JCB

Ubiquitin Links Smoothened to Intraflagellar Transport to Regulate Hedgehog Signaling

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January 30, 2020

Re: JCB manuscript #201912104

Dr. Gregory J Pazour
University of Massachusetts Medical School
Program in Molecular Medicine
Suite 213, Biotech II
373 Plantation Street
Worcester, MA 01605

Dear Dr. Pazour,

Thank you for submitting your manuscript entitled "Ubiquitin Links Smoothened to Intraflagellar Transport to Regulate Hedgehog Signaling". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

The majority of criticisms may be addressed in the text. Reviewer 1 (point 6) is concerned about conclusions based on ubiquitin fusions without experimental support for direct monoubiquitination of Smo occurring physiologically. This is a key claim to substantiate for resubmission.

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

GENERAL GUIDELINES:

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

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

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

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

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

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

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

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

Sincerely,

Maureen Barr, Ph.D.
Monitoring Editor

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

Journal of Cell Biology

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

In their manuscript, "Ubiquitin links Smoothened to intraflagellar transport to regulate Hedgehog signaling", Desai et al. explore the relationship between ubiquitination and trafficking of Smo. Since the discovery that Smo enriches in cilia upon pathway stimulation, the field has studied the mechanisms involved in ciliary entry, enrichment and exit of Smo, particularly in relation to Smo activation. Desai et al. propose that Smo exit from cilia requires ubiquitination and IFT transport. They identify the residues on intracellular loop 3 of Smo that are ubiquitinated and demonstrate that ubiquitination is both necessary and sufficient for Smo ciliary exit. The authors propose a model where an unidentified ubiquitin ligase is inhibited and removed from cilia when Shh (or Ihh/Dhh) ligand is present. These data move the field forward in revealing a ubiquitin-based regulatory mechanism of Smo trafficking. Furthermore, these data suggest a conservation of Smo ubiquitination that has shifted to a cilia-dependent process in vertebrates- potentially shedding light on the evolution of how cilia and Hh got linked. Overall, the data are well controlled although the statistical analysis must be redone using appropriate tests to establish that the conclusions herein are correct (See point #5). As overexpression is well established to drive Smo into cilia, the authors are commended for switching to appropriate promoters on their constructs.
This work has the potential to be quite important in the field's mechanistic understanding of the relationship between cilia, Smo regulation and mammalian Hh signaling. Several points need to be addressed.
Major Concerns.
(1) Transient localization, accumulation/enrichment, exit and activation of Smo are distinct yet are not consistently articulated as such in the manuscript. Throughout the manuscript the authors need to articulate whether Smo cannot "localize" or cannot "accumulate" in cilia. An example of this is at the start of page 17 where the authors state "...mgrn1 a strong candidate to be the enzyme

