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Peer Review File

Kinesin-14 HSET and KlpA are non-processive microtubule motors with load-dependent power strokes

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:

The authors use optical tweezers to investigate the mechanical properties of two kinesin-14 motors, HSET and KlpA. HSET and its Drosophila ortholog ncd have been the subject of a number of studies, and the authors fail to find the processivity seen in a recent study from the Lang lab. KlpA is notable because it is bidirectional, enabled by its microtubule-binding tail, and the authors make the first force measurements on this motor and find that the motor is nonprocessive under load and has a low maximum force, which is not unexpected, but good to have in the literature.

Although the manuscript is well written, the overall logic and organization of the paper is not entirely clear, and the four figures are not particularly well linked; the paper feels more like four separate results. Partly because of this and partly due to the lack of a single notable result in the paper, the paper is somewhat lacking in its impact. Finally, the title does not accurately describe the results because, although different powerstroke sizes are seen for different loads for HSET, this experiment is not carried out for KlpA.

One of the main results from the paper is the finding that HSET is nonprocessive under load and generates ~0.5 pN. The Lang lab (Reinemann et al) previously showed in a rather compelling manner that full-length HSET is processive in an optical trap. They identified clear 8 nm steps, they showed that the step durations are exponentially distributed, consistent with a single rate-limiting step, and they found that the stepping rate slowed at elevated loads. In the present study the authors fail to find processive stepping of HSET in a trap, they find a stall force that is roughly half of the Reinemann work, and they show that with multiple motors the beads show clear runs and higher forces. However, the higher forces and clear runs with high motor densities on the beads is expected. It does provide evidence (along with the motor:bead dilution data that fit n=1) show convincingly that the authors are in the single-motor regime. However, that is not sufficient to settle the argument and conclusively show that the Reinemann HSET processivity is due to multiple motors. Lack of processivity is diminished function, and so it's formally possible that the lack of processivity observed is because one head is dead, the dimerization domain is soft, the buffer conditions weaken the affinity, or other reasons. (The authors point this out in Line 60 in the introduction, but they do not settle the argument in the results.). If the authors could, for instance, show that under multi-motor conditions there are 8 nm steps with exponentially distributed durations and the forces in this case were 1.1 pN, then they would have a strong argument that the Reinemann processivity is due to a multimotor effect. With the current data, the authors do not have enough to make this claim that the Reinemann result is an artifact.

The other main result is the authors claim that HSET has load-dependent powerstroke size. However, I don't feel that the results support this conclusion. If the trap stiffness is sufficiently low, then it is expected that a full 9 nm step is seen (based on the previous cryoEM work from the Endres). And the authors find that with stiffer traps, the measured displacements are smaller. One interpretation is that the neck-coil lever arm is flexible and so in the catalytic core the conformational change (perhaps neck linker/cover bundle docking) occurs but the lever bends and doesn't cause the full displacement. A second interpretation is that the force is preventing full neck linker/cover bundle docking in the catalytic core and the lever arm does a partial rotation and that is what is measured. However, any series compliance between the lever arm and the centroid of the bead will diminish the measured 'apparent' powerstroke measured by the bead. These compliances include the N-terminal coiled-coil region, the N-terminal tail, and the anti-GFP antibody. Thus, the smaller measured bead displacements with higher trap stiffnesses do not conclusively show that the HSET powerstroke is smaller at elevated loads.

The last principal result from this work is identifying conditions in in vitro motility assays in which the velocity increases rather than decreases with increasing motor density. The authors conclude

that previous findings of a decrease in speed with increasing motor density results from the kinesin-14 tail acting as a brake when motors are adsorbed to the surface in such a way that some of them are adsorbed head down and tail up. This is a good detail to get in the literature, but it is essentially an experimental artifact of previous work and in my mind doesn't tell us a lot of new information about kinesin-14 function. I agree that faster speed with increasing motor density is a prediction for a non-processive motor, and the result does go in line with higher densities of kinesin-14 motors generating higher forces. But the impact of this result is arguable.

The concluding point that the authors make is that kinesin-14 are non-processive motors and they work in teams like myosin-II motors. This is pretty much already the consensus I would argue about how kinesin-14 motors work. Thus, the somewhat loosely tethered parts of the paper, the fact that some of the claims are not fully supported by the data, and the lack of a single focal new and exciting result in the paper work together to dampen my enthusiasm for this work.

Minor comments:
1) Line 39: Based on the Lang lab work, there is evidence that ncd is in fact processive and so this is not entirely true.
2) Line 93-95: The authors conclude that on its own KlpA is processive but under load it is not. However, it is possible that binding to the bead makes the motor nonprocessive, not the load per se. To rule this out, the authors should show that single KlpA bound to a bead maintain their processivity in the absence of load.
3) Line 107 (also Line 264): I don't believe the authors are fairly representing how kinesin-1 motors can work together to generate high forces. I would say the consensus is that groups of dynein work together better to generate higher forces as a team, but kinesin-1 clearly does generate elevated stall forces as a team (or even pairs). Work from Mike Diehl's lab should be cited and discussed, this arguably the most relevant work toward this question.
4) Line 118: says 'weak processivity' of HSET, contradicting statement on line 39. This is an example of how there are uncertainties in what questions precisely the authors are asking in this manuscript.
5) Lines 125-135: The presence of 3 and 4 photobleaching steps is actually a strong argument that there are dimers of dimers in the prep. The question of co-localization of isolated dimers is easy to test statistically based on the density of spots and the size of the point-spread function. Perhaps there are errors in photobleaching step identification and other measurement errors her, but overall and especially in the absence of some positive control showing what it looks like for tetramer forming motors, these data are pretty weak.
6) Paragraph starting on Line 249. This is an interesting point relevant to bidirectionality of KlpA, but the current manuscript does not address bidirectionality and the paragraph is extraneous.
7) Fig 1F and 2J – the absolute concentrations on the x-axis are relevant and thus it is inappropriate to plot them as categories – the data should be plotted in an x-y axis with proper spacing of points on x-axis.

Reviewer #2:
Remarks to the Author:
The manuscript by Liu and coworkers reports on the interaction of the kinesin-14 motors HSET and KlpA with microtubules using optical trapping and gliding assays supplemented with TIRF microscopy measurements. In trapping assays, under single-molecule conditions (about 10 pM), they observed the binding and unbinding of motors without any processive motion against the load of the optical trap. The average binding force was about 0.5 pN that increased somewhat with trap stiffness. The average displacement upon binding decreased with trap stiffness which the authors interpret as a load-dependent power stroke. When trapping microspheres were incubated with additional motors that do not have a specific binding tag on the microspheres, the authors observed processive motion against forces of a few piconewtons. Also, TIRF measurements show that at 1 nM motor concentration, motor aggregates move processively towards the microtubule

minus end. Gliding assays show that with increasing density of motors microtubule gliding speeds decrease. Since a tail-truncation mutant does not show this decrease, but potentially a modest increase in speed, the authors conclude that the diffusive interaction of the tail leads to friction that slows down the gliding and that cooperativity of the motors could increase the speed. While the manuscript has several very interesting findings, some of these need some additional control experiments to rule out alternative explanations as pointed out below. The manuscript sheds light on how kinesin-14 motors may work in the mitotic spindle. In summary, I recommend publication after a revision that addresses my concerns below.

Major point:
1. My main concern is interpreting the "power stroke". For non-processive muscle myosin, the three-bead assay was developed to distinguish binding/unbinding from an ATP-driven power stroke. Such experiments are typically done in a force-clamp mode where power-stroke displacements can be measured as a function of force. Here, several questions remain open and alternative explanations are possible. First, how was the directionality of the displacement with respect to the microtubule polarity measured? Are all displacements in the same direction? Please, show longer traces with many events. Is there any evidence that displacements are due to ATP hydrolysis? Binding should precede the power stroke. Detecting the binding of the motor should be possible by, for example, measuring a reduction in the positional standard deviation. Or, as done in the three-bead assays, a reduction in a small amplitude oscillation might be used to detect binding. Subsequently, according to the proposed power-stroke mechanism (Fig. 3A), the "power-stroke" displacement should be detectable. The time between binding and displacement should depend on ATP concentration. In particular, at low ATP concentrations, binding and power-stroke should be distinguishable. Using slowly hydrolyzable ATP analogs might be another approach.

An alternative explanation for the observations might be that the kinesin simply binds and unbinds without a power stroke. It is impossible to center a trap directly over a canonical kinesin binding site. Thus, any binding is intrinsically associated with a displacement both along the microtubule axis and perpendicular to it. Please, along with the above-mentioned longer traces that show multiple events, also show the perpendicular microsphere displacements. Apparent directionality or bias of binding events may also arise due to an intrinsic bias of the motor (e.g. the motor might be very susceptible to detachment under assisting loads that no events are detected vs. prolonged binding under hindering loads).

The reduction in displacement with trap stiffness may be simply due to a more confined Brownian motion. The expected rms positional fluctuations are x_rms = sqrt{k_B*T/kappa} whereby k_B is the Boltzmann constant, T is the absolute temperature, and kappa is the trap stiffness. For kappa = 0.05 pN/nm, the microsphere fluctuates by about x_rms = 9 nm. This value matches very well the observed mean displacement and also the expected width of the distribution (Fig. 3C). For kappa = 0.3 pN/nm, x_rms = 3.7 nm, somewhat larger compared to the 1.9 nm of what the authors measured. Together with an offset from the trap position relative to the binding site, values are within reasonable agreement.

Furthermore, there is an additional effect that can cause an erroneous displacement arising from the rocking motion of the microsphere when binding. Since the motor stalk plus GFP and antibody form a flexible linker, initial displacements after binding and loading may be largely due to the rocking motion of the microsphere and pulling the linker taught. Since forces are acting through the center of the microsphere, motions are amplified by a long lever, i.e. the 500 nm radius of the microsphere. Thus, it is unclear how much of the displacement is due to a potential motion of the motor and how much is due to the rocking motion of the microsphere. In a force-clamp experiment, such artifacts do not arise.

Do the authors have any evidence that at the roughly 10 pM concentration used for the single-molecule force measurements, motors indeed are dimers? What was the motor concentration for the bleaching step measurements (Fig. 2G)? At very low concentrations, motors may not be

dimers. Monomers would also not be processive.

Minor points:
2. How was the trap calibrated?

3. What was the data acquisition rate? Was there an anti-alias filter?

4. How was the data processed? What is the bandwidth with which the data is displayed?

5. For the mCherry-tagged motor experiments, the authors argue that motors oligomerize with the GFP-tagged motors. However, no direct evidence is provided. Please provide some TIRF kymographs of oligomerized GFP- and mCherry-tagged motors moving together along microtubules. The force measurements are not convincing since the increased force is about the "single motor force" of 0.5 pN plus the force of the non-specifically attached motors. For example, in Suppl. Fig. 2, the differences between non-specifically attached motors to GFP-motors plus mCherry motors were 0.67 pN, 0.61 pN, and 0.35 pN which on average is 0.54 pN. This average force difference matches the single-motor force. Thus, forces may be the addition of GFP-tagged motors plus the non-specifically attached ones without any direct oligomerization. In analogy to Fig. 1E and Fig. 2I, please also provide force traces for microspheres that had only the mCherry motors bound. Did all supplemented motors bind to the microspheres? In particular for the high concentrations, likely a lot of motors are still in solution. These motors will also interact with the microtubules and may change the motility of the microsphere-attached motors. Please provide some TIRF images of the used mixtures to illustrate how many mCherry motors are interacting with the microtubules during these experiments.

