Hedgehog-induced ciliary trafficking of kinesin-4 motor KIF7 requires intraflagellar transport but not KIF7's microtubule binding

Yang Yue, Martin Engelke, T. Lynne Blasius, and Kristen Verhey

Corresponding author(s): Kristen Verhey, University of Michigan Medical School

Review Timeline:

Submission Date:	2021-04-28
Editorial Decision:	2021-06-29
Revision Received:	2021-08-31
Editorial Decision:	2021-10-01
Revision Received:	2021-10-14
Accepted:	2021-10-18

Editor-in-Chief: Matthew Welch

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.)

RE: Manuscript #E21-04-0215
TITLE: Hedgehog-induced ciliary trafficking of kinesin-4 motor KIF7 requires intraflagellar transport but not KIF7 microtubule binding

Dear Dr. Verhey:

First of all, I apologize for the extreme delay and thank you for your patience. We had to make a change of Monitoring Editors and I will now be handling your manuscript.

You'll see that your manuscript was sent to two reviewers, both of whom provide thorough and thoughtful commentary. They note the work has the potential to provide mechanistic insight into the role of Kif7 in cilia and its contributions to Hedgehog signaling. However, both also raise a number of concerns that need to be addressed. In particular, both feel that the evidence provided is not sufficient to distinguish between the multiple models proposed. Both also provide thoughtful suggestions as to how you might address their concerns.

Once you've had a chance to look over these comments feel free to contact me with any questions. We look forward to evaluating a revised manuscript that addresses the points raised.

Sincerely,

Matthew Welch
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Verhey,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office

mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Yue et al examines the mechanisms by which the atypical (non-motile) kinesin Kif7 becomes enriched at the ciliary tip specifically upon activation of Hh signaling. They propose 4 models by which this could occur. That Hh stimulation: 1) may increase access of Kif7 into the cilium 2) that it may increase loading of Kif7 onto IFT trains 3) that it may increase Kif7 microtubule binding or 4) that it may increase Kif7 interaction with some anchoring protein found at the ciliary tip. They generate a number of mutants to test this model: a mutant that is locked to microtubules, a mutant that cannot bind microtubules, and two different mutant fused to the motor domains motile kinesins. They find that both microtubule locked and non-binding variants are able to traffick to the ciliary tip, with the non-binding variant able to respond to Hh signaling. They also show that blocking IFT trafficking blocks localization of Kif7 to the ciliary tip. Overall the work has the potential to provide significant mechanistic insight into the role of Kif7 in cilia and its contributions to Hh signaling, but a number of key points remain to be addressed prior to publication.

Major points:
The authors don't seem to fully consider the fact that the models they propose to account for Kif7 accumulation may not be mutually exclusive. They conclude that their data support model 2 and exclude models 1 and 3. Though model 4 is mentioned, there is little data either supporting or excluding it. However, I'm not totally convinced models 1 and 3 can be completely excluded: Could the fusion of Kif7 to the motor domains of Kif2c or Kif21 alter access to the cilium in and of themselves, for example? And with respect to model 3, blocking microtubule binding did allow some access of Kif7 into the cilium, which I agree is interesting and surprising. However, the noMT mutant was impaired in its ability to become enriched in the cilium upon Hh stimulation- this is the only mutant tested that was impaired in this way. That seems to suggest that microtubule binding plays at least some role in the Hh dependent enrichment. To get at whether microtubule binding might contribute partially redundantly along with IFT trafficking to Kif7 localization the authors should assess the localization of the noMT and rigor mutants in the contexts of the inhibitable Kinesin 2 motor (ie the experiment in Figure 5). I'm particularly interested in the rigor mutant fits with a model where IFT is driving most of Kif7 localization in response to Hh.

Relatedly, the noMT condition is at least somewhat impaired in its localization to the cilium upon SAG stimulation, yet this mutant fully rescues the Gli localization phenotype in the Kif7-/- cells. What are explanations for this? In other mutants, the localization of Gli2 or Gli3 to the ciliary tip seems linked to the degree to which that Kif7 mutant localizes to the ciliary tip, so the noMT mutant represents the one departure from this.

Another gap in the model is that there isn't direct evidence that Kif7 is trafficked by IFT. Some live imaging data showing Kif7 trafficking with IFT in this way would strengthen this argument.

Additional points:
A large number of comparisons are being done in Fig. 3, so a t-test is not really appropriate. 2 way Anova with a post-hoc test would be better.

In figure 4, it would be good to show representative images for Gli2 and Gli3 rather than having this in the supplemental data. The number of samples is also very small- just 2 replicates for some conditions. Given that this is a key piece of data, the level of rigor here should be improved with additional samples.

Line 90- what does it mean that it's uninhibited? Lack of autoinhibition? Some more context here would be helpful. We are referred to a submitted manuscript as a reference for Kif7 autoinhibition which makes this hard to evaluate. In general, the truncated mutant of Kif7 is mentioned at the beginning when the different binding mutants are introduced, but they aren't really used in subsequent experiments, and so how they come into play is somewhat confusing for a non-kinesin motor specialist. Perhaps they are controls? Clarity would improve readability.

Reviewer #2 (Remarks to the Author):

Summary:
In this article, Yue and colleagues investigate the localization of different mutants and chimera of the kinesin-4 motor KIF7 to primary cilia in pharmacologically treated cells to stimulate the Hedgehog signaling pathway. In fixed cells KIF7 can be visualized at the ciliary tip, similar to the GLI transcription factors that are the main effectors of Hedeghog signaling. KIF7 as well as GLIs accumulate at the ciliary tip after Hedgehog pathway activation, yet, neither the significance nor the molecular mechanism for accumulation are clear at this point. KIF7 is an unusual kinesin as it appears to bind statically to microtubules in vitro, while other

kinesins, such as the major ciliary kinesin-2 transports proteins in an anterograde fashion by so-called intraflagellar transport (IFT). The authors "consider four models for how Hh stimulation results in an accumulation of KIF at the tip of the primary cilium" and are trying to dissect this mechanism by transient transfection of different KIF7 variants into fibroblasts in vitro, followed by subsequent immunofluorescence microscopy analysis.

While testing different models is of high scientific value, the authors' experimental approach has major flaws, and their conclusions are merely interpretations of their data that lack substantial evidence. Some of the proposed models are ruled out prematurely, such that most models still appear valid and the main insight of this study is delivered in the title of the manuscript, reading: "...KIF7 requires intraflagellar transport but not its own microtubule binding". This conclusion is indeed supported by the findings presented here and the abstract focuses on these stronger parts that make up about a quarter of this manuscript. However, as the authors seem to focus on testing different models of regulated KIF7 transport into cilia, from which they draw potentially false conclusions, I do not recommend publication of this manuscript in its current form.