that regulates the ciliary localization of Smo but the variable amount of Smo accumulation in cilia suggests..." This wording suggests a role of ubiquitin in Smo entry, but data in Figure 4 suggest that loss of ubiquitin alone does not disrupt Smo entry, only Smo exit. Clarity on this will improve the logic of the experiments and conclusions.
(2) The proposed model details the role of ubiquitin in the Hh-dependent and endogenous mechanisms that regulate Smo in cilia, but most of the data in this manuscript involve Smo agonist SAG, not Hh ligands. SAG does not activate Smo via the endogenous pathway as it bypasses Hh and Ptch regulation of Smo by directly binding Smo. Therefore, the proposed model of decreased Smo ubiquitination by the endogenous pathway is not tested in the majority of the experiments. While the authors need not repeat every experiment, they have Shh-conditioned media (Figure 6) so could directly test their model. Given the nature of SAG, Shh-conditioned media may reveal more variation in Smo enrichment phenotypes, especially in cells expressing Smo mutants (Figure 5D).
(3) Along these same lines, the authors have a unique opportunity to dissect the multistep process of Smo activation and enrichment in cilia. The field has long considered regulation of Smo to be a multistep process, enrichment in cilia and activation can be uncoupled by pharmacological agents (Rohatgi et al 2007). Another small molecule Smo ligand is cyclopamine. Cyclopamine enriches Smo in cilia, but functions antagonistically, preventing Smo from activating the Hh pathway. In their system, the authors can shed light on how cyclopamine is able to separate Smo enrichment and activation. Perhaps cyclopamine allows Smo to bypass ubiquitination, allowing it to enrich in cilia. Moreover, SANT-1, which does not enrich Smo in cilia and antagonizes Hh pathway activity, may promote Smo ubiquitination, preventing it from enriching in cilia. If so, perhaps Pyr41 would prevent SANT-1 inhibition of Smo accumulation of cilia. Such experiments would enable the authors to build more complete data to support their model.
(4) The proposed model is that Smo ubiquitination is occurring in the cilium, however as it stands this has not been tested. The authors should discuss the possibility that ubiquitination of Smo does not occur in the cilium.
(5) The statistical tests used are not appropriate. A one-way ANOVA is for comparisons of 3 or more groups separated by a single independent variable. In many cases the data in this manuscript are defined by two independent variables, therefore the data should be analyzed by a two-way ANOVA. For instance, in Figure 1B the experiment has two cell types (variable 1) treated with control or SAG media (variable 2). To make comparisons within and among groups, these data should be analyzed by two-way ANOVA. In these cases, post-hoc analysis will require a different test than Tukey's HSD.
(6) There are several issues with figure 6, which is central to the proposed model. The authors should cite evidence that HA-Ub can be efficiently ligated to proteins. Furthermore, the authors need to explain why the smear observed on the blot probed for HA-Ub is larger than 250kD. The bands without or with Shh do not appear to have entered the gel. The biggest concern is that It is not at all clear why the lysates in A and C, both of which got IP'd with anti-FLAG so are argued to represent Smo-Flag protein, would display such distinct sizes when blotted with anti-Flag and anti-HA antibody. Shouldn't the HA blot be detecting the Smo-Flag protein and thus be the same size? Furthermore, if the distinction between the Smo-Flag blots in A and C is the ubiquitination, shouldn't the size difference reveal the Ub-dependent size shift? The Smo-Flag and Smo noK-Flag transfected samples should be run on the same gel so they can be directly compared (and explained). Finally, is the quantification normalized to total protein?
(7) In the text, the authors state that "SSTR3-Flag-Ub does not accumulate to significant levels in cilia of either cell line (wild type and Ift127-/- IMCD3 cells)." However, in Figure 2D the data show a statistically significant increase of SSTR3-flag-Ub in Ift27-/- cells compared to wild type, identified by a single asterisk *. Is the data statistically significant and the interpretation that the enrichment is not biologically significant? The authors should clarify.
Minor concerns:

The authors should explain how the Ub tag they use works- especially its ability to be polyubiquitinated and that the "no K" version in figure 5 retains a single ubiquitin. Assume the reader is unfamiliar.
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The authors should check they are using standardized nomenclature and that proteins are properly designated and consistent throughout the manuscript. For example, "hedgehog" would be Drosophila protein and not vertebrate "Hedgehog" (as hh was identified as a recessive mutation in fly and the mammalian genes were identified by homology). There are also some instances of "smoothened" instead of "Smo" as it had previously been abbreviated.
While requesting a plasmid from the lab is facilitated by using the Pazour lab plasmid cataloging numbers, they interfere with the reader following the logic when used to label figures and legends. Table 1 in the methods is quite clear and will be useful for requests. However, within figures and figure legends the exact protein expressed should be clearly identified, which will enable the reader to follow the logic of the experiment.
The methods section states that data were arcsine transformed. Were all percentage data treated in this manner? Are the graphed values presented transformed or untransformed? The authors should clarify and provide justification for this method of data transformation.
Acetylated tubulin is listed in the antibody table but is not used in the present manuscript.
Figure 3H is quantification of IMDC3 wildtype and Ift27-/- cells expressing SmoPi or SmoPi-Ub, representative images of these data should be included. Moreover, there is no 3H callout in the text. Perhaps the data should be moved to supplement?
Is Figure 6C mislabeled?

