The mechanism of DNA unwinding by the eukaryotic replicative helicase

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Accurate DNA replication is tightly regulated in eukaryotes to ensure genome stability during cell division and is performed by the multi-protein replisome. At the core an AAA+ heterohexameric complex, Mcm2-7, together with GINS and Cdc45 form the active replicative helicase Cdc45/Mcm2-7/GINS (CMG). It is not clear how this replicative ring helicase translocates on, and unwinds, DNA. We measure real-time dynamics of purified recombinant Drosophila melanogaster CMG unwinding DNA with single-molecule magnetic tweezers. Our data demonstrates that CMG exhibits a biased random walk, not the expected unidirectional motion. Through building a kinetic model we find CMG may enter up to three paused states rather than unwinding, and should these be prevented, in vivo fork rates would be recovered in vitro. We propose a mechanism in which CMG couples ATP hydrolysis to unwinding by acting as a lazy Brownian ratchet, thus providing quantitative understanding of the central process in eukaryotic DNA replication.
Healthy cell division requires accurate DNA duplication to maintain genome stability which is accomplished through complex coordination of proteins forming a replisome, travelling along and replicating parental DNA. To provide templates for synthesis, DNA strands are first separated by NTP hydrolysing helicase proteins, invariably forming a toroidal complex, a mechanism common across all domains of life. Eukaryotic replisome construction and disassembly is tightly regulated\(^{2,3}\); an AAA+ ATPase hetero-hexameric minichromosome maintenance complex (Mcm) 2–7 loads as a double hexamer on double-stranded DNA (dsDNA)\(^4\) at origins of replication before additional firing factors aid formation of the active replicative helicase Cdc45/Mcm2-7/GINS (CMG)\(^5\)\(^\text{-}^7\). Further proteins are recruited to the replication fork, including polymerases, Toh1, Csm3, Mcr1, and PCNA, all required to achieve maximum rates of replisome progression\(^8\). A concerted effort is underway to understand the molecular mechanisms of origin firing, from Mcm2-7 recruitment, through CMG activation, to elongation\(^9\)\(^\text{-}^10\).

CMG, thought to be conserved across archaea and eukaryotes\(^11\), is a ring-shaped helicase formed by the superfamily six Mcm2-7 hexamer with distinct C- and N-terminal domains forming two tiers\(^12\), and GINS and Cdc45 plugging a gate between Mcm2 and Mcm5\(^12\)\(^\text{-}^13\), preventing the opening of the ring without additional factors\(^14\). Notably, other replicative helicases, including SV40 large T antigen, can load from a monomeric state and open the hexameric ring dynamically\(^15\)\(^\text{-}^16\). How CMG couples ATP hydrolysis to unwinding is unclear and an understanding of the translocation, hence unwinding mechanism, post activation, remains elusive, although analogous helicases give indications. Structural studies of papillomavirus DNA replicative ring helicase E1 suggest a cyclic escort translocation mechanism, where the helicase walks along ssDNA, a Brownian motor\(^24\). Structural studies of CMG reveal only two distinct conformers, not the expected six\(^13\) leading to proposals between Mcm2 and Mcm5\(^12\)\(^\text{-}^13\), preventing the opening of the ring without additional factors\(^14\). Notably, other replicative helicases, including SV40 large T antigen, can load from a monomeric state and open the hexameric ring dynamically\(^15\)\(^\text{-}^16\). Translocation proceeds on single-stranded DNA (ssDNA) with 3′–5′ polarity, unwinding via steric exclusion\(^5\)\(^\text{-}^7\)\(^,^18\), excluding the lagging-strand template outside, and threading the leading-strand template through the helicase\(^13\)\(^,^19\).

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Little is known regarding the dynamics or kinetics of CMG as it unwinds DNA\(^9\)\(^,^14\). Our work measures single CMGs unwinding dsDNA in real-time with high precision. We find linear unwinding rates low compared to replication fork rates measured in cells and the helicase exhibits unexpectedly complex dynamics. To explain our data we build a quantitative kinetic model to underpin a biophysical description of the unwinding mechanism and establish that CMG unwinds DNA via a biased random walk with propensity to pause. Without this understanding of the foundation of the eukaryotic replisome, further insight into how replication dysfunction can be a major source of genome instability will be slowed.

Results

Tracking DNA unwinding with magnetic tweezers. Helicase activity of purified recombinant Drosophila melanogaster (DmCMG) (Fig. 1a) was confirmed with bulk model fork unwinding assays\(^18\)\(^,^26\).

The substrate was incubated with DmCMG in the presence of ATP\(\gamma\)S and unwinding initiated by addition of ATP. DmCMG demonstrated helicase activity (Fig. 1b), matching previous work\(^6\).

We remove ensemble averaging of classical biochemical techniques by employing multiplexed single-molecule magnetic tweezers\(^27\) (Fig. 1c, Supplementary Fig. 1a). A single 2.7 kilobase (kb) dsDNA molecule, with a polyT 3′ flap for high affinity binding\(^6\)\(^,^26\), is tethered between a PEylated glass surface via biotin/streptavidin binding and a super-paramagnetic microsphere via digoxigenin/anti-digoxigenin binding. CMG is drawn into the sample chamber in loading buffer, containing ATP\(\gamma\)S, and given time to bind the 3′ flap. Next ~4–5 chamber volumes of temperature-equilibrated CMG-free running buffer, containing ATP, is drawn through the chamber (Fig. 1d). This ensures only previously loaded CMG remains in the sample chamber, preventing multiple helicases acting on single DNA substrates. Constant force is applied with a cuboid magnet pair such that conversion from dsDNA to ssDNA increases the microsphere vertical displacement (Supplementary Fig. 1b). After subtraction of the position of reference microspheres stuck to the glass cover-slip\(^28\) and low-pass filtering to 0.17 Hz, displacement is measured with 3.2 ± 1.2 nm (n = 3, all uncertainties standard deviation, unless otherwise stated) standard deviation over 91 mins (Supplementary Fig. 2). This displacement is converted to base pairs unwound; a proxy for helicase position. Using forces between 20 and 40 pN prevent re-annealing behind the helicase and permits spontaneous re-annealing from the ss-dsDNA junction, ahead of the helicase (Supplementary Note 1). Measuring this displacement through time gives a CMG unwinding trajectory. Upon analysis, ~10% of single DNA molecules show activity, demonstrating it is unlikely more than a single helicase acts (further detail in Supplementary Note 2).

