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Strand separation suffices to establish a long-lived, foolproof DNA–protein lock at the Tus–*Ter* replication fork barrier

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ABSTRACT

The bidirectional replication of a circular chromosome by many bacteria necessitates proper termination to avoid the head-on collision of the opposing replisomes. In *E. coli*, replisome progression beyond the termination site is prevented by Tus proteins bound to asymmetric *Ter* sites. While structural evidence suggests that strand separation on the blocking (non-permissive) side of Tus–*Ter* triggers roadblock formation, biochemical evidence indicates roles for protein–protein interactions. Here, we perform DNA unzipping experiments demonstrating that non-permissively oriented Tus–*Ter* forms a tight lock also in the absence of replicative proteins. Conversely, permissively oriented Tus–*Ter* allows nearly unhindered strand separation. We quantify the lock strength and demonstrate the existence of several intermediate lock states that are severely impacted by mutations in the Tus lock domain, but not by mutations in the Tus DNA-binding domain. Lock formation is 100% efficient and highly specific. Since these efficiencies exceed those reported for the *E. coli* replisome itself, we postulate that protein–protein interactions may actually *disrupt* rather than promote proper lock formation.

INTRODUCTION

DNA replication in *Escherichia coli* initiates bidirectionally at *oriC,* creating two replication forks that proceed around the circular 4.6 Mbp chromosome in opposite directions. The forks progress at an average speed of 1 kbp/s until they meet again at the terminus region opposite the origin. As the replication forks approach the terminus, each encounters five 23 bp *Ter* DNA sites bound in a specific orientation by a 36 kDa DNA binding protein called Tus[1–4], and proceeds unhindered. However, when a replication fork continues beyond these sites, Tus–*Ter* is approached from the opposite direction (**Fig. 1a**). This triggers Tus–*Ter* to form a tightly locked complex, thereby bringing the replication fork to a halt[1,5–7]. Each *Ter* site is non-palindromic, does not contain any direct repeats and has a strictly conserved GC6 base pair followed by a highly conserved 13 base-pair core region. Tus is a monomeric protein that forms a simple 1:1 complex with *Ter*[8] (**Fig. 1b**). The structure of the Tus–*TerA* complex shows that many of the conserved residues among the *Ter* sites make base-specific contacts with the protein[4,9]. The Tus–*TerB* complex has a reported dissociation constant (K_D) of

0.3 pM as well as a half-life of 550 min in 150 mM potassium glutamate[10], and a K_D of 44 pM in 50 mM NaCl[11]. This renders it the most stable complex known between a monomeric sequence-specific DNA-binding protein and a duplex DNA recognition sequence.

A long-standing issue regards the manner in which the asymmetric blockage at Tus–*Ter* comes about. Does Tus itself function as a molecular roadblock, locking itself onto the *Ter* DNA as the DNA replication machinery approaches, or are there specific protein–protein interactions that lead to the polar arrest of the replisome? Studies investigating the specificity of this roadblock to motor proteins other than its natural target have presented contradictory results. On one hand, various studies imply specific protein–protein interactions between Tus and the replicative helicase, DnaB. For example, Andersen *et al.* showed that while the functionally similar but structurally unrelated *Bacillus subtilis* replication termination system also works well in *E. coli*, Tus–*Ter* is very much more effective in its natural host[12,13]. Sahoo *et al.* found that Tus–*Ter* blocks DnaB but not the Rep helicase *in vitro*[14], and evidence has been obtained for specific interactions between DnaB and Tus through yeast two-hybrid analysis[15]. On the other hand, there are numerous studies pointing towards a protein-independent polar blocking mechanism. For example, it was shown that RNA chain elongation catalyzed by T7, SP6 and *E. coli* RNA polymerases is impeded by Tus–*Ter* in a polar manner[16,17]. Lee *et al.* found that the non-permissive face of Tus–*TerB* blocked the actions of the DnaB, UvrD, Rep and PriA helicases, indicating low specificity of helicase blocking activity[18,19]. Similar results were obtained for SV40 large T antigen helicase[20,21].

In 2006, Mulcair *et al.* shed light on this elusive asymmetric molecular roadblock through surface plasmon resonance (SPR) studies of dissociation of Tus from forked *TerB* oligonucleotides, supported by a crystal structure of a forked, "locked" Tus–*Ter* complex. This locked complex exhibited significant structural changes at the fork-blocking (non-permissive) face in comparison with the ds*TerA*-bound, but not locked, structure elucidated a decade earlier[4,22]. The locked conformation revealed that of the ssDNA bases in the forked *Ter* region (*Ter* bases 1–7), the highly conserved C6 base is flipped out of the helical DNA axis and into the protein (**Fig. 1c**). In this conformation the C6 base undergoes tight interactions with several amino acids, of which the most prominent are I79, F140, H144, G149 and L150 (**Fig. 1d**). These amino acid residues of the Tus lock domain were distinctly different from those involved in sequence recognition and binding affinity[23]. Based on these observations, it was proposed that the Tus–*Ter* system is the molecular analog of a mousetrap: the trap is set by Tus binding to *Ter* in an oriented fashion, and is triggered by strand separation by the approaching replication machinery[22].

The mousetrap model has two major implications. First, it suggests that *Ter* binding and lock formation are not only two different mechanisms, but can also be ascribed to different domains of Tus. This means that a

mutation in one domain might affect the properties of only that domain. Secondly, lock formation through strand separation could occur independently of any specific protein–protein interactions. Nevertheless, Bastia *et al.* produced convincing evidence that translocation of DnaB on dsDNA in the absence of unwinding is sufficient to provoke polar arrest[24]. Although this result does not require it, these authors proposed an alternative model in which the DnaB helicase binds specifically to Tus and argued that locked complex formation might act as a backup mechanism when protein–protein interaction fails. At least, their data suggest that the mousetrap is likely not sufficient on its own.