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

Desai et al. Ubiquitin Links Smoothened to Intraflagellar Transport to Regulate Hedgehog Signaling

The authors analyze the mechanism by which smoothened (smo) is removed from cilia prior to activation of the hedgehog (hh) pathway. Building on previous studies from the Pazour lab, this work demonstrates that smo export from cilia by the IFT27-BBS pathway depends on ubiquitination of smo. In detail, the authors show that addition of one ubiquitinin (Ub) to smo is sufficient to avoid accumulation of smo-ub in cilia during activation of the pathway and that this process requires IFT27 and the BBSome When cells are treated with an inhibitor of the E1 ub activating enzyme, smo-FLAG accumulates in cilia without pathway activation and a smo constructs lacking two lysine residues in the intracellular loop 3, the likely sites of smo ubiquitination, fail to exit cilia upon hh pathway activation. Further, the authors show that endogenous ubiquitination of a "wild-type" smo-FLAG is greatly reduced upon activation of the pathway. Taken together, this study provides clear evidence that cells prevent smo from accumulating in cilia prior to pathway activation by its ubiquitination, which triggers its removal from cilia in a process depending on IFT27, LZTTL1, and BBS2. This pathway is novel as it is distinct from the removal of other GPCRs (e.g., Sstr3), which also involves ubiquitination but is independent of the IFT27/BBS machinery.
Hedgehog signaling is an important developmental signaling pathway that requires signaling proteins to move in and out of cilia. However, our understanding of these transports is only emerging. Thus, I believe that this work is a significant step forward and suited for publication in the journal. The work is clear, provides several independent lines of evidence that smo ubiquitination is a prerequisite for its removal from cilia by IFT27/BBS; necessary controls were included. I have only a

few comments, which the authors might want to address.

1) A puzzling observation is that SmoM2 and SmoPi, which mimic the activated form of Smo, do not require IFT-BBS for removal from cilia when expressed in fusions with ubiquitinin. As described in the paper, these mutations alter the confirmation of Smo and SmoM2i could be removed from cilia by an IFT27/BBS-independent retrieval pathway. Because these mutations likely reconfigure Smoothened's putative BBSome-binding site the authors suggest that the altered smo will then be able to bind to other IFT proteins. However, SmoM2 export still depends on the presence of ubiquitinin. Control Smo is ubiquitinated without pathway activation raising the question why SmoM2 is not ubiquitinated and removed from cilia without pathway activation. Is it possible that SmoM2 is no longer a substrate for the ub ligase due to its altered configuration (as briefly mentioned on p.11)? This could be tested using the same approach as in Fig. 6 but using the SmoM2-Flag.

The model in Fig. 7 suggests that Smo is not ubiquitinated once the pathway is active because the required ub ligase is coupled to patched and removed together with patched from cilia. This would be not necessary if Smo activation simply prevents it from being a substrate for the ubiquitination system due to its altered confirmation.

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p. 17: 2nd to last line of discussion: sentence fragment.

p.16: both crispr and CRISPR is used.

Fig. S3A: The western shows plenty of bands corresponding to smo and its ubiquitinated forms. The band or bands based on which smo decay was calculated, should be indicated.

Some figures are rather small (Fig. 1A, 3D) making it difficult to see the cilia (and absence of ciliary signals); high mag inserts might help.

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March 17, 2020

Maureen Barr, Ph.D., Monitoring Editor
Marie Anne O'Donnell, Ph.D., Scientific Editor
Journal of Cell Biology

Dear Drs. Barr and O'Donnell,

My co-authors and I would like to thank you and the reviewers for your efforts on behalf of our manuscript. We appreciate the detailed comments and suggestions for improvement. As detailed on the following pages, we made extensive edits in response to the comments. In particular, you pointed out that we should address point 6 of reviewer 1. To address the concern, this we repeated the experiment as suggested and ran both the Smo-Flag and Smo^{noK}-Flag immunoprecipitations on the same gel. In addition, we extended this experiment by examining SmoM2 as suggested by reviewer 2. This was an excellent suggestion and showed that little ubiquitin is coupled to SmoM2 under basal or induced conditions.

