Mechanism of transcription modulation by the transcription-repair coupling factor

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Abstract

Elongation by RNA polymerase is extensively modulated by accessory factors. The transcription-repair coupling factor (TRCF) recognizes distressed RNAPs and either rescues transcription or initiates transcription termination. Precisely how TRCFs choose to execute either outcome remains unclear. With *Escherichia coli* as a model, we used single-molecule assays to study dynamic modulation of elongation by Mfd, the bacterial TRCF. We found that nucleotide-bound Mfd chaperones the elongation complex (EC) into a catalytically active state, presenting the EC with an opportunity to restart transcription. After long-lived residence in this catalytically poised state, ATP hydrolysis by Mfd remodels the EC through an irreversible process leading to loss of the RNA transcript. Further, electron cryo-microscopy revealed that the motor domain of Mfd binds and partially melts DNA containing a template strand overhang. The results explain pathway choice determining the fate of the elongation complex and provide a molecular mechanism for transcription modulation by TRCF.

Introduction

Gene expression involves synthesis of RNA transcripts by RNA polymerases across all domains of life. During elongation, RNA polymerase (RNAP) maintains a 9 – 10 nucleotide RNA:DNA hybrid within the transcription bubble through the templated addition of ribonucleotides (reviewed by (Belogurov and Artsimovitch, 2019)). Processive nucleotide addition cycles result in the growth of the RNA transcript that is then extruded from the polymerase through the exit channel, accompanied by translocation of the polymerase along the template. Finally, during termination, nucleotide addition ceases and RNAP is displaced from the template DNA and recycled for subsequent rounds of transcription.

Elongation is punctuated by sequence-dependent pausing, stalling at sites of damage on the DNA template or at sites of roadblocks in the form of DNA-bound proteins. Prolonged residence in paused states can lead to escape into backtracked states in which the catalytic site of RNAP loses register with the 3' end of the RNA transcript, allowing the newly synthesized transcript to invade the secondary channel of RNAP (Abdelkareem et al., 2019; Gabizon et al., 2018; Komissarova and Kashlev, 1997). Rescue from such backtracked states is catalyzed by processivity factors such as GreA and GreB (Abdelkareem et al., 2019; Borukhov et al., 1993). Elongation is modulated by the universally conserved elongation/pause factor, the bacterial NusG, homologous with Spt5 in eukaryotes (reviewed by (Wang and Artsimovitch, 2020)). In *Escherichia coli*, NusG maintains processive transcription (Burova et al., 1995) by suppressing backtracking through contacts on the upstream DNA (Herbert et al., 2010; Kang et al., 2018).

Elongation is also modulated by the transcription-repair coupling factor (TRCF) resulting in either transcription termination or rescue. The bacterial TRCF – Mfd is a multi-domain adaptor protein consisting of 7 domains connected by flexible linkers (Deaconescu et al., 2006). Domains 1a/2/1b form the UvrB–homology module that bears structural homology to the nucleotide excision repair protein UvrB and enables contacts with the repair protein UvrA. Domain 3 serves as a structural element playing a role in orienting the UvrB-homology module during the activation of the protein (Brugger et

al., 2020), following interactions of domain 4, the RNAP interaction domain, with distressed RNAP (Kang et al., 2021). Domains 5 and 6 together form the motor domain, representing a superfamily II ATPase that exhibits structural homology to the RecG translocase (Mahdi et al., 2003). Domain 7 is an auto-inhibitory domain that contacts the UvrB-homology module to maintain it in a closed conformation, suppressing promiscuous DNA binding of Mfd in the absence of interactions with RNAP.

Through molecular biology, biochemical (Chambers et al., 2003; Haines et al., 2014; Manelyte et al., 2010; Park et al., 2002; Selby and Sancar, 1993a, b; Smith et al., 2012; Smith et al., 2007), and structural methods (Brugger et al., 2020; Deaconescu et al., 2006; Kang et al., 2021; Shi et al., 2020), as well as single-molecule (Fan et al., 2016; Graves et al., 2015; Ho Wan et al., 2012; Le et al., 2017) and live-cell imaging techniques (Ghodke et al., 2020; Ho et al., 2018, 2020), a picture has been formed of the bacterial TRCF Mfd acting in a complex role in modulating transcription activity, including at hard-to-transcribe regions (Ragheb et al., 2021). At sites of DNA damage, Mfd orchestrates premature transcription termination leading to an enhancement in repair of DNA damage in the template strand – a phenomenon known as transcription-coupled DNA repair (Fan et al., 2016; Ghodke et al., 2020; Haines et al., 2014; Ho et al., 2020; Mellon and Hanawalt, 1989; Selby and Sancar, 1993a). In bacterial cells, the UvrA₂B nucleotide excision repair machinery is recruited to the site of distressed RNAPs by Mfd, resulting in the site-specific loading of the damage verification enzyme UvrB (Ho et al., 2020). This ability to recruit DNA-repair machinery at sites of lesion-stalled RNAP to promote ATP-dependent premature transcription termination is a property that is shared by eukaryotic functional homologs of Mfd – the yeast Rad26 (Duan et al., 2020; Xu et al., 2017) and human CSB (van der Weegen et al., 2020). Mutations in CSB protein result in deficient repair machinery recruitment causing Cockayne syndrome in humans (Mayne and Lehmann, 1982; Troelstra et al., 1992; Venema et al., 1990).

In addition to initiating transcription termination, the three best-studied TRCFs also serve as elongation factors. Like Rad26 (Xu et al., 2017) and CSB (Selby and Sancar, 1997), Mfd has also been shown to rescue transcription at pause sites *in vitro* through a ‘release-and-catch up’ mechanism (Le et al., 2017; Park et al., 2002). Forward translocation of the elongation complex (EC) is central to TRCF

activities including rescue from backtracked states, and transcription termination (Le et al., 2017; Park et al., 2002; Selby and Sancar, 1997; Xu et al., 2017). Considering their membership in the class of superfamily 2 helicases (Byrd and Raney, 2012), the structural homology of the TRCF motor domains and a similar mode of engagement with the elongation complex (EC) between Mfd (Brugger et al., 2020; Kang et al., 2021; Shi et al., 2020) and Rad26 (Xu et al., 2017), it is plausible that the molecular mechanisms underlying these outcomes are conserved.

However, precisely how TRCF activity results in transcription versus rescue is unknown. Two models have been proposed to explain forward translocation of RNAP by TRCFs: first, the TRCF ‘pushes’ RNAP forward while translocating on dsDNA as proposed for Mfd (Park et al., 2002), and second, the TRCF pulls the ssDNA template to effectively translocate the polymerase forward (Xu et al., 2017). Recent structures of *Ec*Mfd approaching an *in vitro* reconstituted EC assembled on a non-complementary bubble have demonstrated that following initial recognition of the EC, Mfd translocates forward until it bumps into the RNAP, pushing the polymerase and resulting in global remodeling of the EC (Kang et al., 2021). The structure of yeast Rad26 bound to upstream DNA in the RNA Pol II EC (Xu et al., 2017) reveals direct contacts between dsDNA-bound Rad26 and ssDNA template strand. Based on the homology to the Swi2/Snf2-family core ATPase domains, and the mechanism of Snf2 in chromatin remodeling, it has been proposed that Rad26 pulls template DNA in the Pol II EC (Xu et al., 2017). Structures of DNA-bound Mfd have established the homology of its motor domains to ATPases involved in chromatin remodeling (most notably Snf2) (Brugger et al., 2020). Since available structures depict initial global remodeling of the EC but not transcription termination leading to disassembly of the EC accompanied by loss of the transcript (Kang et al., 2021), it remains to be seen whether a similar Mfd-ssDNA complex forms during transcription termination. Further, while it is clear that displacement of the polymerase in the absence of downstream binding partners of Mfd is a slow event requiring several minutes *in vitro* (Graves et al., 2015; Ho Wan et al., 2012), the sequence of events leading to loss of the transcript, and its residence time in the EC during this remodeling remains unclear.

Here, we set out to understand how Mfd dynamically remodels the EC by monitoring the fate of the RNA transcript under various scenarios. We developed a single-molecule Förster resonance energy transfer (smFRET)-based assay that directly visualizes dynamic changes in the distance between donor-labeled upstream DNA and acceptor-labelled RNA transcript forming the transcription bubble. We reconstituted catalytically active ECs *in situ* and monitored the FRET signal from surface-immobilized molecules under conditions of partial or complete nucleotide starvation, and probed global remodeling of the EC by NusG and Mfd on the timescale of minutes. NusG efficiently rescued ECs from catalytically inactive states consistent with its role as an elongation factor. On the other hand, Mfd was able to rescue ECs from catalytically inactive states into a long-lived catalytically poised state, prior to continued ATP-hydrolysis dependent forward translocation consistent with transcription termination. This catalytically poised state acts as a ‘molecular timer’, presenting the EC with an opportunity to restart transcription. Further biophysical and structural biology studies collectively reveal that in addition to the previously described dsDNA binding mode, Mfd makes contacts with ssDNA on DNA substrates mimicking the transcription bubble.

Results

Rationale for a smFRET assay to study transcription elongation

We monitored transcription elongation by assembling functional ECs on dye-labeled fully-complementary transcription bubbles *in situ* by adopting a previously described strategy to study RNA Pol II dynamics (Andrecka et al., 2008) in combination with well-established approaches to reconstitute functional *E. coli* ECs (Komissarova et al., 2003). Specifically, we introduced a Cy3 donor dye on the upstream DNA and a Cy5 acceptor on the RNA (Figure 1A) at sites that are predicted to exhibit high smFRET signals within the dynamic range of the technique. Three features of this strategy enable precise dissection of the dynamics of molecular mechanisms of Mfd-dependent remodeling of the EC: (i) the use of synthetic RNA and DNA nucleotides enables site-specific introduction of dyes in the phosphodiester backbone that serve as FRET pairs, and along with it, the ability to probe conformational changes between various parts of this complex (ii) smFRET affords sub-second

temporal resolution (200 ms) enabling reliable measurements of dynamic interactions on the timescale of seconds to minutes, and (iii) the ability to specify RNA sequences that cannot hybridize with upstream DNA limits extensive backtracking of ECs. RNAP in the elongation phase can exist as a backtracked complex (where the 3' end of the transcript is extruded into the secondary channel (Abdelkareem et al., 2019)), a pre-translocated conformation prior to nucleic acid addition, and a post-translocated conformation following nucleic acid incorporation. Since backtracking requires hybridization of the RNA transcript with the upstream DNA, the choice of a non-complementary RNA sequence is expected to minimize the heterogeneity in the observed population of ECs in this assay.

