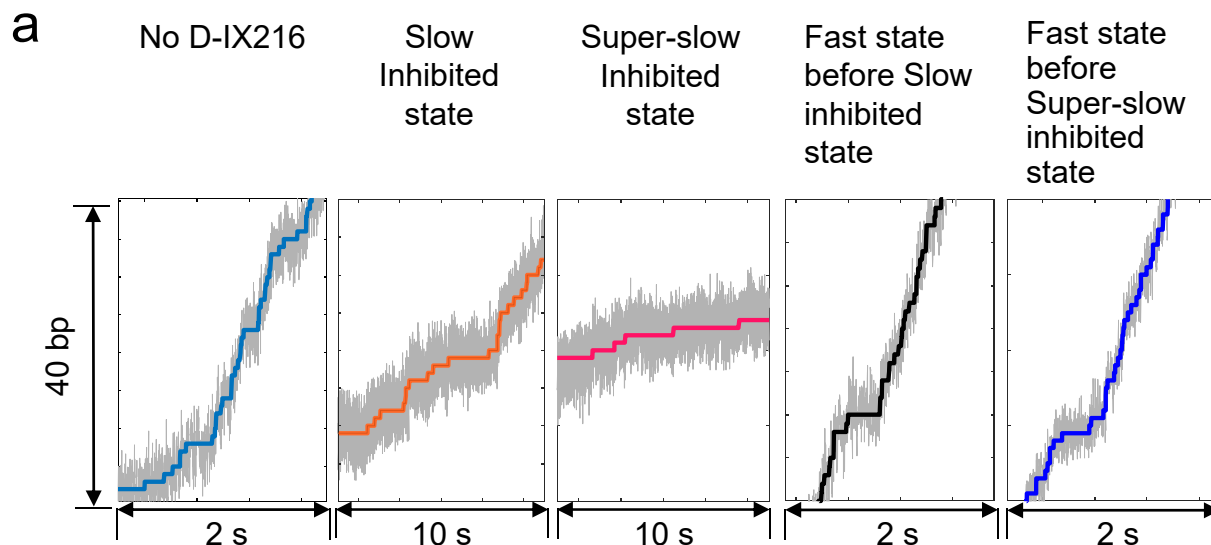
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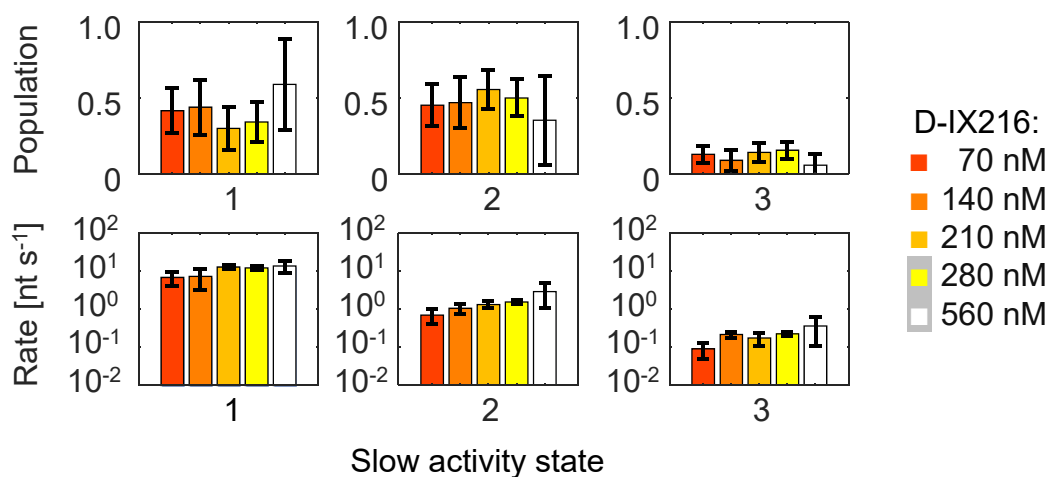


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