In this study, we use the quantitative power of high-throughput single-molecule approaches to address both of the key implications of the Tus–*Ter* mousetrap model, and to dissect the overall mechanism of lock formation. By applying mechanical force to unwind a DNA hairpin containing a single *TerB* site, we mimic replisome-mediated DNA unwinding and directly show that strand separation alone can trigger the non-permissively oriented Tus–*TerB* to form a strong and long-lived lock. Remarkably, the Tus–*Ter* lock formed in 100% of our hairpin opening attempts under low force regimes. This is in stark contrast to Tus–*TerB* in the permissive orientation: here strand separation proceeds virtually unhindered, as *in vivo*. We are able to quantify the lock strength by measuring the lifetimes of the Tus–*TerB* complex under different force regimes. These experiments reveal that at high forces, dissociation of Tus still exhibits pauses in 100% of events, but Tus dissociation occurs on three (or more) characteristic timescales, suggesting that strand separation at high forces partitions the Tus–*TerB* structure into thermodynamically trapped sub-structures. We argue that the shorter-lived sub-structures correspond to intermediates in the process of full lock formation during replisomal strand separation, and that the longest-lived structure is the full lock.

By using this approach, we show that mutations in the lock domain can severely affect the lock characteristics. For example, H144, a residue deep in the Tus lock pocket, determines the strength of interaction of the Tus–*Ter* lock: force-dependent lifetimes of H144A decreased more profoundly than those of any of the other single-site mutations tested. F140, located at the side of the lock pocket, is found to play a key role in lock formation: the probability of seeing a full lock state disappeared for F140A. Interestingly, residue E49, located outside the lock domain and thought to play a pivotal role in the specific interaction of Tus with DnaB, is shown also to play a role in lock formation in the absence of any replication machinery: E49K displays a marked decrease in the probability of lock formation very similar to that of F140A, though now the chance to form a full lock state is significantly higher than for F140A. Conversely, neither the lock probability nor the force-dependent lifetime is affected significantly when Tus is mutated in the DNA-binding domain at the permissive face of the complex.

Our results strongly validate the molecular mousetrap model[22] by showing that Tus–*Ter* causes polar arrest of strand separation in the absence of any replication-related proteins. The interaction formed is extremely efficient and specific, and depends largely on interaction of the C6 base with the Tus lock pocket. Added to this, our results shed light on the lock formation mechanism and reveal how DNA processing enzymes could still influence lock formation in a non-specific manner. Our assay resolves the controversy that still surrounds this protein–DNA complex by providing direct insight into how different DNA processing enzymes in a head-on collision with Tus–*Ter* can exhibit varying blocking efficiencies, in particular by modulating the probability of lock formation through (non-specific) steric hindrance.

RESULTS

We mimic DNA replication fork progression using DNA hairpins containing a single *Ter* site

To investigate and dissect the mechanism of asymmetric replication fork blockage by *Ter*-bound Tus, we aimed to measure the dynamics of lock formation in an isolated fashion, i.e. in the absence of any other proteins. Since single-molecule, force-based assays can controllably invoke the double-stranded DNA unwinding that normally accompanies DNA replication, we set up a single-molecule assay using magnetic tweezers and DNA hairpins. Our experiments comprise three DNA hairpin designs with specific sequences inserted at their midpoints: the first hairpin contains a single *TerB* site in the permissive orientation (**Fig. 2a**); the second has the *TerB* site inverted, forming the non-permissive orientation (**Fig. 2b**); and lastly, the third hairpin contains a *TerB* site in the non-permissive orientation, but now including a point mutation at the GC6 site in which the highly conserved C6 base has been replaced by a guanine (GC-flip) (**Supplementary Fig. 1b**). Using this assay, we are able to mimic replication fork progression towards the Tus–*Ter* site by mechanically opening the DNA hairpin through pulling force only, without the need for any protein other than Tus. As the mousetrap model suggests a purely mechanical interaction of Tus–*Ter* upon strand separation, it predicts that in our setup lock formation should still occur in one direction (non-permissive) but not the other (permissive). Conversely, if protein-protein interactions are essential for proper lock formation, lock formation should be at most infrequent in our assay. Our tweezers-based assay is readily equipped to detect lock formation due to the difference in extension between a fully opened hairpin and one that is blocked halfway.

Asymmetric blocking of strand separation by Tus–*Ter* is invoked through mechanical unzipping of DNA hairpins

At low forces (<16 pN), base-paired DNA is energetically more favorable than single-stranded DNA, so the hairpin remains closed[25–27]. Upon increasing the force (>16 pN) in the absence of Tus, the hairpin opens, which can be seen as a rapid increase in extension for both the permissive and the non-permissive hairpin

(**Fig. 2c,d**, red traces). Repeating this experiment in the presence of Tus, the outcome is almost identical for the permissive *TerB* hairpin (**Fig. 2c**, blue trace): here Tus–*TerB* interaction leaves only a transient signal upon hairpin opening (**Supplementary Fig. 2h**). In contrast, the results are very different for the non-permissive hairpin (**Fig. 2d**, blue trace): here the maximal extension in the presence of Tus is only half of the fully opened hairpin, indicating that strand separation is blocked exactly at the *TerB* site (see Methods and **Supplementary Fig. 1a**). Strikingly, this behavior was observed for non-permissive hairpins in 100% of the experiments at 50 mM KCl and a Tus concentration of 2 nM. This universal blocking is observed despite the fact that in our experiments the effective speed at which the DNA helix is unwound is ~30 kbp·s^{-1} (**Supplementary Fig. 1c**), at least 20-fold faster than any replisome would unwind DNA. Increasing the force to 60 pN shows that the available ssDNA is stretched in a similar fashion as during the experiment in the absence of Tus, but now with extension remaining half of the fully opened hairpin at the same force. This demonstrates that the Tus–*Ter* lock remains in place at forces up to 60 pN and shows the remarkable strength of this locked complex. This experiment thus validates the protein–protein independency proposed by Mulcair *et al.*[22] by demonstrating that there is no need for direct protein–protein interaction to trigger lock formation. It also strongly suggests that this mechanism alone is readily equipped for the task of blocking an approaching replication fork, other helicases and transcription machinery alike.