Major comments:
• One major flaw in the authors' interpretation (as well as choice of experimental strategy) is that they seem to equal MT-binding with cilia tip binding. The ability to bind to MT +ends is met by so-called +TIP proteins, none of which contains a classical MT-binding domain found in kinesins. Hence, the KIF7 variants that alter MT-binding (and mobility) are interesting but do not address the original question. Thus, -unlike stated by the authors- their "model 3" cannot be ruled out based on the fact that the MT-binding domain in KIF7 is dispensable for tip accumulation. In fact, the authors give evidence that KIF7 may be transported by IFT, which indeed makes direct MT-binding of KIF7 obsolete. Nonetheless, wild type KIF7 does accumulate at cilia tips, which may well be based on an increase in affinity of KIF7 for MT +ends in response to Hh signal.
• In the "motile" KIF7 variants presented here, the main motor domain is replaced with the motor domains of kinesin-1 family members, such that one can argue that their main property has been changed and the term "motile KIF7" is somewhat misleading. Hence, the statement "the finding that motile variants of KIF7 accumulate in the cilium without pathway activation rules out the possibility that Hh signaling increases the ability of KIF7 to access the ciliary compartment" (lines 152-154) is also an over-interpretation. It rules out that the chimeric proteins (that are much more closely related to kinesin-1 family members) are regulated in such a way. In fact, these findings highlight that the motor domain in KIF7 is critically important and may well be regulated in a Hh-dependent manner. These findings further question the usefulness of these chimera to study KIF7 function. Additional minor point: The increased localization of "motile" KIF7 variants to cilia in response to Hh stimulation does not appear to be significant and any conclusions from these data should be phrased accordingly.
• Based on the above, it cannot be ruled out that there is a Hh-dependent increase in the ability of KIF7 variants to access cilia. Hence, in contrast to the authors' conclusions their "model 1" cannot be ruled out. Moreover, as the affinity to MT +ends may be altered in a Hh-dependent manner, their "model 3" cannot be excluded either. To unambiguously rule out models 1 and 3 and further support models 2 and 4, which appear to be the authors' favorite, additional evidence is required.

Minor point:
• The simplest explanation for many observations in this study is that there is a constant fraction of KIF7 (or its variants, as well as GLI proteins) that localize to the cilia tip and that differences in the protein levels may lead to an apparent increase at the cilia tip. Hence, assessing the expression levels of the individual proteins is absolutely critical, and the authors should show that the protein levels after transient expression of the different KIF7 variants, GLI2 and GLI3 are equal (detecting the epitope tags by Western Blotting may be a valid option).
• It is somewhat unclear how cilia tip localization of KIF7 (and GLIs) is defined. The authors should give a metric. If cilia tip localization of proteins is based on subjective assessment, the authors should try to implement a more unbiased way based on quantitative assessment of fluorescence intensities, such that the data stands on solid ground and can be reproduced more easily.
• The "rigor" mutant appears to give different results for the cilia tip localization of GLI2 vs. GLI3 (see Figure 4), which is unexpected. Interestingly, the data presented for this mutant appears to be the only analysis (in Figure 4) based on three independent experiments. Given the spread of the data presented a third replicate for all mutants would be helpful, such that error assessment and statistical testing could be performed to strengthen the authors' conclusions.
• When discussing different motilities of ciliary motor proteins (lines 246-247) the authors should also discuss the different ciliary kinesin motors found in C. elegans, which have been reported exhibit different velocities ,and compare to KIF7 and kinesin-2 in their system.
• "Immotile behavior" should be rephrased.

Significance:
• The chemogenetic "i3Ai3B" system that allows the authors to draw the conclusion as stated in the title was published in Engelke et al., 2019, where it was used in a beautiful study to analyze KIF17's function in IFT. The findings on KIF7(WT) presented here are an interesting additional observation made possible by this powerful system, however, the follow up experiments lack the rigorousness required to draw strong conclusions, such as ruling out various proposed models of KIF7 accumulation at cilia tips.
• I believe the manuscript in its current form is of limited value to the community as it appears to be of interest mainly for a

specialist readership. Most importantly, however, the specialist readership will also recognize that the authors fail to adequately dissect the proposed models due to over-interpretation of the presented data, such that they will be left with an interesting observation that KIF7 cilia localization appears independent of its microtubule binding domain. Whether this is sufficient for publication in MBoC is unclear.

• In order to substantiate the observations and give additional evidence for IFT-dependent transport of KIF7 (model 2), the authors could try to visualize and quantitate IFT of the KIF7 variants -admittedly, a challenging task. However, this would dramatically increase the significance of the study with new possibilities to mechanistically dissect the process.

• Another possibility to increase the significance of this study would be to assess the functional consequences of the different KIF7 chimera for Hh signaling.

We thank the reviewers for their thorough and thoughtful commentary. In response, we have performed new experiments and rewritten portions of the text (indicated by red text in manuscript). We believe we have addressed all of the concerns as indicated in our point-by-point response below (reviewer comments in black text, our responses in red text). We are grateful for the feedback and believe the changes have improved the manuscript and our story.

Reviewer #1 (Remarks to the Author):

Yue et al examines the mechanisms by which the atypical (non-motile) kinesin Kif7 becomes enriched at the ciliary tip specifically upon activation of Hh signaling. They propose 4 models by which this could occur. That Hh stimulation: 1) may increase access of Kif7 into the cilium 2) that it may increase loading of Kif7 onto IFT trains 3) that it may increase Kif7 microtubule binding or 4) that it may increase Kif7 interaction with some anchoring protein found at the ciliary tip. They generate a number of mutants to test this model: a mutant that is locked to microtubules, a mutant that cannot bind microtubules, and two different mutant fused to the motor domains motile kinesins. They find that both microtubule locked and non-binding variants are able to traffick to the ciliary tip, with the non-binding variant able to respond to Hh signaling. They also show that blocking IFT trafficking blocks localization of Kif7 to the ciliary tip. Overall the work has the potential to provide significant mechanistic insight into the role of Kif7 in cilia and its contributions to Hh signaling, but a number of key points remain to be addressed prior to publication.

Major points:
The authors don't seem to fully consider the fact that the models they propose to account for Kif7 accumulation may not be mutually exclusive.

Response: We thank the reviewer for this important comment. We agree that the models may not be mutually exclusive and have adjusted the text of the manuscript (lines 81-82, 270-273) to reflect this point of view.

They conclude that their data support model 2 and exclude models 1 and 3. Though model 4 is mentioned, there is little data either supporting or excluding it. However, I'm not totally convinced models 1 and 3 can be completely excluded: Could the fusion of Kif7 to the motor domains of Kif2c or Kif21 alter access to the cilium in and of themselves, for example?

Response: The reviewer raises an important point. We do not believe that the motor domains of KIF5C or KIF21A could alter access to the cilium in and of themselves based on three experimental findings. First, KIF21A does not localize to the primary cilium whereas KIF5C can be found at cilium tip in the basal and Hh-stimulated states as shown in a new supplemental figure (new Figure S4). We have updated the text to reflect this new information (lines 119-125).

Second, our recent publication in the Journal of Cell Science (Blasius et al., 2021) shows that the truncated KIF7(1-558) localizes along the shaft of the primary cilium in the basal state, suggesting the motor domain of KIF7 can access to the cilium. We have updated the text to

reflect this new information (lines 172-174). Third, KIF7's localization to the tip of the cilium requires sequences in its C-terminal coiled-coil segment (Blasius et al., 2021). Thus, the KIF21A and KIF5C motor domains merely provide the fusion proteins with the ability to move along cytosolic microtubules (KIF21A and KIF5C) and axonemal microtubules (KIF5C). We have altered the text (lines 125-129) to reflect this important point.

We agree that at this point, we cannot provide data supporting or excluding model 4 and had mentioned this in the text (lines 270-273).