6. Fig. 2B: How was the relative motor concentration determined?

7. Fig. 4: Please provide concentrations used during incubation instead of dilutions. For Fig. 4E, please plot speed vs. the incubation concentration and fit a line to the data. Is the slope significant? It is stated in the abstract and final discussion, but apart from presenting the data, there is no further analysis or statistical test of the significance. Also, the conclusion in l. 217 that an increase in ionic strength weakens the interaction between the tail and microtubule is too early at that point. Only with the additional measurements presented further on such an interpretation seems reasonable. Also, nothing can be said about the strength of the interaction, for example, "the tail strongly interacts with MTs through ionic interactions". It can only be concluded that there is a stronger dependence on the ionic strength. For the strength of the interaction, the authors would have to perform force measurements.

8. l. 171: Please provide a reference for the cryoEM structure of Ncd.

9. Fig. 3: "biphasic behavior" - there is only one data point at 0.04 pN/nm. Please measure also at 0.01 and 0.02 pN/nm whether there is indeed a different behavior for trap stiffness smaller than 0.05 pN/nm.

10. Fig. 1C: The displacement noise is too low compared to the expected value of x_rms for the used trap stiffness. How was the data low-passed filtered? The question applies to all trapping data.

11. How were the maximum forces determined? What were the criteria? Was it the true maximum of the (unfiltered) Brownian motion? Was it the average? Was it the maximum of the filtered data? Over how many data points or what time was it averaged?

12. For the insets in Fig. 1C and 2C, please provide labels and ticks for the axes.

13. Fig. 2G & H. Please provide some fluorescence intensity traces that show the bleaching steps.

How was the MT polarity determined in the kymograph?

14. Fig. 4: The authors argue that the diffusive interactions of the motor tails cause the observed slow down with increasing motor density. However, the relative ratio of force-generating motors and tail-interacting motors should not change with the density. As on average the relative number of motors counterbalance the friction caused by the same relative number of tails, one would not expect an increased friction with density. Also since speeds are very low, friction forces are expected to be very small. Based on the data the authors have in Fig. 3A, they could determine the friction based on the measured diffusion coefficient of the motors in Fig. 3A. and their speed. Most likely friction is not limiting, but other effects.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors use optical tweezers to investigate the mechanical properties of two kinesin-14 motors, HSET and KlpA. HSET and its Drosophila ortholog ncd have been the subject of a number of studies, and the authors fail to find the processivity seen in a recent study from the Lang lab. KlpA is notable because it is bidirectional, enabled by its microtubule-binding tail, and the authors make the first force measurements on this motor and find that the motor is nonprocessive under load and has a low maximum force, which is not unexpected, but good to have in the literature.

Although the manuscript is well written, the overall logic and organization of the paper is not entirely clear, and the four figures are not particularly well linked; the paper feels more like four separate results. Partly because of this and partly due to the lack of a single notable result in the paper, the paper is somewhat lacking in its impact.

⇒ We respectfully note the reviewer's concerns regarding the organization and the perceived impact of our findings. We believe our manuscript presents several novel and significant discoveries that challenge existing paradigms. Contrary to what has been suggested by the Lang lab, we provide evidence that HSET is non-processive and capable of performing only single power strokes. Additionally, our work is the first to demonstrate that the power strokes of Kinesin-14 motors are load-dependent. In our revised manuscript, we also present data that show that the lever-arm lengths of Kinesin-14 motors are considerably larger than previously thought, reaching up to approximately 25 nm for full-length HSET and KlpA. To address the reviewer's comments on the coherence of the paper, we have taken steps to improve the flow of our manuscript. We have also added a large number of new experimental results, which collectively support the central thesis of our study. We believe that these changes have substantively improved the manuscript's clarity and underscore the impact of our findings.

Finally, the title does not accurately describe the results because, although different powerstroke sizes are seen for different loads for HSET, this experiment is not carried out for KlpA.

⇒ We appreciate the reviewer's observation regarding the title's alignment with our reported results. To address this, we have included new data that characterize KlpA's power-stroke size in relation to the trap stiffness (see new Suppl. Figure 14). This additional data ensures that our findings for both HSET and KlpA are comprehensively represented, making the title an accurate reflection of the manuscript's content.

One of the main results from the paper is the finding that HSET is nonprocessive under load and generates ~0.5 pN. The Lang lab (Reinemann et al) previously showed in a rather compelling manner that full-length HSET is processive in an optical trap.

⇒ We acknowledge the findings of Reinemann et al. (Curr. Biol. 2018) regarding the processivity of HSET. However, it is important to note that their study did not demonstrate single-molecule behavior. Specifically, Reinemann et al. did not conclusively address the

potential for motor aggregation/oligomerization, nor did they measure the fraction of moving beads in relation to motor concentration—a methodology we employed in our study (see Fig. 2B in our manuscript). Furthermore, the authors utilized beads coated with anti-His antibodies and incubated them for one hour with His-tagged motor proteins. This extended incubation period could significantly increase the likelihood of motor protein oligomerization and the formation of multi-motor complexes. Given Kinesin-14's tendency towards oligomerization and cooperative behavior (see Suppl. Fig. 6), such prolonged incubation may have influenced their experimental results. The authors' conclusion of single-molecule activity primarily relied on observations of trapping beads advancing in ~8 nm increments and the characterization of dwell times between steps by a single exponential function. We argue that these criteria alone do not suffice to confirm single-molecule behavior definitively.

To thoroughly address this point, our revised manuscript includes new data clearly demonstrating that multiple HSET motors are required to generate forces exceeding 1 pN. Crucially, our results show that while teams of HSET motors need to act cooperatively, the movement of beads up to 1.5 pN can be characterized by ~8 nm increments, and the intervals between these steps fit a single exponential distribution (see new Suppl. Figure 12). This offers a more detailed and nuanced understanding of HSET's behavior under load, significantly enriching the current knowledge in this field.

They identified clear 8 nm steps, they showed that the step durations are exponentially distributed, consistent with a single rate-limiting step, and they found that the stepping rate slowed at elevated loads. In the present study the authors fail to find processive stepping of HSET in a trap, they find a stall force that is roughly half of the Reinemann work, and they show that with multiple motors the beads show clear runs and higher forces. However, the higher forces and clear runs with high motor densities on the beads is expected. It does provide evidence (along with the motor:bead dilution data that fit n=1) show convincingly that the authors are in the single-motor regime. However, that is not sufficient to settle the argument and conclusively show that the Reinemann HSET processivity is due to multiple motors. Lack of processivity is diminished function, and so it's formally possible that the lack of processivity observed is because one head is dead, the dimerization domain is soft, the buffer conditions weaken the affinity, or other reasons. (The authors point this out in Line 60 in the introduction, but they do not settle the argument in the results.). If the authors could, for instance, show that under multi-motor conditions there are 8 nm steps with exponentially distributed durations and the forces in this case were 1.1 pN, then they would have a strong argument that the Reinemann processivity is due to a multimotor effect. With the current data, the authors do not have enough to make this claim that the Reinemann result is an artifact.

⇒ We appreciate the detailed feedback and have performed the analysis suggested by the reviewer. Our results indeed show that force generation up to 1.5 pN by multiple HSET motors is characterized by ~8 nm increments and exponentially distributed dwell times between steps, as highlighted in our revised manuscript. This aligns with our findings and bolsters our argument regarding multi-motor effects.

Additionally, it's crucial to consider the biological context of our results. The HSET ortholog Ncd is known to be non-processive, which is consistent with our findings for HSET and KlpA. This supports our assertion that the sliding of anti-parallel microtubules (MTs) by Kinesin-14 motors, like HSET, is driven by non-processive motors with a low duty ratio. This characteristic fits the biological function of these motors more accurately.

In response to the reviewer's concerns about potential alternative explanations for the observed lack of processivity, such as inactive motor heads or buffer conditions, we have meticulously addressed these factors in our experimental design and analysis. We optimized our experimental conditions, including the buffer composition. Additionally, we implemented an extra MT-binding and -release purification step, which was specifically designed to remove inactive motors, thereby ensuring robust motor activity. The consistency of our findings across various experimental setups—which include optical trapping experiments, fluorescence imaging of single and multiple Kinesin-14 motors, and MT-gliding assays—significantly diminishes the likelihood that our observations are mere artifacts resulting from reduced motor function. We believe that our comprehensive methodology, augmented by the new data and analysis presented, compellingly argues against the notion that the non-processivity observed in our study stems from experimental artifacts or motor malfunction.

The other main result is the authors claim that HSET has load-dependent powerstroke size. However, I don't feel that the results support this conclusion. If the trap stiffness is sufficiently low, then it is expected that a full 9 nm step is seen (based on the previous cryoEM work from the Endres).

⇒ We acknowledge the reviewer's perspective on our claim regarding HSET's load-dependent power stroke size. In our manuscript, we demonstrate that at the lowest trap stiffness, HSET exhibits a power stroke of approximately 8 nm (Fig. 3C, left). Further analysis, which includes adjustments for the compliance of the motor and motor-bead linkage, reveals a corrected power-stroke size of approximately 25 nm for full-length HSET at the lowest trap stiffness tested. This aligns with findings by Endres et al. (Nature 2006), who estimated a ~16 nm power stroke for a tail-truncated Ncd (see Fig. 2b in their paper). Considering the longer tails in full-length HSET and KlpA, we hypothesize that the extended coiled-coil regions significantly contribute to the power-stroke size of Kinesin-14 motors. Our additional experiments with varied tail lengths in KlpA and Ncd (which we now include as well) support this hypothesis. These experiments reveal increased power-stroke sizes with longer tails (see new Suppl. Figures 14 and 18). Consequently, our results indicate a considerably larger lever-arm length for Kinesin-14 motors than previously understood, corroborating the coiled-coil predictions made by AlphaFold (new Suppl. Figure 16).

And the authors find that with stiffer traps, the measured displacements are smaller. One interpretation is that the neck-coil lever arm is flexible and so in the catalytic core the conformational change (perhaps neck linker/cover bundle docking) occurs but the lever bends and doesn't cause the full displacement. A second interpretation is that the force is preventing full neck linker/cover bundle docking in the catalytic core and the lever arm does a partial rotation and that is what is measured.

However, any series compliance between the lever arm and the centroid of the bead will diminish the measured 'apparent' powerstroke measured by the bead. These compliances include the N-terminal coiled-coil region, the N-terminal tail, and the anti-GFP antibody. Thus, the smaller measured bead displacements with higher trap stiffnesses do not conclusively show that the HSET powerstroke is smaller at elevated loads.

⇒ We appreciate the reviewer's insightful observations. It is essential to clarify that Kinesin-14 motors, unlike Kinesin-1, do not utilize a neck-linker conformational change and the formation of

a cover-neck bundle to drive motility. Instead, they achieve MT minus-end-directed motility through the rotation of the dimerizing coiled-coil. In response to the reviewer's point about potential series compliance influencing our measurements, we have undertaken a comprehensive analysis of the combined compliances of the motor and motor-bead linkage at each trap stiffness. This has allowed us to correct the measured displacements accordingly. These adjustments ensure that our observations and conclusions regarding the HSET and KlpA power strokes under various loads are based on accurate estimates. In addition, we have performed a series of tail truncation experiments both on KlpA and Ncd, which demonstrate the correlation between tail length and power-stroke size.

