

**Reviewed Preprint**

v1 • November 12, 2025

Not revised

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**Competing interests:** No

competing interests declared

**Funding:** See [page 16](#)

**Reviewing editor:** Kerry Bloom, The University of North Carolina at Chapel Hill, United States

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# DNA tensiometer reveals catch-bond detachment kinetics of kinesin-1, -2 and -3

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## eLife Assessment

The use of DNA tethers is a **useful** advance for studying how motor proteins respond to load. The authors use a **convincing** methodology to investigate the detachment and reattachment kinetics of kinesin-1, 2, and 3 motors against loads oriented parallel to the microtubule. As the manuscript stands, the conclusions drawn from the experiments, as well as the overall interpretation of the results, are **incompletely** supported by the presented data, and the novelty over previous reports appears less clear.

<https://doi.org/10.7554/eLife.108837.1.sa4>

## Abstract

Bidirectional cargo transport by kinesin and dynein is essential for cell viability and defects are linked to neurodegenerative diseases. Computational modeling suggests that the load-dependent off-rate is the strongest determinant of which motor ‘wins’ a kinesin-dynein tug-of-war, and optical tweezer experiments find family- dependent differences in the sensitivity of detachment to load, with kinesin-3 > kinesin-2 > kinesin-1. However, in reconstituted kinesin-dynein pairs *vitro*, all three kinesin families compete nearly equally well against dynein. Modeling and experiments have confirmed that vertical forces inherent to the large trapping beads enhance kinesin-1 dissociation rates. In *vivo*, vertical forces are expected to range from negligible to dominant, depending on cargo and microtubule geometries. To investigate the detachment and reattachment kinetics of kinesin-1, 2 and 3 motors against loads oriented parallel to the microtubule, we created a DNA tensiometer comprising a DNA entropic spring attached to the microtubule on one end and a motor on the other. Kinesin dissociation rates at stall were slower than detachment rates during unloaded runs, and the complex reattachment kinetics were consistent with a weakly-bound ‘slip’ state preceding detachment. Kinesin-3 behaviors under load suggested that long KIF1A run lengths result from the concatenation of multiple short runs connected by diffusive episodes. Stochastic simulations were able to recapitulate the load-dependent detachment and reattachment kinetics for all three motors and provide direct comparison of key transition rates between families. These results provide insight into how kinesin-1, -2 and -3 families transport cargo in complex cellular geometries and compete against dynein during bidirectional transport.

## Introduction

Bidirectional cargo transport by kinesin and dynein motors is essential for cell viability, and disruptions in transport are linked to neurological diseases including hereditary spastic paraplegia, microcephaly and amyotrophic lateral sclerosis <sup>1–8</sup>. It has been established that kinesin and dynein, which move in opposite directions along microtubules, are often bound simultaneously to the same cargo <sup>9–12</sup>. This has led to the ‘tug-of-war’ model, in which the direction of cargo movement is determined by which team of motors dominates <sup>9,13–17</sup>. How well

motors compete is determined by their load- dependent motor properties along with multiple regulation mechanisms, many of which are still emerging <sup>13,18–21</sup>. Furthermore, the large range of cargo sizes and the complexity of microtubule organization in cells means that motors are subjected to forces both parallel and perpendicular to their microtubule track, which can have differing effects on their mechanochemistry.

Intuitively, a motor's effectiveness in transporting cargo rests on its ability to remain bound to its microtubule track. Consistent with this, computational simulations have found that the load-dependent off-rate of a motor is the most important determinant of how well a kinesin competes against dynein in bidirectional transport <sup>22,23</sup>. Single-bead optical tweezers have found that the transport motors kinesin-1, -2, and -3 all act as slip bonds, defined as load accelerating their detachment rate. Their propensity to detach under load varies strongly by family, with relative load sensitivity kinesin-3 > kinesin-2 > kinesin-1 <sup>24–27</sup>. Based on this behavior, it was surprising that when kinesin-1 was linked to dynein, complexes moved at near-zero speeds for up to tens of seconds, much longer than predicted based on previously measured kinesin-1 off-rates <sup>25,28,29</sup>. Moreover, kinesin-1, -2, and -3 all fared equally well against dynein, contrary to the differing load-dependent detachment rates measured in single-bead optical tweezer experiments <sup>30</sup>.

Recent work suggests a solution to this paradox, namely that the ~micron scale beads used for optical trapping result in significant forces oriented perpendicular to the microtubule as the motor pulls against the force of the trap. First, the load-dependent dissociation rate from single-bead optical trapping was accounted for by a model in which the effects of horizontal loads on detachment is highly asymmetric and vertical loads play a dominant role in detachment, particularly against hindering loads <sup>31</sup>.

Second, when a three-bead optical trapping geometry was used (in which the motor is raised up on a pedestal bead and the microtubule was held by beads attached to either end of the microtubule) motors remained bound longer than in the single-bead geometry <sup>24,32</sup>. Third, when kinesin-1 motors were connected to a microtubule by a micron-long segment of DNA, very long residence times were observed, consistent with catch-bond behavior, defined as the off-rate slowing with load <sup>33</sup>. In cells, kinesin and dynein transport cargoes that range from tens of nm in diameter (like vesicles), where motor forces are expected to be aligned parallel to the microtubule, up to several microns (like mitochondria and nuclei), where vertical forces are expected to be much larger. Thus, understanding the influence of vertical and horizontal forces on transport motors is important for understanding the mechanics underlying bidirectional transport in cells.

The goal of the present study was to characterize the load-dependent detachment kinetics of kinesin-1, -2 and -3 motors in a geometry that eliminates vertical forces inherent in traditional optical trapping studies. Building on previous approaches, we used double stranded DNA (dsDNA), which acts as an entropic spring to resist the pN-level forces generated by the motors <sup>33–38</sup>. We found that kinesin-1, -2, and -3 all remained at stall for multiple seconds before releasing, which is substantially longer than the unloaded run times for kinesin-1 and -2. This behavior of slower off-rates under load is defined as a 'catch bond' and contrasts with the normal 'slip bond' behavior load- accelerated off-rates seen previously for kinesin <sup>39</sup>. Following the termination of a stall, motors reengaged with the microtubule with complex kinetics that were consistent with a 'slip' state that preceded full detachment. To compare the key transition rates that determined the family-specific motor behaviors, we developed a stochastic model that was able to recapitulate the experimental results for all three motors.

## Results

### Constructing a motor-bound DNA tensiometer

To study motor performance against a resistive load oriented parallel to the microtubule, we constructed a DNA tensiometer consisting of a ~ 1 μm strand of dsDNA attached to the microtubule on one end and a motor on the other (Figure 1A [↗](#)). We used TIRF microscopy to

visualize the motor moving against the entropic elasticity of the DNA spring. Due to the nonlinear elasticity of the DNA (Figure 1B [34,35](#)), the motor moves under minimal load until it stretches the DNA to near its contour length, at which point it stalls (Figure 1C-E [36](#)).

Our DNA-motor tensiometer consists of a 3,009 bp (999 nm contour length) dsDNA ‘spring’ that was synthesized by PCR using a biotinylated forward primer for attachment to the microtubule and a reverse primer containing a 3’ overhang for motor attachment (details in Methods). We investigated members of the three dominant families of kinesin transport motors, kinesin-1 (*Drosophila melanogaster* KHC), kinesin-2 (*Mus musculus* Kif3A), and kinesin-3 (*Rattus norvegicus* Kif1A) used in our previous kinesin-dynein study [30](#). In each case, the motor and neck linker domains were fused to the stable neck-coil domain (residues 345-406) of kinesin-1, followed by EGFP, a SNAP tag, and His6 tag, as described previously [30](#). This dimerization strategy avoids any autoinhibition and family-dependent differences in neck-coil stability, thus enabling the most direct comparison of family-dependent motor properties. Motors were conjugated to an oligonucleotide complementary to the 3’ overhang of the dsDNA spring via their C-terminal SNAP tag. The DNA tensiometer complex (Figure 1A [37](#)) was created in a flow cell by sequentially flowing in biotinylated microtubules, neutravidin, biotinylated dsDNA, and Qdot-functionalized motors containing the complementary oligo (described fully in Methods).

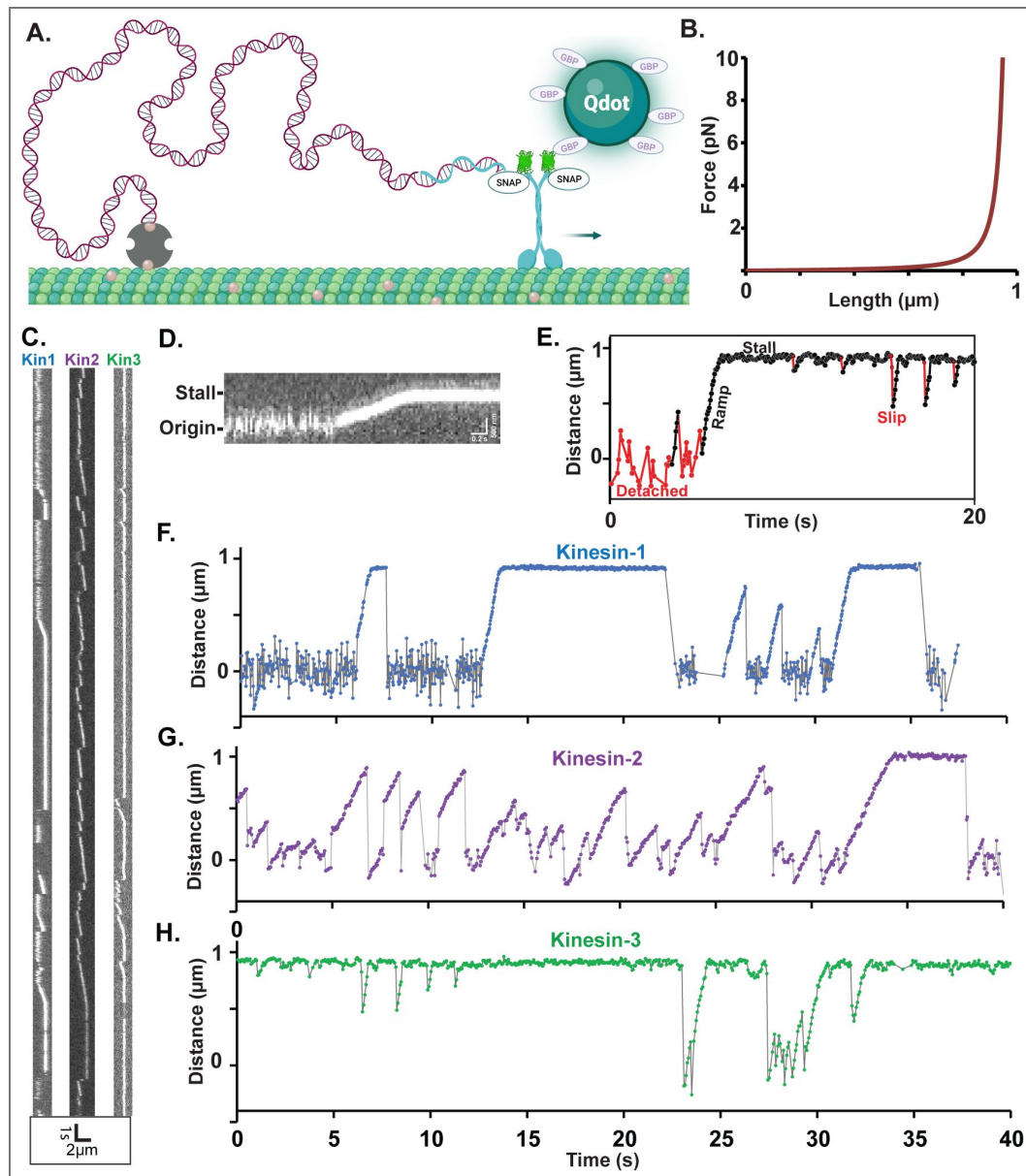
The resulting dsDNA tensiometer kymographs (Figure 1C [38](#)) show a reproducible behavior of moving, stalling, and returning to origin multiple times, which contrasts with the singular attachment, unidirectional movement and detachment of motors not bound by DNA (Figure S1). Because motors are tethered to the microtubule by the flexible DNA, large fluctuations around the origin are observed when the motor is detached (Figure 1C-H [39](#)). Consistent with these fluctuations, initial attachment points were variable and roughly normally distributed with a standard deviation of 145 nm (Figure S2). Upon engagement with the microtubule, the motor walks at a steady velocity, consistent with the expected nonlinear stiffness of the dsDNA tether (Figure 1B [40](#)), until it either disengages or reaches a stall state. Stalls are terminated either by the motor slipping backwards a short distance and restarting a new ramp, or by the motor fully disengaging and returning to the baseline (Figure 1E-H [41](#) and Figure S3). To confirm that motors are indeed extending the DNA and that Qdots are not enabling multi-motor assemblies, we incorporated Cy5-dCTP into the dsDNA and left the Qdots out of the reaction. In this case, clear extensions of the DNA spring could be observed, and the stall durations were of similar duration (Figure S4). In all subsequent experiments dsDNA was labeled with a low concentration of Cy5-dCTP to confirm colocalization of the DNA and the microtubule before collecting tensiometer data.