And with respect to model 3, blocking microtubule binding did allow some access of Kif7 into the cilium, which I agree is interesting and surprising. However, the noMT mutant was impaired in its ability to become enriched in the cilium upon Hh stimulation- this is the only mutant tested that was impaired in this way. That seems to suggest that microtubule binding plays at least some role in the Hh dependent enrichment. To get at whether microtubule binding might contribute partially redundantly along with IFT trafficking to Kif7 localization the authors should assess the localization of the noMT and rigor mutants in the contexts of the inhibitable Kinesin 2 motor (ie the experiment in Figure 5). I'm particularly interested in the rigor mutant fits with a model where IFT is driving most of Kif7 localization in response to Hh.

Response: We agree this is an important experiment. We have performed new experiments in *Kif3A/Kif3B-/-* cells expressing the inhibitable kinesin-2 KIF3A/KIF3B/KAP motor (i3A/i3B). As seen in the new Figure 5, inhibition of IFT blocks the Hh-induced ciliary tip localization of both the KIF7(FL)^{noMT} and KIF7(FL)^{rigor} versions of KIF7. This experiment suggests that microtubule binding is not partially redundant in driving KIF7 localization in response to Hh stimulation.

Relatedly, the noMT condition is at least somewhat impaired in its localization to the cilium upon SAG stimulation, yet this mutant fully rescues the Gli localization phenotype in the Kif7-/- cells. What are explanations for this? In other mutants, the localization of Gli2 or Gli3 to the ciliary tip seems linked to the degree to which that Kif7 mutant localizes to the ciliary tip, so the noMT mutant represents the one departure from this.

Response: We agree that the noMT variant shows reduced localization to the cilium tip in both the basal and stimulated states as compared to the WT protein, yet its Hh-stimulated increase in cilium tip localization is similar to that of WT and its ability to drive Hh-stimulated Gli2 and Gli3 ciliary localization is similar to that of WT. While these findings support our conclusion that the ability of KIF7 to bind to microtubules is dispensable for its function in promoting ciliary localization of the Gli proteins, we do not presently have a molecular explanation for this. One possibility is that only a minimal level of KIF7 is needed for full Gli localization to the tip of the primary cilium. Its also possible that KIF7 and the Gli proteins have different recycling out of the cilium that is not reflected in our static images but impacts the steady-state levels of these proteins in the cilium. We plan to carry out future experiments that can shed light on this interesting phenomenon.

Another gap in the model is that there isn't direct evidence that Kif7 is trafficked by IFT. Some live imaging data showing Kif7 trafficking with IFT in this way would strengthen this argument.

Response: We agree that live imaging data showing KIF7 trafficking with IFT would be ideal. We have tried many experiments to visualize KIF7 movement in cilia including a variety of expression systems, fluorescent protein tags, photoactivatable Halo ligands, etc. We have also tried a variety of experimental approaches to visualize other kinesins in the cilium such as the KIF3A/KIF3B/KAP IFT motor itself and the related kinesin-2 KIF17. Unfortunately, we have only been successful at live imaging of IFT88, a component of the IFT particles (Engelke et al., 2019). The inability to track kinesins in the cilium is likely due to issues with the signal-to-noise of the fluorescence signals. One issue is that the high cytoplasmic background of the expressed kinesins makes it very difficult to see any signal in the cilium. In comparison, the cytoplasmic background of IFT88 is negligible and the cilium-localized signal is thus amenable to live imaging. Another issue is that the kinesin proteins may be present in too few copies per IFT particle for them to be visualized and tracked in live-cell imaging experiments. In comparison, IFT88 may be present in multiple copies per IFT particle.

Additional points:
A large number of comparisons are being done in Fig. 3, so a t-test is not really appropriate. 2 way Anova with a post-hoc test would be better.

Response: We have performed a one-way ANOVA with post-hoc Dunnett's test.

In figure 4, it would be good to show representative images for Gli2 and Gli3 rather than having this in the supplemental data. The number of samples is also very small- just 2 replicates for some conditions. Given that this is a key piece of data, the level of rigor here should be improved with additional samples.

Response: We thank the reviewer for this suggestion. We have moved the images of Gli2 and Gli3 from the supplemental data to Figure 4. And we have repeated the experiments to ensure all samples have at least three replicates.

Line 90- what does it mean that it's uninhibited? Lack of autoinhibition? Some more context here would be helpful. We are referred to a submitted manuscript as a reference for Kif7 autoinhibition which makes this hard to evaluate. In general, the truncated mutant of Kif7 is mentioned at the beginning when the different binding mutants are introduced, but they aren't really used in subsequent experiments, and so how they come into play is somewhat confusing for a non-kinesin motor specialist. Perhaps they are controls? Clarity would improve readability.

Response: We apologize for the confusion. Yes, uninhibited means lack of autoinhibition. The manuscript that was referred to as submitted is now published in the Journal of Cell Science

(Blasius et al., 2021). We have altered the text (lines 90-95) in an effort to improve readability for non-kinesin specialists.

Reviewer #2 (Remarks to the Author):

Summary:
In this article, Yue and colleagues investigate the localization of different mutants and chimera of the kinesin-4 motor KIF7 to primary cilia in pharmacologically treated cells to stimulate the Hedgehog signaling pathway. In fixed cells KIF7 can be visualized at the ciliary tip, similar to the GLI transcription factors that are the main effectors of Hedeghog signaling. KIF7 as well as GLIs accumulate at the ciliary tip after Hedgehog pathway activation, yet, neither the significance nor the molecular mechanism for accumulation are clear at this point. KIF7 is an unusual kinesin as it appears to bind statically to microtubules in vitro, while other kinesins, such as the major ciliary kinesin-2 transports proteins in an anterograde fashion by so-called intraflagellar transport (IFT). The authors "consider four models for how Hh stimulation results in an accumulation of KIF at the tip of the primary cilium" and are trying to dissect this mechanism by transient transfection of different KIF7 variants into fibroblasts in vitro, followed by subsequent immunofluorescence microscopy analysis.

While testing different models is of high scientific value, the authors' experimental approach has major flaws, and their conclusions are merely interpretations of their data that lack substantial evidence. Some of the proposed models are ruled out prematurely, such that most models still appear valid and the main insight of this study is delivered in the title of the manuscript, reading: "...KIF7 requires intraflagellar transport but not its own microtubule binding". This conclusion is indeed supported by the findings presented here and the abstract focuses on these stronger parts that make up about a quarter of this manuscript. However, as the authors seem to focus on testing different models of regulated KIF7 transport into cilia, from which they draw potentially false conclusions, I do not recommend publication of this manuscript in its current form.

Major comments:
• One major flaw in the authors' interpretation (as well as choice of experimental strategy) is that they seem to equal MT-binding with cilia tip binding. The ability to bind to MT +ends is met by so-called +TIP proteins, none of which contains a classical MT-binding domain found in kinesins. Hence, the KIF7 variants that alter MT-binding (and mobility) are interesting but do not address the original question. Thus, -unlike stated by the authors- their "model 3" cannot be ruled out based on the fact that the MT-binding domain in KIF7 is dispensable for tip accumulation. In fact, the authors give evidence that KIF7 may be transported by IFT, which indeed makes direct MT-binding of KIF7 obsolete. Nonetheless, wild type KIF7 does accumulate at cilia tips, which may well be based on an increase in affinity of KIF7 for MT +ends in response to Hh signal.

Response: We respectfully disagree. We do not “equal MT-binding with cilium tip binding”. We are in fact directly testing whether KIF7’s MT-binding is required for its Hh-induced cilium tip localization.