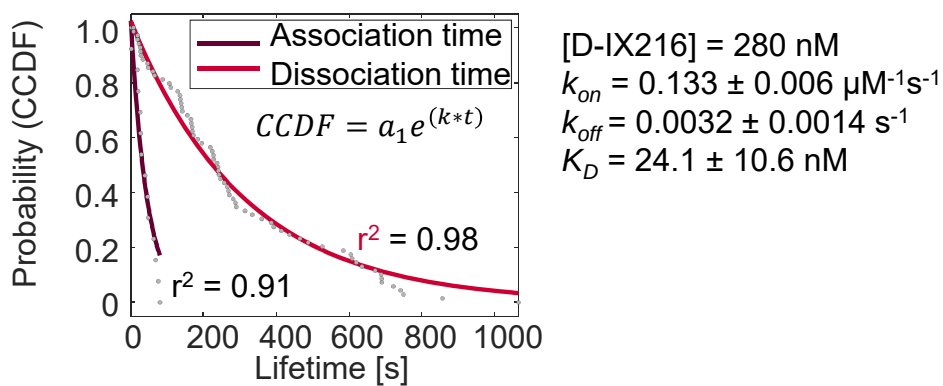
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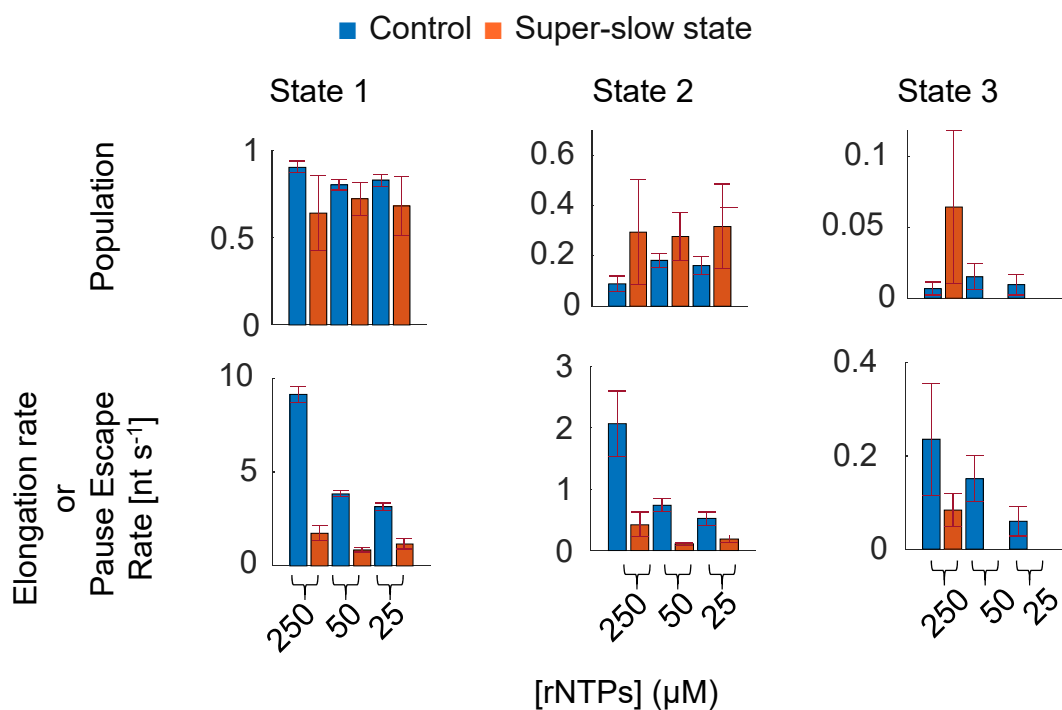
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