JCB

Developmentally Regulated GTP binding protein 1 modulates ciliogenesis via Dishevelled

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Re: JCB manuscript #201811147

Dr. Ira Daar
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Dear Ira -

We have now received reviews from our external referees for your manuscript "Dvl modulates ciliogenesis via an interaction with Developmentally Regulated GTP binding protein 1". As you will see, the reviews are somewhat mixed. Both find the discovery of Drg1 binding to Dvl, and its role in ciliogenesis to be of interest, but they also highlight a number of issues, some of which can be dealt with by simple changes or additions to the text, but others will require additional experimental work to address. Unfortunately, based on these reviews, we cannot accept the manuscript for JCB in its present form. However, we would be pleased to consider a revised manuscript that addresses the key points of the reviews.

Reviewer #1 raises several issues with the biochemical analysis - for instance, the lack of information on the relative amounts for input lanes versus IP lanes, and the question of direct versus indirect binding of Dvl to Drg1. However, I do not think that measuring Kds and stoichiometry are necessary for this study. This reviewer also mentions the lack of binding of Drg2. I looked at the sequences of the two isoforms, and interestingly, the region you map in Drg1 that is essential for Dvl binding is very similar in Drg2:

DRG1: likefkyalvwgssvk

DRG2: ltsqfkyalvwgtstk

Does this mean that this region is necessary but not sufficient for Dvl binding? It would be very interesting to know if it is in fact sufficient for binding Dvl. I think in the revised manuscript it would be helpful and informative to discuss in more detail the implications of the domain mapping. For example, how similar are the DEP+ C sequences between the Dvl isoforms? What other proteins bind to the same region of Dvl? As this is a novel interaction it would seem appropriate to discuss this in more depth.

Reviewr #1 also suggests that - given the statement in the abstract - it is important to actually measure the length and number of cilia in the MCCs. I do not think that cilia functional assays are required, but I agree that a rescue using an activated form of Daam1 would be valuable to support your model.
I noticed that although on p17 you state that Daam1-GFP decorates the basal bodies, the opposite appears to be true in Figure 4C - there appears to be no significant overlap between Daam1-GFP

and centrin-RFP. Reviewer #2 also commented on this point, and noted that whereas centrin-RFP distribution in MCCs appears to require Drg1, Dvl2-cterm-GFP is still colocalized with centrin-RFP (Fig. 4A) in Drg1 MO-containing cells, so these results need to be carefully re-evaluated in terms of your proposed mechanism. This point is particularly relevant because the reviewer proposes an alternative model in which there is a failure of MCC progenitors to get to the surface, which would suppress their differentiation - thus suggesting a migration defect rather than a direct effect on basal body docking. This reviewer also feels that the T1000D Drg1 mutant is not adequate to conclude that GTPase activity is dispensable for its function in ciliogenesis.

Finally, I noted that the manuscript begins with the identification of Drg1 binding from MS analysis of Dvl2 immunoprecipitation. However, no information is provided in the results or methods about how the MS was performed, and no data are provided about the number of peptides, coverage or other parameters of the analysis. Since the experiment is the basis for the study I think it is important to provide some factual information about it, for instance in the supplementary data section.

Overall, there are a number of key points that I believe will require further experimental work. This will inevitably increase the length of the manuscript, which we suggest might be resubmitted as a full article. However, there are many places throughout the manuscript that seem unnecessarily wordy and could be made more concise. Please note that along with the revised manuscript we would also need a point-by-point response to all of the reviewer comments.

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 a Report is < 20,000, 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.

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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 or call (212) 327-8588.

Sincerely,

Ian Macara, Ph.D.
Editor
Journal of Cell Biology

Andrea L. Marat, Ph.D.
Scientific Editor
Journal of Cell Biology

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

In their manuscript entitled "Dvl modulates ciliogenesis via an interaction with Developmentally Regulated GTP binding protein 1" Lee et al present their characterization of Drg1 as a novel factor important for ciliogenesis in multiciliated cells (MCCs). Specifically, the authors report:

• Physical associations within a Drg1:Dvl2:Daam1:RhoA network, including mapping determinants of these associations in Drg1 and Dvl2 (DEP+C).
• Localization of Drg1 to the basal body region in MCCs
• Characterization of Drg1 loss of function affects ciliogenesis in MCCs, including analysis basal body anchoring polarization, RhoA activation and apical actin network formation, and recruitment of Dvl2 and Daam1 to basal body regions.

By characterizing a novel factor involved in ciliogenesis, this manuscript will be of interest to researchers studying the biology of cilia and polarization in development. However, in its current state I feel that the authors are premature in many of their conclusions and that additional controls and analysis will be required in order for the authors to substantiate many of the claims made in this manuscript. Furthermore, I feel the short article format prevents the authors from significantly developing the main lines of experimentation presented in this study, which ultimately limits the ability to draw specific conclusions about the physiological functions of Drg1. This is underscored by their model (Figure 4F) - which is intriguing but very complex with many facets not fully justified by the data in its current form. I believe this manuscript can be developed into a form that might justify this model, but it will require additional controls and experiments that are probably better accommodated in the article format.

Major Points:

1. The biochemical experiments provided in FIG1 and FIG4 do not justify conclusions about the proposed interaction network.
• In experiments using exogenous overexpression to probe interactions in the Drg1-Dvl2-Daam1-

RhoA network the authors should provide quantification of the extent of overexpression (compared to endogenous expression) of bait and prey proteins.
• Percentage of input loaded should also be added to provide a reference. The authors should also make an attempt to estimate the stoichiometry of interactions in this network.
• From the data provided, the authors cannot make any determinations about whether the physical association between Drg1 and Dvl2 (or Dvl3) is direct or indirect (although the authors assume this interaction is direct). To justify this conclusion, the authors should determine if recombinant purified Drg1 can interact with recombinant purified Dvl2 (or Dvl3), which should be feasible especially if the authors use the minimal interaction domains reported. Reconstitution of this interaction would also allow the authors to report a Kd for the Drg1-Dvl2 interaction. Such analysis would significantly contribute to the rigor of biochemical characterization provided in this study.
• The authors clearly show that Drg2 does not have a role in ciliogenesis in MCCs, but does Drg2 interact with Dvl(1,2,3)? If not, is this due to lack of conservation in the region of Drg1 that contains amino acids 329-344? And if Drg2 does interact with Dvl(1,2,3), could this indicate regulation of ciliogenesis in a different context (i.e., not MCCs)? Following up on this would clearly be beyond the scope of this study - but the authors should at least test if Drg2 interacts with Dvl(1-3).