The reviewer is incorrect in stating that the "ability to bind to MT +ends is met by so-called +TIP proteins". While +TIP proteins bind preferentially to the plus ends of MTs, so do several members of the kinesin superfamily. Particularly relevant is previous work showing that KIF7 binds preferentially to the plus ends of microtubules and tracks growing plus ends in a purified system *(i.e.* absence of +TIP proteins) (He et al 2014, Jiang et al 2019). The He et al. 2014 paper went on to propose that KIF7's ability to bind selectively to MT plus ends is required for its ability to organize the cilium tip compartment.

Thus, our original question was, and still is, whether KIF7's ability to bind to microtubules is required for it to accumulate at the cilium tip in response to Hh stimulation. As the reviewer states, we demonstrate that "the MT-binding domain in KIF7 is dispensable for tip accumulation." We thus conclude that a) MT-binding does NOT equal cilium tip binding and b) KIF7's role at the cilium tip does not require its own ability to bind to microtubules.

Note that our recent publication (Blasius et al., 2021) demonstrates that Hh signal does not increase the affinity of KIF7 for MT +ends and this is included in the text (lines 151-153).

• In the "motile" KIF7 variants presented here, the main motor domain is replaced with the motor domains of kinesin-1 family members, such that one can argue that their main property has been changed and the term "motile KIF7" is somewhat misleading. Hence, the statement "the finding that motile variants of KIF7 accumulate in the cilium without pathway activation rules out the possibility that Hh signaling increases the ability of KIF7 to access the ciliary compartment" (lines 152-154) is also an over-interpretation. It rules out that the chimeric proteins (that are much more closely related to kinesin-1 family members) are regulated in such a way. In fact, these findings highlight that the motor domain in KIF7 is critically important and may well be regulated in a Hh-dependent manner. These findings further question the usefulness of these chimera to study KIF7 function. Additional minor point: The increased localization of "motile" KIF7 variants to cilia in response to Hh stimulation does not appear to be significant and any conclusions from these data should be phrased accordingly.

Response: We respectfully disagree that the term "motile KIF7 is somewhat misleading". By replacing the motor domain of the kinesin-4 KIF7 (which can bind to microtubules but not move along microtubules) with the motor domains of two different motile kinesins (the kinesin-4 protein KIF21A or the kinesin-1 protein KIF5C), we have generated motile versions of KIF7.

The reviewer is incorrect in stating that the chimeric proteins are "much more closely related to kinesin-1 family members". In fact, the KIF7(FL)$^{motile\_21A}$ variant does not contain any sequences from any kinesin-1 family member. The chimeric proteins contain the motor domains (~350 aa) of KIF21A or KIF5C fused to the coiled-coil and tail domains (~1000 aa) of KIF7. Thus, the chimeric proteins are mainly comprised of sequences from KIF7 including the regions required for a) cilium tip localization (Blasius et al., 2021), b) binding to Gli transcription factors (Cheung et al 2009, Endoh-Yamagami et. al 2009), and c) dephosphorylation by the concerted action of liprin-α1 (PPFIA1) and phosphatase PP2A. We have included new text (lines 119-129) and a new schematic (new Figure 2A) to clarify this point for the reader.

Additional minor point: we agree that the Hh-induced localization of the motile KIF7 variants is not significant. Thus, our only conclusions from these results concern the access to the cilium in the unstimulated state as described on lines 168-171: “Thus, access of KIF7 to the ciliary compartment is not restricted in the unstimulated state. These results suggest that a Hh-induced increase in the ability of KIF7 to access the ciliary compartment (model 1, Figure 1) is unlikely to be the primary mechanism by which Hh stimulation causes an increase in KIF7 localization to the cilium tip.”

• Based on the above, it cannot be ruled out that there is a Hh-dependent increase in the ability of KIF7 variants to access cilia. Hence, in contrast to the authors' conclusions their "model 1" cannot be ruled out. Moreover, as the affinity to MT +ends may be altered in a Hh-dependent manner, their "model 3" cannot be excluded either. To unambiguously rule out models 1 and 3 and further support models 2 and 4, which appear to be the authors' favorite, additional evidence is required.

Response: Based on our above responses concerning the motile KIF7 variants, we feel we can rule out a Hh-dependent increase in the ability of KIF7 variants to access cilia. Additional evidence against model 1 can be found in our recent work (Blasius et al., 2021) where we demonstrate that 1) the truncated KIF7(1-558) localizes along the shaft of the primary cilium, suggesting that KIF7 is not restricted from accessing the cilium in the unstimulated state (lines 172-174). Additional evidence against model 3 also comes from our recent work (Blasius et al., 2021) where we demonstrate that the affinity of KIF7 for microtubule plus ends is not altered in a Hh-dependent manner (lines 151-153).

Minor point:
• The simplest explanation for many observations in this study is that there is a constant fraction of KIF7 (or its variants, as well as GLI proteins) that localize to the cilia tip and that differences in the protein levels may lead to an apparent increase at the cilia tip. Hence, assessing the expression levels of the individual proteins is absolutely critical, and the authors should show that the protein levels after transient expression of the different KIF7 variants, GLI2 and GLI3 are equal (detecting the epitope tags by Western Blotting may be a valid option).

Response: Assessing the expression levels of KIF7 requires quantitative fluorescence microscopy of individual cells and cannot be assessed by western blotting across the population of cells. We have carried out quantitative fluorescence microscopy and demonstrated that differences in localization to cytosolic microtubules and to the cilium tip are not caused by differences in expression level of KIF7 (see below Supplemental Figure 1 of Blasius et al., 2021). We have added this information to the Material and Methods (lines 340-342).