Tus–*Ter* lock properties can be quantified by measuring constant-force barrier lifetimes

We measured the distribution of constant-force lock lifetimes for all protein and *Ter* variants (**Fig. 2e**), taking advantage of the natural force clamp mode and multiplexing capacity of magnetic tweezers. The distributions are highly reproducible and contain force-specific as well as mutant-specific signatures (**Fig. 3b,c**; **Supplementary Fig. 2a–c**). A main feature of all lock lifetime distributions is that they are (multi-)exponentially distributed, reflecting the stochastic nature of lock rupture (**Fig. 2f**). By applying the Bayes-Schwartz information criterion (BIC)[28] to the datasets, we find that the distributions contain evidence of two or three exponentially distributed states, depending on the type of Tus–*TerB* interaction investigated. We use maximum likelihood estimation to fit the data (**Fig. 2f**)[29], and obtain confidence intervals through bootstrapping[30] as described previously.[31] As these high-force measurements place a large tension on the DNA tethers, choosing the right force is a trade-off between tether lifetime[32] and the ability to resolve the different lock states (**Supplementary Fig. 1d**). Typically, we chose the force such that 3-exponential datasets exhibited a first short-lived exponential with a lifetime of ~1 s, a second exponential with a lifetime on the order of 10 s and a third, long-lived exponential on the order of 100 s.

Lock breakage shows different degrees of lock formation

We next set out to assess the force-dependent lifetimes of wild-type (wt) Tus–*TerB*. As we now more closely examine locking behavior, it is convenient to consider this system through a lock and key analogy, where the C6 base is the key that fits into the Tus lock pocket (**Fig. 3a**). In this analogy, the interaction between wt Tus and *TerB* should provide a signature analogous to a perfect match between key and keyhole (**Fig. 3a**, wt Tus–*TerB*), hence *a priori* we expect the force-dependent lifetimes to be significantly higher than lifetimes imposed by binding without locking. We found the force-dependent lifetimes of the wt Tus–*TerB* lock to be distributed over three states, with the longest-lived exponential distribution having a lifetime of ~720 s at 59 pN (**Fig. 3b,c**, purple circles) and individual lifetimes reaching up to a remarkable 2000 s at this force. The shortest-lived exponential state at 59 pN had a lifetime of ~1 s, and the intermediate state a lifetime of ~30 s. The lifetimes of all three states decrease in a concerted fashion as the force is increased, with the longest-lived distribution having a lifetime of 54 s at 93 pN (**Fig. 3c, Supplementary Table 3**). We also observe a force dependence in the probability of forming the longest-lived state: while at 93 pN there is a mere 7% chance a lifetime will belong to the longest-lived state, this probability increases to 73% at 59 pN (**Fig. 3d**). Conversely, trapping the system in one of the shorter-lived states becomes progressively more unlikely as force is decreased and suggests that the longest-lived state predominates at low forces (i.e. at forces likely to be exerted by the replisome). The force-dependent probability of all states also indicates that the first two states likely represent intermediate conformations that occur at all forces, including during replisome-mediated strand separation, while the longest-lived state is the full lock; thus, when the magnetic tweezers exert their highest forces, they simply prevent the short-lived conformations from proceeding to the fully-locked state prior to lock rupture.

When changing the orientation of *TerB* on the hairpin from the non-permissive to the permissive orientation, we observe lifetimes barely above the detection limit of our assay: lifetimes of permissive wt Tus–*TerB* obey a single-exponential distribution with a mean of 0.8 s at 19 pN (**Fig. 3c**, purple square; **Supplementary Fig. 2h**), and are too short to be detected at higher forces. Note that there is no single force at which both non-permissive and permissive lifetimes can both be measured: the lifetimes of non-permissive wtTus–*Ter* become too long for practical measurements at 19 pN (see **Supplementary Fig. 1d**). This implies that none of the states we find for the non-permissive orientation can be attributed solely to the binding of Tus in the absence of lock formation. To further investigate the origin of the observed states, we can compare the changes in lifetime and probability invoked by any type of change, wherein such changes can include alternations in ionic strength or a mutation in Tus and/or *TerB*, as we will now show.

Lock lifetimes are insensitive to ionic strength

As the reported dissociation constant (K_D) of the Tus–ds*TerB* complex has been shown to be highly salt-dependent[23], we investigated whether *lock formation* also exhibits a strong salt dependence. We observed that the fraction of rupture events recorded with a lifetime below our cutoff time of 10^{-2} s (i.e., the fraction of open hairpins at t = 0 s) increased from 0% at 50 mM to 14% at 350 mM KCl, while during these experiments care was taken to keep [Tus] well above (at least an order of magnitude) the reported salt-dependent K_D, thereby ensuring the continuous binding of Tus to *Ter*. Concomitantly, we observed that the lifetimes of the two longest-lived exponentials for wt Tus remain virtually unaffected when increasing the [KCl] from 50 to 350 mM, indicating that the lock *strength* is hardly affected by salt concentration (**Supplementary Fig. 2d–f**). In contrast, the reported K_D of the Tus–ds*TerB* complex increases from ~10^{-13} to ~10^{-8} M within the 50 to 350 mM range, a change of five orders of magnitude. Taking these observations into account, we conclude that though the rate of lock formation is slightly affected by ionic screening, once the lock is formed its strength remains unaffected. This is in accord with the SPR data of Mulcair *et al.*[22]