## Kinesin-1 and -2 act as catch-bonds at stall

The first question we addressed was: what are the detachment rates of kinesin-1, -2 and -3 motors at stall? The load-dependence of protein-protein interactions can be described as a slip-bond [40](#), defined as a faster off-rate under load; an ideal bond, defined as an off-rate that is independent of load; or a catch-bond, in which the off-rate is slower under load [39](#). Single-bead optical trapping studies consistently find slip-bond characteristics for kinesin-1, 2 and 3 [25,27,41](#), whereas dynein off-rates have been described as a slip-bond or catch-bond [42–45](#).

We define stall duration as the time that a motor stalls against the hindering load of fully extended DNA, without further detectable stepping. Stalls are terminated by the motor detectably (>60 nm) slipping backwards or by disengaging and returning to the origin (Figure 1E [46](#)). Although we don’t directly measure the stall force, based on the predicted force-extension curve of the dsDNA (Figure 1B [47](#)), the displacements are consistent with the 4–6 pN stall forces for kinesin-1, -2 and -3 measured using optical traps [27,46–50](#). Stall durations were compared to the unloaded single-motor run durations determined from TIRF kymograph analysis (Figure S1).

To compare unloaded to stall off-rates, cumulative distributions of the run and stall durations were plotted for each motor and fit with a single exponential function (Figure 2 [51](#)). The kinesin-1 tensiometer stall duration time constant was 3.01 s, with 95% confidence intervals (CI) of 2.30 to 3.79 s determined via bootstrapping in MEMLET with 1000 iterations) [51](#) (N= 78 stalls). In contrast, the kinesin-1 unloaded run duration time constant, measured by a traditional TIRF assay, was 1.04



**Figure 1. Experimental Design and Raw Data from Motor-DNA Tensiometers.**

(A) Schematic of a motor-DNA tensiometer, consisting of a dsDNA (burgundy) connected on one end to a kinesin motor through a complimentary oligo (blue), and on the other end to the MT using biotin-avidin (tan and gray, respectively). A Qdot functionalized with GFP binding protein nanobodies is attached to the motor's GFP tag and used to track motor position. (Not to scale; motor and Qdot are both  $\sim 20$  nm and DNA is  $\sim 1$  micron) (B) Predicted force extension curve for a worm-like chain 3009 bp dsDNA based on a 50 nm persistence length. (C) Representative kymographs of motor-DNA tensiometers for kinesins-1, -2 and -3. (D) Enlarged kymograph showing diffusion around the origin, ramp, and stall. (E) Example distance vs. time trace (kinesin-3), highlighting detached durations (red), ramps and stalls (black) where the motor has pulled the DNA taut, and transient slips during stall (red). (F-H) Representative distance vs. time plots for kinesin-1 (F), kinesin-2 (G) and kinesin-3 (H), corresponding to the kymographs in (C). Further examples are shown in Figure S3.

s, (95% CI of 0.79 to 1.30 s; N= 59) (Figure 2A). Stall durations longer than unloaded run durations indicate that load slows the off-rate, the definition of a catch-bond<sup>39</sup>. Similarly, the kinesin-2 tensiometer stall duration time constant of 2.83 s (95% CI of 2.03 to 3.79 s; N= 50) was longer than its unloaded run duration of 1.07 s (95% CI of 0.85 to 1.35 s; N= 87), also indicating a catch-bond. Conversely, the kinesin-3 tensiometer stall duration time constant of 1.89 s (95% CI of 1.53 to 2.31 s; N= 140) was shorter than its unloaded run duration of 2.74 s (95% CI of 2.33 to 3.17 s; N= 106), indicating a slip-bond characteristic by this definition.

## Kinesin-3 detaches readily under low load

To determine whether sub-stall hindering loads affect motor detachment rates, we compared tensiometer ramp durations to the tensiometer stall and unloaded run durations (Figure 3). We defined ramp durations as the time the motor spends walking against the DNA spring before a slip or detachment, or before reaching stall. Although the dsDNA force-extension curve (Figure 1B) predicts negligible loads until the DNA is close to fully extended, there are still non-zero loads imposed during the ramp phase that may affect motor detachment. Based on 10-20% slower ramp velocities relative to unloaded velocities for each motor, we estimated the apparent force to be ~1 pN (Table S1). To estimate the true detachment rate during the ramp phase in a way that takes into account both the observed detachments and ramps that successfully reach stall, we used a Markov process model, coupled with Bayesian inference methods (detailed in Supplementary Material) to estimate a duration parameter,  $\tau$ , equivalent to the inverse of the detachment rate constant during a ramp. Each increment of time is considered to be an independent opportunity to detach while assuming a constant detachment rate; hence the probability of staying attached to the microtubule through a segment of duration  $\Delta$  is  $e^{-\Delta/\tau}$ . Using this method, ramp duration parameters,  $\tau$ , were calculated for each motor, along with 95% credible regions. Finally, to allow for proper comparison, we performed a similar analysis to obtain the stall and unloaded duration parameters along with their 95% credible regions (Figure 3). The stall and unloaded durations were similar to estimates from curve fitting in Figure 2 (Table S2).

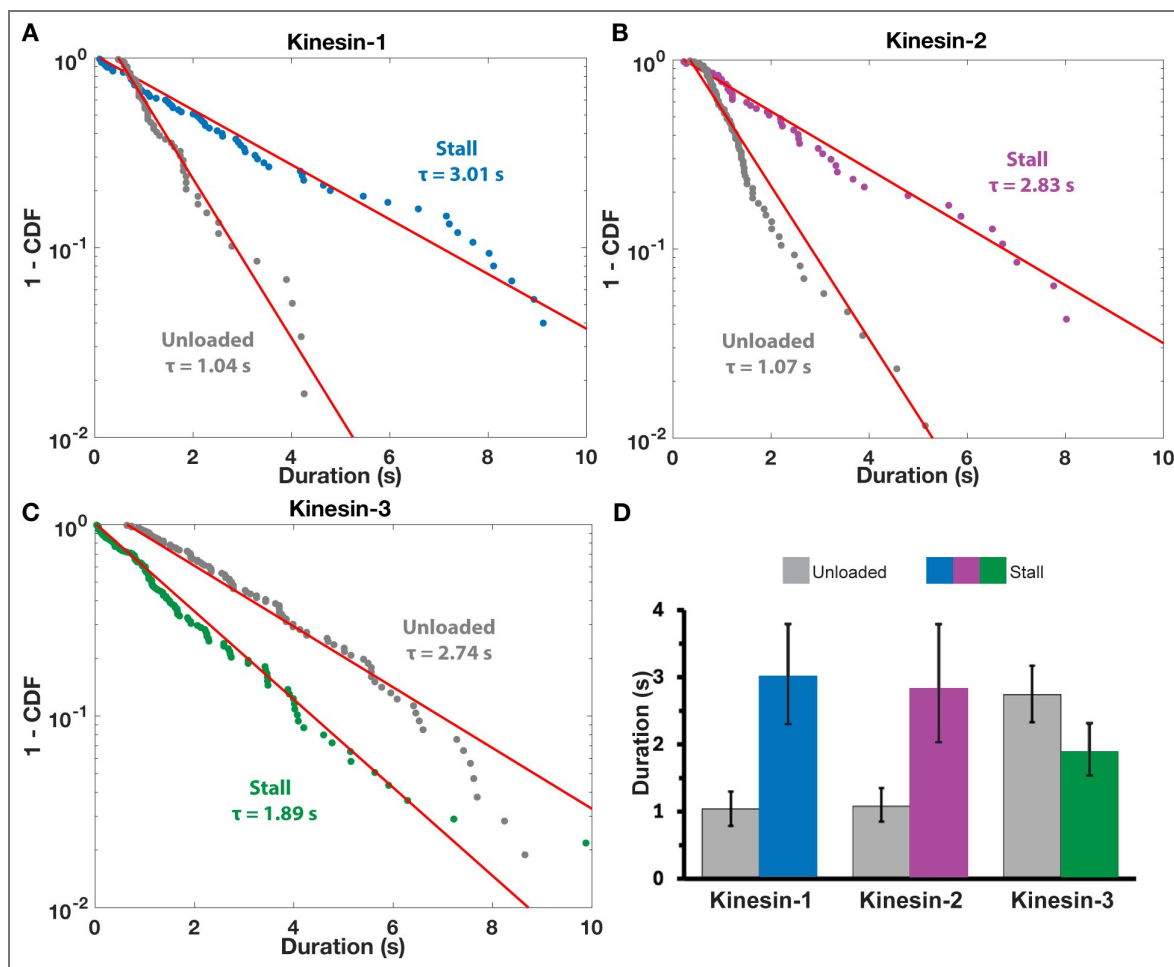
The simplest prediction is that against the low loads experienced during ramps, the detachment rate should match the unloaded detachment rate. This was the case for kinesin-2, where the ramp duration of 0.97 s was within 95% CI of the unloaded run duration of 1.08 s (Figure 3B, Table S2). In contrast, the kinesin-1 ramp duration of 2.49 s was much closer to the stall duration (3.05 s) than the unloaded run duration (1.05 s) (Figure 3A). To test whether the ramp duration was affected by the DNA tether, we carried out a control experiment in which the DNA tether was linked to the kinesin-1 motor but not the microtubule. The run duration in that case was 1.40 s, slightly longer than the motor alone, but less than the kinesin-1 ramp duration (Figure S6). One possible explanation for the longer kinesin-1 ramp is that the catch-bond character of kinesin-1 engages at low loads rather than rising proportionally to load or engaging only near stall.

The most notable ramp behavior was seen in kinesin-3, where the ramp duration of 0.75 s was nearly four-fold shorter than the unloaded run duration (2.76 s) and was more than two-fold shorter than the stall duration (1.90 s) (Figure 3C). As expanded on in the Discussion, the positively charged ‘K-loop’ in the kinesin-3 motor KIF1A is known to interact electrostatically with the negatively charged C-terminal tail of tubulin<sup>52–54</sup>; thus, it is reasonable that even the low loads imposed during ramps are sufficient to overcome these weak electrostatic interactions. The ramp duration is arguably the best definition of the time before KIF1A motors enter a partially dissociated ‘slip’ state, meaning that the observed unloaded durations represent a concatenation of multiple shorter runs interspersed by short diffusive events. Notably, by defining the ramp duration as the motor’s behavior under low load, kinesin-3 can be classified as a catch bond because high load (stall) durations are longer than low load (ramp) durations.