The last principal result from this work is identifying conditions in in vitro motility assays in which the velocity increases rather than decreases with increasing motor density. The authors conclude that previous findings of a decrease in speed with increasing motor density results from the kinesin-14 tail acting as a brake when motors are adsorbed to the surface in such a way that some of them are adsorbed head down and tail up. This is a good detail to get in the literature, but it is essentially an experimental artifact of previous work and in my mind doesn't tell us a lot of new information about kinesin-14 function. I agree that faster speed with increasing motor density is a prediction for a non-processive motor, and the result does go in line with higher densities of kinesin-14 motors generating higher forces. But the impact of this result is arguable.

⇒ We appreciate the reviewer's perspective on our work and the identification of conditions under which the velocity in *in vitro* motility assays increases with motor density. While we acknowledge that our findings clarify an experimental artifact observed in previous studies—specifically, the effect of the Kinesin-14 tail acting as a brake when motors are adsorbed head down and tail up—we believe this insight significantly advances our understanding of Kinesin-14 function. We also validated our hypothesis by testing several new constructs of Ncd and KlpA, providing additional evidence that the brake-acting tail is shared among the Kinesin-14 motors (see Fig. 4 and new Suppl. Fig. 23).

Furthermore, our results do not merely highlight an experimental nuance; they fundamentally challenge and expand upon existing knowledge. By demonstrating that increased motor density correlates with faster speeds and higher forces in Kinesin-14 motors, we provide novel insights into their non-processive nature and cooperative action. This understanding is crucial for interpreting their role in cellular processes, particularly the sliding of anti-parallel MTs within the mitotic spindle. Thus, while the correction of an experimental artifact is a component of our findings, the broader impact lies in the new light shed on the fundamental mechanisms of Kinesin-14 motors. This contribution is significant not only for the field of cytoskeletal motor proteins but also for the general cell biological community.

The concluding point that the authors make is that kinesin-14 are non-processive motors and they work in teams like myosin-II motors. This is pretty much already the consensus I would argue about how kinesin-14 motors work.

⇒ We respectfully note that, contrary to the reviewer's assertion, there has not been a consensus regarding the functional characteristics of Kinesin-14 motors. Prior to our study, the prevailing view, as presented in the only published paper on HSET's force generation, was that HSET is processive. Our work challenges this perspective by conclusively demonstrating that HSET, like Ncd, is non-processive. This finding significantly contributes to the field by correcting

a previously held assumption and providing a deeper understanding of the mechanism of Kinesin-14 motors. By establishing that HSET as well as KlpA operates in a non-processive manner, our research adds a crucial piece of information to the existing knowledge about motor proteins and their roles in cellular processes.

Thus, the somewhat loosely tethered parts of the paper, the fact that some of the claims are not fully supported by the data, and the lack of a single focal new and exciting result in the paper work together to dampen my enthusiasm for this work.

⇒ We thank the reviewer for their candid assessment and understand their concerns regarding the perceived cohesion and impact of our paper. We believe, however, that the collective findings presented in our manuscript represent significant advancements in our understanding of Kinesin-14 motors.

First, we address and correct a prevailing misconception in the field by demonstrating that HSET, contrary to previous assertions, is non-processive. This finding alone challenges existing paradigms and provides a new perspective on the functionality of Kinesin-14 motors.

Second, our discovery of a significantly larger power stroke in Kinesin-14 motors represents not just a minor adjustment to existing knowledge but a fundamental shift in understanding their mechanics. This insight has far-reaching implications for our comprehension of the motility and mechanics of motor proteins with lever arms composed of coiled-coils and their roles in cellular processes.

Third, we demonstrate that the power stroke of the Kinesin-14 motors KlpA, HSET and Ncd is unidirectional (along the long MT axis) and occurs in one step. This contrasts with the previous conclusion that MT binding of Ncd causes a ~2 nm displacement in the absence of ATP and that the power stroke has a normal component (deCastro et al., Nat. Cell Biol. 2000).

Finally, we have carefully considered the reviewer's comments on the structure and presentation of our paper. In response, we have made concerted efforts to improve the logical flow, ensuring that each part of the paper contributes meaningfully to a cohesive whole. We believe these revisions enhance the paper's clarity and highlight the interconnectedness of our findings, thereby reinforcing the overall impact and novelty of our work.

In conclusion, while we acknowledge the reviewer's reservations, we respectfully assert that our study makes a substantial contribution to the field by overturning existing beliefs and introducing new insights into the behavior of Kinesin-14 motors.

Minor comments:

1) Line 39: Based on the Lang lab work, there is evidence that ncd is in fact processive and so this is not entirely true.

⇒ We appreciate the reviewer's feedback. We understand that the reference was intended for the HSET results from the Lang lab, not Ncd. The general statement regarding the non-processivity of Kinesin-14 members has been removed. However, we would like to highlight that our manuscript offers significant evidence supporting the non-processive nature of these motor proteins. This evidence will provide a solid foundation for future research, enabling subsequent

studies to confidently reference these findings in their introductions.

2) Line 93-95: The authors conclude that on its own KlpA is processive but under load it is not. However, it is possible that binding to the bead makes the motor nonprocessive, not the load per se. To rule this out, the authors should show that single KlpA bound to a bead maintain their processivity in the absence of load.

⇒ We meant that the anchoring of the MT-binding tail domain to the optical trapping beads alone causes KlpA to be non-processive. To directly address this point, we have added experiments that demonstrate that single KlpA molecules are incapable of moving beads along MTs in the absence of load (when the trap is deactivated following force generation), while beads bound to several KlpA molecules support the processive motion of beads along MTs (see lines 97-105 in the revised manuscript and new Suppl. Fig. 3). Our new experiments therefore show that cargo attachment alone and not the applied load cause KlpA to be non-processive.

3) Line 107 (also Line 264): I don't believe the authors are fairly representing how kinesin-1 motors can work together to generate high forces. I would say the consensus is that groups of dynein work together better to generate higher forces as a team, but kinesin-1 clearly does generate elevated stall forces as a team (or even pairs). Work from Mike Diehl's lab should be cited and discussed, this arguably the most relevant work toward this question.

⇒ Thank you for your valuable input. We acknowledge that there is existing literature demonstrating Kinesin-1's capacity to generate somewhat higher forces collectively, albeit with minimal dependence on the number of motors involved. This is supported by studies such as those by Furuta et al. (PNAS, 2013) and Elshenawy et al. (Nat. Chem. Biol., 2019), which align with the findings of Mike Diehl's group (Jamison et al., Biophys. J., 2010, Fig. 1D). In response to your suggestion, we have now cited the work from Mike Diehl's group at both line 125 and line 313 of our revised manuscript.

4) Line 118: says 'weak processivity' of HSET, contradicting statement on line 39. This is an example of how there are uncertainties in what questions precisely the authors are asking in this manuscript.

⇒ We have resolved this contradiction by revising our statement in line 39 (see revised lines 40 to 43). Please also see our response above to point 1 of the reviewer's Minor Comments.

5) Lines 125-135: The presence of 3 and 4 photobleaching steps is actually a strong argument that there are dimers of dimers in the prep. The question of co-localization of isolated dimers is easy to test statistically based on the density of spots and the size of the point-spread function. Perhaps there are errors in photobleaching step identification and other measurement errors her, but overall and especially in the absence of some positive control showing what it looks like for tetramer forming motors, these data are pretty weak.

⇒ We appreciate the reviewer's insights regarding our photobleaching analysis. Our data clearly indicate that the predominant form of HSET molecules in our preparations are dimers,

with oligomers of higher order accounting for less than 16% of the total. This is explicitly demonstrated in our observations of HSET and KlpA oligomerization, which increases with motor concentration, as shown in Figures 1E, 2H, 2I, and the new Supplementary Figure 6 of our manuscript.

To further substantiate our findings, we have conducted a native gel analysis, reinforcing the conclusion that HSET predominantly exists as dimers. This additional evidence can be found in lines 140 and 141 of the revised manuscript and in the new Supplementary Figure 10. We believe this comprehensive approach addresses the concerns raised and strengthens the validity of our conclusions regarding HSET oligomerization.

6) Paragraph starting on Line 249. This is an interesting point relevant to bidirectionality of KlpA, but the current manuscript does not address bidirectionality and the paragraph is extraneous.

⇒ Our discussion emphasizes the tail's critical role in MT-binding and power-stroke execution, as well as its flexibility, which is also essential for directing the movement of Kinesin-14 motors. It is therefore important to mention in the Discussion that the flexibility of the tail has an impact on motor function.

7) Fig 1F and 2J – the absolute concentrations on the x-axis are relevant and thus it is inappropriate to plot them as categories – the data should be plotted in an x-y axis with proper spacing of points on x-axis.

⇒ We appreciate the reviewer's attention to the details of our data presentation. The format of Figures 1F and 2J was chosen to reflect the experimental design as described in lines 101-114 of our previous submission. In this design, beads were pre-coated with GFP-tagged motors, which were bound to anti-GFP antibodies, to achieve a baseline where a maximum of 30% of the trapping beads showed force generation. This was followed by the addition of varying concentrations of mCherry-tagged motors, which should oligomerize with the GFP-tagged motors. The absolute concentrations of the added mCherry-tagged motors are indeed specified in the figures. Each data point corresponds to force measurements performed at these exact concentrations, thus the forces measured are plotted directly against the corresponding concentrations of added mCherry-tagged motors. Our intention was to clearly demonstrate the impact of each incremental addition of mCherry-tagged motors on force generation. We believe that this categorical representation provides the most straightforward visualization of the relationship between the concentration of added mCherry-tagged motors and the resultant force output.

Reviewer #2 (Remarks to the Author):

The manuscript by Liu and coworkers reports on the interaction of the kinesin-14 motors HSET and KlpA with microtubules using optical trapping and gliding assays supplemented with TIRF microscopy measurements. In trapping assays, under single-molecule conditions (about 10 pM), they observed the binding and unbinding of motors without any processive motion against the load of the optical trap. The average binding force was about 0.5 pN that increased somewhat

with trap stiffness. The average displacement upon binding decreased with trap stiffness which the authors interpret as a load-dependent power stroke. When trapping microspheres were incubated with additional motors that do not have a specific binding tag on the microspheres, the authors observed processive motion against forces of a few piconewtons. Also, TIRF measurements show that at 1 nM motor concentration, motor aggregates move processively towards the microtubule minus end. Gliding assays show that with increasing density of motors microtubule gliding speeds decrease. Since a tail-truncation mutant does not show this decrease, but potentially a modest increase in speed, the authors conclude that the diffusive interaction of the tail leads to friction that slows down the gliding and that cooperativity of the motors could increase the speed. While the manuscript has several very interesting findings, some of these need some additional control experiments to rule out alternative explanations as pointed out below. The manuscript sheds light on how kinesin-14 motors may work in the mitotic spindle. In summary, I recommend publication after a revision that addresses my concerns below.

⇒ We thank the reviewer for their positive assessment of our manuscript and for the insightful comments and suggestions provided. We are encouraged by the recognition of the interesting findings in our study. In agreement with the reviewer's suggestions, we have conducted additional control experiments, the results of which are included in our revised manuscript. We believe that addressing the raised concerns has not only strengthened the validity of our results but also enhanced the overall impact of our study.