The binding and lock domains are two independent entities

Next we assess the extent to which we can treat binding and locking domains as two separate entities. The crystal structure of the locked wt Tus–*TerB* shows that DNA sequence recognition and binding can be largely attributed to a DNA binding domain that primarily consists of 2 antiparallel β-strands that interact with the major groove of *Ter* DNA (**Fig. 1b**). The lock domain is the area where the C6 base specifically interacts with several amino acid residues (**Fig. 1d and Supplementary Fig. 3**). Site-specific mutation in the DNA binding domain (Q250A, **Supplementary Fig 3e**) is known to result in a sharp increase of the K_D[23], but whether it affects lock kinetics is unknown. In our magnetic tweezers experiments, this binding domain mutant Q250A exhibits lifetime distributions that are identical to those of wt at the same forces (**Fig. 3c**, cyan). From this, we conclude that lock formation is not significantly affected by a change in the binding domain far away from the lock. We see no correlation between Tus K_D and lock strength, and therefore the lock and binding domains can be treated as two separate entities.

The C6 base is crucial for lock formation, and decoupling lock formation from mechanical probing does not alter state populations

We subsequently set out to examine the effect that changing the key, i.e. the C6 base (**Fig. 3a,** switch from green to red key), has on the wt Tus–*Ter* lifetimes. A single base pair inversion of the *TerB* sequence at position 6, which modifies the *Ter* key from C to G, was reported to profoundly affect the locking efficiency, as expected from the model of Mulcair *et al.*[22] In our experiments, inversion of GC6 indeed has a dramatic effect on the lifetimes (**Fig. 3c**, purple triangles), as the dominant lifetime is no greater than 1 s at 40 pN. By

comparison, the dominant lifetime of wt Tus–*TerB* is at least two orders of magnitude higher based on extrapolation of the lifetimes of the fully locked state observed in the range 59–93 pN (**Fig. 3c**, purple circles and solid lines). Despite the severe decrease in the observed lifetimes, the G6 conformation continues to impose an increased barrier to hairpin opening, as the lifetimes remain well above those found for the binding in the absence of lock formation (**Fig. 3c**, purple square). For wt Tus with the modified key, the BIC further reveals the presence of just two exponentially distributed lifetimes (**Supplementary Fig. 2g**, purple), with the longest lifetime decreasing from 39 s to 0.7 s in the 29–40 pN range (**Fig. 3c**, purple triangles and dashed line). The latter state might correspond to the first, short-lived state observed for wt Tus–*TerB*, although this does not appear very likely since even in this force range it is already shorter-lived than the wt first state (1.3 s at 59 pN). We also assessed whether uncoupling lock formation from mechanical probing (by creating a hairpin containing an unpaired region of 5 bases containing C6, see **Supplementary Fig. 1b**) would populate the fully locked state even at high forces, as Mulcair et al. have shown that this 5-base mismatch dramatically increases the affinity of the Tus–*Ter* complex compared to fully duplex DNA.[22] We show, however, that the resulting state-probabilities are identical to that of normal wt Tus–*Ter* (**Fig. 4c**), indicating pre-formation of the lock does not alter the population of the states.

Principal features of the Tus-*Ter* lock mechanism are revealed through mutations in or near the lock domain

To investigate how the *Ter* key enters the Tus lock, we performed experiments on a series of Tus mutants that are mutated in or near the lock domain. Two amino acid residues H144 and F140 are situated directly in the lock domain, and based on the crystal structure their roles differ in a subtle, though significant manner. Amino acid residue H144 lies deep within the lock pocket and interacts only with the C6 base. Replacement of this histidine by alanine will remove the imidazole ring as well as a positive charge, leaving a cavity deep within the pocket (**Supplementary Fig. 3b**, in blue). In our lock-and-key representation, we depict this by changing the inner shape of the lock (**Fig. 3a**, light blue). Amino acid residue F140 lies closer to the outer edge of the lock pocket than H144. F140 still interacts with C6, and a stacking interaction of the phenyl ring with the adjacent A7 base is also present (**Supplementary Fig. 3c,** in orange). Removal of the phenyl ring in the F140A mutant will thus lead to a gap at the edge of the lock pocket. In the schematic representation, we depict this as a widening of the keyhole while keeping the inner shape of the lock intact (**Fig. 3a**, orange). Amino acid residue E49, which has been linked to the putative specific protein–protein interaction between Tus and the *E. coli* DnaB helicase, lies just outside the lock domain (**Supplementary Fig. 3d**, in green). Though the shape of the lock pocket remains unaffected by this mutation (**Fig. 3a**, green keyhole identical to purple wt), we include mutant E49K in our tests as it has been reported to be deficient in polar replication fork arrest[14,15].

Lock mutant F140A prevents Tus–*Ter* forming a fully locked state and makes the lock less specific for C6

Measurements on the lock-domain mutant F140A show a marked decrease in lifetimes at 59 pN of pulling force (**Fig. 3b**, orange). Upon fitting the distribution we find that the longest-lived exponential now has a lifetime of ~55 s, compared to 720 s for wt Tus at the same force (**Fig. 3c**, orange circles and solid line). We also observe that this third, longest-lived state has all but disappeared: the probability of entering this state is reduced from 73% for wt Tus to 1.8% for F140A in the same regime (**Fig. 3b**,orange: note the absence of counts >100 s; **Fig. 3e**, orange bar in 'full lock' column). Thus, F140 appears to give rise to the third, long-lived state observed in Tus species with an intact lock pocket, and as such plays a key role in the probability of forming a fully locked state. Similar to wt Tus, the probability has a clear force-dependence: decreasing the force to 47 pN increases full lock probability to 31% (**Fig. 3d**, orange). Combining F140A with the mutated *Ter* site (**Fig. 3a**, red key with orange lock) also further reduces the force-dependent lifetimes markedly, but somewhat surprisingly the resulting force-dependent lifetimes exceed those of wt Tus with the mutated *Ter* site (**Fig. 3c**, orange triangles and dashed line; **Supplementary Fig. 2g**, orange). This apparent increase in lock strength in the presence of an altered key indicates that mutation of F140 leads to a decreased specificity for having only the highly conserved C6 base interact with the lock.

