JCB

HOOK3 is a scaffold for the opposite-polarity microtubule-based motors cytoplasmic dynein and KIF1C

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Re: JCB manuscript #201812170

Dr. Samara Reck-Peterson
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La Jolla, CA 92093

Dear Dr. Reck-Peterson,

Thank you for submitting your manuscript entitled "HOOK3 is a scaffold for the opposite-polarity microtubule-based motors cytoplasmic dynein and KIF1C". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers are interested in how bidirectional transport is controlled by the simultaneous binding of these motors to HOOK3. In addition to the specific concerns about the data presented that should be addressed to substantiate the paper's main claims, a revision should mainly focus on establishing physiological relevance.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu.

Sincerely,

Tarun Kapoor, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Kendrick et al. follow up on a previously identified interaction between the dynein activator HOOK3 and the kinesin-3 motor KIF1C by demonstrating the formation of a co-complex of KIF1C with HOOK3 and dynein/dynactin. While HOOK3 is known to activate dynein, the binding of HOOK3 to KIF1C does not affect the processive movement of the kinesin-3 motor. The authors propose that linking dynein and kinesin motors by dynein activating adaptors may be a general mechanism to regulate bidirectional motility.

The manuscript is clearly written and presented, and describes a potentially interesting finding - that a co-complex of HOOK3, KIF1C and dynein/dynactin can be formed. However, the overall novelty and impact of the work is limited, as detailed below. Most significantly, this work is limited to in vitro experiments, and provides no insights into the possible role of HOOK3 in mediating bidirectional transport in cells. Thus, it falls short of the impact expected for a publication in JCB, and is more appropriate for publication in another journal.

1. The authors have described the HOOK3-KIF1C interaction in a previous publication; the additional pulldowns here are confirmatory but not novel. They map the interaction domain on KIF1C, but not very precisely on HOOK3, and they primarily rely on truncations rather than point mutations to perturb the interaction.
2. The demonstration that HOOK3, either truncated or full-length, activates dynein has been published by multiple labs, and thus is confirmatory and can be moved to the supplement.
3. Cellular or in vivo data are completely lacking, so it is unclear how relevant these observations are to trafficking in cells. It should be straightforward to test the effects of truncation and point

mutations disrupting these interactions on organelle motility, and this would be required to meet the expected standard for a JCB publication.
4. No data are included describing the relative frequencies of motility in either direction - in the mixed motor assays, which motor predominates?
5. How is HOOK3 regulated to favor either kinesin-driven or dynein-driven motility?
6. The authors propose that scaffolding of dynein and kinesin motors may be a general principle of activating adaptors. This would be a stronger point if the authors were to include supportive data from BICD2 or BICDL1 experiments - the authors note that BICDL1 has previously been shown to interact with KIF1C so this may be the obvious candidate to focus on.

Additional minor points:
1. The authors should take more effort to cite primary publications rather than reviews when possible.
2. The authors should more precisely state the number of observations per condition, for example in Fig. 6D, which indicates a very broad range of n values (16-238).

In summary, this work is clean but not particularly novel or impactful, as it replicates previously published findings on the interaction of HOOK3 with KIF1C and the activation of dynein by HOOK3. Significantly, no data are provided to support the possible import of this mechanism in cells, despite the generation of engineered cell lines that could readily be used for live imaging experiments. Given this lack, the work in its current form is better suited to a biochemical or biophysical journal such as JBC or J. Biophys.

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, Kendrick et al. investigate the coupling of a kinesin and dynein mediated through the HOOK3 adaptor protein. The authors report that HOOK3 specifically binds a kinesin-3 family member Kif1c, and that this binding does not affect the velocity or the run length of the motor. Further, the authors map this interaction to specific regions in each of the proteins. Finally, the authors show that HOOK3 can serve as a scaffold, binding to both Kif1c and dynein, and supporting motility driven by either one or the other motor. The manuscript is straightforward and clearly written. The analysis of the tripartite complex is relatively limited and could be expanded on. In general, it would be interesting to determine the stoichiometry of individual proteins in observed complexes. While the authors demonstrate that the tripartite complexes can form, and can move in either direction, it is not clear whether such minimal complexes indeed assemble within cells, thus the physiological relevance is somewhat elusive.

Specific comments:

Figure 2.
The authors report that HOOK3 binding does not affect the motility properties of the Kif1c motors. However, it appears that the authors did not quantify the frequency of non-motile or pausing motors with or without HOOK3, which are readily seen in their kymographs. This may be particularly relevant in the context of the competing paper, which reports that Kif1c is autoinhibited on its own. Similarly, the quantification of Kif1c microtubule-landing rates in the presence and absence of HOOK3 would be beneficial.

The photobleaching analysis (Figure S1) yields that the majority of motile Kif1c molecules are dimers. Is this also the case with HOOK3 bound? Note that the fluorescence intensity of Kif1c

traces shown in kymographs (Figure 2A &2D) appears highly variable, with some traces looking much more intense than others. These examples seem to contradict the photobleaching analysis?

Similarly, while the authors report that HOOK3 has no effect on the Kif1c velocity, the kymograph in Figure 2D shows HOOK3-associated tracks significantly faster than the Kif1c alone track? Is this kymograph not representative?

Figure 3.
Given the reported interaction of HOOK3CT with Kif1C, it would be useful (and presumably easy to generate this short purified construct) to show colocalization of this construct with Kif1C as a positive control.