Major point:

1. My main concern is interpreting the "power stroke". For non-processive muscle myosin, the three-bead assay was developed to distinguish binding/unbinding from an ATP-driven power stroke. Such experiments are typically done in a force-clamp mode where power-stroke displacements can be measured as a function of force. Here, several questions remain open and alternative explanations are possible.

First, how was the directionality of the displacement with respect to the microtubule polarity measured?

⇒ We appreciate the reviewer's query regarding the measurement of displacement directionality in relation to MT polarity. In our experiments, we used polarity-marked MTs to determine the direction of the displacement. We realize that this information was inadvertently omitted from the methods section of our initial manuscript. To rectify this, we have now included a new section titled ‘Polymerization of polarity-marked microtubules' in the Methods section of our revised manuscript, providing a detailed description of this procedure.

Are all displacements in the same direction?

⇒ Yes, all displacements observed in our study occurred exclusively in the minus-end direction of the MTs. This detail is explicitly stated in the manuscript, providing clarity on the directional nature of the displacements. We have included this information in the first section “KlpA is non-processive under load” for easy reference.

Please, show longer traces with many events.

⇒ We appreciate the reviewer's request for more comprehensive data. In response, we have included longer traces featuring multiple events for all three studied motors (HSET, KlpA, and Ncd) in the Supplementary Material of our revised manuscript (see new Suppl. Figs. 2, 8, 13, 19, and 21). These extended traces provide a more detailed view of the motor interactions and enhance the robustness of our findings.

Is there any evidence that displacements are due to ATP hydrolysis?

Binding should precede the power stroke. Detecting the binding of the motor should be possible by, for example, measuring a reduction in the positional standard deviation. Or, as done in the three-bead assays, a reduction in a small amplitude oscillation might be used to detect binding. Subsequently, according to the proposed power-stroke mechanism (Fig. 3A), the "power-stroke" displacement should be detectable. The time between binding and displacement should depend on ATP concentration. In particular, at low ATP concentrations, binding and power-stroke should be distinguishable. Using slowly hydrolyzable ATP analogs might be another approach.

⇒ We appreciate the reviewer's thoughtful recommendation to correlate motor displacement with ATP hydrolysis. Prompted by this suggestion, we have performed additional experiments where ATP was depleted using apyrase. These ATP/ADP-depleted conditions revealed that KlpA binding to MTs does not result in any measurable displacement, as shown in the new Supplementary Figure 4. Displacements were only observed when ATP was present, thereby affirming that the power strokes we report are indeed driven by ATP. This experimental update solidifies the linkage between ATP presence and the initiation of the power stroke, in line with the mechanism depicted in Figure 3A.

An alternative explanation for the observations might be that the kinesin simply binds and unbinds without a power stroke. It is impossible to center a trap directly over a canonical kinesin binding site. Thus, any binding is intrinsically associated with a displacement both along the microtubule axis and perpendicular to it. Please, along with the above-mentioned longer traces that show multiple events, also show the perpendicular microsphere displacements. Apparent directionality or bias of binding events may also arise due to an intrinsic bias of the motor (e.g. the motor might be very susceptible to detachment under assisting loads that no events are detected vs. prolonged binding under hindering loads).

⇒ We are thankful for the reviewer's comments. To address the concerns raised, we have extended our experimental analysis beyond the presence and absence of nucleotides. Our additional data now include segments of traces that distinctly demonstrate the ATP-driven power strokes of KlpA, HSET, and Ncd. These strokes occur exclusively along the longitudinal axis of the microtubule, with no detectable displacement in the perpendicular direction, as depicted in the newly added Supplementary Figure 5. This evidence clearly indicates that the binding of Kinesin-14 motors to MTs results in unidirectional displacements along the microtubule axis, rather than in random, bidirectional movements. Consequently, our findings support the specificity of the ATP-driven power stroke over the alternative hypothesis of non-directional binding and unbinding.

*The reduction in displacement with trap stiffness may be simply due to a more confined Brownian motion. The expected rms positional fluctuations are x_rms = sqrt{k_B*T/kappa} whereby k_B is the Boltzmann constant, T is the absolute temperature, and kappa is the trap stiffness. For kappa = 0.05 pN/nm, the microsphere fluctuates by about x_rms = 9 nm. This value matches very well the observed mean displacement and also the expected width of the distribution (Fig. 3C). For kappa = 0.3 pN/nm, x_rms = 3.7 nm, somewhat larger compared to the 1.9 nm of what the authors measured. Together with an offset from the trap position relative to the binding site, values are within reasonable agreement.*

⇒ We appreciate the reviewer's consideration of Brownian motion as a potential explanation for our observations. However, our data clearly indicate that Brownian motion cannot account for the measured displacements. Firstly, the displacements we observed were consistently in the direction of the MT's minus-end, whereas Brownian motion would result in random displacements in both directions. Secondly, our new data demonstrate that these unidirectional displacements are dependent on ATP, further supporting a mechanism driven by active motor function rather than passive thermal fluctuations.

Additionally, our new results demonstrate that the displacement magnitude is influenced by the tail length of Kinesin-14 motors. This dependency is inconsistent with a model based solely on Brownian motion and instead supports the concept of a single, tail-dependent power stroke. Our findings align with previous optical trapping studies on Ncd by Endow and Higuchi (Science, 2000) and de Castro et al. (Nature Cell Biology, 2000), yet our approach offers distinct advancements. While Endow and Higuchi specifically bound Ncd motors to trapping beads via a biotin tag at the N-terminus, their study did not account for the compliance of the motor and bead linkage. Additionally, de Castro et al. employed non-specific binding of Ncd motors to trapping beads, a method likely to yield shorter ATP-dependent displacements, not directly correlated with tail length. To address these methodological nuances and further strengthen our conclusions, we have conducted additional force measurements on Ncd. These new data, included in the new Supplementary Figures 5C, 20, and 21, provide a more comprehensive understanding of the motor function, and underscore the significance of our findings in the context of Kinesin-14 motor research.

Furthermore, there is an additional effect that can cause an erroneous displacement arising from the rocking motion of the microsphere when binding. Since the motor stalk plus GFP and antibody form a flexible linker, initial displacements after binding and loading may be largely due to the rocking motion of the microsphere and pulling the linker taught. Since forces are acting through the center of the microsphere, motions are amplified by a long lever, i.e. the 500 nm radius of the microsphere. Thus, it is unclear how much of the displacement is due to a potential motion of the motor and how much is due to the rocking motion of the microsphere. In a force-clamp experiment, such artifacts do not arise.

⇒ We appreciate these further comments from the reviewer regarding the potential influence of microsphere rocking motion on displacement measurements. The additional experiments we have conducted and described earlier, particularly the ones demonstrating ATP dependence and unidirectionality of the measured displacements, directly address this concern. By confirming that the displacements are ATP-dependent and consistently unidirectional, we demonstrate that the observed movements are not merely artifacts of microsphere rocking. Furthermore, our corrections for the compliance of the motor and bead linkage help to ensure that the displacements we measured are reflective of motor movement rather than the force

applied by the optical trap. This comprehensive approach strengthens the validity of our findings and mitigates concerns about potential artifacts arising from the experimental design.

Do the authors have any evidence that at the roughly 10 pM concentration used for the single-molecule force measurements, motors indeed are dimers? What was the motor concentration for the bleaching step measurements (Fig. 2G)? At very low concentrations, motors may not be dimers. Monomers would also not be processive.

⇒ We thank the reviewer for highlighting the importance of confirming the oligomeric state of the motors at the concentrations used in our experiments. To address this, we have repeated the photo-bleaching experiments using the same motor concentration as that employed for the single-molecule optical trapping experiments (10 pM). The results from these repeated experiments, now included in the updated Figure 2G, confirm that HSET predominantly exists as a dimer under these conditions. In addition, we have run a native gel, which also shows that HSET forms predominantly a dimer. These results are presented in the new Supplementary Figure 10. Together, these experiments comprehensively demonstrate that HSET operates as a dimer in the conditions used for our single-molecule studies, supporting our conclusions about its functionality and processivity.

Minor points:

2. How was the trap calibrated?

⇒ We utilized the LUMICKS' C-Trap system for our study, which features an active calibration method. During lateral calibration, this method involves oscillating a 1-μm bead held in the trap near the cover-glass surface. The calibration process encompasses analyzing the resulting Lorentzian power spectrum. A critical aspect of this analysis is the identification of the isolated spike in the power spectrum, which is a direct consequence of the bead's oscillations. By employing this method, one ensures precise and accurate calibration of the optical trap. This approach is particularly beneficial as it eliminates the need for user-supplied specifications like bead diameter or height, especially for measurements conducted in close proximity to the cover glass.

3. What was the data acquisition rate? Was there an anti-alias filter?

⇒ In our study, the data acquisition utilized an analog-to-digital conversion based on a sigma-delta system. The C-Trap's base sampling frequency operates at 20 MHz, which is then digitally down-sampled by a factor of 256. This down-sampling results in a final output sample rate of 78.125 kHz. Additionally, the C-Trap system incorporates a digital anti-aliasing filter. This filter creates a passband with a maximum frequency of 31.25 kHz. The C-Trap only fits the data within this passband region.

4. How was the data processed? What is the bandwidth with which the data is displayed?

⇒ After acquisition, data sampled at 78.125 kHz are securely transferred to a data server and subsequently processed using our custom MATLAB program. Within this program, an averaging window with a width of 200 data points is applied to the data before analysis to facilitate

visualization. This smoothing process helps in reducing noise while preserving the key features of the data. The calculation of the average distance from the trapping center—which indicates the size of the power-stroke—and the variance in position during the post-power stroke phase—which is used to calculate the motor's compliance—are based on the original, non-averaged data. Moreover, the corresponding force generated is also derived from the unprocessed dataset to ensure accuracy. We have now included a detailed description of our data processing methodology in the newly added 'Analysis of optical trapping data' section of the methods. We believe these details provide a comprehensive understanding of our data processing and display approaches.

5. For the mCherry-tagged motor experiments, the authors argue that motors oligomerize with the GFP-tagged motors. However, no direct evidence is provided. Please provide some TIRF kymographs of oligomerized GFP- and mCherry-tagged motors moving together along microtubules.

⇒ In response to the reviewer's request for direct evidence of oligomerization between GFP- and mCherry-tagged motors, we have conducted dual-color Total Internal Reflection Fluorescence (TIRF) microscopy experiments using equal molar amounts of both motor types. The results of these experiments are now presented in the new Supplementary Figure 6. The kymograph included in this figure clearly demonstrates that GFP- and mCherry-tagged motors do indeed oligomerize and move together along MTs, as suggested. We believe this additional data directly addresses the reviewer's concern and further supports our conclusions regarding the oligomerization and cooperative function of these motors.

The force measurements are not convincing since the increased force is about the "single motor force" of 0.5 pN plus the force of the non-specifically attached motors. For example, in Suppl. Fig. 2, the differences between non-specifically attached motors to GFP-motors plus mCherry motors were 0.67 pN, 0.61 pN, and 0.35 pN which on average is 0.54 pN. This average force difference matches the single-motor force. Thus, forces may be the addition of GFP-tagged motors plus the non-specifically attached ones without any direct oligomerization.

