Strand separation establishes a sustained lock at the Tus–Ter replication fork barrier

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The bidirectional replication of a circular chromosome by many bacteria necessitates proper termination to avoid the head-on collision of the opposing replisomes. In Escherichia coli, replisome progression beyond the termination site is prevented by Tus proteins bound to asymmetric Ter sites. Structural evidence indicates that strand separation on the blocking (nonpermissive) side of Tus–Ter triggers roadblock formation, but biochemical evidence also suggests roles for protein-protein interactions. Here DNA unzipping experiments demonstrate that nonpermissively oriented Tus–Ter forms a tight lock in the absence of replicative proteins, whereas permissively oriented Tus–Ter allows nearly unhindered strand separation. Quantifying the lock strength reveals the existence of several intermediate lock states that are impacted by mutations in the lock domain but not by mutations in the DNA-binding domain. Lock formation is highly specific and exceeds reported in vivo efficiencies. We postulate that protein-protein interactions may actually hinder, rather than promote, proper lock formation.

DNA replication in E. 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. As the replication forks approach the terminus, each encounters five 23-bp Ter DNA sites (out of a total of ten, denoted TerA–E; Fig. 1a) bound in a specific orientation by a 36-kDa DNA-binding protein called Tus3–8 and proceeds unhindered. However, when a replication fork continues beyond the terminus, Tus–Ter is approached from the opposite direction (Fig. 1a), triggering it to form a tightly locked complex and bringing the replication fork to a halt9–12. Each Ter site is nonpalindromic, does not contain any direct repeats and has a strictly conserved G-C6 base pair followed by a highly conserved 13-bp core region. Tus is a monomeric protein that forms a simple 1:1 complex with Ter (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 protein10,13. The Tus–TerB complex has a reported dissociation constant (Kd) of 44 pM in 50 mM NaCl10. 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? On one hand, various studies imply specific protein-protein interactions between Tus and the replicative helicase DnaB. Tus–Ter is much more effective in its natural host, for instance, while the functionally similar but structurally unrelated Bacillus subtilis replication termination system works well in E. coli11,12. Tus–Ter blocks DnaB, but not the Rep helicase, in vitro13, and evidence from yeast two-hybrid analysis shows specific interactions between DnaB and Tus14. On the other hand, ample evidence suggests a protein-independent polar blocking mechanism. For example, RNA chain elongation catalyzed by T7, SP6 and E. coli RNA polymerases is impeded by Tus–Ter in a polar manner15,16. Tus–TerB also blocks the actions of the UvrD, Rep, PriA and SV40 large T antigen helicases, indicating low specificity for DnaB alone17–20.

In 2006 light was shed on this molecular roadblock through a surface plasmon resonance (SPR) study of the dissociation of Tus from forked TerB oligonucleotides, which was supported by a crystal structure of a forked, ‘locked’ Tus–Ter complex21. This locked complex exhibits significant structural differences at the fork-blocking (nonpermissive) face in comparison with the dsTerA-bound, but not locked, Tus structure elucidated a decade earlier1. The locked conformation reveals that, of the single-stranded (ss) DNA 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 (Fig. 1d). These Tus lock domain residues are distinctly different from those involved in sequence recognition and binding affinity22. It was therefore 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 triggered by strand separation invoked by the approaching replication machinery21.

The mousetrap model has two major implications. First, it suggests that binding and lock formation are two different mechanisms that can be ascribed to different domains of Tus. Second, lock formation through strand separation could occur independently of any specific protein-protein interactions. Nevertheless, convincing evidence arose that translocation of DnaB on double-stranded (ds) DNA in the absence of unwinding is sufficient to provoke polar arrest23. Although this result did not require it, these authors proposed an alternative model in which the DnaB helicase binds specifically to Tus. They argued that formation of the locked complex may act as a backup mechanism when protein-protein interaction fails but may not be sufficient on its own.

