JCB

Rab7 regulates primary cilia disassembly through cilia excision

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Review Timeline:

Submission Date:	2018-11-26
Editorial Decision:	2019-01-07
Revision Received:	2019-07-07
Editorial Decision:	2019-08-06
Revision Received:	2019-09-05
Editorial Decision:	2019-09-16
Revision Received:	2019-09-21

Monitoring Editor: Monica Bettencourt-Dias

Scientific Editor: Melina Casadio

Transaction Report:

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

DOI: https://doi.org/N/A

January 7, 2019

Re: JCB manuscript #201811136

Dr. Hui-Yan Li
National Center of Biomedical Analysis
27,taiping road
Beijing 100850
China

Dear Dr. Li,

Thank you for submitting your manuscript entitled "Rab7 regulates primary cilia disassembly through cilia decapitation". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that while the reviewers find a potential role for Rab7 in cilia disassembly interesting, the consensus opinion is that it is currently not sufficiently supported by the data. Providing the level of experimental support required to substantiate your main findings appears more substantial than can be addressed in a typical revision period. Therefore, if you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic, I would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. In addition to the additional controls required by the reviewers, if you wish to resubmit it is essential that you show convincing localization of Rab7 in cilia (including nucleotide-dependency), using TIRF or super-resolution. Furthermore, you must clarify the binding and relationship between Rab7 and actin, and rule out alternative mechanisms for the action of Rab7 on actin/in cilia disassembly as suggested by the reviewers.

Because successful resubmission would involve a substantial new data set, if you would like to resubmit this work to JCB, please contact the journal office to discuss a revision plan, or you may submit a plan as an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Monica Bettencourt-Dias, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

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

This manuscript presents the unexpected finding that Rab7 promotes the secretion of extracellular vesicles at the tip of primary cilia by locally promoting actin polymerization. Several parallels have been made between cilia and endosomes in the past* and it is quite interesting to find that a small GTPase known for its role in late endosome maturation is now playing a role at the tip of the cilium. The

*cilia start out as endosomes as beautifully demonstrated by Sorokin (JCB 1962) and the endosomal GTPases Rab11 and Rab8 participate in ciliary trafficking.

The data are for the most part of high quality and the conclusions are well supported. The logic is relatively well articulated and I believe that this Report will be of general interest to the readership of the JCB. The manuscript is well-suited for a Report as it presents an original finding that will stimulate future research, in particular on the ciliary effectors of Rab7.

Major comments:

First, the evidence that Rab7 enters the cilium (Fig. 5A) is not sufficiently persuasive to gain acceptance. There is a considerable number of Rab7 foci in the cell and the close proximity of one such focus to the cilium in a couple of imaging planes could happen by chance. The methods lack sufficient details to determine whether Fig. 5A shows a single optical section or a projection. The authors are encouraged to generate a stable cell line expressing GFP-SMO and Cherry-Rab7 in order to minimize the expression levels of Rab7 and diminish the risk of associated artifactual localization. The use of TIRF may allow for much clearer results as the signal from intracellular organelles will be largely invisible. Alternatively, imaging cilia that are well detached from the cell may allow for the convincing demanstration that Rab7 appears in cilia before ectocytosis.

Second, the binding of Rab7 to actin (Fig. 5C and D) needs to be better controlled. Promiscuous binding to actin is not such a rare occurrence and powerful negative control are needed before one can conclude that Rab7 associates with actin. Other GTPases closely related to Rab7 hold be used as negative controls. As the authors are proposing the Rab7-GTP promotes actin polymerization in cilia, one predicts that Rab7-GTP but not Rab7-GDP will bind actin. The authors have the tools to conduct such experiments and their conclusions will benefit from further biochemical experimentation.

Third, the general scholarship needs attention.
P.12 'We assume that the cilia decapitation taking place in quiescent cells may maintain the equilibrium between cilia assembly and disassembly, avoiding abnormal ciliogenesis during quiescence.'
The authors must cite {Long, H., Zhang, F., Xu, N., Liu, G., Diener, D.R., Rosenbaum, J.L., Huang, K., 2016. Comparative Analysis of Ciliary Membranes and Ectosomes. Current biology

https://doi.org/10.1016/j.cub.2016.09.055} as the paper that first suggested ectocytosis as a mechanism for cilium shortening.

P.4 'Interestingly, a recent study provided more direct evidence of the role of actin dynamics in cilia disassembly, demonstrating that F-actin can polymerise in primary cilia to excise cilia tips for cilia decapitation'
Here the authors need to cite {Nager, A.R., Goldstein, J.S., Herranz-Pérez, V., Portran, D., Ye, F., García-Verdugo, J.M., Nachury, M.V., 2017. An Actin Network Dispatches Ciliary GPCRs into Extracellular Vesicles to Modulate Signaling. Cell 168, 252-263.e14. https://doi.org/10.1016/j.cell.2016.11.036} as this paper arrived a similar conclusions as Phua et al. regarding the role of actin in ectocytosis.

P.2 'demonstrate that Rab7 depletion significantly suppresses cilia tip excision, referred to as cilia decapitation, which is newly identified as required for cilia disassembly.'
This sentence gives the impression that the authors have discovered cilia decapitation in the current manuscript.

Finally, the use of the term 'cilia decapitation' may be frowned upon by those who would prefer not to increase the complexity of the scientific vocabulary unless it necessary. Rosenbaum et al. have used the term ectosomes since 2013 (PMID: 23623554) and the term has been generally accepted by the cilia community. The extracellular vesicle community prefers the term microvesicles but this latter term cannot be declined into an active verb. While decapitation may sound cute, it does not add anything to ectocytosis and the authors would be well advised to use the term 'ectocytosis' throughout.

Minor comments:

The authors may wish to cite {Mick, D.U., Rodrigues, R.B., Leib, R.D., Adams, C.M., Chien, A.S., Gygi, S.P., Nachury, M.V., 2015. Proteomics of Primary Cilia by Proximity Labeling. Developmental Cell 35, 497-512. https://doi.org/10.1016/j.devcel.2015.10.015} which had identified Rab7 in their cilia proteome. Rab7 in the cilia proteome seemed like an anomaly at the time and it is quite interesting to see this identification validated a posteriori.