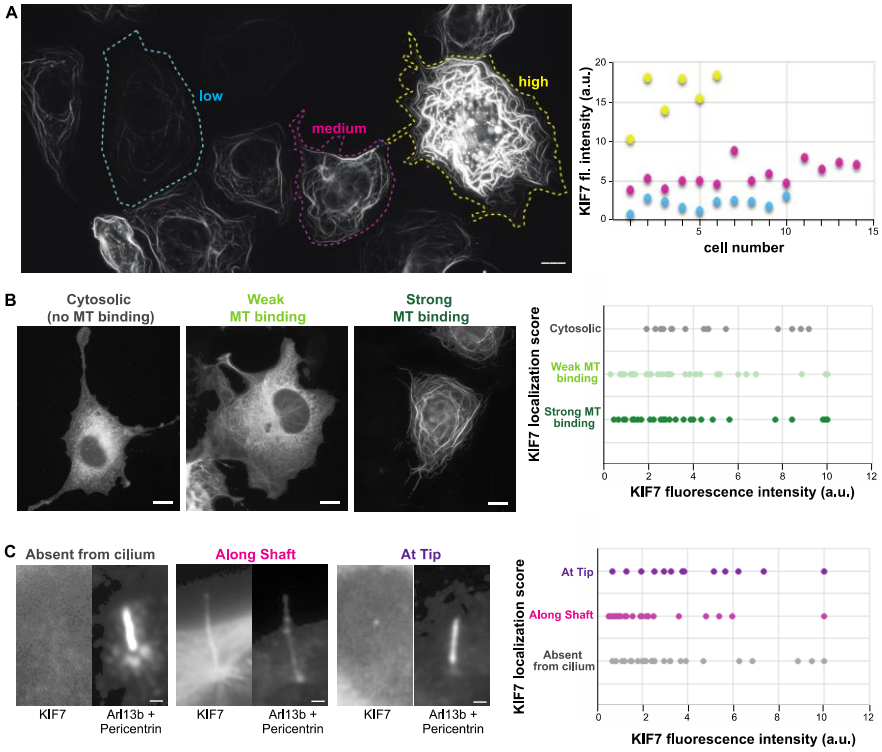

Figure S1. KIF7 localization does not correlate with level of expression. (A) Left, representative image of COS-7 cells expressing KIF7(1-1114). Scale bars, 10 μm. Right, plot of average KIF7(1-1114) fluorescence intensity per cell for 30 randomly chosen cells. Expression was defined as high, medium, or low expression and only cells with medium or low expression were included in the analysis. (B) Criteria for microtubule (MT) localization. Left, representative images of MT localization phenotypes in COS-7 cells. Scale bars, 10 μm. If the expressed protein exhibited a cytoplasmic/diffuse localization, this was scored as 'no MT binding'. If the expressed protein could be seen both in the cytoplasm and on microtubules, this was scored as 'weak MT binding'. If the exogenous protein could only be seen on MTs, this was scored as 'strong MT binding'. Right, plot of average KIF7 fluorescence intensity across MT binding phenotypes. Each spot represents the average KIF7 fluorescence intensity of one cell across a total of 70 randomly selected cells. (C) Criteria for cilium localization. Left, representative images of cilium localization phenotypes in NIH-3T3 cells. Scale bars, 1 μm. If the expressed protein could not be detected in the cilium, this was scored as 'absent from cilium'. If the expressed protein was observed at the base of the cilium (defined by presence of pericentrin staining), this was scored as 'at base' (not shown). If the expressed protein was observed at the tip of the cilium (opposite end as pericentrin staining), the localization was scored as 'at tip'. If the expressed protein was distributed uniformly along the cilium, the localization was scored as 'along shaft'. Right, plot of average KIF7 fluorescence intensity across cilium localization phenotypes. Each spot represents the average KIF7 fluorescence intensity of one cell across a total of 60 randomly selected cells.

• It is somewhat unclear how cilia tip localization of KIF7 (and GLIs) is defined. The authors should give a metric. If cilia tip localization of proteins is based on subjective assessment, the authors should try to implement a more unbiased way based on quantitative assessment of fluorescence intensities, such that the data stands on solid ground and can be reproduced more easily.

Response: We apologize that the quantification was not adequately described and have added new text to the Materials and Methods section (lines 344-349).

• The "rigor" mutant appears to give different results for the cilia tip localization of GLI2 vs. GLI3 (see Figure 4), which is unexpected. Interestingly, the data presented for this mutant appears to be the only analysis (in Figure 4) based on three independent experiments. Given the spread of the data presented a third replicate for all mutants would be helpful, such that error assessment and statistical testing could be performed to strengthen the authors' conclusions.

Response: We thank the reviewer for this important point. We have now repeated the experiments in Figure 4 to be sure that each mutant is assessed in at least three independent experiments and statistical testing finds that the rigor mutant is able to facilitate the tip localization of Gli2 and Gli3.

• When discussing different motilities of ciliary motor proteins (lines 246-247) the authors should also discuss the different ciliary kinesin motors found in C. elegans, which have been reported exhibit different velocities ,and compare to KIF7 and kinesin-2 in their system.

Response: We are unsure what point the reviewer is trying to make since we do not discuss "different motilities of ciliary motor proteins". We only state that "several other kinesins have been identified to play a role in ciliary transport and/or assembly (Lechtreck, 2015; He *et al.*, 2017; Reilly and Benmerah, 2019)" (lines 217-220).

The velocity of KIF7 is irrelevant since this is an immotile kinesin, as shown by several groups (He et al, 2014; Yue et al, 2019, Jiang et al 2019). As to kinesin-2 in our system, we do not discuss the velocities of the mammalian kinesin-2 motors, and the velocities of the *C* elegans kinesin-2 motors thus seem irrelevant.

• "Immotile behavior" should be rephrased.

Response: Unless the reviewer has a better suggestion, we believe the term "immotile behavior" accurately describes the behavior of the immotile kinesin KIF7.

Significance:
• The chemogenetic "i3Ai3B" system that allows the authors to draw the conclusion as stated in the title was published in Engelke et al., 2019, where it was used in a beautiful study to analyze KIF17's function in IFT. The findings on KIF7(WT) presented here are an interesting additional observation made possible by this powerful system, however, the follow up experiments lack the rigorousness required to draw strong conclusions, such as ruling out various proposed models of KIF7 accumulation at cilia tips.

Response: We have carried out additional experiments and rewritten portions of the text to build on the powerful i3Ai3B system (new Figure 5, lines 236-245).

• I believe the manuscript in its current form is of limited value to the community as it appears to be of interest mainly for a specialist readership. Most importantly, however, the specialist readership will also recognize that the authors fail to adequately dissect the proposed models due to over-interpretation of the presented data, such that they will be left with an interesting observation that KIF7 cilia localization appears independent of its microtubule binding domain. Whether this is sufficient for publication in MBoC is unclear.

Response: We believe the manuscript will be of interest to researchers studying formation and function of the primary cilium, mechanisms of Hedgehog signaling, the functions of kinesin motors, and regulation of microtubule dynamics.

• In order to substantiate the observations and give additional evidence for IFT-dependent transport of KIF7 (model 2), the authors could try to visualize and quantitate IFT of the KIF7 variants -admittedly, a challenging task. However, this would dramatically increase the significance of the study with new possibilities to mechanistically dissect the process.

Response: We agree that live imaging data showing KIF7 trafficking with IFT would be ideal. We have tried many experiments to visualize KIF7 movement in cilia including a variety of expression systems, fluorescent protein tags, photoactivatable Halo ligands, etc. We have also tried a variety of experimental approaches to visualize other kinesins in the cilium such as the KIF3A/KIF3B/KAP IFT motor itself and the related kinesin-2 KIF17. Unfortunately, we have only been successful at live imaging of IFT88, a component of the IFT particles (Engelke et al., 2019). The inability to track kinesins in the cilium is likely due to issues with the signal-to-noise of the fluorescence signals. One issue is that the high cytoplasmic background of the expressed kinesins makes it very difficult to see any signal in the cilium. In comparison, the cytoplasmic background of IFT88 is negligible and the cilium-localized signal is thus amenable to live imaging. Another issue is that the kinesin proteins may be present in too few copies per IFT particle for them to be visualized and tracked in live-cell imaging experiments. In comparison, IFT88 may be present in multiple copies per IFT particle.

• Another possibility to increase the significance of this study would be to assess the functional consequences of the different KIF7 chimera for Hh signaling.

Response: We thank the reviewer for the suggestion. In the future, we plan to examine how KIF7's MT-binding ability impacts its role in Hh signaling, especially with respect to Gli protein processing and turnover and changes in gene expression. Since these experiments require biochemical assays (not microscopy-based assays), we first need to generate stable cell lines expressing each of the KIF7 variants described in this manuscript.

RE: Manuscript #E21-04-0215R
TITLE: "Hedgehog-induced ciliary trafficking of kinesin-4 motor KIF7 requires intraflagellar transport but not KIF7's microtubule binding"

Dear Dr. Verhey:

Thank you sending your revised manuscript to MBoC.