Figure 4.
Although the mean velocity and run lengths of dynein appear the same regardless of the activating HOOK3 construct, the pausing behavior looks qualitatively different. Here again, it would be beneficial to quantify non-motile and pausing motor molecules for any potential differences induced by the activating constructs.

Figure 5.
The schematic in Figure 5A including cargo is confusing, since the authors provide no evidence for either the model in which cargo is or isn't mediating the interaction. I would suggest dropping this schematic, and combining Figures 5 &6 into a single figure characterizing the complex.

Figure 6.
This is the most interesting figure of the paper, but it leaves much to be desired.
For example, what is the frequency of individual complexes (i.e. how many tracks have HOOK3 complexed with one or the other motor, and how many have all three components? For that matter, how are the used concentrations of individual components decided?). It appears that HOOK3 rarely associates with Kif1c, as compared to dynein.

What is the oligomeric state of HOOK3? Is it a homodimer? Or can a single HOOK3 monomer bind both motors?

Along those lines, what is the stoichiometry of individual components in complexes? Are there multiple copies of any of the protein species? Fluorescence intensity analysis, size exclusion, or other appropriate techniques could provide insight into this important question.

It is surprising that no directionality switching of the full scaffolded complexes is observed. What is the total number and length of measured tracks of the tripartite complexes?

For the scaffolded tripartite complexes, what is the order of molecules in the complex assembly? What would happen if HOOK3 was preincubated with Kif1C and dynein was subsequently added? Or if strongly bound kinesin state was induced (AMPPNP or motor-dead)?

It would be beneficial to show controls using both motors in the absence of HOOK3.

Minor comments:
Figure 2C. Tau is an odd choice of a variable name representing length.

Figure 2C. The authors do not specify how they polymerized 80+ um long microtubules that were presumably used in the run length analysis?

Figure 3. HOOK3 channels in Figure 3C and 3F appear very different, with a multitude of brief lattice-association events (or passing clusters?) observed with the chimeric construct. Is there any explanation for this?

Figure 4.
Histograms in B, E, H should use the same bin sizes.
x-axis in C, F and I should have the same range. Again 'tau' is an awkward choice.
It appears that the dynein and HOOK3 channel colors are switched in the merged image.

Figure 6.
Are the individual channels mixed up? Why is HOOK3 processively moving in 6B without motor-association?

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, the authors demonstrate that the dynein-dynactin activating adaptor HOOK3 can also bind to KIF1C and trigger formation of a complex with both motors. Surprisingly, such complexes still move unidirectionally, either to the microtubule plus or minus end.

How teams of opposite polarity motors are recruited to cargoes and how their motility is controlled are exciting and long-standing questions in the field of microtubule-based transport. In recent years, the role of adaptor proteins in activating the dynein-dynactin complex became apparent and several of these adaptor proteins were suggested to also bind to kinesins. This raised several important questions, such as: is the binding between adaptors and opposite polarity motors mutually exclusive or can both motors bind at the same time. In case of the latter, what is the dynamics of the tripartite complex that is formed? The hope is that addressing these questions using well-controlled in vitro assays will reveal key mechanisms underlying the control of (bi)directional transport.

Based on their earlier finding that the C-term of the dynein-activating adaptor HOOK3 also interacts with KIF1C (Redwine et al. Elife 2017), the current manuscript reports that KIF1C does not bind HOOK1 or HOOK2 and that HOOK3 does not bind other Kinesin-3s nor Kinesin-1s. The authors map a 14aa region in the KIF1C tail domain responsible for HOOK3 binding and demonstrate that KIF1C can transport HOOK3 over microtubules and that HOOK3 does not seem to affect KIF1C motile properties (run length, speed). While it was already known that the N-term of HOOK3 activates the dynein-dynein complex, the authors here demonstrate that full-length HOOK3 can also do it. Furthermore, they demonstrate that KIF1C and Dynein-dynactin can be bound to HOOK3 at the same time. Remarkably, such complexes still move unidirectionally, either to the microtubule plus or minus end.

While the current manuscript reports and characterizes an important scaffolding complex for intracellular motility, I feel that it currently fails in properly characterizing the most important aspects of it, i.e. the biochemistry and activity of the opposite motor complex. I do recognize that this complex will likely become an important model system for future work and that not all aspects need to be explored in the current work, but as it stands, some central conclusions lack sufficient support and could impede future work. Better characterization will be required before I can recommend

publication in JCB.

* Characterization of opposite motor complexes:

If an adaptor can interact with two different motors, this could lead to different scenarios:
1. Binding is completely mutually exclusive. Motor A can never bind when B is bound, and vice versa.
2. Binding is completely non-exclusive and the motility of individual motors is not regulated by presence of the other (but could be impeded because motors have opposite directionality).
3. Binding is somehow competitive. Again, different scenarios can be foreseen:
a. The affinity between adaptor and motor A is decreased when motor B is bound to the adaptor. The could be mutual or not.
b. Binding affinities are not affected, but the activity of motor A is impeded when motor B is bound. The could be mutual or not.

The authors present evidence against model 1 and seem to support model 2. The small decrease in dynein velocity is explained by antagonizing motility of KIF1C, not by some biochemical regulatory process. Surprisingly, none of the more gradual possibilities (models 3a, 3b) are considered, test or discussed.