⇒ We appreciate the reviewer's concern regarding our multi-motor force measurements. Our study provides robust evidence of oligomerization between GFP- and mCherry-tagged motors, as demonstrated in our initial manuscript, and further reinforced by our new dual-color TIRF microscopy data (new Supplementary Figure 6). This data clearly shows motors oligomerizing, making it reasonable to infer that similar oligomerization occurs when the motors are bound to the bead surface.

Moreover, the average increase in force between beads with only mCherry-tagged motors and those with both mCherry- and GFP-tagged motors is approximately 0.6 pN. However, many individual force measurements significantly exceed this value. For instance, with 29 pM mCherry-tagged motors and pre-bound GFP-tagged motors, we recorded numerous forces above 2 pN, reaching up to approximately 4 pN. This contrasts sharply with the maximal force of less than 2 pN observed for beads with only mCherry-tagged motors. This trend is also evident in experiments with 87 pM mCherry-tagged motors.

These instances of higher force values suggest that the increased forces are not just the sum of the force generated by a single GFP-tagged motor and forces from non-specifically attached motors, but also result from the direct oligomerization of these motors. It is expected that as the

number of mCherry-tagged motors increases, the force difference between mCherry-only beads and beads with a pre-bound GFP-tagged motor plus mCherry-tagged motors diminishes. This is because the trapping beads are likely to carry only one specifically bound GFP-tagged motor, while an increasing number of mCherry-tagged motors bind non-specifically. Therefore, the higher the concentration of mCherry-tagged motors, the smaller the difference becomes between the mCherry-only beads and the beads with a pre-bound GFP-tagged motor plus mCherry-tagged motors.
In analogy to Fig. 1E and Fig. 2I, please also provide force traces for microspheres that had only the mCherry motors bound. Did all supplemented motors bind to the microspheres? In particular for the high concentrations, likely a lot of motors are still in solution. These motors will also interact with the microtubules and may change the motility of the microsphere-attached motors. Please provide some TIRF images of the used mixtures to illustrate how many mCherry motors are interacting with the microtubules during these experiments.

⇒ We appreciate the reviewer's concern regarding the potential impact of unbound mCherry-tagged motors on the force generation observed in our experiments. To mitigate this, we washed the beads with mCherry-tagged motors attached (using centrifugation and buffer resuspension) to minimize the presence of free motors in the solution.

In response to the reviewer's suggestion, we conducted additional TIRF microscopy experiments under the same conditions as our optical trapping experiments. These TIRF experiments show that while some residual mCherry-tagged motors remain in solution, these motors (as a result of their brief non-processive power strokes) do not accumulate on the MT surfaces. We present these findings in the new Supplementary Figure 7B.

These TIRF microscopy results, coupled with our detailed bead washing protocol now included in the methods section 'Optical tweezers assay', affirm that the force generation observed in our study is not influenced by motors interacting with MTs from the solution. Additionally, it's worth noting that any free motors binding to the trapping bead from the solution would enhance the cooperative behavior we report, thereby positively contributing to our measurements.

6. Fig. 2B: How was the relative motor concentration determined?

⇒ For determining the relative motor concentrations in our experiments, we maintained a constant bead concentration while the motor stock solution, which had a pre-adjusted concentration, was stepwise diluted and then added to the bead solution. The absolute motor concentration was not necessary for our analysis since the plotted relative concentration is simply the inverse of the dilution factor of the motor solution. This method allowed us to accurately control and report the relative concentrations of the motors used in each experimental condition.

7. Fig. 4: Please provide concentrations used during incubation instead of dilutions.

⇒ We have updated Figure 4 to include the actual motor concentrations used during incubation as requested by the reviewer.

For Fig. 4E, please plot speed vs. the incubation concentration and fit a line to the data. Is the slope significant? It is stated in the abstract and final discussion, but apart from presenting the data, there is no further analysis or statistical test of the significance.

⇒ We acknowledge the reviewer's request for a more detailed analysis of the data presented in Figure 4E. Upon further examination, we have determined that the increase in velocity, though modest, is statistically significant. Specifically, the p-value for the velocity increase between the first two motor dilutions is less than 0.01, and for the second and third dilutions, it is less than 0.001. This demonstrates a clear trend of increasing velocity with motor concentration. Furthermore, to strengthen our argument, we have included new MT-gliding data with a tail-truncated Ncd construct, which lacks the MT-binding tail sequences. This data, presented in the new Supplementary Figure 23A&B, shows an even more pronounced increase in velocity compared to HSET. The inclusion of these additional results provides a comprehensive view of the relationship between motor density on the glass surface and gliding velocity, supporting our conclusions drawn from the HSET data.

Also, the conclusion in l. 217 that an increase in ionic strength weakens the interaction between the tail and microtubule is too early at that point. Only with the additional measurements presented further on such an interpretation seems reasonable.

Also, nothing can be said about the strength of the interaction, for example, "the tail strongly interacts with MTs through ionic interactions". It can only be concluded that there is a stronger dependence on the ionic strength. For the strength of the interaction, the authors would have to perform force measurements.

⇒ We thank the reviewer for their insightful comments. We agree that our initial conclusions regarding the ionic strength's effect on the interaction between the motor tail and the MT may have been premature in the text. In light of this, we have revised the relevant section to better reflect the progression of our findings and the subsequent interpretations that they support. The revised text can now be found on lines 254 to 273. Additionally, we acknowledge that our characterization of the interaction strength between the tail and the MTs was based on the observed dependency on ionic strength. We recognize that force measurements would be necessary to make definitive statements about the interaction strength, and we have adjusted our language in the manuscript to more accurately represent this. Thank you for bringing this to our attention, ensuring the precision of our study's conclusions.

8. l. 171: Please provide a reference for the cryoEM structure of Ncd.

⇒ Done.

9. Fig. 3: "biphasic behavior" - there is only one data point at 0.04 pN/nm. Please measure also at 0.01 and 0.02 pN/nm whether there is indeed a different behavior for trap stiffness smaller than 0.05 pN/nm.

⇒ We appreciate the reviewer’s attention to the detail regarding the 'biphasic behavior' observed in our study. Following your suggestion, we have performed more HSET force measurements and attempted additional measurements at lower trap stiffnesses of 0.02 pN/nm. Unfortunately, at this lower stiffness value, the intrinsic fluctuations of the trapped bead were too significant, rendering our displacement measurements unreliable. Given these technical constraints, we have decided to only include force measurements for trap stiffnesses ranging from 0.05 to 0.3 pN/nm in our revised manuscript, where the motor-induced displacements are

within the detectable and reliable range. We believe this approach maintains the integrity of our data and provides a clear representation of the motor behavior as a function of trap stiffness.

10. Fig. 1C: The displacement noise is too low compared to the expected value of x_rms for the used trap stiffness. How was the data low-passed filtered? The question applies to all trapping data.

⇒ We are grateful to the reviewer for pointing out the apparent discrepancy in the displacement noise level. To clarify, the data was indeed low-pass filtered to facilitate the detection of power strokes. This was achieved by averaging the data using a sliding window with a width of 200 data points. We understand the importance of transparency in data processing methods and have therefore included a detailed description of this procedure in the 'Analysis of optical trapping data' section of our methods. This addition will provide readers with a complete understanding of the steps taken to analyze the displacement data.

11. How were the maximum forces determined? What were the criteria? Was it the true maximum of the (unfiltered) Brownian motion? Was it the average? Was it the maximum of the filtered data? Over how many data points or what time was it averaged?

⇒ Regarding the determination of maximum forces, we have addressed this in our response to point 10, as you have noted. In the 'Analysis of optical trapping data' section newly added to our methods, we detail the criteria for determining the generated forces. Specifically, we outline the data averaging process (used for visualization only) and the selection criteria for maximum force events.

12. For the insets in Fig. 1C and 2C, please provide labels and ticks for the axes.

⇒ Thank you for pointing out the omission in Figures 1C and 2C. We have now added the appropriate labels and tick marks to the axes in these insets, as requested. We apologize for any inconvenience the absence of this information may have caused and appreciate your attention to detail.

13. Fig. 2G & H. Please provide some fluorescence intensity traces that show the bleaching steps. How was the MT polarity determined in the kymograph?

⇒ Thank you for your queries regarding the photobleaching data and the determination of MT polarity. In response, we have included a selection of fluorescence intensity traces showcasing the photobleaching steps in the new Supplementary Figure 9. Additionally, we have provided a comprehensive description of the methods used to determine MT polarity in the 'Polymerization of polarity-marked microtubules' section of the methods. We believe these additions will offer the necessary clarity and further substantiate the findings presented in our study.

14. Fig. 4: The authors argue that the diffusive interactions of the motor tails cause the observed slow down with increasing motor density. However, the relative ratio of force-generating motors and tail-interacting motors should not change with the density. As on average the relative

number of motors counterbalance the friction caused by the same relative number of tails, one would not expect an increased friction with density.

Also since speeds are very low, friction forces are expected to be very small. Based on the data the authors have in Fig. 3A, they could determine the friction based on the measured diffusion coefficient of the motors in Fig. 3A. and their speed. Most likely friction is not limiting, but other effects.

⇒ We appreciate the reviewer's point regarding the expected frictional forces at increasing motor densities. However, the premise that the effects of force-generating motors and tail-MT interactions counterbalance does not hold due to the significantly different MT affinities of the motor and tail domains. This is detailed in the section 'Kinesin-14 motors work cooperatively to increase the velocity during MT gliding' in the main text. As motor density increases, the proportion of tail domains interacting with the MTs also increases, which disproportionately contributes to the observed reduction in MT-gliding velocity. This is because the tail domains, which have a higher affinity for MTs compared to the motor domains, create a drag that is not offset by the force generation of the motor domains. Consequently, at higher motor concentrations, the cumulative effect of these tail interactions outweighs the force generated by the motor heads, leading to a net slowdown in gliding speed. This conclusion is further corroborated by our experiments with a construct lacking the MT-binding tail domain. In these experiments, the absence of the tail resulted in an increase in MT-gliding speed, aligning with expectations for non-processive, low-duty ratio motors. Reflecting these findings, we have revised the corresponding section of our manuscript to describe these observations more accurately.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:

This is a revision and the authors did a good job of addressing most of the points that I made in my first review. In particular, I thought the flow of the paper was much better, with the different sections tying together much more effectively. I felt that the single- and multi-motor analysis of HSET and KlpA was a solid contribution. Specifically, showing that they are both non-processive but can work together to form processive teams capable of multi pN force generation is a solid piece of work. In my previous review, I felt that the HSET analysis was insufficient; the authors have addressed all of my concerns regarding their claims that HSET is non-processive, and it is a notable contribution since it contrasts from previous work from the Lang lab. Also, the mulimotor gliding showing the effect of the tail is clear and ties nicely to the multi-motor trapping work. Thus, three out of the four main points of the paper I thought were strong. However, I have serious concerns regarding the authors' stiffness corrections that they use to extrapolate the claimed powerstroke to be three-fold the measured (discussed below). That fourth part I thought was solid from a qualitative level, but was flawed from a quantitative level.