The instrument precision is quantified by the Allan deviation\(^29\) of an enzyme-free trajectory (Supplementary Fig. 2). Before low-pass filtering, the Allan deviation shows precision on the order of a nanometre across experimental timescales. The method and apparatus are validated by demonstrating that unwinding by homohexameric ring helicase SV40 large T antigen replicates the speed and features of previous work\(^30\)\(^,^31\) and has features similar to other ring helicases\(^32\)\(^\text{-}^34\) (Supplementary Fig. 3). The protocol remains identical to CMG assays except 110 nm monomer large T antigen was incubated with ATP at 37 °C for 20 mins before introduction to the sample chamber of tethered DNA, with no further chamber washes. In Supplementary Fig. 3a, we demonstrate a typical example of SV40 large T antigen unwinding a 1-kb DNA hairpin and in Supplementary Fig. 3b, unwinding the 3′ flap DNA template used for CMG experiments. The hairpin was not used in CMG experiments as in our hands no unwinding was observed, likely as CMG requires a free 3′ ssDNA tail to bind the DNA construct\(^14\) (Supplementary Fig. 4 and Supplementary Note 3).

The measured trajectories are low-pass filtered to 0.17 Hz (Supplementary Fig. 5a). Following the protocol outlined in Fig. 1d, we observe typical CMG single-molecule unwinding trajectories as shown in Fig. 1e, with no unwinding observed in the absence of CMG or ATP (Supplementary Figs. 6a and 7).

CMG slowly unwinds DNA, non-monotonically with heterogeneity. Figures 1e, f demonstrate the number of base pairs unwind by CMG is non-uniform in rate and does not solely increase. We observed variable unwinding rates and regions where the number of unwound base pairs decreases. This indicates unwinding of DNA by CMG is non-monotonic with heterogeneity in rate found both within, and between, individual enzyme unwinding trajectories.
We observed CMG unwinds DNA slowly compared to previous ring helicase studies. Calculating the mean unwinding rates with linear fits to single-molecule unwinding trajectories, we measured rates between $0.10 \pm 0.08$ bps$^{-1}$ ($n = 19$) and $0.47 \pm 0.56$ bps$^{-1}$ ($n = 75$) over the ATP concentrations and forces applied in our experiments (Fig. 2). Although one should be careful not to heavily weight inferences from this naive approach, this is approximately one to two orders of magnitude slower than this is approximately one to two orders of magnitude slower than replication fork rates observed in eukaryotic cells. The replicative helicase does not attain the speeds necessary for timely genome duplication.

Our observations contrast starkly with the 5′ to 3′ translocating superfamily 4, RecA core, ring replicative helicases of bacteriophage 39–41 and E. coli 32. These have predominantly exhibited monotonic, uniform, and unidirectional unwinding behaviour at ~100 bps$^{-1}$, interspersed with pauses in activity. These rates are within one order of magnitude of replication fork rates observed in live cells and bulk biochemical assays. CMG linear unwinding rates measured in this work are similar to those of other AAA+ helicases. For example, purified SV40 large T antigen shows relatively slow unwinding rates of ~1.5 bps$^{-1}$ (online refs. 30,31) and ~3 bps$^{-1}$ in cell extract in vitro. Furthermore, at high precision E1 shows non-monotonic behaviour, including pausing, forward, and reverse movement as does archaean MCM 35. Similarities in speed and dynamics between these homohexameric helicases and CMG may result from the common AAA+ ATPase motor and 3′ to 5′ translocation direction of superfamily three and six helicases.

Unexpectedly, CMG exhibited not only unwinding and pause-like dynamics but also reverse motion (Fig. 1f). With annealing behind the helicase prevented at high forces spontaneous annealing can only take place from the ss-dsDNA junction. Thus, the reduction in bp unwound indicates CMG has travelled backwards, allowing annealing ahead of the helicase. Reverse motion previously observed in ring helicases is either abrupt and monotonic and ascribed to slipping 21,33,46, a motion considered not to require ATP hydrolysis, or is on the order of single base pairs. Our results showed CMG translocates backwards over prolonged periods, neither abruptly nor over single base pairs, thus cannot be slipping. In this work, rather than attribute to separate mechanisms, we incorporate the reverse motion within a single unifying description, thus the emergence becomes inherent (see below).

We investigated DNA unwinding by individual CMG molecules with varying forces applied to the linear duplex through the lagging-strand template. Passive helicases, which await spontaneous thermal fluctuations to open the duplex, demonstrate higher unwinding rates when larger forces are applied to DNA. Naïvely calculating the linear unwinding rate (Fig. 2a) we observed a wider distribution and increase in mean from 30 pN to 40 pN, yet a decrease in mean and narrower distribution from 20 pN to 30 pN ($t(86) = 5.1, p = 2.2 \times 10^{-6}$ and $t(78) = 2.9$, respectively).
pausing versus activity. To characterise behaviour, this state-
that is susceptible to bias from choice of derivative window size
usually states within the raw trajectory. Instead we employ
considered to be a pause and prevents standard techniques detecting
velocity of CMG is indistinguishable from noise usually con-
the same segment of DNA (Fig. 3a).

Fast continuous unwinding appears as short FPTs, but annealing
of the helicase. Taking the commonly held model of a
NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-019-09896-2 | www.nature.com/naturecommunications

Fig. 2 Mean linear DNA unwinding rate by CMG as a function of force and ATP concentration. a Forces of 20.0, 30.0, and 39.3 pN applied to the linear duplex through the lagging-strand template varies the unwinding rates and distribution with increases at high forces. b ATP concentrations of 0.05, 0.2, and 4 mM varies the unwinding rates and distribution with increasing ATP having higher rates. Error bars are standard deviation

CMG has behaviour beyond that of a simple walker. Activity
and pausing are commonly discriminated between in single-
molecule molecular motor trajectories, using instantaneous
velocity thresholding techniques, to remove pauses or study
the kinetics of the pauses and unwinding bursts. This tech-
nique is susceptible to bias from choice of derivative window size
and mis-assignment of states due to a threshold determining
pausing versus activity. To characterise behaviour, this state-
separated data is binned, a notoriously subjective process, before
usually fit with a Gaussian model a priori. The low instantaneous
velocity of CMG is indistinguishable from noise usually con-
sidered to be a pause and prevents standard techniques detecting
states within the raw trajectory. Instead we employ first-
passage time (FPT) analysis, extended from dwell time distributions,
which also negates the analysis problems described. We measured the time taken for CMG to first unwind
a specific number of base pairs (passage interval, Fig. 3a, 20 bp),
and repeated along the trajectory. To prevent spurious detection
of FPTs we choose the passage interval to be twice the standard
deviation of an enzyme-free trajectory (Supplementary Fig. 6b, c). Thus, the maximum number of bases unwound in each trajectory is used to report mean and standard deviation for all data.

CMG is a biased random walker with multiple distinct pauses. Current suggested mechanisms are unable to explain the observed FPT distributions and reverse motion. With absence of evidence indicating CMG acts unidirectionally (Supplementary Fig. 6d), we describe the observed motion with a one-dimensional hopping model discrete in space and continuous in time, as developed for helicases by Betterton and Jülicher. Pictured in Fig. 3d, the helicase translocates on ssDNA, positioned at the junction
between ss- and dsDNA, by hopping forwards at rate \( r_f \) from \( n \) to \( n + 1 \), and backwards at rate \( r_b \). The resulting biased random walk is governed by the master equation,

\[
\frac{\partial P(n,t|n_0)}{\partial t} = r_b P(n+1,t|n_0) + r_f P(n-1,t|n_0) - (r_f + r_b)P(n,t|n_0),
\]

where \( P(n,t|n_0) \) is the probability of the helicase being at base pair \( n \) at time \( t \), given it started at \( n_0 \) at \( t = 0 \).