P.7 'the level of endogenous Rab7 decreased continuously during ciliogenesis and increased moderately during cilia disassembly, resembling the profile of the established cilia disassembly regulator, AurA (Fig. 2 E).'
The authors need to graph the band intensity before reaching this conclusion. It appears to me that the magnitude of Rab7 accumulation is much less than that of aurA

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

Rab7 regulates primary cilia disassembly through cilia decapitation
The manuscript by Wang et al characterizes how loss of function or gain of function of the small GTPase Rab7 impacts the primary cilium in cultured human RPE-1 cells. Rab7 is known to play multiple roles in different intracellular trafficking events and, not surprisingly, localizes to ER, TGN, late endosomes, lysosomes, and mitochondrial membranes (Jimenez-Orgaz et al EMBO 2018). Here, authors show that Rab7 inhibits ciliogenesis even in the presence of serum (using siRNA) and promotes ciliary disassembly upon serum stimulation (using siRNA). Until this point in the

manuscript, the strategy, data, and interpretations are experimentally sound. After this point, the data does not support the proposed mechanisms of action for Rab7 in ciliary disassembly and, as detailed below, the manuscript is not appropriate for publication in the JCB.
Authors focus on Rab7 action in ciliary decapitation - a newly discovered phenomenon whereby the mammalian ciliary tip is clipped via coordinated actions of phosphoinositides and actin and process that precedes ciliary disassembly. Authors show that Rab7 promotes ciliary decapitation under serum-stimulated but not serum-starved conditions (using siRNA). Next, authors claim that Rab7 is acting in the cilium to regulate actin polymerization at the region of ciliary decapitation. In this section, the supporting data is extremely weak. Figure 5A shows Rab7 localization and use an arrow to argue that Rab7 and Smoothened colocalize. This data is not convincing. Rab7 (red) appears to localize everywhere except the cilium. To make this argument convincing, authors must show the location of WT Rab7, Rab7 active, and Rab7 DN in the RPE-1 cilium labeled with Smoothened, Arl13, acetylated tubulin, and an IFT reporter. Without this essential piece of data, there is no evidence that Rab7 is acting within the cilium.
Likewise, the data for Rab7 acting in the cilium to control F-actin assembly (a Lifeact reporter) is weak. Rab7 is known to control phosphoinositides in other intracellular compartments, PIs regulate ciliary decapitation - the effect of Rab7 on ciliary disassembly via decapitation could be entirely indirect. Authors dismiss a lysosomal role of Rab7 in ciliary disassembly based on pharmacological inhibitors (NH_4Cl and chloroquine), but ignore the multifaceted roles of Rab7 in other intracellular compartments.

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

The manuscript by Wang et al. describes a novel and very interesting finding. The authors report that Rab7 regulates primary cilia disassembly through the regulation of cilia decapitation. This is a novel function of Rab7, which is known as a regulator of late endosome homotypic and heterotypic fusion. The findings about Rab7 regulating primary cilia disassembly through cilia decapitation are strong in general, but the mechanism proposed lacks solid evidence. Indeed, the results shown in Fig. 5 fall short of JCB standards. Therefore, the authors need to strength and extend the mechanistic evidence to reach the standard needed for the manuscript to be accepted in JCB.

Major issues:

1 - In Fig. 1, the authors should attempt a rescue experiment to rule out off-target effects of the siRNAs targeting Rab7 on the percentage of cells with cilia and cilia length. The authors should also assess the dependency on GTP/GDP binding by testing the dominant negative and constitutively active mutants employed in Fig. 3.

2 - In Fig. 3, the distribution of dominant-negative Rab7 is similar to the wt and constitutively active mutant. This is not what is expected from a Rab dominant negative mutant, which does not bind to the membrane and therefore should be cytosolic. An example of the cytosolic distribution of Rab7 dominant negative mutant is shown in Kimura et al. Autophagy. 2007 Sep-Oct;3(5):452-60. Is this an issue with this type of cells? In other words, if the authors use the mutant in HeLa cells, is the distribution similar to what was obtained by other authors? This must be thoroughly determined.

3 - In Fig. 3, the authors use Cytochalasin D to interfere with the actin cytoskeleton (although they do not refer this in the text, as they should). It would be informative to assess if latrunculin A gives the same result, since cytochalasin D inhibits actin-cofilin interaction and cofilin was shown by Phua

et al. to lead to the loss of INPP5E and accumulation of PI(4,5)P2 (see 6c, below).

4 - In Fig. 4, the authors do not show any results with Rab7 Q67L. Is there any reason for this? Did the authors attempt to use this mutant?

5 - In Fig. 4 and 5 the authors use Smoothened, which is a ciliary receptor that normally is only present in cilia upon activation of Shh signaling. Could the authors validate some of the results in cells expressing tagged HTR6, a well-established ciliary receptor?

6 - Regarding Fig. 5, there are several issues:

6a - The localization of Rab7 to cilia is not convincing. The vesicle identified with an arrow could well be outside cilia (underneath or above the focal plane). Adding to this, the signal for Rab7 is saturated and the resolution does not allow to take any conclusion. Significantly better imaging, ideally super-resolution should be done to convincingly show the presence of Rab7 in cilia, which is one of the crucial points that is made.

6b - The co-sedimentation does not allow the authors to take firm conclusions about biochemical interaction, let alone a direct interaction, as the authors claim. One crucial limitation is that cytoskeleton elements such as actin filaments sediment easily. Adding to this, actin is known to be sticky and therefore great care must be taken when interpreting interactions with actin. Several experiments are needed before the authors can take any conclusion: co-IPs (in both ways) should be performed and the dependency of GTP/GDP binding assessed either with mutants or GTPgS/GDP loading. If Rab7 interacts with actin in a GTP-dependent manner, the GDP-bound condition will serve as a perfect negative control. To show a direct interaction, the authors have to use purified proteins and assess if they co-IP. Stringent negative controls are essential.

6c - Regarding the mechanism, Phua et al. showed that cilia undergo decapitation after the loss of INPP5E, which leads to an accumulation of PI(4,5)P2 at the distal tip of cilia. These authors also showed that PI(4,5)P2 accumulation leads to F-actin polymerization. Therefore, the authors should assess if Rab7 regulates the loss of ciliary INPP5E, and/or PI(4,5)P2 accumulation.

Minor issues:

7 - The authors use either a-tubulin or GAPDH as loading controls. Is there any reason for the alternative use of different housekeeping genes instead of just only one throughout the experiments?

8 - The authors have to remove all the references to a direct interaction between Rab7 and actin, unless they convincingly show it (see 6b).

9 - page 4, line 19, "IFT trafficking" is redundant. "IFT" is enough, since it stands for "intraflagellar transport".

10 - A reference is missing in page 6, line 3.

11 - In page 7, line 7, "completely blocked" is a strong statement that is difficult to show in absolute terms. Therefore "blocked" should be used instead.

12 - In page 11, line 10, it should read "is involved..."

Point-to-point responses to the reviewers' concerns.

Reviewer #1:

This manuscript presents the unexpected finding that Rab7 promotes the secretion of extracellular vesicles at the tip of primary cilia by locally promoting actin polymerization. Several parallels have been made between cilia and endosomes in the past and it is quite interesting to find that a small GTPase known for its role in late endosome maturation is now playing a role at the tip of the cilium. The cilia start out as endosomes as beautifully demonstrated by Sorokin (JCB 1962) and the endosomal GTPases Rab11 and Rab8 participate in ciliary trafficking. The data are for the most part of high quality and the conclusions are well supported. The logic is relatively well articulated and I believe that this Report will be of general interest to the readership of the JCB. The manuscript is well-suited for a Report as it presents an original finding that will stimulate future research, in particular on the ciliary effectors of Rab7.