## Motor reengagement kinetics vary between families

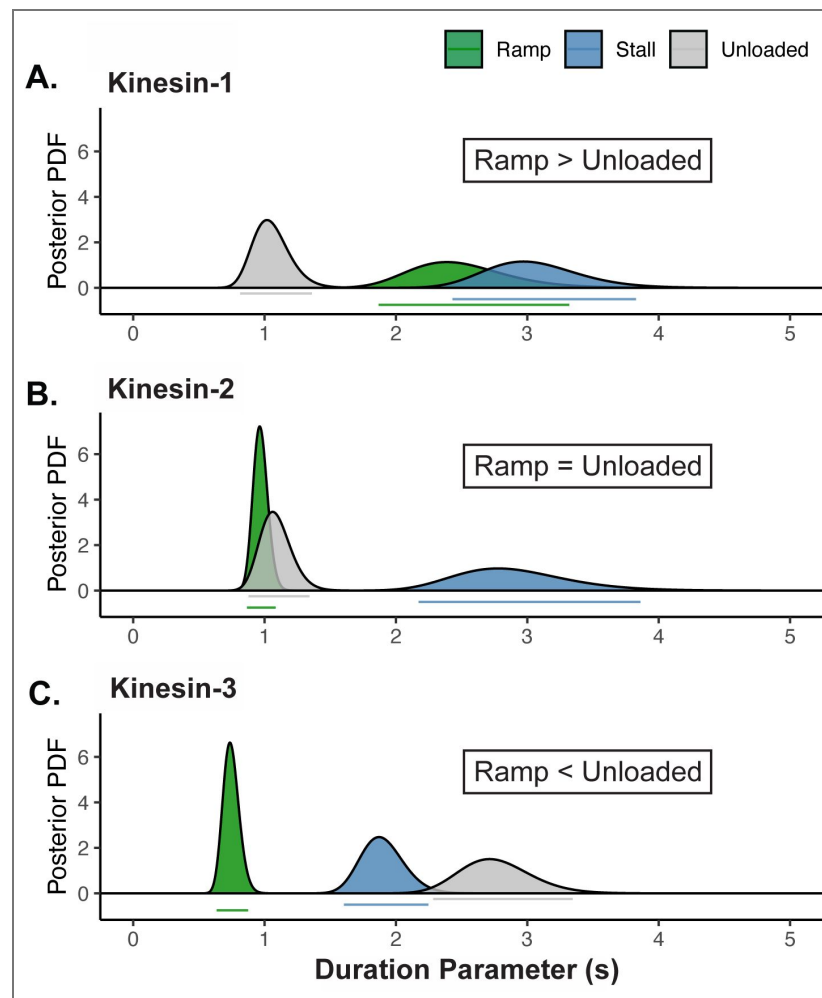
Stall plateaus were terminated by three types of events: 1) small slips that initiated a new ramp, typically within a single frame (~40 ms), 2) the motor returning to the baseline and reengaging rapidly within a few frames (~100 msec), or 3) the motor returning to the baseline for a few





**Figure 2. Tensiometer Stall Durations Indicate Catch-bond Behavior for Kinesin-1 and -2.**

Tensiometer stall durations are plotted for A) kinesin-1 (blue), (B) kinesin-2 (purple), and (C) kinesin-3 (green). Unloaded run durations for each motor are plotted in gray. Distributions were fit with a single exponential function using MEMLET to generate time constants, representing the mean durations. (D) Comparison of unloaded and stall durations for the three motors, with error bars indicating 95% CI. Stall durations >20s were excluded from the fit (three events for kinesin-1 and two events for kinesin-2). Bi-exponential fits of all data including >20 s are shown in Figure S5.



**Figure 3. During Ramps, Kinesin-3 Detaches More Readily Than Under Zero Load.**

Unloaded, ramp, and stall duration parameters were estimated using a Markov process model, coupled with Bayesian inference methods. Curves show the posterior probability distributions of the duration parameters for (A) kinesin-1, (B) kinesin-2 and (C) kinesin-3. Bars below each peak indicate the 95% credible regions for the ramp (green), unloaded (gray) and stall (blue) duration parameters. Notably, the estimated ramp durations are larger, the same, and smaller than the unloaded run durations for kinesin-1, -2, and -3, respectively. For the unloaded and stall durations, this estimation method produces almost identical values as the maximum likelihood estimates in Figure 2 (values provided in Table S2).

seconds before reengaging (Figure 4A). We defined a slip event as a displacement of  $>60$  nm from the plateau (distinguishable from normal small fluctuations at stall; Figure 1E) that recovers before reaching within 400 nm of the baseline (outside the range of normal baseline fluctuations; Figure 1F and S2). These slip events have been observed previously for all three motor families in optical trapping experiments and are proposed to represent an intermediate state in which the motor exits the normal stepping cycle but remains associated with the microtubule<sup>24,55–58</sup>. As an initial analysis, we quantified the fraction of events for each motor (Figure 4A) and found that kinesin-3 had the highest proportion of slip events while kinesin-2 had the lowest proportion. In the context of pulling a large cargo through the viscous cytoplasm or competing against dynein in a tug-of-war, these slip events enable the motor to maintain force generation and, hence are distinct from true detachment events. Thus, we reanalyzed the stall durations for the three motors where slips are not counted and only disengagements where the motor returns to the baseline are counted as stall termination events (Figure 4B & C, Figure S7). By this definition, stall durations were between 1.5 and 3-fold longer for each motor. Notably, the kinesin-3 stall duration in this analysis was longer than its unloaded run duration, defining it as a catch-bond by this measure.

To obtain a more complete picture of the motor reengagement kinetics for each motor, we plotted the time before starting a new ramp (trestarting), including all slips and reattachments (Figure 4D–F). In each case, the distributions included a fast phase and a long tail of slower events. The distributions were fit with a tri-exponential function with the fast phase (30, 130 and 40 msec, respectively) accounting for roughly half of the events (Figure 4 D–F, Table S3). The fast population corresponds to slip and fast reattachment events classified in Figure 4A. The slower phases, which represent detachment events where the motor fluctuated around the baseline before initiating a new ramp, were the fastest for kinesin-3 (time constants of 0.15 and 1.34 s) and the slowest for kinesin-1 (time constants of 1.51 and 20.9 s). Interestingly, the order of the reattachment kinetics (kinesin-3  $>$  kinesin-2  $>$  kinesin-1) and the  $\sim 10$ -fold ratio of kinesin-3 to kinesin-1 match published bimolecular on-rate constants for microtubule binding from stopped-flow experiments (1.1, 4.6, and 17  $\mu\text{M}^{-1} \text{s}^{-1}$  for kinesin-1, -2 and -3, respectively<sup>54,59,60</sup>).

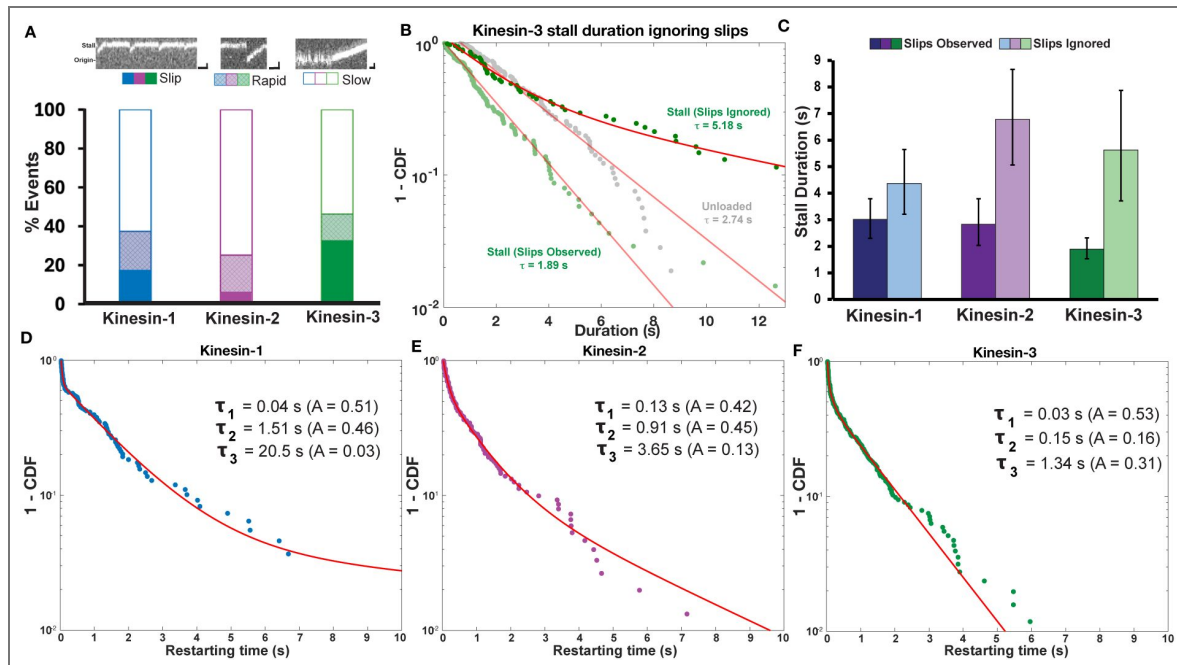
## Simulating Potential Catch-Bond Mechanisms

To compare the motor detachment and reattachment kinetics between the three kinesin transport families, we carried out stochastic simulations of load-dependent motor stepping, unbinding and rebinding. For simplicity, we reduced the chemomechanical cycle down to a single strongly-bound state (ATP and nucleotide-free states) and a single weakly-bound state (ADP and ADP-Pi states) (Figure 5A). Based on the published load and ATP dependencies of substeps in the kinesin-1 chemomechanical cycle<sup>55</sup>, we incorporated a load-dependent strong-to-weak transition,  $k_{s-w}$ . Based on our restarting durations from Figure 4 and previous work<sup>55–58</sup>, we included both a slip state, from which the motor recovers rapidly, and a detached state associated with a slower recovery. Runs or stalls are terminated by transition from the weakly-bound state into the slip state ( $k_{slip}$ ). Based on backstepping rates observed in optical tweezer experiments, we incorporated a load-independent backward stepping rate of  $3 \text{ s}^{-1}$ , meaning that stall is defined as the load at which forward stepping slows to  $3 \text{ s}^{-1}$ <sup>61,62</sup>. For simplicity, we set  $k_{s-w} = k_{w-s}$  at zero load, meaning that the motor spends half of its cycle in each state,<sup>63</sup> with the rates set to match the unloaded velocity for each motor.

Using parameters chosen to match motor behavior under no load (Figure 5B), we were able to reproduce the unloaded run durations for all three motors (Figure 5C–E).

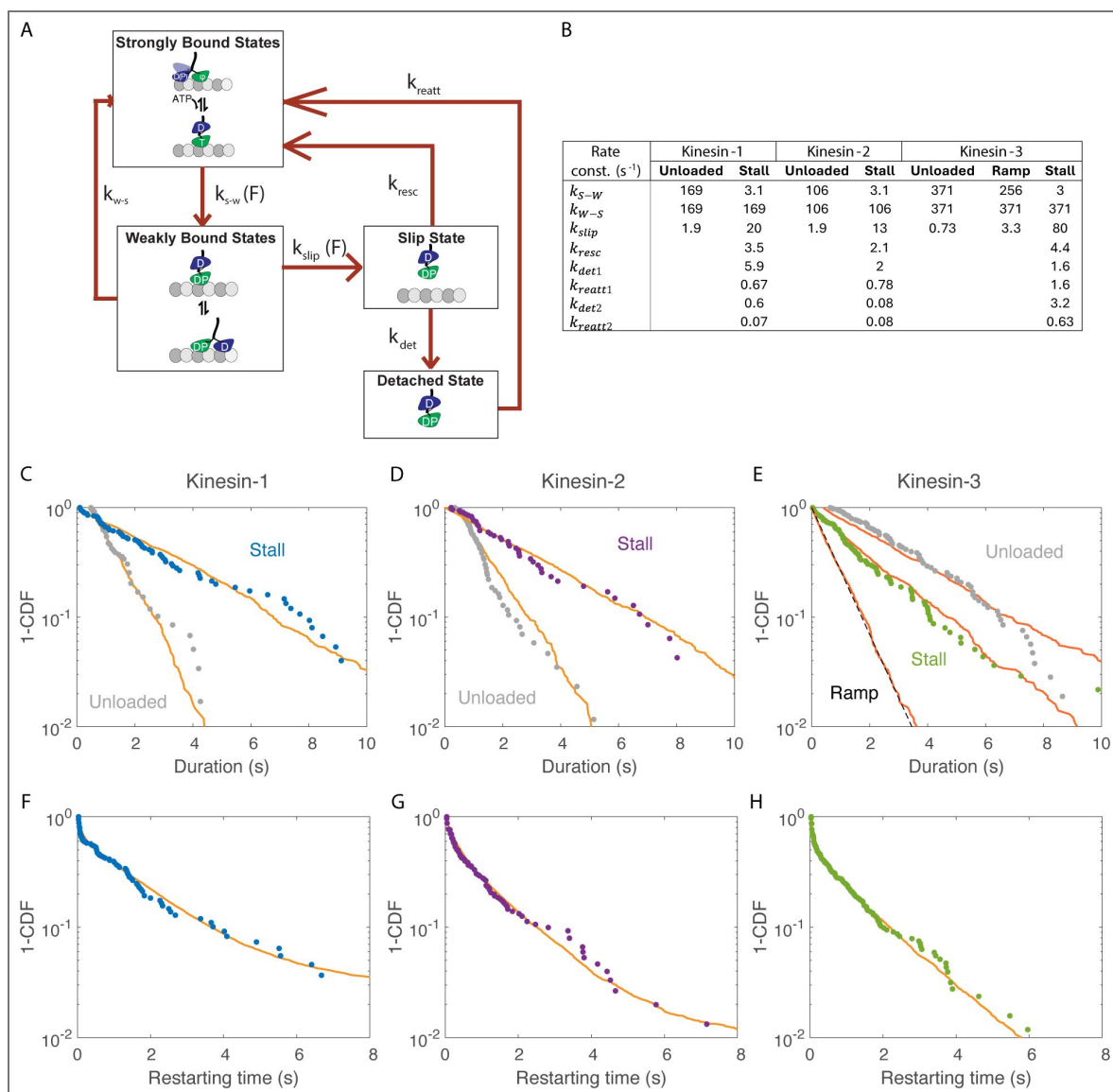
Next, to match the experimental stall durations we incorporated a negative load dependence into the transition out of the strongly-bound state and a positive load dependence into the transition from the weakly-bound state into the slip state. With these parameters (Figure 5B), we were able to reproduce the stall duration distribution for all three motors (Figure 5C–E). Importantly, in this model formulation, dissociation from the weakly-bound state acts as a slip-bond and the