Monitoring transcription elongation by RNAP

Functional RNAP ECs were assembled *in situ* by flowing a solution containing RNAP that had been pre-incubated with the template:RNA hybrid into a microfluidic device with surface immobilized biotinylated non-template DNA. After complex assembly, the flow cell was washed with a high-ionic strength solution (1M KCl) followed by rapid exchange with a buffer solution supporting transcription but without ribonucleotides (NTPs). Subsequent measurements of the smFRET signal between the Cy3 donor and the Cy5 acceptor (see Methods) reveals complexes assembled resided in stable high FRET states centered at 0.9 (Figure 1B upper trace). In addition, a small fraction (15% of 318 trajectories) also exhibited mid-FRET states centered at 0.6 (Figure 1B lower trace). These states were recapitulated in temporal heatmaps (Figure 1C) that are scatterplots describing how the smFRET signal evolves in time for the observed population. These temporal heat maps are a convenient representation presenting an overview of the progress of FRET trajectories over time. Here, the overlaid black lines reflect contours parsing the data into deciles.

Since the majority of the complexes thus assembled exhibited time-FRET trajectories that fluctuate around 0.9 FRET, and a minority exhibited transitions to the 0.6 FRET state, we pursued an unbiased approach to visualize transitions between various FRET states. FRET trajectories were idealized using vbFRET (<http://vbfret.sourceforge.net/>) and the detected states were plotted in transition density plots (TDPs; Supplemental Figure 1A, 'RNAP alone') to visualize interconversion

among different states and the directionality of those changes (Bronson et al., 2009). In this representation, features that are only present in one half of the plot on either side of the diagonal manifest as asymmetric features and denote irreversible transitions between states on the timescale of the observation. As demonstrated by the TDP, the interconversions between the states were reversible, and were restricted to the high FRET regime. Together, the observed symmetry of these peaks and their proximity to the diagonal, and to each other indicate that the complex thus reconstituted transitions between states reversibly. Within the paradigm that FRET changes reflect conformational changes, a simple interpretation of these data is that the complex undergoes relatively minor, but reversible, conformational changes. For convenience, here we assign smFRET signals between 0.7 – 1 as being ‘high’, 0.4 – 0.7 as ‘mid’ and 0 – 0.4 as being ‘low’.

To assign states, and to test that the complexes are catalytically functional, we challenged complexes with deoxyNTPs (dNTPs), ‘wrong’ (or non-complementary) NTPs and the complete set of NTPs. In this assay, transcript elongation is expected to increase the distance between the FRET pair dictated by the geometry of the EC, leading to a drop in FRET. Since dNTPs are selected against incorporation into the transcript (Svetlov et al., 2004), we expected that smFRET signal from the reconstituted ECs would not drop. Consistent with this expectation, complexes exposed to dNTPs revealed a high-FRET population (Figure 1D, 1E). In contrast, non-complementary NTPs converted the population of complexes in the high-FRET states to the mid-FRET states (Figure 1F, G). In both cases, the interconverting populations were found to be symmetric across the diagonal indicating that in the presence of either dNTPs or the wrong NTPs, the ternary complex visits the observed states reversibly (Supplemental Figure 1A).

‘Walking’ the complex by supplying a subset of the ‘correct’ complementary NTPs revealed gradual conversion from the high-FRET to a mid-FRET population (Supplemental Figure 1B–D). When presented with the complete set of NTPs, the ternary complex exhibited unidirectional conversion of the FRET signal from high- to low-FRET values (Figure 1H, 1I and Supplemental Figure 1A). Notably, the transition density plots, and indeed the individual traces, revealed a conversion of the high-FRET

states to the low-FRET states *via* the mid-FRET states.

Collectively, the data indicate that the complexes assembled *in situ* represent functional ternary ECs that exist in a catalytically ‘inactive’ state and a catalytically ‘poised’ state. The observations that complexes first transition to the mid-FRET state (0.6) prior upon addition of NTPs suggests that this represents a catalytically poised state. We tentatively assigned the high-FRET states centered at 0.9 to a catalytically inactive state. This catalytically inactive state may represent a pre-translocated or backtracked conformation of the EC. Considering that the sequence of the 5’ end of the transcript is non-complementary to the upstream DNA, we expect that extensive backtracking is disfavored on this substrate. We propose that the likeliest explanation is that the high- and mid- FRET states detected here represent pre- and catalytically poised post-translocated states accessed by the EC during the nucleotide addition cycle.

NusG promotes residence of ECs in the catalytically poised state

Next, we tested this assignment of states by assessing the response of the complex to the elongation factor – *EcNusG* (Figure 2A), which is predicted to retain ECs in the catalytically poised state. Addition of NusG indeed resulted in an increased occupancy of mid-FRET states accompanied by a reduction of the high-FRET (Figures 1B, 1C *cf.* 2B, 2C). Trajectories revealed a conversion from the high-FRET state into the mid-FRET state and fluctuation around the mid-FRET state (Figure 2B). This behavior confirms our assignment of the mid-FRET state as the catalytically active state. The reverse transition from mid-FRET to high-FRET states that would reflect unbinding of NusG appears to be disfavored in this experimental condition as the detected asymmetric features in the transition density plot are unidirectional (Supplemental Figure 2A). These trajectories are readily explained if binding of NusG restricts conformational rearrangements of the EC.

Incubation with incorrect NTPs in the presence of NusG revealed enrichment of molecules exhibiting the 0.6 FRET state (Figure 2E, 2F), similar to that observed in the case of incorrect NTPs alone (Figure 1F, 1G). Reversible short-lived transitions to low-FRET states were also evident in this data set (Figure 2D, 2E and Supplemental Figure 2A). These transitions may reflect changes in the path

of the upstream DNA that have been detected previously (Kang et al., 2018). Importantly, the complete set of NTPs revealed efficient, irreversible conversion to the low-FRET states (0 – 0.2) from the mid-FRET state (Figure 2F, 2G and Supplemental Figure 2A) in the presence of NusG suggesting processive extension of the transcript through catalysis and associated forward translocation by RNAP.

Together, these observations are consistent with the role of *Ec*NusG as a suppressor of backtracking of RNAP (Burova et al., 1995; Herbert et al., 2010; Kang et al., 2018). Overall, these results indicate that *Ec*NusG promotes residence of the complex in a catalytically poised conformation. The loss of high-FRET states upon binding of NusG supports the interpretation that the high-FRET states represent inactive states.

Remodeling of the elongation complex by Mfd

With the assignment of the high-FRET state as a catalytically inactive state, and the mid-FRET state as a catalytically poised state, we next assessed the interaction of Mfd with ECs. To characterize the binding of Mfd to RNAP, we first measured the affinity of Mfd for pre-assembled ECs, or the dsDNA alone using surface plasmon resonance (SPR). Since Mfd interacts poorly with DNA in bulk assays in the absence or presence of ATP, measurements of binding of purified Mfd to either DNA or the EC were carried out in the presence of slowly hydrolyzable ATP γ S. SPR measurements revealed a dissociation constant (K_D) of 170 ± 20 nM (Supplemental Figure 2B) for 49-mer dsDNA alone, consistent with recent measurements by other groups (Brugger et al., 2020; Le et al., 2017). Binding to reconstituted RNAP ECs on a 49-mer exhibited similar affinity with a K_D of 127 ± 6 nM (Supplemental Figure 2C). These data are consistent with the model that dsDNA serves as a major binding partner for Mfd-ATP γ S in the context of the EC as previously demonstrated (Kang et al., 2021; Le et al., 2017; Park et al., 2002; Shi et al., 2020).

We then investigated how interactions of Mfd with RNAP influence the conformational dynamics of the transcription bubble using our smFRET assay (Figure 3A). Consistent with the lack of stable binding of Mfd with DNA in the absence of ATP, Mfd engaged the EC poorly in the smFRET measurements with 58% of trajectories ($n_{\text{obs}} = 75$) presenting no observable interactions with Mfd

(Figure 3B lower panel). The remaining trajectories exhibited reversible transitions from the high- to mid-FRET states even in the absence of any nucleotide co-factor (Figure 3B upper panel, 3C and Supplemental Figure 3A 'RNAP, Mfd') reflecting attempts at stable engagement of Mfd with the EC. Notably, differently from the EC alone (Supplemental Figure 1A), dynamic trajectories exhibited transitions mainly in the mid-FRET region (Figure 3B upper panel, Supplemental Figure 3A 'RNAP, Mfd').

Mfd-ATP γ S resulted in dynamic ECs visiting additional mid FRET states (Figure 3D, 3E and Supplemental Figure 3A 'RNAP, Mfd, ATP γ S'; 59% of 69 trajectories presented dynamic behavior). ATP γ S alone did not account for these observations (Supplemental Figure 3B). Curiously, Mfd-ATP γ S exhibits quantized transitions in the transition density plot compared to Mfd alone, indicating that binding of Mfd-ATP γ S displaces RNAP forward (Supplemental Figure 3A 'RNAP, Mfd' vs. 'RNAP, Mfd, ATP γ S'). Together the data reveal that Mfd can isomerize RNAP merely upon binding to the nucleotide starved EC. The efficiency of this isomerization is appreciably greater in the presence of ATP γ S.

Treatment of *in situ* assembled ECs with Mfd and ATP revealed four distinct types of trajectories in the data set: (i) static trajectories that exhibited high FRET values throughout the observation reflecting molecules that are presumably unaffected by Mfd-ATP (54/176 observations; 31%), (ii) dynamic trajectories exhibiting high FRET values at the start of the observation resulting in a direct transition to low-FRET states (24/176 trajectories, 14%; Figure 3F lower panel), (iii) dynamic trajectories starting at a high-FRET value but transiting to a low-FRET state through a single mid-FRET intermediate (56/176 trajectories; 32%, Figure 3F upper panel) and (iv) dynamic trajectories that are at the mid-FRET state at the start of the observation (42/176 corresponding to 24%; compared to 14% of observations in the absence of Mfd). Since the dataset presented here represents a pooled data set from several videos collected after Mfd-ATP was introduced to the flow chamber, this last category of trajectories likely reflects ECs that are already being remodeled by Mfd at the start of the observation.