Lock mutant H144A weakens the lock more than any of the other single-site Tus mutants

Replacing H144, the amino acid residue deep inside the lock pocket, by alanine leads to a more substantial decrease in Tus–*Ter* lock lifetimes than F140A (**Fig. 3b,c**, blue circles and solid lines). The dominant lifetime extracted at 59 pN was found to be ~2 s, whereas those of wt and Q250A at the same force lie two orders of magnitude higher. The datasets were found to exhibit lifetimes measurable on experimental timescales over a wide range of forces (24–59 pN), all containing three exponential states. At 59 pN, mutation H144A leads to an even larger drop in the probability of entering the third, longest-lived state than mutation F140A: from 73% for wt Tus this has now become 0.7% (**Fig. 3e**, blue). As observed for F140A and wt Tus, there is also a clear force-dependence of the probability to form a fully locked state for H144A: the probability increases to 26% at 24 pN (**Fig. 3d**, blue line). While mutation H144A is the single-site mutation with the largest effect on the lock lifetimes, the decrease is not as severe as that induced by mutation of the C6 base to a G in combination with wt Tus. When combining the H144A lock mutant with the mutated *TerB* site (**Fig. 3a**, red key with blue lock), the lifetimes were so short that our time resolution only allowed the full distribution to be measured at the lower force of 19 pN. The resulting distribution is not unlike that obtained for permissively oriented wt Tus (0.8 s at 19 pN, **Supplementary Fig. 2h**, blue), with the dominant lifetime being ~0.5 s (**Fig. 3c**, blue circle). This indicates that the lifetimes found for H144A with wt *TerB* result from the specific interactions of the C6 base with the remaining amino acid residues in the lock pocket; further

modification of the key (by flipping the C6 base) within this altered lock results in the loss of all locking interactions.

Mutation E49K leads to a severe decrease in the probability of full lock formation, but the lifetimes are wt-like

Amino acid residue E49 is not part of the lock domain, though it does make a water-mediated hydrogen-bonding contact with the 5'-phosphate of A7 in the locked complex[22]. For E49K, we obtain a distribution of lifetimes at 59 pN that contains the same three exponentially distributed states as wt Tus–*TerB* (**Fig. 3b**, green circles and solid line), with the longest-lived state having a fit lifetime of 933 s (720 s for wt). However, the probability of a given lifetime originating from this state has decreased significantly from 73% for wt to 6% for E49K (**Fig. 3e**). The first two exponentials fully overlap with the two shortest-lived for F140A (**Fig. 3b**, orange). This suggests that the DNA-phosphate interaction with E49 is crucial for guiding the C6 base into its pocket to form the fully locked state. Interestingly, mutating the *TerB* site (**Fig. 3a**, magenta key with green lock) now causes loss of almost all lifetimes above ~1 s for forces above 26 pN, similar to the barrier imposed by Tus–*Ter* in the permissive orientation (**Supplementary Fig. 2g**). However, closer inspection reveals that we continue to observe longer-lived events with a very low probability of ~1.5% (**Supplementary Fig. 2h, Supplementary Table 2**); when extrapolated to higher forces, the longer-lived lifetimes (**Fig. 3c**, green triangles) resemble the much more probable states found for wt Tus interacting with the mutated *Ter* site in the 29–40 pN range (**Fig. 3c**, open purple triangles, **Supplementary Table 2**). This indicates that while the wt lock domain continues to interact with the incorrect G6 key, mutation of E49 renders such an interaction very unlikely. Our observations clearly link the change invoked by E49K to a change in probability of forming the third, fully locked state. As experiments by others have linked this particular mutation of E49 to a deficiency in replication fork arrest[14,15], this may suggest that the first two lock states are likely not sufficient to cause replication arrest *in vivo*.

Given these observations, we propose a kinetic model for wt lock formation containing three states (**Fig. 4a**). In this model, the Tus–*Ter* complex strengthens progressively and irreversibly as passage from one stable state to the next proceeds until the final, fully locked and longest-lived state is reached. Loss of the long-lived lock state as force is increased is not due to slower transitions to stronger lock states, but rather to increased rate of disruption of the weaker lock states as the force-dependent trends in the rates show (**Fig. 4b**).

DISCUSSION

Our results have important implications for understanding of how the Tus–*Ter* lock is formed. We can directly discard the notion that Tus–*Ter* requires specific interaction with DnaB to form a stable lock and

block replication fork progression for extended times. Our results strongly suggest that strand separation followed by specific interaction of the *Ter* C6 base with the Tus lock domain is the only mechanism needed for polar arrest. Evidence for this can be found in the fact that lock probabilities and lifetimes are affected by mutations in the lock domain, or mutation of the C6 base, but not by a mutation in the DNA binding domain. We further observe that mutant E49K, which is hypothesized to be deficient in polar replication fork arrest due to the elimination of specific protein–protein interactions[14,15], gives rise to lifetimes identical to that of wt Tus, only now with a severely decreased probability of entering the longest-lived state. This ties the observed deficiency of *in vivo* fork arrest to the drop in occurrence of the longest-lived state found in our experiments. In other words, the longest-lived state is likely to be the native lock conformation implicated in *in vivo* fork arrest, and amino acid residue E49 is shown to be part of the mechanism that facilitates formation of a tight interaction between C6 and the lock pocket.