**Figure 4. Restart Kinetics for Kinesin-1, -2 and -3.**

(A) Fraction of slip, fast rebinding, and slow rebinding events for each motor, with example kymographs for each (top; scale bars are 0.5  $\mu\text{m}$  and 0.2 s). Solid colors indicate slips during stall, where the motor resumes a new ramp within a single frame ( $\sim 40$  ms), crosshatching indicates rapid reattachment events (100 ms) following fall to baseline, and open bars indicate slow reattachment events with  $>100$  ms fluctuations around baseline. (B) Kinesin-3 stall durations, with unloaded run times in gray, stall durations terminated by slips in light green, and stall durations terminated by falling to the baseline (ignoring slips) in dark green. Unloaded and stall durations (replotted from Figure 2) were fit with single exponential functions in MEMLET. Stall durations ignoring slips were fit with a bi-exponential by least squares ( $p_1 = 2.01$  s [95% CI: 1.52, 2.35 s],  $A_1 = 0.66$  s [0.54, 0.83 s],  $p_2 = 11.0$  s [9.18, 13.70],  $A_2 = 0.33$  [0.23, 0.48]). Weighted average of the two time constants is displayed in plot for comparison to other time constants. Similar results for kinesin-1 and -2 are shown in Figure S7. (C) Comparison of stall durations for kinesins -1, -2 and -3 with slips observed as stall terminations or ignored. (D-F) Distribution of restart times for each motor fit to a tri-exponential (least squares). Confidence intervals of parameters determined by bootstrapping with 1000 iterations are given in Table S3.



**Figure 5. Chemomechanical Model of Proposed Catch-bond Mechanism.**

(A) Diagram of kinesin chemomechanical cycle model consisting of strongly- and weakly-bound states that make up the stepping cycle, and slip and detached states that terminate runs and stalls. Note that two pathways of detachment from the slip state (and reattachment) are incorporated into the model, but only one pathway is shown for simplicity (see Supplementary Methods for details). (B) Table of rate constants used to simulate unloaded and stall durations and restarting times. All rate constants are derived from fits to experimental data, as described in Supplemental Methods.  $k_{s-w}$  and  $k_{slip}$  depended exponentially on load ( $k(F) = k_1 e^{\beta F}$ ) with 8 for  $k_{s-w}$  of -2.7, -2.4, and -3.6 nm and 8 for  $k_{slip}$  of 1.6, 1.3 and 2.7 nm for kinesin-1, -2 and -3, respectively; see also Figure S8A). (C-E) Experimental (symbols) and simulated (lines) unloaded and stall durations. 10,000 events were simulated for each condition and plotted with minimum cutoffs matching experiments. Kinesin-3 ramp durations were taken from parameter estimated in Figure 3. (F-H) Experimental (symbols) and simulated (lines) restart times.

kinesin catch-bond characteristics are achieved by the motor spending a larger fraction of its cycle in the strongly-bound state under increasing loads. To complete our model, we simulated recovery from the slip and detached states.

The rate of rescue from the slip state ( $k_{\text{resc}}$ ) was set based on the duration and relative amplitude of the fast phase of the restarting times in Figure 4 [4](#). We posited that detachment follows the slip state, consistent with previous formulations [55,58](#). The two slower restarting time constants from Figure 4 [4](#) were used to set the reattachment rates,  $k_{\text{reatt}}$  (see Supplementary Methods for details). Using this approach, we were able to reproduce the restarting durations for all three motors (Figure 5F-H [4](#)).

The rate constants derived from this model allow for a comparison of the specific transitions that differ between the three motor families. Three features are notable. First, for kinesin-3 the transition into the slip state at stall,  $k_{\text{slip}}$ , is the fastest of the three families, consistent with the observation by eye of the plateaus (Figure 1H [4](#) and 4A [4](#)) and consistent with previous three-bead optical trapping results [24](#). Second, the rates of rescue from the slip state,  $k_{\text{resc}}$ , for the three motors match within a factor of two. Thus, the fast bimolecular on-rates from stopped flow and the relatively short durations before restarting we observe for kinesin-3 (Figure 4 [4](#)) do not result from a faster reengagement out of the slip state. Third, the slow reattachment rate for kinesin-3 is 10-fold faster than for kinesin-1 and -2. Hence, in this model formulation the fast bimolecular on-rates and short restart durations observed for kinesin-3 result from recovery from a detached state rather than rescue from a slip state.

## Discussion

Understanding how motors respond vectorially to external loads is crucial for understanding cargo transport in complex intracellular geometries and how kinesin motors compete against dynein in bidirectional transport [13,19](#). Optical tweezer experiments have provided many essential details of the kinesin mechanochemical cycle under load; however, the bead diameters needed to achieve substantial trapping forces impose vertical forces on the motors. Using DNA as a nanospring enables mechanical experiments using a standard TIRF microscope and allows for simultaneous monitoring of numerous motor-DNA complexes in a single imaging field. With this geometry, a kinesin motor pulls against the elastic force of a stretched DNA solely in a direction parallel to the microtubule, matching the geometry of vesicles measuring a few tens of nanometers. Similar approaches have been used to study myosin, dynein and kinesin-1 in both single-molecule and gliding assays [33,34,36–38,64](#). The most striking observation was that members of all three kinesin transport families show catch-bond behavior in which off-rates at stall are slower than those at low or zero loads.

Additionally, following disengagement from the microtubule, the three motor families reengaged with the microtubule with complex and variable kinetics.

## Comparison to previous work

Despite the clear slip-bond behavior of kinesin-1 seen in single-bead optical traps, there has been growing evidence that kinesin-1 detachment is sensitive to the direction of load. Motor engagement times were shown to decrease when larger beads were employed in single-bead traps, and to be extended in three-bead geometries that minimize vertical forces [32](#). In a study that employed DNA-tethered kinesin-1 to extract tubulin the microtubule lattice, pulling durations of  $\sim 30$  s were observed at the lowest motor concentrations, indicative of catch-bond behavior [33](#). When kinesin-1 was connected to micron-scale beads through a DNA linker and hydrodynamic forces parallel to the microtubule imposed, dissociation rates were relatively insensitive to loads up to  $\sim 3$  pN, inconsistent with slip-bond characteristics [37](#). The 3 s kinesin-1 stall duration in our tensiometer falls between 30 s value for tubulin pulling and the 1.3 s median engagement time measured in the three-bead trap [32,33](#). In contrast to kinesin-1, kinesin-3 (KIF1A) median engagement durations were found to be similarly short in both the one-bead (69 ms) and three bead (62 ms) geometries [24](#), much shorter than our 1.9 s stall duration in the DNA tensiometer. One

difference may be that very fast (~millisecond) slips observed in the optical tweezer measurements were counted as termination events, and thus limited engagement times. Secondly, the microtubule is held under tension in the three-bead experiment, which may alter the lattice properties. Finally, it can't be ruled out that KIF1A is particularly sensitive to vertical loads and small lateral or vertical forces present in the three-bead geometry are absent in the DNA tensiometer geometry. To date there have been no studies on kinesin-2 where vertical loads are minimized, and in single-bead optical traps the off-rate depends strongly on load<sup>65</sup>.

## Transport kinesins have a catch-bond behavior under hindering loads

What is the mechanism of the observed catch-bond behavior? Cell adhesion proteins such as integrins, selectins, and FimH have been shown to form longer lasting bonds under load, with the proposed mechanisms generally involving an allosteric effect that strengthens the protein:protein interface<sup>66–68</sup>. However, motor proteins are different in that they cycle in a nucleotide-dependent way between strongly- and weakly bound states, offering multiple potential mechanisms for slower dissociation rates under load. For instance, under a few piconewtons of load, Myosin I was shown to dissociate nearly two orders of magnitude slower than in the absence of load, an effect attributed to load-dependent trapping of ADP in the active site that maintained the motor a high-affinity binding state<sup>69</sup>. Dynein was also shown to have catch-bond behavior over certain ranges of resisting loads, though the precise mechanism is unclear<sup>42,43,70,71</sup>.

We interpreted our stepping, detachment, and reattachment results using a model that incorporates a load-dependent strong-to-weak transition and a load-dependent entry into a transient 'slip' state preceding detachment. The key feature of the model is that under load the motor spends an increasing fraction of its hydrolysis cycle in a strongly-bound state that resists dissociation.

## The role of vertical forces in motor detachment

We next asked whether by considering the different geometries we could reconcile our catch-bond observations with previous single-bead optical tweezer kinesin-1 slip-bond measurements that found the kinesin-1 off-rate increased from  $1.11 \text{ s}^{-1}$  at zero load to  $2.67 \text{ s}^{-1}$  at 6 pN<sup>25</sup>. Using a 440 nm bead diameter and estimated motor length of 35 nm that results in the force being imposed on the motor at a  $60^\circ$  angle (Figure S8B), a 6 pN stall force parallel to the microtubule corresponds to a 10 pN force perpendicular to the microtubule<sup>25,31</sup>. A model developed by Khataee and Howard was able to fully account for these geometry-dependent off-rates using a two-step detachment process having catch-bond behavior for parallel loads and slip-bond behavior for vertical loads<sup>31</sup>. Applying that model to our geometry and assuming a purely horizontal load and a 6 pN stall force, the predicted stall duration for kinesin-1 is 77 s, much longer than the 3 s we measure (Figure S8). We approached the detachment process differently, by incorporating a load-dependent transition in the hydrolysis cycle ( $k_S$ -W) and a load-dependent exit from the hydrolysis cycle ( $k_{\text{slip}}$ ). To explore effects of vertical forces using our two-state model, we incorporated both horizontal and vertical loads as accelerating detachment from the weakly-bound state, as follows:

$$k_{\text{slip}}(F) = k_{\text{slip}}^0 e^{\frac{(F_{\parallel}\delta_{\parallel} + F_{\perp}\delta_{\perp})}{k_B T}}$$

Here  $F_{\parallel}$  and  $F_{\perp}$  are the magnitude of the parallel and perpendicular loads and  $\delta_{\parallel}$  and  $\delta_{\perp}$  represent the distance parameters in each direction (Figure S8). Using  $\delta_{\parallel} = 1.61 \text{ nm}$  (Figure 5B<sup>30</sup>), we found that by setting  $\delta_{\perp} = 1.58 \text{ nm}$ , we were able to reproduce the slip bond behavior observed in the single-bead optical trap experiments (Figure S8).

Notably, this model implies that vertical and horizontal forces have similar effects on the transition rate into the slip state. We stress that this model is a hypothesis that needs further testing. Nonetheless, this is a simple formulation that shows that a motor can display either catch bond or slip bond behavior depending on the geometry of the imposed loads.

## Ramps reveal detachment behaviors at low loads

In addition to reporting on the detachment properties at stall, our DNA tensiometer provides new insights into fast rebinding that occurs during unloaded runs of kinesin-3. It has long been appreciated that the kinesin-3 motor KIF1A achieves long run lengths due to electrostatic attraction between its positively charged Loop-12 (K-loop) and the negatively charged C-terminal tail of tubulin <sup>53,54,72–77</sup>. Furthermore, the KIF1A off-rate in ADP, in which the motor diffuses on the microtubule lattice, was found to match the off-rate during processive stepping in ATP <sup>54</sup>. The relatively high microtubule affinity of this weakly-bound state suggests that the motor may be undergoing diffusive episodes between processive runs, while maintaining association with the microtubule.

Our DNA tensiometer offers a way to test the hypothesis that the long, unloaded run lengths of KIF1A are due to a concatenation of shorter runs. Due to the nonlinearity of the dsDNA force-extension curve in our DNA tensiometer, the motor is walking against forces below 1 pN for roughly 90% of the distance to stall (Figure 1B [↗](#)).