Based on size-exclusion chromatography of purified HOOK3, the authors conclude that KIF1C is in a complex that contains HOOK3, dynein and dynactin. While this demonstrates binding of KIF1C and dynein is not mutually exclusive, the exact stoichiometries remain unclear. Controls showing how individual components are running are missing. No analysis has been performed to analysis the distribution of different complexes.
In most examples in Figure 6, HOOK3 is bound to either KIF1C or Dynein-Dynactin. This suggest that binding of KIF1C and Dynein-dynactin could still be competitive in a way and would explain why most events are unidirectional. To test for competitive binding effects, the authors can perform more quantitative binding experiments to test if binding of KIF1C and Dynein is affected by the presence of Dynein and KIF1C, respectively. In addition, the abundance of different complexes during motility event in Figure 6 should be quantified (dynein only, KIF1C only, two-motors).

* Potential regulatory effects of HOOK1:

The authors demonstrate motile events of dynein-dynactin in the presence of HOOK3, demonstrating that FL-HOOK3 can activate this complex. However, how activation efficiency compares with that of activation by the N-term of HOOK3 only remains unknown. It could still be that the N-term only is a much more potent activator. In addition, it could be that binding of KIF1C to HOOK3 makes HOOK3 a much more or a much less potent activator of dynein motility.
Along the same lines: the manuscript reports a small decrease in dynein activity when KIF1C is present in the same complex. To test if this is due to the opposite motility of KIF1C or to the presence of KIF1C in the complex, the authors can test how KIF1C lacking motor domains affects motility.

The authors discuss concurrent work on BioRxiv (Siddiqui et al) that claims that HOOK3 relieves autoinhibition and conclude that the observed differences could be due to protein source, purification protocols and assay conditions. However, the authors did not do any experiments to directly compare the results. The Siddiqui paper reports a twofold increase in motile events in the presence of HOOK3, not an absence of events in the absence of HOOK3. The current manuscript shows that HOOK3 does not alter run length or speed, but did not analyze the number of motile events per second per unit MT in the absence or presence of HOOK3. I would propose that the

authors include these data, which could perhaps be extracted from their existing data sets.

Response to Review

We thank the reviewers for their very helpful comments, which have led to a much-improved manuscript. We address each reviewer point below in detail. The reviewer comments are in blue and our responses are in black font. We have also included a separate version of the manuscript that highlights the major changes (in blue font) throughout the manuscript.

We have added new data requested by the reviewers, the most substantial of which include: 1) a more extensive analysis of the motile properties of KIF1C in the presence and absence of Hook3, which reinforces our conclusion that Hook3 does not activate the motile properties of KIF1C; 2) additional analysis of purified full-length Hook3 activation of dynein/dynactin complexes and complexes containing both dynein/dynactin and kinesin; 3) new experiments examining how the dynein:kinesin ratio affects the motile properties of complexes containing both dynein and kinesin; and 4) a new cellular analysis of Hook3, KIF1C, and dynactin (p150) localization in 293T and U2OS cells, which shows that KIF1C recruits Hook3 to the cellular periphery.

We think this complicated in vitro reconstitution of dynein/dynactin and KIF1C by a physiological scaffold and the ensuing analysis is an important contribution that will be appreciated by those studying molecular machines from cell biology, biophysics, and bioengineering perspectives. This complex of opposite-polarity motors also provides an excellent model system for future studies from a theoretical perspective as well as in cells. We note that this is the first reconstitution using purified components of a physiological scaffold with opposite polarity cargo-transporting motors outside of a fungal system.

Reviewer #1

Kendrick et al. follow up on a previously identified interaction between the dynein activator HOOK3 and the kinesin-3 motor KIF1C by demonstrating the formation of a co-complex of KIF1C with HOOK3 and dynein/dynactin. While HOOK3 is known to activate dynein, the binding of HOOK3 to KIF1C does not affect the processive movement of the kinesin-3 motor. The authors propose that linking dynein and kinesin motors by dynein activating adaptors may be a general mechanism to regulate bidirectional motility.

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1. The authors have described the HOOK3-KIF1C interaction in a previous publication;

the additional pulldowns here are confirmatory but not novel. They map the interaction domain on KIF1C, but not very precisely on HOOK3, and they primarily rely on truncations rather than point mutations to perturb the interaction.

Our previous analysis (Redwine et al., 2017) showed that the carboxy-terminal region of Hook3 could associate with KIF1C when Hook3 was overexpressed in 293T cells. In that work, we presented this as a supplemental figure to support our BioID approach to identify the dynein protein interactome. In our current work we have gone far beyond this initial observation:

a) We show that endogenous Hook3 co-precipitates with endogenous KIF1C.

b) In further support of this interaction, we perform a BioID experiment with endogenous levels of KIF1C by generating KIF1C knockout cells and re-introducing endogenous levels of BioID-tagged KIF1C. In this experiment we identified Hook3 as one of the top hits in the KIF1C protein interactome. We also report the entire KIF1C protein interactome.

c) We identify 14 amino acids in the tail of KIF1C that are required for the Hook3 interaction. We attempted to map this further by making three different double point mutants in that region: R797A/ R801A, D802A/D805A, and W796A/ W804A, but we observed Hook3 binding to KIF1C in all cases. This data has now been added to Figure S2.

d) To monitor the importance of the carboxy-terminal region of Hook3, we generated a Hook3-Hook2 chimera, where the carboxy-terminus of Hook3 is replaced with that from Hook2. We show that this chimera does not bind KIF1C, but still activates dynein/dynactin, and thus is an ideal tool to dissect the role of this region in the context of cells (see below) or in our in vitro experiments. We also attempted to identify a more precise binding site for KIF1C on the carboxy-terminus of Hook3. However, none of the numerous constructs we generated (a subset of which we show in Figure S2) allowed us to do this. As we discuss in the manuscript, this could be due to the interaction site requiring a three-dimensional fold, which would not be amenable to dissection by the linear deletion analysis we performed.