You'll see that I sent it back to both of the original reviewers. One now fully supports publication with minor revisions. The other has remaining concerns, but in my view these can be addressed through minor revisions and clarifications.

Reviewer #1's suggestions are relatively straightforward to address and mainly involve simplifications to the text and minor wording changes. Reviewer #2's comments are somewhat more extensive and can be found in the attached file. Although they have remaining concerns about the extent to which the data support some of the conclusions, it seems to me that these can be addressed by revisions to the tone of the language used to state the conclusions. I encourage you to make appropriate minor revisions to address the concerns of the more skeptical reader, as these will ultimately strengthen the manuscript.

I look forward to receiving a revised manuscript that addresses these remaining concerns.

Sincerely,
Matthew Welch
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Verhey,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):
Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The authors have addressed all of my prior concerns adequately. The following are minor points that I recommend correcting in the final version:

page 3-4, the first paragraph of the results section is a bit hard to follow still. I think bringing up the use of the truncated mutant that isnt' actually part of the paper gets very confusing. I would cut down on some of the backstory and make this as succinct as possible.

Lines 153-154- "These findings call into question previous suggestions that KIF7's cellular function is to regulate microtubule dynamics at the tip of the primary cilium" I find this to be overstated, given that the mutant cilia have axonemal structural defects linked to the microtubules. The statement goes a bit beyond the conclusions of the paper, in my opinion.

Reviewer #2 (Remarks to the Author):

Point-by-point response can be found in the attached file.

We thank the reviewers and the editor for their further time and effort in reviewing our manuscript. Our response to each point is detailed below. The flow of the text is colored as:
black text = reviewers' comments round 1
red text = our response in round 1
blue text = reviewers' comments round 2
magenta text = our response

Changes within the main text are also indicated in magenta text.

Reviewer #1 (Remarks to the Author):

The authors have addressed all of my prior concerns adequately. The following are minor points that I recommend correcting in the final version:

page 3-4, the first paragraph of the results section is a bit hard to follow still. I think bringing up the use of the truncated mutant that isnt' actually part of the paper gets very confusing. I would cut down on some of the backstory and make this as succinct as possible.

Response: We have moved the text describing the truncated KIF7(1-558) to the legends of Figures S1 and S3. As a result, the main text only describes results with the full-length protein and we have thus changed all designations of KIF7(FL) to KIF7 in the text and in the figures.

Lines 153-154- "These findings call into question previous suggestions that KIF7's cellular function is to regulate microtubule dynamics at the tip of the primary cilium" I find this to be overstated, given that the mutant cilia have axonemal structural defects linked to the microtubules. The statement goes a bit beyond the conclusions of the paper, in my opinion.

Response: We have removed the sentence.

Reviewer #2 (Remarks to the Author):

I am happy that Yue and colleagues accepted some of the reviewers' comments to improve their manuscript. Their newly published complementary study (Blasius et al.,2021) adds additional data to be considered. I really appreciate the authors' openness in disagreeing with some of my commentary. Open discussion is the basis for scientific progress, which can only be made by falsifying as well as verifying

hypothetical models as they are presented by the authors. Nevertheless, I still respectfully disagree with some of the conclusions drawn by the authors based on the presented data here. I, therefore, suggest further improvements to the current manuscript (see below in blue writing).

Most importantly, I do not share the authors' conclusion that "Hedgehog stimulation increases the loading of KIF7 and Gli proteins onto intraflagellar transport trains" (lines 43-44), as there is no direct evidence presented. Although this model is only "proposed" in the abstract, I strongly recommend removing this sentence from the abstract. At this point in time, this model still appears too speculative.

Response: We have removed the sentence from the Abstract.

Reviewer #2 (Remarks to the Author):

Summary:
In this article, Yue and colleagues investigate the localization of different mutants and chimera of the kinesin-4 motor KIF7 to primary cilia in pharmacologically treated cells to stimulate the Hedgehog signaling pathway. In fixed cells KIF7 can be visualized at the ciliary tip, similar to the GLI transcription factors that are the main effectors of Hedeghog signaling. KIF7 as well as GLIs accumulate at the ciliary tip after Hedgehog pathway activation, yet, neither the significance nor the molecular mechanism for accumulation are clear at this point. KIF7 is an unusual kinesin as it appears to bind statically to microtubules in vitro, while other kinesins, such as the major ciliary kinesin-2 transports proteins in an anterograde fashion by so-called intraflagellar transport (IFT). The authors "consider four models for how Hh stimulation results in an accumulation of KIF at the tip of the primary cilium" and are trying to dissect this mechanism by transient transfection of different KIF7 variants into fibroblasts in vitro, followed by subsequent immunofluorescence microscopy analysis.

While testing different models is of high scientific value, the authors' experimental approach has major flaws, and their conclusions are merely interpretations of their data that lack substantial evidence. Some of the proposed models are ruled out prematurely, such that most models still appear valid and the main insight of this study is delivered in the title of the manuscript, reading: "...KIF7 requires intraflagellar transport but not its own microtubule binding". This conclusion is indeed supported by the findings presented here and the abstract focuses on these stronger parts that make up about a quarter of this manuscript. However, as the authors seem to focus on testing different models of regulated KIF7 transport into cilia, from which they draw potentially false conclusions, I do not recommend publication of this manuscript in its current form.

Major comments:
• One major flaw in the authors' interpretation (as well as choice of experimental strategy) is that they seem to equal MT-binding with cilia tip binding. The ability to bind to MT +ends is met by so-called +TIP proteins, none of which contains a classical MT-binding domain found in kinesins. Hence, the KIF7 variants that alter MT-binding (and mobility) are interesting but do not address the original question. Thus, -unlike stated by the authors- their "model 3" cannot be ruled out based on the fact that the MT-binding domain in KIF7 is dispensable for tip accumulation. In fact, the authors give evidence that KIF7 may be transported by IFT, which indeed makes direct MT-binding of KIF7 obsolete. Nonetheless, wild

type KIF7 does accumulate at cilia tips, which may well be based on an increase in affinity of KIF7 for MT +ends in response to Hh signal.

Response: We respectfully disagree. We do not "equal MT-binding with cilium tip binding". We are in fact directly testing whether KIF7's MT-binding is required for its Hh-induced cilium tip localization.

The reviewer is incorrect in stating that the "ability to bind to MT +ends is met by so-called +TIP proteins". While +TIP proteins bind preferentially to the plus ends of MTs, so do several members of the kinesin superfamily. Particularly relevant is previous work showing that KIF7 binds preferentially to the plus ends of microtubules and tracks growing plus ends in a purified system (i.e. absence of +TIP proteins) (He et al 2014, Jiang et al 2019). The He et al. 2014 paper went on to propose that KIF7's ability to bind selectively to MT plus ends is required for its ability to organize the cilium tip compartment.

Thus, our original question was, and still is, whether KIF7's ability to bind to microtubules is required for it to accumulate at the cilium tip in response to Hh stimulation. As the reviewer states, we demonstrate that "the MT-binding domain in KIF7 is dispensable for tip accumulation." We thus conclude that a) MT-binding does NOT equal cilium tip binding and b) KIF7's role at the cilium tip does not require its own ability to bind to microtubules.

Note that our recent publication (Blasius et al., 2021) demonstrates that Hh signal does not increase the affinity of KIF7 for MT +ends and this is included in the text (lines 151-153).

