Kinesin-2 motors adapted their stepping behavior for processive transport on axonemes and microtubules

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 31 March 2017

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all three referees highlight the potential interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn. As the reports are below, I will not detail them here, but we think it will be critical to analyze the motility of other kinesin-2 motors (especially the Kif3A/3B motor from Xenopus) on the axonemes, and to examine the motility on suspended microtubules and axonemes where the glass surface does not hinder helical motility (reviewer 1 point 6, reviewer 2 point 5, and reviewer 3 points 4 and 5). Also important will be a more scholarly assessment of the literature, critical consideration of alternative explanations, a more detailed and comprehensive presentation of the results and appropriate statistical analyses of the data.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.
Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify, where applicable, the number ”n” for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? Please provide statistical testing where applicable.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS
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Referee #1:
The manuscript by Stepp et al examines the motility of two members of the kinesin-2 family, C elegans KLP11/KLP20 and Xenopus KLP3A/KLP3B, on two different types of microtubule substrates, single microtubules and axonemes. One subunit of each motor is tagged on the motor domain with a Halo or SNAP tag, observed by TIRF microscopy, and the stepping behavior determined. The authors show that both CeKLP11/KLP20 and XlKLP3A/KLP3B are processive motors—this is not surprising and has been shown for other kinesin-2 motors. Using FIONA, the authors determine that both motors walk along single microtubules with a step size of 13.4-13.5 nm whereas CeKLP11/KLP20 walks along axonemes with a step size of 16 nm. This finding is novel and interesting and contributes to the field. The authors interpret these results to mean that both motors side step when walking along microtubules but CeKLP11/KLP20 walks in a linear manner along axonemal microtubules. The experiments and data are nice but further work is needed to support the strong interpretations. I also have some conceptual issues with the background and framework of the questions.

Conceptual issues:
1. The authors conclude that the kinesin-2 motors side step to adjacent protofilaments when walking towards the plus ends of microtubules (based on the step size in Fig 3 and the motility behavior in Fig 4). The authors suggest that the ability of kinesin-2 motors to side step is an evolutionary advantage for IFT. There are several problems with this argument.

First, there is a priori no reason that motors that walk in a linear fashion along a microtubule would
not make excellent IFT motors. That is, kinesin-1 motors could make excellent IFT motors since they walk in a linear fashion on a microtubule. Indeed, the idea that side stepping of kinesin-2 limits its motility to one side of the microtubule ignores other (perhaps more plausible) mechanisms for restricting kinesin-2 transport to the B-tubule. First, cryo-electron tomography of Tetrahymena cilia has shown that only the B-tubule is available to IFT trains starting their journey at the base of the cilium (Ounjai et al 2013 Curr Biol). A second possibility is that posttranslational modifications of tubulin that are restricted to the A versus B tubule regulate transport by dynein-2 and kinesin-2 (Multigner et al 1996 Biochem, Kuno et al 2010 Curr Biol, Suryavanshi et al 2010 Curr Biol).

Second, IFT in mammalian cells is driven by the KIF3A/KIF3B heterodimeric motor which the authors have shown does not spiral (Brunnbauer et al 2012 Mol Cell) but moves in a linear fashion along the microtubule surface. Clearly, the ability to spiral or sidestep is not universally adapted by IFT motors to restrict their motility to "one side of the doublet microtubule".

2. In the Summary, the authors state that "transport on axonemes is exclusively reserved for the members of the kinesin-2 family" (lines 4-5). This is not an accurate description of the field. A number of kinesins from different families have been shown to localize to cilia and may transport along axonemes, in particular, the kinesin-8 motor KIF19 (Niwa et al 2012 Dev Cell, Wang et al 2016 eLife) and the kinesin-3 KLP-6 (Peden & Barr 2005 Curr Biol, Morsci & Barr 2011 Curr Biol).

3. The delineation of kinesin-2 motors as either ciliary (CeKLP11/KLP20) or cytoplasmic (XIKLP3A/KLP3B) (p. 5, lines 7-9) seems narrow-minded and based on a lack of data, rather than an actual evidence. Most kinesins contribute to multiple transport events in cells and the kinesin-2s probably carry out both ciliary and cytoplasmic transport events in all organisms. Most work on the CeKLP11/KLP20 motors has focused on a role in IFT but this motor likely has additional roles in cytoplasmic transport that have not been studied. And most work on the XIKLP3A/KLP3B motor has focused on cytoplasmic transport processes but this motor almost certainly has a role in IFT in this animal. Importantly, the function of the mammalian kinesin-2 motors has been studied in both ciliary and cytoplasmic transport events.

Experimental issues:

4. The authors state that the measured processivity of CeKLP11/KLP20 and XIKLP3A/KLP3B was "surprising" but recent (uncited) work has shown that mouse KIF3A/KIF3B and KIF3A/KIF3C are "highly processive" (Guzik-Lendrum et al 2015 Biophys J). Thus Fig 1 adds to the data in the field that kinesin-2 motors are processive motors capable of driving long-distance transport.

5. The authors state that the difference in processivity between the previous work (Brunnbauer et al) where the kinesin-2 motors were attached to beads and the present work where the kinesin-2 motors are untethered is due to the tail region of KLP3B (Fig 2 and p. 10, lines 1-4). But there are alternative explanations for the results in Fig 2. It is possible that the reduced processivity of the bead-associated motors is due to the non-specific binding of the motors to the bead. It is also possible that deletion of the KLP3B tail region results in decreased run lengths because the KLP3A tail domain is better able to engage the two motor heads in an autoinhibited state, as seen for kinesin-1 (Hackney et al 2009 Biochem). What happens if both tail regions are deleted?

6. The most interesting data in the manuscript is Fig 3. Here, the authors use FIONA analysis of single-tagged kinesin-2 motors to examine the step size along single microtubules and axonemes. The data in Fig S5 is critical for this and needs to be shown in the main text with magnified views of the steps and individual step sizes indicated. Kymographs of the motility also need to be shown. Furthermore, the motility of XIKLP3A/KLP3B along axonemes needs to be examined in an identical fashion. The ultimate test of the model would be to examine the motility of both CeKLP11/KLP20 and XIKLP3/KLP3B along suspended doublet microtubules to be certain that the motors are incapable of spiraling along doublet microtubules like they do along single microtubules. My suggestion would be to build on this figure to provide a complete picture of kinesin-2 motility along single microtubules and axonemes.

7. the model also needs to be tested by looking at the motility of teams of kinesin-2 motors on single microtubules versus axonemes.
8. The data in Fig 4 are impossible to assess or interpret without images of the microtubules/axonemes and the motors and kymographs of the motility data.

9. Fig 5 is speculative and ignores the finding that kinesin-2 motors are restricted to the B-tubule at least in Tetrahymena cilia (Ounjai et al 2013 Curr Biol). This figure can be deleted and experimental work that bolsters the authors claims should be used to replace this figure

Referee #2:

The manuscript by Stepp et al., examines the processivity of two different kinesin-2 family members, KLP11/20 a c. elegans ciliary motor and KLP3A/B a xenopus cytoplasmic motor. There are two major findings:

1.) Differences in the C-terminal tails of the two heavy chains in the heterodimeric KLP3A/B motor differentially affect processivity.

2.) KLP11/20, like KLP3A/B, takes 13 nm side-steps on individual microtubules, but straight 16 nm steps on axonemes, its natural substrate.

While these are both novel and important findings, both sets of experiments seem incomplete. Indeed, there are two completely different studies going on here, the effects of C-terminal tail variations on KLP3A/B processivity, and the effects of microtubule architecture on KLP 11/20 motility. Yet only the latter hypothesis is covered in the introduction, no rationale is provided for also looking at C-terminal tail contributions until the Results/Discussion section. But then it isn't clear why they are only looking at KLB3A/B and not KLP11/20. Are the KLP11/20 C-terminal tails identical so no differences would be expected, or could the authors also look at KLP11/20 and show that C-terminal tail variations are a general property that arise out of heterodimeric kinesin-2 motors?