The analytical solution for the FPT distribution of the biased
random walk of Eq. 1 is given by the first term of Eq. 2 which
describes a broader peak (Fig. 3b, orange line) in comparison to a
stochastic unidirectional walker (Fig. 3b, green line). This arises
from the possibility of backwards travel and thus increase in
duration of FPTs. However, this description does not explain the
appearance of long FPTs observed in our experimental data

\[ \text{Fig. 2} \]
(Fig. 3b). Given the exponential nature of these times and the propensity of translocating enzymes to pause, we attribute these events to one or more pause-like states, and include them in the FPT probability distribution as the second term in Eq. \(1^{28}\).

Combining the biased random walk unwinding state and probable pause states, we describe the FPT distribution, \(P(\tau)\), for CMG unwinding DNA by:

\[
P(\tau) = w_m \frac{m}{\pi^m} (\frac{\tau}{\tau_b})^{\frac{m}{2}} e^{-\frac{\tau}{\tau_b}} I_n (r_1 + r_b)(1 - \left(\frac{\tau_b - \tau - \tau_f}{\tau_f + \tau_b}\right)^2)^{\frac{m}{2}} + \sum_{i=1}^{q} w_i k_i e^{-k_i \tau}
\]

where \(m\) is the number of bases in the passage interval, \(I_m\) is the modified Bessel function of the \(m^{th}\) kind, \(\tau\) is the FPT, \(w_m\) is the proportion of FPTs attributed to the biased random walk, \(w_i\) describes the proportion of FPTs that are accounted for by pause states, and \(k_i\) are the pause exit rates. The number of distinct pauses, \(q\), is selected through comparison of the Bayesian information criterion (BIC) for increasing numbers of pauses. The mean unwinding velocity is \(v_{\text{mean}} = (r_1 - r_b)\) and the effective diffusion coefficient \(D_{\text{eff}} = \frac{1}{2}(r_1 + r_b)^2\).

Evidence from cryo-EM studies of both *Saccharomyces cerevisiae* (ScCMG) and *Drosophila* (DmCMG) indicate all six Mcms may bind ssDNA in the central channel with two nucleotides per monomer\(^{13,19}\). This does not preclude the possibility of single
nucleotide hopping as used here. Furthermore, our model description can be adapted for arbitrary integer nucleotide hopping without qualitative alteration of the results and interpretation.

We observed up to 3 distinct pauses, \( q \leq 3 \), so for clarity we label the proportion of FPTs attributed to pauses at short, medium, and long timescales as \( w_{\text{short}} \), \( w_{\text{med}} \), and \( w_{\text{long}} \) replacing \( \omega \) respectively. The corresponding exit rates are \( k_{\text{short}} \), \( k_{\text{med}} \), and \( k_{\text{long}} \). Following Dulin et al.\(^{28}\) we report probabilities of entering the biased random walk, \( P_{\text{rw}} \), or short, medium, and long timescale pauses, \( P_{\text{short}}, P_{\text{med}}, \text{and } P_{\text{long}} \), respectively.

The parameters of the model best describing our measured FPTs are determined via maximum likelihood estimation. For the data in Fig. 3b, we overlay the resulting FPT distribution model (blue line) on the experimental FPT distribution of multiple molecules from a single experiment (data combined from multiple experiments is presented in Supplementary Table 1). In the typical example of Fig. 3b we found \( t_1 = 136 \pm 22 \text{ bps}^{-1} \) and \( n_0 = 109 \pm 27 \text{ bps}^{-1} \), giving \( \nu_{\text{mean}} = 27.35 \pm 3.75 \text{ bps}^{-1} \), and \( D_{\text{eff}} = 122 \pm 17 \text{ bps}^{-1} \) at 20 pN and 4 mM ATP. For this example data set the proportion of FPTs contributed by biased random walk unwinding is \( w_{\text{rw}} = 0.26 \pm 0.05 \), while \( w_{\text{short}} = 0.15 \pm 0.12 \), \( w_{\text{med}} = 0.45 \pm 0.10 \) and \( w_{\text{long}} = 0.14 \pm 0.06 \). The probability of entering the biased random walk state, \( P_{\text{rw}} \), is 0.935 \pm 0.002, and short, \( P_{\text{short}} \), medium, \( P_{\text{med}} \), and long, \( P_{\text{long}} \), timescale pauses, \( 0.02 \pm 0.01, 0.04 \pm 0.02, \text{ and } 0.007 \pm 0.010, \) respectively. Finally, the exit rates from the short, \( k_{\text{short}} \), medium, \( k_{\text{med}} \), and long, \( k_{\text{long}} \) Pauses are \( 0.034 \pm 0.013 \text{ s}^{-1}, 0.0078 \pm 0.0030 \text{ s}^{-1}, \) and \( 0.0004 \pm 0.0004 \text{ s}^{-1}, \) respectively. Full statistics and number of replicates are given in Supplementary Table 1. In Fig. 3c we plot further examples of FPT distributions at two forces demonstrating changes in the distribution at different timescales. At 20 pN, an increase in propensity to pause at short timescales correlates with a reduction in the FPT probability density of performing the biased random walk (the short time peak), when compared to 30 pN.

Remarkably, we can describe the dynamic motion observed in Fig. 1e, f using a relatively simple stochastic model. Elegantly, the periods of reverse translocation are an inherent consequence of the model, without an additional separate mechanism, due to the finite probability of the helicase hopping backwards.

The trajectories used to calculate the linear unwinding rates in Fig. 2 underwent FPT analysis and the resulting parameter estimations plotted in Fig. 4 and Supplementary Fig. 9. The proportion of FPTs ascribed to pausing (\( w_{\text{short}} + w_{\text{med}} + w_{\text{long}} \)) accounts for between 0.43 \pm 0.05 and 0.84 \pm 0.04 of FPTs observed. The proportion of FPTs attributed to CMG performing the biased random walk of unwinding, \( w_{\text{rw}} \), ranges between 0.16 \pm 0.02 and 0.57 \pm 0.02. Thus, surprisingly, the FPT can be \( \geq 2 \) times more likely to be dominated by a pause state rather than biased random walk unwinding. The probability of entering a random walk state, \( P_{\text{rw}} \), is \( \geq 0.91, \) and the likelihood of entering one of the three pause states is \( \leq 0.09, \) as displayed in Fig. 4a, b. The mean rate of exit from the pause states, over all observations, is \( 0.015 \pm 0.02 \text{ s}^{-1}, \) quantitatively demonstrating the helicase frequently enters long lived pauses.