We characterized the trajectories describing efficient and stepwise conversion of the population of ECs from the high- to the mid-FRET state, with further conversion to low-FRET states for

individual ECs (Figure 3F, upper panel). The intermediate step was randomly distributed in the range of FRET from 0.35 to 0.5 (Supplemental Figure 3C). Strikingly, the lifetime of this mid-FRET state did not exhibit a dependence on the concentration of ATP in the range from 10 μ M to 10 mM (Supplemental Figure 3D). To further explore the nature of these transitions, we visualized them in a transition density plot (Supplemental Figure 3A, 'RNAP, Mfd, ATP'; entire data set is plotted here). Notably, irreversible transitions manifesting as asymmetric features in these plots appeared mainly as transitions from the mid-FRET to low-FRET states (Supplemental Figure 3A, 'RNAP, Mfd, ATP', red ellipse). As a control, titration of ATP in the absence of Mfd resulted in RNAP complexes exhibiting primarily a high-FRET population, with a gradual increase in the mid-FRET state at millimolar concentrations (Supplemental Figure 3E). Compared to the wild-type, the Mfd(R953A) mutant (Chambers et al., 2003), known to be proficient in ATP hydrolysis while strikingly deficient in RNAP displacement and transcription termination exhibited inefficient conversion to the low-FRET states (Figure 3H, 3I and Supplemental Figure 3A 'RNAP, Mfd(R953A), ATP'). Notably, unlike wild-type Mfd, irreversible transitions were observed mainly in the high- to mid- FRET regime (Supplemental Figure 3A 'RNAP, Mfd(R953A), ATP', red ellipse). Intriguingly, Mfd(R953A)-ATP exhibited a pattern of quantized transitions that is similar to that of Mfd-ATP γ S in the transition density plots (Supplemental Figure 3A, compare 'RNAP, Mfd, ATP γ S' and 'RNAP, Mfd(R953A), ATP'), suggesting that the mutation in this residue serves to reveal distinct aspects of Mfd's binding to the EC.

Together, the data indicate that both wild-type and translocase deficient Mfd rescue RNAP from the catalytically inactive (potentially pre-translocated or weakly backtracked) states accessible in our substrate to catalytically-poised states, albeit with varying efficiencies. However, only the wild-type Mfd can further process the EC in the presence of ATP. The low-FRET states observed in our system represent circumstances in which the distance between the donor-acceptor pair is outside the dynamic range of efficient FRET as might be expected when the RNAP is forward translocated well past the stall site and/or the Cy5-labelled RNA transcript is lost. The observation that the EC fails to return to the mid- or high-FRET states rules out a scenario where this low-FRET state simply reflects

an over-wound transcription bubble, or a bent upstream DNA conformation as is seen in the case of NusG (Kang et al., 2018). Hence, this loss of FRET is most consistent with an irreversible loss of RNA. In this model, Mfd(R953A) fails to displace the transcript from the EC, consistent with prior findings (Chambers et al., 2003).

Mfd contacts template ssDNA

Structures of Mfd bound to dsDNA and ECs containing non-complementary transcription bubbles have revealed a series of translocation intermediates that are formed prior to and leading up to stable engagement of Mfd and initial disruption of the EC (Kang et al., 2021; Shi et al., 2020). Currently, little is known about the intermediate states that follow this initial contact. We set out to identify whether Mfd makes contacts with ssDNA in substrates mimicking the upstream edge of the transcription bubble by employing a combination of approaches: First, we employed smFRET experiments to detect whether and how Mfd contacts template ssDNA. Second, we visualized Mfd bound to a primed DNA template using cryo-EM.

To detect ATP-dependent contacts of Mfd with ssDNA we employed a previously described smFRET assay to study translocase activity (Park et al., 2010). We used a DNA substrate comprised of an 18-mer dsDNA duplex (mimicking upstream DNA) and a 5' dT₄₀ overhang mimicking the template DNA (Figure 4A) with a FRET pair positioned on either end of the ssDNA overhang (Table 1: bio_AS18_Cy5: Cy3_dT40_S18, see Materials and Methods). In smFRET experiments, this substrate exhibited an average FRET of 0.33 ± 0.15 (Figure 4B) in the absence of Mfd reflecting rapid sampling of various conformations accessed by the entropic coil formed by ssDNA (Figure 4C and Supplemental Figure 4A 'template overhang DNA'). Mfd binds this substrate with a K_D of 390 ± 10 nM (see Supplemental Information and Supplemental Figure 4B–D).

In the presence of ATP γ S, the smFRET signal from the Mfd-bound substrate revealed additional distinct populations at higher FRET values of 0.6 and 0.8 (Figure 4D, 4E and Supplemental Figure 4A 'Mfd, ATP γ S'). Transitions among these states were further enhanced in the presence of ATP as the substrate exhibited a continuous spectrum of mid- and high-FRET states (Figure 4F, 4G and

Supplemental Figure 4A ‘Mfd, ATP’) in addition to the distinct states detected in the presence of ATP γ S. In this case, the higher FRET states were only transiently accessed indicating that ATP hydrolysis plays a role in exiting from these high-FRET states to low-FRET states. This observation is consistent with a picture in which Mfd-ATP contacts the ssDNA to bring the dyes close to each other resulting in a high FRET signal, and subsequent ATP hydrolysis induces a lower-affinity conformation in which Mfd-ADP binds the ssDNA very weakly resulting in a return to the baseline FRET value of 0.33.

In the presence of the ATPase-deficient but DNA-binding competent E730Q mutant of Mfd (Deaconescu et al., 2012; Ho et al., 2018), this template exhibited high-FRET states extremely rarely compared to the wild-type protein (Supplemental Figure 4F, 4G). Therefore, the observation that Mfd(E730Q)-ATP is unable to stably engage with ssDNA, unlike Mfd-ATP γ S suggests that the Mfd(E730Q)-ATP and Mfd-ATP γ S pre-hydrolytic states are sufficiently different that only the wild-type Mfd retains the ability to engage ssDNA efficiently.

The RNAP-displacement deficient R953A mutant (Chambers et al., 2003) did not exhibit high FRET states in the presence of ATP, indicating that the ability to hydrolyze ATP alone is insufficient to engage ssDNA on primed DNA templates (Figure 4H, 4I). Therefore, we conclude that both the ability to hydrolyze bound ATP and the presence of an intact R953 are essential for Mfd to engage the ssDNA template DNA overhang. We propose that R953 serves to ‘hook’ ssDNA, and ATP binding serves to ‘pull’ ssDNA closer to the junction resulting in the high FRET signal detected in this assay with wild-type Mfd. We note that the recent structures of Mfd translocating on dsDNA indicate that R953 along with key residues in the translocation motif (TRG: translocation in RecG (Mahdi et al., 2003)) of Mfd, specifically R929 and Q963, contacts the non-template strand in dsDNA. From these structures it is not immediately obvious how the template strand overhang is contacted by Mfd in the smFRET assays. The results presented here uncover a new role for R953 in making critical contacts with the template ssDNA distinct from its previously described role in translocation on dsDNA.

Intriguingly, each of the three Mfd variants also exhibited a distinct sub-population of molecules centered at ~0.1 FRET. Here, whereas ATP γ S promoted the formation of this state almost

immediately, Mfd (or mutants) in the presence of ATP entered this state in a time window ~5-20 seconds after introduction into the flow cell (Figure 4E, 4G, 4I and Supplemental Figure 4H). The presence of the low-FRET peak and the excursions to high-FRET states suggested that multiple independent events may underlie the FRET signal observed here. Whereas all conditions involving Mfd (or mutant) exhibited the low-FRET peak, the excursions to the high FRET states were only observed for wild type Mfd in the presence of ATP or non-hydrolyzable analog.

Mfd melts dsDNA upon contacting template ssDNA

To further understand how Mfd binds to 5' overhang-containing DNA, we visualized Mfd bound to the dye labelled smFRET substrate by cryo-EM (Table 1: bio_AS18_Cy5: Cy3_dT40_S18, see Materials and Methods). Full-length Mfd bound to the primed-template substrate in the presence of transition-state analog ADP-AlF_x was subjected to single particle cryo-EM analysis (Figure 5A) and a ~5.2 Å resolution map was obtained (real resolution ~8 Å, Supplemental Figure 5A–C). This reconstruction yielded electron density that could at best accommodate the motor domains of Mfd (residues 575–956 representing domains 5 and 6); other domains are presumably flexible and could not be seen in the electron density maps.

The cryo-EM map reconstructed here suggests that the dsDNA on the upstream side of the motor domains and the placement of the motor domains with respect to dsDNA is consistent with previously reported complexes (Brugger et al., 2020; Kang et al., 2021) (Figure 5B). However, density for residues 917 onward, containing the TRG motif, appeared to be absent from the side of domains 5 and 6, and the density of the DNA as it passes from the upstream side to the downstream side becomes inconsistent with dsDNA (Figure 5B). Using molecular dynamics flexible fitting (Singharoy et al., 2016) to equilibrate the structure in density suggests that the TRG motif has dropped into the path of the dsDNA (yellow ribbon, Figure 5C), resulting in the partial melting of the duplex. The motif forms an arch that interacts with the template DNA (Figure 5C, 5D). There are precedents for conformational plasticity in the TRG motif: the pair of helices forming the bulk of the TRG motif are almost orthogonal in the apo-structure of Mfd (PDB ID 2EYQ) with the sulfate group from 4-(2-hydroxyethyl)-1-piperazine

ethanesulfonic acid bound between the helices, and are parallel in the complex with ADP- AlF_x and dsDNA (PDB 6XEO). These movements are attributable to both to shifts in domains 5 and 6 relative to each other and to movements of the helices relative to each other. The conformation of domains 5 and 6 suggested here may represent a mode of engagement whereby Mfd grabs and stabilizes the template DNA in the upstream edge of the transcription bubble.

The model suggests that Mfd partially melts about five nucleotides of the dsDNA near the junction forming a 'TRCF bubble' (Figure 5E). Residues R953 and R929 line the path of the template ssDNA three and six nucleotides away from the junction respectively (Figure 5C, 5F). ADP- AlF_x -bound Mfd scrunches the template ssDNA (Figure 5D–F), explaining the basis of the high-FRET signal observed in the smFRET experiments (Figure 4). The proximity of R953 to the ssDNA in the model also explains how R953 can hook the template ssDNA in an ATP-dependent manner (Figure 5C, 5F) and in case of the distressed EC, transform the energy of ATP binding and hydrolysis into forward translocation of RNAP.