Consistent with this, motor velocities before stall were nearly constant (Figure 1D [↗](#) and S3), and averaged ~15% slower than unloaded velocities (Table S1), which corresponds to ~1 pN of force if the force-velocity relationship is linear. Using a Bayesian Inference approach that takes into account motors that dissociate during the ramps as well as those that complete ramps by achieving stall, we measured a nearly four-fold faster KIF1A detachment rate during ramps than under zero load (Figure 3 [↗](#)). If, under zero load, the long runs observed were actually a concatenation of a series of shorter runs connected by diffusive weakly-bound events, the diffusive state would likely be unable to withstand even the sub-pN forces from the DNA spring <sup>76</sup>. For instance, based on a  $0.044 \mu\text{m}^2/\text{s}$  diffusion coefficient (equivalent to a  $\sim 0.1 \text{ pN}\cdot\text{s}/\mu\text{m}$  drag coefficient <sup>76,78</sup>), if the motor were in a weakly-bound state for 10 ms, a 1 pN force would pull the motor back 100 nm. Thus, in considering whether KIF1A acts as a catch bond, we used this ramp duration as the best approximation for the true run unloaded length in the absence of diffusive events.

The ramp durations of kinesin-1 and kinesin-2 also provide insights into how load alters their interactions with microtubules. For kinesin-2, the predicted ramp duration was not statistically different from the unloaded run duration, suggesting that unloaded runs do not include short diffusive episodes. Interestingly the predicted ramp duration for kinesin-1 was nearly the same as the stall duration and much longer than the unloaded duration. One possibility is that the catch-bond effect of hindering load comes into play at low loads and not only at stall where the motor has slowed considerably.

## Motor slips and detachments reflect different processes

Because the DNA tensiometer tethers the motor near the microtubule, such that repeated binding and unbinding events occur, it enables comparison of family-dependent differences in kinesin rebinding kinetics. In addition to clear detachment events, rapid slip and recovery events were observed for all three motors, with highest frequency for kinesin-3 and lowest frequency for kinesin-1. Backwards slipping while maintaining association with the microtubule was first seen for kinesin-8 motors, which are highly processive yet generate only small forces <sup>56</sup>. Similar backward slips at stall were observed for kinesin-1, with kinetics that suggested a transition such as phosphate release precedes dissociation <sup>58</sup>. Subsequent higher resolution work, enabled by small Germanium nanoparticles, revealed a staircase pattern during these slips with ~8 nm steps of mean duration 73  $\mu\text{s}$ , suggesting that the motor was transiently interacting with each tubulin subunit as it slipped backward. Similar slips have also been observed for kinesin-2 and two kinesin-3 family members, KIF1A and KIF1C <sup>24,57</sup>.

There is some dispute in the literature regarding the kinetics of kinesin-1 recovery from the slip state. Using a single-bead trap, Sudhakar found that 80% of restart events were slips with a time constant of 128 ms <sup>55</sup>, whereas Toleikis measured slip recoveries that were essentially at the limit of detection (~1 ms) <sup>58</sup>. Using a three-bead trap, Pyrpassopoulos measured a 10 ms slip time constant for kinesin-1 <sup>32</sup>. Our 40 ms slip time constant, which accounts for half of recovery events,

is limited by the camera frame rate, and thus is likely an overestimate. In the three-bead geometry, kinesin-3 (KIF1A) slips recovered with a time constant of 1 ms<sup>24</sup>, faster than the 30 msec (upper limit estimate) we observe. Thus, the precise recovery rate is dependent on the detailed measurement and analysis used. In our DNA tensiometer results, the higher frequency of slips for kinesin-3 relative to kinesin-1 is seen by direct counting (Figure 4A<sup>24</sup>), by the large enhanced stall duration when slips are not counted as termination events (Figure 4C<sup>24</sup>), and by the fast kslip parameter under load in the kinesin-3 model (Figure 5B<sup>24</sup>).

## Catch-bond behavior provides insights into tug-of-war with dynein

In previous simulations of kinesin-dynein bidirectional transport, we found that the strongest determinants of kinesin's ability to compete against dynein were the load-dependent motor dissociation rate and the motor rebinding rate<sup>22,23</sup>. Simply put, if motors detach, then the opposing motor wins. The finding here that all three dominant kinesin transport families display catch-bond behavior at stall necessitates a reevaluation of how motors function during a tug-of-war. There is evidence that dynein forms a catch bond or at least an ideal (load independent) bond<sup>14,42,43,70,71,79</sup>; thus, kinesins and dyneins are primed to strongly oppose one another.

The catch bond results here help to explain previous in vitro work in which one kinesin and one dynein were connected through a complementary ssDNA<sup>14,28,30</sup>. It was found that the motor pairs had periods of near-zero velocity that lasted for many seconds, considerably longer than kinesin's unloaded off-rate. Furthermore, kinesin-2 and kinesin-3 also showed these sustained slow tug-of-war periods despite their reported faster load-dependent off-rates from optical tweezer studies. The functional catch-bond behavior observed here provides a simple explanation for these sustained kinesin-dynein stalemates.

Importantly, the load-dependent off-rates of both kinesins and dynein are expected to depend on the cargo geometry. A 30 nm vesicle would lead to forces on the motor nearly parallel to the microtubule surface, whereas when transporting a micron-scale mitochondria the vertical forces would be larger than the horizontal forces. Cargo geometry and stiffness are also expected to play a role; for instance, deformation of a cargo, either due to compliance of the cargo or to multiple motors pulling on it will tend to reduce vertical force components on the motors. The present work emphasizes that along with motor type, motor number, motor autoinhibition, and the growing list of regulatory proteins, the geometry with kinesin and dynein engage in a tug-of-war can be an important determinant of the speed and direction of cargo transport in cells.

## Methods

### DNA Tensiometer Construction

For the dsDNA spring, a 5' biotinylated forward primer (5'-5Biosg/TGC CTC CGT GTA AGG GGG AT-3') and a reverse primer with a 5' overhang (5'-GGG CCA TCG CCA ATT GGA GTA /idSp/ GTG AGT TAA AGT TGT ACT CGA GTT TGT GTC CAA GAA -3') were used to create a 3009 bp dsDNA by PCR from plasmid *Mus Musculus* BicD2- sf-GFP (aa 25-425) in pet28a. The abasic Int 1',2'-Dideoxyribose spacer (idSp) creates an overhang by terminating the polymerase. All oligonucleotides were purchased from IDT. Each 50 µL PCR reaction contained: 1x Phusion HF buffer, 198 µM dNTPs, 2 µM dCTP-Cy5, 0.5 µM primers, 3 ng template DNA and 1 U/50 µL HF Phusion Polymerase. Fluorescent dsDNA used in Figure S4 had 10 µM dCTP-Cy5 and 190 µM dNTPs. The PCR reaction was carried out in a thermal cycler with the following procedure: 98°C for 30 s, then 45 cycles of 98°C for 10 s, 58°C for 30 s and 72°C for 1.5 min, then lastly 72°C for 5 min. The product was purified using a NucleoSpin® PCR clean-up kit and the concentration determined by absorbance on a Nanodrop 2000c Spectrophotometer.

DNA bands were visualized on a 1% agarose gel with ethidium bromide staining.



## Motor-Microtubule-Tensiometer Assembly

Motors were bacterially expressed, purified and linked through its SNAP tag to an oligonucleotide (5'-TAC TCC AAT TGG CGA TGG CCC / 3AmMC6T/-3') complementary to the dsDNA overhang. Details of motor expression, purification and labeling, as well as tubulin biotinylation and polymerization are given in Supplementary Information. The DNA tensiometer was assembled on the microtubule as follows. The following three buffers are made on the same day of the experiment: C2AT (BRB80, 10  $\mu$ M Taxol, 2 mM MgATP, 2 mg/mL Casein), 2AT (BRB80, 10  $\mu$ M Taxol, 2 mM MgATP) and Imaging Solution (BRB80, 10  $\mu$ M Taxol, 2 mg/mL Casein, 2 mM MgATP, 20 mM D-glucose, 0.02 mg/mL Glucose oxidase, 0.008 mg/mL Catalase, 0.5% BME and 2 mg/mL BSA). Full-length rigor kinesin was used to attach microtubules to the coverglass <sup>63</sup>.

Tensiometers were created in the flow cell using the following work flow: C2AT, 5 min > Rigor kinesin, 5 min > C2AT wash > BioMT, 5 min > 2AT > 8 nM Neutravidin, 5 min > 2AT > 10 nM Bio-dsDNA-Overhang, 5 min > C2AT > 4 nM KinesinMotor + 40nM Qdot-GBP (pre incubated in tube on ice for >15 min) in imaging solution, 10 min > Imaging solution wash. Note that because casein can contain free biotin, casein-free 2AT buffer was used during avidin-biotin binding steps. Following assembly, the Qdot connected to the motor was imaged on a custom-built TIRF microscope, described previously <sup>80</sup>. Raw data were typically collected at 25 fps (range of 20-40 fps) on a Photometrics Prime 95B camera.

## Data Analysis

Movies were uploaded into FIESTA software <sup>81</sup> and Qdot intensities were tracked using a symmetric 2-D gaussian function to obtain x,y,t data for each Qdot. When drift correction was needed, TetraSpeck™ Fluorescent Microspheres (Thermo) and immobile Qdots were used as fiducial markers. The smallest position errors at stall in FIESTA fitting were 3-4 nm, which matched the positional error of Qdot-labeled motors stuck to microtubules in AMPPNP. Points with position errors greater than 20 nm were excluded because they often involved clearly spurious position estimates. Notably, many tensiometers had small segments of missing data due to the Qdot fluctuating out of the TIRF field or blinking; these occurred most often during periods when the motors were detached from the microtubule.

After obtaining X and Y positions of linear motor tracks in Fiesta, we rotated and translated the data in Excel to generate X versus t traces. The apparent origin was determined by averaging the points where the motor is fluctuating on its tether. In rare instances where no fluctuation was observed, the approximate origin was calculated by averaging the starting positions of all the ramps within the tensiometer (Figure 1 [↗](#)). We then measured the ramp time, distance traveled, stall durations, reattachment times and starting positions. Tensiometers occasionally ended with the Qdot signal going dark, denoting either bleaching or failure of the Qdot-motor or motor-DNA connection.

Notably, no clear instances of motor-Qdots walking past the plateau point (denoting the tensiometer breaking) were observed. Stalls that terminated due to the tensiometer going dark or the video ending were excluded from analysis.

## Stochastic Modeling

Kinesin run and stall durations were simulated by using a modified version of published stochastic model of kinesin stepping <sup>23,30</sup>. A motor is either in a strongly- bound state or a weakly-bound state (Figure 5A [↗](#)). At each timepoint, a motor in the strongly bound state can transition into the weakly-bound state with a first order transition rate constant,  $k_{112}$  or step backward by 8 nm with a constant rate,  $k_{345} = 3 \text{ s}^{-1}$ . A motor in the weakly-bound state can complete an 8-nm forward step by transitioning back to the strongly-bound state with rate constant,  $k_{211}$ , or it can disengage from the microtubule with transition rate,  $k_{\text{off}}$ .

For simplicity, we set  $k_{112} = k_{211}$  at zero load. Load-dependent transition rates were defined as:

$$k(F) = k^0 * e^{\frac{F\delta}{k_B T}}$$

where  $k^0$  is the unloaded transition rate,  $\delta$  is the characteristic distance parameter and  $k_B T$  is the Boltzmann's constant multiplied by the absolute temperature, equal to 4.1 pN-nm at 25° C. Stall force was set to 6 pN. Unloaded and stall durations were simulated by starting the motor in strongly-bound state and continuing until it transitioned into the slip state, with 1000 simulations for each condition. Restart times were simulated by starting the motor in the slip state. From there, the motor can reengage by transitioning into the strongly-bound state or switch to one of two detached states having either a slow or fast recovery rate. Restart simulations were run for 10,000 iterations. Model parameters were constrained by experimental data, described fully in Supplementary Methods.

## Acknowledgements

This work was originally conceived as part of a NIH-funded collaborative modeling project to Will Hancock, Scott McKinley, John Fricks, and Peter Kramer (R01GM122082). We thank Qingzhou Feng, Scott Pflumm, and Adheshwari Ramesh for early efforts on this project and all members of the Hancock Lab for helpful discussions. This work was funded by NIH Grant R35GM139568 to W.O.H.. C.R.N. was supported by NIH postdoctoral fellowship F32GM149114, R.J. was supported by NIH Training Grant T32GM108563, and S.A.M. was supported by the NSF-Simons Southeast Center for Mathematics and Biology (SCMB) through grant NSF-DMS1764406 and Simons Foundation-SFARI 594594.