2. One of the strengths of this manuscript is in the characterization of ciliogenesis defects associated with loss of Drg1 function in MCCs - but even here, the study would benefit from additional controls and analysis:
• The abstract states: "The loss of Drg1 or disruption of its interaction with Dvl reduces the length and number of cilia along with displaying defects in basal body migration and docking to the apical surface of multiciliated cells (MCCs)." To justify this statement, the authors should measure the length and number of cilia in MCCs. The authors do measure cilium length in gastrocoel roof plate of Xenopus embryos (Figure 3A) - but they never quantify cilia number in the GRP, and they make neither measurement for MCCs. It seems very important to include these two measurements in their analysis of ciliogenesis of MCCs, since this is a critical conclusion that is communicated in the abstract.
• Although the authors provide several experiments to describe the morphological defects in ciliogenesis observed upon Drg1 knockdown, they do not perform any experiments to characterize cilia function in the absence of Drg1. The authors should consider measuring fluid flow across the embryo epithelium, or measure coordinated cilia beating on MCC cells. This would be particularly important for drawing conclusions about the Drg1 T100D mutant (GTPase dead) since it is possible that Drg1 GTPase activity is important for cilia function (despite being dispensable for cilia morphology).
• If the major function of Drg1 is to assist Dvl2 in the activation of Daam1 then expressing an activated form of Daam1 (reported in Liu et al., 2008) should bypass the requirement for Drg1, and suppress Drg1 MO knockdown phenotypes. This type of experiment is critical if the authors want to justify their model.
• Based on the images provided, it looks like maybe Drg1 knockdown may affect the size of epithelial cells in the Xenopus embryo. If true, this is something the authors should probably discuss in their manuscript.

3. The experiment shown in Figure 3B is important, but I am unclear on a couple of details for this experiment. First, why is there a huge difference in total GFP signal in control and Drg1 morphant embryos? I would guess this a single Z plane taken from a confocal stack - and if the entire stack were projected the GFP intensity would be similar in control and Drg1 knockdown cells, but that isn't clear from the description of the experiment. Perhaps this would be clarified if the authors would include a blot for expression of the RBD-GFP construct. If there is a large differential in expression of the RBD-GFP in control and Drg1 knockdown embryos then it may be difficult to draw

conclusions from these experiments. Second, the authors do not describe how they normalize their fluorescence intensity measurements in the graph to the right of Figure 3B. Without a better description of how these measurements were made and normalized it is difficult to assess the significance of this data.

Minor points:

1. I don't think the title accurately communicates the pith of this story - which is more about Drg1 and its role in ciliogenesis.

2. In instances where quantification of fluorescence microscopy images is provided (FIG 2A, 3A-D, and S2D-F) it is unclear how the data is normalized across samples. For example, in Figures 3C and 3D the p-cofilin and p-LIMK signals should be normalized to total cofilin and total LIMK, respectively, in order to conclude that Drg1 specifically contributes to the activation of RhoA effector pathways (as opposed to affecting expression of cofilin or LIMK in MCCs).

3. For protein domain schematics (as in FIG 1D) it would be helpful if specific amino acid positions were enumerated (i.e., show where specific deletions were made - this was done for FIG 1E and it is very helpful).

4. For exogenous coIP experiments (e.g., FIG 1A and 1C) additional experimental details should be provided. Presumably affinity tags are used to purify baits, but this is not reflected in figure labels. (i.e., should "IP Drg1" be "IP V5"?) This would help clarify the figure and the way the experiments were done.

5. In many figures, the authors fail to detect endogenous proteins. For example, in FIG 1D if the authors are blotting using Dvl2 antibody why do they fail to detect endogenous Dvl2? Also, why does Dvl2 migrate as a doublet in some experiments (FIG 1D and 1E) but as a single band in others (FIG 1A and 1C).

6. The authors do not mention the model (Figure 4F) in the manuscript text.

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

This manuscript implicates Drg1, a developmentally regulated GTPase, in the control of Dishevelled (Dvl)-mediated ciliogenesis in multiciliated cells (MCCs) in the Xenopus epidermis. The authors discovered that Drg1 associates with Dvl and both proteins colocalize at the basal bodies in MCCs. Drg1 depletion is reported to cause multiple defects, including Dvl and Daam1 mislocalization (at the basal bodies), rotational basal body polarity, basal body docking, cilia growth, apical actin distribution and RhoA and LIMK activation in MCCs. Based on Drg1 mutant analysis, the authors suggest that Drg1 GTPase activity is not required for the described role in ciliogenesis and propose that the effect of Drg1 is through the apical actin meshwork needed for basal body docking.

This work contains a large body of experimental evidence supporting a novel role of Drg1 in apical actin remodelling and cilia formation in MCCs, which is appropriate for JCB readers. The majority of the reported experiments are well executed and have the necessary controls, although some conclusions require further validation. I hope that the authors can revise the paper by addressing several key points listed below.

1. The authors identified Dvl2 DEP-Cterm as the domain interacting with Drg1. Because the localization Dvl2 C-terminus is regulated by Drg1 at the basal body, it would be important to show that this protein fragment (rather than DEP) is the one associating with Drg1.
2. The conclusions that the localizations of Dvl and Daam1 depend on Drg1 are not compelling. Whereas centrin-RFP distribution in MCCs appears to require Drg1, Dvl2-cterm-GFP is still colocalized with centrin-RFP (Fig. 4A) in Drg1 MO-containing cells. In contrast, Daam1-GFP does not colocalize with centrin-RFP (Fig. 4C). Therefore, the results presented in Fig. 4C cannot be explained by the direct interaction of Dvl and Daam1. This needs to be sorted out.
3. It is unclear how protein localization at the basal body relates to the observed defects in RhoA activity and apical actin localization. The authors propose a direct effect on basal body docking. An alternative explanation is a cell migration defect, as suggested by their data. Fig. 2A and Suppl. Fig. 3B show that the groups treated with Drg1 MO, especially in combination with Δ329-344, appear to have diminished number of MCCs coming to the surface. This possibility is suggested by equal cell size characteristic of goblet cells, or the presence of very small cells that could correspond to poorly integrated MCCs. Failure of MCC progenitors to get to the surface would suppress their differentiation, resulting in defective basal body positioning and ciliogenesis.
4. The mild change in the enzymatic activity of T100D Drg1 shown in the cited paper is not sufficient to justify the conclusion that Drg1 does not require its GTPase activity for the observed effects. Since T100D may retain sufficient activity to rescue Drg1 knockdown phenotypes, introducing another mutation inactivating the enzyme would be warranted.
5. Some of the pulldown panels (e.g., Fig. 1D and E, top panels) should have formal controls for nonspecific 'stickiness' of the proteins of interest. Alternatively, they could be removed. The conclusions would still stand because the pulldowns have been performed in reciprocal experiments.
6. Minor: Fig. 1A is mislabelled.