In this study, we used 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 a mechanical force to unwind a DNA
hairpin containing a single TerB site, we mimicked replisome-mediated DNA unwinding and directly showed that strand separation alone could trigger the nonpermissively oriented Tus–Ter to form a strong and long-lived lock. Remarkably, the Tus–Ter lock formed in 100% of our hairpin-opening attempts. This was in contrast to Tus–Ter in the permissive orientation, where strand separation proceeded virtually unhindered. We quantified lock strength by measuring the lifetimes of the Tus–Ter complex at different forces. These experiments revealed that at high forces Tus dissociation occurred on three (or more) characteristic timescales, suggesting that strand separation at high forces partitioned the Tus–Ter structure into thermodynamically trapped substructures. We argue that the shorter-lived substructures correspond to intermediates in the process of full lock formation during replisomal strand separation and that the longest-lived structure is the full lock.

Our results strongly validated the molecular mousetrap model by showing that Tus–Ter caused polar arrest of strand separation in the absence of any replication-related proteins. We showed that the interaction was efficient and was not limited by the rate of C6 flipping and finding the lock pocket. Using specific mutants we were able to discriminate DNA-binding and locking domains in this system. Residue H144, located deep in the Tus lock domain, 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, was found to be involved in the specificity of the lock pocket for a C base. Notably, a mutation in residue E49, which is located outside the lock domain and thought to play a pivotal role in the specific interaction of Tus with DNA, displayed a marked decrease in the probability of lock formation even though the lock lifetime was identical to that of wild-type (WT) Tus–Ter. This showed that E49 plays a crucial role in guiding C6 to the lock domain and that interfering with specific residues surrounding the Tus lock modulates the probability of forming a tightly locked Tus–Ter complex. Conversely, a mutation in the DNA-binding domain at the permissive face of the complex did not affect locking behavior. As in vivo experiments point toward probabilities of molecular-motor arrest substantially below those found here, we hypothesize that, instead of forming the basis of promoting polar arrest, protein-protein interactions may actually perform the opposite function of hindering proper lock formation. 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 (nonspecific) steric hindrance.

**RESULTS**

**Mimicking replication fork progression using DNA hairpins**

We set up a single-molecule assay using magnetic tweezers and DNA hairpins, which allowed us to controllably invoke the dsDNA unwinding that normally accompanies DNA replication, only now in the absence of the replication proteins. Our experiments initially comprised three DNA hairpin designs with specific sequences inserted at their midpoints: the first hairpin contained a single TerB site in the permissive orientation (Fig. 2a); the second had the TerB site inverted, forming the nonpermissive orientation (Fig. 2b); and lastly, the third hairpin contained a TerB site in the nonpermissive orientation, but included a point mutation at the G-C6 site in which the highly conserved C6 base was replaced by a guanine (referred to here as the ‘GC flip’) (Supplementary Results, Supplementary Fig. 1b). As the mousetrap model suggests a purely mechanical interaction for Tus–Ter upon strand separation, it predicts that, in our setup, lock formation should still occur in one direction (nonpermissive) but not the other (permissive). If protein-protein interactions are essential for proper lock formation, the phenomenon should, at most, be infrequent in our assay. We detected lock formation by measuring the difference in extension between a hairpin that is fully opened and one that is blocked halfway.

**Tus–Ter blocks force-induced unwinding**

At low forces (<16 pN), base-paired DNA is energetically more favorable than ssDNA, so the hairpin remains closed. Upon increasing the force (>16 pN) in the absence of Tus, the hairpin opens, and this could be seen as a rapid increase in extension for both the permissive and the nonpermissive hairpins (Fig. 2c,d, red traces). Repeating this experiment in the presence of Tus resulted in an almost identical outcome for the permissive Ter hairpin (Fig. 2c, blue trace). Here the Tus–Ter interaction left only a transient signal upon hairpin opening (Supplementary Fig. 2h). In contrast, the results were very different for the nonpermissive hairpin (Fig. 2d, blue trace)—here the maximal extension in the presence of Tus was only half of the fully opened hairpin, indicating that strand separation was blocked exactly at the Ter site (see Online Methods and Supplementary Fig. 1a). This behavior was observed for nonpermissive hairpins in 100% of the experiments at 50 mM KCl and a Tus concentration of 2 nM. Increasing the ionic strength to 350 mM resulted in a modest decrease in the occurrence of blocking, but did not affect the lock strength (Supplementary Fig. 2d–f). The high efficiency of lock formation still occurred despite the fact that in our experiments the DNA helix was unwound at a rate of ~30 kbp/s (Supplementary Fig. 1c), which is at least tenfold faster than any replisome would unwind DNA. Increasing the force showed that the Tus–Ter lock could remain in place at forces up to 60 pN, demonstrating the remarkable strength of this locked complex. This experiment thus validated the proposed protein-protein independence for fork arrest and strongly suggested that the Tus–Ter locking mechanism alone is readily equipped for the task of blocking an approaching replication fork, other helicases and the transcription machinery alike.
second exponential with a lifetime on the order of 10 s and a third, long-lived exponential on the order of 100 s.