I want to apologize if some commentary has upset the authors, I did not mean to be disrespectful, but my misunderstanding might reflect the fact that certain points could have been presented more clearly to non-kinesin experts. I am very thankful for pointing out additional literature, which was not available at time of review (Blasius et al., 2021) or slipped my attention (Jiang et al., 2019).

Interestingly, after studying Jiang et al., 2019, I noted that the question of whether MT-binding of KIF7 is required for tip binding has been addressed in this study and they found MT-binding to be required for cilium-tip localization -particularly during Hedgehog signaling (based on a KIF7 mutant with ~8fold lower affinity to MTs). Since the authors now present new data suggesting that tip localization of KIF7 appears to be independent of MT-binding, it would be helpful to discuss this seemingly contrasting finding, and point out more clearly why they think their study is superior over previous studies.

Response: We have added new text to discuss these seemingly contrasting findings (lines 142-148).

• In the "motile" KIF7 variants presented here, the main motor domain is replaced with the motor domains of kinesin-1 family members, such that one can argue that their main property has been changed and the term "motile KIF7" is somewhat misleading. Hence, the statement "the finding that motile variants of KIF7 accumulate in the cilium without pathway activation rules out the possibility that Hh signaling increases the ability of KIF7 to access the ciliary compartment" (lines 152-154) is also an over-interpretation. It rules out that the chimeric proteins (that are much more closely related to kinesin-1 family members) are regulated in such a way. In fact, these findings highlight that the motor domain in KIF7 is critically important and may well be regulated in a Hh-dependent manner. These findings further question the usefulness of these chimera to study KIF7 function. Additional minor point:

The increased localization of "motile" KIF7 variants to cilia in response to Hh stimulation does not appear to be significant and any conclusions from these data should be phrased accordingly.

Response: We respectfully disagree that the term "motile KIF7 is somewhat misleading". By replacing the motor domain of the kinesin-4 KIF7 (which can bind to microtubules but not move along microtubules) with the motor domains of two different motile kinesins (the kinesin-4 protein KIF21A or the kinesin-1 protein KIF5C), we have generated motile versions of KIF7.

The reviewer is incorrect in stating that the chimeric proteins are "much more closely related to kinesin-1 family members". In fact, the KIF7(FL) $^{motile\_21A}$ variant does not contain any sequences from any kinesin-1 family member. The chimeric proteins contain the motor domains (~350 aa) of KIF21A or KIF5C fused to the coiled-coil and tail domains (~1000 aa) of KIF7. Thus, the chimeric proteins are mainly comprised of sequences from KIF7 including the regions required for a) cilium tip localization (Blasius et al., 2021), b) binding to Gli transcription factors (Cheung et al 2009, Endoh-Yamagami et. al 2009), and c) dephosphorylation by the concerted action of liprin-α1 (PPFIA1) and phosphatase PP2A. We have included new text (lines 119-129) and a new schematic (new Figure 2A) to clarify this point for the reader.

While I also respectfully disagree about the semantics, I do think that including the schematic clearly helps non-specialists to grasp the introduced changes in KIF7. I am afraid my phrasing could have been more precise: on a whole sequence level the KIF7 variants contain more sequence elements of KIF7, of course. However, the central motor domain -if exchanged for KIF5C- resembles a kinesin-1 chimera. We agree that the KIF21A domain keeps the chimera a kinesin-4 family member, for which the term "mobile KIF7" is more adequate than for the KIF5C fusion.

Nevertheless, the gist of the criticism here is: The authors do not show convincing data (to this reviewer) to rule out the proposed models. More specifically, they do not show that Hedgehog signaling does NOT increase the ability of KIF7 to access the ciliary compartment, which is of course hard to show with the presented experimental approaches. Their data using "motile KIF7", which have introduced changes to an absolutely essential domain that changes basic characteristics does not "rule out" the possibility that indeed Hedgehog signals increase the ability of a wild type KIF7 to access the ciliary compartment. Therefore, I still insist that the authors' "model 3" cannot be ruled out based on their interpretation of one line of experiments. To unambiguously rule out "model 3" would require additional experiments, such as live cell microscopy, which probably is beyond the scope of this study.

Response: We agree that further work would be required to unambiguously rule out model 3. We thus do not state that we have ruled out model 3 but rather state that the presented data do not support model 3.

Additional minor point: we agree that the Hh-induced localization of the motile KIF7 variants is not significant. Thus, our only conclusions from these results concern the access to the cilium in the unstimulated state as described on lines 168-171: "Thus, access of KIF7 to the ciliary compartment is not restricted in the unstimulated state. These results suggest that a Hh-induced increase in the ability of KIF7 to access the ciliary compartment (model 1, Figure 1) is unlikely to be the primary mechanism by which Hh stimulation causes an increase in KIF7 localization to the cilium tip."

• Based on the above, it cannot be ruled out that there is a Hh-dependent increase in the ability of KIF7 variants to access cilia. Hence, in contrast to the authors' conclusions their "model 1" cannot be ruled out. Moreover, as the affinity to MT +ends may be altered in a Hh-dependent manner, their "model 3" cannot be excluded either. To unambiguously rule out models 1 and 3 and further support models 2 and 4, which appear to be the authors' favorite, additional evidence is required.

Response: Based on our above responses concerning the motile KIF7 variants, we feel we can rule out a Hh-dependent increase in the ability of KIF7 variants to access cilia. Additional evidence against model 1 can be found in our recent work (Blasius et al., 2021) where we demonstrate that 1) the truncated KIF7(1-558) localizes along the shaft of the primary cilium, suggesting that KIF7 is not restricted from accessing the cilium in the unstimulated state (lines 172-174). Additional evidence against model 3 also comes from our recent work (Blasius et al., 2021) where we demonstrate that the affinity of KIF7 for microtubule plus ends is not altered in a Hh-dependent manner (lines 151-153).

I respectfully disagree. Even if the authors _feel_ they can rule out a Hh-dependent increase in the ability of KIF7 to access cilia, this is not fully supported by the presented data. Such statements should therefore be avoided and rephrased. If the authors “suggest the scenario unlikely”, this will give the reader space for their own interpretation -if they can follow the logic.

Response: We had removed the words “rule out” from the manuscript in response to the reviewer’s comments in round 1. We thus have not made any further alterations to the text as we only use the words “strongly support” or “do not support” or “argue against” to indicate whether the findings are consistent with a particular model presented in Figure 1.

Minor point:
• The simplest explanation for many observations in this study is that there is a constant fraction of KIF7 (or its variants, as well as GLI proteins) that localize to the cilia tip and that differences in the protein levels may lead to an apparent increase at the cilia tip. Hence, assessing the expression levels of the individual proteins is absolutely critical, and the authors should show that the protein levels after transient expression of the different KIF7 variants, GLI2 and GLI3 are equal (detecting the epitope tags by Western Blotting may be a valid option).

Response: Assessing the expression levels of KIF7 requires quantitative fluorescence microscopy of individual cells and cannot be assessed by western blotting across the population of cells. We have carried out quantitative fluorescence microscopy and demonstrated that differences in localization to cytosolic microtubules and to the cilium tip are not caused by differences in expression level of KIF7 (see below Supplemental Figure 1 of Blasius et al., 2021). We have added this information to the Material and Methods (lines 340-342).

This is an impressive experiment (of their new, complementary study) and clearly argues against my hypothesis. The authors may want to add this information as a note in the main text of this study.

Response: We have now added this information to the main text (lines 132-133).