Dear Dr. Macara, Dr. Marat, and Reviewers,

We are submitting a revised manuscript (#201811147R) that is now entitled **"Developmentally Regulated GTP binding protein 1 modulates ciliogenesis via an interaction with Dishevelled 1**". We greatly appreciate the suggestions and comments of the editor and reviewers, and a number of new experiments have been performed to address the concerns that were raised. We feel that in this revised paper, our use of a combination of loss-of-function (endogenous) experiments, replacement experiments (knockdown of endogenous protein and re-expression at carefully titrated levels), and *in vivo* and *ex vivo* assays in *Xenopus* embryos provide mechanistic insight into how Drg1 functionally affects ciliogenesis and the apical actin network. We are grateful to the reviewers whose suggestions have led to a more thorough assessment of Drg1 and its role in this process, and to strengthen the claims of our paper.

The concerns of reviewers and editor have been addressed below:

Reviewer #1:

In their manuscript entitled "Dvl modulates ciliogenesis via an interaction with Developmentally Regulated GTP binding protein 1" Lee et al present their characterization of Drg1 as a novel factor important for ciliogenesis in multiciliated cells (MCCs). Specifically, the authors report:

• Physical associations within a Drg1:Dvl2:Daam1:RhoA network, including mapping determinants of these associations in Drg1 and Dvl2 (DEP+C).
• Localization of Drg1 to the basal body region in MCCs
• Characterization of Drg1 loss of function affects ciliogenesis in MCCs, including analysis basal body anchoring polarization, RhoA activation and apical actin network formation, and recruitment of Dvl2 and Daam1 to basal body regions.

By characterizing a novel factor involved in ciliogenesis, this manuscript will be of interest to researchers studying the biology of cilia and polarization in development. However, in its current state I feel that the authors are premature in many of their conclusions and that additional controls and analysis will be required in order for the authors to substantiate many of the claims made in this manuscript. Furthermore, I feel the short article format prevents the authors from significantly developing the main lines of experimentation presented in this study, which ultimately limits the ability to draw specific conclusions about the physiological functions of Drg1. This is underscored by their model (Figure 4F) - which is intriguing but very complex with many facets not fully justified by the data in its current form. I believe this manuscript can be developed into a form that might justify this model, but it will require additional controls and experiments that are probably better accommodated in the article format.

We greatly appreciate the reviewer's general comments and have performed more experiments to address the issues outlined below.

Major Points:

1. The biochemical experiments provided in FIG1 and FIG4 do not justify conclusions about the proposed interaction network.
• In experiments using exogenous overexpression to probe interactions in the Drg1-Dvl2-Daam1-RhoA network the authors should provide quantification of the extent of overexpression (compared to endogenous expression) of bait and prey proteins.

Since the antibodies required to detect endogenous *Xenopus* proteins are not available, it is challenging to provide quantification of the extent of overexpression of bait and prey proteins compared with endogenous expression. One way we try to circumvent these concerns is in experiments where we knockdown and replace proteins with wild-type or mutant versions. In these cases, which are probably our most potent experiments, we use the minimum level of expression to perform a rescue with the wild-type construct, while expressing the same level of the mutant as judged by Western analysis. However, as an additional approach we expressed exogenously tagged proteins, where we have compared the relative interaction affinity among proteins. For example, we tested whether a protein (e.g. Drg1-Flag) has an interaction preference to the other binding partners (e.g. Dvl2-HA and Daam1-HA) under conditions where similar expression levels are obtained for Dvl2-HA and Daam1-HA etc. Here is a brief summary of these Co-IP tests:

1) Drg1-Flag interaction with Dvl2-HA or Daam1-HA : expressing similar amounts of Dvl2-HA and Daam1-HA, Drg1-Flag displays a more robust interaction with Dvl2-HA than Daam1-HA.

2) Dvl2-V5 interaction with Drg1-HA or Daam1-HA : expressing similar amounts of Drg1-HA and Daam1-HA, Dvl2-V5 has a slightly stronger interaction with Drg1-HA than Daam1-HA.

3) Daam1-HA interaction with Drg1-V5 or Dvl2-V5 : expressing similar amounts of Drg1-V5 and Dvl2-V5, Daam1-HA has a stronger interaction with Dvl2-V5 than Drg1-V5.

These results support a role for Dvl2 bridging the interaction between Drg1 and Daam1, and although a Dvl2-Drg1 complex formation might be preferred to a Dvl2-Daam1 complex formation it does not appear that there are major differences that would lead to large stoichiometric differences among the proteins of the complex. We include this data in Fig. 6A-C.

• Percentage of input loaded should also be added to provide a reference. The authors should also make an attempt to estimate the stoichiometry of interactions in this network.

We now include a statement that the approximate percentage of input loaded (depending upon the experiment the input is between 2.5-10%). For exogenously expressed protein interaction experiments, 2.5% of input is loaded. For endogenous protein interactions, 5% of the input is loaded. For the *in vitro* binding assays, the input percentage is 10%. The percentages are described in the figures and legends. Again, with our current available tools, it would be extremely difficult to address the stoichiometry of the interaction network further.

• From the data provided, the authors cannot make any determinations about whether the physical association between Drg1 and Dvl2 (or Dvl3) is direct or indirect (although the authors assume this interaction is direct). To justify this conclusion, the authors should determine if recombinant purified Drg1 can interact with recombinant purified Dvl2 (or Dvl3), which should be feasible especially if the authors use the minimal interaction domains reported. Reconstitution of this interaction would also allow the authors to report a Kd for the Drg1-Dvl2 interaction. Such analysis would significantly contribute to the rigor of biochemical characterization provided in this study.