Conversely, why do they only look at KLP11/20 motility on axonemes? On one hand, it is understandable that axonemes are the natural substrate for KLP11/20 and not KLP3A/B, but is the ability of KLP11/20 to adjust its stepping mechanism on axonemes a unique specialization of that motor or a general property of all kinesin-2 motors? That important question could be answered if the authors also looked KLP3A/B motility on axonemes.

There are also issues with how the processive run length data is analyzed. Given the data is exponentially distributed, it is not clear what is being presented in terms of standard deviation or standard error, and how these were calculated. It is also not clear which statistical tests were used and if they are appropriate for comparing exponentially distributed data. Bootstrapping algorithms have been useful in similar studies (https://www.ncbi.nlm.nih.gov/pubmed/24940781; https://www.ncbi.nlm.nih.gov/pubmed/23790373).

Furthermore, the authors, with no statistical test to validate the claim, claim that truncation of the B-subunit tail reduces the processivity relative to the A-subunit, without comparing the results to the wild-type construct used in Figure 1. It seems that with both C-terminal tails intact, an intermediate run length value is obtained - is this true? If so, what does it mean? And do these changes in run length correspond to changes in true processive run lengths, or do the C-terminal tails introduce some sort of electrostatic tethering or diffusive behavior between processive runs? Kymographs and/or mean-squared displacement of the motile behavior in these experiments would be useful.

There are a couple of recent papers regarding protofilament switching behavior of kinesin-1 (https://www.ncbi.nlm.nih.gov/pubmed/25954882) and kinesin-2 (https://www.ncbi.nlm.nih.gov/pubmed/28267259) that should be included in the discussion.

Finally, the explanation of how axonemes promote 16 nm steps of KLP11/20 isn't clear, at least to someone who is not familiar with the structure of the flagella and the process of IFT. As presented, the proposed mechanism seems highly speculative. Are there other possible explanations? Could this model actually be tested?
Referee #3:

Stepp et al have used FIONA on two types of Kinesin-2, the ciliary KLP11/20 from C. elegans and the cytoplasmic KLP3A/B from X. laevis, each fluorescently labelled on one head. They find a difference between the step sizes on microtubules versus axonemes, and interpret this to mean that on axonemes, the left-biased off-axis component of stepping for kinesin-2 is suppressed. They speculate that this suppression of off-axis stepping is due to physical blockade of the sidestepping by an impenetrable line of crosslinks between the A and B tubes of the axonemal peripheral doublets. Whilst this is in principle interesting unfortunately I think neither the finding of a different step size nor the interpretation are sufficiently well supported.

1. Abstract sentence 2 is inaccurate -should read "reserved for kinesins from the kinesin-2 family"

2. Abstract "ability to self-organise on the MT lattice" the arguments necessary to understand this phrase have not yet been made. I suggest: "the inherent ability of the kinesin-2 to side-track on the microtubule lattice restricts the motor to one side of the microtubule doublet in axonemes."

3. P5 L8 "At the example of" please clarify; I think this should read "By studying"

4. Data. Microtubules and axonemes are down on a surface, so how can the motors move in a helical path around the microtubule periphery? In principle these trajectory data (Figure 4) actually gainsay the central idea in the paper, that a physical barrier (in this case the glass surface) can block sidestepping.

5. Data. It looks to me that the assignment of steps and step sizes is being very much forced on these data by the fitting algorithm. Look for example at the bottom left panel in figure 4. In this situation small differences in the average step size will appear if one dataset is slightly more noisy than another, especially if the rules for what constitutes a step are adjusted by varying the window size and t-threshold taken to indicate a step. I think robust conclusions cannot be drawn without very much more careful controls - for example by fitting kinesin-1 data on the two different substrates and showing that the the same stepsize is obtained with different levels of noise. For the step fitting, we need to know the threshold and window size employed, if these were varied, and if so, on what basis.

6. Interpretation. The authors argue that off axis stepping to the left would confine kinesin-2 to one side of the B-tubules. Is there evidence that addresses whether motors can transfer from the A-tubule to the B tubule and vice versa? If the impenetrable barrier in the model is speculative then please say so. If I'm missing something then the authors need to help me and other readers understand.

7. Given the measured average stepsize, it should be possible to predict the pitch of the helical path?

1st Revision - authors' response 27 June 2017

Point-to-point response to the referee comments

Referee #1:

The manuscript by Stepp et al examines the motility of two members of the kinesin-2 family, C elegans KLP11/KLP20 and Xenopus KLP3A/KLP3B, on two different types of microtubule substrates, single microtubules and axonemes. One subunit of each motor is tagged on the motor domain with a Halo or SNAP tag, observed by TIRF microscopy, and the stepping behavior determined. The authors show that both CeKLP11/KLP20 and XlKLP3A/KLP3B are processive motors - this is not surprising and has been shown for other kinesin-2 motors. Using FIONA, the authors determine that both motors walk along single microtubules with a step size of 13.4-13.5 nm whereas CeKLP11/KLP20 walks along axonemes with a step size of 16 nm. This finding is novel and interesting and contributes to the field. The authors interpret these results to mean that both motors side step when walking along single microtubules but CeKLP11/KLP20 walks in a linear manner along axonemal microtubules. The experiments and data are nice but further work is needed to support the strong interpretations. I also have some conceptual issues with the background and framework of the questions.
Conceptual issues:

1. The authors conclude that the kinesin-2 motors side step to adjacent protofilaments when walking towards the plus ends of microtubules (based on the step size in Fig 3 and the motility behavior in Fig 4). The authors suggest that the ability of kinesin-2 motors to side step is an evolutionary advantage for IFT. There are several problems with this argument.

First, there is a priori no reason that motors that walk in a linear fashion along a microtubule would not make excellent IFT motors. That is, kinesin-1 motors could make excellent IFT motors since they walk in a linear fashion on a microtubule. Indeed, the idea that side stepping of kinesin-2 limits its motility to one side of the microtubule ignores other (perhaps more plausible) mechanisms for restricting kinesin-2 transport to the B-tubule. First, cryo-electron tomography of Tetrahymena cilia has shown that only the B-tubule is available to IFT trains starting their journey at the base of the cilium (Ounjai et al 2013 Curr Biol). A second possibility is that posttranslational modifications of tubulin that are restricted to the A versus B tubule regulate transport by dynein-2 and kinesin-2 (Mültigner et al 1996 Biochem, Kuno et al 2010 Curr Biol, Suryavanshi et al 2010 Curr Biol). We thank our referee for the detailed comments. By no means did we intend to disregard other models. Besides, the models don’t need to be mutually exclusive, and we now discuss these points in the revised manuscript.

Nevertheless, the level of experimental evidence in support of some of the mentioned models may not be satisfying in each case. Structural analysis of the CPC from Tetrahymena pyriformis by Ounjai et al. led the authors to suggest that “the outer channel may serve as a tunnel for the IFT trains” because “the larger section of the CPC pore is adjacent to the B-tubule of the doublet” that appears big enough to accommodate IFT trains. However, we note that there is actually no experimental evidence for this model because no structural data pinpointed where exactly the IFT trains start their journey. Moreover, it is difficult to envision how retrograde trains would pass the membrane since it is claimed to be blocked. So it remains unclear how the IFT would deal with the two-way traffic on the same microtubule doublet as seen recently in C. reinhardtii by Stepanek et al., Science 2016. To our knowledge, IFT in Tetrahymena pyriformis has not yet been visualized and it is not known whether this organism has specialized to enable CPC passage only on the B-tubule as proposed by Ounjai et al.