I hope that our edits satisfy your concerns and we look forward to seeing this paper in press.

Best regards,

Gregory J Pazour, PhD

In their manuscript, "Ubiquitin links Smoothened to intraflagellar transport to regulate Hedgehog signaling", Desai et al. explore the relationship between ubiquitination and trafficking of Smo. Since the discovery that Smo enriches in cilia upon pathway stimulation, the field has studied the mechanisms involved in ciliary entry, enrichment and exit of Smo, particularly in relation to Smo activation. Desai et al. propose that Smo exit from cilia requires ubiquitination and IFT transport. They identify the residues on intracellular loop 3 of Smo that are ubiquitinated and demonstrate that ubiquitination is both necessary and sufficient for Smo ciliary exit. The authors propose a model where an unidentified ubiquitin ligase is inhibited and removed from cilia when Shh (or Ihh/Dhh) ligand is present. These data move the field forward in revealing a ubiquitin-based regulatory mechanism of Smo trafficking. Furthermore, these data suggest a conservation of Smo ubiquitination that has shifted to a cilia-dependent process in vertebrates- potentially shedding light on the evolution of how cilia and Hh got linked. Overall, the data are well controlled although the statistical analysis must be redone using appropriate tests to establish that the conclusions herein are correct (See point #5). As overexpression is well established to drive Smo into cilia, the authors are commended for switching to appropriate promoters on their constructs.

This work has the potential to be quite important in the field's mechanistic understanding of the relationship between cilia, Smo regulation and mammalian Hh signaling.

Thank you!

Several points need to be addressed.

Major Concerns.

(1) Transient localization, accumulation/enrichment, exit and activation of Smo are distinct yet are not consistently articulated as such in the manuscript. Throughout the manuscript the authors need to articulate whether Smo cannot "localize" or cannot "accumulate" in cilia. An example of this is at the start of page 17 where the authors state "...mgrn1 a strong candidate to be the enzyme that regulates the ciliary localization of Smo but the variable amount of Smo accumulation in cilia suggests..." This wording suggests a role of ubiquitin in Smo entry, but data in Figure 4 suggest that loss of ubiquitin alone does not disrupt Smo entry, only Smo exit. Clarity on this will improve the logic of the experiments and conclusions.

We have modified the introduction to make this point stronger. It now reads: ”We previously showed that Ift25 and Ift27, which are subunits of IFT-B, are not required for ciliary assembly. Instead, these two IFTs work with the adaptor protein Lztfl1 and the BBSome to regulate hedgehog signaling by facilitating the removal of Smo from cilia at the basal state and Ptch1 after pathway activation (Eguether et al., 2018; Eguether et al., 2014; Keady et al., 2012).

In this work, we explore the mechanism underlying the dynamics of Smo localization to cilia and find that the removal of Smo from cilia is regulated by ubiquitination.”

In addition, we changed “localization” to “accumulation” throughout the manuscript as the reviewer is correct that “localization” could imply delivery to cilia.

(2) The proposed model details the role of ubiquitin in the Hh-dependent and endogenous mechanisms that regulate Smo in cilia, but most of the data in this manuscript involve Smo agonist SAG, not Hh ligands. SAG does not activate Smo via the endogenous pathway as it bypasses Hh and Ptch regulation

of Smo by directly binding Smo. Therefore, the proposed model of decreased Smo ubiquitination by the endogenous pathway is not tested in the majority of the experiments. While the authors need not repeat every experiment, they have Shh-conditioned media (Figure 6) so could directly test their model. Given the nature of SAG, Shh-conditioned media may reveal more variation in Smo enrichment phenotypes, especially in cells expressing Smo mutants (Figure 5D).

The reviewer is correct that SHH-conditioned medium would probe the pathway upstream of Smo but we are looking at Smo localization so SAG is appropriate. To ensure that something unexpected was not occurring, we tested the effects of SHH-condition medium on wild type cells expressing Smo-Flag or Smo-Flag-Ub. The results were as expected and this data was added to supplemental figure 1. In 5D, the SAG treatment is being used to tell us what percentage of the cells express our Smo construct and so SAG is more appropriate than SHH.