To address the reviewer's comment, we performed an *in vitro* binding assay. We used purified recombinant GST-tagged proteins consisting of Drg1-WT, Drg1-Δ329-344, and Drg1-TGS domain. Dvl2-HA recombinant proteins were purified from the complex of GST-Dvl2-HA/Glutathione resin by treating with Thrombin protease. Consistent with the interaction domain mapping result (Fig. 1E and S1C), the *in vitro* binding assay indicates that amino acid region aa 329-344 of Drg1 is required for an interaction with Dvl, and the TGS domain is sufficient for the formation of the Dvl-Drg1 complex. The data are included in Fig. 1F.

• The authors clearly show that Drg2 does not have a role in ciliogenesis in MCCs, but does Drg2 interact with Dvl(1,2,3)? If not, is this due to lack of conservation in the region of Drg1 that contains amino acids 329-344? And if Drg2 does interact with Dvl(1,2,3), could this indicate regulation of ciliogenesis in a different context (i.e., not MCCs)? Following up on this would clearly be beyond the scope of this study - but the authors should at least test if Drg2 interacts with Dvl(1-3).

The reviewer makes a very interesting point. As the reviewer suggested we tested both exogenous and endogenous protein interactions between Drg2 and Dvls (1-3). With regard to exogenous protein interactions, Dvl2 and Dvl3 appear to associate with Drg2. In contrast, we failed to observe an interaction between the endogenous Drg2 and Dvl2 or Dvl3 in HT29 cells. One similar phenomenon was related in a previous report (Ishikawa et al., 2009). In this case, only overexpressed Drg1 associates with Dfrp2 (DRG family regulatory protein 2), while endogenous Drg1 does not bind to endogenous Dfrp2. The ability to bind when over-expressed is likely due to the high amino acid sequence homology between Drg1 and Drg2.

Although Drg1 and Drg2 have approximately 55% amino acid sequence identity, the known interaction partners are not the same. There is also evidence that the two family members may not be localized to the same region within a cell and it is reported that these proteins may have some related as well as distinct physiological roles in cells. For example, whereas Drg1 is present in the polysomal component with Dfrp1 (DRG family regulatory protein 1), Drg2 forms a complex with Dfrp2 that is not found in the ribosomal fractions (Ishikawa et al., 2009). Another functional distinction is that Drg2, as an endosomal protein, interacts and modulates Rab5 and transferrin recycling (Mani et al., 2016).

We now include the Co-IP of exogenous and endogenous proteins in Fig S2F & G. We discuss these results and implications in the Discussion section of the manuscript.

2. One of the strengths of this manuscript is in the characterization of ciliogenesis defects associated with loss of Drg1 function in MCCs - but even here, the study would benefit from additional controls and analysis:
• The abstract states: "The loss of Drg1 or disruption of its interaction with Dvl reduces the length and number of cilia along with displaying defects in basal body migration and docking to the apical surface of multiciliated cells (MCCs)." To justify this statement, the authors should measure the length and number

of cilia in MCCs. The authors do measure cilium length in gastrocoel roof plate of Xenopus embryos (Figure 3A) - but they never quantify cilia number in the GRP, and they make neither measurement for MCCs. It seems very important to include these two measurements in their analysis of ciliogenesis of MCCs, since this is a critical conclusion that is communicated in the abstract.

We agree with reviewer that an accurate assessment of the length and number of cilia in both MCCs and the GRP may be revealing. We have now included an account of the cilia length and number in the MCCs and GRP. Compared with control GRPs, the number of ciliated cells in the Drg1 morphant GRPs are reduce by 28%. The expression of WT Drg1 restored the ciliated cell population to 93% of controls. However, the Δ329-344 mutant did not restore the reduced ciliated cell population. Likewise, the average length of cilium in the GRP is reduced from 8.36 to 3.68μm upon Drg1 knockdown, which is partially rescued to 6.8μm by WT Drg1 expression, but the Δ329-344 mutant failed to restore the reduced cilium length.

As for the MCCs, the average length of cilium in control MCCs is 11.9um. The average length of Drg1 morphant cilia is 3.03um, and morpholino resistant Drg1 expression partially rescues the length of cilia to 6.47um. The population of cilia in the MCCs is also decreased by 44% in the Drg1 morphants, and the re-introduction of Drg1 partly restores the number of cilia to 85% of the control MCCs. Taken together, the loss of Drg1 affects the length and number of cilia in both MCCs and GRP, but with a somewhat more robust effect on the MCCs. We now include the data in Fig. 2H (length of cilia in GRP), Fig. 2I (number of ciliated cells in GRPs), Fig. 2C (length of cilia in MCCs), and Fig. 2D (number of cilia in MCCs).

• *Although the authors provide several experiments to describe the morphological defects in ciliogenesis observed upon Drg1 knockdown, they do not perform any experiments to characterize cilia function in the absence of Drg1. The authors should consider measuring fluid flow across the embryo epithelium, or measure coordinated cilia beating on MCC cells. This would be particularly important for drawing conclusions about the Drg1 T100D mutant (GTPase dead) since it is possible that Drg1 GTPase activity is important for cilia function (despite being dispensable for cilia morphology).*

The reviewer makes an interesting point, thus we measured the velocity of fluid flow over the epidermis of embryos. For visualization of fluid flow, fluorescent beads were employed and the velocity was manually measured by tracking individual beads along the body axis according to a previous established method (Werner and Mitchell, 2013). There was marked decrease in the velocity of bead movement over the epidermis of Drg1 morphants relative to the control embryos, suggesting that the decreased length and number of cilia in MCCs leads to functional defects of MCCs. Re-expression of the wild-type Drg1 rescues the fluid flow and this data is presented in Fig. 2F and videos 1-3.
With regard to the newly generated GTPase mutant (S78N-T100D), we found that expression of this mutant in the Drg1 morphant was able to functionally restore ciliary flow and is presented in Fig S4 E, videos 4-7.

• *If the major function of Drg1 is to assist Dvl2 in the activation of Daam1 then expressing an activated form of Daam1 (reported in Liu et al., 2008) should bypass the requirement for Drg1, and suppress Drg1 MO knockdown phenotypes. This type of experiment is critical if the authors want to justify their model.*

The reviewer suggests a very thoughtful experiment to test the model. We used a C-terminal fragment of Daam1 (as reported in Lie et al., 2008) as an active form of Daam1, and C-Daam1 encoding mRNAs

were co-injected with MOs and/or mRNAs. The acetylated tubulin staining in MCCs shows that active Daam1 expression successfully restores the reduced acetylated tubulin staining observed upon Drg1 knockdown, suggesting that active Daam1 is sufficient to bypass the requirement for Drg1. That is likely due to restoration of the apical actin meshwork since phalloidin staining shows a rescue of apical actin levels similar to control MCCs. We now include this data in Fig 7C and 7D.