Our experiments also demonstrate that flipping of the C6 base and subsequent interactions with the Tus lock pocket is not a rate-limiting step in the lock formation process. In fact we see little force-dependence in the inter-state rates (k_{12} and k_{23}, **Figure 4a**,**b**), and the force-dependence of state probabilities is caused solely through their competition with force-dependent state exit rates (k_{10}, k_{20} and k_{30}). As force influences the speed of strand separation and thus the time available for C6 flipping, while not affecting the inter-state transfer rates, the C6 flipping is likely not rate-limiting at the comparatively low unwinding velocities (**Supplementary Fig. 1c**) of the *E. coli* replisome.

This notion is strengthened further by our observation that pre-formation of the lock yields a distribution of lifetimes identical to Tus–*Ter* without pre-formed lock (**Fig. 4e**). In the pre-formed lock situation, it can be assumed that the C6 base has reached its equilibrium lock position. Our results imply that our pulling experiment provides sufficient time for this equilibration, even though the pulling experiment as a whole is a system in non-equilibrium. This is directly understood by comparing the typical timescales of DNA unwinding and the timescale of molecular rearrangement of the DNA bases upon disruption of Watson-Crick base pairing. The unwinding rate of DNA by a replisome is of the order of 1 kb/s, and in our pulling experiments this rate is around 30 kb/s. Typical molecular single-bond rotations are known to take place on femtosecond to picosecond timescales, and larger scale rotations (e.g. rotation of a nucleotide) occurring in the nanosecond to microsecond range.[33–35] This implies that there is at least a 3 order of magnitude difference between the rate of unwinding and the rate at which flipping of C6 and concomitant molecular rearrangements take place. In other words, there is ample time for C6 to reach its equilibrium locked conformation in our experiments. The identical experimental outcomes also rule out possible effects of a difference in conformation between our pulling experiments and lock formation in the absence of tension.

Simply, in our pulling experiments, we trap the system into one of three possible states and observe the lifetime distribution corresponding to that particular state, whereas in the tension-free situation there is likely to be exchange between the different states.

The interpretation that the longest-lived state is the native or full lock state implies that the probability of full lock formation is actually significantly lower than 100% at the highest forces measured, as we have shown (**Fig. 4d**, purple). So even though the probability of observing transient blocking of hairpin strand separation at the highest forces in our experiments is 100%, this does not directly correlate with full lock formation. The force-dependent trends in lock probability do however suggest that the full lock is the dominant state at low forces for wt Tus, and e.g. suggest that the mutant with the lowest full lock probability (H144A), may still have a significant chance of blocking an approaching replisome. A more direct link between our probabilities and *in vivo* arrest efficiencies will require knowledge of e.g. the amount of work performed by a replisome.

The difference between the high efficiency of reaching the full-lock state in our experiments and the lower efficiencies of replisome arrest observed *in vivo* must therefore have a cause arising from interactions not captured in our experiments. These interactions might be invoked by the presence of an enzyme running into Tus–*Ter*: steric effects, i.e. functional protein–protein interaction, could then be the cause of the observed discrepancy in efficiency. So instead of being the basis of fork arrest, functional interaction could have an antagonistic effect *in vivo*. Our experiments with mutant E49K suggest a possible mechanism: as the mutation in the Tus protein modulates the probability of forming the fully locked state without affecting the lifetime of the lock, it is not unthinkable that an enzyme running into Tus–*Ter* could invoke a similar effect through functionally interacting with that same residue. Our experiments with wt Tus and E49K then respectively set the upper (no interaction, thus high lock probability) and lower (E49 function completely disrupted, low lock probability) boundary of blocking probabilities. Two different enzymes that run into non-permissive Tus–*Ter* can then in turn have their own characteristic probabilities of being blocked due to the different ways these enzymes (non-specifically) interact with amino acid residues such as E49 upon collision; i.e. functional interaction might prevent lock formation from happening.

It remains to be determined to what extent the two shortest-lived lock states are capable of causing arrest of DNA-processing enzymes, since there is currently no way to link the probabilities associated with each state to the probability of *in vivo* arrest. It is clear, however, that these two ‘lesser’ lock states still pose a significant barrier to strand separation, much more so than the mere binding of Tus alone.

In summary, we have directly shown the great efficiency with which the locked Tus–*Ter* complex is formed in one orientation, but not the other. We further show that lock formation is not dependent on specific interaction with the replicative proteins native to the host cell, while we argue that steric effects can hinder lock formation in an enzyme-dependent manner. Using specific mutants, we are able to discriminate DNA binding and locking domains in this system. H144 is a critical amino acid residue that influences the lifetimes of all three lock states, while E49 mainly guides the C6 base into the fully locked state. Combining mutations in the lock domain with the inverted GC6 base pair, we find that for mutant H144A the locking capability is completely dismantled while mutant E49K only decreases the probability of the state found for wt Tus with the GC flipped *TerB*. This strongly points to that the lock pocket specifically allows entry of only a C-base, only by which then tight interaction with H144 becomes possible. We conclude that mutating F140 at the edge of the lock pocket decreases the specificity for the C6 base relative to G.

The wt Tus–*Ter* lock is extremely robust and rather insensitive to ionic strength, as opposed to the binding affinity of Tus–*Ter*. As the interaction between a 23 bp sequence and a relatively small 36 kDa protein is so specific, fail-safe and subject to modulation through straightforward site-directed mutagenesis, we foresee that this system can be readily exploited in practical ways: for instance, by using Tus–*Ter* as a fail-safe transcription roadblock to produce RNA aptamers in a high-throughput RNA-protein affinity screening assay[36]. Indeed, as most *in vivo* experiments thus far point towards molecular motor arrest probabilities significantly below 1, we hypothesize that protein–protein interactions, rather than forming the basis of promoting polar arrest, might actually perform the opposite function of *disrupting* proper lock formation.