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Reviewer 1 comment 1. First, the evidence that Rab7 enters the cilium (Fig. 5A) is not sufficiently persuasive to gain acceptance. There is a considerable number of Rab7 foci in the cell and the close proximity of one such focus to the cilium in a couple of imaging planes could happen by chance. The methods lack sufficient details to determine whether Fig. 5A shows a single optical section or a projection. The authors are encouraged to generate a stable cell line expressing GFP-SMO and Cherry-Rab7 in order to minimize the expression levels of Rab7 and diminish the risk of associated artifactual localization. The use of TIRF may allow for much clearer results as the signal from intracellular organelles will be largely invisible. Alternatively, imaging cilia that are well detached from the cell may allow for the convincing demanstration that Rab7 appears in cilia before ectocytosis.

Response: Many thanks for the constructive suggestions. To diminish the risk of

associated artifactual localization, we have transfected low levels of Rab7and applied another ciliary marker HTR6 to show the localization of Rab7 in cilia. We have obtained much clearer results that Rab7 transiently localized at the cilia tip excision site, which is shown in a single optical section (in Fig. 5A in our revised vesion). In addition, as primary cilia do not constantly adhere to the underlying substratum, the use of TIRF often defocus of the cilia section during observation in our experiment. So we choosed to use inverted fluorescence microscope with UltraView spinning-disc confocal scanner unit instead to obtain optical sections of primary cilia.

Reviewer 1 comment 2. Second, the binding of Rab7 to actin (Fig. 5C and D) needs to be better controlled. Promiscuous binding to actin is not such a rare occurrence and powerful negative control are needed before one can conclude that Rab7 associates with actin. Other GTPases closely related to Rab7 hold be used as negative controls. As the authors are proposing the Rab7-GTP promotes actin polymerization in cilia, one predicts that Rab7-GTP but not Rab7-GDP will bind actin. The authors have the tools to conduct such experiments and their conclusions will benefit from further biochemical experimentation.

Response: Many thanks for the suggestions. To confirm the interaction between actin and Rab7, we have performed *in vitro* binding assays using purified actin and Rab7 mutant proteins. Also, we have used the dominant negative mutant of Rab7 (T22N) as a negative control. The result shows that Rab7-Q67L but not Rab7-T22N could bind actin (Fig. 5D in the revised version), suggesting that Rab7 interacts with actin in a GTP dependent manner.

Reviewer 1 comment 3. Third, the general scholarship needs attention.

P.12 'We assume that the cilia decapitation taking place in quiescent cells may maintain the equilibrium between cilia assembly and disassembly, avoiding abnormal ciliogenesis during quiescence.'

The authors must cite {Long, H., Zhang, F., Xu, N., Liu, G., Diener, D.R., Rosenbaum, J.L., Huang, K., 2016. Comparative Analysis of Ciliary Membranes and Ectosomes. Current biology https://doi.org/10.1016/j.cub.2016.09.055} as the paper that first suggested ectocytosis as a mechanism for cilium shortening.

Response: Thanks for the suggestion. We have cited this elegant paper in our revised manuscript.

P.4 'Interestingly, a recent study provided more direct evidence of the role of actin dynamics in cilia disassembly, demonstrating that F-actin can polymerise in primary cilia to excise cilia tips for cilia decapitation'.

Here the authors need to cite {Nager, A.R., Goldstein, J.S., Herranz-Pérez, V., Portran, D., Ye, F., García-Verdugo, J.M., Nachury, M.V., 2017. An Actin Network Dispatches Ciliary GPCRs into Extracellular Vesicles to Modulate Signaling. Cell 168, 252-263.e14. https://doi.org/10.1016/j.cell.2016.11.036} as this paper arrived a similar conclusions as Phua et al. regarding the role of actin in ectocytosis.

Response: Thanks for the suggestion. We have cited this elegant paper in our revised manuscript.

P.2 'demonstrate that Rab7 depletion significantly suppresses cilia tip excision, referred to as cilia decapitation, which is newly identified as required for cilia disassembly.'

This sentence gives the impression that the authors have discovered cilia decapitation in the current manuscript.

Response: Thanks for the suggestion. We have modified our description in our revised manuscript as 'demonstrate that Rab7 depletion significantly suppresses cilia tip excision, referred to as cilia decapitation, which has been identified as required for cilia disassembly.'

Reviewer 1 comment 4. The use of the term 'cilia decapitation' may be frowned upon by those who would prefer not to increase the complexity of the scientific vocabulary unless it necessary. Rosenbaum et al. have used the term ectosomes since 2013 (PMID: 23623554) and the term has been generally accepted by the cilia community. The extracellular vesicle community prefers the term microvesicles but this latter term cannot be declined into an active verb. While decapitation may sound cute, it does not add anything to ectocytosis and the authors would be well advised to use the term 'ectocytosis' throughout.

Response: We are grateful to the reviewer for these suggestions. In our revised version, we have used the term 'ectocytosis' instead of 'decapitation'.

Reviewer 1 Minor comments.

Reviewer 1 comment 5. The authors may wish to cite {Mick, D.U., Rodrigues, R.B., Leib, R.D., Adams, C.M., Chien, A.S., Gygi, S.P., Nachury, M.V., 2015. Proteomics of Primary Cilia by Proximity Labeling. Developmental Cell 35, 497-512. https://doi.org/10.1016/j.devcel.2015.10.015} which had identified Rab7 in their cilia proteome. Rab7 in the cilia proteome seemed like an anomaly at the time and it is quite interesting to see this identification validated a posteriori.

Response: Thanks for the suggestion. This information is very useful for us and will strengthen our findings. We have cited this elegant paper in our revised manuscript.

Reviewer 1 comment 6. P.7 'the level of endogenous Rab7 decreased continuously during ciliogenesis and increased moderately during cilia disassembly, resembling the profile of the established cilia disassembly regulator, AurA (Fig. 2 E).'

The authors need to graph the band intensity before reaching this conclusion. It appears to me that the magnitude of Rab7 accumulation is much less than that of AurA

Response: Thanks for this suggestion. We have graphed the band intensity with Image

J software to better show the expression profiles of Rab7 during ciliogensis and cilia disassembly in Fig. 1E in our revised version. The result shows that the level of endogenous Rab7 decreases continuously during ciliogenesis and increases moderately during cilia disassembly, resembling the profile of the established cilia disassembly regulator, AurA.

Reviewer #2:

Rab7 regulates primary cilia disassembly through cilia decapitation

The manuscript by Wang et al characterizes how loss of function or gain of function of the small GTPase Rab7 impacts the primary cilium in cultured human RPE-1 cells. Rab7 is known to play multiple roles in different intracellular trafficking events and, not surprisingly, localizes to ER, TGN, late endosomes, lysosomes, and mitochondrial membranes (Jimenez-Orgaz et al EMBO 2018). Here, authors show that Rab7 inhibits ciliogenesis even in the presence of serum (using siRNA) and promotes ciliary disassembly upon serum stimulation (using siRNA). Until this point in the manuscript, the strategy, data, and interpretations are experimentally sound. After this point, the data does not support the proposed mechanisms of action for Rab7 in ciliary disassembly and, as detailed below, the manuscript is not appropriate for publication in the JCB.