e) We show that the interaction between Hook3 and KIF1C is specific. Hook3 does not interact with the three kinesin-1 proteins in the human genome (KIF5A, KIF5B, and KIF5C). Nor does it interact with the two most closely related kinesin-3 proteins in the human genome (KIF1A and KIF1B). In addition, we show that KIF1C does not interact with the two other Hook proteins in the human genome: Hook1 and Hook2.

f) Using a single-molecule motility assay we show that Hook3 interacts with moving KIF1C and we robustly characterize the motile properties of KIF1C with and without Hook3.

g) We have added new data using our KIF1C knockout 293T cells showing that when KIF1C is expressed in these cells it recruits Hook3 to the cell periphery. Furthermore, we show that when we express the KIF1C mutant that cannot bind Hook3, Hook3 is no longer recruited to the cell periphery. We also show in human U2OS cells that the carboxy-terminus of Hook3 is required for the KIF1C-dependent localization of Hook3 to the cell periphery. Importantly these experiments show that KIF1C and Hook3 interact in a cellular environment.

2. The demonstration that HOOK3, either truncated or full-length, activates dynein has been published by multiple labs, and thus is confirmatory and can be moved to the supplement.

Apologies, we did not intend to imply that Hook3 has not been studied previously and did cite in our original manuscript experiments using truncated human Hook3 (McKenney et al., 2014; Schroeder and Vale, 2016) and full-length human Hook3 isolated from cell extracts (Olenick et al., 2016). Our advance is to *purify full-length Hook3 to homogeneity* and characterize the motility of the human dynein/ dynactin complex with pure components. We have added additional text in the manuscript to clarify this. We think our purification of full-length Hook3 and its use in in vitro motility experiments with dynein/dynactin is also important because it indicates that, as purified, Hook3 is not autoinhibited, as has been observed for another dynein/dynactin activator, BICD2. We have clarified this point in the text.

3. Cellular or in vivo data are completely lacking, so it is unclear how relevant these observations are to trafficking in cells. It should be straightforward to test the effects of truncation and point mutations disrupting these interactions on organelle motility, and this would be required to meet the expected standard for a JCB publication.

We agree that cellular data would contribute to this work. We have now added a new figure (Figure 6) to address this. Specifically, as described in point 1g above we find that KIF1C expression leads to the relocalization of Hook3 and that this relocalization requires the 14 amino acids of KIF1C that we identified as the binding site for Hook3. Furthermore, the carboxy-terminus of Hook3 is required for the KIF1C-mediated localization of Hook3 to the cell periphery. We did not observe KIF1C-dependent relocalization of the dynactin subunit, p150 to the cell periphery.

We interpret this result as indicating that KIF1C and Hook3 can interact in a cellular environment and that a potential consequence of this interaction could be to remove Hook3 from the pool of available dynein/dynactin activators. It is also possible that in another cell type we would observe recruitment of dynein/dynactin to the cell periphery. We made these KIF1C knockout cell lines with the intent of performing biochemistry (such as the BioID experiment), not cell biological experiments. 293T cells are not ideal for imaging due their small size, rather disordered microtubule cytoskeleton, and lack of strong polarity compared to other cell types. Going forward we will work with other cell types to further investigate this, but given the time to generate new CRISPR cell lines we feel that this is beyond the scope of this manuscript and the revision timeline. Ideal cell types for this would be those where KIF1C has been better-studied, such as epithelial cells (Kopp et al., 2006; Theisen et al., 2012), fibroblasts (Efimova et al., 2014) or neurons (Lipka et al., 2016).

We have added a more extensive discussion of the models for Hook3's interaction with KIF1C and dynein/dynactin, all of which will be testable in the future, but will require significantly more time:

a) KIF1C removes Hook3 from the cellular pool of activators available for dynein/dynactin. Essentially acting as a negative regulator of dynein/dynactin/Hook3 cargo motility. Our new data showing that KIF1C recruits Hook3 to the cell periphery, but does not recruit dynactin supports this idea, at least in 293T cells.

b) KIF1C and dynein/dynactin share a common cargo(s) and are linked to that cargo via Hook3.

c) KIF1C recycles dynein/dynactin complexes to microtubule plus ends via the Hook3 scaffold and/ or dynein/dynactin recycles KIF1C toward microtubule minus ends via the Hook3 scaffold.

4. No data are included describing the relative frequencies of motility in either direction - in the mixed motor assays, which motor predominates?

Thank you for the suggestion to include this. We have added new data to Figure 5D that shows the relative frequencies of motility in each direction. We have also added new data to Figure 5E that shows the percentage of processive events in the mixed motor assays when different ratios of KIF1C to dynein/dynactin are used.

5. How is HOOK3 regulated to favor either kinesin-driven or dynein-driven motility?

We are very interested in understanding this question. Our new data presented in Figure 5E shows that the number of dual motor events increased as we increased the KIF1C concentration relative to the dynein/dynactin concentration. This concentration-dependence could imply that dynein/dynactin has a higher affinity for Hook3 compared to KIF1C; we will need to increase our protein yields to carefully analyze this, something that we hope to do in a future study and that we feel is beyond the scope of the current study. We think it is also possible that an unidentified regulatory factor is required to regulate directionality. This is something we will explore in the future as well. Initial candidates to explore would include the Hook3 interacting proteins FTS and FHIP, which we have not yet purified in the lab. We discuss these points in the discussion.