In examining Tus 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 nonpermissive Ter should provide a signature analogous to a perfect match between key and keyhole (Fig. 3a, WT Tus–Ter). We found the force-dependent dwell times of the WT Tus–Ter 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). The shortest-lived exponential state at 59 pN had a lifetime of ~1 s, and the intermediate state had a lifetime of ~30 s. The lifetimes of all three states decreased in a concerted fashion as the force was increased, with the longest-lived distribution having a lifetime of 54 s at 93 pN (Fig. 3c and Supplementary Table 1). We also observed a force-dependent probability of forming the longest-lived state: although at 93 pN there was a mere 7% chance for a dwell time to belong to the longest-lived state, this probability increased to 73% at 59 pN (Fig. 3d,e). Conversely, trapping of the system in one of the shorter-lived states became progressively less likely as the force was decreased (Supplementary Fig. 2i). The force-dependent probability of all states also indicated that the first two states are likely to represent intermediate conformations that occur at all forces, while the longest-lived state represents the full lock. Thus when the magnetic tweezers exerted their highest forces, they prevented the short-lived conformations from proceeding to the fully locked state, while the longest-lived state predominated at low forces.

Experiments on permissive WT Tus–Ter resulted in sharply reduced dwell times that obeyed a single-exponential distribution with a mean of 0.8 s at 19 pN (Fig. 3c, purple square) and Supplementary Fig. 2h; at higher forces dwell times were too short to be detected. In fact, there was no single force at which both nonpermissive and permissive dwell times could be measured; the dwell times of nonpermissive WT Tus–Ter became too long at 19 pN for practical measurements (Supplementary Fig. 1d). This implies that none of the states we found for the nonpermissive orientation can be attributed solely to binding by Tus. To further investigate the origin of the observed states, we compared the changes in lifetime and probability invoked by mutations in Tus and/or TerB.

A binding-domain mutation does not hamper lock formation

The crystal structure of the locked WT Tus–Ter shows that DNA sequence recognition and binding can be largely attributed to a Tus DNA-binding domain that primarily consists of two antiparallel β-strands that interact with the major groove of Ter DNA (Fig. 1b). A site-specific mutation in the DNA-binding domain (Q250A, Supplementary Fig. 3e) is known to result in a sharp increase in the Tus–TerB Kd, but whether it affects lock kinetics is unknown. In our experiments, Q250A exhibited dwell time distributions very similar to those of WT Tus at the same forces (Fig. 3c, cyan). We saw no correlation between the Kd of Tus–dsTerB and lock strength. From this we concluded that lock formation is not severely affected by a change in Tus’s DNA-binding domain.

C6 base is crucial but not rate-limiting for lock formation

We subsequently set out to examine the effect that changing the key (the C6 base) (Fig. 3a, switch from green to magenta key) had on WT Tus–Ter lifetimes. A single-base-pair inversion of the TerB sequence at position 6 profoundly affects the fork-arrest efficiency31. In our experiments inversion of G-C6 indeed had a dramatic effect on the lifetimes (Fig. 3c, purple triangles), as the dominant lifetime was no greater than 1 s at 40 pN. By comparison the dominant lifetime of WT Tus–Ter was at least two orders of magnitude higher, as indicated by extrapolation of the lifetimes of the fully locked state observed in the 59–93 pN range (Fig. 3c, purple circles). Despite
the decrease in observed lifetimes, the G6 Ter site continued to impose an increased barrier to hairpin opening because the lifetimes remained well above those found for binding of WT Tus only (Fig. 3c, purple square). For WT Tus with the modified key, we found two states (Supplementary Fig. 2g, purple), with the longest-lived lifetime decreasing from 39 to 0.7 s in the 29–40 pN range (Fig. 3c, purple triangles). We also assessed whether uncoupling lock formation from mechanical probing (by creating a hairpin with an unpaired region of five bases containing C6; see Supplementary Figure 1b) would populate the fully locked state even at high forces, as it is known that this 5-base mismatch dramatically increases the affinity of the Tus–Ter complex\(^1\). The resulting state probabilities, however, were identical to those of normal WT Tus–Ter (Fig. 3a), indicating that preformation of the lock did not alter the occupancy of the different states.