Reviewer 2 comment 1. Authors focus on Rab7 action in ciliary decapitation - a newly discovered phenomenon whereby the mammalian ciliary tip is clipped via coordinated actions of phosphoinositides and actin and process that precedes ciliary disassembly. Authors show that Rab7 promotes ciliary decapitation under serum-stimulated but not serum-starved conditions (using siRNA). Next, authors claim that Rab7 is acting in the cilium to regulate actin polymerization at the region of ciliary decapitation. In this section, the supporting data is extremely weak. Figure 5A shows Rab7 localization and use an arrow to argue that Rab7 and Smoothened colocalize. This data is not convincing. Rab7 (red) appears to localize everywhere except the cilium. To make this argument convincing, authors must show the location of WT Rab7, Rab7 active, and Rab7 DN in the RPE-1 cilium labeled with Smoothened, Arl13, acetylated tubulin, and an IFT reporter. Without this essential piece of data, there is no evidence that Rab7 is acting within the cilium.

Response: Thanks for the critical and constructive suggestions. To diminish the risk of

associated artifactual localization, we have transfected low levels of Rab7and applied another ciliary marker HTR6 to show the localization of Rab7 in cilia. We have obtained much clearer results that Rab7 transiently localized at the cilia tip excision site, which is shown in a single optical section (Fig. 5A in our revised vesion).

Reviewer 2 comment 2. The data for Rab7 acting in the cilium to control F-actin assembly (a Lifeact reporter) is weak. Rab7 is known to control phosphoinositides in other intracellular compartments, PIs regulate ciliary decapitation - the effect of Rab7 on ciliary disassembly via decapitation could be entirely indirect. Authors dismiss a lysosomal role of Rab7 in ciliary disassembly based on pharmacological inhibitors (NH4Cl and chloroquine), but ignore the multifaceted roles of Rab7 in other intracellular compartments.

Response: Thanks for raising these important points. To confirm the interaction between actin and Rab7, we have performed in vitro binding assays using purified actin and Rab7 mutant proteins. We have used the dominant negative mutant of Rab7 (T22N) as a negative control. The result shows that Rab7-Q67L but not Rab7-T22N could bind actin (Fig. 5D in the revised version), suggesting that Rab7 interacts with actin in a GTP dependent manner.

The reviewer's second concern is that "*the effect of Rab7 on ciliary disassembly via decapitation could be entirely indirect"*. As described by the reviewer, Rab7 is well-known to play roles in controlling phosphoinositides switching in intracellular compartments through its effectors PI3K and WDR91, such as PI, PI(3)P and PI(3,5)P2 (Stein et al., *Traffic*, 2003; Liu et al., *JCB*, 2017). However, it is unclear whether these phosphoinositides could participate in ciliary decapitation. According to previous reports, PI(4)P/PI(4,5)P2 switching is reported to be involved in this process. In order to exclude that Rab7 regulates ciliary decapitation indirectly through PIs, we have explored the effect of Rab7 knockdown during serum stimulation and found no obvious difference in ciliary reduction of INPP5E and ciliary accumulation of PI(4,5)P2, which regulates the PI(4)P/PI(4,5)P2 switching in cilia, between Rab7 knockdown cells and

control cells (Fig. S3 in the revised version). Additionally, to rule out whether Rab7 regulates cilia disassembly depending on its localization on other intracellular compartments, we knocked down VPS35 to inhibit Rab7 localization to ER and mitochondrial membranes (Jimenez-Orgaz et al., *EMBO*, 2018). However, VPS35 knock-down had no effect on cilia disassembly (Fig. S1 C-E in the revised version), suggesting that the role of Rab7 in cilia disassembly unlikely depends on its localization to ER or mitochondrial membranes.

Reviewer #3:

The manuscript by Wang et al. describes a novel and very interesting finding. The authors report that Rab7 regulates primary cilia disassembly through the regulation of cilia decapitation. This is a novel function of Rab7, which is known as a regulator of late endosome homotypic and heterotypic fusion. The findings about Rab7 regulating primary cilia disassembly through cilia decapitation are strong in general, but the mechanism proposed lacks solid evidence. Indeed, the results shown in Fig. 5 fall short of JCB standards. Therefore, the authors need to strength and extend the mechanistic evidence to reach the standard needed for the manuscript to be accepted in JCB.

Major issues:

Reviewer 3 comment 1. In Fig. 1, the authors should attempt a rescue experiment to rule out off-target effects of the siRNAs targeting Rab7 on the percentage of cells with cilia and cilia length. The authors should also assess the dependency on GTP/GDP binding by testing the dominant negative and constitutively active mutants employed in Fig. 3.

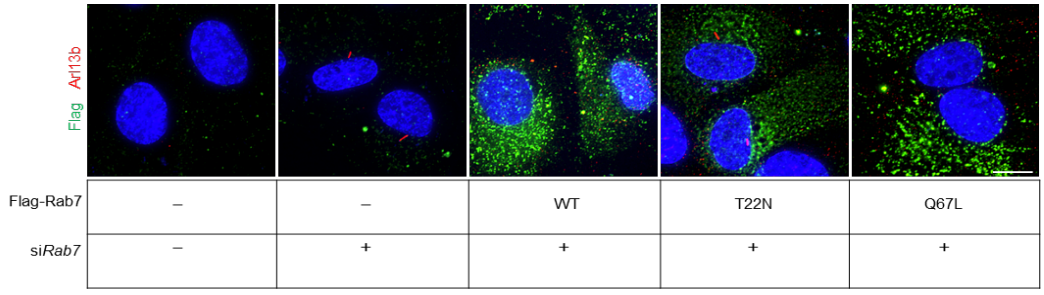
Response: Thanks for the suggestions. To rule out off-target effects of siRNA and assess the dependency on GTP/GDP binding of Rab7 in Fig.1, we have performed rescue experiments by using Rab7 mutants. The results show that wild-type Rab7 and Q67L mutant could efficiently rescue the spontaneous ciliogenesis and prolonged cilia length in Rab7 knockdown cells, while the dominant negative Rab7 mutant, T22N, failed to rescue these phenotypes (Fig. S1 A and B in the revised manuscript), which suggests the requirement of active Rab7 in proper maintenance of ciliogenesis.