## Additional information

### Author Contributions

S.A.M. and W.O.H. conceived of original idea, C.R.N., R.J. and W.O.H. designed research; C.R.N., R.J. and T.M. performed research; C.R.N., T.M. and S.A.M. analyzed data; C.R.N., T.M., S.A.M., and W.O.H. wrote the paper.

### Funding

| Funder                        | Grant reference number |
|-------------------------------|------------------------|
| National Institutes of Health | R35GM139568            |
| National Institutes of Health | F32GM149114            |
| National Institutes of Health | T32GM108563            |
| National Science Foundation   | DMS1764406             |
| Simons Foundation             | 594594                 |

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## Additional files

[Supplementary Data](#) 

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## Peer reviews

### Reviewer #1 (Public review):

#### Summary:

Noell et al have presented a careful study of the dissociation kinetics of Kinesin (1,2,3) classes of motors moving in vitro on a microtubule. These motors move against the opposing force from a ~1 micron DNA strand (DNA tensiometer) that is tethered to the microtubule and also bound to the motor via specific linkages (Figure 1A). The authors compare the time for which motors remain attached to the microtubule when they are tethered to the DNA, versus when they are not. If the former is longer, the interpretation is that the force on the motor from the stretched DNA (presumed to be working solely along the length of the microtubule) causes the motor's detachment rate from the microtubule to be reduced. Thus, the specific motor exhibits "catch-bond" like behaviour.

#### Strengths:

The motivation is good - to understand how kinesin competes against dynein through the possible activation of a catch bond. Experiments are well done, and there is an effort to model the results theoretically.

#### Weaknesses:

The motivation of these studies is to understand how kinesin (1/2/3) motors would behave when they are pitted in a tug of war against dynein motors as they transport cargo in a bidirectional manner on microtubules. Earlier work on dynein and kinesin motors using optical tweezers has suggested that dynein shows a catch bond phenomenon, whereas such signatures were not seen for kinesin. Based on their data with the DNA tensiometer, the authors would like to claim that (i) Kinesin1 and Kinesin2 also show catch-bonding and (ii) the earlier results using optical traps suffer from vertical forces, which complicates the catch-bond interpretation.

While the motivation of this work is reasonable, and the experiments are careful, I find significant issues that the authors have not addressed:

(1) Figure 1B shows the PREDICTED force-extension curve for DNA based on a worm-like chain model. Where is the experimental evidence for this curve? This issue is crucial because the F-E curve will decide how and when a catch-bond is induced (if at all it is) as the motor moves against the tensiometer. Unless this is actually measured by some other means, I find it hard to accept all the results based on Figure 1B.

(2) The authors can correct me on this, but I believe that all the catch-bond studies using optical traps have exerted a load force that exceeds the actual force generated by the motor. For example, see Figure 2 in reference 42 (Kunwar et al). It is in this regime (load force > force from motor) that the dissociation rate is reduced (catch-bond is activated). Such a regime is never reached in the DNA tensiometer study because of the very construction of the experiment. I am very surprised that this point is overlooked in this manuscript. I am therefore not even sure that the present experiments even induce a catch-bond (in the sense reported for earlier papers).

(3) I appreciate the concerns about the Vertical force from the optical trap. But that leads to the following questions that have not at all been addressed in this paper:

(i) Why is the Vertical force only a problem for Kinesins, and not a problem for the dynein studies?

(ii) The authors state that "With this geometry, a kinesin motor pulls against the elastic force of a stretched DNA solely in a direction parallel to the microtubule". Is this really true? What matters is not just how the kinesin pulls the DNA, but also how the DNA pulls on the kinesin. In Figure 1A, what is the guarantee that the DNA is oriented only in the plane of the paper? In fact, the DNA could even be bending transiently in a manner that it pulls the kinesin motor UPWARDS (Vertical force). How are the authors sure that the reaction force between DNA and kinesin is oriented SOLELY along the microtubule?

(4) For this study to be really impactful and for some of the above concerns to be addressed, the data should also have included DNA tensiometer experiments with Dynein. I wonder why this was not done?

While I do like several aspects of the paper, I do not believe that the conclusions are supported by the data presented in this paper for the reasons stated above.

<https://doi.org/10.7554/eLife.108837.1.sa3>

## Reviewer #2 (Public review):

### Summary:

To investigate the detachment and reattachment kinetics of kinesin-1, 2, and 3 motors against loads oriented parallel to the microtubule, the authors used a DNA tensiometer approach comprising a DNA entropic spring attached to the microtubule on one end and a motor on the other. They found that for kinesin-1 and kinesin-2, the dissociation rates at stall were smaller than the detachment rates during unloaded runs. With regard to the complex reattachment kinetics found in the experiments, the authors argue that these findings were consistent with a weakly-bound 'slip' state preceding motor dissociation from the microtubule. The behavior of kinesin-3 was different and (by the definition of the authors) only showed prolonged "detachment" rates when disregarding some of the slip events. The authors performed stochastic simulations that recapitulate the load-dependent detachment and reattachment kinetics for all three motors. They argue that the presented results provide insight into how kinesin-1, -2, and -3 families transport cargo in complex cellular geometries and compete against dynein during bidirectional transport.

### Strengths:

The present study is timely, as significant concerns have been raised previously about studying motor kinetics in optical (single-bead) traps where significant vertical forces are present. Moreover, the obtained data are of high quality, and the experimental procedures are clearly described.

### Weaknesses:

However, in the present version of the manuscript, the conclusions drawn from the experiments, the overall interpretation of the results, and the novelty over previous reports appear less clear.

### Major comments:

(1) The use of the term "catch bond" is misleading, as the authors do not really mean consistently a catch bond in the classical sense (i.e., a protein-protein interaction having a dissociation rate that decreases with load). Instead, what they mean is that after motor detachment (i.e., after a motor protein dissociating from a tubulin protein), there is a slip state during which the reattachment rate is higher as compared to a motor diffusing in solution. While this may indeed influence the dynamics of bidirectional cargo transport (e.g., during tug-of-war events), the used terms (detachment (with or without slip?), dissociation,

rescue, ...) need to be better defined and the results discussed in the context of these definitions. It is very unsatisfactory at the moment, for example, that kinesin-3 is at first not classified as a catch bond, but later on (after tweaking the definitions) it is. In essence, the typical slip/catch bond nomenclature used for protein-protein interaction is not readily applicable for motors with slippage.

(2) The authors define the stall duration as the time at full load, terminated by >60 nm slips/detachments. Isn't that a problem? Smaller slips are not detected/considered... but are also indicative of a motor dissociation event, i.e., the end of a stall. What is the distribution of the slip distances? If the slip distances follow an exponential decay, a large number of short slips are expected, and the presented data (neglecting those short slips) would be highly distorted.

(3) Along the same line: Why do the authors compare the stall duration (without including the time it took the motor to reach stall) to the unloaded single motor run durations? Shouldn't the times of the runs be included?

(4) At many places, it appears too simple that for the biologically relevant processes, mainly/only the load-dependent off-rates of the motors matter. The stall forces and the kind of motor-cargo linkage (e.g., rigid vs. diffusive) do likely also matter. For example: "In the context of pulling a large cargo through the viscous cytoplasm or competing against dynein in a tug-of-war, these slip events enable the motor to maintain force generation and, hence, are distinct from true detachment events." I disagree. The kinesin force at reattachment (after slippage) is much smaller than at stall. What helps, however, is that due to the geometry of being held close to the microtubule (either by the DNA in the present case or by the cargo in vivo) the attachment rate is much higher. Note also that upon DNA relaxation, the motor is likely kept close to the microtubule surface, while, for example, when bound to a vesicle, the motor may diffuse away from the microtubule quickly (e.g., reference 20).

(5) Why were all motors linked to the neck-coil domain of kinesin-1? Couldn't it be that for normal function, the different coils matter? Autoinhibition can also be circumvented by consistently shortening the constructs.

(6) I am worried about the neutravidin on the microtubules, which may act as roadblocks (e.g. DOI: 10.1039/b803585g), slip termination sites (maybe without the neutravidin, the rescue rate would be much lower?), and potentially also DNA-interaction sites? At 8 nM neutravidin and the given level of biotinylation, what density of neutravidin do the authors expect on their microtubules? Can the authors rule out that the observed stall events are predominantly the result of a kinesin motor being stopped after a short slippage event at a neutravidin molecule?

(7) Also, the unloaded runs should be performed on the same microtubules as in the DNA experiments, i.e., with neutravidin. Otherwise, I do not see how the values can be compared.

(8) If, as stated, "a portion of kinesin-3 unloaded run durations were limited by the length of the microtubules, meaning the unloaded duration is a lower limit." corrections (such as Kaplan-Meier) should be applied, DOI: 10.1016/j.bpj.2017.09.024.

(9) Shouldn't Kaplan-Meier also be applied to the ramp durations ... as a ramp may also artificially end upon stall? Also, doesn't the comparison between ramp and stall duration have a problem, as each stall is preceded by a ramp ...and the (maximum) ramp times will depend on the speed of the motor? Kinesin-3 is the fastest motor and will reach stall much faster than kinesin-1. Isn't it obvious that the stall durations are longer than the ramp duration (as seen for all three motors in Figure 3)?

(10) It is not clear what is seen in Figure S6A: It looks like only single motors (green, w/o a DNA molecule) are walking ... Note: the influence of the attached DNA onto the stepping

duration of a motor may depend on the DNA conformation (stretched and near to the microtubule (with neutravidin!) in the tethered case and spherically coiled in the untethered case).

(11) Along this line: While the run time of kinesin-1 with DNA (1.4 s) is significantly shorter than the stall time (3.0 s), it is still larger than the unloaded run time (1.0 s). What do the authors think is the origin of this increase?

(12) "The simplest prediction is that against the low loads experienced during ramps, the detachment rate should match the unloaded detachment rate." I disagree. I would already expect a slight increase.

(13) Isn't the model over-defined by fitting the values for the load-dependence of the strong-to-weak transition and fitting the load dependence into the transition to the slip state?

(14) "When kinesin-1 was tethered to a glass coverslip via a DNA linker and hydrodynamic forces were imposed on an associated microtubule, kinesin-1 dissociation rates were relatively insensitive to loads up to ~3 pN, inconsistent with slip-bond characteristics (37)." This statement appears not to be true. In reference 37, very similar to the geometry reported here, the microtubules were fixed on the surface, and the stepping of single kinesin motors attached to large beads (to which defined forces were applied by hydrodynamics) via long DNA linkers was studied. In fact, quite a number of statements made in the present manuscript have been made already in ref. 37 (see in particular sections 2.6 and 2.7), and the authors may consider putting their results better into this context in the Introduction and Discussion. It is also noteworthy to discuss that the (admittedly limited) data in ref. 37 does not indicate a "catch-bond" behavior but rather an insensitivity to force over a defined range of forces.

<https://doi.org/10.7554/eLife.108837.1.sa2>

### Reviewer #3 (Public review):

#### Summary:

Several recent findings indicate that forces perpendicular to the microtubule accelerate kinesin unbinding, where perpendicular and axial forces were analyzed using the geometry in a single-bead optical trapping assay (Khataee and Howard, 2019), comparison between single-bead and dumbbell assay measurements (Pyrpassopoulos et al., 2020), and comparison of single-bead optical trap measurements with and without a DNA tether (Hensley and Yildiz, 2025).

Here, the authors devise an assay to exert forces along the microtubule axis by tethering kinesin to the microtubule via a dsDNA tether. They compared the behavior of kinesin-1, -2, and -3 when pulling against the DNA tether. In line with previous optical trapping measurements, kinesin unbinding is less sensitive to forces when the forces are aligned with the microtubule axis. Surprisingly, the authors find that both kinesin-1 and -2 detach from the microtubule more slowly when stalled against the DNA tether than in unloaded conditions, indicating that these motors act as catch bonds in response to axial loads. Axial loads accelerate kinesin-3 detachment. However, kinesin-3 reattaches quickly to maintain forces. For all three kinesins, the authors observe weakly attached states where the motor briefly slips along the microtubule before continuing a processive run.