(3) Along these same lines, the authors have a unique opportunity to dissect the multistep process of Smo activation and enrichment in cilia. The field has long considered regulation of Smo to be a multistep process, enrichment in cilia and activation can be uncoupled by pharmacological agents (Rohatgi et al 2007). Another small molecule Smo ligand is cyclopamine. Cyclopamine enriches Smo in cilia, but functions antagonistically, preventing Smo from activating the Hh pathway. In their system, the authors can shed light on how cyclopamine is able to separate Smo enrichment and activation. Perhaps cyclopamine allows Smo to bypass ubiquitination, allowing it to enrich in cilia. Moreover, SANT-1, which does not enrich Smo in cilia and antagonizes Hh pathway activity, may promote Smo ubiquitination, preventing it from enriching in cilia. If so, perhaps Pyr41 would prevent SANT-1 inhibition of Smo accumulation of cilia. Such experiments would enable the authors to build more complete data to support their model.

These experiments are interesting, but I think they are more complicated than presented here. These experiments will require significant controls to ensure that the results are due to effects on ubiquitination of Smo and not effects on Smo ciliary delivery or turnover of Smo and should be left for a future study.

Reviewer two suggested a version of this experiment where we examine the ubiquitination of SmoM2. We find that SmoM2 does not incorporate significant amounts HA-Ub and the amount is not changed by SHH treatment. This data was added to Figure 6.

(4) The proposed model is that Smo ubiquitination is occurring in the cilium, however as it stands this has not been tested. The authors should discuss the possibility that ubiquitination of Smo does not occur in the cilium.

We have added the point that models could be built with Smo ubiquitination occurring outside of the cilium.

(5) The statistical tests used are not appropriate. A one-way ANOVA is for comparisons of 3 or more groups separated by a single independent variable. In many cases the data in this manuscript are defined by two independent variables, therefore the data should be analyzed by a two-way ANOVA. For instance, in Figure 1B the experiment has two cell types (variable 1) treated with control or SAG media (variable 2). To make comparisons within and among groups, these data should be analyzed by two-way ANOVA. In these cases, post-hoc analysis will require a different test than Tukey's HSD.

All statistics were redone using one, two or three-way ANOVA as appropriate and the test was included in the figure legend. A few p-values changed from what was previously reported but no conclusions were affected.

(6) There are several issues with figure 6, which is central to the proposed model. The authors should cite evidence that HA-Ub can be efficiently ligated to proteins. Furthermore, the authors need to explain why the smear observed on the blot probed for HA-Ub is larger than 250kD. The bands without or with Shh do not appear to have entered the gel. The biggest concern is that It is not at all clear why the lysates in A and C, both of which got IP'd with anti-FLAG so are argued to represent Smo-Flag protein, would display such distinct sizes when blotted with anti-Flag and anti-HA antibody. Shouldn't the HA blot be detecting the Smo-Flag protein and thus be the same size? Furthermore, if the distinction between the Smo-Flag blots in A and C is the ubiquitination, shouldn't the size difference reveal the Ub-dependent size shift? The Smo-Flag and Smo noK-Flag transfected samples should be run on the same gel so they can be directly compared (and explained). Finally, is the quantification normalized to total protein?

We have added a reference to the HA-Ub construct that we used. This is a commonly used reagent (Google Scholar identified 4910 publications containing the word "HA-ubiquitin") and is well established to function as we describe.

As requested by the reviewer, we have repeated this experiment with Smo-Flag, Smo^{noK}-Flag and SmoM2 on the same blots. We would not expect to see exactly the same pattern with both Flag and HA as the band intensity is related to the number of epitopes present at that position on the gel. The two main Smo bands detected by Flag are thought to be non-ubiquitinated forms of Smo and so would not be detected by the HA antibody. The larger bands would be detected by both Flag and HA but the intensity of the two would vary with HA going up as more ubiquitin molecules are added. Thus, we would expect the bands near the top of the gel to be stronger with HA than with Flag. In addition, the protein is undergoing degradation that further complicates the pattern.