**Probing mechanism via 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 altered in or near the lock domain. Two amino acid residues, H144 and F140, are situated directly in the lock domain, and the crystal structure\(^2\) suggests that their roles differ in a subtle, though significant, manner. Residue H144 lies deep within the lock pocket and interacts only with the C6 base. The H144A mutation removes 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 depicted 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 does 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 would thus lead to a gap at the edge of the lock pocket, which we depict as a widening of the keyhole (Fig. 3a, orange). Residue E49, linked to the putative specific protein–protein interaction between Tus and the E. coli DnaB helicase\(^1\), lies just outside the lock domain (Supplementary Fig. 3d, in green), although it does make a water-mediated hydrogen-bonding contact with the 5′ phosphate of A7 in the locked complex\(^2\). The shape of the lock pocket remained unaffected by this mutation (Fig. 3a, green keyhole identical to WT).

**F140A affects specificity and H144A affects strength of the lock**

We found that mutant F140A showed a marked decrease in dwell times at 59 pN (Fig. 3b, orange). Fitting revealed that the longest-lived exponential now had a lifetime of \(\sim 55\) s, compared to 720 s for WT Tus at the same force (Fig. 3c, orange circles). We also observed that this third, longest-lived state had all but disappeared as the probability of entering this state was 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) and Fig. 3e (orange bar in ’full lock’ column)). Thus F140 appeared to give rise to the third, long-lived state observed in the Tus species with an intact lock pocket and, as such, seems to play a role in the probability of forming a fully locked state. Similarly to that of WT Tus, F140A’s probability of attaining the full lock state exhibited a clear force-dependence: decreasing the force to 47 pN increased full lock probability to 31% (Fig. 3d, orange). Combining F140A with the mutated Ter site (Fig. 3a, magenta key with orange lock) further reduced the force-dependent lifetimes, but the resulting force-dependent lifetimes exceeded those of WT Tus with the mutated Ter site (Fig. 3c, orange triangles and Supplementary Fig. 2g, orange circles). This apparent increase in lock strength in the presence of an altered key indicated that mutation of F140 led to a decreased specificity for allowing only the C6 base into the lock.

Replacing H144 led to a more substantial decrease in Tus–Ter lock dwell times than resulted from the F140A mutation (Fig. 3b,c, blue circles). The dominant lifetime extracted at 59 pN was found to be \(\sim 2\) s, whereas those of WT Tus and Q250A at the same force were two orders of magnitude higher. The data sets were found to exhibit lifetimes measurable over a wide range of forces (24–59 pN), and all retained three exponential states. At 59 pN, H144A led to a larger drop in the probability of entering the third, longest-lived state than that for F140A, from 73% for WT Tus to 0.7% for H144A (Fig. 3e, blue). As observed for F140A and WT Tus, there was also a clear force-dependence in the probability to form a fully locked state for H144A: the probability increased to 26% at 24 pN (Fig. 3d, blue line). While H144A is the single-site mutation with the greatest effect on lock lifetimes, the decrease was not as severe as that for WT Tus with the G6 Ter site. When combining the H144A lock mutant with the mutated Ter site (Fig. 3a, magenta key with blue lock),
for F140A (Fig. 3b, orange circles). This suggested that the DNA-phosphate interaction with E49 is crucial for guiding the C6 base into the Tus lock pocket to form the fully locked state. Mutating the TerB site (Fig. 3a, magenta key with green lock) caused a loss of almost all dwell times 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 revealed longer-lived events with a low probability of ~1.5% (Supplementary Fig. 2h and Supplementary Table 2). When extrapolated to higher forces, the longer lifetimes (Fig. 3c, green triangles) resembled the much more probable states found for the interaction between WT Tus and the mutated Ter site in the 29–40 pN range (Fig. 3c, purple triangles, and Supplementary Table 2). This indicated that while the WT lock domain continued to interact with the incorrect C6 key, mutation of E49 rendered such an interaction unlikely. Our observations clearly link the change invoked by E49K to a change in the probability of forming the third, fully locked state.