Several highly conserved basic residues (H842, R846, R848, H890, H897, R900; Figure 5E, residues indicated as blue spheres) line the path of the template ssDNA along with absolutely conserved polar (N817, T868) (Figure 5G, residues indicated as yellow spheres) and aromatic residues (Y816, F891) (Figure 5G, residues indicated as green spheres) capable of base stacking interactions or H-bonding interactions with ssDNA (Figure 5G, residues indicated as spheres). A cluster of highly conserved basic residues in domain 5 (R685, K690, H711, K712, H732, R733, R737, K739, R761) (Figure 5G, blue spheres) along with polar (T710) (Figure 5G, yellow spheres) and aromatic (F734) (Figure 5G, green spheres) residues comprise the surface in contact with the non-template DNA strand (Figure 5E). The binding mode proposed here is partially validated by prior observations implicating several key conserved residues in domain 6 (Y816, N817, H842, T868 and R887) as well as domain 5 (R685, K690, T710, H711 and K712) implicated in DNA binding (Brugger et al., 2020; Le et al., 2017). Consistent with this scenario, Mfd-ATP γ S binds a 49-mer dsDNA substrate containing a non-complementary bubble of 9 nucleotides with an affinity of 395 ± 7 nM as assessed by SPR (Supplemental Figure 5D).

Together, the findings describe how Mfd contacts DNA substrates mimicking the upstream edge of the transcription bubble: the cryo-EM data suggest that Mfd binds and partially melts the dsDNA near the junction, contacting the unpaired template ssDNA via the conserved R929 and R953 residues in the TRG motif, and the smFRET experiments reveal that Mfd dynamically cycles between high- and low-affinity states in an ATP-hydrolysis dependent manner on the template ssDNA overhang, pulling and scrunching the template strand in this process.

Discussion

In this work, we established that a ‘molecular timer’ intermediate formed by Mfd and the transcription elongation complex determines the fate of the elongation complex. Our attempts to understand this intermediate resulted in the discovery of a novel DNA-binding mode in which Mfd binds ssDNA in a transcription bubble mimic. Together, the results provide unprecedented insight into the transcription termination reaction and enable us to propose a comprehensive model describing how events unfold after initial engagement of the elongation complex with the transcription-repair coupling factor.

Interactions of Mfd with RNAP are complex. ATP-dependent remodeling of the EC by Mfd proceeds via multiple stages: In the first stage, Mfd recognizes a distressed polymerase and is recruited to the EC (Ho et al., 2018; Ho Wan et al., 2012; Le et al., 2017; Park et al., 2002; Proshkin and Mironov, 2016) through a conserved RNAP-interacting domain (Park et al., 2002) (Figure 6A). Mfd can recognize extensively backtracked (Le et al., 2017), reversibly paused as well as stalled complexes (Proshkin and Mironov, 2016). In the second stage, EC-tethered Mfd undergoes a series of concerted conformational changes (Smith et al., 2012) that enable it to translocate on upstream dsDNA and ‘load’ onto the EC (Kang et al., 2021; Shi et al., 2020) (Figure 6B). In the third stage, Mfd either reactivates paused transcription (Le et al., 2017; Park et al., 2002), or terminates transcription (Ho Wan et al., 2012; Selby and Sancar, 1993a). Alongside these activities, Mfd recruits its binding partners UvrA₂B to the site during transcription-coupled DNA repair (Fan et al., 2016; Ghodke et al., 2020; Ho et al., 2018, 2020; Selby and Sancar, 1993a).

Our studies shed light on hitherto mysterious aspects of Mfd-RNAP interactions central to this

reaction and present a new model for resolution of a critical intermediate. The smFRET-based elongation modulation assay revealed that Mfd can engage the EC in an ATP independent manner and manifest transient, reversible conformational changes in the EC. Stable association requires ATP or at minimum non-hydrolysable ATP, likely required to impart a dsDNA binding conformation in the motor domain. Indeed, a recent study reported the structural basis of ATP-dependent translocation of Mfd following initial recognition of distressed elongation complexes leading to docking of Mfd with the upstream edge of the transcription bubble (Kang et al., 2021). Our findings agree well with these findings (Figure 6 A – C).

Following a period of dynamic remodeling in which the EC transits from high- to mid-FRET states, Mfd-ATP, Mfd-ATP γ S and Mfd(R953A)-ATP each chaperone the EC into a single long-lived gating intermediate (Figures 3 and 6D) hovering in the mid-FRET range controlling the transition to low-FRET states. Efficient entry into this state is granted to Mfd in the presence of ATP or ATP γ S, whereas Mfd(R953A)-ATP enters this state only inefficiently. The observation that Mfd-ATP γ S is sufficient to promote entry into this state indicates that binding of ATP alone is a sufficient requirement for the formation of the gating intermediate.

The suggestion that Mfd-ADP-AlF₄ might bind and melt primed DNA templates to unwind dsDNA (Figure 5), provides insight into this transition. Only 13 nucleotides of the 18-mer DNA template are consistent with dsDNA, with the remainder being partially melted. The lack of observations of dsDNA melting in a recently reported structure (Brugger et al., 2020) suggests that Mfd requires access to unpaired ssDNA *via* critical residues in the TRG motif to melt the dsDNA.

We propose that in the context of the upstream dsDNA, ATP- or non-hydrolyzable analog bound - Mfd partially melts about five nucleotides of dsDNA to produce a bubble, henceforth referred to as the ‘TRCF bubble’ (Figure 5). Considering the three nucleotide proximity of the binding site of Mfd to the upstream edge of the transcription bubble (Kang et al., 2021), the upstream DNA may further melt allowing the TRCF bubble to fuse with the transcription bubble to produce the isomeric TRCF-transcription (‘TRCF-T’) bubble (Figure 6D). Since Mfd has not been reported to melt dsDNA

lacking an overhang, a more likely possibility is that Mfd merely extends the transcription bubble by further melting the upstream edge, resulting in essentially a TRCF-T bubble.

Binding of Mfd to bubble substrates has hitherto escaped extensive characterization, although we documented that Mfd binds a nine nucleotide bubble with a dissociation constant that is only two-fold weaker compared to dsDNA, consistent with this cryo EM–derived model. Several independent pieces of evidence in the literature support our finding: (i) A previous study investigating specificity of binding of Mfd to a 12 nucleotide bubble reported a comparable but not enhanced binding affinity to a 12–nucleotide bubble substrate (Selby and Sancar, 1995). This observation is consistent with our finding that binding to bubble substrates is only marginally weaker than to dsDNA. (ii) Although the authors do not interpret the observations as a TRCF-T bubble, Mfd-dependent exposed ssDNA in the 20 nucleotides coinciding with Mfd’s footprint in the upstream DNA is evident in potassium permanganate sensitivity assays aimed at monitoring Mfd-dependent rewinding of the transcription bubble (Ho Wan et al., 2012), and (iii) ATP_γS–mediated a 12–base pair movement in the footprint of bound Mfd toward the EC in unzipping mapper experiments (Le et al., 2017). This effect was attributed by the authors to a nucleotide dependent conformational change in Mfd, consistent with the formation of the TRCF-T bubble described here.

Our cryo-EM derived model suggests that R953 and R929 line the path of the template overhang (Figure 5). The observation that Mfd(R953A)-ATP fails to stably engage the ssDNA overhang in our smFRET study supports a model in which this residue (potentially along with other residues in the translocation motif) serves to stabilize the fork at the downstream edge of the TRCF bubble, or template ssDNA in the TRCF-T bubble. We propose that binding of R953 to the ssDNA template occurs simultaneously with the melting of the upstream dsDNA. Evidently along with a role in DNA translocation, R953 has a newly discovered role in hooking and stabilizing ssDNA. Our structure also predicts a critical role for R929 in this activity.

A clear prediction from our model is that ATP– (or non-hydrolyzable analog)-bound Mfd contacts upstream DNA in the EC and this results in the formation of an extended TRCF-T bubble and

a contact through R953. Although available structures of Mfd-[ATP or non-hydrolyzable analog]-EC do not report melting of the upstream DNA or a TRCF-T bubble, and a structure of Mfd-EC bound to the TRCF-T bubble is not as yet forthcoming, precisely such an intermediate has been observed in the Rad26-Pol II structure where an extended TRCF-T bubble of 17 nucleotides is observed (Xu et al., 2017). The conserved motor domains of Rad26 contact three nucleotides each from partially unwound upstream DNA in this structure. Analogous to R953 in Mfd, R613 in Rad26 makes a contact with ssDNA in the TRCF-T bubble. These similarities between the prototypical members of this class of transcription-coupled DNA repair factors hint to a more profound conservation of mechanism of action.

We propose that the formation of the TRCF-T bubble results in forward translocation of the EC (or at minimum restricts backtracking of the EC) leading to the formation of the catalytically poised state of the RNAP. Here, in the presence of the correct NTP and an intact R953, resumption of transcription would lead to faster forward translocation of RNAP leaving behind Mfd-ATP bound to the TRCF bubble (Figure 6E). In effect this finding explains how Mfd can ‘release’ distressed polymerases in the so-called ‘release and catch-up’ model for transcription rescue (Le et al., 2017). A prediction of our model is that Mfd bound to a non-hydrolyzable ATP analog should be sufficient to induce transcription rescue.

To understand what happens once this gating intermediate (Figure 6D) forms, we must first consider that exit from this intermediate is signified by a loss of FRET. To what might this irreversible loss of FRET correspond? This catalytically poised intermediate formed prior to irreversible remodeling of the EC is directly comparable to the Mfd-concentration independent intermediate detected in nanomanipulation assays (Ho Wan et al., 2012). However, two key differences are immediately apparent: first, whereas the lifetimes of the intermediate detected in our FRET studies follows a single exponential distribution with a lifetime of 6 s (reflecting a process with a single dominant rate-limiting step), the lifetimes of the intermediate observed in the previous study exhibit a Gaussian distribution (reflecting a stepwise process with several comparable reaction rates). Second, the lifetime of the

intermediate in the nanomanipulation assays (335 ± 3 s) is much longer than the lifetime of the intermediate detected here (6 s). To reconcile these differences, we note that the nanomanipulation assays reflect the extension of the supercoiled DNA as a proxy for rewinding of the transcription bubble, while our smFRET measurements reflect the distance between the dye pair positioned on the upstream DNA and the RNA transcript. Thus loss of FRET observed once the complex exits from the gating intermediate can reflect either loss of the transcript, or forward translocation of the polymerase to a point where the acceptor is no longer within the dynamic range of the technique. Since the lifetime of the intermediate formed during complete rewinding of the transcription bubble (335 s) (Ho Wan et al., 2012) is much longer than measured here, the irreversible process detected here likely describes loss of the RNA transcript (Figure 6D, F). This interpretation is supported by previous observations that loss of RNA from the EC occurs rapidly after Mfd binds the EC (Graves et al., 2015).