• Based on the images provided, it looks like maybe Drg1 knockdown may affect the size of epithelial cells in the Xenopus embryo. If true, this is something the authors should probably discuss in their manuscript.

To address reviewer's concern, we measured the size of the apical surface of MCCs in control and Drg1 morphants. Even in a single embryo, the apical size of MCCs is heterogenous and the measured average size of apical surface of MCCs did not appear to differ between control and Drg1 morphant embryos. This data was quantified and is now included in Fig. 2E.

3. The experiment shown in Figure 3B is important, but I am unclear on a couple of details for this experiment. First, why is there a huge difference in total GFP signal in control and Drg1 morphant embryos? I would guess this a single Z plane taken from a confocal stack - and if the entire stack were projected the GFP intensity would be similar in control and Drg1 knockdown cells, but that isn't clear from the description of the experiment. Perhaps this would be clarified if the authors would include a blot for expression of the RBD-GFP construct. If there is a large differential in expression of the RBD-GFP in control and Drg1 knockdown embryos then it may be difficult to draw conclusions from these experiments.

To address the reviewer's comment and clarify the issue as suggested, we performed a Western blot to examine the expression of the RBD-GFP. The expression level of the RBD-GFP in the Drg1 knockdown embryos is not significantly different from the control embryos. We now include a Western blot in Figure 4B. We also describe that we took images from the subapical region, 2.5 um below the surface.

Second, the authors do not describe how they normalize their fluorescence intensity measurements in the graph to the right of Figure 3B. Without a better description of how these measurements were made and normalized it is difficult to assess the significance of this data.

We now state that the RBD-GFP intensity of the experimental groups was expressed as a percentage relative to the control group, which is included in Figure 4B legend.

Minor points:

1. I don't think the title accurately communicates the pith of this story - which is more about Drg1 and its role in ciliogenesis.

We agree with the reviewer and we have revised the title to "Developmentally Regulated GTP binding protein 1 modulates ciliogenesis via an interaction with Dishevelled".

2. In instances where quantification of fluorescence microscopy images is provided (FIG 2A, 3A-D, and S2D-F) it is unclear how the data is normalized across samples. For example, in Figures 3C and 3D the p-

cofilin and p-LIMK signals should be normalized to total cofilin and total LIMK, respectively, in order to conclude that Drg1 specifically contributes to the activation of RhoA effector pathways (as opposed to affecting expression of cofilin or LIMK in MCCs).

As the reviewer mentions, immunofluorescence of phospho-proteins should be normalized to immunostaining of the endogenous proteins. However, many endogenous proteins in *Xenopus* are not detected by the available antibodies. We are unable to perform immunofluorescence to obtain endogenous protein levels of cofilin and LIMK1 due to this limitation. Thus, we carefully normalized phospho-cofilin and phospho-LIMK1 signals of the experimental groups to that of control groups.

Although we were unable to examine the total endogenous protein levels, we assessed whether the amount of exogenous cofilin and LIMK1 fluctuates when Drg1 expression is inhibited. To do so, we titrated the minimum amount of mRNAs encoding cofilin-HA or LIMK1-HA that could be detected by IF, and co-injected these with MOs and other mRNAs. Western blotting and/or immunostaining (using an HA probe) was performed to test whether these proteins changed with the Drg1 knockdown. Immunostaining shows that cofilin-HA signal intensity is not altered upon Drg1 knockdown. Also, the Western blot analysis indicates that Drg1 knockdown does not cause the exogenous cofilin and LIMK1 protein levels to change. Although limited and indirect information is obtained from these tests, there is no indication that protein levels are affected by the Drg1 MO. We now include the data in Fig. S3 C.

3. For protein domain schematics (as in FIG 1D) it would be helpful if specific amino acid positions were enumerated (i.e., show where specific deletions were made - this was done for FIG 1E and it is very helpful).

We have now indicated the amino acid positions in Fig 1D.

4. For exogenous coIP experiments (e.g., FIG 1A and 1C) additional experimental details should be provided. Presumably affinity tags are used to purify baits, but this is not reflected in figure labels. (i.e., should "IP Drg1" be "IP V5"?) This would help clarify the figure and the way the experiments were done.

We agree with the reviewer. To clarify the figures, we changed the protein name to the tag. Also, all exogenous proteins were labeled as “protein name + tag” (e.g. Drg1-V5), while endogenous proteins are labeled with there names (e.g. Drg1).

5. In many figures, the authors fail to detect endogenous proteins. For example, in FIG 1D if the authors are blotting using Dvl2 antibody why do they fail to detect endogenous Dvl2? Also, why does Dvl2 migrate as a doublet in some experiments (FIG 1D and 1E) but as a single band in others (FIG 1A and 1C).

The proteins labeled in Fig 1D are exogenously tagged proteins. We have now corrected the mislabeling in Fig 1D.

The number of Dvl2 bands shown in blots depends on the gel percentage, protein amount, post-translational modification (e.g. phosphorylation), and film exposure time. When overexpressed (Fig 1A, Fig 1D and Fig1E), Dvl2 has 2 bands as shown in Western blots, however endogenous Dvl2 (Fig 1C) usually displays one major band with a faint minor band in Western blots - using Dvl2 antibody (Cell signaling antibody).

6. The authors do not mention the model (Figure 4F) in the manuscript text.

Answer-

The reviewer makes an excellent point.
We now include an explanation of the model in the Discussion and have moved the model to Fig. S5 H:

Reviewer #3:

This manuscript implicates Drg1, a developmentally regulated GTPase, in the control of Dishevelled (Dvl)-mediated ciliogenesis in multiciliated cells (MCCs) in the Xenopus epidermis. The authors discovered that Drg1 associates with Dvl and both proteins colocalize at the basal bodies in MCCs. Drg1 depletion is reported to cause multiple defects, including Dvl and Daam1 mislocalization (at the basal bodies), rotational basal body polarity, basal body docking, cilia growth, apical actin distribution and RhoA and LIMK activation in MCCs. Based on Drg1 mutant analysis, the authors suggest that Drg1 GTPase activity is not required for the described role in ciliogenesis and propose that the effect of Drg1 is through the apical actin meshwork needed for basal body docking.