6. The authors propose that scaffolding of dynein and kinesin motors may be a general principle of activating adaptors. This would be a stronger point if the authors were to include supportive data from BICD2 or BICDL1 experiments - the authors note that BICDL1 has previously been shown to interact with KIF1C so this may be the obvious candidate to focus on.

Point well taken. We are very interested in this, but in the time frame allowed for this revision we have not been able to test other activating adaptors ourselves. We cite and describe the work that has reported these interactions. We have removed this statement from the abstract.

Additional minor points:

1. The authors should take more effort to cite primary publications rather than reviews when possible.

We have modified the text to include more primary references.

2. The authors should more precisely state the number of observations per condition, for example in Fig. 6D, which indicates a very broad range of n values (16-238).

Good point. We have now listed the n values for all conditions in the figure legends.

In summary, this work is clean but not particularly novel or impactful, as it replicates previously published findings on the interaction of HOOK3 with KIF1C and the activation of dynein by HOOK3. Significantly, no data are provided to support the possible import of this mechanism in cells, despite the generation of engineered cell lines that could readily be used for live imaging experiments. Given this lack, the work in its current form is better suited to a biochemical or biophysical journal such as JBC or J. Biophys.

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Figure 2.
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examples seem to contradict the photobleaching analysis?

Similarly, while the authors report that HOOK3 has no effect on the Kif1c velocity, the kymograph in Figure 2D shows HOOK3-associated tracks significantly faster than the Kif1c alone track? Is this kymograph not representative?

We thank the reviewer for these insightful comments. To fully address the reviewer's comments, we collected additional data and performed additional analysis. We have now modified figures 1, 2 and supplementary figure 1, as well as the manuscript to add the following analyses. Overall, we observe that KIF1C motility is not activated by the presence of Hook3.
We added the following analysis:

a) Landing rates of KIF1C with and without Hook3.
b) Pausing frequency of KIF1C with and without Hook3.
c) The number of processive, static, and diffusive events of KIF1C with and without Hook3.
d) KIF1C step-wise bleaching analysis in the presence and absence of Hook3. This analysis indicates that KIF1C exists predominantly as a dimer with a small number of oligomers present. This distribution is unaffected by the presence of Hook3.
e) We also replaced the kymographs in figure 2. We realized that those that we originally chose were not representative of most of our data.

Figure 3.
Given the reported interaction of HOOK3CT with Kif1C, it would be useful (and presumably easy to generate this short purified construct) to show colocalization of this construct with Kif1C as a positive control.

In our view the Hook3/Hook2 chimera is a nice control in the single-molecule experiment because it demonstrates that the carboxy-terminus of Hook3, but not another related Hook protein is required for the KIF1C interaction. Importantly, in cells we show that the CT of Hook3 alone is sufficient to target Hook3 to sites of KIF1C localization (new Figure 6).

Figure 4.
Although the mean velocity and run lengths of dynein appear the same regardless of the activating HOOK3 construct, the pausing behavior looks qualitatively different. Here again, it would be beneficial to quantify non-motile and pausing motor molecules for any potential differences induced by the activating constructs.

Thank you for this suggestion. We have added this analysis to Figure S3.

Figure 5.
The schematic in Figure 5A including cargo is confusing, since the authors provide no evidence for either the model in which cargo is or isn't mediating the interaction. I would suggest dropping this schematic, and combining Figures 5 &6 into a single figure

characterizing the complex.

We see your point and have removed the schematic. We have removed Figure 5 entirely, moved Figure 6 up (now Figure 5), and generated new cellular data for a new Figure 6.

Figure 6.
This is the most interesting figure of the paper, but it leaves much to be desired. For example, what is the frequency of individual complexes (i.e. how many tracks have HOOK3 complexed with one or the other motor, and how many have all three components? For that matter, how are the used concentrations of individual components decided?). It appears that HOOK3 rarely associates with Kif1c, as compared to dynein.

This figure is now figure 5. We have extended our analysis of the three-color in vitro assays to include the frequency of each individual complex. We have also determined the run lengths of each individual complex. In all three-color in vitro assays, we chose to use equimolar concentrations of each motor to allow for easier interpretation of the multiple complexes detected in this experiment. We have now also repeated these experiments with different ratios of KIF1C to dynein/dynactin. We find that increased concentrations of KIF1C lead to more plus-end-directed dual motor runs. All of this new analysis is included in Figure 5 and Figure S4.

What is the oligomeric state of HOOK3? Is it a homodimer? Or can a single HOOK3 monomer bind both motors? Along those lines, what is the stoichiometry of individual components in complexes? Are there multiple copies of any of the protein species? Fluorescence intensity analysis, size exclusion, or other appropriate techniques could provide insight into this important question.

Photo-bleaching analysis suggests that our purified KIF1C exists predominantly as a dimer, in the presence or absence of Hook3. We propose that KIF1C is likely binding a Hook3 homodimer based on previous structural data showing that Hook proteins, as well as other dynein activating adaptors, form dimers in complex with dynein and dynactin (Lee et al., 2018; Urnavicius et al., 2015). We have now referenced these papers in the manuscript. To fully address the stoichiometry of Hook3 scaffolded complexes, more detailed analysis is required. However, we are currently unable to perform such analysis due to relatively low protein purification yields.