Reviewer 3 comment 2. In Fig. 3, the distribution of dominant-negative Rab7 is similar to the wt and constitutively active mutant. This is not what is expected from a Rab dominant negative mutant, which does not bind to the membrane and therefore should be cytosolic. An example of the cytosolic distribution of Rab7 dominant negative mutant

is shown in Kimura et al. Autophagy. 2007 Sep-Oct;3(5):452-60. Is this an issue with this type of cells? In other words, if the authors use the mutant in HeLa cells, is the distribution similar to what was obtained by other authors? This must be thoroughly determined.

Response: Thanks for the reviewer's keen observation. We apologize for unclear description on these images. In our original manuscript, in order to show cilia clearly, the representative images in Fig. 3A were processed by iterative constrained deconvolution (SoftWoRx, Applied Precision Instruments). In the raw images without deconvolution, we find that the distribution of Rab7 dominant negative mutant, T22N, looks different from the membrane associated distribution of wild-type and the dominant active mutant Q67L. The distribution of T22N seems cytosolic and not binding to the membrane, which is in consistent with previous reports (**Figure for reviewer #3**). (Also shown in Fig. 3A in the revised version).

a.



b.

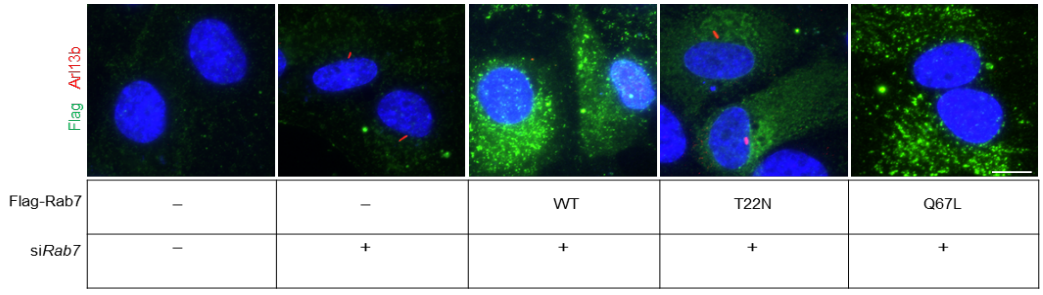


Figure for reviewer #3. a, Deconvoluted images in Fig. 3A. b, Raw images of Fig. 3A

without deconvolution. Quiescent RPE-1 cells transfected with the indicated siRNAs and plasmids were subjected to serum re-stimulation for 24 h. Cells were stained with Flag (green) and Arl13b (red). Nuclei were stained with Hoechst (blue). WT, wild type Rab7; T22N, dominant negative mutant of Rab7; Q67L, dominant active mutant of Rab7. Scale bar, 10 μm.

Reviewer 3 comment 3. In Fig. 3, the authors use Cytochalasin D to interfere with the actin cytoskeleton (although they do not refer this in the text, as they should). It would be informative to assess if latrunculin A gives the same result, since cytochalasin D inhibits actin-cofilin interaction and cofilin was shown by Phua et al. to lead to the loss of INPP5E and accumulation of PI(4,5)P2 (see 6c, below).

Response: Thanks for the constructive suggestion. To rule out the possible indirect effect of cytochalasin D on cilia decapitation through cofilin, we have applied latrunculin A instead to interfere with the actin cytoskeleton, which also shows inhibition of cilia decapitation during serum stimulation (Fig. 4 A,B in the revised version).

Reviewer 3 comment 4. In Fig. 4, the authors do not show any results with Rab7 Q67L. Is there any reason for this? Did the authors attempt to use this mutant?

Response: Thanks for the constructive suggestion. In our original manuscript, since the effect of the dominant active Rab7 Q67L on cilia disassembly is similar to Rab7 wide-type in Fig. 3, we only applied wide-type and the dominant negative T22N for further experiments in Fig. 4. In addition, in Fig.4 of our revised version, we have also shown that Rab7-Q67L could rescue the inhibitory effect of Rab7 knockdown on cilia decapitation.

Reviewer 3 comment 5. In Fig. 4 and 5 the authors use Smoothened, which is a ciliary receptor that normally is only present in cilia upon activation of Shh signaling. Could the authors validate some of the results in cells expressing tagged HTR6, a well-

established ciliary receptor?

Response: We apologize for the lack of detailed description of the cell line stably expressing Smoothened. This cell line stably expresses constitutive active W535L mutant of Smoothened which is constantly present in cilia and is kindly provided by Christopher J. Westlake (Quanlong Lu et al., *NCB,* 2015). We have added this information in our revised manuscript. In addition, according to the suggestion, we have applied another ciliary marker HTR6 and showed that Rab7 could localize to cilia during cilia decapitation in Fig. 5A in our revised version.

Reviewer 3 comment 6. Regarding Fig. 5, there are several issues:

6a - The localization of Rab7 to cilia is not convincing. The vesicle identified with an arrow could well be outside cilia (underneath or above the focal plane). Adding to this, the signal for Rab7 is saturated and the resolution does not allow to take any conclusion. Significantly better imaging, ideally super-resolution should be done to convincingly show the presence of Rab7 in cilia, which is one of the crucial points that is made.

Response: Many thanks for your suggestions. To diminish the risk of associated artifactual localization, we have transfected low levels of Rab7and applied another ciliary marker HTR6 to show the localization of Rab7 in cilia. We have obtained much clearer results that Rab7 transiently localized at the cilia tip excision site, which is shown in a single optical section (Fig. 5A in our revised vesion). In addition, owing to the phototoxicity and photobleaching when using super-resolution microscope, the cells and primary cilia do not exhibit normally during observation in our experiment. So we choosed to use inverted fluorescence microscope with UltraView spinning-disc confocal scanner unit instead to obtain optical sections of primary cilia.

6b - The co-sedimentation does not allow the authors to take firm conclusions about biochemical interaction, let alone a direct interaction, as the authors claim. One crucial limitation is that cytoskeleton elements such as actin filaments sediment easily. Adding

to this, actin is known to be sticky and therefore great care must be taken when interpreting interactions with actin. Several experiments are needed before the authors can take any conclusion: co-IPs (in both ways) should be performed and the dependency of GTP/GDP binding assessed either with mutants or GTPgS/GDP loading. If Rab7 interacts with actin in a GTP-dependent manner, the GDP-bound condition will serve as a perfect negative control. To show a direct interaction, the authors have to use purified proteins and assess if they co-IP. Stringent negative controls are essential.

Response: Many thanks for the suggestions. To confirm the interaction between actin and Rab7, we have performed in vitro binding assays using purified actin and Rab7 mutant proteins. We have used the dominant negative mutant of Rab7 (T22N) as a negative control. The result shows that Rab7-Q67L but not Rab7-T22N could bind actin (Fig. 5D in the revised version), suggesting that Rab7 interacts with actin in a GTP dependent manner.

6c - Regarding the mechanism, Phua et al. showed that cilia undergo decapitation after the loss of INPP5E, which leads to an accumulation of PI(4,5)P2 at the distal tip of cilia. These authors also showed that PI(4,5)P2 accumulation leads to F-actin polymerization. Therefore, the authors should assess if Rab7 regulates the loss of ciliary INPP5E, and/or PI(4,5)P2 accumulation.