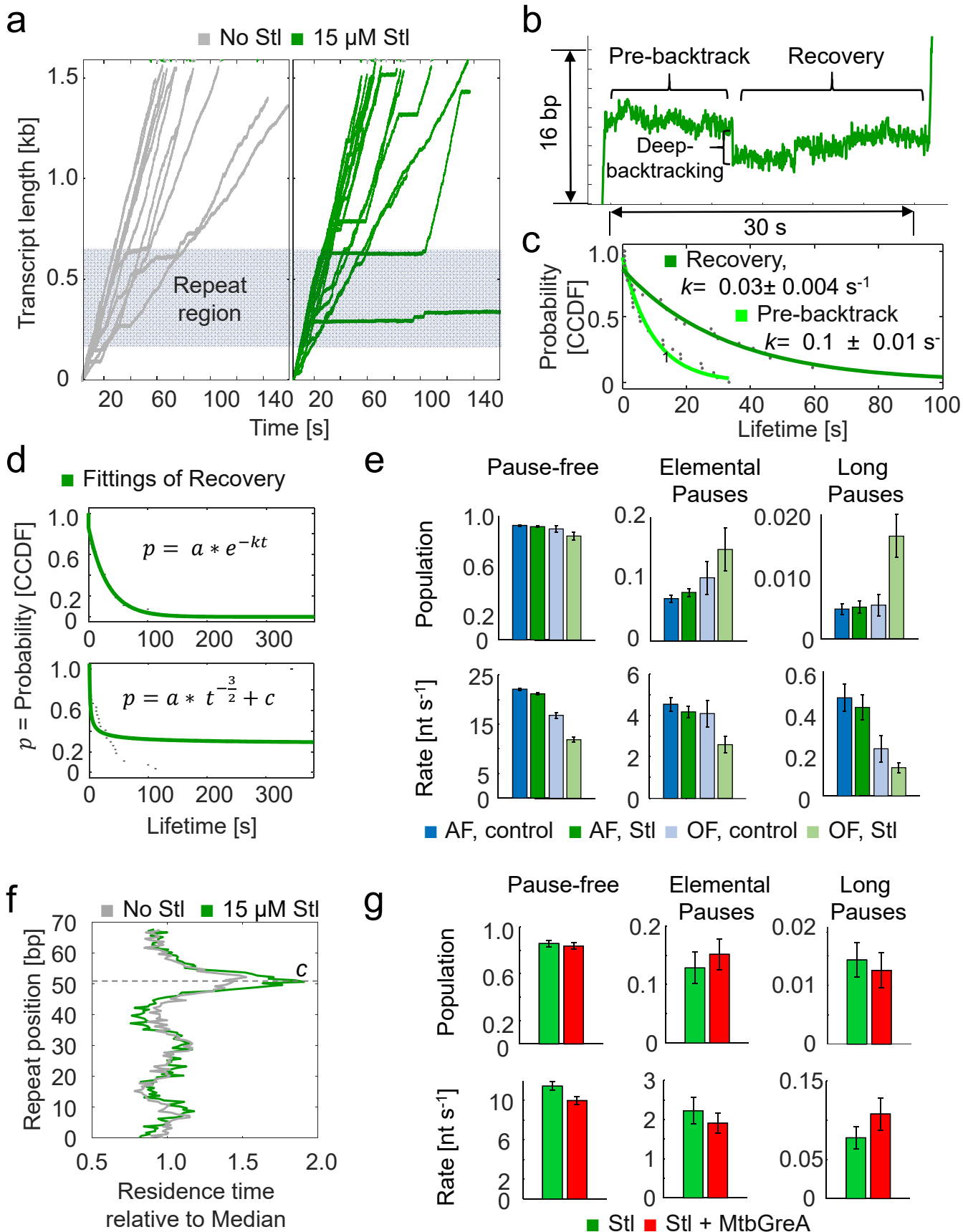


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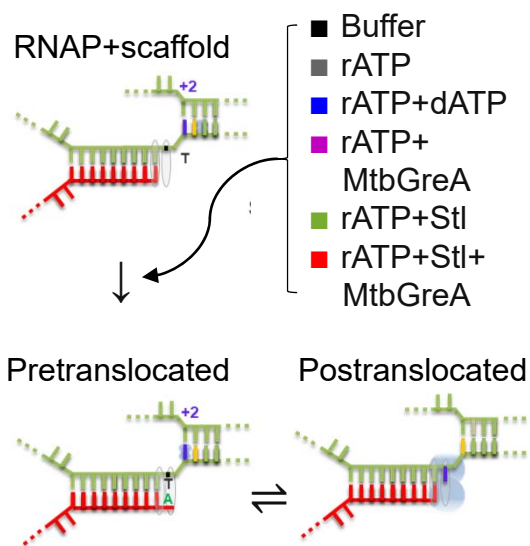


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