Regarding the powerstroke size estimates, I felt that there was an opportunity lost for using other approaches to estimate the powerstroke size. For instance, the velocity data in Fig S23C, coupled with the alpha-fold estimates of the coiled-coil length (or an approximation of 1.5A/residue) enables a calculation of the estimated powerstroke. (This is analgous to the Endres ncd work.) Additionally, by analyzing the distribution of the single-motor binding durations trapping traces, they could get an estimate of the cycling rate. These could all be combined to estimate the duty ratio. These are just suggestions, but they provide a different approach to estimating powerstroke size than the approach using a large correction factor that the authors used.

I have concerns with the extrapolations made in the powerstroke determination. It is reasonable to expect that there is a compliance between the motor domain and the bead, and that the bead may rotate, which effectively adds more compliance. This compliance would result in a smaller measured step size than the true powerstroke the motor takes. The authors use a variance analysis to estimate the summed motor and trap stiffnesses. At different force plateau values, the variance is different, resulting in an apparent increasing motor stiffness with force (Fig. S14A and S15). The authors don't say this explicitly, but this implies a nonlinear elasticity of the motor. That is not unsurprising. It is somewhat odd that the stiffness goes to zero at zero force; a normal spring doesn't do this. Be that as it may, it is very difficult to understand how this property of the motor, its nonlinear stiffness, appears to change when the trap stiffness changes (the different slopes for different stiffness values in Fig. S14A and S15). How can that be? If the analysis were sound, a consistent measured motor stiffness (either linear or nonlinear) at different trap stiffnesses would serve as a consistency check that the measurements are sound.

Based on the questionable assumptions regarding stiffness and the fact that the assumptions lead to a three-fold change from the measured to the extrapolated powerstroke size, I think the authors should treat their measured displacements and foces much more conservatively. The qualitative effect, arguing the true powerstroke is larger than the measured discplacement, is there. However, a valid quantification of the powerstroke size requires either more detailed analysis with controls or perhaps a three-bead geometry used for myosin. This is an important point because the authors make a lot out of their 25 nm powerstroke estimate throughout the manuscript.

Specific comments:
Lines 107-109. The authors state that they rule out any sideward displacements. However, their analysis is very cursory, visual analysis of a few traces, and even in one of those (first FL-HSET trace in Fig S5. This contrasts with the detailed analysis done previously, such as in the deCastro

paper. Thus, to make this claim, the authors must carry out a more complete and rigorous analysis.

Line 125. What is the evidence that mCherry does not bind to anti-GFP antibodies? There is clearly a lot of mCherry motor binding, and the fluorescent proteins share ancestors, and so it isn't out of the question.

Line 126: I think "conclusively" is excessive here.

Reviewer #2:
Remarks to the Author:
In the revised manuscript, Liu and coworkers have addressed all of my concerns and I generally support publication of the manuscript after addressing a few points as outlined in the following.

Major point:
1. While a compliance correction, in principle, is reasonable, it should be self-consistent. Currently, it is not. The authors correct the measured lateral displacement based on the series of compliance of the optical spring and motor stalk/linker (while I favor that the correction is left out completely, the authors should have cited their reference 48 for the correction as it precedes ref. 54). The authors find that the spring constant of the motor increases linearly with force and depends on the trap stiffness (Fig. S14A, Fig. S15, Fig. S18A, and Fig. S20A). While the former is in principle possible (the increase appears to be very large, about 10-fold for a small force of 1 pN), the latter indicates that the correction must be wrong. The motor compliance is a material property of the motor itself and cannot depend on the settings of the measurement device (the trap stiffness). Thus, the correction does not capture the physics of the system under investigation. A similar argument may apply to the power stroke itself. After correcting for the compliance/geometry, the power stroke should also be a constant in analogy to the step size of kinesin that is 8 nm after a compliance correction and independent of the trap stiffness used for the measurement (as shown in their ref. 48 and by many others). Thus, based in particular on the first argument, the correction cannot be correct and should be by all means omitted. Otherwise, I do not support the publication of the manuscript.

One likely reason why the simple compliance correction is flawed is that the geometry of the system is not properly accounted for. The authors argue that the coiled-coil of the stalk pivots around the motor heads by about 70 deg in analogy to ncd. For HSET and KlpA, the starting angle and range might be different and in particular not symmetric about the optical axis of the optical trap. Since the stalk rotates around the motor head, the authors probe a combination of the bending and stretching stiffness of the coiled coil (tangential and normal forces relative to the arc that the end of the coiled-coil traces in the absence of load). Since the measurements here are parallel to the microtubule axis, they are a vectorial combination of tangential and normal forces. Based on the persistence length of coiled-coils of a few 100 nanometers, their bending stiffness is at least 0.1 pN/nm for a 25-nm-long coiled coil comparable to the trap stiffness of the authors. For the 0.5 pN generated by the motor, the stalk should bend by at most 5 nm. For such small displacements, one would not expect much of a force dependence of the bending stiffness (certainly not a factor 10). The stretching stiffness of the coiled-coil is much larger as the bonds need to be stretched and most likely can be considered to be an inextensible rod. With increasing trap stiffness, the authors measured a reduced displacement. Thus, the angular position of the stalk is different for each trap stiffness. And, because the angle is different, the relative amounts of the tangential and normal force vectors are different, and concurrently the relative amounts of bending and stretching stiffness that are probed and that contribute to the overall fluctuations. Furthermore, for the same reason, the distance from the surface of the tethered bead might be different, i.e. depend on trap stiffness, constraining the lateral pivoting motion by a different amount. This constrained pivoting or tethered motion would be a simple explanation for the

somewhat reduced variance (at most 20 percent smaller standard deviation based on Fig. S14). This explanation would lead to reduced variance without any change in motor compliance. It is simply the geometry. Furthermore, due to the fairly stiff motor stalk, rotational and translational motion of the bead are coupled. This coupling may also lead to a reduced variance. An analogous effect was reported for magnetic tweezers when the DNA is attached off-axis (see Klaue & Seidel, Phys. Rev. Lett. 102, 028302 (2009)). While in the case of the magnetic tweezers, the torsional stiffness is due to the bead, here, it is due to the torsional stiffness of the motor stalk. The math and physics of the system are equivalent and changes in the displacement standard deviation of 20 percent seem plausible (much smaller than the changes reported for the magnetic tweezers). The authors would have to calculate & solve the coupled differential equations for translational and rotational motion to properly account for any change in variance and potentially correct the displacements of their power strokes. Since this correction is probably beyond the scope of the present manuscript, the authors should not make any corrections and only discuss potential corrections. As it stands, the analysis and correction are not self-consistent and cannot be true.

The dependence of power stroke displacement on the various motors still indicates that the motors do a power stroke by a rotating lever arm. How the length of the tail domains translates into trap-measured displacements depends on the detailed geometry that is unknown (beginning with the starting and end angle of the power stroke). A proper analysis would have to include motions and forces in the z-axis (it is unclear what the forces and displacements in the z-axis are - based on Fig. S4, fluctuations in z are non-symmetric indicating that the authors push down on the glass surface with the typical large offset that needs to be subtracted in the z-axis not properly accounted for - for all other measurements, it is unclear what the axial motions are).

Minor points:
2. Fig. S3B: The zero-force position seems wrong. In the beginning, the baseline appears to be at around 0.6 pN, which would imply that the bead (and motors) have reversed direction in the end (at about 55 s).

3. Fig. S5: Why do all of the diagonal motor events gradually increase in force while for the x- and y-direction, they are more or less instantaneous power strokes?

4. Line 125: please rephrase the statement that mCherry does not bind to anti-GFP antibodies. Fig. S7A does demonstrate significant force production when mCherry-KlpA is incubated with GFP antibody beads. Thus, motors are bound to beads either non-specifically or via the antibodies. What it is exactly is unclear.

5. For the compliance correction, the authors used the variance in position during the post-power stroke relative to the calculated variance of the free bead based on the calibrated trap stiffness and the equipartition theorem. The authors should have used the reference variance in the proximity of the power stroke (a certain time window before and after) instead. In this manner, they can properly account for any local changes in trap stiffness or drift. Yet, since the authors should omit the correction completely (changes in variance likely have a different origin than motor compliance), this point is not relevant.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This is a revision and the authors did a good job of addressing most of the points that I made in my first review. In particular, I thought the flow of the paper was much better, with the different sections tying together much more effectively. I felt that the single- and multi-motor analysis of HSET and KlpA was a solid contribution. Specifically, showing that they are both non-processive but can work together to form processive teams capable of multi pN force generation is a solid piece of work. In my previous review, I felt that the HSET analysis was insufficient; the authors have addressed all of my concerns regarding their claims that HSET is non-processive, and it is a notable contribution since it contrasts from previous work from the Lang lab. Also, the mulimotor gliding showing the effect of the tail is clear and ties nicely to the multi-motor trapping work. Thus, three out of the four main points of the paper I thought were strong. However, I have serious concerns regarding the authors' stiffness corrections that they use to extrapolate the claimed powerstroke to be three-fold the measured (discussed below). That fourth part I thought was solid from a qualitative level, but was flawed from a quantitative level.

⇒ We appreciate the reviewer's thorough assessment of our revised manuscript and their acknowledgment of the improvements made in response to their previous feedback. Regarding the concerns raised about the stiffness corrections used in our study to extrapolate the claimed powerstroke to be three-fold the measured, we acknowledge the importance of this issue, which we address below.

Regarding the powerstroke size estimates, I felt that there was an opportunity lost for using other approaches to estimate the powerstroke size. For instance, the velocity data in Fig S23C, coupled with the alpha-fold estimates of the coiled-coil length (or an approximation of 1.5A/residue) enables a calculation of the estimated powerstroke. (This is analgous to the Endres ncd work.)

⇒ We indeed considered this approach. However, as we wrote already in our revision on page 10 and 11, lines 194-197, the velocity from microtubule-gliding assays with non-processive, low-duty ratio motors cannot be used to determine the power-stroke size as the velocity depends not only on the catalytic rate and power-stroke length but also on the density of motors bound to the cover glass. The power-stroke sizes estimated by Endres et al. are indeed not accurate.

Additionally, by analyzing the distribution of the single-motor binding durations trapping traces, they could get an estimate of the cycling rate. These could all be combined to estimate the duty ratio. These are just suggestions, but they provide a different approach to estimating powerstroke size than the approach using a large correction factor that the authors used.

⇒ We also considered this approach. However, since microtubule binding does not induce detectable displacements in the absence of ATP (as shown in Supplementary Figure 4), it remains unclear precisely when ADP releases, ATP binds, or whether motor detachment occurs before or after ATP hydrolysis and/or Pi release. Consequently, determining the catalytic rate solely from binding durations would require additional experiments beyond the scope of our current study.

I have concerns with the extrapolations made in the powerstroke determination. It is reasonable to expect that there is a compliance between the motor domain and the bead, and that the bead may rotate, which effectively adds more compliance. This compliance would result in a smaller measured step size than the true powerstroke the motor takes. The authors use a variance analysis to estimate the summed motor and trap stiffnesses. At different force plateau values, the variance is different, resulting in an apparent increasing motor stiffness with force (Fig. S14A and S15). The authors don't say this explicitly, but this implies a nonlinear elasticity Hof the motor. That is not unsurprising. It is somewhat odd that the stiffness goes to zero at zero force; a normal spring doesn't do this. Be that as it may, it is very difficult to understand how this property of the motor, its nonlinear stiffness, appears to change when the trap stiffness changes (the different slopes for different stiffness values in Fig. S14A and S15). How can that be? If the analysis were sound, a consistent measured motor stiffness (either linear or nonlinear) at different trap stiffnesses would serve as a consistency check that the measurements are sound.