It is surprising that no directionality switching of the full scaffolded complexes is observed. What is the total number and length of measured tracks of the tripartite complexes?

We also found this interesting and suggest in the discussion that additional factors may be required to regulate directionality. We have also added to the manuscript the number of runs observed and their run lengths for the tripartite complexes (Figure S4). We

analyzed a total of 856 runs when both dynein and KIF1C were present in the motility chamber; 71 of these contained both motors, but none switched direction.

For the scaffolded tripartite complexes, what is the order of molecules in the complex assembly? What would happen if HOOK3 was preincubated with Kif1C and dynein was subsequently added? Or if strongly bound kinesin state was induced (AMPPNP or motor-dead)?

In each experiment, dynein, dynactin, Hook3, and KIF1C were preincubated on ice for 10 minutes prior to imaging. In our hands, the order of protein addition or preincubation of Hook3 with KIF1C before dynein and dynactin addition did not affect complex behavior. This information is in the methods. Similarly, the presence of AMP-PNP, instead of ATP in the reaction mixture did not affect the formation of Hook3/KIF1C complexes or dual-motor complexes.

We also purified a KIF1C motor-dead construct (KIF1C-rigor, G251A) that binds strongly to microtubules but does not move processively (Nakata and Hirokawa, 1995). However, we were unable to perform single-molecule in vitro experiments with this mutant protein because the KIF1C-rigor concentrations that would allow for Hook3 binding to KIF1C were too high to visualize single runs in our assays due to the very strong interaction of KIF1C-rigor with microtubules.

It would be beneficial to show controls using both motors in the absence of HOOK3.

Good point. We have added this to supplementary Figure S4.

Minor comments:

Figure 2C. Tau is an odd choice of a variable name representing length.

Tau is a mean decay constant and represents the fitted run length of the reported data. We have now collected additional data and represented our run length analysis as 1-cumulative frequency, as is common in the field. We also added a more detailed explanation to the figure legends and methods and replaced “tau” with “run length” in all figure legends where run length is reported.

Figure 2C. The authors do not specify how they polymerized 80+ um long microtubules that were presumably used in the run length analysis?

Microtubules of this length were very rare. However, we have also added more detailed information for how we prepare microtubules prior to performing in vitro motility assays.

Figure 3. HOOK3 channels in Figure 3C and 3F appear very different, with a multitude of brief lattice-association events (or passing clusters?) observed with the chimeric construct. Is there any explanation for this?

We looked back through all of our kymographs and realized that these brief lattice-association events were not representative of our data. We replaced the images in Figure 3 with images more representative of our data.

Figure 4.
Histograms in B, E, H should use the same bin sizes.
x-axis in C, F and I should have the same range. Again 'tau' is an awkward choice.
It appears that the dynein and HOOK3 channel colors are switched in the merged image.

We adjusted the bin size and x-axis range for the histograms. We also marked in the figure and figure legends the specific colors used for representing the data in each channel. See above for a discussion of tau.

Figure 6.
Are the individual channels mixed up? Why is HOOK3 processively moving in 6B without motor-association?

Thanks for catching this! We have fixed this.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, the authors demonstrate that the dynein-dynactin activating adaptor HOOK3 can also bind to KIF1C and trigger formation of a complex with both motors. Surprisingly, such complexes still move unidirectionally, either to the microtubule plus or minus end.

How teams of opposite polarity motors are recruited to cargoes and how their motility is controlled are exciting and long-standing questions in the field of microtubule-based transport. In recent years, the role of adaptor proteins in activating the dynein-dynactin complex became apparent and several of these adaptor proteins were suggested to also bind to kinesins. This raised several important questions, such as: is the binding between adaptors and opposite polarity motors mutually exclusive or can both motors bind at the same time. In case of the latter, what is the dynamics of the tripartite complex that is formed? The hope is that addressing these questions using well-controlled in vitro assays will reveal key mechanisms underlying the control of (bi)directional transport.

Based on their earlier finding that the C-term of the dynein-activating adaptor HOOK3 also interacts with KIF1C (Redwine et al. Elife 2017), the current manuscript reports that KIF1C does not bind HOOK1 or HOOK2 and that HOOK3 does not bind other Kinesin-3s nor Kinesin-1s. The authors map a 14aa region in the KIF1C tail domain responsible for HOOK3 binding and demonstrate that KIF1C can transport HOOK3 over microtubules and that HOOK3 does not seem to affect KIF1C motile properties (run length, speed). While it was already known that the N-term of HOOK3 activates the dynein-dynein complex, the authors here demonstrate that full-length HOOK3 can also

do it. Furthermore, they demonstrate that KIF1C and Dynein-dynactin can be bound to HOOK3 at the same time. Remarkably, such complexes still move unidirectionally, either to the microtubule plus or minus end.

While the current manuscript reports and characterizes an important scaffolding complex for intracellular motility, I feel that it currently fails in properly characterizing the most important aspects of it, i.e. the biochemistry and activity of the opposite motor complex. I do recognize that this complex will likely become an important model system for future work and that not all aspects need to be explored in the current work, but as it stands, some central conclusions lack sufficient support and could impede future work. Better characterization will be required before I can recommend publication in JCB.

* Characterization of opposite motor complexes:

If an adaptor can interact with two different motors, this could lead to different scenarios:
1. Binding is completely mutually exclusive. Motor A can never bind when B is bound, and vice versa.
2. Binding is completely non-exclusive and the motility of individual motors is not regulated by presence of the other (but could be impeded because motors have opposite directionality).
3. Binding is somehow competitive. Again, different scenarios can be foreseen:
a. The affinity between adaptor and motor A is decreased when motor B is bound to the adaptor. The could be mutual or not.
b. Binding affinities are not affected, but the activity of motor A is impeded when motor B is bound. The could be mutual or not.