The mean unwinding velocity, \( \nu_{\text{mean}} \), may be interpreted as a pause-free unwinding rate, describing the peak in FPT distribution alone, measured between 18 \pm 13 \text{ bps}^{-1} \) and \( 32 \pm 27 \text{ bps}^{-1} \) for our data (Fig. 4c, d). Considered alone, these compare well with expected eukaryotic replication fork rates of \( \sim 10-50 \text{ bps}^{-1} \) in cells and the maximal in vitro replication rates of 24 \text{ bps}^{-1} \) (online ref. \(^{8}\)). However, the largely constant \( \nu_{\text{mean}} \) does not explain the change in linear unwinding rates (Fig. 2a) as a function of force. The decrease in probability of entering a short pause (Fig. 4a and Supplementary Fig. 9) and increased rate of exiting (Fig. 4e) as a function of force is most likely responsible for speed up. Similarly, for increasing ATP concentration the increase in pause exit rates is likely responsible for the decrease in linear unwinding rate (Fig. 4f and Supplementary Fig. 9). Our data indicates that increasing force and ATP concentration do not increase the helicase random walk forward bias but do reduce the probability of pause entry and increase rate of exit, changing the average speed of the helicase.

The resulting estimates for the parameters of our model (Fig. 4 and Supplementary Fig. 9), show that CMG undergoes a diffusive random walk with a bias towards forward translocation acting to unwind DNA, with a proclivity to pause on at least two discrete timescales.

**CMG activity is an interplay between unwinding and pausing.** The probability of entering medium and long pause states is constant with force but entrance into the biased random walk or short pause are affected (Fig. 4a and Supplementary Fig. 9a, c, e, g). For decreasing ATP concentration, the long timescale pause disappears, together with a reduction in the likelihood of entering the medium timescale pause. The exit rate from a medium pause also decreases with decreasing ATP concentration (Fig. 4f and Supplementary Fig. 9l, n). The rate of short pause exit, \( k_{\text{short}} \), increases with ATP concentration, while decreasing with force between 20 pN and 30 pN before increasing for 40 pN (Fig. 4e, f and Supplementary Fig. 9k, l). Force and ATP regulate the entrance and exit from pauses demonstrating the intricate interplay between mechanics and ATP hydrolysis.

**Discussion**

We have demonstrated the molecular motor CMG exhibits dynamics of a biased random walk, with a propensity to pause. This more complex, yet elegant, description forgoes the necessity for linear or deterministic mechanisms, such as coordinated escort.

It remains unclear whether this is a universal feature of ring helicases or peculiar to CMG. The relatively slow linear unwinding rates of CMG may allow sufficient sampling of the activity to uncover the behaviour, but for ring helicases acting at 100 \text{ bps}^{-1}, such as DnaB\(^{32}\), the activity may be under-sampled. Indeed, it has been suggested that backstepping is a universal feature of helicases\(^{61}\); a topic for further study.

The low linear unwinding rate observed is surprising and our work explains the origin of these long timescales. Firstly, the helicase undergoes a biased random walk; the magnitude of the bias governs the velocity of the helicase; 18 to 32 \text{ bps}^{-1}. It is the relatively small bias, due to the finite backward hopping rate, that contributes to low speed. Secondly, the frequent entrance into long-lived pause states further reduces the overall speed.

Collaborative coupling between helicase and polymerase has been observed to speed up forks by 3–30 fold in prokaryotic\(^{62}\) and bacteriophage\(^{45,63}\) systems and single-molecule work has shown reduction in pausing\(^{63}\). Bulk biochemical studies of the eukaryotic minimal replisome observed rates of 4.4 \text{ bps}^{-1} in vitro\(^{64}\) and inclusion of further replisome components increases this to 24 \text{ bps}^{-1} (online ref. \(^{8}\)). Single-molecule studies of ScCMG within minimal replisomes have shown replication with polymerase \( \varepsilon \) occurs at 5.4 \text{ bps}^{-1}, the inclusion of Mcm10 increases this to 11.9 \text{ bps}^{-1}, and Mrc1-Tof1-Csm3 further to 21.1 \text{ bps}^{-1} (online ref. \(^{9}\)). Given eukaryotic replication is more tightly regulated than viral and bacterial counterparts, it may not be surprising that eukaryotes need factors beyond a coupled polymerase to achieve endogenous replication fork rates.

We hypothesise additional eukaryotic replisome factors such as polymerase \( \varepsilon \), RPA, Mrc1, Csm3, Tof1, or Mcm10, not present in
our study, increase the forward bias of the random walk, or reduce the probability of entering, or increase the exit rate from, a pause, or a combination of both. In particular, one would expect leading strand synthesis to severely impair any reverse motion currently observed. All would increase the linear fork rate measured.

Our quantitative kinetic model describing the behaviour of CMG as it translocates along ssDNA as a biased random walker with pausing explains well the heterogenous, non-monotonic and low speed unwinding observed. To better understand the molecular mechanism of CMG, we wished to establish a biophysically sensible description that explains how ATP hydrolysis is coupled to unwinding and gives rise to such dynamics. Our model must be consistent with unwinding occurring in a crowded Brownian environment. We exclude the colloquial power-stroke, which describes a movement driven by a conformational change that releases free energy, as it has been shown to be irrelevant in determining the directionality and thermodynamic properties of all chemically driven molecular motors.

The two conformational states observed in CMG structures have been suggested to represent the compact and extended shape of an inch-worm like mechanism or an open paused state and closed translocation state. We propose these two conformational states alter the affinity for DNA (and the specificity for ATP) allowing the motor to act as a Brownian ratchet. Such translocation mechanisms can be mapped onto the biased random walk master equation (Eq. 1) describing the motion observed here, and only two DNA affinity states are required.

To facilitate a Brownian ratchet we propose, in the weakly bound state, open conformer, CMG may diffuse along DNA due to bdcfe15202530354045Force (pN)10−310−210−1100ProbabilityFig. 4 Model parameters of Eq. 2 extracted from maximum likelihood estimation of experimentally measured first-passage times for varying force and ATP concentration. Probability of entering the biased random walk state (brown squares), a short (orange circles), medium (cyan diamonds), or a long (blue crosses) timescale pause as a function of a force and b ATP concentration. Mean velocity (red squares) and effective diffusion (blue circles) as a function of c force and d ATP concentration. Rate of exit from short (dark grey squares), medium (grey circles) or long (light grey diamonds) timescale pause as a function of e force and f ATP concentration. All error bars are standard deviations from 1000 bootstraps.
to a weak free energy potential. This motion is unbiased and depicted in Fig. 5a. Upon ATP binding or hydrolysis CMG undergoes allosteric structural changes that alter DNA affinity, increasing the free energy potential of interaction with DNA. In this state, CMG remains at the bottom of the potential well, fixed in position. The asymmetry in the potential means that upon cycling between these two states through ATP hydrolysis CMG is more likely to be in the \((n+1)\)st potential well representing the next base along, giving rise to a bias towards the unwinding direction.