An important consequence of entry into this gating intermediate is that a finite time window is available beyond which, transcription restart is no longer possible. The prolonged residence in the catalytically poised state prior to irreversible remodeling of the EC by Mfd may serve to provide an opportunity to restart transcription at sites of pausing. This observation explains how Mfd can orchestrate transcription restart (Le et al., 2017) or termination (Ho Wan et al., 2012) via a kinetic proofreading mechanism (Hopfield, 1974) whereby paused ECs can be substrates for transcription restart, and irreversibly or DNA damage-stalled ECs are substrates for transcription termination. In effect, this gating intermediate serves as a molecular timer determining the fate of the EC.

It can be deduced that resolution of this gating intermediate during transcription termination proceeds via at least partial, and potentially complete, shrinking of the TRCF-T bubble leaving behind Mfd bound to the TRCF bubble. How might these events play out? Our results demonstrate that the lifetime of this state is only 6 s at 20 °C. While entry into this state is dependent on the presence of ATP, the observation that this lifetime is independent of ATP concentration in the range from 10 μ M to 10 mM, suggests that hydrolysis of stoichiometric amounts of ATP, may be sufficient to promote exit from this state. Further, an intact R953 is required for exit from this state. Since dissociation of

the 9-nt RNA:DNA hybrid (possessing a melting temperature of 15 °C) is expected to be spontaneous at this temperature in the absence of stabilizing interactions with the EC, the loss of the transcript signals the resolution of the molecular timer, accompanied by the formation of an intermediate where RNAP is extensively remodeled, and the TRCF-T bubble is partially or completely rewound. The cryo-EM derived model presented here sheds light on how Mfd may scrunch template DNA in this process.

A model for transcription termination that accounts for these observations is as follows: Entry into the gating intermediate reflects a configuration in which Mfd-ATP binds the EC on an extended TRCF-T bubble intermediate in which the 3'-OH of the RNA transcript is aligned with the active site of the polymerase, and R953 hooks the template ssDNA. Based on the structures of translocation intermediates of Mfd (Kang et al., 2021), we propose that ATP hydrolysis leads to unhooking of ssDNA (Figure 4), completing the pull stroke. This in turn leads to re-zipping of the upstream edge of the TRCF bubble and advancement of the TRCF bubble toward the EC. This ATP hydrolysis event triggers catastrophic remodeling of the EC (Figure 3 and Supplemental Figure 3), exposing the template-RNA (potentially through a rearrangement of the β' lid of RNAP) and enabling its rapid dissociation from the former EC. While this model awaits further validation, a prediction is that Mfd must possess an annealase activity as has been documented for the human homolog CSB (Muftuoglu et al., 2006).

What happens to Mfd after this ATP hydrolysis event? Single-molecule nanomanipulation assays monitoring the extension of the DNA during the resolution of the Mfd-RNAP intermediate suggest that the transcription bubble shrinks by two-thirds of its size (Graves et al., 2015; Ho Wan et al., 2012). The observation that the transcription bubble was rapidly rewound upon interaction with Mfd led the authors to suggest that this residual DNA extension reflects "bending/wrapping interactions". Alternately, this residual DNA extension might reflect Mfd binding to a TRCF bubble and translocating forward via continued ATP hydrolysis.

Mfd recruits the repair machinery to the site of distressed polymerases during transcription-coupled nucleotide excision repair. During global genomic nucleotide excision repair, the damage recognition enzyme UvrA₂ partially melts putative damage containing dsDNA and loads two copies of

the damage verification enzyme UvrB at the site (Kisker et al., 2013). Two UvrB helicases then examine the unpaired ssDNA with single-nucleotide resolution to precisely identify the damaged strand (Jaciuk et al., 2020; Pakotiprapha et al., 2012). In contrast, during transcription coupled DNA repair, Mfd recruits UvrA₂B to the site of the distressed polymerase (Ho et al., 2020). Compared to global genomic nucleotide excision repair, only a single copy of UvrB is recruited to a site of damage by Mfd through interactions with UvrA₂. Thus, sequestration of the damage-containing template by Mfd, as suggested here, raises the intriguing possibility that Mfd possesses the intrinsic ability to faithfully convey the identity of the damaged strand to complete the handoff to a single UvrB via the adaptor UvrA₂. Further elucidation of transcription-coupled DNA repair intermediates will shed light on this model.

Exposed ssDNA in the TRCF bubble may be more susceptible to DNA damage, and consistent with this expectation, overexpression of Mfd was found to sensitize cells to 4-nitroquinoline, ultraviolet light and nitrofurazone (Kamarthapu et al., 2016). Based on the findings of our study, we can propose a model in which high cellular concentrations of Mfd lead to indiscriminate binding of Mfd to ECs, leading to deposition of TRCF bubble-bound translocating Mfd molecules in the wake of transcription elongation. Treatment with DNA damaging agents may serve to damage the exposed nucleobases in single-stranded DNA. Thus, the TRCF bubble may explain a hitherto mysterious property of Mfd: the ability to promote mutagenesis (Ragheb et al., 2019).

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Author Contributions

Data curation: BP, ZQ, SJ, SB, HG; Data analysis: BP, ZQ, SJ, HG, AJO, PJJ; Writing—Original draft: HG; Writing—review and editing: all authors; Resources: ND and AvO; Conceptualization: BP and HG;

572 Supervision: HG.

573 **Declaration of Interests**

574 No competing interests.

575

Materials and Methods

Proteins used in this study

Purification of RNA polymerase

RNA polymerase was purified essentially as described previously (Svetlov and Artsimovitch, 2015). pIA900 was a generous gift from Irina Artsimovitch (Addgene #104401)(Svetlov and Artsimovitch, 2015). pIA900 was transformed into BL21(λ DE3)*recA*⁻ cells. A single colony was isolated and streaked on an ampicillin plate (100 mg L⁻¹). Four clones were then tested for small scale protein expression (25 mL) and were found to produce similar levels of overexpressed RNAP. Colony 1 was then used for overproduction. 6 L of culture were inoculated with an overnight culture of colony 1 and shaken for 2.5 h at 37 °C to reach A_{595} = 0.6, followed by induction with a final concentration of 1 mM IPTG. Following induction, cultures were grown for an additional 3 h followed by pelleting. Overproduced protein was purified from cells following the exact protocols described in (Svetlov and Artsimovitch, 2015). Following chromatographic separation over the Mono-Q column, two fractions were isolated, one corresponding to the RNAP core complex, and a second smaller fraction corresponding to the holoenzyme. For this work, the RNAP core complex was isolated at a concentration of 35.7 mg mL⁻¹ corresponding to 92 μ M in storage buffer (50 mM Tris/HCl pH 7.6, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 50% v/v glycerol). Purified protein was stored at -80 °C following snap freezing with liquid N₂. Aliquots in use were stored at -20 °C.

Overproduction and purification of Mfd variants

Sequence verified *mfd-ypet*, *mfd(E730Q)-ypet* and *mfd(R953A)-ypet* genes were obtained from Aldevron. Plasmids for overexpression of Mfd, Mfd(E730Q) and Mfd(R953A) were created as follows: first, *mfd* or mutant gene inserts (lacking the *ypet* gene) were amplified using PCR from the custom synthesized constructs using the primers (pETMCSII_Mfd_F and pETMCSII_Mfd_R; Table 1). PCR products were digested with *Bam*HI and *Eco*RI and purified over a Qiagen miniprep kit. The *mfd* (or mutant) fragments were inserted between the same restriction sites in vector pSH1017 (Hamdan et al., 2002), previously isolated using electrophoretic separation and an extraction from a 1% (w/v)

agarose gel. Ligations were performed with T4 DNA ligase at 16 °C overnight. Reactions were then transformed into *E. coli* DH5 α cells, colonies were screened for the presence of insert by colony PCR and intact *mfd* sequences in plasmids pHG4137 (coding for wildtype Mfd), pHG42305 (Mfd(R953A)) and pHG42306 (Mfd(E730Q)) were confirmed by sequencing.

A generalized procedure for the expression and purification of Mfd variants used in this study is best exemplified by the methods used in the production of Mfd(E730Q). *E. coli* strain BL21(λ DE3)*recA*/pHG42306 (E730Q) was grown at 37 °C in LB medium supplemented with thymine (50 mg L⁻¹) and ampicillin (200 mg L⁻¹) to A₆₀₀ = 0.6. To induce Mfd(E730Q) overproduction, 1 mM IPTG was added to the shaking culture. Cultures were grown for a further 3 h, and then chilled in ice. Cells were harvested by centrifugation (11,000 x *g*; 6 min), frozen in liquid N₂ and stored at -80 °C.

After thawing, cells (17.1 g, from 6 L of culture) were resuspended in 120 mL lysis buffer (50 mM Tris/HCl, pH 7.6, 2 mM dithiothreitol, 0.5 mM EDTA, 20 mM spermidine) and lysed by two passages through a French press operated at 12000 psi. Cell debris was removed from the lysate by centrifugation (35,000 x *g*; 30 min) to yield the soluble Fraction I. Proteins in Fraction I that were then precipitated by addition of solid ammonium sulfate (0.38 g mL⁻¹), followed by stirring for 30 min, were collected by centrifugation (38,000 x *g*; 30 min) and dissolved in 45 mL buffer A (50 mM Tris/HCl, pH 7.6, 2 mM dithiothreitol, 0.5 mM EDTA, 10% v/v glycerol) containing 180 mM NaCl. The solution was dialyzed against 2 L of the same buffer to yield Fraction II.

Fraction II was divided into two sub-fractions (23 mL each), which were successively applied at 1 mL min⁻¹ onto a column (2.5 x 15 cm) of Toyopearl DEAE-650M resin that had been equilibrated in buffer A + 190 mM NaCl. Fractions containing proteins that did not bind to the resin (50 mL per sub-fraction; 100 mL in total) were pooled and dialyzed against two changes of 2 L of buffer A to yield Fraction III.

The dialysate (Fraction III) was loaded at 1 mL min⁻¹ onto the same DEAE column that had been equilibrated with buffer A. After the column was washed with 80 mL of the same buffer, Mfd(E730Q) was eluted at 2.5 mL min⁻¹ using a linear gradient (700 mL) of 0 – 500 mM NaCl in buffer A. The

Mfd(E730Q) eluted in a single, relatively narrow, peak centered around 80 mM NaCl. Fractions containing Mfd(E730Q) (40 mL) were pooled and dialyzed against four changes of 1.2 L of buffer B (50 mM Na/MES, pH 6.5, 2 mM dithiothreitol, 0.5 mM EDTA, 10% v/v glycerol) to yield Fraction IV.