This work contains a large body of experimental evidence supporting a novel role of Drg1 in apical actin remodelling and cilia formation in MCCs, which is appropriate for JCB readers. The majority of the reported experiments are well executed and have the necessary controls, although some conclusions require further validation. I hope that the authors can revise the paper by addressing several key points listed below.

1. The authors identified Dvl2 DEP-Cterm as the domain interacting with Drg1. Because the localization Dvl2 C-terminus is regulated by Drg1 at the basal body, it would be important to show that this protein fragment (rather than DEP) is the one associating with Drg1.

The reviewer's point is well taken. We conducted Co-IPs using the C-terminus of Dvl2 and determined that the C-terminus was sufficient to interact with Drg1. However, it is worth noting that the interaction between Drg1 and the C-terminus of Dvl2 was not as strong as the interaction between Drg1 and full-length Dvl2. The data are included in Fig. 5B.

2. The conclusions that the localizations of Dvl and Daam1 depend on Drg1 are not compelling. Whereas centrin-RFP distribution in MCCs appears to require Drg1, Dvl2-cterm-GFP is still colocalized with centrin-RFP (Fig. 4A) in Drg1 MO-containing cells. In contrast, Daam1-GFP does not colocalize with centrin-RFP (Fig. 4C). Therefore, the results presented in Fig. 4C cannot be explained by the direct interaction of Dvl and Daam1. This needs to be sorted out.

The reviewer makes an excellent point and we have tried to address this issue despite the technical limitations it poses. While the localization of C-term fragment of Dvl2 is likely to be less restricted to the centrin-RFP and slightly more diffuse upon Drg1 knockdown, a considerable amount of C-term of Dvl2 still co-localizes to the centrin-RFP. This data suggests that while Drg1 colocalizes to Dvl2 in MCCs, Drg1 may not be a major player in the modulation of Dvl localization to the basal body area. However, the reintroduction of WT Drg1 (but not Δ329-344) restores the basal body localization of Dvl2 c-term, like in the control, the interaction between Drg1 and Dvl2 may be necessary in part to stabilize the Dvl2 localization in the MCCs. We more clearly state this in the results.

Regarding the Daam1 localization in MCCs, Daam1 localized to the apical and subapical area of MCCs, but in a rather diffuse manner and is quite consistent with the previous reported localization data (Yasunaga et al., 2015 - Fig 4A and Fig S3G; and also Fig. S5 D and Fig. S5E in our manuscript). Daam1 contains several domains including an FH (formin-homology) and DAD (diaphanous autoregulatory domain; necessary for an interaction with Dvl). Since Daam1 associates with actin filaments through the actin binding region within the FH2 domain, the broad localization pattern of Daam1 in an MCC may not be unexpected. To address the association with Dvl at the rootlet, we took advantage of the DAD domain and generated a Dad- mCherry fusion. This construct shows Daam1 localization to the basal body-rootlet area. We observed the co-localization of DAD-mCherry with GFP-CLAMP at the rootlet where a major portion of Dvl localizes in MCCs (Fig. 6E). Drg1-GFP also co-localized with DAD-mCherry protein in MCCs, and the N-term-mCherry protein (lacking the DAD) was used as control (Fig S5 F). These data suggest that a portion of Daam1 associates with Dvl2 is found in the basal body area. This allowed us to then test whether MO-mediated loss of Drg1 is important for Daam1 localization to basal body area. The results led to a much clearer indication that in the absence of Drg1, DAD-mCherry no longer localized to the basal body region, and this was phenocopied by using Dvl2 and Dvl3 MOs. Moreover, re-introducing an MO-resistant Wild-type Drg1 RNA substantially rescued the localization of the DAD-mCherry construct to the basal bodies. The data support the model and other biochemical data indicating that Drg1-Dvl-Daam1 interaction regulates ciliogenesis and are now presented in Fig. 6 A-E and Fig. S5 B & S5F. The data displaying expression of the full-length Daam1-GFP construct is now presented in Fig. S5 D & E.

3. It is unclear how protein localization at the basal body relates to the observed defects in RhoA activity and apical actin localization. The authors propose a direct effect on basal body docking. An alternative explanation is a cell migration defect, as suggested by their data. Fig. 2A and Suppl. Fig. 3B show that the groups treated with Drg1 MO, especially in combination with Δ329-344, appear to have diminished number of MCCs coming to the surface. This possibility is suggested by equal cell size characteristic of goblet cells, or the presence of very small cells that could correspond to poorly integrated MCCs. Failure of MCC progenitors to get to the surface would suppress their differentiation, resulting in defective basal body positioning and ciliogenesis.

The reviewer proposes a very interesting and plausible alternative explanation for the effects on RhoA activity. Thus, to test the possibility of a delay or defect in MCC migration to the surface upon Drg1 knockdown, we fixed the embryos at stage 25 and transverse sectioned them, followed by α-tubulin immunostaining. α-tubulin is robustly expressed in MCC progenitors. When compared with control MO-injected embryos, Drg1 morphants did not show any defects in MCC migration, and similar to control morphants, MCCs successfully reached the surface of epidermis. We now present this data in Fig. S3 A.

4. The mild change in the enzymatic activity of T100D Drg1 shown in the cited paper is not sufficient to justify the conclusion that Drg1 does not require its GTPase activity for the observed effects. Since T100D may retain sufficient activity to rescue Drg1 knockdown phenotypes, introducing another mutation inactivating the enzyme would be warranted.

We agree with the reviewer. Although the T100D mutant is reported to decrease the GTPase activity by 2/3 of normal, the residual enzyme activity of the mutant could be sufficient to function as a GTPase. To prevent this possibility, we introduced another mutation at S78 (S78N), resulting in a double mutant to more effectively inactivate the GTPase. We tested whether the double mutant can rescue the Drg1 knockdown phenotypes and whether Dvl can bind to the S78N-T100D mutants. S78N-T100D mutant

results are as follows: 1) the expression of S78N-T100D at levels similar to wild-type is sufficient to rescue the Drg1 knockdown ciliogenesis phenotype; 2) S78N-T100D mutant protein associates with Dvl. Thus, these results suggest that the role of Drg1 in ciliogenesis is independent of its GTPase activity; 3) we performed a ciliary fluid flow assay to determine whether the GTPase activity, while not being necessary for cilia formation, may be required for proper function. This assay showed no impairment of function, indicating GTPase activity is not necessary for ciliogenesis. The data are included in Fig. S4 E, videos 4-7.