• It is somewhat unclear how cilia tip localization of KIF7 (and GLIs) is defined. The authors should give a metric. If cilia tip localization of proteins is based on subjective assessment, the authors should try to implement a more unbiased way based on quantitative assessment of fluorescence intensities, such that the data stands on solid ground and can be reproduced more easily.

Response: We apologize that the quantification was not adequately described and have added new text to the Materials and Methods section (lines 344-349).

If the analysis was done as rigorously as now stated in the Materials and Methods section, I suggest the average intensities after background correction should be used as a more quantitative measure for tip localization (rather than the binary "positive" vs. "negative", for which I consider a >0 cutoff inadequate)? The more quantitative data would clearly strengthen the manuscript.

Response: The major effect of Hh stimulation is an increase in the percentage of cells with KIF7 (and Gli proteins) at the tip of the primary cilium, not a change in their average intensities. We thus report the data as the percentage of cells with KIF7 or Gli2 or Gli3 at the cilium tip in the unstimulated vs Hh-stimulated states.

• The "rigor" mutant appears to give different results for the cilia tip localization of GLI2 vs. GLI3 (see Figure 4), which is unexpected. Interestingly, the data presented for this mutant appears to be the only analysis (in Figure 4) based on three independent experiments. Given the spread of the data presented a third replicate for all mutants would be helpful, such that error assessment and statistical testing could be performed to strengthen the authors' conclusions.

Response: We thank the reviewer for this important point. We have now repeated the experiments in Figure 4 to be sure that each mutant is assessed in at least three independent experiments and statistical testing finds that the rigor mutant is able to facilitate the tip localization of Gli2 and Gli3.

• When discussing different motilities of ciliary motor proteins (lines 246-247) the authors should also discuss the different ciliary kinesin motors found in C. elegans, which have been reported exhibit different velocities ,and compare to KIF7 and kinesin-2 in their system.

Response: We are unsure what point the reviewer is trying to make since we do not discuss "different motilities of ciliary motor proteins". We only state that "several other kinesins have been identified to play a role in ciliary transport and/or assembly (Lechtreck, 2015; He et al., 2017; Reilly and Benmerah, 2019)" (lines 217-220).

The velocity of KIF7 is irrelevant since this is an immotile kinesin, as shown by several groups (He et al, 2014; Yue et al, 2019, Jiang et al 2019). As to kinesin-2 in our system, we do not discuss the velocities of the mammalian kinesin-2 motors, and the velocities of the C elegans kinesin-2 motors thus seem irrelevant.

• "Immotile behavior" should be rephrased.

Response: Unless the reviewer has a better suggestion, we believe the term “immotile behavior” accurately describes the behavior of the immotile kinesin KIF7.

The phrase seems to be common to kinesin specialists and can be kept if these are the only readers addressed. To this reviewer it seems odd that the phrase “immotile behavior” is used instead of the simpler “immotility”, but this should be left to the authors to decide.

Response: We appreciate the reviewer’s perspective but prefer to keep the phrase “immotile behavior”

Significance:
• The chemogenetic "i3Ai3B" system that allows the authors to draw the conclusion as stated in the title was published in Engelke et al., 2019, where it was used in a beautiful study to analyze KIF17's function in IFT. The findings on KIF7(WT) presented here are an interesting additional observation made possible by this powerful system, however, the follow up experiments lack the rigorousness required to draw strong conclusions, such as ruling out various proposed models of KIF7 accumulation at cilia tips.

Response: We have carried out additional experiments and rewritten portions of the text to build on the powerful i3Ai3B system (new Figure 5, lines 236-245).

Interesting new data, indeed, but it reinforces my earlier notion about the misleading nomenclature. “Motile_5C” and “motile_21A” KIF7 variants do not behave like kinesin-4 members anymore, so the comparison is somewhat ambiguous.

Response: We are not sure what the reviewer means by the statement “do not behave like kinesin-4 members anymore.” Kinesin-4s constitute a broad class of kinesins so it would be difficult to pin down their behavior. For example, KIF4 is a motile kinesin that is commonly referred to as chromokinesin as it contributes to chromosome congression in mammalian cell division. KIF7 is immotile and functions in Gli localization to the cilium tip and proteolytic processing upon Hh simulation. KIF27 is a slow motile kinesin that functions in motile cilia. KIF21A and KIF21B regulate microtubule dynamics and play important roles in neural outgrowth and centrosome relocalization upon T cell stimulation, respectively.

• I believe the manuscript in its current form is of limited value to the community as it appears to be of interest mainly for a specialist readership. Most importantly, however, the specialist readership will also recognize that the authors fail to adequately dissect the proposed models due to over-interpretation of the presented data, such that they will be left with an interesting observation that KIF7 cilia localization appears independent of its microtubule binding domain. Whether this is sufficient for publication in MBoC is unclear.

Response: We believe the manuscript will be of interest to researchers studying formation and function of the primary cilium, mechanisms of Hedgehog signaling, the functions of kinesin motors, and regulation of microtubule dynamics.

• In order to substantiate the observations and give additional evidence for IFT-dependent transport of KIF7 (model 2), the authors could try to visualize and quantitate IFT of the KIF7 variants -admittedly, a challenging task. However, this would dramatically increase the significance of the study with new possibilities to mechanistically dissect the process.

Response: We agree that live imaging data showing KIF7 trafficking with IFT would be ideal. We have tried many experiments to visualize KIF7 movement in cilia including a variety of expression systems, fluorescent protein tags, photoactivatable Halo ligands, etc. We have also tried a variety of experimental approaches to visualize other kinesins in the cilium such as the KIF3A/KIF3B/KAP IFT motor itself and the related kinesin-2 KIF17. Unfortunately, we have only been successful at live imaging of IFT88, a component of the IFT particles (Engelke et al., 2019). The inability to track kinesins in the cilium is likely due to issues with the signal-to-noise of the fluorescence signals. One issue is that the high cytoplasmic background of the expressed kinesins makes it very difficult to see any signal in the cilium. In comparison, the cytoplasmic background of IFT88 is negligible and the cilium-localized signal is thus amenable to live imaging. Another issue is that the kinesin proteins may be present in too few copies per IFT particle for them to be visualized and tracked in live-cell imaging experiments. In comparison, IFT88 may be present in multiple copies per IFT particle.

• Another possibility to increase the significance of this study would be to assess the functional consequences of the different KIF7 chimera for Hh signaling.

Response: We thank the reviewer for the suggestion. In the future, we plan to examine how KIF7's MT-binding ability impacts its role in Hh signaling, especially with respect to Gli protein processing and turnover and changes in gene expression. Since these experiments require biochemical assays (not microscopy-based assays), we first need to generate stable cell lines expressing each of the KIF7 variants described in this manuscript.

These points are well taken.

Response: Thank you.

RE: Manuscript #E21-04-0215RR
TITLE: "Hedgehog-induced ciliary trafficking of kinesin-4 motor KIF7 requires intraflagellar transport but not KIF7's microtubule binding"

Dear Dr. Verhey:

Thanks for submitting your revised manuscript to MBoC. I am pleased to accept it for publication.

Sincerely,
Matthew Welch
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Verhey:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

Would you like to see an image related to your accepted manuscript on the cover of MBoC? Please contact the MBoC Editorial Office at mboc@ascb.org to learn how to submit an image.

Authors of Articles and Brief Communications are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