Fraction IV (40 mL) was loaded at 1.5 mL/min onto a 2.5 x 13 cm SP Sepharose FastFlow column (GE Healthcare) that had been equilibrated with buffer B. After washing the column with 80 mL of the same buffer B, Mfd(E730Q) was eluted at 2.5 mL min⁻¹ using a linear gradient (700 mL) of 0–500 mM NaCl in buffer B. The Mfd(E730Q) eluted in a single peak centered around 100 mM NaCl. Fractions containing highly purified Mfd(E730Q) were pooled and dialyzed against four changes of 1.2 L of storage buffer (40 mM Tris/HCl, pH 7.6, 100 mM NaCl, 4 mM dithiothreitol, 0.5 mM EDTA, 30% v/v glycerol) to yield final product containing 130 mg of protein at a final concentration of 46.7 μM. The same protocol yielded 51.5 mg of Mfd(R953A) at a final concentration of 18 μM. Final protein preparations were snap frozen in liquid N₂ and stored at –80 °C.

In case of wildtype Mfd, a modified purification procedure was used whereby the fractions containing Mfd from the second DEAE (40 mL) column were pooled and directly applied at 1 mL min⁻¹ onto a 2.5 x 10 cm heparin Sepharose 4B that had been equilibrated with buffer A + 50 mM NaCl. Mfd was eluted at 1 mL min⁻¹, in a broad peak centered around ~120 mM NaCl, using a linear gradient (450 mL) of 50–400 mM NaCl in buffer A. Fractions containing highly purified Mfd were pooled and dialyzed against 2 L of storage buffer (40 mM Tris/HCl, pH 7.6, 100 mM NaCl, 4 mM dithiothreitol, 0.5 mM EDTA, 25% v/v glycerol) to yield 115 mg of protein at a final concentration of 40 μM. Purified Mfd was snap frozen in liquid N₂ and aliquots were stored at –80 °C.

NusG was a generous gift from Prof Robert Landick (University of Wisconsin-Madison) (Kang et al., 2018).

651 **Table 1: Nucleic Acids used in this study**

Experiment	DNA	Sequence
pETMCSII_Mf d_F	Cloning primer	GTT TAA TCG GAT CCT AAG GAG GTT AAT TCC CGC TAT GCC TGA ACA ATA TCG TTA TAC G
pETMCSII_Mf d_R	Cloning primer	GGGAGCTCGAATTCTTAAGCGATCGCGTTCTCT
smFRET (Figures 1–3)	Non- template (NT70_bio)	ATC GAG CAA CTA CTC AGA CAG CAC TAC TGC GAC TTA CAG ACA TCG AGA GGG TAA TGG CGA ATA GCA CTG A /3 bioTEG/
smFRET (Figures 1–3)	Template (T70_31Cy3)	TCA GTG CTA TTC GCC ATT ACC CTC TCG ATG T/iCy3/CT GTA AGT CGC AGT AGT GCT GTC TGA GTA GTT GCT CGAT
smFRET (Figures 1–3)	RNA(R15_4 Cy5)	rArUrArU/iCy5/rArU rArUrC rGrArG rArGrG
smFRET (Figure 4)	bio_AS18_C y5	/5Biosg/ TGG CGA CGG CAG CGA GGC/3Cy5Sp/
smFRET (Figure 4)	Cy3_dT40_ S18	/5Cy3/TT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTG CCT CGC TGC CGT CGC CA
SPR (Supplement al Figure 4)	bio_AS18	/5Biosg/ TGG CGA CGG CAG CGA GGC
SPR	dT40_S18	TT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTG CCT

(Supplemental Figure 4)		CGC TGC CGT CGC CA
SPR (Supplemental Figure 4)	bio_dT40_S18	/Biosg/TT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTG CCT CGC TGC CGT CGC CA
SPR (Supplemental Figure 4)	AS18	TGG CGA CGG CAG CGA GGC
SPR (Supplemental Figure 2)	TS_49	TCA GTG CTA TTC GCC ATT ACC CTC TCG ATG T/iCy3/CT GTA AGT CGC AGT AGT G
SPR (Supplemental Figure 2)	NT_49_bio	CAC TAC TGC GAC TTA CAG ACA TCG AGA GGG TAA TGG CGA ATA GCA CTG A /3 bioTEG/
SPR (Supplemental Figure 5)	NT_49_bio_bubble	CAC TAC TGC GAC TTA CAG ACT TTT TTT TTG TAA TGG CGA ATA GCA CTG A /3BioTEG/
SPR (Supplemental Figure 2)	RNA_15	rArUrAr UrArU rArUrC rGrArG rArGrG

652

653 All oligonucleotides were purchased from Integrated DNA Technologies (IDT, Singapore). Primed DNA

654 template for smFRET and cryo-EM was first annealed by hybridizing bio_AS18_Cy5 and Cy3_dT40_S18

655 in 10 mM Tris/HCl 7.6, 50 mM NaCl, 5 mM MgCl₂ at a final concentration of 10 μM of Cy3_dT40_S18

and 1.05-fold excess of the bio_AS18_Cy5 complementary strand.

Single molecule FRET measurements

smFRET TIRF microscope setup:

A home built objective-type TIRF microscope based on a Nikon TiE2 model was used to record single molecule movies. FRET was measured by excitation with a 532 nm laser and the emissions at 555 and 647 nm were collected using a band-pass filter at 555 nm and a long-pass filter at 650 nm. Scattered light was removed by using a 560 nm long pass filter. Cy3 and Cy5 signals were separated by a 638 nm dichroic using photometrics dual view (DV-2) and both signals were focused onto a CCD camera (Hamamatsu C9 100–13), simultaneously. Data were collected at 5 frames s⁻¹.

Preparation of flow cells for imaging:

Coverslips were prepared as described previously (Johnston et al., 2020). Quartz coverslips were cleaned with 100% ethanol and 1 mM KOH. Aminosilanization of cleaned coverslips was carried out using a 1% (v/v) (3-aminopropyl)triethoxy silane (Alfa Aesar, A10668, UK) solution in acetone. PEGylation was carried out by incubating a mixture of biotinPEG-SVA MW 5000 and mPEG-SVA MW 5000 (Laysan Bio, AL) at a ratio of 1:10 prepared in 50 mM MOPS pH 7.5 solution on the top of the silanized coverslip for 3–4 h. PEGylated coverslips were stored under dry nitrogen gas at –20 °C.

Neutravidin solution was prepared in transcription buffer (1 × TB; 20 mM Tris/HCl pH 8.0, 40 mM KCl, 5 mM MgCl₂ and 1 mM β-mercaptoethanol) and spread on the top of dry a PEGylated coverslip followed by a 10 min incubation. Sample flow chambers were created by sandwiching polydimethylsiloxane (PDMS) on the top of the streptavidin coated coverslip. Then, blocking buffer (1 × TB + 0.25% (v/v) Tween 20) was injected into the channel to reduce non-specific binding of proteins on the surface followed by 10–15 min incubation. This was then followed by washing with 1 × TB to remove unbound Tween 20.

In situ reconstitution of ECs for smFRET measurements:

Protocols described by (Komissarova et al., 2003) were adapted for *in situ* reconstitution of ECs in single-molecule FRET studies. 1 μM template DNA was annealed to 3 μM RNA primer in 1 × TB by

heating to 65 °C for 5 min followed by 3 °C drops in temperature every 2 min until the reaction temperature reached 27 °C, followed by a hold step at 25 °C. Typically, 200 µL of reaction were prepared at a time. To form the RNAP:template (T70_31Cy3):RNA(R15_4Cy5) complex, 0.22 µL of 92 µM RNAP was incubated with 2 µL of template:RNA hybrid in 1 × TB at room temperature for 30 min. Meanwhile, 200 µL of 100 pM of NT70_bio (Table 1) were immobilized on the neutravidin-coated surface. Following this, the RNAP:template:RNA mixture was diluted serially 6000 × in a final suspension of 200 µL of 1 × TB and introduced into the flow cell and incubated for at least 5 min to promote annealing of the template DNA to the biotinylated non-template DNA resulting in formation of the EC. At the end of this incubation step, the flow cell was washed with 200 µL of 1 × TB + 1 M KCl and subsequently with 1 × TB supplemented with an oxygen-scavenging system (OSS) consisting of protocatechuic acid (2.5 mM) and protocatechuate-3,4-dioxygenase (50 nM) to reduce photo-bleaching of the fluorophores and 2 mM Trolox to reduce photo-blinking of dyes, followed by imaging. All elongation experiments in Figures 1 and 2 were performed at 100 µM of each NTP or dNTP (where indicated). smFRET experiments involving NusG were performed at 500 nM of NusG in 1 × TB (Figure 2). smFRET experiments involving wildtype Mfd (Figures 3 and 4) were performed at 500 nM of Mfd in 1 × TB. Experiments involving Mfd(R953A) (Figure 3 and 4) and Mfd(E730Q) (Figure 4) were performed at 1 µM of Mfd mutant in 1 × TB. Concentrations of ATP and ATPγS were at 1 mM and 0.5 mM respectively unless otherwise specified.

Primed DNA template (Figure 4):

Primed DNA substrate (bio_AS18_Cy5: Cy3_dT40_S18) used in smFRET experiments in Figure 4 was diluted to 50 pM in 200 µL of 1 × TB prior to immobilization in the flow cell.

Data Analysis

Single-molecule data acquisition was carried out using NIS element software. Then, single-molecule intensity time trajectories and FRET-time trajectories were generated in MASH-FRET software (<https://rna-fretools.github.io/MASH-FRET>). Further data analysis and figures are plotted using custom written Python scripts.

708

709 **Surface plasmon resonance**

710 Activation of SPR chip:

711 SPR experiments were performed using a BIAcore T200 instrument (GE Healthcare) using a
712 streptavidin (SA) coated sensor chip to study the binding kinetics of Mfd to reconstituted ECs, or
713 various DNA substrates. All experiments were carried out at 20 °C with a flow rate of 5 $\mu\text{L min}^{-1}$, unless
714 specified otherwise. The SA chip was activated with three sequential 1 min injections of 1 M NaCl, 50
715 mM NaOH, then stabilized by 1 min treatment with 1 M MgCl_2 .