The authors present evidence against model 1 and seem to support model 2. The small decrease in dynein velocity is explained by antagonizing motility of KIF1C, not by some biochemical regulatory process. Surprisingly, none of the more gradual possibilities (models 3a, 3b) are considered, test or discussed.

Thank you for pointing this out. We should have discussed this more thoroughly and have now added additional discussion to the manuscript to address these points.

Based on size-exclusion chromatography of purified HOOK3, the authors conclude that KIF1C is in a complex that contains HOOK3, dynein and dynactin. While this demonstrates binding of KIF1C and dynein is not mutually exclusive, the exact stoichiometries remain unclear. Controls showing how individual components are running are missing. No analysis has been performed to analysis the distribution of different complexes.

This is an important point. We would like to clarify that the size exclusion analysis in former Figure 5 was performed on an immunoprecipitated HA-FLAG-Hook3 sample, rather than purified proteins. We apologize for the confusion. The analysis as it stands does not address the stoichiometry of the complex. Because we would need larger

amounts of pure protein to do a proper SEC-MALS experiment, we decided to remove this figure (former Figure 5). It will take a considerable amount of time to improve our expression protocols and purification protein yields, which would be required to do this experiment. We think this is important, but beyond the scope of the current work.

We note that our single-molecule reconstitution clearly shows that a complex forms between dynein/dynactin, Hook3 and KIF1C and that this interaction requires the carboxy-terminus of Hook3 (based on the Hook3/Hook2 chimera studies) to bind to a 14 amino acid region in the tail of KIF1C.

In most examples in Figure 6, HOOK3 is bound to either KIF1C or Dynein-Dynactin. This suggest that binding of KIF1C and Dynein-dynactin could still be competitive in a way and would explain why most events are unidirectional. To test for competitive binding effects, the authors can perform more quantitative binding experiments to test if binding of KIF1C and Dynein is affected by the presence of Dynein and KIF1C, respectively. In addition, the abundance of different complexes during motility event in Figure 6 should be quantified (dynein only, KIF1C only, two-motors).

We have now quantified the abundance of each complex and included that in what is now Figure 5.

To test if binding of Hook3 to each motor is affected by the presence of the other motor, we collected additional single-molecule in vitro data with increasing concentrations of KIF1C in the context of dual-motor complexes (Figure 5E and S4F-G). This analysis shows that increasing the amount of KIF1C leads to a decrease in the percentage of minus-end-directed events. We propose that the affinity of KIF1C for Hook3 may be weaker than that of dynein/dynactin for Hook3. It is also possible that Hook3 binding to one motor affects the affinity of binding by the other motor, which would influence the formation of dual-motor complexes. However, detailed analysis of competitive binding events with Hook3, dynein, dynactin and KIF1C will require higher protein concentrations of each component. At this point, given our relatively low protein purification yields we were unable to perform such an analysis.

* Potential regulatory effects of HOOK1:

The authors demonstrate motile events of dynein-dynactin in the presence of HOOK3, demonstrating that FL-HOOK3 can activate this complex. However, how activation efficiency compares with that of activation by the N-term of HOOK3 only remains unknown. It could still be that the N-term only is a much more potent activator. In addition, it could be that binding of KIF1C to HOOK3 makes HOOK3 a much more or a much less potent activator of dynein motility.

We have extended our analysis of dynein/ dynactin/ Hook3^{FL} and dynein/ dynactin/ Hook3^{NT} complexes. This analysis now includes velocity, run length, pausing frequency, and number of processive, diffusive, and static events and suggests that there is no

difference in the activation capabilities between these Hook3 constructs. This new data is in Figure S3.

Our additional analysis of dual-motor complexes performed with increasing concentrations of KIF1C (see previous reply and Figure 5E and S4F-G) shows a decrease in minus-end-directed runs when increasing concentrations of KIF1C are used. See previous reply for discussion of this point.

Along the same lines: the manuscript reports a small decrease in dynein activity when KIF1C is present in the same complex. To test if this is due to the opposite motility of KIF1C or to the presence of KIF1C in the complex, the authors can test how KIF1C lacking motor domains affects motility.

This is a great idea. We generated a KIF1C construct lacking the motor domain. However, this construct proved difficult to purify and we were unable to obtain protein of the concentration and purity required for these experiments.

The authors discuss concurrent work on BioRxiv (Siddiqui et al) that claims that HOOK3 relieves autoinhibition and conclude that the observed differences could be due to protein source, purification protocols and assay conditions. However, the authors did not do any experiments to directly compare the results. The Siddiqui paper reports a twofold increase in motile events in the presence of HOOK3, not an absence of events in the absence of HOOK3. The current manuscript shows that HOOK3 does not alter run length or speed, but did not analyze the number of motile events per second per unit MT in the absence or presence of HOOK3. I would propose that the authors include these data, which could perhaps be extracted from their existing data sets.

See response to reviewer #2 and modified Figure 2.

References

Efimova, N., Grimaldi, A., Bachmann, A., Frye, K., Zhu, X., Feoktistov, A., Straube, A., and Kaverina, I. (2014). Podosome-regulating kinesin KIF1C translocates to the cell periphery in a CLASP-dependent manner. J. Cell. Sci. *127*, 5179–5188.