#### Strengths:

These observations suggest that the conventional view that kinesins act as slip bonds under load, as concluded from single-bead optical trapping measurements where perpendicular loads are present due to the force being exerted on the centroid of a large (relative to the

kinesin) bead, needs to be reconsidered. Understanding the effect of force on the association kinetics of kinesin has important implications for intracellular transport, where the force-dependent detachment governs how kinesins interact with other kinesins and opposing dynein motors (Muller et al., 2008; Kunwar et al., 2011; Ohashi et al., 2018; Gicking et al., 2022) on vesicular cargoes.

Weaknesses:

The authors attribute the differences in the behaviour of kinesins when pulling against a DNA tether compared to an optical trap to the differences in the perpendicular forces. However, the compliance is also much different in these two experiments. The optical trap acts like a  $\sim$  linear spring with stiffness  $\sim 0.05$  pN/nm. The dsDNA tether is an entropic spring, with negligible stiffness at low extensions and very high compliance once the tether is extended to its contour length (Fig. 1B). The effect of the compliance on the results should be addressed in the manuscript.

Compared to an optical trapping assay, the motors are also tethered closer to the microtubule in this geometry. In an optical trap assay, the bead could rotate when the kinesin is not bound. The authors should discuss how this tethering is expected to affect the kinesin reattachment and slipping. While likely outside the scope of this study, it would be interesting to compare the static tether used here with a dynamic tether like MAP7 or the CAP-GLY domain of p150glued.

In the single-molecule extension traces (Figure 1F-H; S3), the kinesin-2 traces often show jumps in position at the beginning of runs (e.g., the four runs from  $\sim 4$ -13 s in Fig. 1G). These jumps are not apparent in the kinesin-1 and -3 traces. What is the explanation? Is kinesin-2 binding accelerated by resisting loads more strongly than kinesin-1 and -3?

When comparing the durations of unloaded and stall events (Fig. 2), there is a potential for bias in the measurement, where very long unloaded runs cannot be observed due to the limited length of the microtubule (Thompson, Hoeprich, and Berger, 2013), while the duration of tethered runs is only limited by photobleaching. Was the possible censoring of the results addressed in the analysis?

The mathematical model is helpful in interpreting the data. To assess how the "slip" state contributes to the association kinetics, it would be helpful to compare the proposed model with a similar model with no slip state. Could the slips be explained by fast reattachments from the detached state?

<https://doi.org/10.7554/eLife.108837.1.sa1>

## Author response:

### Reviewer 1 (Public review):

*(1) Figure 1B shows the PREDICTED force-extension curve for DNA based on a worm-like chain model. Where is the experimental evidence for this curve? This issue is crucial because the F-E curve will decide how and when a catch-bond is induced (if at all it is) as the motor moves against the tensiometer. Unless this is actually measured by some other means, I find it hard to accept all the results based on Figure 1B.*

The Worm-Like-Chain model for the elasticity of DNA was established by early work from the Bustamante lab (Smith et al., 1992) and Marko and Siggia (Marko and Siggia, 1995), and was further validated and refined by the Block lab (Bouchiat et al., 1999; Wang et al., 1997). The 50 nm persistence length is the consensus value, and was shown to be independent of force and extension in Figure 3 of Bouchiat et al (Bouchiat et al., 1999). However, we would like to stress that for our conclusions, the precise details of the Force-Extension relationship of our dsDNA

are immaterial. The key point is that the motor stretches the DNA and stalls when it reaches its stall force. Our claim of the catch-bond character of kinesin is based on the longer duration at stall compared to the run duration in the absence of load. Provided that the motor is indeed stalling because it has stretched out the DNA (which is strongly supported by the repeated stalling around the predicted extension corresponding to ~6 pN of force), then the stall duration depends on neither the precise value for the extension nor the precise value of the force at stall.

*(2) The authors can correct me on this, but I believe that all the catch-bond studies using optical traps have exerted a load force that exceeds the actual force generated by the motor. For example, see Figure 2 in reference 42 (Kunwar et al). It is in this regime (load force > force from motor) that the dissociation rate is reduced (catch-bond is activated). Such a regime is never reached in the DNA tensiometer study because of the very construction of the experiment. I am very surprised that this point is overlooked in this manuscript. I am therefore not even sure that the present experiments even induce a catch-bond (in the sense reported for earlier papers).*

It is true that Kunwar et al measured binding durations at super-stall loads and used that to conclude that dynein does act as a catch-bond (but kinesin does not) (Kunwar et al., 2011). However, we would like to correct the reviewer on this one. This approach of exerting super-stall forces and measuring binding durations is in fact less common than the approach of allowing the motor to walk up to stall and measuring the binding duration. This ‘fixed trap’ approach has been used to show catch-bond behavior of dynein (Leidel et al., 2012; Rai et al., 2013) and kinesin (Kuo et al., 2022; Pyrpassopoulos et al., 2020). For the non-processive motor Myosin I, a dynamic force clamp was used to keep the actin filament in place while the myosin generated a single step (Laakso et al., 2008). Because the motor generates the force, these are not superstall forces either.

*(3) I appreciate the concerns about the Vertical force from the optical trap. But that leads to the following questions that have not at all been addressed in this paper:*

*(i) Why is the Vertical force only a problem for Kinesins, and not a problem for the dynein studies?*

Actually, we do not claim that vertical force is not a problem for dynein; our data do not speak to this question. There is debate in the literature as to whether dynein has catch bond behavior in the traditional single-bead optical trap geometry - while some studies have measured dynein catch bond behavior (Kunwar et al., 2011; Leidel et al., 2012; Rai et al., 2013), others have found that dynein has slip-bond or ideal-bond behavior (Ezber et al., 2020; Nicholas et al., 2015; Rao et al., 2019). This discrepancy may relate to vertical forces, but not in an obvious way.

*(ii) The authors state that "With this geometry, a kinesin motor pulls against the elastic force of a stretched DNA solely in a direction parallel to the microtubule". Is this really true? What matters is not just how the kinesin pulls the DNA, but also how the DNA pulls on the kinesin. In Figure 1A, what is the guarantee that the DNA is oriented only in the plane of the paper? In fact, the DNA could even be bending transiently in a manner that it pulls the kinesin motor UPWARDS (Vertical force). How are the authors sure that the reaction force between DNA and kinesin is oriented SOLELY along the microtubule?*

We acknowledge that “solely” is an absolute term that is too strong to describe our geometry. We will soften this term in our revision to “nearly parallel to the microtubule”. In the Geometry Calculations section of Supplementary Methods, we calculate that if the motor and streptavidin are on the same protofilament, the vertical force will be <1% of the horizontal force. We also note that if the motor is on a different protofilament, there will be lateral forces and forces perpendicular to the microtubule surface, except they are oriented toward



rather than away from the microtubule. The DNA can surely bend due to thermal forces, but because inertia plays a negligible role at the nanoscale (Howard, 2001; Purcell, 1977), any resulting upward forces will only be thermal forces, which the motor is already subjected to at all times.

*(4) For this study to be really impactful and for some of the above concerns to be addressed, the data should also have included DNA tensiometer experiments with Dynein. I wonder why this was not done?*

As much as we would love to fully characterize dynein here, this paper is about kinesin and it took a substantial effort. The dynein work merits a stand-alone paper.

*While I do like several aspects of the paper, I do not believe that the conclusions are supported by the data presented in this paper for the reasons stated above.*

The three key points the reviewer makes are the validity of the worm-like-chain model, the question of superstall loads, and the role of DNA bending in generating vertical forces. We hope that we have fully addressed these concerns in our responses above.

#### **Reviewer #2 (Public review):**

##### *Major comments:*

*(1) The use of the term "catch bond" is misleading, as the authors do not really mean consistently a catch bond in the classical sense (i.e., a protein-protein interaction having a dissociation rate that decreases with load). Instead, what they mean is that after motor detachment (i.e., after a motor protein dissociating from a tubulin protein), there is a slip state during which the reattachment rate is higher as compared to a motor diffusing in solution. While this may indeed influence the dynamics of bidirectional cargo transport (e.g., during tug-of-war events), the used terms (detachment (with or without slip?), dissociation, rescue, ...) need to be better defined and the results discussed in the context of these definitions. It is very unsatisfactory at the moment, for example, that kinesin-3 is at first not classified as a catch bond, but later on (after tweaking the definitions) it is. In essence, the typical slip/catch bond nomenclature used for protein-protein interaction is not readily applicable for motors with slippage.*

We appreciate the reviewer's point and we will work to streamline and define terms in our revision.

*(2) The authors define the stall duration as the time at full load, terminated by >60 nm slips/detachments. Isn't that a problem? Smaller slips are not detected/considered... but are also indicative of a motor dissociation event, i.e., the end of a stall. What is the distribution of the slip distances? If the slip distances follow an exponential decay, a large number of short slips are expected, and the presented data (neglecting those short slips) would be highly distorted.*

The reviewer brings up a good point that there may be undetected slips. To address this question, we plotted the distribution of slip distances for kinesin-3, which by far had the most slip events. As the reviewer suggested, it is indeed an exponential distribution. Our preliminary analysis suggests that roughly 20% of events are missed due to this 60 nm cutoff. This will change our unloaded duration numbers slightly, but this will not alter our conclusions.

*(3) Along the same line: Why do the authors compare the stall duration (without including the time it took the motor to reach stall) to the unloaded single motor run durations? Shouldn't the times of the runs be included?*

The elastic force of the DNA spring is variable as the motor steps up to stall, and so if we included the entire run duration then it would be difficult to specify what force we were comparing to unloaded. More importantly, if we assume that any stepping and detachment behavior is history independent, then it is mathematically proper to take any arbitrary starting point (such as when the motor reaches stall), start the clock there, and measure the distribution of detachments durations relative to that starting point.

More importantly, what we do in Fig. 3 is to separate out the ramps from the stalls and, using a statistical model, we compute a separate duration parameter (which is the inverse of the off-rate) for the ramp and the stall. What we find is that the relationship between ramp, stall, and unloaded durations is different for the three motors, which is interesting in itself.

*(4) At many places, it appears too simple that for the biologically relevant processes, mainly/only the load-dependent off-rates of the motors matter. The stall forces and the kind of motor-cargo linkage (e.g., rigid vs. diffusive) do likely also matter. For example: "In the context of pulling a large cargo through the viscous cytoplasm or competing against dynein in a tug-of-war, these slip events enable the motor to maintain force generation and, hence, are distinct from true detachment events." I disagree. The kinesin force at reattachment (after slippage) is much smaller than at stall. What helps, however, is that due to the geometry of being held close to the microtubule (either by the DNA in the present case or by the cargo in vivo) the attachment rate is much higher. Note also that upon DNA relaxation, the motor is likely kept close to the microtubule surface, while, for example, when bound to a vesicle, the motor may diffuse away from the microtubule quickly (e.g., reference 20).*

We appreciate the reviewer's detailed thinking here, and we offer our perspective. As to the first point, we agree that the stall force is relevant and that the rigidity of the motor-cargo linkage will play a role. The goal of the sentence on pulling cargo that the reviewer highlights is to set up our analysis of slips, which we define as rearward displacements that don't return to the baseline before force generation resumes. We agree that force after slippage is much smaller than at stall, and we plan to clarify that section of text. However, as shown in the model diagram in Fig. 5, we differentiate between the slip state (and recovery from this slip state) and the detached state (and reattachment from this detached state). This delineation is important because, as the reviewer points out, if we are measuring detachment and reattachment with our DNA tensiometer, then the geometry of a vesicle in a cell will be different and diffusion away from the microtubule or elastic recoil perpendicular to the microtubule will suppress this reattachment.

Our evidence for a slip state in which the motor maintains association with the microtubule comes from optical trapping work by Tokelís et al (Tokelís et al., 2020) and Sudhakar et al (Sudhakar et al., 2021). In particular, Sudhakar used small, high index Germanium microspheres that had a low drag coefficient. They showed that during 'slip' events, the relaxation time constant of the bead back to the center of the trap was nearly 10-fold slower than the trap response time, consistent with the motor exerting drag on the microtubule. (With larger beads, the drag of the bead swamps the motor-microtubule friction.) Another piece of support for the motor maintaining association during a slip is work by Ramaiya et al. who used birefringent microspheres to exert and measure rotational torque during kinesin stepping (Ramaiya et al., 2017). In most traces, when the motor returned to baseline following a stall, the torque was dissipated as well, consistent with a 'detached' state. However, a slip event is shown in S18a where the motor slips backward while maintaining torque. This is best explained by the motor slipping backward in a state where the heads are associated with the microtubule (at least sufficiently to resist rotational forces). Thus, we term the resumption after slip to be a rescue from the slip state rather than a reattachment from the detached state.