As a Brownian ratchet requires no rotary nucleotide firing or coordinated escort, only alterations in DNA affinity, this model agrees with the observation of functional asymmetry in CMG, where ATPase activity of two subunits can be mutated without abrogating helicase activity. Instead we suggest a stochastic/probabilistic principle, as proposed previously for AAA+ motors, where allosteric changes provide the required difference in DNA affinity. The lack of requirement for a sequential mechanism may also explain the ability of Mcm4,6,7 to act as a helicase, although with relatively poor activity.

The inherent plasticity of Brownian ratchet mechanisms in ATP hydrolysis timing and robustness against major errors in the hexameric core would be beneficial for maintaining genome stability. For example, accurate DNA replication requires bypassing of DNA lesions and roadblocks as no further helicase loading occurs after G1 phase of the cell cycle and only already loaded origins are fired in S phase. Similarly, the ability of the replisome core to exhibit complex dynamics and move backwards seems essential for fork reversal and remodelling. Our suggested mechanistic model does not require the existence of six conformational states, in agreement with the lack of multiple rotational states observed in Cryo-EM studies of DmCMG and ScCMG on various DNA templates. The long timescale FPTs identified as pause states exist outside the Brownian ratchet described motion and hinder helicase progression at endogenous rates. Consequently, we label CMG a lazy Brownian ratchet and suggest the kinetic scheme for helicase action shown in Fig. 5c. There are several kinetic schemes with different pathways that may explain our data (Supplementary Fig. 10). Here, striking a balance with simplicity, we consider the scheme consists of a Brownian ratchet for unwinding that may enter one of three pauses. The kinetics governing these processes are dependent on force applied to DNA and the ATP concentration.

![Diagram](https://example.com/diagram.png)
To the best of our knowledge there are no known causes of pausing during DNA unwinding by replicative helicases and the origin of those observed here cannot be established from our current work. We coarsely categorise them into either force and ATP concentration dependent or solely ATP concentration dependent (Fig. 5c). Cryo-EM of ScCMG bound to fork template with roadblocks on both strands shows dsDNA can partially enter CMG19. Perhaps this represents a non-productive pause state, which must be exited before unwinding can resume, or is prevented by additional factors such as Mcm10. Our data shows larger forces increase the likelihood of performing a biased random walk, hence unwinding, decreases the probability of entering a short pause, and increases the rate of exit (Fig. 4 and Supplementary Fig. 9), indicating a disruption to the lagging-strand template may inhibit pausing. This correlates with previous work demonstrating a decrease in time spent pausing by DnaB as increasing force is applied to the leading (non-tracking) strand. Further studies to achieve higher precision and hidden Markov modelling would help elucidate pause origins and kinetic pathways.

There are limitations to our study. Due to finite time resolution we miss shorter timescale events. We are also limited in spatial resolution as the efficiency with which we obtain DNA unwinding events means we must utilise low magnification to multiplex our measurements over 100 s of molecules to obtain sufficient statistics. Although we use purified recombinant DenCMG, that binds to the DNA fork substrate in a non-physiological manner, it is unlikely that the dynamics of CMG activated from a fired origin would behave differently, as recombinant CMG can support full replication.

The eukaryotic replicative helicase unwinds DNA by executing a biased random walk with frequent entrance into one of three long lifetime pauses. We find a pause-free unwinding rate would recover in vivo replication fork rates in vitro, leading to speculation that additional factors not present in our study, for example, polymerase ε, Mcm10, Mrc1, or Csm3/Tof1, may alter unwinding mechanism kinetics, as found for leading strand synthesis.

We propose a lazy Brownian ratchet is the simplest model to explain our observations. Remarkably, the relatively simple stochastic model can recapitulate and elegantly explain the heterogeneous dynamics, without requiring careful sequential escort of the DNA.

It is far more plausible the replicative helicase evolved to take advantage of the stochastic and energetic nature of Brownian motion rather than fight against it to perform a perfectly deterministic and sequential walking mechanism. Future higher precision work may refine the model and provide a quantitative description of the free energy landscape along which CMG translocates. Many details of replisome component operation remain to be understood. Our framework provides a means to investigate how these components interact and function to produce the dynamics and kinetics observed.

**Methods**

**Microscopy.** The magnetic tweezers hardware was custom built. All apparatus was, at minimum, switched on several hours before experiments began and allowed to equilibrate to 23 °C, to minimise vertical drift in the experiment. The brightfield microscope was built on a vibration isolated optical table (Thorlabs, B7590 Nexus Breadboard, PFA51505 Active Isolation Frame) surrounded by a light tight isolation chamber for temperature and air flow stability. Illumination was provided by a 530 nm LED (Thorlabs, M300D2) without Köhler illumination using only collimation by an aspheric f = 201 mm condenser lens (Thorlabs, ACL2520U-A). A Nikon 50x NA 0.90 microscope objective lens (Nikon, MRLO1502) in conjunction with a 2” f = 200 mm achromatic doublet tube lens (Thorlabs, AC508-200-A) imaged the sample plane onto a Falcon2 12 M camera (Teledyne DALSA, FA-80-12M1H) via a LaView compatible frame grabber (National Instruments, NI PCIe-1433, 781169-01). The sample chamber was mounted on an x,y translation stage (Märzhäuser, 00-30-101-0000) and clamped in position with homemade stage clips. The microscope objective was mounted in a z-axis piezo stage (Physik Instrumente, PD722Z1AQ) for focus adjustment.

**Magnetic tweezers.** Two neodymium cube magnets (supermagnet.de, W-05-N50-G) are epoxied onto a microscope cover slide (Fisher Scientific, 12332098) with magnetic moments pointing vertically in opposite directions using two aluminium spacers to provide the desired magnet gap. An optically clear window of glass is left to allow illumination light to pass through onto the sample chamber. This magnet pair is mounted on a vertical translation stage (Physik Instrumente, M-112.1DG) to allow force alteration. The stage mounted magnet pair and illumination source are mounted on an x,y translation stage (Thorlabs, MT1B/M) for alignment above the microscope objective centre.

**Fluidics.** The outlets of the pre-prepared sample chamber was connected to a six-way selection valve (VWR, 560-0166) via additional tubing (VWR, 554-2962) and hubless needles (Hamilton, 21 G, 22021-01). The six-way valve was connected to a glass syringe (Hamilton 1000 series, 26211-U) via a three-way stopcock (Cole-Parmer, WZ-30600-02). Using such a six-way valve allows facile switching of multiple sample chambers. The syringe was withdrawn using a syringe pump (Harvard Apparatus, 704504).