⇒ We acknowledge the reviewer's observation regarding the varying slopes of the stiffness-versus-force curves for different trap stiffnesses. Initially, we attributed these differences to increasing loading rates, which rise with increasing trap stiffness (i.e., increasing force per unit displacement). However, following a suggestion from Reviewer 2 to measure the position variance before and after the power stroke, we identified a growing discrepancy between trap stiffness derived from measured position variance (using the equipartition theorem) and that from active calibration, particularly evident at higher trap stiffnesses (Fig. 1).

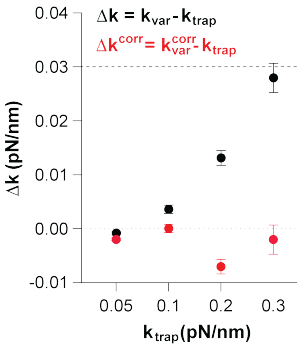

Figure 1 Error Δk between k_{var} and k_{trap} with and without correction for the parasitic filtering effect of the PSD.

Notably, the trap stiffness derived from position variance before and after a power stroke aligned with that obtained from active calibration for the lowest trap stiffness of 0.05 pN/nm (Figure 1). While discrepancies remained relatively small with higher trap stiffnesses (the difference Δk between the variance-based trap stiffness k_{var} and the trap stiffness derived from active calibration k_{trap} is less than 10% at the largest used trap stiffness of 0.3 pN/nm, see Fig. 1), these differences were sufficient to cause the slope of the stiffness-versus-force curves to increase with trap stiffness.

By measuring the position variance before and after power strokes across all trap stiffnesses, we confirmed that all stiffness-versus-force data overlapped, irrespective of trap stiffness (see Figure 2 below, with the linear fits and the new manuscript figures 3D, S13C, S16C, and S18C with the modeling of the data with a worm-like chain (WLC) model discussed below). Consequently, we now present only one graph showing KlpA's stiffness-versus-force data for all trap stiffness to demonstrate that the motor's stiffness is independent of the trap stiffness (Fig. 3D). For all other motor constructs, we utilize the stiffness-versus-force data acquired at 0.05 pN/nm (Supplementary figures 13C, 16C, and 18C). This adjustment ensures consistency and reliability in our measurements.

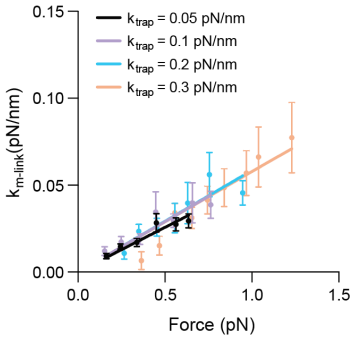

Figure 2 Stiffness of motor and bead-linkage as a function of force for four different trap stiffness.

Additionally, using a MATLAB script provided by LUMICKS that accounts for the parasitic filtering of the position-sensitive detector (PSD), largely removes the discrepancy between variance-based and active calibration-derived trap stiffness (Figure 1). Thus, active calibration (which takes into account the parasitic filtering effect) provides an accurate trap stiffness for force and distance measurements, while variance analysis before and during the power stroke offers an accurate estimate for the combined compliances of motor and bead linkage. We detail our new variance-analysis approach in the updated Methods section, "Calculation of the compliance of the motor- and GFP-antibody bead linkage and correction of the power stroke".

To address both the reviewer's query about the motor's non-linear stiffness and the impression that the stiffness approaches zero at zero force, we now apply a non-linear WLC model to describe our stiffness data. By introducing a minimal, non-zero stiffness, $k_{\text{m-link}}^{\text{min}}$, the WLC model describes our data very well (see new Figures 3D, S13C, S16C, and S18C) and results in estimated load-free power strokes that agree well with the power stroke sizes suggested by AlphaFold (Suppl. Fig. 23). We describe the details of this approach and the underlying equations, which include the use of a Boltzmann distribution (instead of using a simple harmonic potential) to account for the non-linear nature of the motor and bead-linkage, in the Methods section.

Based on the questionable assumptions regarding stiffness and the fact that the assumptions lead to a three-fold change from the measured to the extrapolated powerstroke size, I think the authors should treat their measured displacements and foces much more conservatively. The qualitative effect, arguing the true powerstroke is larger than the measured discplacement, is there. However, a valid quantification of the powerstroke size requires either more detailed analysis with controls or perhaps a three-bead geometry used for myosin. This is an important point because the authors make a lot out of their 25 nm powerstroke estimate throughout the manuscript.

⇒ With the changes we have made and described above, our data and analyses are now fully self-consistent. Specifically, modeling our experimental data using the non-linear WLC model results in estimated load-free power strokes for all four motor constructs. These estimates align well with the coiled-coil lever-arm lengths predicted by

AlphaFold and have been experimentally validated through coiled-coil truncation experiments. These findings demonstrate that the entire predicted length of the dimerizing coiled-coils contribute to the power stroke of Kinesin-14 motors.

Specific comments:

Lines 107-109. The authors state that they rule out any sideward displacements. However, their analysis is very cursory, visual analysis of a few traces, and even in one of those (first FL-HSET trace in Fig S5. This contrasts with the detailed analysis done previously, such as in the deCastro paper. Thus, to make this claim, the authors must carry out a more complete and rigorous analysis.

⇒ To address this comment, we conducted a correlation analysis for the power strokes depicted in the previous Supplementary Figure 5. This analysis demonstrates that a significant Pearson correlation is only observed when the microtubule is oriented diagonally, consistent with a unidirectional power stroke toward the microtubule minus end resulting in measurable displacements in both the x and y directions. In cases where microtubules are oriented either horizontally or vertically, the obtained Pearson correlation value, *r*, is close to zero. This pattern holds true for all analyzed power strokes (see new Fig. 1E and Suppl. Fig. 7). We have included a statement regarding these findings in the main text and the corresponding figure legends. In addition, we are discussing the possible reason why the deCastro paper reported sidewards displacements in the Discussion section.

Line 125. What is the evidence that mCherry does not bind to anti-GFP antibodies? There is clearly a lot of mCherry motor binding, and the fluorescent proteins share ancestors, and so it isn't out of the question.

⇒ While we consistently observe an increase in force generation in the presence of mCherry-KlpA when trapping beads are pre-bound to a single GFP-tagged motor (new Supp. Fig. 5A), suggesting that the affinity of mCherry-tagged motors for the anti-GFP antibodies may be lower compared to GFP-tagged motors, it's important to note that this observation does not definitively demonstrate that mCherry does not bind to anti-GFP antibodies. Additionally, the potential for oligomerization between GFP-tagged and mCherry-tagged motors further complicates the interpretation (new Fig. 1F). In response to the reviewer's comment, we have revised this line and removed the statement suggesting that mCherry does not bind to anti-GFP antibodies.

Line 126: I think "conclusively" is excessive here.

⇒ We have deleted the word "conclusively" and have rephrased the corresponding lines.

Reviewer #2 (Remarks to the Author):

In the revised manuscript, Liu and coworkers have addressed all of my concerns and I generally support publication of the manuscript after addressing a few points as outlined in the following.

Major point:

1. While a compliance correction, in principle, is reasonable, it should be self-consistent. Currently, it is not. The authors correct the measured lateral displacement based on the series of compliance of the optical spring and motor stalk/linker (while I favor that the correction is left out completely, the authors should have cited their reference 48 for the correction as it precedes ref. 54). The authors find that the spring constant of the motor increases linearly with force and depends on the trap stiffness (Fig. S14A, Fig. S15, Fig. S18A, and Fig. S20A). While the former is in principle possible (the increase appears to be very large, about 10-fold for a small force of 1 pN), the latter indicates that the correction must be wrong. The motor compliance is a material property of the motor itself and cannot depend on the settings of the measurement device (the trap stiffness). Thus, the correction does not capture the physics of the system under investigation.

⇒ We appreciate Reviewer 2's thorough examination of our work and their valuable feedback. Firstly, we acknowledge the oversight in not citing reference 48 by Svoboda et al. (Nature, 1993) in our method section. We have now rectified this by including the citation alongside reference 54 by Coppin et al. (PNAS, 1997). This ensures proper acknowledgment of the authors' contributions while providing sufficient details for the compliance correction. We have updated the manuscript accordingly to reflect this change.

Regarding the concern raised about the dependence of the stiffness-versus-force plots on trap stiffness, we initially attributed this to the increasing loading rate associated with higher trap stiffness. However, upon reevaluation prompted by the insightful comments from both Reviewer 1 and Reviewer 2, we conducted further analysis. Specifically, by directly comparing the position variance of the trapping bead before and during the power stroke, we found that the stiffness-versus-force plots indeed become independent of trap stiffness (see updated Fig. 3D). This observation aligns with the expected physical behavior and underscores the robustness of our findings.

A similar argument may apply to the power stroke itself. After correcting for the compliance/geometry, the power stroke should also be a constant in analogy to the step size of kinesin that is 8 nm after a compliance correction and independent of the trap stiffness used for the measurement (as shown in their ref. 48 and by many others). Thus, based in particular on the first argument, the correction cannot be correct and should be by all means omitted. Otherwise, I do not support the publication of the manuscript.

⇒ We thank the reviewer for this insightful comment. Unlike Kinesin-1, which takes processive steps along microtubules directly related to the spacing of tubulin subunits (where compliance correction indeed results in the correct tubulin spacing), Kinesin-14 motors undergo single power strokes independent of tubulin spacing. Instead, similar to an arm-wrestler, Kinesin-14 motors perform power strokes that depend on the length of the lever arm and the applied load.

With this in mind, we used the non-linear stiffness analysis (described above in response to Reviewer 1) to estimate a "load-free" power stroke for the motors. This approach asks what power stroke the motors could theoretically perform if the lever arm movement occurred in the absence of load (i.e., without bending and stretching of the lever arm). While the measured motor displacements reveal that load physically impairs the lever arm movements as expected (Fig. 3C, S13B, S16B and S18B), modeling the motor's stiffness using the variance in the proximity of the power stroke as a reference—suggested by the reviewer in Minor Point 5—indeed results in estimated load-free power strokes that are independent of the trap stiffness (Fig. 3E).