716 Immobilization of 49-mer dsDNA substrates (Supplemental Figure 2):

717 49-mer dsDNA substrate was prepared by hybridizing NT_49_bio (final concentration of 1 μM) with
718 two-fold excess of TS_49 in 1 $\times$ TB in a final volume of 120 μL by heating in a heat block at 90 °C for 5
719 min followed by a slow cooling step until the room temperature was reached. NT_49_bio:TS_49
720 dsDNA substrate was resuspended in 1 $\times$ SPR buffer (30 mM Tris/HCl, pH 7.6, 40 mM KCl, 5 mM MgCl_2 ,
721 0.005% (v/v) surfactant P20, 0.25 mM EDTA, 0.5 mM dithiothreitol) to a final concentration of 2.5 nM
722 (of the biotinylated oligo) and introduced onto the SPR chip for immobilization, followed by a wash
723 step with 1M KCl. The signal from the dsDNA template corresponded to 106 RUs. Measurement of the
724 Mfd binding affinity for the immobilized dsDNA ligand was performed as described below.

725 Immobilization of reconstituted EC on biotinylated 49-mer dsDNA (Supplemental Figure 2):

726 The RNAP:template (TS_49):RNA(RNA_15) complex was formed using the procedure described for *in*
727 *situ* reconstitution of ECs for smFRET measurements. Following this, biotinylated non-template strand
728 (NT49_bio; 10 μL of 10 μM working stock in water) was added to the mixture and the reaction was
729 incubated at room temperature for 15 min. Next, 50 μL of nickel resin suspension were washed and
730 equilibrated with 1 $\times$ TB. This resin resuspended in 50 μL of 1 $\times$ TB was then added to the mixture
731 containing the RNAP, template:RNA hybrid and non-template strand followed by incubation at 37 °C
732 for 15 min to allow binding of His-tagged RNAP complexes to the resin. The remaining steps were then
733 performed at room temperature. First, supernatant from this suspension was isolated by

centrifugation on a mini-benchtop centrifuge for 10 s. The pelleted resin was washed with 150 μ L of 1 $\times$ TB supplemented with 1 M KCl for 2 min. This suspension was then subjected to centrifugation and the supernatant was discarded. Following this, 150 μ L of elution buffer (1 $\times$ TB + 500 mM imidazole) were then applied to the resin followed by gentle mixing by pipetting. Finally, the suspension was centrifuged and the eluate containing reconstituted elongation complexes was collected. All reactions containing RNAP were performed in LoBind microcentrifuge tubes (Eppendorf). The eluate was then applied to a size exclusion column (Wyatt SEC analytical column 030S5) previously equilibrated with 30 mM Tris/HCl, pH 7.6, 150 mM KCl, 15% (v/v) glycerol, 0.5 mM EDTA, 1 mM dithiothreitol to separate biotinylated elongation complexes from RNAP alone and collected in 200 μ L fractions. Fraction 7 (F7) was found to contain the elongation complex as verified by smFRET studies. Immobilization of these reconstituted ECs was performed by resuspending 40 μ L of F7 in 200 μ L of SPR buffer and introduction into the flow cell. Immobilization was stopped when the signal reached 1380 RU over the background. This was followed by treatment with 1M KCl (10 μ L min⁻¹) that served to displace incorrectly assembled or non-specifically bound RNAP from the chip surface. The resulting signal corresponded to 550 RU over the background. ECs thus immobilized were found to be stable for several days. Measurement of Mfd binding affinity for the immobilized EC ligand was performed as described below.

Immobilization of primed DNA (Supplemental Figure 4):

Primed DNA templates were assembled *in situ* (on the chip surface) in two stages. First, the biotinylated strand was immobilized in three of the four channels in the chip. Single-stranded biotinylated DNA substrates (bio_dT40_S18, bio_AS18, and bio_AS18_Cy5) were diluted to 1 nM in SPR buffer and immobilized in channels 2, 3, and 4 respectively. This corresponded to 150 RUs of bio_dT40_S18 (channel 2), 75 RUs of bio_AS18 (channel 3), and 80 RUs of bio_AS18_Cy5 (channel 4). Next, the complementary non-biotinylated strand (2 μ M in 1 $\times$ SPR buffer) was introduced into the flow cell to hybridize the template *in situ* at 5 μ L min⁻¹. This led to a further increase in signal corresponding to 39 RUs (channel 2, complementary strand: AS18), 138 RUs (channel 3,

complementary strand: dT40_S18) and 115 RUs (channel 4, complementary strand: Cy3_dT40_S18) upon complete hybridization. Following this step, the flow cell was washed with 1 M MgCl₂. Measurement of Mfd binding affinity to immobilized primed DNA template was performed as described below.

Titration of Mfd (Supplemental Figures 2 and 4):

Following the immobilization of the DNA substrates or the EC, in each case, binding studies were performed by injecting increasing amounts of Mfd in the indicated concentration range in the SPR buffer supplemented with 0.5 mM ATP_γS. For measurements of Mfd binding to dsDNA (Supplemental Figure 2: NT_49_bio: TS_49), reconstituted EC (Supplemental Figure 2) and primed DNA substrates, an optimized concentration range of [0, 10, 20, 40, 80, 160, 320, 640] μM of Mfd was used. For measurements of Mfd binding to ssDNA substrate (bio_dT40_S18), an optimized concentration range of [0, 0.25, 0.5, 1, 2, 4, 8, 16] μM was used.

Two conditioning steps of flushing the flow cell with 300 mM KCl for 30 s at 10 μL min⁻¹ were carried out prior to injection of Mfd. Mfd-ATP_γS was flowed in for 150 s in the association phase, and dissociation was observed for 200 s in 1 × SPR buffer lacking any Mfd or nucleotide cofactor.

Analysis of sensorgrams:

Sensorgrams were subtracted from the signal from unmodified flow cell (Flow channel 1), then zero-subtracted (injection at 0 μM Mfd or mutant) and R_{eq} values, generated by averaging response values in the sensorgram's steady-state region from the appropriate Mfd concentration range, fit against [Mfd] using a 1:1 steady state affinity (SSA) model incorporated in the BIAevaluation software 4.0.1:

$$R_{eq} = R_{max} \left(\frac{[A]}{[A] + K_D} \right) \dots\dots\dots (1)$$

where R_{max} corresponds to the response when all the immobilized DNA ligands on the surface are saturated with the analyte A, K_D is the dissociation constant, and [A] is the concentration of analyte (Mfd or mutant) in solution. In general, raw sensorgrams and fit curves (where applicable) were exported using Excel, then plotted in Origin, and final Figures prepared in Adobe Illustrator CC.

Sample preparation for electron microscopy

Electron microscopy data acquisition:

30 μL of Mfd stock (40 μM) were dialyzed overnight in 1 L of 1 $\times$ TB at 4 $^{\circ}\text{C}$ in a Slida-A-lyzer mini dialysis unit with a 3,000 MWCO (Thermo Fisher) to reduce the amount of glycerol in the sample. Following this, protein was recovered and concentrated to a 1.67 mg mL^{-1} . For the sample with ADP- AlF_x , ADP, NaF and AlCl_3 were added to the mixture of Mfd (7.8 μL at 1.6 mg mL^{-1}) and fluorescent oligonucleotides (10 μL of 10 μM stock) to final concentrations of 2, 5 and 0.5 mM, respectively. This reaction mixture was incubated at 37 $^{\circ}\text{C}$ for 5 min to allow binding of Mfd to the template. The samples were centrifuged at 20,000 $\times g$ for 10 min at 4 $^{\circ}\text{C}$ to clear any precipitate before grid preparation. Samples (3 μL) were applied to glow-discharged Quantifoil R 1.2/1.3, Au 400 grids. The grids were blotted at 6 $^{\circ}\text{C}$ for 3.5 s at 100% humidity with no extra blot force on an FEI Vitrobot Mark IV and plunged into liquid ethane. The grids were stored in liquid nitrogen until use.

Electron microscopy data acquisition:

Data were collected using FEI Talos Arctica microscope at 200 kV equipped with a Falcon III (FEI) camera in electron counting mode. Micrographs were acquired at a nominal defocus range of -1.0 to $-1.9 \mu\text{m}$, with a pixel size of 0.74 $\text{\AA}$ and a total dose of 50 $\text{e}^- \text{\AA}^{-2}$ accumulated over 50 s and fractionated across 50 frames.

Image processing of EM micrographs

Data were processed in cryoSPARC v2 (Punjani et al., 2017). For the sample with ADP- AlF_x , 472 images were manually selected after motion correction and CTF estimation for final processing. 110562 particles were selected after 2D classification and subjected to 3D classification in *ab initio* reconstruction followed by heterogeneous refinement using four *ab initio* classes. 31718 particles from class 3 were used for 3D refinement, which resulted in a 3D reconstruction of 5.2 $\text{\AA}$ (0.143 FSC gold standard).

Model fitting

Molecular dynamics with explicit solvent was used to fit the model of Mfd on primer DNA into the cryo-EM density map (Singharoy et al., 2016). The starting model was comprised of the Mfd motor domain (PDB ID 2eyq) with idealized B-DNA with the same sequence as used for the cryo-EM experiments but with a dT(12) overhang instead of dT(40). The system was equilibrated for 10 ns. The resulting model was then used as the basis for modelling the complex in COOT (Emsley et al., 2010) with minimal distortions to protein secondary structure and DNA. The model was then subjected to restrained positional refinement in phenix real_space_refine (Liebschner et al., 2019). The final model is comprised of 382 residues from Mfd and 47 nucleotide residues. Model was visualized in Chimera(Pettersen et al., 2004).

Figure legends

Figure 1: Reconstitution of a functional EC.

(A) ECs were assembled on dye-labelled nucleic acids by flowing in pre-incubated RNAP-template:RNA complexes into a flow cell with immobilized biotinylated non-template strand (EC shown in grey). Following initial immobilization, a 1 M KCl wash step was introduced to remove poorly hybridized ECs from the surface. Here, only complexes containing both the Cy3-labelled template strand and Cy5-labelled transcript will be observed when stably associated with the biotinylated non-template strand (shown in orange). Two example FRET trajectories and temporal heat maps are provided for RNAP (B, C) (n = 318 molecules), RNAP in the presence of dNTPs (D, E) (n = 80 molecules), RNAP with non-complementary ('wrong') NTPs (F, G) (n = 179 molecules) and the full set of NTPs (H, I) (n = 81 molecules) respectively. Fits to detected states in the FRET trajectories are shown in red. The sequence of the four next correct incorporations is G, U, A and A.

Supplemental Figure 1: Walking the EC with subsets of correct rNTPs. *Related to Figure 1.*

(A) Transition density plots for RNAP alone (n = 318 molecules), in the presence of dNTPs (n = 80 molecules), with non-complementary ('wrong') NTPs (n = 179 molecules) and the full set of NTPs (n = 81 molecules) respectively.