On the basis of our observations, we propose a kinetic model for WT lock formation involving three states (Fig. 4b and Supplementary Fig. 4). In this model, the Tus–Ter complex strengthens progressively and irreversibly upon passage from one stable state to the next until the final, fully locked and longest-lived state is reached. Fitting this kinetic model to the data revealed that loss of the long-lived lock state as the force is increased is not due to slower transitions to stronger lock states (Fig. 4c, 17 and 27) but rather to increased rates of disruption (k30 and k31) of the lock states, as the force-dependent trends in these rates showed.

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 by mutation of the C6 base, but not by a mutation in the DNA-binding domain. We further observed that mutant E49K, which is hypothesized to be deficient in polar replication-fork arrest due to the elimination of specific protein–protein interactions9–13, gave rise to lifetimes identical to that of WT Tus, only now with a severely decreased probability of entering the longest-lived state. This tied the observed deficiency of in vivo fork arrest to the decrease 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 we showed residue E49 to be part of the mechanism that facilitates formation of a tight interaction between C6 and the lock pocket.

Our experiments also demonstrated that the flipping of the C6 base and Ter’s subsequent interactions with the Tus lock pocket is not a rate-limiting step in the lock formation process. This is supported by our observation that there was little force-dependence in the inter-state rates (k10 and k20) (Fig. 4b,c); this means that the force-dependence of state probabilities was caused solely by the force-dependent state exit rates (k30, k31 and k32). 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 further strengthened by our observation that preformation of the lock yielded a distribution of dwell times identical to those of normal Tus–Ter (Fig. 4a). In the preformed lock situation, it could be assumed that the C6 base has reached its equilibrium lock position. Our results imply that our pulling
experiment provided sufficient time for this equilibration, although the pulling experiment as a whole is a system in nonequilibrium. This can be 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 (Fig. 4d). The unwinding rate of DNA by a replisome is of the order of 1 kbp/s, and in our pulling experiments this rate is ~30 kbp/s. Typical molecular single-bond rotations are known to take place on femtosecond to picosecond timescales, with larger-scale motions such as lock formation likely occurring in the nanosecond to microsecond range\(^{23-25}\). This implies that there is at least several orders of magnitude of difference between the rate of unwinding and the rate at which flipping of C6 and concomitant molecular rearrangements take place, leaving ample time for C6 to reach its equilibrium-locked conformation.

The interpretation that the longest-lived state is the native, or full lock, state implies the probability of native lock formation is substantially lower than 100% at the highest forces measured (Fig. 3d, purple). The force-dependent lock probabilities do, however, suggest that the full lock is the dominant state at low forces for WT Tus, and suggest that the mutant with the lowest full-lock probability (H144A) might still have a significant chance of blocking an approaching replisome. Identifying a more direct link between our probabilities and in vivo arrest efficiencies would require knowledge of, for example, the amount of work performed by a replisome. It remains to be determined to what extent the two shortest-lived lock states are capable of causing arrest of DNA-processing enzymes, although the reported replisome arrest deficiency of E49K\(^{11,14}\) and our observation that E49K affected only the longest-lived state suggests that these intermediate states are not sufficient to block replication fork progression. It is clear, though, that these two ‘lesser’ lock states still pose a significant barrier to strand separation, much more so than the mere binding of Tus alone.

The difference between the high efficiency of reaching the full-lock state that we observed and the lower efficiencies of replisome arrest observed in vivo\(^{12}\) must 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, and steric effects (through functional protein–protein interactions) could then be the cause of the observed decrease in efficiency. Thus, instead of providing the basis of fork arrest, functional interactions could have an antagonistic effect in vivo. Our experiments with mutant E49K suggested a possible mechanism: just as the mutation in the Tus protein modulates the probability of forming the fully locked state without affecting the lifetime of the lock, an enzyme running into Tus–Ter could invoke a similar effect through functionally interacting with that same residue. Our experiments with WT and E49K Tus, respectively, thus set the upper (no interaction, high lock probability) and lower (E49 function completely disrupted, low lock probability) boundaries of blocking probabilities. Two different enzymes that run into nonpermissive Tus–Ter could then, in turn, have their own characteristic probabilities of being blocked due to their the differential modes of nonspecific interaction with Tus residues (such as E49) upon collision.