Kopp, P., Lammers, R., Aepfelbacher, M., Woehlke, G., Rudel, T., Machuy, N., Steffen, W., and Linder, S. (2006). The kinesin KIF1C and microtubule plus ends regulate podosome dynamics in macrophages. Mol. Biol. Cell *17*, 2811–2823.

Lee, I.-G., Olenick, M.A., Boczkowska, M., Franzini-Armstrong, C., Holzbaur, E.L.F., and Dominguez, R. (2018). A conserved interaction of the dynein light intermediate chain with dynein-dynactin effectors necessary for processivity. Nat Commun *9*, 986.

Lipka, J., Kapitein, L.C., Jaworski, J., and Hoogenraad, C.C. (2016). Microtubule-binding protein doublecortin-like kinase 1 (DCLK1) guides kinesin-3-mediated cargo transport to dendrites. Embo J. *35*, 302–318.

McKenney, R.J., Huynh, W., Tanenbaum, M.E., Bhabha, G., and Vale, R.D. (2014). Activation of cytoplasmic dynein motility by dynactin-cargo adapter complexes. Science *345*, 337–341.

Nakata, T., and Hirokawa, N. (1995). Point mutation of adenosine triphosphate-binding motif generated rigor kinesin that selectively blocks anterograde lysosome membrane transport. J. Cell Biol. *131*, 1039–1053.

Olenick, M.A., Tokito, M., Boczkowska, M., Dominguez, R., and Holzbaur, E.L.F. (2016). Hook Adaptors Induce Unidirectional Processive Motility by Enhancing the Dynein-Dynactin Interaction. The Journal of Biological Chemistry *291*, 18239–18251.

Redwine, W.B., DeSantis, M.E., Hollyer, I., Htet, Z.M., Tran, P.T., Swanson, S.K., Florens, L., Washburn, M.P., and Reck-Peterson, S.L. (2017). The human cytoplasmic dynein interactome reveals novel activators of motility. Elife *6*, e28257.

Schroeder, C.M., and Vale, R.D. (2016). Assembly and activation of dynein-dynactin by the cargo adaptor protein Hook3. J. Cell Biol. *214*, 309–318.

Theisen, U., Straube, E., and Straube, A. (2012). Directional persistence of migrating cells requires Kif1C-mediated stabilization of trailing adhesions. Dev. Cell *23*, 1153–1166.

Urnavicius, L., Zhang, K., Diamant, A.G., Motz, C., Schlager, M.A., Yu, M., Patel, N.A., Robinson, C.V., and Carter, A.P. (2015). The structure of the dynactin complex and its interaction with dynein. Science *347*, 1441–1446.

June 3, 2019

RE: JCB Manuscript #201812170R

Dr. Samara Reck-Peterson
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Dear Dr. Reck-Peterson:

Thank you for submitting your revised manuscript entitled "HOOK3 is a scaffold for the opposite-polarity microtubule-based motors cytoplasmic dynein and KIF1C". We would be happy to publish your paper in JCB provided revisions to the text and summary are made to discuss the remaining points raised by Reviewer #3. Figure 6 is a good start to addressing the physiological relevance of the Hook3 and KIF1C interaction but perhaps does not address Reviewer#1's concerns about the physiological relevance of Hook3's proposed role in regulation of bidirectional transport. However, we feel that Reviewer #1's comments can be addressed by clearly stating the limitations of the cellular studies in the text and abstract. Please also address the following formatting changes:

- add MW markers to Fig 1F and 1G, 3B and 3E, S2E and S2F

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Reviewer #1 (Comments to the Authors (Required)):

The authors have provided a thoughtful response to the reviews, including the clean-up of a number of experiments. But unfortunately, due to a combination of technical limitations and a lack of consideration of alternative approaches, they have not further developed the biological implications of the study. For example, they state that their knockout cell line is not ideal for studying the cellular function of the interactions they are reporting, but they could readily have tried transient knockdown approaches in other cell types.

In the absence of any further significant biological data, I think this work is best suited to a more biophysical or biochemical journal, such as BJ or JBC.

Reviewer #2 (Comments to the Authors (Required)):

In the revised manuscript, Kendrick et al. have addressed many of the reviewers' concerns. They performed additional characterization of tracks, more analysis of the tripartite complex, as well as included an investigation of the Kif1c/Hook3 interaction in cells. While many questions remain, the current manuscript presents a solid step towards our understanding of physiologically relevant complexes of opposite-polarity microtubule motors.

Reviewer #3 (Comments to the Authors (Required)):

In this revised manuscript, the authors addressed most of my comments. They reformatted the data in a more convincing way and added several experiments and analyses. I highly recommend publication, provided the authors satisfactorily address the comment below.

1. The authors now added a quantification of the different complexes formed. This revealed that the number of complexes that have both kinesin and dynein is very low. Most complexes have either kinesin or dynein and only 8% of complexes have both motors (71/856). In addition, adding more kinesin gives more kinesin-hook complexes, at the expense of dynein-hook complexes. This strongly suggests some form of competition, even though binding is not entirely mutually exclusive. Yet the authors write in their conclusion "Our three-color single-molecule experiments show that Hook3, KIF1C, and dynein/dynactin exist in a complex together." This part of the discussion should be more balanced and highlight their finding that most complexes have one of the two motors and that dynein can be competed of by adding more kinesin. This is an important and interesting finding that should not be washed away by pushing the fact that they managed to observe some tripartite complexes.