5. Some of the pulldown panels (e.g., Fig. 1D and E, top panels) should have formal controls for nonspecific 'stickiness' of the proteins of interest. Alternatively, they could be removed. The conclusions would still stand because the pulldowns have been performed in reciprocal experiments.

As suggested by the reviewer, we performed the Co-IPs including the formal controls. These data are now presented in Fig. 1D, 1E, and 5B.

6. Minor: Fig. 1A is mislabelled.

Thanks for catching this mislabeling. We have corrected it.

All the reviewers' assessments are much appreciated.
We look forward to a positive assessment of this revised manuscript.

Sincerely,

Ira Daar, Ph.D.
Senior Investigator, Chief
Cancer & Developmental Biology Laboratory
NCI, NIH

May 21, 2019

Re: JCB manuscript #201811147R

Dr. Ira Daar
National Cancer Institute
Cancer & Developmental Biology Laboratory
Bld. 560 rm. 12-88
Bld. 560 rm. 12-88
Frederick, MD 21702

Dear Ira -

We have received the comments from our 2 external referees for your revised manuscript "Developmentally Regulated GTP binding protein 1 modulates ciliogenesis through an interaction with Dishevelled". I am pleased to let you know that both reviewers felt that you had addressed most of the issues they had raised and that the paper is significantly strengthened. Overall, this is a very interesting study. However, reviewer #2 does raise one concern, about whether or not the data show a significant change in the apical surface area or number of MCCs, in Drg1 morphants, and feels that this issue is critical for discrimination of the underlying mechanism. My take on this is that, in Figure S3B, it is not possible to distinguish MCCs from other cell types so the number cannot be estimated. As this concern was brought up in the initial round of review, we believe it needs to be addressed before final acceptance for publication. At the same time, there are a couple of minor comments from this reviewer that could easily be addressed in the text, and I feel it would be helpful to readers if the brightness of some of the figures (Fig 4 B, C, D, and Fig S2 D,E) could be heightened - the images are barely visible in my copy of the PDF.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Ian Macara, Ph.D.
Editor
Journal of Cell Biology

Andrea L. Marat, Ph.D.

Scientific Editor
Journal of Cell Biology

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

In their revised manuscript, Lee et al. have rigorously addressed all the points raised in review. Several additions and improvements are presented that significantly strengthen the manuscript, including:
• Recombinant protein interaction analysis, which justifies the claim that the interaction between Dvl2 and Drg1 is direct.
• Improved characterization of the cilia defect in mutants, including measurements of cilia length and number in MCCs and GRP. Furthermore, the addition of functional data using a fluid flow velocity measurements is a nice addition, and the provided movies do an excellent job of illustrating the functional phenotype.
• Use of a constitutively-active Daam1 variant that suppresses phenotypes associated with loss of Drg1 is an excellent addition to the manuscript that clearly demonstrates the ciliary defects observed in the absence of Drg1 are due to defects in Daam1 activation.
The result is a very rigorous study that will be of significant interest to the broad readership of JCB. I have only a few minor comments/suggestions:
1. The last sentence of the introduction (page 7) appears to be a run-on, and should probably be re-worded.
2. Figure 1D (and 5B): I find the schematic at the top illustrating the different deletion constructs to be confusing. I suspect this was an error in exporting the graphic and should look more like FIG S1A.
3. Figure 1F: For the GST-Drg1-TGS recombinant protein, presumably the fusion protein around 35 kDa is the expected MW for that fusion protein. What is the identity of the higher MW (~80-90 kDa) species that also appears to be a GST fusion protein? The authors should probably comment on this at least in the legend.
4. For the text describing Figure 4B: Although it seems likely the signal corresponds to RhoA, the Rhotekin domain will bind GTP-bound RhoA, RhoB, or RhoC.

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

The revision successfully addressed many raised points, although one concern remains. Multiciliated cells (MCCs) are significantly smaller than non-intercalating cells allowing them to be distinguished from other skin cells. In Fig. S3B, the tissue morphology indicates that Drg1 MO causes incomplete or delayed apical emergence of MCCs, whereas the group injected with Drg1 MO + Δ329-344 RNA hardly shows any MCCs at the surface. This figure and some panels in Figs. 2B, 4C, 4D appear to contradict the authors' claim that the apical surface of MCCs and their numbers are not altered in Drg1 morphants (new figures Fig.2E and Fig. S3A). As a result, the reader is left wondering whether the observed changes are due a direct effect on ciliogenesis or abnormal MCC intercalation and differentiation. This issue is critical for the discrimination of the underlying mechanisms and should be resolved.

Because embryonic epidermis consists of multiple cell types, specific markers are needed to confirm that the observed changes take place in MCCs rather than another cell type. The use of a-tubulin or equivalent marker in these experiments should help clarify what is going on. Furthermore, stages

20-22 are more appropriate for studying MCC intercalation than stages 25-27, at which the delayed cells might 'catch up' with their faster unmanipulated counterparts.

Minor: It would be appropriate to describe the morphological phenotype that accompanies the observed defects in ciliation. For example, are there any left-right defects that are often associated with cilia abnormalities?

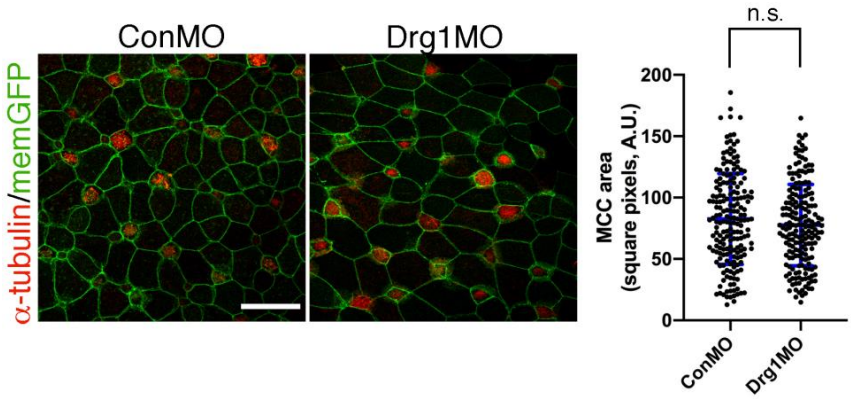
Dear Andrea and Ian,

We have now addressed all of the reviewers' and your comments and concerns, and we are really pleased with the final product, and we hope you will be as well.