One likely reason why the simple compliance correction is flawed is that the geometry of the system is not properly accounted for. The authors argue that the coiled-coil of the stalk pivots around the motor heads by about 70 deg in analogy to ncd. For HSET and KlpA, the starting angle and range might be different and in particular not symmetric about the optical axis of the optical trap. Since the stalk rotates around the motor head, the authors probe a combination of the bending and stretching stiffness of the coiled coil (tangential and normal forces relative to the arc that the end of the coiled-coil traces in the absence of load). Since the measurements here are parallel to the microtubule axis, they are a vectorial combination of tangential and normal forces. Based on the persistence length of coiled-coils of a few 100 nanometers, their bending stiffness is at least 0.1 pN/nm for a 25-nm-long coiled coil comparable to the trap stiffness of the authors. For the 0.5 pN generated by the motor, the stalk should bend by at most 5 nm. For such small displacements, one would not expect much of a force dependence of the bending stiffness (certainly not a factor 10). The stretching stiffness of the coiled-coil is much larger as the bonds need to be stretched and most likely can be considered to be an inextensible rod. With increasing trap stiffness, the authors measured a reduced displacement. Thus, the angular position of the stalk is different for each trap stiffness. And, because the angle is different, the relative amounts of the tangential and normal force vectors are different, and concurrently the relative amounts of bending and stretching stiffness that are probed and that contribute to the overall fluctuations. Furthermore, for the same reason, the distance from the surface of the tethered bead might be different, i.e. depend on trap stiffness, constraining the lateral pivoting motion by a different amount. This constrained pivoting or tethered motion would be a simple explanation for the somewhat reduced variance (at most 20 percent smaller standard deviation based on Fig. S14). This explanation would lead to reduced variance without any change in motor compliance. It is simply the geometry. Furthermore, due to the fairly stiff motor stalk, rotational and translational motion of the bead are coupled. This coupling may also lead to a reduced variance. An analogous effect was reported for magnetic tweezers when the DNA is attached off-axis (see Klaue & Seidel, Phys. Rev. Lett. 102, 028302 (2009)). While in the case of the magnetic tweezers, the torsional stiffness is due to the bead, here, it is due to the

torsional stiffness of the motor stalk. The math and physics of the system are equivalent and changes in the displacement standard deviation of 20 percent seem plausible (much smaller than the changes reported for the magnetic tweezers). The authors would have to calculate & solve the coupled differential equations for translational and rotational motion to properly account for any change in variance and potentially correct the displacements of their power strokes. Since this correction is probably beyond the scope of the present manuscript, the authors should not make any corrections and only discuss potential corrections. As it stands, the analysis and correction are not self-consistent and cannot be true.

The dependence of power stroke displacement on the various motors still indicates that the motors do a power stroke by a rotating lever arm. How the length of the tail domains translates into trap-measured displacements depends on the detailed geometry that is unknown (beginning with the starting and end angle of the power stroke). A proper analysis would have to include motions and forces in the z-axis (it is unclear what the forces and displacements in the z-axis are - based on Fig. S4, fluctuations in z are non-symmetric indicating that the authors push down on the glass surface with the typical large offset that needs to be subtracted in the z-axis not properly accounted for - for all other measurements, it is unclear what the axial motions are).

⇒ We appreciate the thorough analysis provided by Reviewer 2 and have taken their comments into careful consideration in our further revision. In response to the concerns raised regarding the compliance correction and the factors influencing the measurable power stroke, we have made significant efforts to address all issues, whether directly or indirectly.

The combined compliances exhibited by the lever arm, motor domain, and bead linkage, manifested through both bending of the coiled-coil and potentially some stretching of the motor domain, dimerizing coiled-coil, and bead linkage, can be conceptualized as a "black box." Within this framework, the lever arm, along with the motor and bead-linkage, acts as a cohesive unit, displacing the bead by a measurable distance. The mechanical property of the black box can be well characterized by a non-linear force-extension profile of the motor and bead-linkage as described in the response to the comments of Reviewer 1 above (see also Fig. 3D).

While we acknowledge that factors such as the rotation of the trapping bead and changes in the distance between the bead and the microtubule surface may influence the measurable power stroke, our analysis suggests that these effects only lead to an underestimation of the actual power stroke (see Supplementary Figure 20). Consequently, we explicitly state in our manuscript that our measurements represent the lower bounds of the actual power strokes.

Moreover, we find substantial agreement between our measured and corrected power-stroke sizes and the length of the coiled-coils predicted by AlphaFold (see Supplementary Figure 14). Additionally, our coiled-coil truncation studies validate both the predicted and measured lever-arm length. Thus, while our measurements provide only the lower bounds of the true lever-arm length, we are confident in their overall accuracy.

Finally, it is important to note that any additional so far unaccounted physical processes that could potentially reduce the variance of the position fluctuations during an experiment and therefore affect our compliance correction, must be rather insignificant, as the correction of the variance-based calculation of the trap stiffness for the parasitic filtering of the PSD already largely accounts for the discrepancy between the active calibration (which takes into account the parasitic filtering effect) and the Equipartition theorem-based calculation using the position variance (see Figure 1 above).

We believe these clarifications enhance the understanding of our methodology and strengthen the robustness and accuracy of our findings. We thank Reviewer 2 for their valuable feedback, which has significantly contributed to the improvement of our manuscript.

Minor points:

2. Fig. S3B: The zero-force position seems wrong. In the beginning, the baseline appears to be at around 0.6 pN, which would imply that the bead (and motors) have reversed direction in the end (at about 55 s).

⇒ We appreciate the reviewer's observation regarding Supplementary Figure 3B. It's crucial to recognize that the bead is coated with a high concentration of motors, leading to a diverse range of behaviors due to the asynchronous binding of motors to the microtubule. In the depicted example, these behaviors may include premature detachment at forces ranging from 0.5 to 3 pN and stalling at approximately 3 pN. Furthermore, compromised or inactive motors

may act as passive linkages between the bead and the microtubule, limiting the force generation capacity of active motors. This phenomenon is evident during the time period from 3 to 13 seconds, where the bead briefly reaches approximately 1 pN before quickly returning to zero force. This behavior results in the appearance of a baseline of around 0.6 pN during the time period from 3 to 13 seconds. However, the baseline, which is only reached once all motors disengage from the microtubule, is zero. While instances of the baseline reaching zero can be observed intermittently during the first 14 seconds, it becomes notably prominent at the end of the entire trace. \
3. Fig. S5: Why do all of the diagonal motor events gradually increase in force while for the x- and y-direction, they are more or less instantaneous power strokes?

⇒ The reviewer raises an interesting point regarding Supplementary Figure S5 (now Fig. 1E and Supp. Fig. 7A&B). It appears that the forces gradually increase in the diagonal motor events; however, this is solely due to the overlapped traces. To demonstrate this, we have separated the traces in Figure 3 below.

4. Line 125: please rephrase the statement that mCherry does not bind to anti-GFP antibodies. Fig. S7A does demonstrate significant force production when mCherry-KlpA is incubated with GFP antibody beads. sThus, motors are bound to beads either non-specifically or via the antibodies. What it is exactly is unclear.

⇒ We appreciate the reviewer's comment regarding the binding of mCherry-tagged motors to the beads coated with anti-GFP antibodies. As suggested, we have revised the corresponding sentence to accurately reflect the observation that motors may bind to the beads either non-specifically or through oligomerization of the mCherry-tagged motors with the pre-bound GFP-tagged motor (see lines 124-130 of the revised manuscript).

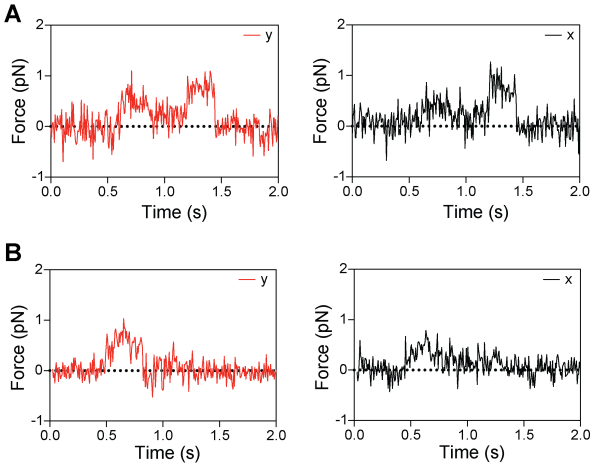

Figure 3. Example unidirectional power strokes on a diagonally oriented microtubule.

5. For the compliance correction, the authors used the variance in position during the post-power stroke relative to the calculated variance of the free bead based on the calibrated trap stiffness and the equipartition theorem. The authors should have used the reference variance in the proximity of the power stroke (a certain time window before and after) instead. In this manner, they can properly account for any local changes in trap stiffness or drift. Yet, since the authors should omit the correction completely (changes in variance likely have a different origin than motor compliance), this point is not relevant.

⇒ We highly appreciate the suggestion from the reviewer. Following their advice, we conducted the suggested analysis using the reference variance in the proximity of the power stroke. As a result, we observed that the stiffness-versus-force data now overlap for all trap stiffnesses as expected (see new Figure 3D). With this correction and the modeling of our stiffness data with a non-linear WLC model, our data are now self-consistent. See also our responses to Reviewer 1.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have addressed my comments.

Reviewer #2:
Remarks to the Author:
In the second revision, the authors have sufficiently addressed my major concern. Still, the WLC model does not seem to be a good model for a protein (or its stalk) that is expected to be a coiled-coil, i.e. a stiff rod, coupled via GFP to an antibody - two more stiff elements with potential (elastic) hinges in between (maybe more reminiscent of a jointed chain consisting of a few elements). The arbitrarily introduced offset and large contour length fit value also indicate that the model does not describe the physics of the system well. Yet, as a phenomenological approach, the model appears to represent the data well so that a compliance correction can be made in a self-consistent manner. The correction for the antibody and GFP also seems rather arbitrary without any knowledge of how these molecules are bound to the surface of the microspheres and relative to each other. The authors should address/point out some more of the shortcomings of their model and assumption in the main discussion. Also, the authors should provide information/details/references (in the methods section) on the active calibration that was not mentioned previously. Please, also correct the typo in line 682. After the authors have addressed these minor points, I support the manuscript's publication.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed my comments.

Response: We thank Reviewer #1 for the positive assessment of our revised manuscript.

Reviewer #2 (Remarks to the Author):

In the second revision, the authors have sufficiently addressed my major concern. Still, the WLC model does not seem to be a good model for a protein (or its stalk) that is expected to be a coiled-coil, i.e. a stiff rod, coupled via GFP to an antibody - two more stiff elements with potential (elastic) hinges in between (maybe more reminiscent of a jointed chain consisting of a few elements). The arbitrarily introduced offset and large contour length fit value also indicate that the model does not describe the physics of the system well.

Response: Contrary to Reviewer 2's statement, the contour lengths of the coiled-coil lever arms of the Kinesin-14 motors we obtained from the fitted WLC model are very close to the contour lengths measured for coiled-coils of the same length using atomic force microscopy measurements. We have included this information in the Methods section where we discuss contour lengths (lines 697-704). While we don't disagree that other non-linear force extension models could more accurately describe our data, based on the agreement of the coiled-coil predictions from AlphaFold and our lever-arm truncation and optical tweezers studies, we don't think that other models will result in significantly improved estimates for the lever-arm lengths of Kinesin-14 motors. However, to address this point, we have added two sentences about the choice of models to the discussion (lines 370-375).

Yet, as a phenomenological approach, the model appears to represent the data well so that a compliance correction can be made in a self-consistent manner.

Response: We agree that our data are self-consistent.

The correction for the antibody and GFP also seems rather arbitrary without any knowledge of how these molecules are bound to the surface of the microspheres and relative to each other. The authors should address/point out some more of the shortcomings of their model and assumption in the main discussion.

Response: We have added two sentences to the Discussion to address this point. Please see lines 370-375 of the updated manuscript. Additionally, we previously included a discussion about the assumption of the length of the GFP -antibody complex in the main text (lines 207-212). These discussions address the comment of Reviewer 2.

Also, the authors should provide information/details/references (in the methods section) on the active calibration that was not mentioned previously.

Response: We have added the requested reference to the methods section (line 649).

Please, also correct the typo in line 682. After the authors have addressed these minor points, I support the manuscript's publication.

Response: We have corrected the typo.