Work by Suryavanshi et al indeed showed that the post-translational modification ( poly-glutamylation) occurs preferentially on the B-tubule in Tetrahymena thermophila. The authors concluded that the poly-glutamylation is involved in the process of force generation via inner dynein arms. Similarly, Kuno et al also linked poly-glutamylation to the motility of inner dynein arms in C. reinhardtii. We note however, that these findings do not directly relate to the antero- and retrograde IFT because dynein inner arms are not involved in IFT. (Dynein-2 powers retrograde IFT on the A tubule (Stepanek et al., 2016) whereas dynein inner and outer arms are involved in ciliary beating.). While it is true that early structural evidence points to the differentially posttranslationally modified A and B-tubules (e.g. Multigner et al 1996, Kann et al., 1998, Huitorel et al., 2002), and the motility of some kinesin motors can be modulated by post-translational modifications (e.g. Suryavanshi et al., 2010, Kuno et al., 2010., Sirajuddin et al., 2014, Barisic et al., 2015; Nirschl et al., 2016), so far no evidence has been collected that demonstrates that post-translational modifications on A and B-tubules control the dynein- and kinesin-2-dependent two-way traffic as recently observed by Stepanek et al. Indeed Stepanek et al., stated in their publication “Nevertheless, the effect of such modifications on the control of IFT remains unclear in vivo”

The simple model proposed in our work specifically addresses the mechanism of the two-way traffic on the same doublet microtubule as seen by Stepanek et al. Unfortunately, too little is known about the specifics of the IFT in different organisms. How general is the two-way traffic on the same double microtubule as observed in C. reinhardtii (Stepanek et al., 2016)? Are the A- and B-tubules used differently in different doublet microtubules as proposed for T. pyriformis (Quajin et al., 2013)? Is IFT restricted to only a subset of doublet microtubules as seen in T. brucei (Absalon et al., 2008)?

To better reflect the points raised above, we updated our manuscript accordingly by now discussing these options (see p. 4, l. 16-22 and p. 5, l. 4-11).
Second, IFT in mammalian cells is driven by the KIF3A/KIF3B heterodimeric motor which the authors have shown does not spiral (Brunnbauer et al 2012 Mol Cell) but moves in a linear fashion along the microtubule surface. Clearly, the ability to spiral or sidestep is not universally adapted by IFT motors to restrict their motility to "one side of the doublet microtubule".

This is true, however we note that the respective pitches observed in multiple motor assays as done previously (Brunnbauer et al, Mol Cell 2012) and at the single molecule level (our current manuscript) differ by an order of magnitude (see Figure EV 3 and new Figure 4), so it cannot be ruled out that single kinesin-2 from mouse displays a handedness as well. Consistent with this possibility, previous work on truncated, single kinesin-2 motors from mouse displayed side-stepping on microtubules (Hoeprich et al, Traffic 2017). Indeed, the intriguing and non-trivial question here is how the ensemble of motors influence each other's side-stepping behavior on microtubules. We made the following changes to the manuscript to discuss this point (see comment by referee #2 on page 9 and manuscript p10, l. 11-12 and l. 18-20).

2. In the Summary, the authors state that "transport on axonemes is exclusively reserved for the members of the kinesin-2 family" (lines 4-5). This is not an accurate description of the field. A number of kinesins from different families have been shown to localize to cilia and may transport along axonemes, in particular, the kinesin-8 motor KIF19 (Niwa et al 2012 Dev Cell, Wang et al 2016 eLife) and the kinesin-3 KLP-6 (Peden & Barr 2005 Curr Biol, Morsci & Barr 2011 Curr Biol).

We apologize for this confusion. We did not mean to suggest that the kinesin-2 family of motors are the only kinesins of the cilium. Instead, what we intended to say was that the kinesin-2 motors are the main work-horses of the plus-end directed IFT that builds and maintains the cilium. This is exemplified by the fact that kinesin-2 loss-of-function mutants fail to generate cilia, but not the loss-of-function mutants of KIF19 and KLP-6 motors (see below).

Work by Niwa et al and Wang et al showed that KIF19 is localized to the ciliary tip and is proposed to function as plus-end depolymerizing motor as its loss-of-function leads to elongated cilia.

However, the plus-end directed motility of KIF19 appears to be too slow (~20nm/s) to reliably function as an IFT motor (Niwa et al 2012 Dev Cell). Indeed, the loss of KIF19 function does not eliminate the cilium (unlike elimination of the kinesin-2 motor) but leads to abnormal ciliary length. Work by Morsci et al., identified the KLP-6 to be expressed in a small sub-set of male sensory cilia of C. elegans and proposed a regulatory role of KLP-6 on the kinesin-2 motors. Loss of KLP-6 function also did not eliminate the cilium but impaired sensory function (Peden et al., 2005). We have updated our manuscript to clarify this issue. (See new Summary page 2) Please also see coment by referee #3 point 2 on page 10.

3. The delineation of kinesin-2 motors as either ciliary (CeKLP11/KLP20) or cytoplasmic (XIKLP3A/KLP3B) (p. 5, lines 7-9) seems narrow-minded and based on a lack of data, rather than an actual evidence. Most kinesins contribute to multiple transport events in cells and the kinesin-2s probably carry out both ciliary and cytoplasmic transport events in all organisms. Most work on the CeKLP11/KLP20 motors has focused on a role in IFT but this motor likely has additional roles in cytoplasmic transport than have not been studied. And most work on the XIKLP3A/KLP3B motor has focused on cytoplasmic transport processes but this motor almost certainly has a role in IFT in this animal. Importantly, the function of the mammalian kinesin-2 motors has been studied in both ciliary and cytoplasmic transport events.

Again, we apologize for this confusion. We did not mean to suggest that CeKLP11/KLP20 and XIKLP3A/KLP3B motors are working exclusively on axonemes and microtubules, respectively. We have modified our manuscript to clarify this point. (see p. 6, l. 7-10 and p. 7, l. 4-7)

Experimental issues:

4. The authors state that the measured processivity of CeKLP11/KLP20 and XIKLP3A/KLP3B was "surprising" but recent (uncited) work has shown that mouse KIF3A/KIF3B and KIF3A/KIF3C are "highly processive" (Guzik-Lendrum et al 2015 Biophys J). Thus Fig 1 adds to the data in the field that kinesin-2 motors are processive motors capable of driving long-distance transport.

Our referee is right. We note, however, that work by Guzik-Lendrum et al used artificially dimerized motor head domains, whereas we used full length enzymes. Importantly, their construct lacked the C- terminal domains, which, as we show, further increases the run length of the motor (new Figure 2). Our results thus predict that the run length of the full length mouse kinesin-2 will
further increase the motor’s processivity. We have modified our manuscript to also include this comparison. (see p.4, l. 6-11 and p. 9, l. 16-19) Please also see comment by referee #2 on page 8.

5. The authors state that the difference in processivity between the previous work (Brunnbauer et al) where the kinesin-2 motors were attached to beads and the present work where the kinesin-2 motors are untethered is due to the tail region of KLP3B (Fig 2 and p. 10, lines 1-4). But there are alternative explanations for the results in Fig 2. It is possible that the reduced processivity of the bead-associated motors is due to the non-specific binding of the motors to the bead. It is also possible that deletion of the KLP3B tail region results in decreased run lengths because the KLP3A tail domain is better able to engage the two motor heads in an autoinhibited state, as seen for kinesin-1 (Hackney et al 2009 Biochem). What happens if both tail regions are deleted? The point raised by our referee is correct as we cannot distinguish between disengagement of the C-terminal tail region and unspecific attachment of the full-length kinesin-2 to a bead. Regardless, the binding of the kinesin-2 to the bead appears to substantially curtail the processivity of the motor. Indeed, the experiment suggested by our referee to also analyze the behavior of the motor with both tail region removed (see new Fig. 2), further corroborated our conclusion that only the KLP3B distal tail is involved in influencing the processivity of the KLP3A/B motor. If the KLP3A were able to better disengage the two motor heads in the absence of the KLP3B tail, the removal of KLP3A tail in addition to KLP3B tail should have rescued the processivity of the heterodimeric KLP3A/B motor, which we did not observe (see new Fig. 2). We have included this important point into the manuscript and thank our referee for suggesting the experiment that leads to the clearer conclusion of the KLP3B tail’s impact on the processivity of kinesin-2. (see p. 9, l. 11-16)

6. The most interesting data in the manuscript is Fig 3. Here, the authors use FIONA analysis of single-tagged kinesin-2 motors to examine the step size along single microtubules and axonemes. The data in Fig S5 is critical for this and needs to be shown in the main text with magnified views of the steps and individual step sizes indicated. Kymographs of the motility also need to be shown. Furthermore, the motility of XIKLP3A/KLP3B along axonemes needs to be examined in an identical fashion. The ultimate test of the model would be to examine the motility of both CeKLP11/KLP20 and XIKLP3/KLP3B along suspended doublet microtubules to be certain that the motors are incapable of spiraling along doublet microtubules like they do along single microtubules. My suggestion would be to build on this figure to provide a complete picture of kinesin-2 motility along single microtubules and axonemes.