**Magnetic tweezers control software.** Control of the objective vertical position (focus) and magnet vertical position (force) was performed with custom written LabVIEW code. An interactive camera capture and microscope tracking programme was used to perform the experiments. The salient features of this software are the tracking in real-time of several microsphere positions using the algorithm described by van Loenhout et al.11, and the export of individual image data as an mp4 to 58 fps directly to a hard drive. Code and software is available at github.com/danielburnham

**Microsphere tracking software.** Tracking of the microspheres for final analysis was performed with custom written MATLAB (Mathworks, 2015a-2017a) code based on the algorithm described by van Loenhout et al.11. This is available for download at github.com/danielburnham

**Force calibration.** The force applied by the two neodymium cube magnets is calibrated as a function of displacement from the sample chamber using the equipartition method25 and corrected for the finite exposure time.26 We used two different sized gaps between the magnet pairs, one of 0.5 mm and one of 1.5 mm to provide low and high range forces.

**dsDNA to ssDNA extension calibration.** To convert the microsphere displacement, hence DNA extension, to number of base pairs unwind we performed force extension measurements of the dsDNA unwinding template and a denatured ssDNA sample, in CMG running buffer. The results are plotted in Supplementary Fig. 1b, and values used with

\[ n_{bp} = \frac{z_{meas}(F) - z_{ss}(F)}{F_{ds}} \]  

(3)

to calculate base pairs unwound, where \( z_{meas}(F) \) is the measured DNA extension, \( z_{ss}(F) \) and \( z_{ds}(F) \) are the extensions of fully ds- and ssDNA respectively, and \( n_{bp} \) is the length of the template in base pairs.

**sdDNA was formed by denaturing dsDNA. Stock DNA (~10 mg/dl) was diluted 5-fold in Milli-Q. One microliter of this diluted sample was further diluted 10-fold into 500 mM NaOH, heated at 37 °C for 10 mins and kept on ice. The resulting ssDNA is used in place of stock template DNA in the conjugation of DNA to microspheres.**

**Sample chamber.** The sample chamber consists of three parts; a gasket, a functionalised coverslip, and a coverglass with holes. All following steps at room temperature unless stated otherwise.

Gaskets were cut with a scalpel from double-sided self-adhesive sheets (TESA SE, TESA 4965) to 24 x 40 mm rectangles with three 3 x 30 mm rectangles cut out. Glass coverslips were functionalized by first placing five 24 x 40 x 0.17 mm coverslips (Hecht-Assistent, 41013125) in a staining jar (Sigma-Aldrich, S5641). The coverslips were immersed in ethanol and sonicated in an ultrasonic bath (Branson, M3800-E, 142-0133) for 30 minutes, rinsed with Milli-Q water (Millipore), then sonicated in 1 M KOH for 30 minutes, and again rinsed in Milli-Q. The ethanol, Milli-Q, KOH, Milli-Q steps were repeated before finally being left immersed in Milli-Q.

Milli-Q was decanted from the jar and the coverslips rinsed with acetone three times. Upon each rinse the acetone was carefully decanted over the inside of the jar lid to remove any traces of water. During the third rinse the coverslips were sonicated for 10 min in a water bath. Finally, the coverslips were immersed in fresh acetone.

Each coverslip was next treated to add silica reference microspheres permanently to the glass. Tracking of the position of such stuck microspheres
describes the motion of the coverslip. Three micrometer diameter silica microspheres (Bangs Laboratories, SS0501) were diluted 1:100 in ethanol and a 4-µl elongated bead of the suspension placed along the short edge of the coverslip before being swiped along the coverslip long axis with a pipette tip. The coverslip was heated on a hot plate for 10 mins at 70 °C and then placed back in acetone in the staining jar.

With hairpin, 2% (v/v) saline solution was prepared by mixing 2 ml of 3-aminopropyltriethoxysilane (Sigma-Aldrich, A3648) in 100 ml acetone. The acetone from the staining jar was decanted as described above and the jar refilled with the 2% saline solution. The jars were then shaken horizontally in all directions for 120 seconds. Next the jar was rapidly filled with ~2 volumes of Milli-Q (without decantation). The staining jar was then filled, shaken, and decanted 6 times with Milli-Q before leaving the coverslips immersed in Milli-Q.

The coverslips were placed on concertina aluminium foil and placed in an oven at 110 °C for 1 hour.

The backing paper of the previously made gaskets are removed and placed adhesively side up on a bench before placing the now dry coverslip silanised down onto the glass. Gentle pressure is applied to seal the glass to the coverslip/gasket combination is placed on top. The plastic adhesive tape removed and placed adhesive side up on a bench.

The coverslip/window has a slightly curved, concave shape and includes a 568-bp duplex handle was amplified from within plasmid pUC19 using forward primer oligo 1 and reverse primer oligo 2. The primers were designed to hybridize to the coding region of the lambda DNA (NEB, N3011L) and amplify a 2711-bp duplex. The PCR was carried out in the presence of modified dUTP, digoxigenin-11-dUTP (Roche, 1109388910) or biotin-16-dUTP (Enzo, ENZ-42811), at a molar ratio of 1:20 with unmodified dNTP mix. Each handle was PCR amplified by PCR sample purification using electroelution with 12-14 kDa MWCO dialysis tubing (VWR, 734-0672). The gel slice was removed from the dialysis tubing and used for unwinding studies.

The lambda DNA (NEB, N3011L) was phosphorylated with T4 Polynucleotide Kinase (NEB, M0201S) for 4 hours at 37 °C and then heated at 70 °C for 20 mins. To ligate the biotin incorporated handle to lambda DNA – 10-fold molar excess of the biotin handle was ligated with the lambda DNA to 65 °C and allowed to cool to room temperature on a heat block before ligation (T4 Ligase, NEB, M0202L) overnight. The lambda DNA template was purified using ethidium bromide staining, excised, and purified using electroelution with 12-14 kDa MWCO dialysis tubing (VWR, 734-0672). The gel slice was removed from the dialysis tubing and the sample dialysed against 10 mM Tris pH 8 overnight at 4 °C. Finally, DNA was concentrated with Vivaspin 50 10 kDa MWCO (Vivascience, VS0102).

**Unwinding template.** The 3′ flap template for unwinding studies was constructed from five parts. A 2711-bp duplex was PCR amplified from within plasmid pUC19 using forward primer oligo 6 and reverse primer oligo 7 using Phusion polymerase (NEB, M0530L). The PCR was carried out in the presence of modified dUTP, digoxigenin-11-dUTP (Roche, 1109388910) or biotin-16-dUTP (Enzo, ENZ-42811), at a molar ratio of 1:20 with unmodified dNTP mix. Each handle was PCR amplified by PCR sample purification using electroelution with 12-14 kDa MWCO dialysis tubing (VWR, 734-0672). Finally, DNA was concentrated with Vivaspin 50 3-kDa MWCO (Vivascience, VS0192).

A 3′ foot probe was formed by placing equimolar ratios of oligo 12, oligo 13, and oligo 14 in 10 mM Tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA and heating to 85 °C for 10 mins, then allowed to anneal by cooling to room temperature. Bands from an 8% PAGE gel and gel purified using electroelution with 12-14 kDa MWCO dialysis tubing (VWR, 734-0672). DNA was concentrated with Vivaspin 500 3-kDa MWCO (Vivascience, VS0192).