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METHODS

Methods and any associated references are available in the online version of the paper.

References


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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.v.L., R.J. and N.H.D. designed, and B.C. and T.v.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., N.E.D. and N.H.D. wrote the paper.

Competing financial interests
The authors declare no competing financial interests.

Additional information
Supplementary information, chemical compound information and chemical probe information is available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to N.H.D.
ONLINE METHODS

DNA hairpins. Plasmids pTER and pTER_Rev, containing the TerB site in either the nonpermisive or permissive orientation, respectively, and flanked by phase A sequences, were obtained from Invitrogen. Plasmid pTER_mutant, encoding mutated TerB (G6→G6) was generated from pTER by site-directed mutagenesis using primers 1 and 2 (primer sequences are listed in the next paragraph). Hairpins were constructed in a multistep process (Supplementary Fig. 1). First, 1-kb fragments containing the TerB site were amplified from the three pTER plasmids using primers 3 and 4. These fragments were digested with the nonpalindromic restriction enzyme BsaI (New England BioLabs Inc., Ipswich, MA) and ligated at one end with a 42-bp oligonucleotide to form a U-turn (oligonucleotide 5). To create a 1-kb fragment containing a 5-base mismatch between bases 3–7 in the Ter site, two fragments of 500 bp were generated by PCR using pTER as template and primer combinations 3 and 12 and 14, respectively. These fragments were digested with BsaI and ligated to each end of the annealed primer pair 14 and containing the wobble. Hairpin handles were created by PCR amplification of the 1.2-kb pBluescript SK+ (Stratagene–Agilent Technologies, Santa Clara, CA) fragment using primers 6 and 7 in the presence of either biotin-16-UTP or digoxigenin-11-UTP (Roche Diagnostics, Basel, Switzerland). Prior to ligation to spacer oligonucleotides, handles were digested with either BamHI or NotI. The upper spacer of the hairpin was generated by annealing 5′-phosphorylated oligonucleotides 8 and 9 and ligating this double-stranded DNA fragment to the NotI-digested biotin-labeled handle. The lower spacer was made by annealing 5′-phosphorylated primers 10 and 11 and ligationing to the BamHI-digested digoxigenin-labeled handle. Finally, the overhangs of these handle-spacer constructs were allowed to anneal to form a short (50-bp) stem with a 5′-GCAA overhang that was ligated to the complementary BsaI site of the 1-kb TerB fragment. Oligonucleotides were obtained from Biologeo B.V., Nijmegen, the Netherlands and from Ella Biotech GmbH, Martinsried, Germany.