To finish the point, with the complex geometry of a vesicle, during slip events the motor remains associated with the microtubule and hence primed for recovery. This recovery rate is expected to be the same as for the DNA tensiometer. Following a detachment, however, we agree that there will likely be a higher probability of reattachment in the DNA tensiometer due to proximity effects, whereas with a vesicle any elastic recoil or ‘rolling’ will pull the detached motor away from the microtubule, suppressing reattachment. We plan to clarify these points in the text of the revision.

*(5) Why were all motors linked to the neck-coil domain of kinesin-1? Couldn't it be that for normal function, the different coils matter? Autoinhibition can also be circumvented by consistently shortening the constructs.*

We chose this dimerization approach to focus on how the mechanochemical properties of kinesins vary between the three dominant transport families. We agree that in cells, autoinhibition of both kinesins and dynein likely play roles in regulating bidirectional transport, as will the activity of other regulatory proteins. The native coiled-coils may act as ‘shock absorbers’ due to their compliance, or they might slow the motor reattachment rate due to the relatively large search volumes created by their long lengths (10s of nm). These are topics for future work. By using the neck-coil domain of kinesin-1 for all three motors, we eliminate any differences in autoinhibition or other regulation between the three kinesin families and focus solely on differences in the mechanochemistry of their motor domains.

*(6) I am worried about the neutravidin on the microtubules, which may act as roadblocks (e.g. DOI: 10.1039/b803585g), slip termination sites (maybe without the neutravidin, the rescue rate would be much lower?), and potentially also DNA-interaction sites? At 8 nM neutravidin and the given level of biotinylation, what density of neutravidin do the authors expect on their microtubules? Can the authors rule out that the observed stall events are predominantly the result of a kinesin motor being stopped after a short slippage event at a neutravidin molecule?*

We will address these points in our revision.

*(7) Also, the unloaded runs should be performed on the same microtubules as in the DNA experiments, i.e., with neutravidin. Otherwise, I do not see how the values can be compared.*

We will address this point in our revision.

*(8) If, as stated, "a portion of kinesin-3 unloaded run durations were limited by the length of the microtubules, meaning the unloaded duration is a lower limit." corrections (such as Kaplan-Meier) should be applied, DOI: 10.1016/j.bpj.2017.09.024.*

*(9) Shouldn't Kaplan-Meier also be applied to the ramp durations ... as a ramp may also artificially end upon stall? Also, doesn't the comparison between ramp and stall duration have a problem, as each stall is preceded by a ramp ...and the (maximum) ramp times will depend on the speed of the motor? Kinesin-3 is the fastest motor and will reach stall much faster than kinesin-1. Isn't it obvious that the stall durations are longer than the ramp duration (as seen for all three motors in Figure 3)?*

The reviewer rightly notes the many challenges in estimating the motor off-rates during ramps. To estimate ramp off-rates and as an independent approach to calculating the unloaded and stall durations, we developed a Markov model coupled with Bayesian inference methods to estimate a duration parameter (equivalent to the inverse of the off-rate) for the unloaded, ramp, and stall duration distributions. With the ramps, we have left censoring due to the difficulty in detecting the start of the ramps in the fluctuating baseline, and we have right censoring due to reaching stall (with different censoring of the ramp duration for the

three motors due to their different speeds). The Markov model assumes a constant detachment probability and history independence, and thus is robust even in the face of left and right censoring (details in the Supplementary section). This approach is preferred over Kaplan-Meier because, although these non-parametric methods make no assumptions for the distribution, they require the user to know exactly where the start time is.

Regarding the potential underestimate of the kinesin-3 unloaded run duration due to finite microtubule lengths. The first point is that the unloaded duration data in Fig. 2C are quite linear up to 6 s and are well fit by the single-exponential fit (the points above 6s don't affect the fit very much). The second point is that when we used our Markov model (which is robust against right censoring) to estimate the unloaded and stall durations, the results agreed with the single-exponential fits very well (Table S2). For instance, the single-exponential fit for the kinesin-3 unloaded duration was 2.74 s (2.33 – 3.17 s 95% CI) and the estimate from the Markov model was 2.76 (2.28 – 3.34 s 95% CI). Thus, we chose not to make any corrections due to finite microtubule lengths.

(10) *It is not clear what is seen in Figure S6A: It looks like only single motors (green, w/o a DNA molecule) are walking ... Note: the influence of the attached DNA onto the stepping duration of a motor may depend on the DNA conformation (stretched and near to the microtubule (with neutravidin!) in the tethered case and spherically coiled in the untethered case).*

In Figure S6A kymograph, the green traces are GFP-labeled kinesin-1 without DNA attached (which are in excess) and the red diagonal trace is a motor with DNA attached. There are also two faint horizontal red traces, which are labeled DNA diffusing by (smearing over a large area during a single frame). Panel S6B shows run durations of motors with DNA attached. We agree that the DNA conformation will differ if it is attached and stretched (more linear) versus simply being transported (random coil), but by its nature this control experiment is only addressing random coil DNA.

(11) *Along this line: While the run time of kinesin-1 with DNA (1.4 s) is significantly shorter than the stall time (3.0 s), it is still larger than the unloaded run time (1.0 s). What do the authors think is the origin of this increase?*

Our interpretation of the unloaded kinesin-DNA result is that the much slower diffusion constant of the DNA relative to the motor alone enables motors to transiently detach and rebind before the DNA cargo has diffused away, thus extending the run duration. In contrast, such detachment events for motors alone normally result in the motor diffusing away from the microtubule, terminating the run. This argument has been used to reconcile the longer single-motor run lengths in the gliding assay versus the bead assay (Block et al., 1990). Notably, this slower diffusion constant should not play a role in the DNA tensiometer geometry because if the motor transiently detaches, then it will be pulled backward by the elastic forces of the DNA and detected as a slip or detachment event. We will address this point in the revision.

(12) *"The simplest prediction is that against the low loads experienced during ramps, the detachment rate should match the unloaded detachment rate." I disagree. I would already expect a slight increase.*

Agreed. We will change this text to: "The prediction for a slip bond is that against the low loads experienced during ramps, the detachment rate should be equal to or faster than the unloaded detachment rate."

(13) *Isn't the model over-defined by fitting the values for the load-dependence of the strong-to-weak transition and fitting the load dependence into the transition to the slip state?*

Essentially, yes, it is overdefined, but that is essentially by design and it is still very useful. Our goal here was to make as simple a model as possible that could account for the data and use it to compare model parameters for the different motor families. Ignoring the complexity of the slip and detached states, a model with a strong and weak state in the stepping cycle and a single transition out of the stepping cycle is the simplest formulation possible. And having rate constants ( $k_{S-W}$  and  $k_{slip}$  in our case) that vary exponentially with load makes thermodynamic sense for modeling mechanochemistry (Howard, 2001). Thus, we were pleasantly surprised that this bare-bones model could recapitulate the unloaded and stall durations for all three motors (Fig. 5C-E).

(14) "When kinesin-1 was tethered to a glass coverslip via a DNA linker and hydrodynamic forces were imposed on an associated microtubule, kinesin-1 dissociation rates were relatively insensitive to loads up to ~3 pN, inconsistent with slip-bond characteristics (37)." This statement appears not to be true. In reference 37, very similar to the geometry reported here, the microtubules were fixed on the surface, and the stepping of single kinesin motors attached to large beads (to which defined forces were applied by hydrodynamics) via long DNA linkers was studied. In fact, quite a number of statements made in the present manuscript have been made already in ref. 37 (see in particular sections 2.6 and 2.7), and the authors may consider putting their results better into this context in the Introduction and Discussion. It is also noteworthy to discuss that the (admittedly limited) data in ref. 37 does not indicate a "catch-bond" behavior but rather an insensitivity to force over a defined range of forces.

The reviewer misquoted our sentence. The actual wording of the sentence was: "When kinesin-1 was connected to micron-scale beads through a DNA linker and hydrodynamic forces parallel to the microtubule imposed, dissociation rates were relatively insensitive to loads up to ~3 pN, inconsistent with slip-bond characteristics (Urbanska et al., 2021)." The sentence the reviewer quoted was in a previous version that is available on BioRxiv and perhaps they were reading that version. Nonetheless, in the revision we will note in the Discussion that this behavior was indicative of an ideal bond (not a catch-bond), and we will also add a sentence in the Introduction highlighting this work.

**Reviewer #3 (Public review):**

The authors attribute the differences in the behaviour of kinesins when pulling against a DNA tether compared to an optical trap to the differences in the perpendicular forces. However, the compliance is also much different in these two experiments. The optical trap acts like a ~ linear spring with stiffness ~ 0.05 pN/nm. The dsDNA tether is an entropic spring, with negligible stiffness at low extensions and very high compliance once the tether is extended to its contour length (Fig. 1B). The effect of the compliance on the results should be addressed in the manuscript.

This is an interesting point. To address it, we calculated the predicted stiffness of the dsDNA by taking the slope of theoretical force-extension curve in Fig. 1B. Below 650 nm extension, the stiffness is <0.001 pN/nM; it reaches 0.01 pN/nM at 855 nm, and at 960 nm where the force is 6 pN the stiffness is roughly 0.2 pN/nm. That value is higher than the quoted 0.05 pN/nm trap stiffness, but for reference, at this stiffness, an 8 nm step leads to a 1.6 pN jump in force, which is reasonable. Importantly, the stiffness of kinesin motors has been estimated to be in the range of 0.3 pN (Coppin et al., 1996; Coppin et al., 1997). Granted, this stiffness is also nonlinear, but what this means is that even at stall, our dsDNA tether has a similar predicted compliance to the motor that is pulling on it. We will address this point in our revision.

Compared to an optical trapping assay, the motors are also tethered closer to the microtubule in this geometry. In an optical trap assay, the bead could rotate when the kinesin is not bound. The authors should discuss how this tethering is expected to affect



*the kinesin reattachment and slipping. While likely outside the scope of this study, it would be interesting to compare the static tether used here with a dynamic tether like MAP7 or the CAP-GLY domain of p150glued.*

Please see our response to Reviewer #2 Major Comment #4 above, which asks this same question in the context of intracellular cargo. We plan to address this in our revision. Regarding a dynamic tether, we agree that's interesting – there are kinesins that have a second, non-canonical binding site that achieves this tethering (ncd and Cin8); p150glued likely does this naturally for dynein-dynactin-activator complexes; and we speculated in a review some years ago (Hancock, 2014) that during bidirectional transport kinesin and dynein may act as dynamic tethers for one another when not engaged, enhancing the activity of the opposing motor.

*In the single-molecule extension traces (Figure 1F-H; S3), the kinesin-2 traces often show jumps in position at the beginning of runs (e.g., the four runs from ~4-13 s in Fig. 1G). These jumps are not apparent in the kinesin-1 and -3 traces. What is the explanation? Is kinesin-2 binding accelerated by resisting loads more strongly than kinesin-1 and -3?*

Due to the compliance of the dsDNA, the 95% limits for the initial attachment position are  $\pm 290$  nm (Fig. S2). Thus, some apparent 'jumps' from the detached state are expected. We will take a closer look at why there are jumps for kinesin-2 that aren't apparent for kinesin-1 or -3.

*When comparing the durations of unloaded and stall events (Fig. 2), there is a potential for bias in the measurement, where very long unloaded runs cannot be observed due to the limited length of the microtubule (Thompson, Hoeprich, and Berger, 2013), while the duration of tethered runs is only limited by photobleaching. Was the possible censoring of the results addressed in the analysis?*

Yes. Please see response to Reviewer #2 points (8) and (9) above.

*The mathematical model is helpful in interpreting the data. To assess how the "slip" state contributes to the association kinetics, it would be helpful to compare the proposed model with a similar model with no slip state. Could the slips be explained by fast reattachments from the detached state?*

In the model, the slip state and the detached states are conceptually similar; they only differ in the sequence (slip to detached) and the transition rates into and out of them. The simple answer is: yes, the slips could be explained by fast reattachments from the detached state. In that case, the slip state and recovery could be called a "detached state with fast reattachment kinetics". However, the key data for defining the kinetics of the slip and detached states is the distribution of Recovery times shown in Fig. 4D-F, which required a triple exponential to account for all of the data. If we simplified the model by eliminating the slip state and incorporating fast reattachment from a single detached state, then the distribution of Recovery times would be a single-exponential with a time constant equivalent to  $t_1$ , which would be a poor fit to the experimental distributions in Fig. 4D-F.

We appreciate the efforts and helpful suggestions of all three reviewers and the Editor.

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<https://doi.org/10.7554/eLife.108837.1.sa0>