The 3′ footprint was ligated (T4 Ligase, M0202L) to the 3′ foot probe at 10:1 molar ratio, overnight by cycling through the temperatures 16 °C, 20 °C and 25 °C in 1 hour repetitions. The same was repeated for the biotin handle and spacer 2. Each were separated on 1% agarose gel before gel extraction and purification. These two products were ligated (T4 Ligase, NEB, M0202L) to the 2.7-kb template at room temperature for 2 hours at equimolar ratio. The final product was separated on a 14% agarose gel before being purified and stored at −20 °C.

**Bulb unwinding template.** Equimolar ratio of oligos 18 and 19 were mixed in STE buffer and incubated at 85 °C for 3 mins then allowed to cool to room temperature. After separation on 3% agarose gel the band corresponding to the fork template was excised and gel purified. Subsequent to purification, the template was radiolabelled with [α-32P]-ATP at 5′ ends using T4 PNK. To eliminate excess [α-32P]-ATP the sample was passed through MicroSpin G50 columns (GE Healthcare, 27-5330-01) previously equilibrated with 10 mM Tris-HCl, pH 8.0, 20 mM NaCl.

**Hairpin template for large T antigen experiments.** The hairpin template for unwinding studies was constructed from five parts. A 1046-bp duplex was PCR amplified from within plasmid pUC19 using forward primer oligo 20 and reverse primer oligo 21 using Phusion polymerase (NEB, M0530L). The PCR was carried out in the presence of modified dUTP, digoxigenin-11-dUTP (Roche, 1109388910) or biotin-16-dUTP (Enzo, ENZ-42811) previously equilibrated with 10 mM Tris-HCl, pH 8.0, 20 mM NaCl.

**Hairpin template for large T antigen experiments.** The hairpin template for unwinding studies was constructed from five parts. A 1046-bp duplex was PCR amplified from within plasmid pUC19 using forward primer oligo 20 and reverse primer oligo 21 using Phusion polymerase (NEB, M0530L). The PCR was carried out in the presence of modified dUTP, digoxigenin-11-dUTP (Roche, 1109388910) previously equilibrated with 10 mM Tris-HCl, pH 8.0, 20 mM NaCl.
extracted from an 8% PAGE gel and purified using electrodialysis with 12-14 kDa MWCO dialysis tubing (VWR, 734-0672). Finally, DNA was concentrated with Vivaspin 500 3 kDa MWCO.

A Lower Linker was constructed by heating equimolar ratios of oligo 17 and oligo 23 in 10 mM Tris-HCl (pH 8), 0.1% NaCl, 1 mM EDTA at 85 °C for 10 mins, then allowed to anneal by cooling to room temperature. Bands were extracted from an 8% PAGE gel and purified using electrodialysis with 12-14 kDa MWCO dialysis tubing. Finally, DNA was concentrated with Vivaspin 500 3 kDa MWCO.

The digoxigenin handle was ligated (T4 Ligase, NEB, M0202L) at 1:3:30 molar ratio, overnight by cycling extraction and purification consecutively, each for 1 hour. The sample was separated on 1% agarose gel before extraction and purification and into 10 mM Tris-HCl, pH 8, 0.1% NaCl to 10 mM.

The final hairpin was formed by ligating the 1 kb template, the handle/linkers, and oligo 24 (T4 Ligase, NEB, M0202L) at 1:30 molar ratio, overnight by cycling through the temperatures 16 °C, 20 °C and 25 °C, 1 hour repetitions. The final product was separated on 1% agarose gel before being purified.

Protein purification. SV40 large T antigen was expressed in insect cells and purified using a monoclonal antibody as described previously15. Briefly, full length SV40 large T-antigen gene was cloned into pFastBac1 (ThermoFisher), which was used to make the baculovirus. SF21 cells maintained in SF-900-III were infected with the virus (2 x 10^6 pfu/ml) using an MOI of 0.1 for 72 hours. The 1 L Cultures grown in 5 L Flasks. Antibody PA419 was coupled to Protein A sepharose beads (5 mg/ml) in PBS, incubated overnight, rotating in a cold room. Beads were washed with 15 ml 0.1 M sodium borate, pH 9.0, and re-suspended in 2 ml 20 mM of 0.0125g/ml dimethyl pimelimidate was mixed with the beads and incubated, rotating, at room temperature for 1 hour. Coupling reaction was halted by incubating beads in 0.2 M ethanamine, pH 8.0, rotating for 1 hr at room temperature.

Cell pellets were re-suspended in 10 pellet volumes of L-Tag re-suspension buffer and incubated on ice for 15 mins. The suspension was centrifuged at 25,000 x g for 15 mins. 0.5 volumes of L-Tag neutralisation buffer was added and mixed. Sample was first loaded onto protein A only column, equilibrated with L-Tag loading buffer. Flow-through was then loaded onto PA419-coujugated protein A column equilibrated with L-Tag loading buffer. The column was washed with 50 ml L-Tag loading buffer, then 50 ml of L-Tag wash buffer, followed by 20 ml L-Tag EG buffer.

Large T-antigen was eluted with 5–10 ml of L-Tag elution buffer before dialysis overnight in L-Tag dialysis buffer. 11 subunits of DmCMG (plasmids provided by Costa Lab) were co-expressed using the baculovirus expression system11. Expression and purification of the complex were carried out as described in Abid Ali et al.13 and Ilves et al.14. With minor changes the method is outlined here.

Briefly, following band generation for each subunit of DmCMG, SF21 cells (Structural Biology, STP, The Francis Crick Institute) were used for the initial transfection and in the subsequent virus amplification stage to make P2 stocks using serum-free SF-900TM III SFM insect cell medium (Invitrogen, 10902-096). Virus was amplified to make a P3 stock by inoculating 100 ml of Sf9 cell cultures (Cell Services, STP, The Francis Crick Institute) with 20 µl of diluted stock DNA construct (−10 ng/µl stock dialuted 500x fold with MilliQ) and added left on a tube rotator (VWR, 445-2102, Stuart S3B) at room temperature and 10 rpm for 20 mins to prevent sedimentation.

The DNA conjugated microspheres are separated with a magnet and supernatant removed before re-suspension in 200 µl of blocking buffer. This buffer is removed in the same manner and replaced with 40 µl blocking buffer.

To bind DNA-conjugated microspheres to the sample chamber surface the sample chamber is first removed from 4 °C, mounted on the microscope and allowed to reach temperature equilibrium. The outlet tubing is connected to the six-way valve and the inlet tubing is connected to the pump. Once the inlet tubing is filled with 40 µl of CMG loading buffer, 100 µl of CMG complex was separated with a 20 ml 100-550 mM KCl gradient using MonoQ PC 1.6/5GL (GE Healthcare) column equilibrated with buffer C-150-14 kDa MWCO dialysis buffer for 2 hours. Aliquots were flash frozen using liquid nitrogen and kept in −80 °C. Protein purification was checked by SDS-PAGE – original gel image given in Supplementary Fig. 11a.