As suggested by our referee, we have analyzed the stepping behavior of the XIKLP3A/KLP3B motor on axonemes (See new Figure 3 and comment by referee #2 on page 8). We also added magnified views of the respective steps (Fig. 3, bottom panel). We propose to include the old Fig. S 5 into the Expanded View format as Figure EV 1. The respective kymographs are included in the Fig. S 6. (see p. 11, l. 20 to p. 12, l. 3)

Our referee suggested to test the behavior of our motors on suspended doublet microtubules. To this end, we have digested the sea urchin axonemes and isolated individual doublets. Due to the intrinsic curvature, doublet microtubules adopt a ‘piggy tail’ conformation when in solution (Kamiya et al, Biophys J 2005). In contrast to singlet microtubules, the curvature of the doublets has prevented our attempts to suspend them and to track our motors on the suspended doublets.

In addition, and as requested by our Editor, we also tried to accomplish a tracking on suspended singlet microtubules. Indeed, we succeeded in suspending a singlet microtubule. First, we checked the stability of this setup by measuring the frame to frame movement of the part of the bridge suspended between gold platforms. We recorded the bridge movement at the same cycle time as we collect kinesin traces. The mobility of the suspended bridge increases a lot compared to a microtubule bound to the surface via a biotin/streptavidin system. To see if the additional noise derived from the bridge would thwart efforts to track single kinesins by FIONA, we simulated data by adding noise from the movement data of the bridge to the y-displacement of one of the paths of a single kinesin walking on kinesins stuck to the cover slip via biotin attachment. The simulations show that a precise tracking of motor trajectories is expected to be greatly hampered due to the additional noise. We conclude that despite our best efforts to suspend single and double microtubules within the TIRF field the requested experiments are technically not feasible at this point.

(Figures for Referees not shown.)
Because building microtubule bridges failed due to the intrinsic noise of the setup, we aimed to perform an alternative experiment that did not require the non-trivial task of building bridges within the TIRF field. Our model proposes that the straight path observed on axonemes (See new Fig. 4A) is imposed by the presence of structural demarcations (particularly the inter-doublet connector nexin) between the doublet microtubules (see model Figure 5). Thus, removal of these demarcations (Brokaw CJ, Science 1980) is predicted to enable the motor to spiral again.

In preliminary results, this was exactly what we have observed on isolated doublet microtubules lacking the demarcations. We could not repeat the experiment much to our regret due to lack of sea urchin sperm, which we obtain from our collaborators when they go on expeditions to collect the animals. Nevertheless, the results are highly promising and fully support our conclusions.

(Figures for Referees not shown.)

7. the model also needs to be tested by looking at the motility of teams of kinesin-2 motors on single microtubules versus axonemes.

In our previous work (Brunnbauer et al., Mol Cell 2012), we have already analyzed the behavior of teams of kinesin-2 motors on suspended single microtubules. Indeed, these teams also spiral around the MT, fully consistent with all our conclusions. The significance of this work for the model in our current manuscript (see Figure 5) is (a) teams of kinesin-2 motors (KLP11/20 and KLP3A/B) also take side-steps and (b) side-stepping results in an exclusively left-handed spiraling of motor-covered beads around the suspended microtubules. Here, the number of motors engaging on the microtubule surface are unknown (a rough estimate is given in Figure R 1).

Figure R 1: Estimate of engaged motors in the bead on microtubule geometry.

It might be expected that the axoneme structure also inhibits spiralling if multiple motors are present on the cargo. For the large beads used in optical tweezer assays, however, the distance of the bead to neighbouring doublets is not significantly different between two doublet microtubules (see Figure R 2). Under these circumstances the inter-doublet connections may no longer serve as a barrier as it does for single motors and, presumably, smaller cargoes. Because such spiraling would not report any meaningful parameters (given that it is impossible to define the number and geometry of motors attached to the bead), we have not pushed these experiments with multiple motors on suspended axonemes in Brunnbauer et al., Mol Cell 2012.
8. The data in Fig 4 are impossible to assess or interpret without images of the microtubules/axonemes and the motors and kymographs of the motility data. We have addressed the concerns of our referee in the new Figure 4, Figure EV 2 and Figure S 8. (see p. 12, l. 7-12)

9. Fig 5 is speculative and ignores the finding that kinesin-2 motors are restricted to the B-tubule at least in Tetrahymena cilia (Ounjai et al 2013 Curr Biol). This figure can be deleted and experimental work that bolsters the authors claims should be used to replace this figure. As requested, we included additional experimental work that bolsters our claims into the manuscript (Figures 2, 3 and 4, also Figures EV 2 and additional supplementary figures). We do feel however, that Figure 5 helps the reader to understand our proposed model more easily. Given that our referees #2 and #3 did not request the removal of Figure 5, we opted to keep this figure, but we would be happy to move it to the ‘expanded view’ if deemed necessary. We note, that we also discuss now other possible models (see above) to provide a balanced discussion of the current state of possible models (see p. 5, l. 4-11).

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Referee #2:

The manuscript by Stepp et al., examines the processivity of two different kinesin-2 family members, KLP11/20 a c. elegans ciliary motor and KLP3A/B a xenopus cytoplasmic motor. There are two major findings:

1.) Differences in the C-terminal tails of the two heavy chains in the heterodimeric KLP3A/B motor differentially affect processivity.

2.) KLP11/20, like KLP3A/B, takes 13 nm side-steps on individual microtubules, but straight 16 nm steps on axonemes, its natural substrate.

While these are both novel and important findings, both sets of experiments seem incomplete. Indeed, there are two completely different studies going on here, the effects of C-terminal tail variations on KLP3A/B processivity, and the effects of microtubule architecture on KLP 11/20 motility. Yet only the latter hypothesis is covered in the introduction, no rationale is provided for also looking at C-terminal tail contributions until the Results/Discussion section. But then it isn't clear why they are only looking at KLB3A/B and not KLP11/20. Are the KLP11/20 C-terminal tails identical so no differences would be expected, or could the authors also look at KLP11/20 and show that C-terminal tail variations are a general property that arise out of heterodimeric kinesin-2 motors? As we discussed in the manuscript, the processivity displayed by the two kinesin-2 motors was unexpected based on the available literature. The main reason we described only the KLP3A/B and not the KLP11/20 motor is because we repeatedly failed to clone the truncated KLP11 subunit. Given that the processivity of the KLP3A/B substantially exceeded that of kinesin-1, we felt that we at least have to try to understand the reason even if it was limited to the KLP3A/B motor. Indeed, with the new Figure 2, we now show that the distal random coil of the 3B subunit is responsible for
the increased processivity of the heterodimeric KLP3A/B (see also our response to Referee #1, page 3 point 5). Whether this is a general property of all heterodimeric kinesin-2 motors remains to be tested case by case in future experiments. We note that the basic transport parameters of motor proteins such as velocity, processivity, or stall forces are being used for theoretical models that describe motor-dependent transport processes in vitro and in vivo. Even though not part of our main message, the findings presented here raise the cautionary flag that transport parameters may be significantly altered when these motors are derivatized, for example by attachment to beads (see p. 9, l. 11-16) We have updated our manuscript to also include a general introduction about the processivity of kinesin-2 motors. (see p. 4, l. 6-11)