The Ub dependent shift described by the reviewer is usually only detected for the first few added ubiquitin molecules and then the protein smears out due to heterogeneity of chain length and lack of ability of acrylamide gels to resolve small additions to high molecular weight proteins.

(7) In the text, the authors state that "SSTR3-Flag-Ub does not accumulate to significant levels in cilia of either cell line (wild type and Ift127-/- IMCD3 cells)." However, in Figure 2D the data show a statistically significant increase of SSTR3-flag-Ub in Ift27-/- cells compared to wild type, identified by a single asterisk *. Is the data statistically significant and the interpretation that the enrichment is not biologically significant? The authors should clarify.

The reviewer is correct that it is statistically significant and is correct that our interpretation is that it is not biologically relevant. We have changed the sentence to:

"To determine how other GPCRs behave, we tagged the somatostatin receptor Sstr3 with Flag and Ub. Sstr3-Flag is highly enriched in both control and IMCD3Ift27-/- cilia while Sstr3-Flag-Ub is not highly enriched in cilia of either cell line (Figure 2C,D)."

Minor concerns:

The authors should explain how the Ub tag they use works- especially its ability to be polyubiquitinated and that the "no K" version in figure 5 retains a single ubiquitin. Assume the reader is unfamiliar.

We have added a figure to the supplement that contains diagrams of the various constructs with lysines marked.

Are the data in Supplemental Figure 1 and Figure 1C acquired using the same parameters? Specifically, Smo-Flag and Smo-Flag-Ub are detectable in the cell body in Figure 1C, but absent in Supplemental Figure 1. As both constructs should be detectable by Smo antibody, they would be expected to reveal the same cell body staining.

As described, Figure 1C used an anti-Flag antibody while Supplemental Figure 1 (now S1C) used antibody against Smo so we would not expect them to look the same. The point that we are trying to make in the supplemental figure 1C is that even though cilia on cells expressing Smo-Flag-Ub do not label with Flag, they do label with antibody against Smo.

The authors should check they are using standardized nomenclature and that proteins are properly designated and consistent throughout the manuscript. For example, "hedgehog" would be Drosophila protein and not vertebrate "Hedgehog" (as hh was identified as a recessive mutation in fly and the mammalian genes were identified by homology). There are also some instances of "smoothened" instead of "Smo" as it had previously been abbreviated.

“hedgehog” was changed to “Hedgehog” and Smoothened was changed to Smo where appropriate.

While requesting a plasmid from the lab is facilitated by using the Pazour lab plasmid cataloging numbers, they interfere with the reader following the logic when used to label figures and legends. Table 1 in the methods is quite clear and will be useful for requests. However, within figures and figure legends the exact protein expressed should be clearly identified, which will enable the reader to follow the logic of the experiment.

We did our best to precisely label the proteins used. However, there are so many slight variations that we do not feel that we can accurately and concisely represent what is in used in each experiment without providing the numbers.

The methods section states that data were arcsine transformed. Were all percentage data treated in this manner? Are the graphed values presented transformed or untransformed? The authors should clarify and provide justification for this method of data transformation.

The statistical methods have been redone without arcsine transformation and this has been removed. The test used is included in the figure legends.

Acetylated tubulin is listed in the antibody table but is not used in the present manuscript.

Removed from the table.

Figure 3H is quantification of IMDC3 wildtype and Ift27-/- cells expressing SmoPi or SmoPi-Ub, representative images of these data should be included. Moreover, there is no 3H callout in the text. Perhaps the data should be moved to supplement?

Images have been added (now 3H) and 3H and 3I are called out in the text.

Is Figure 6C mislabeled?

Not that we can see.