Single-molecule DNA unwinding assay. To bind DNA constructs to magnetic microspheres it should be noted that values with * indicate parameters that need to be adjusted to obtain an optimal number of single DNA molecules tethered between glass surface and microscope at an ideal density. In total, 1 µl of anti-digoxigenin conjugated magnetic microspheres were re-suspended in 100 µl blocking buffer and the supernatant removed after separation with a magnet. This step was repeated. Next the microspheres where re-suspended in 100 µl binding buffer and the supernatant removed after separation. After re-suspension in 4 µl of blocking buffer, 1 µl of diluted stock DNA construct (−10 ng/µl stock dialuted 500x fold with MilliQ) was added left and isolated the flow system. 200 µl of blocking buffer is drawn through the chamber at 20 µl min^-1 with judicious flicking of the inlet and outlet tubing for the first 100 µl to remove trapped air. The magnetic force is set to 0 pN and with the flow stopped and isolated the inlet tubing is placed in the DNA conjugated microsphere sample before flowing at 20 µl min^-1 until a high density of microspheres is present in the chamber. The flow is stopped and isolated to ensure no microsphere movement due to leaks or capacitance in the fluidics and the inlet placed back in blocking buffer.

Finally, the force is increased and a field of view chosen with the maximum number of single DNA-microspheres at an ideal density for analysis, and with at least two reference microspheres. If necessary the procedure is repeated to increase the density of available DNA molecules.

A look up table (LUT) is created to allow sub-pixel vertical tracking of microspheres. Once a sufficient number of DNA conjugated microspheres are tethered to the glass surface, 100 µl of CMG loading buffer is drawn into the sample chamber at 20 µl min^-1. The force is set to that to be used in the experiment. The focus is set to just above a force stretched DNA molecule and microsphere and then stepped in 100 nm increments above the focus a distance greater than the contour length of the DNA construct. At each step a single image is taken and saved for later analysis.

CMG unwinding assay. With the flow turned off and isolated the force is set at 7 pN, then 0 pN, and back to 7 pN, each for ~25 s. This data is later used to both

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screen for DNA molecules of the correct length and as a point of reference for the absolute position of the sample chamber bottom. The magnet force is set to 8 pN.

Stock CMG is taken directly from −80 °C freezer and diluted to 42 nM in CMG loading buffer. The sample is directly drawn into the flow chamber at 20 µl min⁻¹. After 8 mins at 8 pN the magnet force is set to 3.5 pN for a further 15 mins. The force is then set to that required and left for ~70 seconds to record a reference point. Next, 40–50 µl (equal to 4–5 flow chamber volumes) of temperature equilibrated CMG running buffer is drawn into the flow chamber and the flow turned off. The experiment is left for up to 2 hours before ending, having collected ~0.5 million image frames.

Any used sample chamber is not used again.

SV40 large T antigen unwinding assay. For the hairpin template assay the flow is turned off and isolated and force is set at 9 pN. The DNA tethers are identified as hairpins by varying the force between 4 pN and 25 pN to open and close the construct. The force is returned to 9 pN. For the linear template assay the flow is turned off and isolated and the force is set at 7 pN, then 0 pN, and back to 7 pN, each for ~25 s. The force is finally set to 20 pN.

For both constructs stock large T antigen is diluted to 110 nM (monomer) into 20 µl CMG running buffer. The sample is heated at 37 °C for 20 mins before being drawn into the flow chamber at 20 µl min⁻¹. The flow is again isolated and data recorded.

Data analysis. Raw images saved to hard disk go through several steps in an analysis workflow. The salient points are discussed here.

All microspheres that exhibit expected behaviour and have the expected length under a high to low force transition are tracked through time from the image frames exported during the experiment. The resulting z position trajectories have the z position of the mean of at least two reference microspheres subtracted point for point and set to the zero level of the pre-unwinding trajectory. The raw z position data is low pass filtered using a 6 s moving mean average. This 6 s filter size is selected from studying the enzyme-free motion of the DNA-tethered microsphere at 20 pN force. The sum of the squared difference between raw and filtered data points is plotted as a function of filter size (Supplementary Fig. 5a). At a certain filter size the benefit of further increases has diminishing returns and only serves to smooth the signal rather than increasing the signal to noise ratio. As such a filter size is chosen from the point at which the sum of differences between raw and filtered data reaches a plateau.

Mean linear velocities of unwinding are found through a linear fit to the filtered data. The passage interval is chosen by determining twice the standard deviation of the signal when filtered with the filter size chosen above. For illustration, we plot in Supplementary Fig. 5b how the standard deviation, σ, of an 80 s enzyme-free trajectory at 20 pN varies as a function of filter size. For the 6 s filter size chosen above σ = 10 bp. To avoid spurious interval crossing and hence first passage times the passage interval, m, is set to 20 bp = 2σ. The time taken for the trajectory to first pass each consecutive interval was measured until the end of the trajectory was reached. All first-passage times under the same conditions were concatenated and used in the fitting procedure. The parameters extracted are resistant to changes in passage interval, m, beyond the associated uncertainties, as the choice of interval for raw data analysis is reflected in the model fitting with Eq. 2.

The model first-passage time probability distribution (Eq. 2) was used to find maximum likelihood estimators for the model parameters. The maximum likelihood estimation is performed using fmincon in MATLAB (Mathworks, 2014a–2017a). The choice of model, unidirectional walker or biased random walk, as well as the number of phases to include was made by calculating the Bayesian Information Criterion (BIC) for each and selecting the model with the minimum BIC as the best fit. This criterion penalises for model complexity, i.e., the more parameters, the larger the BIC. Greater detail on fitting multi parameter models can be found in references 56,84 and those within.

For display of the data the first-passage times were binned, normalised by bin width and total samples and plotted. The model parameters found throughMLE are used to overlay the resulting analytical expression for the model best describing our data. We note that the model is not fit to the histogram.

The standard deviation error bars on the experimental first-passage time data bins and the standard deviation reported with estimated parameter values were calculated through bootstrapping with replacement 1000 times.

Buffers. A list of buffers and contents are given in the Supplementary Methods.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Data of this study are available from the authors upon reasonable request.

Code availability. Custom computer code used in this work is available for download at github.com/danburnham/LabVIEW (National Instruments, 2012) was used for instrument control and MATLAB (Mathworks, 2015a–2017a) for analysis.

Received: 26 April 2018 Accepted: 5 April 2019
Published online: 14 May 2019

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Author contributions

D.R.B. and H.Y. conceived and designed the study. D.R.B. built the apparatus, wrote the software, and performed experiments and ran analysis. H.B.K. purified DmrCMG and performed bulk unwinding assay. D.R.B., R.B.H., and H.Y. designed the FPT model. D.R.B. and H.Y. interpreted results and wrote the paper with input from H.B.K. and R.B.H.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-09896-2.

Competing interests: The authors declare no competing interests.

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Journal peer review information: Nature Communications thanks Vincent Croquette and other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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