(B) Schematic of *in situ* assembled FRET pair labelled ECs incubated with GTP (the next correct NTP) (n = 63 molecules) or with GTP, ATP and UTP (n = 87 molecules). Temporal heat maps of the reaction for experiments conducted in the presence of GTP (C) or GTP, ATP and UTP (D) are presented here.

Figure 2: Interactions between NusG and the EC

(A) Schematic of FRET pair labelled ECs incubated with NusG (purple). Example FRET trajectories and temporal heat maps for RNAP and NusG alone (B, C) (n = 461 molecules), RNAP and NusG in the presence of wrong NTPs (D, E) (n = 268 molecules), and RNAP and NusG with the full set of NTPs (F, G)

(n = 425 molecules). Here, NusG promotes the residence of the EC in a catalytically poised state (EC shown in blue).

Supplemental Figure 2: Measurement of binding affinity of Mfd for dsDNA and the EC. Related to Figures 2 and 3.

(A) Transition density plots for RNAP and NusG alone (n = 461 molecules), RNAP and NusG in the presence of wrong NTPs (n = 268 molecules), and RNAP and NusG with the full set of NTPs (n = 425 molecules). Here, NusG promotes the residence of the EC in a catalytically poised state (EC shown in blue). Red ellipse highlights unidirectional transitions.

Schematic and SPR sensorgrams show association (150 s) and dissociation phases of serially-diluted 10 – 640 μ M Mfd-ATP γ S, including a 0 μ M control for (B) biotinylated 49-mer dsDNA, and (C) *in vitro* reconstituted EC on biotinylated 49-mer DNA flown into the SPR chip. Responses at equilibrium averaged over the steady region of sensorgrams (R_{eq}) were fit (insets) using a steady-state affinity (SSA) model to derive values of K_D (as indicated). Errors are *S.E* of the fit.

Figure 3: Interactions between Mfd and the EC.

(A) Schematic of FRET pair labelled surface immobilized ECs incubated with Mfd in solution. Two example FRET trajectories and temporal heat maps are presented for RNAP and Mfd alone (B, C) (n = 75 molecules), RNAP and Mfd in the presence of 1 mM ATP γ S (D, E) (n = 68 molecules), RNAP and Mfd in the presence of ATP (F, G) (n = 174 molecules), and RNAP and DNA-binding competent but translocase-deficient Mfd(R953A) in the presence of 1 mM ATP (H, I) (n = 234 molecules).

Supplemental Figure 3: Interactions of ATP with the EC alone or in the presence of Mfd. Related to Figure 3.

(A) Transition density plots for RNAP and Mfd alone (n = 75 molecules), RNAP and Mfd in the presence of 1 mM ATP γ S (n = 68 molecules), RNAP and Mfd in the presence of ATP (n = 174 molecules), and

867 RNAP and DNA-binding competent but translocase-deficient Mfd(R953A) in the presence of 1 mM ATP
868 (n = 234 molecules). Red ellipses highlight irreversible transitions.

869 (B) Temporal heat map (upper panel) and transition density plot (lower panel) of FRET pair labelled
870 RNAP EC in the presence of 0.5 mM ATP γ S alone (n = 124 molecules).

871 (C) Distribution of the catalytically-poised mid-FRET state for the indicated concentrations of ATP and

872 (D) Dependence of the lifetime (τ_{off}) of mid-FRET state for Mfd-RNAP ECs as a function of ATP
873 concentration in the range from 10 μ M to 1 mM ATP (n = 43, 24, 77, 32 molecules respectively). Plots
874 represent dwell-time distributions of the surviving fraction of the population in the mid-FRET state fit
875 to a single exponential decay (red curve) to the data. Errors represent S.E to the fit.

876 (E) Temporal heat maps of FRET pair labelled RNAP EC when titrated with ATP in the range of 0.1 μ M
877 to 1 mM (n = 151, 82, 81, 76, 157 molecules respectively) as indicated.

878 **Figure 4: Interactions between Mfd and a primed-DNA template**

879 (A) Schematic of FRET pair labelled primed DNA template comprised of an 18-mer dsDNA and a 5' 40-
880 mer poly-dT overhang to assess interactions of Mfd with ssDNA. Two example FRET trajectories,
881 temporal heat maps and transition density plots for primed DNA alone (B, C) (n = 100 molecules), Mfd
882 in the presence of ATP γ S (D, E) (n = 94 molecules), Mfd in the presence of ATP (F, G) (n = 203
883 molecules), and Mfd(R953A) in the presence of ATP (H, I) (n = 105 molecules) are presented here.

884 **Supplemental Figure 4: Interactions between Mfd and a primed-DNA template. Related to Figure 4.**

885 (A) Transition density plots for FRET pair labelled primed DNA alone (n = 100 molecules), or bound to
886 Mfd in the presence of ATP γ S (n = 94 molecules), or ATP (n = 203 molecules), and Mfd(R953A) in the
887 presence of ATP (n = 105 molecules) are presented here. DNA substrate is the same as described in
888 Figure 4.

Schematic and SPR sensorgrams show association (150 s) and dissociation phases of serially-diluted 10 – 640 μM Mfd-ATP γS , including a 0 μM control for (B) primed DNA substrate (biotinylated 18-mer dsDNA with dT₄₀ overhang) (C) “flipped” primed DNA substrate (18-mer dsDNA with biotinylated dT₄₀ overhang) (D) FRET pair labelled primed DNA substrate (biotinylated 18-mer dsDNA with dT₄₀ overhang) and (E) 58-mer ssDNA containing the dT₄₀ sequence. Responses at equilibrium averaged over the steady region of sensorgrams (R_{eq}) were fit (insets) using a steady-state affinity (SSA) model to derive values of K_D (as indicated). Errors are *S.E* of the fit.

(F) Temporal heat map and (G) transition density plot for ATPase mutant Mfd(E730Q) binding to FRET pair labelled primed DNA template used in Figure 4 ($n = 103$ molecules).

(H) For convenience, kernel density estimations of the ensemble FRET distributions read as projections along the ordinate in the heat-maps presented in Figure 4 (panels C, E, G, I) are presented to enable direct visual comparisons.

Figure 5: Interactions between Mfd and primed DNA

(A) Views of the cryo-EM reconstruction of Mfd bound to the FRET pair labelled DNA substrate (used in Figure 4).

(B) Front view of the molecular model derived from the cryo-EM reconstruction showing domain 5 (tan), domain6 (olive), template (dark red) and non-template (pink) DNA. Mfd (residues 575-956) and DNA (template: residues 28-58; non-template: 1-18) are shown in surface representation to illustrate the path of the DNA.

(C) Top view of molecular model showing TRG motif straddling the DNA. The unpaired 5' poly dT overhang is colored as follows: dT1–dT3 (pink), dT4–dT6 (salmon), dT7–dT9 (sandy brown) and dT11–dT12 (brown). R953 and R929 are positioned to contact the ssDNA overhang at 3 and 6 residues away from the junction respectively.

(D) Model suggests dsDNA near the junction is melted to form the TRCF bubble. (F) Path of the DNA is lined by highly conserved residues in Mfd across bacteria: basic residues (Lys, Arg, His) are shown in blue, polar residues (Thr, Asn) are shown in gold, and aromatic residues (Phe, Tyr) are shown in green. Domain 5 corresponds to residues 549 – 780, and domain 6 corresponds to residues 781-990 in Mfd. Labels are provided in (G).

Supplemental Figure 5: EM reconstruction of Mfd. Related to Figure 5.

(A) Overview of image processing using CryoSPARC. 110562 particles were selected after 2D classification for *ab initio* reconstruction and heterogeneous refinement leading to four classes of 3D reconstructions. Of these, 31718 particles that yielded the most complete reconstruction were subjected to homogenous refinement to generate the map presented here. The colormap applied to the reconstruction represents local resolution. (B) Gold-standard Fourier shell correlation curves of the reconstruction reported a resolution of 5.2 Å (C) angular distribution of particle projections (D) Measurement of dissociation constant of Mfd for a 49-mer dsDNA template containing a 9 nucleotide non-complementary bubble where the complementary residues were replaced by dT₉. Errors reflect S.E of the fit.

Figure 6: Model for Mfd-mediated transcription modulation

Mfd interacts with the (A) elongation complex weakly in the absence of ATP, however, (B, C) ATP binding and hydrolysis are required for stable association of Mfd with the EC. Following, a brief period of ATP-hydrolysis dependent translocation of Mfd on upstream dsDNA, Mfd loads on to the EC. This results in chaperoning of the EC from catalytically inactive state(s) into a catalytically poised molecular timer intermediate (D) with a lifetime of ~6 s. In this ATP-bound state, we propose that Mfd melts the upstream DNA to form an extended transcription-repair coupling factor – transcription (“TRCF-T”) bubble. In this state R953 contacts the template ssDNA in the TRCF-T bubble. Melting of this upstream DNA is sufficient to promote residence of the EC in a catalytically poised state. (E) In the presence of

937 NTPs on an undamaged template, transcription can be resumed. (F) On damaged templates or under
 938 conditions of nucleotide starvation or in the presence of impediments to the progress of the EC, the
 939 bound ATP is hydrolyzed to promote transcription termination. In our experiments this is seen as the
 940 loss of RNA. We propose that in either case continued translocation occurs in a manner in which the
 941 ATPase activity of Mfd translocates the TRCF bubble forward until Mfd interacts with its interacting
 942 partners in the repair machinery.

943

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1110 Supplemental Information

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1112 Mechanism of transcription modulation by the transcription-repair coupling factor

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1124 **Supplemental Note 1**

1125 SPR measurements of the strength of interaction between Mfd and this DNA substrate in the presence
 1126 of ATP γ S revealed a dissociation constant that is somewhat higher ($K_D = 300 \pm 10$ nM) than that of
 1127 dsDNA (170 ± 20 nM; Supplemental Figure 4B–D compared to Supplemental Figure 2B). To monitor
 1128 changes in the distance between the junction and the 5' end, we introduced a FRET pair on either end
 1129 of the ssDNA overhang (Figure 4A). SPR measurements revealed a binding affinity of 390 ± 10 nM for
 1130 the binding of Mfd to the FRET pair labelled substrate (Supplemental Figure 4D).

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Supplemental Figure 1

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Figure 2

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Supplemental Figure 2

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Figure 3

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Supplemental Figure 3

Figure 4

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Supplemental Figure 4

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Figure 5

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Supplemental Figure 5

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Figure 6