Primers. Sequences of primers for PCR amplification of pTER plasmids and oligonucleotides that contribute to the structure of the hairpin were as follows: primer 1, 5′-CACTGGCATCAATTTAAACATATGTGTAAGATGGTATAT-3′; primer 2, 5′-ATATATCACTTATGGAAATACAGATTTTATTTATAGCAGACCTGCTGTGGTG-3′; primer 3, 5′-CCTGCGGTCTGCTGTTACCGTCACCAAAATTACGGTC-3′; primer 4, 5′-CCATCTGTTCTCTCAGTTGATTACAGCAGACGGACAGGCTCGTGAAGGCGG-3′; primer 5, 5′-CTCTAAGCTGCGCCGAGGCCAGCGGGATAGCCTGGCTGGTTGCAG-3′; primer 6, 5′-GACCAAGGATGGGGTGTATTGCTG-3′; primer 7, 5′-CAATGGCTGGCAACAGAGGAGGC-3′; primer 8, 5′-GGCCAGGCGACCCTCGTGTCGCCGCATACTATTCTCCAGATGACCGTGGTT-3′; primer 9, 5′-GGCCAGCAATGCTTATGTACATGAAATGTTGGTGCGGCACCGAGTGTCTCTCTGCATTCTCCTACACCGGTGATGTTAATCGGCTGCGATCGCCGCCGGCCG-3′; primer 10, 5′-GATCCTGCTGCTCAATAGCCGCTGATTCGATTTGCCGATGGCCT-3′; primer 11, 5′-GCAAGGAGTCTTAAAGAGCGATCAGTCATTTCTGACAGTACAGTGCCG-3′; primer 12, 5′-CTCTGCGGTCTGCTGTTACCGTCACCAAAATTACGGTC-3′; primer 13, 5′-CTCTGCGGTCTGCTGTTACCGTCACCAAAATTACGGTC-3′; primer 14, 5′-PCGCTACTTTTGTACAAAATACGCTGCCATTACATCGCTATCCGATGCTGTTGCACGCACAGGCGATGTTGATGCACGCTGCTGATTCGATTTGCCGATGGCCT-3′; primer 15, 5′-PTCTGCAAACCCTGCTTTGTACAAAATACGCTGCCATTACATCGCTATCCGATGCTGTTGCACGCACAGGCGATGTTGATGCACGCTGCTGATTCGATTTGCCGATGGCCT-3′. N-terminally His-tagged Tus proteins were prepared as described21,22; their concentrations were determined spectrophotometrically (ε280 = 39,700 M−1·cm−1).

Magnetic tweezers—experimental configuration. The magnetic tweezers implementation used in this study has been described23,24. In short, light transmitted through the sample was collected by an oil-immersion objective (Olympus UPLSAP060XO 60×, numerical aperture (NA) = 1.35, Olympus, USA) and projected onto a 12-megapixel CMOS camera (Falcon FA-80-12M1H, Teledyne Dalsa, Canada) with a sampling frequency of 58 Hz at full field of view, or higher when cropped. A 2-inch 200-mm tube lens between objective and camera made the effective magnification 67×. The applied magnetic field was generated by a pair of vertically aligned permanent neodymium-iron-boron magnets (SuperMagnet, Switzerland) separated by a distance of 1.0 or 0.5 mm and suspended on a motorized stage (M-126.FD2, 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.FD, 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 tweezers36. In short, tracking of the x,y,z coordinates is performed using center-of-mass computation followed by a further refinement using the quad-ritation 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 prerecorded LUT of radial profiles. After subtraction of the reference bead position to correct for instrumental drift, the x, y and z positions 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 aliasing23,24.

Sample preparation and data acquisition. The sample preparation used in this study has been described in detail elsewhere21. In short, the DNA hairpins (final concentration –50 pg/µl) were mixed and incubated for 2 min with 20 µl streptavidin-coated paramagnetic polystyrene 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. Nontethered beads were removed by flushing with 1 ml Tris buffer, applying a high (30–40 pN) force while rotating the magnets (10 r.p.m.), and followed by flushing with more buffer until all nontethered 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 1,000-fold from stock (to ~10 nM) unless high salt concentrations required higher concentrations. Data were acquired at 100 Hz with a 10-ms acquisition time. Force-extension curves were obtained through changing the magnet position in an exponential fashion such as the force change was linear. Constant-force dwell time experiments were obtained by lowering the magnets in a linear fashion (10 mm/s) to the desired distance. The dwell time 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 open state.

Data analysis and statistical procedure. Rupture of the Tus–Ter lock results in a sudden opening of the DNA hairpin: rupture points were easily identified as a sharp peak in the derivative of the z-trace. The dwell-time distribution

$$P(t) = \sum_{i=1}^{M} A_i e^{-t/\tau_i}$$  

(1)

with M number of exponentials (as determined by the Bayes–Schwarz information criterion25) is fit to the data set containing N experimentally collected dwell times {tᵢ} by minimizing the likelihood function24

$$L = \sum_{i=1}^{N} \ln P(t_i)$$  

(2)

with respect to rates and probabilistic weights (Supplementary Results, equations (2) and (3)). We calculated the errors in our parameter estimates by bootstrapping the system 1,000 times, and reported the one-sigma confidence intervals (1 - σ CI) among the bootstrapped data sets (Supplementary Tables 1 and 2).