METHODS

DNA hairpins. Plasmids pTER and pTER_Rev, containing the *TerB* site in either the non-permissive or permissive orientation respectively, and flanked by phage λ sequences, were obtained from Invitrogen. Plasmid pTER_mutant (*TerB*-C^6>G) was generated from pTER by site-directed mutagenesis using primers 1 and 2 (primer sequences are in **Supplementary Table 1**). Hairpins were constructed in a multi-step process (**Supplementary Fig. 1**). First, 1 kb fragments containing the *TerB* site were amplified from the three pTER plasmids using primers 3 and 4 (**Table S1**). These fragments were digested with *Bsa*I and ligated at one end with a 42-bp oligonucleotide to form a U-turn (oligonucleotide 5, **Table S1**). Hairpin handles were created by PCR amplification of a 1.2 kb pBluescript SK+ (Stratagene/Agilent Technologies, Inc, Santa Clara, CA) fragment using primers 6 and 7 (**Table S1**) in the presence of either biotin-16-dUTP (Roche Diagnostics, Basel, Switzerland), digoxigenin-11-dUTP (Roche Diagnostics, Basel, Switzerland) or aminoallyl-dUTP (Thermo Scientific, Waltham, MA). Prior to ligation to spacer oligonucleotides, handles were digested with either

*Bam*HI or *Not*I. The upper spacer of the hairpin was generated by annealing 5'-phosphorylated oligonucleotides 8 and 9 (**Table S1**) together and ligating this double stranded DNA fragment to the *Not*I-digested biotin-labelled handle. The lower spacer was made by annealing 5'-phosphorylated primers 10 and 11 (**Table S1**) together and ligating them to the *Bam*HI-digested digoxigenin or aminoallyl-labelled handle. Finally the overhang of these handle/spacer constructs were allowed to anneal to form a short (50-bp) stem with a *Bsa*I overhang that was ligated to the free *Bsa*I site of the 1 kb *TerB* fragment. All oligonucleotides were obtained from Biolegio B.V., Nijmegen, The Netherlands.

Tus proteins. N-terminally His_6-tagged Tus and mutant derivatives were prepared as described[22,23]; their concentrations were determined spectrophotometrically (ε_{280} = 39,700 $M^{-1}cm^{-1}$).

Magnetic tweezers – experimental configuration. The magnetic tweezers implementation used in this study has been described[31,32,37]. In short, light transmitted through the sample was collected by an oil-immersion objective (Apochromat 100X, NA = 1.25, Olympus, USA) and projected onto a 1.4 MP CMOS camera (Falcon 1.4M, Teledyne Dalsa, Canada) with a sampling frequency of 100 Hz. The applied magnetic field was generated by a pair of vertically aligned permanent neodymium-iron-boron magnets (SuperMagnete, Switzerland) separated by a distance of 1.0 mm, suspended on a motorized stage (M-126.PD2, Physik Instrumente, Germany) above the flow cell. Additionally, the magnet pair could be rotated about the illumination axis by an applied DC servo step motor (C-150.PD, Physik Instrumente, Germany).

Data processing. Image processing of the collected light was used to track the real-time position of both surface-attached reference beads and superparamagnetic beads coupled to DNA tethers in three dimensions. We implemented custom written software in C++, CUDA and LabView (2011, National Instruments Corporation, USA) that is suited for high-throughput tracking in magnetic tweezers[37]. In short, tracking of the *x*,*y* coordinates is performed using center-of-mass computation followed by a further refinement using the quadrant interpolation algorithm. Localization of the bead's *z*-coordinate is achieved by creating a radial profile using the refined *x*,*y* coordinates, and comparing this profile to a pre-recorded LUT of radial profiles. After subtraction of the reference bead position to correct for instrumental drift, the *x*, *y* and *z* position of the DNA-tethered beads were determined with a spatial accuracy of <3 nm. The upward stretching forces on the DNA tethers by the superparamagnetic beads were calibrated from analysis of the extent of its Brownian motion, whereby spectral corrections were employed to correct for camera blur and aliasing[38,39].

Sample preparation and data acquisition. The sample preparation used in this study has been described in detail elsewhere[32]. In short, the DNA hairpins (final concentration ~50 pg/µl) were mixed and incubated for 2

min with 20 µl streptavidin-coated paramagnetic polystyrole beads (M270 Dynabeads) at room temperature in TRIS buffer (50 mM Tris-HCl pH 7.9, 50 mM KCl, 0.1 mM EDTA, 0.01% Triton X-100). The supernatant was replaced by 50 µl TRIS buffer followed by a 15 min incubation of the bead-DNA solution in the flow cell containing an anti-digoxigenin-coated nitrocellulose surface. Non-tethered beads were removed by flushing with 1 ml TRIS buffer, applying a high (30–40 pN) force while rotating the magnets (10 rpm), followed by flushing with more buffer until all non-tethered beads had been flushed out. All KCl buffers used in this study exclusively contained 50 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 0.01% Triton X-100 unless noted otherwise. Tus proteins were diluted 10^3-fold from stock (to ~10 nM) unless high salt concentrations required higher concentrations. Data were acquired at 100 Hz, 10 ms acquisition time. Force-extension curves were obtained through changing the magnet position in an exponential fashion such that the force change was linear. Constant-force lifetime experiments were obtained by lowering the magnets in a linear fashion (10 mm/s) to the desired distance. The lifetime is the time measured between arrival of the magnets at their final position and the further opening of the hairpin from the locked to the fully opened state.