Response: Thanks for raising this important point. In order to exclude that Rab7 regulates ciliary decapitation indirectly through INPP5E and PI(4,5)P2, we have explored the effect of Rab7 knockdown during serum stimulation and found no obvious difference in ciliary reduction of INPP5E and ciliary accumulation of PI(4,5)P2 between Rab7 knockdown cells and control cells (Fig. S3 in the revised version).

Reviewer 3 Minor comments.

Reviewer 3 comment 7. The authors use either a-tubulin or GAPDH as loading controls. Is there any reason for the alternative use of different housekeeping genes instead of

just only one throughout the experiments?

Response: We apologize for showing the different loading controls in different experiments. As the expression level of α-tubulin and GAPDH varies in cells, we often detect both of them in the same experiment. According to the suggestion, we have shown GAPDH as the consistent loading control in our revised version.

Reviewer 3 comment 8. The authors have to remove all the references to a direct interaction between Rab7 and actin, unless they convincingly show it (see 6b).

Response: Thanks for the suggestions. We have removed these references as suggested.

Reviewer 3 comment 9. page 4, line 19, "IFT trafficking" is redundant. "IFT" is enough, since it stands for "intraflagellar transport".

Response: Thanks for this kind reminder. We have corrected this description in our revised manuscript.

Reviewer 3 comment 10. A reference is missing in page 6, line 3.

Response: We apologize for the unclear description. The data mentioned in page 6, line 3 is only a little part of our separate study, which has not been completed and submitted, so that no reference could be cited currently.

Reviewer 3 comment 11. In page 7, line 7, "completely blocked" is a strong statement that is difficult to show in absolute terms. Therefore "blocked" should be used instead.

Response: Thanks for this kind reminder. We have used “blocked” instead in our revised manuscript.

Reviewer 3 comment 12. In page 11, line 10, it should read "is involved..."

Response: Thanks for this kind reminder. We have corrected this grammar mistake in

our revised manuscript.

August 6, 2019

Re: JCB manuscript #201811136R-A

Dr. Hui-Yan Li
National Center of Biomedical Analysis
27,taiping road
Beijing 100850
China

Dear Dr. Li,

Thank you for submitting your revised manuscript entitled "Rab7 regulates primary cilia disassembly through cilia excision" and thank you for your patience with the re-review process. The manuscript has been seen by the original reviewers, whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

We and the reviewers appreciated the new data presented in revision to bolster the Rab7 localization data, the binding relationship between Rab7 and actin, and to test the alternative mechanisms suggested by the reviewers. You will see that Revs#1 and #3 now support publication pending a text edit for Rev#1 and a few minor changes per Rev#3. Rev#2 is however still quite critical of the work due to several issues:

- Rev#2's major issue is that the full range of requested localization studies of WT Rab7, Rab7 active mutant, and Rab7 DN in the RPE-1 cilium labeled with Smoothened, Arl13, acetylated tubulin, and an IF were not provided, hence, in this referee's view, the claim that Rab7 regulates ectocytosis and ciliary excision is not sufficiently supported by the data. The reviewer insists that a ciliary base and an axonemal marker are essential to draw conclusions about Rab7's localization and cites other published works that use such series of markers to bolster their claims. We discussed these points internally in detail and also obtained input from Rev#1. Moreover, we discussed the recent PLOS Genetics paper from the Stearns lab brought up by Rev#2, suggesting that whole cilium shedding is a possible process for disassembly. We appreciate that this work does not entirely exclude the hypothesis that the whole cilium is shed by fractionating in a series of small vesicles and we do not feel that it would be entirely fair to you to evaluate the data in the context of a paper published during the review process. However, commenting on the recent work in this field in the manuscript text seems appropriate and important. In addition, using a base and a ciliary membrane marker to orient cilia in imaging analyses is reasonable and important to strengthen the localization data, as previously requested during the review process.

- Rev#2 has other requests - such as using negative stain immunogold TEM of isolated vesicles with a series of markers for cilium labeling to establish ectocytosis is occurring in figure 4 and providing controls in 3A for Flag-Rab7 WT, T22N, Q67L (no siRab7). We appreciate these valid points. However, we are also mindful of our long-standing policy not to move the bar between rounds of review. These experimental issues were not raised previously and we note that the other referees find the conclusions supported despite the lack of these experiments, so upon editorial discussions, we will not require new data to address these points for publication in JCB.

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 we will expect to make a final decision without additional reviewer input upon resubmission. The revision should be focused on addressing Revs #1 and #3's points in full, discussing recent work in response to Rev#2's comments and providing the previously requested imaging analyses for Rab7 with both base and ciliary membrane markers.

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. Please feel free to contact us to discuss these final changes or if you anticipate any issues addressing these final points.

Thank you for this interesting contribution to the 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,

Monica Bettencourt-Dias, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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

The authors have done a terrific job addressing my concerns. The new imaging data in Fig. 5 and make a convincing case for Rab7 targeting to cilia prior to the initiation of ectocytosis. The interaction of actin specifically with Rab7-GTP considerably strengthens this aspect of the paper. This will be an excellent report for JCB.

One small edit that I would suggest for clarity's sake is to change the sentence 'We assume that the cilia ectocytosis taking place in quiescent cells may maintain the equilibrium between cilia assembly and disassembly, avoiding abnormal ciliogenesis during quiescence (Long et al., 2016).' to 'We assume that the cilia ectocytosis taking place in quiescent cells may maintain the equilibrium between cilia assembly and disassembly to keep length constant (Long et al., 2016).'

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

Cilia excision and cilia ectocytosis are two different processes. The rapid removal of cilia is referred to in the literature as deciliation, ciliary excision, shedding, deflagellation, or flagellar autonomy. Ectocytosis is budding from the plasma membrane and shedding of extracellular vesicles. Authors present data suggesting distal severing of the cilium, there is no data showing a role for Rab7 in ectocytosis. Figures 4, 5, and 7 only show a ciliary membrane marker. A ciliary base and axonemal marker are critical to draw conclusions about ciliary excision. Per my original review: "to make this argument convincing, authors must show the location of WT Rab7, Rab7 active, and Rab7 DN in