Editor Comments:

Dear Ira -

We have received the comments from our 2 external referees for your revised manuscript "Developmentally Regulated GTP binding protein 1 modulates ciliogenesis through an interaction with Dishevelled". I am pleased to let you know that both reviewers felt that you had addressed most of the issues they had raised and that the paper is significantly strengthened. Overall, this is a very interesting study. However, reviewer #2 does raise one concern, about whether or not the data show a significant change in the apical surface area or number of MCCs, in Drg1 morphants, and feels that this issue is critical for discrimination of the underlying mechanism. My take on this is that, in Figure S3B, it is not possible to distinguish MCCs from other cell types so the number cannot be estimated. As this concern was brought up in the initial round of review, we believe it needs to be addressed before final acceptance for publication.



Now Figure S3B. The indicated morpholinos and mem-GFP RNAs were injected to two ventral blastomeres at 4-cell stage embryos. a-tubulin IF was performed with stage 22 embryos, and the surface area of epidermal MCCs was measured. The number of measured MCCs, n=190; embryos per group, n=8; two-tailed unpaired t test. Error bars indicate ± SD; scale bar, 50um.

I believe the reviewer was confused by the image in supplemental Figure S3B. We agree with the editor that in this image, there is no clear way to determine which cells are the MCCs. Moreover, size differences in this case could be due to a more anterior or posterior portion of the embryo being sampled. We did not use a marker since the point of the experiment was to examine the apical actin meshwork in controls and morphants.

However, we have now performed α-tubulin IF using stage 22 embryos with approximately the same AP positioning and this data is embedded in this response letter (above) and is now presented as Fig. S3B in the manuscript. Although the average surface area of control MCCs (82.5) is only slightly larger (~6% larger) than the average surface area of Drg1 morphant MCCs (77.6), the difference in MCC surface area between control and Drg1 morphants is not statistically significant (p=0.1713). Collectively, the data in Fig. 2e that measures the surface area of MCCs in Drg1 KO and controls at stage 27, Fig S3a which shows no effect on migration of MCCs, and this new data (Fig. S3 B) support the concept that Drg1 knockdown does not have a significant impact on the migration of MCC progenitors to the surface epidermis. Thus, the compromised multi-ciliation upon Drg1 knockdown is independent of the MCC progenitor migration.

At the same time, there are a couple of minor comments from this reviewer that could easily be addressed in the text, and I feel it would be helpful to readers if the brightness of some of the figures (Fig 4 B, C, D, and Fig S2 D,E) could be heightened - the images are barely visible in my copy of the PDF.

We have increased the brightness on the images, and the minor text adjustments have been made.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments.

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I have only a few minor comments/suggestions:

1. The last sentence of the introduction (page 7) appears to be a run-on, and should probably be re-worded.

We have corrected the sentence.

2. Figure 1D (and 5B): I find the schematic at the top illustrating the different deletion constructs to be confusing. I suspect this was an error in exporting the graphic and should look more like FIG S1A.

It appears that here was some problem with the PDF conversion where one layer of the schematic was lost – causing confusion. We believe we have corrected this issue along with an error in the amino acid numbering.

3. Figure 1F: For the GST-Drg1-TGS recombinant protein, presumably the fusion protein around 35 kDa is the expected MW for that fusion protein. What is the identity of the higher MW (~80-90 kDa) species that also appears to be a GST fusion protein? The authors should probably comment on this at least in the legend.

The isolated TGS domain can form a dimer (*Loveland et.al., 2016*). The band appearing at MW 80Kda for the GST-Drg1-TGS recombinant protein is likely a dimer of TGS:TGS. We include this information in the legend.

4. For the text describing Figure 4B: Although it seems likely the signal corresponds to RhoA, the Rhotekin domain will bind GTP-bound RhoA, RhoB, or RhoC.

As the reviewer suggests, the Rhotekin domain binds GTP-bound RhoA, RhoB, or RhoC. However, all of Rho GTPases are not activated during multiciliation. Among Rho family proteins, RhoA and RhoB are activated by Foxj1 expression in differentiated mTECs (cultured mouse tracheal epithelial cells forming) forming multicilia (*Pan et al., 2007*). Also, the Rho GTPase family proteins have differences in function and location. For example, RhoB is localized

primarily in endosomes (*Wheeler and Ridley, 2004*). Despite their functional and locational differences, however, we cannot rule out the potential shared role of RhoA and RhoB in multiciliation. Thus, we switched RhoA to Rho in the Results and Discussion sections related to Figure 4 for clarity.

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

The revision successfully addressed many raised points, although one concern remains. Multiciliated cells (MCCs) are significantly smaller than non-intercalating cells allowing them to be distinguished from other skin cells. In Fig. S3B, the tissue morphology indicates that Drg1 MO causes incomplete or delayed apical emergence of MCCs, whereas the group injected with Drg1 MO + Δ329-344 RNA hardly shows any MCCs at the surface. This figure and some panels in Figs. 2B, 4C, 4D appear to contradict the authors' claim that the apical surface of MCCs and their numbers are not altered in Drg1 morphants (new figures Fig.2E and Fig. S3A). As a result, the reader is left wondering whether the observed changes are due a direct effect on ciliogenesis or abnormal MCC intercalation and differentiation. This issue is critical for the discrimination of the underlying mechanisms and should be resolved.

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This is answered in the response to “Editors comments” above (bottom of page 1, and top of page 2).

Minor: It would be appropriate to describe the morphological phenotype that accompanies the observed defects in ciliation. For example, are there any left-right defects that are often associated with cilia abnormalities?

At the end of the Discussion section, we briefly describe the phenotypes and how they relate to the known ciliary phenotypes and hedgehog signaling. Since *JCB* discourages using “data not shown”, we have added images relating to the phenotypes as Fig. S5 I.

We greatly appreciate your patience and suggestions during this process.

Sincerely,

Ira

June 4, 2019

RE: JCB Manuscript #201811147RR

Dr. Ira Daar
National Cancer Institute
Cancer & Developmental Biology Laboratory
Bld. 560 rm. 12-88
Bld. 560 rm. 12-88
Frederick, MD 21702

Dear Dr. Daar:

Thank you for submitting your revised manuscript entitled "Developmentally Regulated GTP binding protein 1 modulates ciliogenesis via an interaction with Dishevelled". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles 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.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, * including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Ian Macara, Ph.D.
Editor
Journal of Cell Biology

Andrea L. Marat, Ph.D.
Scientific Editor
Journal of Cell Biology