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AUTHOR CONTRIBUTIONS

N.E.D and N.H.D. designed the research. B.A.B. and N.H.D. designed the experiments. D.D. designed and assembled the magnetic tweezers apparatus. B.A.B. performed the experiments. B.A.B., B.C., T.L., R.J. and N.H.D. designed, and B.C. and T.L. made the DNA hairpin constructs. Z-Q.X. and S.J. purified the Tus proteins. B.A.B. and M.D. analyzed the data. M.D. developed the application of MLE to force spectroscopy data. B.A.B., D.D., M.D. and N.H.D. interpreted the data. S.J. and N.E.D. contributed to discussions concerning the model and *in vivo* observations. B.A.B. and N.H.D. wrote the paper.

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FIGURE CAPTIONS

Figure 1 The Tus–*Ter* complex structure and domains. (**a**) Location and orientation (turquoise for permissive, red for non-permissive face) of 23 base pair *Ter* sites in the *E. coli* chromosome. (**b**) The crystal structure of the locked Tus–*Ter* complex (PDB ID: 2I06) with a schematic representation directly below showing the protein has a DNA binding domain located mainly in the two antiparallel β-strands interacting with the major groove of *Ter* DNA (dark blue for base-specific interactions, light blue for non-specific interactions), as well as a lock domain (orange) where several amino acid residues interact specifically with C6 upon strand separation (**c**, **d** orange).

Figure 2 Magnetic tweezers assay used to quantify Tus–*Ter* lock mechanism. Schematic overview of a permissive (**a**, **c**) and a non-permissive (**b**, **d**) Tus–*Ter* experiment. (**a**) In the permissive experiment, force-induced DNA strand separation causes weakening of the interaction between Tus DNA-binding domain and the *Ter* site, with subsequent disruption of the Tus–*Ter* interaction as a result. (**c**) In our MT DNA hairpin experiment this implies full opening of the hairpin, yielding almost identical force extension curves for hairpins with or without Tus (blue and red respectively, dotted lines represent reannealing of hairpin during force decrease). (**b**) With the non-permissively oriented *Ter* on a DNA hairpin, strand separation will first cause the C6 base to flip into the high-affinity protein-binding pocket of Tus, resulting in a locked Tus–*Ter* complex. In other words, strand separation in our non-permissive hairpin will be blocked in the presence of Tus, resulting in an extension that is halved (**d**, blue) compared to the same hairpin in the absence of Tus (**d**, red). (**e**) To quantify lock behavior we measure lock lifetimes under constant force. (**f**) This yields a multi-exponential distribution of lifetimes (black circles) to which we fit our kinetic model (red, see text, **Fig. 4a** and **Supplementary Fig. 4** for explanation).

Figure 3 The effect of Tus mutations on lock formation. (**a**) To investigate the locking mechanism of Tus–*Ter*, we measure the force dependent lifetimes of various Tus proteins mutated in or near the lock domain (depicted as a keyhole) forming a locked complex with either *TerB* (green key) or *TerB* with a mutated GC6 base pair (magenta key). Wt Tus (purple) and *TerB* (green key) are a perfect match, H144A (blue) is a mutation deep within the lock (blue, cavity behind keyhole becomes larger), F140A is a mutation at the edge of the lock pocket (orange, keyhole becomes larger) and E49K is a mutation that lies outside the lock pocket (green, keyhole identical to wt). (**b**) The distribution of lifetimes of wt Tus (purple, N = 94), the three lock-domain mutants (H144A: blue, N = 642, F140A: orange, N = 344; E49K: green, N = 323) and binding domain mutant Q250A (cyan, N = 90) when bound to *TerB* (green key) at 59 pN (circles are data, solid lines are fits) allow us to investigate the roles of these specific amino acids residues in lock formation. (**c**) The trends in

force-dependent lifetimes of the eight lock domain investigations depicted in (**a**), as well as those of binding domain mutant Q250A (cyan) and wt Tus in the permissive orientation (purple square) can be compared. Shown is the lifetime of the longest-lived exponential; solid lines and circles are trends with *TerB*, while dashed lines and triangles are with mutated *TerB.* Error bars indicate the 1-σ confidence interval. (**d**) The probability of entering the third, full lock state also decreases with force (same color scheme as (**c**). Error bars indicate the 1-σ confidence interval). (**e**) The state-associated probabilities extracted (supplementary equations 1–4, Table S2). By comparing the state probabilities of each Tus species on *TerB* at 59 pN (purple, cyan, green, orange and blue bars respectively represent wt, Q250A, E49K, F140A and H144A. Error bars represent the 1-σ confidence intervals), we see that the probability of entering the third, longest-lived state decreases significantly upon mutation of the lock domain, but not the DNA binding domain (Q250A).

Figure 4 Modeling Tus–*Ter* lock formation and extracting state-associated probabilities. (**a**) By fitting our 3-state exponential model (**Supplementary Information 4**) to the datasets, allows us to extract the kinetic rates (b) associated with the observed exponential states (Supplementary equations 5–7, Table S3). We observe an exponential increase in all state exit rates (k_{10}, k_{20}, k_{30}; yellow, orange and red, respectively) with increasing force, while the rates connecting states 1 and 2 (cyan) as well as states 2 and 3 (cyan, fill) remain roughly constant (error bars indicate 1-σ confidence intervals). (**c**) State probabilities extracted through fitting the lifetime distributions at 59 pN for wt Tus–*Ter* (purple, same data as **Fig. 3e**) and wt Tus on the Ter site containing the mismatched bases 3-7 (yellow). The inset shows the force-extension curves of both types of hairpins, where the force at which the hairpin returns to its fully closed state consistently lies in the entropic regime (i.e. the forces at which ssDNA is no longer fully stretched) for the mismatch hairpin (yellow arrow, ~ 7 pN), while for the normal hairpin the closing force lies significantly higher (purple arrow, ~ 13 pN).