the RPE-1 cilium labeled with Smoothened, Arl13, acetylated tubulin, and an IFT reporter." Authors did not address this criticism, which becomes more important now that authors propose that Rab7 regulates ectocytosis and ciliary excision.
Authors are directed to the following work by the Stearns lab (originally posted on BioRxIv August 2018) describing mammalian ciliary loss. In this paper, Mirvis et al use a combination of markers to visualize the cilium: a marker for the ciliary base, a marker for the axoneme, and a marker for the ciliary membrane.
Primary cilium loss in mammalian cells occurs predominantly by whole-cilium shedding.Mirvis M, Siemers KA, Nelson WJ, Stearns TP.PLoS Biol. 2019 Jul 17;17(7):e3000381.
Phua et al 2017 coined the term ciliary decapitation, which is distal severing/excision of the cilium. Phua et al use combinations of ciliary base, ciliary axoneme, and ciliary membrane to analyze distal ciliary excision.
Nager et al 2017 characterize ectocytosis in mammalian cilia. Nager et al use cilia base, ciliary axoneme, and ciliary membrane markers to analyze the shedding of GPCRs from the ciliary tip. To prove a particle is an extracellular vesicle, the gold standard in the field is to isolate EVs and perform negative stain immunogold EM. Nager et al showed SSTR3 in EVs purified from IMCD3 culture supernatant.
Figure 3A is missing the controls for Flag-Rab7 WT, T22N, Q67L (no siRab7). This data is important and would show Rab7 localization relative to Arl13b
Figure 4: "ectocytosis" is evaluated by a single marker GFP-SMO. To draw this conclusion, authors need to show negative stain immunogold TEM of isolated vesicles and to use other markers for proteins that label the cilium but not ectosomes.
Figures 5 & 7. The same concerns remain regarding Rab7 ciliary localization. Rab7 localizes everywhere except the cilium. At 3:35, Authors use an arrowhead to point out a bright spot of Rab7 in the region of a cilium labeled by 5HT6, but it is impossible to orient tip of cilium versus base without a transition zone/basal body marker. If Rab7 is localized at the area of ciliary excision, how do authors know the position relative to the base and tip of the cilium?

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

The authors reviewed extensively the manuscript and performed new experiments that answer almost all the reviewers' concerns. As a result of the new experiments performed, the claims and conclusions were significantly strengthened.

Remaining points:

1- In the abstract, the expression "completely blocked" remains and should be replaced by "blocked";

2- The rescue experiment should be shown in Fig. 1, alongside representative immunofluorescence images, since it is an important experiment;

3- On the contrary, Fig. 3D could be supplementary;

4- The results obtained with latrunculin A must be described in the text;

5- In the legend of Fig. 5C, what is the meaning of "two percent of these samples were detected by western blot..."?

6- The "n" in each condition of Fig. S3B should be referred, as well as what do the bars represent, as in other similar plots of the manuscript;

7- More importantly about Fig. S3, the authors claim that "there is no obvious difference..." but one can see an accumulation of PI(4,5)P2 colocalizing with 5HT6 presumably at the base of cilia. Therefore, the authors should do some quantification and account for the differences in the cilia size (this applies also to Fig. S3B);

8- The dot plots (Fig. 1E, S1B and S3B) must have error bars.

Dear Dr. Monica Bettencourt-Dias and Dr. Melina Casadio,

We really appreciate your continuing support and your suggestions on further revision of our manuscript (#201811136R-A). We are glad to see that both you and the reviewers find our study interesting and give us the opportunity to an additional short round of revision. Here we have made the revision with additional data following your suggestions, which are able to address all of the concerns raised by you and the reviewers. The following is a detailed response:

1. In our revised manuscript, we have addressed Rev#1's and #3's points in full, including a text editing for Rev#1 and a few minor changes for Rev#3.
2. You suggested that "using a base and a ciliary membrane marker to orient cilia in imaging analyses is reasonable and important to strengthen the localization data". You also suggested that we will not need to add new data to address other points from Rev#2.

   We really appreciated for your professional and reasonable suggestions. To address this important issue, we applied both base and ciliary membrane markers to orient cilia in imaging analyses, which clearly showed the localization of Rab7 at ciliary tip (Fig. S2C).
3. Following your and Rev#2's suggestions, we have cited and discussed recent works regarding on whole cilium shedding.

I hope that you and the reviewers will find these responses and revisions satisfactory, and consider our revised manuscript for publication in *Journal of Cell Biology*.

Attached please find our point-to-point response to the reviewers' concerns.

Thank you again for your generous help and I am looking forward to hearing from you.

Sincerely yours,

Huiyan Li, PhD

National Center of Biomedical Analysis (NCBA)

27# Taiping Rd, Haidian District, Beijing 100850, P.R. China.

Tel. 86-10-66930344

Point-to-point responses to the reviewers' concerns.

Reviewer #1 comments:

The authors have done a terrific job addressing my concerns. The new imaging data in Fig. 5 and make a convincing case for Rab7 targeting to cilia prior to the initiation of ectocytosis. The interaction of actin specifically with Rab7-GTP considerably strengthens this aspect of the paper. This will be an excellent report for JCB.

One small edit that I would suggest for clarity's sake is to change the sentence 'We assume that the cilia ectocytosis taking place in quiescent cells may maintain the equilibrium between cilia assembly and disassembly, avoiding abnormal ciliogenesis during quiescence (Long et al., 2016).' to 'We assume that the cilia ectocytosis taking place in quiescent cells may maintain the equilibrium between cilia assembly and disassembly to keep length constant (Long et al., 2016).'

Response: Many thanks for your kind suggestion. We have modified our description in our revised manuscript as you suggested (Page 12, Line 20).

Reviewer #2 comments:

Cilia excision and cilia ectocytosis are two different processes. The rapid removal of cilia is referred to in the literature as deciliation, ciliary excision, shedding, deflagellation, or flagellar autonomy. Ectocytosis is budding from the plasma membrane and shedding of extracellular vesicles. Authors present data suggesting distal severing of the cilium, there is no data showing a role for Rab7 in ectocytosis. Figures 4, 5, and 7 only show a ciliary membrane marker. A ciliary base and axonemal marker are critical to draw conclusions about ciliary excision. Per my original review: "to make this argument convincing, authors must show the location of WT Rab7, Rab7 active, and Rab7 DN in the RPE-1 cilium labeled with Smoothened, Arl13, acetylated tubulin, and an IFT reporter." Authors did not address this criticism, which becomes

more important now that authors propose that Rab7 regulates ectocytosis and ciliary excision.

Authors are directed to the following work by the Stearns lab (originally posted on BioRxIv August 2018) describing mammalian ciliary loss. In this paper, Mirvis et al use a combination of markers to visualize the cilium: a marker for the ciliary base, a marker for the axoneme, and a marker for the ciliary membrane.

Primary cilium loss in mammalian cells occurs predominantly by whole-cilium shedding. Mirvis M, Siemers KA, Nelson WJ, Stearns TP.PLoS Biol. 2019 Jul 17;17(7):e3000381.

Phua et al 2017 coined the term ciliary decapitation, which is distal severing/excision of the cilium. Phua et al use combinations of ciliary base, ciliary axoneme, and ciliary membrane to analyze distal ciliary excision.

Nager et al 2017 characterize ectocytosis in mammalian cilia. Nager et al use cilia base, ciliary axoneme, and ciliary membrane markers to analyze the shedding of GPCRs from the ciliary tip. To prove a particle is an extracellular vesicle, the gold standard in the field is to isolate EVs and perform negative stain immunogold EM. Nager et al showed SSTR3 in EVs purified from IMCD3 culture supernatant.