Conversely, why do they only look at KLP11/20 motility on axonemes? On one hand, it is understandable that axonemes are the natural substrate for KLP11/20 and not KLP3A/B, but is the ability of KLP11/20 to adjust its stepping mechanism on axonemes a unique specialization of that motor or a general property of all kinesin-2 motors? That important question could be answered if the authors also looked KLP3A/B motility on axonemes. This point has also been raised by referee #1, point 6 on page 4. Indeed, the KLP3A/B motor showed the same behavior as the KLP11/20 (See new Figure 3). We speculate that other kinesin-2 motors will show the same behavior on axonemes. However, we have experimentally analyzed only KLP3A/B and KLP11/20 motors. (see p. 11, l. 20 to p. 12, l. 3)

There are also issues with how the processive run length data is analyzed. Given the data is exponentially distributed, it is not clear what is being presented in terms of standard deviation or standard error, and how these were calculated. It is also not clear which statistical tests were used and if they are appropriate for comparing exponentially distributed data. Bootstrapping algorithms have been useful in similar studies (https://www.ncbi.nlm.nih.gov/pubmed/24940781; https://www.ncbi.nlm.nih.gov/pubmed/23790373). Furthermore, the authors, with no statistical test to validate the claim, claim that truncation of the B-subunit tail reduces the processivity relative to the A-subunit, without comparing the results to the wild-type construct used in Figure 1. It seems that with both C-terminal tails intact, an intermediate run length value is obtained - is this true? If so, what does it mean? And do these changes in run length correspond to changes in true processive run lengths, or do the C-terminal tails introduce some sort of electrostatic tethering or diffusive behavior between processive runs? Kymographs and/or mean-squared displacement of the motile behavior in these experiments would be useful. We apologize for this negligence. Please see our updated Materials and Methods (p. 24, l. 18-21). We included statistical tests. The results can now be found in the new Figure 2. Kymographs of the full-length KLP3A/B are now presented in Figure S 3. In addition, the description of single molecule TIRF assays in the Materials and Methods section has been restructured for a better overview.

There are a couple of recent papers regarding protofilament switching behavior of kinesin-1 (https://www.ncbi.nlm.nih.gov/pubmed/25954882) and kinesin-2 (https://www.ncbi.nlm.nih.gov/pubmed/28267259) that should be included in the discussion. We have included the work cited by our referee into the manuscript. (see p. 10, l. 11-12 and l. 18-20)

Finally, the explanation of how axonemes promote 16 nm steps of KLP11/20 isn't clear, at least to someone who is not familiar with the structure of the flagella and the process of IFT. As presented, the proposed mechanism seems highly speculative. Are there other possible explanations? Could this model actually be tested? In response to referee #1, point 1, we now discuss other possible explanations (see above p. 1 & 2 and manuscript p. 5, l. 4-11 and p.12, l. 21-22). Below we show an example of the inter-doublet connector nexin depicting how densely the space between the doublet microtubules is packed (Figure R 3 A and B). In fact, the position of the nexin arm interaction to the B-tubule (B4-B5 protofilament) matches the lowest position where anterograde IFT trains are found (Figure R 3 C, arrow highlighted in red).
Our model proposes that the motors cannot overcome these inter-doublet connections. The path of a (left-handed) side stepping motor is therefore restricted along these structural barriers on the left of the B-tubule enforcing a straight path (new Figure 4A) and explaining the on-axis 16nm steps (new Fig. 3C and D). Please also see our preliminary results showing the response of the motor when these structural barriers are removed (Referee #1, point 6 on page 4).

Unfortunately, testing the model in vivo is not simple. So far, Stepanek et al are the only ones that give a detailed description of how large antero- and retrograde IFT trains actually move along the axonemes in C. reinhardtii. Therefore, the most “straight forward” experiment to test our model would be to engineer a Fla8/10 motor (kinesin-2 of C. reinhardtii) that can no longer take side-steps on microtubules (our unpublished work shows that Fla8/10 motors also spiral around a suspended microtubule). This will most likely require a fla8 and fla10 double-knockout background (which to our knowledge does not yet exists) because the neck region of both subunits needs to be modified to restrict side-stepping as we showed using the KRP85/95 kinesin-2 from S. purpuratus (Brunnbauer et al., Mol Cell 2012). Clearly, the biggest challenge would be to engineer two kinesin-2 subunits (Fla8 and Fla10) that also rescue the knockout cell line in vivo as previous changes made to the necklinker/neck regions in one of the two subunits of kinesin-2 failed to rescue IFT in C. elegans (Pan et al, BBRC 2010). However, we feel that these experiments may go beyond the scope of a short report and certainly could not be completed within the time frame of a typical revision. (see p. 5, l. 4-11)

Referee #3:

Stepp et al have used FIONA on two types of Kinesin-2, the ciliary KLP11/20 from C. elegans and the cytoplasmic KLP3A/B from X. laevis, each fluorescently labelled on one head. They find a difference between the step sizes on microtubules versus axonemes, and interpret this to mean that on axonemes, the left-biased off-axis component of stepping for kinesin-2 is suppressed. They speculate that this suppression of off-axis stepping is due to physical blockade of the sidestepping by an impenetrable line of crosslinks between the A and B tubes of the axonemal peripheral doublets. Whilst this is in principle interesting unfortunately I think neither the finding of a different step size nor the interpretation are sufficiently well supported.

1. Abstract sentence 2 is inaccurate -should read "reserved for kinesins from the kinesin-2 family"
   We have updated the manuscript accordingly (new Summary page 2). Please also see comment by referee #1 point 2 on page 2.

2. Abstract "ability to self-organise on the MT lattice" the arguments necessary to understand this phrase have not yet been made. I suggest: "the inherent ability of the kinesin-2 to side-track on the microtubule lattice restricts the motor to one side of the microtubule doublet in axonemes."
   We have updated the manuscript accordingly (new Summary page 2).

3. P5 L8 "At the example of" please clarify; I think this should read "By studying"
We have updated the manuscript accordingly (p.6, l. 6).

4. Data. Microtubules and axonemes are down on a surface, so how can the motors move in a helical path around the microtubule periphery? In principle these trajectory data (Figure 4) actually gainsay the central idea in the paper, that a physical barrier (in this case the glass surface) can block sidestepping.

The inter-doublet connections in axonemes consist of a very densely-packed network of protein structures (in particular nexin and other dynein arms) that leaves no appreciable free space for the motor to penetrate this structure (Oda et al, Mol Biol Cell 2013 and Ishikawa, Cilia 2015). Removal of these inter-doublet connections predicts that the spiraling behavior of kinesin-2 motor is rescued which we indeed observed in preliminary experiments on doublet microtubules that were digested out of the axonemes (see our response to referee #1, point 6).

In contrast, the connection of the microtubules to the coverslip is realized via individual comparatively far-spread Biotin-Streptavidin (10 nm spherical) bonds (see Figure R 4).

Furthermore, the surface for binding is uneven due to the employment of biotinylated BSA for the binding and normal BSA (14 nm x 4 nm x 4 nm ellipsoid) for surface passivation. This geometry is expected to lead to a much more penetrable structure when compared to axonemes. We therefore interpret the sinusoidal path seen on the microtubules as spiraling events with the motors passing between the microtubules and the coverslip.

**Figure R 4: Comparison of two different barrier characteristics.**

To further strengthen our claim that the motors are indeed able to pass through the space between the microtubule and the coverslip, we confirmed the correlation between the observed step size and the characteristic trajectories of the motors. Assuming a 50 % probability of sidestepping was sufficient to recapitulate the experimentally determined step size of the motor (Figure EV 3 and new Figure 3A and B). Remarkably, this assumption alone sufficed to also recapitulate the motors characteristic trajectories observed above (see Figure R 5 and response to point 7 for more detail on the simulation).
Figure R 5: Comparison of simulated to measured spiraling data.
For the simulated run shown in Figure R 7, we also plotted a theoretical kymograph (Figure R 8, left blue). The motor stays on one position for 5 frames and a frame is converted to 0.5 s for the plot. The bi-modal features (fast and slow segments) of the trace are readily visible. These features are recapitulated in actual measurements for motors walking on microtubules (Figure R 8, right). Bimodality of the kymograph data is much less pronounced or even absent on axonemes (Fig. EV 2 A and simulated Figure R 8 red). These results provide another indication that the motors can take sidesteps in this setup.

Figure R 6: Simulated (left) and observed (right) kymographs both display the bi-modal characteristics.
5. Data. It looks to me that the assignment of steps and step sizes is being very much forced on these data by the fitting algorithm. Look for example at the bottom left panel in figure 4. In this situation small differences in the average step size will appear if one dataset is slightly more noisy than another, especially if the rules for what constitutes a step are adjusted by varying the window size and t-threshold taken to indicate a step. I think robust conclusions cannot be drawn without very much more careful controls - for example by fitting kinesin-1 data on the two different substrates and showing that the same stepsize is obtained with different levels of noise. For the step fitting, we need to know the threshold and window size employed, if these were varied, and if so, on what basis.
As suggested by our referee, we tested the robustness of the step size detection for different noise levels (Figure R 9). Gaussian noise was added to the measured distance data (blue traces) and the step detection algorithm was performed on the modified data without changing the parameters. The width of the Gaussian distribution for the noise was varied between 0 and 16.1 nm. The value on the x-axis is calculated by the mean distance of the detected steps (red) from the actual data (blue). This value increases for higher noise levels (0 to 16.1 nm in steps of 1.34 nm). The x-value of the first data point for every series indicates the level of noise present in the data. The y-axis shows the mean step size as obtained from the Gaussian fit of the respective step size histograms. (a, b and c) Show traces with added noise (blue, 0, 8 and 16 nm width) and the detected steps (red).

The threshold for the step detection algorithm is not changed and was chosen to be 1.7 for the ‘MinPeakProminence’ parameter of the “findpeaks” function in Matlab, which is performed directly on the t-values. The window size was adjusted to the speed of the motors and was 13 frames for 11/20 on AX, 11 for 11/20 on MT, 11 for 3A/B on AX and 25 for 3A/B on MT. A wider window tends to miss small steps. We also added this information to the Materials and Methods section of the manuscript (see p. 25, l. 18-23)

The analysis provided following arguments against a decisive influence of an overly sensitive step detection algorithm on the mean step size measured.

- The step sizes are reasonably stable considering the addition of noise up to the observed step size.
- The distance between the datasets for axonemes and microtubules is constant for all noise levels.
- The wider window for 3A/B on microtubules underlines the smaller detected step size.
- The noise is similar for the same motor on different filaments (e.g. KLP11/20 on axoneme versus microtubule) but differs between the motors (e.g. KLP11/20 versus KLP3A/B on microtubules). The noise in the data therefore depends more on the characteristics of the construct (dye, Snap/Halo-Tag, motor) and not so much on the filament it is measured on.

Figure R 7: Behavior of the step detection algorithm for noisy data.

We would have liked to confirm this result with data on kinesin-1 molecules. Unfortunately, we failed to introduce a SNAP-tag into the Kinesin-1 motor (Brunnbauer et al, 2012) that would have allowed us to do these sophisticated experiments. Lastly, the description of single molecule TIRF assays in the Materials and Methods section have been restructured for a better overview.

6. Interpretation. The authors argue that off axis stepping to the left would confine kinesin-2 to one side of the B-tubules. Is there evidence that addresses whether motors can transfer from the A-tubule to the B tubule and vice versa? If the impenetrable barrier in the model is speculative then please say so. If I'm missing something then the authors need to help me and other readers understand.
To our knowledge, there is no evidence that directly demonstrates the motors’ crossing between the A- and B-tubules on the doublet microtubule in vivo or in vitro. Structural evidence so far argues for a highly densely packed space between the respective doublet microtubules (also see our response to referee #2 on page 9), but so far there is no direct demonstration that this structural barrier between the doublet microtubules can be penetrated. Our results argue that at least single kinesin motors cannot. However, please see our response to referee #1, point 6, where we have removed this structural barrier in our preliminary experiments. We have updated our manuscript to better explain these points. Please note that we indeed only suggest that demarcations are impenetrable based on our analysis of step sizes on microtubules versus axonemes and available structural work (see p. 12, l. 20 to p. 13, l. 2).

7. Given the measured average stepsize, it should be possible to predict the pitch of the helical path? As suggested, we simulated the pitch. The stepping of a motor was modelled on a cylinder-shaped geometry with binding sites distributed as expected for a microtubule lattice (Wade 1991). A sidestep probability of 50% was assumed and 200 steps were simulated (new Figure EV 3). The data was projected to a plane to simulate a microscope image. For the histogram, step sizes were blurred by a Gaussian distribution of width 3 nm as estimated detection accuracy. This simple model was sufficient to recapitulate the experimentally determined step size of the motor shown in Figure 3A and B. The pitch for a 13 protofilament microtubule can then be calculated from the mean step size (x) and the probability for sidesteps (p). The resulting mean pitch of 345 nm agrees well with the experimentally obtained walking paths of the motors on microtubules (see new Figure 4). However, the pitch can only be regarded as an ensemble parameter due to the stochastic nature of the sidestepping. (see p. 12, l. 14-19 and Figure EV 3)

2nd Editorial Decision 31 July 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study (you will find enclosed below). All three referees now support the publication of your study in EMBO reports. However, referees #1 and #3 have remaining points, we ask you to address in a final revised version of the manuscript.

After cross-commenting and discussing the points of referee #1 with the other referees, we will not require additional experiments with kinesin-1 (in case you have obtained such data in the meantime, we of course ask you to include them). However, we ask you to address the points of referee #1 and the minor point of referee #3 by text changes, by adding further explanations or requested information, and by extending the discussion. Please also address these concerns in a detailed point-by-point response.

I also have the following editorial requests that need to be addressed.

Please provide all figure files according to our guidelines:
E.g., figures should not have landscape format.

Please add a short (not more than 8 words) running title to the title page.

Please combine all the references to one single reference section.

Please re-format the Appendix. The Appendix should include a table of content on the first page with page numbers, all figures/tables and their legends. Please follow the nomenclature Appendix Figure/Table Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

The labelling in Fig. 3 is confusing, as 3A-D is used twice. Please arrange this differently.

Can the raw data of the single molecule assays (e.g. the movies) be deposited at a public database?
Finally, the synopsis image provided is not very comprehensive. We rather would prefer a scheme summarising the findings (maybe similar to Fig. 5).

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS
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Referee #1:

The revised manuscript by Stepp et al is greatly improved and I thank the reviewers for their extensive response with many additional explanations. The authors show that two different members of the kinesin-2 family, KLP3A/3B from Xenopus and KLP11/20 from C. elegans, are processive motors (Figure 1) and that the C-terminal tail domain can influence the motility properties (velocity and processivity) of KLP3A/3B (Figure 2). They use single molecule imaging and FIONA to show that both motors walk with 13 nm steps along single microtubules but 16 nm steps along axonemes (Figure 3) and that they walk in a helical fashion on microtubules and a straight path on axonemes (Figure 4). I still feel that there are two different stories here that should be split up and individually strengthened. Unfortunately I still have major issues with the experiments and interpretations. Thus, I do not believe the manuscript is yet ready for publication.

Experimental issues:

1. The addition of the statistical comparisons of run length are helpful but I don't understand how the run length data were obtained or analyzed. Given that they are applying FIONA (one nanometer accuracy), why throw out all of the data with run length less than one micrometer? How fast are the authors imaging? Assuming at least 10 Hz given the microscopy setup, this decision seems to ignore a vast amount of the data and overestimate the run lengths. The general approach in the field is to exclude data less than the resolution of the imaging. My guess is that by throwing out the run lengths less than 1 micrometer, they are overestimating the run lengths and the results may not be significantly different between the constructs if these results are included in the analysis.

2. Thank you for the description on how motors can have helical motility on surface-attached microtubules (response to reviewer 3). This information would likely be critical for the reader as well. But helical motility depends on the pitch of the microtubules and this depends on the number of protofilaments. No information is provided about the source of the tubulin and the polymerization conditions for microtubules. Depending on the conditions, the authors could have mostly 12 protofilament microtubules where any motor would move in a helical fashion due to the pitch of the microtubule or mostly 13 protofilament microtubules where only motors that can sidestep can move in a helical fashion (or a number of other possibilities). Overall, the conclusion of helical motion needs to be bolstered by additional experiments. One approach would be to compare the motility of these kinesin-2 motors to kinesin-1. The explanation to reviewer 3 that the authors were unable to clone a kinesin-1 with a SNAP tag are not sufficient given the ability in 2017 to clone by mail or gene synthesis. Another approach would be to polymerize microtubules under conditions that lead to different protofilament numbers and compare the ability of unlabeled motors to rotate microtubules in gliding assays (e.g. Mitra et al 2015 PLoS ONE).

3. Given the fact that the authors can observe side-stepping on single microtubules (and thus helical motion), why don't the motors move this way along the axonemes until they come to the nexin/dynein blockage? This is emblematic of my primary concern with the data that the authors have reached a conclusion without considering alternative explanations. In my opinion, the observed straight motility and larger step size along the axonemes is due to straight walking of the motor along straight 13 protofilament microtubules whereas the observed helical motility and smaller step size along the single microtubules is due to straight walking of the motor along helical 12 protofilament microtubules. This possibility is not considered by the authors and cannot be excluded based on the data presented in the manuscript.

Other issues:
1. The Introduction section is quite long (almost as long as the Results and Discussion). Please edit to be more concise.

2. The finding that the kinesin-2 motors are processive should not have been "surprising" (p. 8) given the work published in 2015 by Guzik-Lendrum et al.

3. The statement that "the asymmetric nature of this effect provides one rationale why nature combined two distinct subunits into one heterodimeric motor" (p. 9-10) is an overinterpretation. Kinesin-1 uses one tail segment to inhibit its homodimeric motor domain (Hackney et al 2009). Clearly a heterodimeric motor is not needed to get an asymmetric autoinhibition mechanism.

4. Please remove the delineation of KLP11/20 as ciliary and KLP3A/B as cytoplasmic motors (p. 11)

Referee #2:

This is a resubmission that has been extensively revised. The authors are to be commended for doing such a thorough job of addressing the reviewers' concerns. Additional experiments, statistical analyses, and figures strengthen the authors' conclusions. Furthermore, the manuscript has been rewritten to clarify its goals and interpretation of its results. This is a solid paper that significantly extends our understanding of the function of kinesin-2 class motors that is worthy of publication in EMBO Reports.

Referee #3:

The authors have worked hard and effectively to address all the points I raised. The ms is dramatically better documented, coherent, convincing and exciting and I would now be happy to see this interesting evidence for a possible 'keep left' sorting mechanism in anterograde IFT transport published in EMBO Reports.

In the abstract, perhaps avoid ambiguity by simply writing that kinesin-2 'is essential' for long range IFT?

2nd Revision - authors’ response 03 August 2017

Point-by-point response

Referee #1:

The revised manuscript by Stepp et al is greatly improved and I thank the reviewers for their extensive response with many additional explanations. The authors show that two different members of the kinesin-2 family, KLP3A/3B from Xenopus and KLP11/20 from C. elegans, are processive motors (Figure 1) and that the C-terminal tail domain can influence the motility properties (velocity and processivity) of KLP3A/3B (Figure 2). They use single molecule imaging and FIONA to show that both motors walk with 13 nm steps along single microtubules but 16 nm steps along axonemes (Figure 3) and that they walk in a helical fashion on microtubules and a straight path on axonemes (Figure 4). I still feel that there are two different stories here that should be split up and individually strengthened. Unfortunately I still have major issues with the experiments and interpretations. Thus, I do not believe the manuscript is yet ready for publication.

We regret that we could not convince our referee with our extensive revision. Below we address the issues raised by our referee to clarify remaining concerns.

Experimental issues:

1. The addition of the statistical comparisons of run length are helpful but I don't understand how the
run length data were obtained or analyzed. Given that they are applying FIONA (one nanometer accuracy), why throw out all of the data with run length less than one micrometer? How fast are the authors imaging? Assuming at least 10 Hz given the microscopy setup, this decision seems to ignore a vast amount of the data and overestimate the run lengths. The general approach in the field is to exclude data less than the resolution of the imaging. My guess is that by throwing out the run lengths less than 1 micrometer, they are overestimating the run lengths and the results may not be significantly different between the constructs if these results are included in the analysis.

We record runs at saturating ATP concentrations with a cycle time of 206 ms (p. 16, l. 20 and p. 17, l. 7). As stated in the Materials and Methods, we consider the truncation of our data in the fitting algorithm. In our experience this procedure is reliable, especially if the resulting run length is longer than the truncation parameter. The probability of a misinterpretation of an event as a processive run is higher for shorter runs. We therefore chose to truncate at 1 µm and consider this in the fitting procedure.

We tested our algorithm by fitting distributions with known parameters (see Figure below). Columns have the same parameter µ for the exponential distribution and Rows have the same truncation parameter. For truncation, all values lower than the indicated threshold are disregarded. Our algorithm was then used to estimate the parameter µ of the distribution. The result is given +− the 95% confidence intervals. N-numbers were chosen to be close to the lowest ones we obtained for the motors described in the study.

Run lengths are typically slightly underestimated but stable for truncations up to 1 µm.

2. Thank you for the description on how motors can have helical motility on surface-attached microtubules (response to reviewer 3). This information would likely be critical for the reader as well. But helical motility depends on the pitch of the microtubules and this depends on the number of protofilaments. No information is provided about the source of the tubulin and the polymerization conditions for microtubules. Depending on the conditions, the authors could have mostly 12 protofilament microtubules where any motor would move in a helical fashion due to the pitch of the microtubule or mostly 13 protofilament microtubules where only motors that can sidestep can move in a helical fashion (or a number of other possibilities). Overall, the conclusion of helical motion needs to be bolstered by additional experiments. One approach would be to compare the motility of these kinesin-2 motors to kinesin-1. The explanation to reviewer 3 that the authors were unable to clone a kinesin-1 with a SNAP tag are not sufficient given the ability in 2017 to clone by mail or gene synthesis. Another approach would be to polymerize microtubules under conditions that lead to
different protofilament numbers and compare the ability of unlabeled motors to rotate microtubules in gliding assays (e.g. Mitra et al 2015 PLoS ONE).

We acknowledge the lack of detailed information in the Materials and Methods about our microtubules and we added the respective information (p. 16 l. 6-9). The procedure of tubulin isolation and polymerization is essentially the same as our group has used previously (Brunnbauer et al, Molecular Cell 2012). In fact, most of the microtubules in that study consisted of 14 protofilaments. The left-handed pitch for the supertwist observed in these microtubules is around 6 µm, which is an order of magnitude bigger than the pitches we observed in our study. We are therefore certain, that the behaviour we observe is not due to a supertwist in the microtubule lattice. The method for Mitra et al is interesting but it is an experiment where many motors are involved that we find hard to relate directly to our experiments. The authors also mention that the pitch varies depending on the motor concentration present on the coverslip.

3. Given the fact that the authors can observe side-stepping on single microtubules (and thus helical motion), why don't the motors move this way along the axonemes until they come to the nexin/dynein blockage? This is emblematic of my primary concern with the data that the authors have reached a conclusion without considering alternative explanations. In my opinion, the observed straight motility and larger step size along the axonemes is due to straight walking of the motor along straight 13 protofilament microtubules whereas the observed helical motility and smaller step size along the single microtubules is due to straight walking of the motor along helical 12 protofilament microtubules. This possibility is not considered by the authors and cannot be excluded based on the data presented in the manuscript.

We included the figure showing the geometry of the Streptavidin/Biotin binding to the Appendix (Appendix Figure S9) into the manuscript (p. 12, l. 18)

We in fact see some traces with a kink at the beginning of the run that could represent the effect described above. To separate this from a random (e.g. drift) event is not trivial due to its non-repetitive nature. At these low ATP-concentrations and because we aim to minimize background fluorescence in our experiment, we observe mostly motors that are already bound to the filament. In other words, most motors have already reached the nexin blockage when we start observation.

Other issues:

1. The Introduction section is quite long (almost as long as the Results and Discussion). Please edit to be more concise.

We note that we have incorporated considerably more information into the introduction based on the suggestions of our reviewers during the revision process. However, if deemed necessary we can remove the additional information.

2. The finding that the kinesin-2 motors are processive should not have been "surprising" (p. 8) given the work published in 2015 by Guzik-Lendrum et al.

We no longer describe the processivity as surprising and mention the paper by Guzik-Lendrum directly in this paragraph. (p.8, l. 9-13)

3. The statement that "the asymmetric nature of this effect provides one rationale why nature combined two distinct subunits into one heterodimeric motor" (p. 9-10) is an overinterpretation. Kinesin-1 uses one tail segment to inhibit its homodimeric motor domain (Hackney et al 2009). Clearly a heterodimeric motor is not needed to get an asymmetric autoinhibition mechanism.

We apologize for this confusion please see page 10 l. 1-4.

4. Please remove the delineation of KLP11/20 as ciliary and KLP3A/B as cytoplasmic motors (p. 11)

We have removed the above-mentioned distinction in the manuscript (p. 11, l. 8-10).
Referee #2:

This is a resubmission that has been extensively revised. The authors are to be commended for doing such a thorough job of addressing the reviewers' concerns. Additional experiments, statistical analyses, and figures strengthen the authors' conclusions. Furthermore, the manuscript has been rewritten to clarify its goals and interpretation of its results. This is a solid paper that significantly extends our understanding of the function of kinesin-2 class motors that is worthy of publication in EMBO Reports.

We thank our referee for his/her contributions to our work and constructive criticism that significantly improved our manuscript.

Referee #3:

The authors have worked hard and effectively to address all the points I raised. The ms is dramatically better documented, coherent, convincing and exciting and I would now be happy to see this interesting evidence for a possible 'keep left' sorting mechanism in anterograde IFT transport published in EMBO Reports.

We thank our referee for his/her contributions and constructive criticism that significantly improved our manuscript.

In the abstract, perhaps avoid ambiguity by simply writing that kinesin-2 'is essential' for long range IFT?

We adjusted the abstract accordingly. (Abstract l. 4&5)

3rd Editorial Decision

09 August 2017

Thank you for the submission of your revised research manuscript to EMBO reports. Thanks for the submission of your revised manuscript. I think that the referee points have now been sufficiently addressed.

However, I would like to ask you for some final editorial changes:

1. A slight change of the title (present tense): Kinesin-2 motors adapt their stepping behavior for processive transport on axonemes and microtubules

2. Please shorten the introduction as suggested by referee #1. I would suggest to shorten it to half of the length.

3. The arrangement of Figure 3 is still very confusing. Please use labels A-H to label the single panels and clearly indicate in the legend what each panel shows.

4. Could the figure shown in the point-by-point response be included in the manuscript (maybe in the appendix)?

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

3rd Revision – authors' response

11 August 2017

Thank you for your support in finalizing the manuscript.
Below I address the points you raised.

1. *A slight change of the title (present tense): Kinesin-2 motors adapt their stepping behavior for processive transport on axonemes and microtubules*

   We have up-dated the title accordingly.

2. *Please shorten the introduction as suggested by referee #1. I would suggest to shorten it to half of the length.*

   The introduction is now substantially shortened (by ~40%). Given that our referee #1 remained critical, we made sure to keep all the suggestions of our referee #1. To also satisfy the final remarks of our referee #1, we have up-dated the results & discussion part to remove the “surprising” increase in processivity as well as our statement concerning the “asymmetry of the kinesin-2 regulation”. We have further eliminated any spelling errors and imprecise expressions in the document. If deemed necessary, we are happy to provide a detailed list of changes we made.

3. *The arrangement of Figure 3 is still very confusing. Please use labels A-H to label the single panels and clearly indicate in the legend what each panel shows.*

   Willi L. Stepp has up-dated the Figure 3 according to your feedback. Please let us know if any additional changes are required.

4. *Could the figure shown in the point-by-point response be included in the manuscript (maybe in the appendix)?*

   The new Appendix Figure S4 is now included.

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Accepted 14 August 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.
You must complete all cells with a pink background.

Useful Links for Completing this Form

A: Figures

1. Data

The data shown in figures should satisfy the following conditions:

- Data are presented in a clear, concise, and accurate way.
- Figures are labeled appropriately, and axis titles are clear.
- Legends are provided for all graphs.
- Graphs include error bars to indicate variability.
- Graphs are scaled appropriately to the range of the data.
- Graphs are labeled with units.
- Graphs are in color when appropriate.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- A description of the experimental setup and results.
- A description of the statistical analysis performed.
- A description of any biases or limitations.

3. Statistics and general methods

- The statistical tests used are reported.
- The assumptions of the tests are met.
- The results are presented graphically.
- The error bars are labeled.
- The statistical significance is indicated.
- The sample size is adequate.
- The experiment is repeated.

4. Animal models

- The species, strain, gender, and age of the animals are reported.
- The in vivo model is appropriate.
- The experiment is conducted in compliance with the ARRIVE guidelines.

5. Human subjects

- The informed consent of the subjects is obtained.
- The ethical clearance is obtained.
- The data are de-identified.

6. Consent

- The consent of the subjects is obtained.
- The ethical clearance is obtained.
- The data are de-identified.

Glossary

- Antibodies
- Proteins
- Enzymes
- Enzyme inhibitors
- Kinetics
- Half-life
- Pharmacokinetics
- Pharmacodynamics
- Bioavailability
- Absorption
- Distribution
- Metabolism
- Excretion
- Clearance
- Area under the curve
- Peak concentration
- Time to peak concentration
- Time to half-life
- Time to maximum effect
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1. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in the study and deposited in a public database (e.g., GSE39462, Gene Expression Omnibus, dbGaP, PRIDE, etc.) See author guidelines, under Reporting Guidelines. Please confirm you have followed these guidelines.

2. The datasets generated during and/or analyzed during the current study are available from the corresponding author on request.

3. A "Data Availability" section is included in the submitted manuscript.

4. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right).

5. Report the clinical trial registration number (at top right).

6. Data deposition in a public repository is mandatory for:
   a. Protein, DNA and RNA sequences
   b. Macromolecular structures
   c. Crystallographic data for small molecules
   d. Functional genomics data
   e. Proteomics and molecular interactions

7. Authors are strongly encouraged to follow the MIRIAM guidelines and deposit their model in a public database such as Biomodels (see link list at top right).

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9. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the deposition in a public database (e.g., RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PX000208, etc.) Please refer to our author guidelines for 'Data Deposition'.

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