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Figure 4: "ectocytosis" is evaluated by a single marker GFP-SMO. To draw this conclusion, authors need to show negative stain immunogold TEM of isolated vesicles and to use other markers for proteins that label the cilium but not ectosomes.

Figures 5 & 7. The same concerns remain regarding Rab7 ciliary localization. Rab7 localizes everywhere except the cilium. At 3:35, Authors use an arrowhead to point out a bright spot of Rab7 in the region of a cilium labeled by 5HT6, but it is impossible to

orient tip of cilium versus base without a transition zone/basal body marker. If Rab7 is localized at the area of ciliary excision, how do authors know the position relative to the base and tip of the cilium?

Response: Many thanks for your suggestions. According to your suggestion, to make sure whether the relative position of Rab7 is near to the base or tip of the cilium, we applied both base and ciliary membrane markers to orient cilia and found that Rab7 indeed localize to the tip of primary cilia. We have added this new data in Fig. S2C in our revised manuscript. In our original manuscript, to confirm the localization of Rab7 in primary cilia, we applied two ciliary markers (5HT6 and SMO) to couples of imaging analyses. These data also showed that Rab7 could localize at the ciliary excision site.

Due to the technical issue, it is hard to orient cilia with axonemal marker. Phua et al 2017, Nager et al 2017, and Mirvis et al 2019 were also unable to apply axonemal marker to orient cilia in their live-cell imaging experiments. In addition, we speculated that Rab7 localization to primary cilia is possibly acute and transient. Thus, it is hard to show its localization in fixed cells. Meanwhile, as Rab7 is widely expressed in cells, it is reasonable to show its localization somewhere else besides primary cilia.

Phua et al found that ciliary ectocytosis was as an initiating step during cilia-loss process in RPE-1 and IMCD-3 cells (Phua et al., 2017). However, Mirvis et al reported that whole-cilium shedding in IMCD3 cells mainly occurs during cilia loss (Mirvis et al., 2019). We speculate that the difference might be result from the different cultured or imaging conditions. We have added this discussion in our manuscript (Page 9, Line 16).

Reviewer #3:

The authors reviewed extensively the manuscript and performed new experiments that answer almost all the reviewers' concerns. As a result of the new experiments performed, the claims and conclusions were significantly strengthened.

Reviewer 3 comment 1. In the abstract, the expression "completely blocked" remains and should be replaced by "blocked";

Response: Many thanks for your kind reminder. We have changed "completely blocked" to “blocked” in our revised manuscript (Page 2, Line 8).

Reviewer 3 comment 2. The rescue experiment should be shown in Fig. 1, alongside representative immunofluorescence images, since it is an important experiment;

Response: Many thanks for your suggestions. Following your suggestion, we have moved the rescue experiments from supplementary data to Fig. 1, alongside representative immunofluorescence images (Fig. 1, E-H).

Reviewer 3 comment 3. On the contrary, Fig. 3D could be supplementary;

Response: Thanks for your suggestions. We have moved the data of original Fig. 3D to supplementary Fig. S1 A in our revised manuscript.

Reviewer 3 comment 4. The results obtained with latrunculin A must be described in the text;

Response: Thanks for your kind reminder. We have added the description on latrunculin A into our text (Page 9, Line 7) as “*Meanwhile, latrunculin A, an inhibitor of actin polymerisation, could restrict cilia ectocytosis after serum treatment (Fig. 4, A and B). The similar result was also observed in previous study (Phua et al., 2017).*”

Reviewer 3 comment 5. In the legend of Fig. 5C, what is the meaning of "two percent

of these samples were detected by western blot..."?

Response: We apologize for the unclear description. The same samples in Fig. 5C were detected by both coomassie blue staining (upper panel) and western blot (lower panel). As western blot is relatively sensitive, the samples that we used in western blot experiments is amount to 2% of that in Coomassie blue staining experiments. We have added these detailed description in the legends of Fig. 5C.

Reviewer 3 comment 6. The "n" in each condition of Fig. S3B should be referred, as well as what do the bars represent, as in other similar plots of the manuscript;

Response: Thank you for your kind reminder. We have added these information that "Data are means ± s.d." and "n = 24, 20, 16, 20 cells, respectively" in the legends of Fig. S3B.

Reviewer 3 comment 7. More importantly about Fig. S3, the authors claim that "there is no obvious difference..." but one can see an accumulation of PI(4,5)P2 colocalizing with 5HT6 presumably at the base of cilia. Therefore, the authors should do some quantification and account for the differences in the cilia size (this applies also to Fig. S3B);

Response: We appreciated for your suggestions. In our revised manuscript, we have quantified the PI(4,5)P2 immunofluorescence signal intensity in primary cilia in Fig. S3D and modified our description (Page 11, Line 5) that "*The results show that Rab7 knockdown did not abolish the ciliary reduction of Inpp5e and ciliary accumulation of PI(4,5)P2 (Fig. S3, A-C).*"

As for the cilia size, the length of cilia is indeed regulated by Rab7 through controling the ciliary disassembly. Under serum starved condition, the cilia of Rab7 knockdown cells are about twenty percent (1μm) longer than that of control cells. However, after serum stimulation, control cells undergoing cilia decapitation shows much shorter cilia, as cilia decapitation and disassembly are inhibited in Rab7 knockdown cells.

Reviewer 3 comment 8. The dot plots (Fig. 1E, S1B and S3B) must have error bars.

Response: Thanks for your kind reminder. We have added error bars into the dot plots, including original Fig. 1E, S1B and S3B (Fig. 1C, 1G and S3B respectively in our revised version).

September 16, 2019

RE: JCB Manuscript #201811136RR

Dr. Hui-Yan Li
National Center of Biomedical Analysis
27,taiping road
Beijing 100850
China

Dear Dr. Li,

Thank you for submitting your revised manuscript entitled "Rab7 regulates primary cilia disassembly through cilia excision". We have assessed your responses to the final review comments and the related data additions. We find them largely satisfactory and 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.

1) JCB Reports have a combined "Results and Discussion" section - please be sure to submit the final files incorporating this change.

2) eTOC summary: A 40-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.
- Please include an eTOC summary statement on the title page at resubmission. It should start with "First author name(s) et al..." to match our preferred style.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 1AD (magnifications), S1B (magnifications)
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add molecular weight with unit labels on the following panels: 1BC, 2DE, 3CE, 4D, 5CD, S1A, S2F

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 indicate n/sample size/how many experiments the data are representative of: 5BF, S2B, S3C

5) 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.
- Please include the basic genetic features for all cell lines and constructs, even if published in other work or provided by colleagues.
- More information about how passive cilia breaking was excluded, even if described in other

published papers.
- 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.).

6) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
- Please include ~1 brief descriptive sentence per supplemental item.

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.**

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).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- 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 the Journal of Cell Biology.

Sincerely,

Monica Bettencourt-Dias, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology