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

Desai et al. Ubiquitin Links Smoothened to Intraflagellar Transport to Regulate Hedgehog Signaling

The authors analyze the mechanism by which smoothened (smo) is removed from cilia prior to activation of the hedgehog (hh) pathway. Building on previous studies from the Pazour lab, this work demonstrates that smo export from cilia by the IFT27-BBS pathway depends on ubiquitination of smo. In detail, the authors show that addition of one ubiquitinin (Ub) to smo is sufficient to avoid accumulation of smo-ub in cilia during activation of the pathway and that this process requires IFT27 and the BBSome When cells are treated with an inhibitor of the E1 ub activating enzyme, smo-FLAG accumulates in cilia without pathway activation and a smo constructs lacking two lysine residues in the intracellular loop 3, the likely sites of smo ubiquitination, fail to exit cilia upon hh pathway activation. Further, the authors show that endogenous ubiquitination of a "wild-type" smo-FLAG is greatly reduced upon activation of the pathway. Taken together, this study provides clear evidence that cells prevent smo from accumulating in cilia prior to pathway activation by its ubiquitination, which triggers its removal from cilia in a process depending on IFT27, LZTTL1, and BBS2. This pathway is novel as it is distinct from the removal of other GPCRs (e.g., Sstr3), which also involves ubiquitination but is independent of the IFT27/BBS machinery.

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Excellent idea, we immunoprecipitated SmoM2 co expressed with HA-Ub and found that SmoM2 did not incorporate significant amounts of HA-Ub. Furthermore, the amount was similar with and without SHH treatment. This data is now included in Figure 6. Thanks for the suggestion.

The model in Fig. 7 suggests that Smo is not ubiquitinated once the pathway is active because the required ub ligase is coupled to patched and removed together with patched from cilia. This would be not necessary if Smo activation simply prevents it from being a substrate for the ubiquitination system due to its altered confirmation.

We have added this point to the discussion.

2) The model (Fig. 7) shows smo-Ub attaching to cilia via the Ub entity. However, I interpret the data as showing that parts of smo itself, in addition to Ub, are required for IFT27/BBS binding and removal from cilia. Changes in smo itself (as those in SmoM2) prevent it from using the IFT27/BBS pathway even when smo carries ub suggesting that the protein backbone is critical for interaction with IFT27/BBS. If this is correct, the model should reflect this.

I think the reviewer means "attaching to IFT" rather than "attaching to cilia." We have changed our model to show interactions between the BBSome and Smo in addition to the connection through Ub and have added a reference to the Seo work showing Smo binding to the BBSome.

p. 17: 2nd to last line of discussion: sentence fragment.

Rewritten to say "Perhaps the binding of ligand to Ptch1 activates an E3 ligase that promotes Ptch1 removal from cilia."

p.16: both crispr and CRISPR is used.

Changed all to CRISPR

Fig. S3A: The western shows plenty of bands corresponding to smo and its ubiquitinated forms. The band or bands based on which smo decay was calculated, should be indicated.

A bracket was added to the figure and the figure legend was updated.

Some figures are rather small (Fig. 1A, 3D) making it difficult to see the cilia (and absence of ciliary signals); high mag inserts might help.

April 5, 2020

RE: JCB Manuscript #201912104R

Dr. Gregory J Pazour
University of Massachusetts Medical School
Program in Molecular Medicine
Suite 213, Biotech II
373 Plantation Street
Worcester, MA 01605

Dear Dr. Pazour:

Thank you for submitting your revised manuscript entitled "Ubiquitin Links Smoothened to Intraflagellar Transport to Regulate Hedgehog Signaling". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

- Provide the main and supplementary texts as separate, editable .doc or .docx files
- Provide main and supplementary figures as separate, editable files according to the instructions for authors on JCB's website *paying particular attention to the guidelines for preparing images and blots at sufficient resolution for screening and production*
- Provide tables as excel files
- Format references for JCB
- Add paragraph after the Materials and Methods section briefly summarizing all "Online Supplementary Materials"
- Add conflict of interest statement to the Acknowledgements section
- Add author contributions

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

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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.**

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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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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

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,

Maureen Barr, Ph.D.
Monitoring Editor

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

Journal of Cell Biology

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

The authors satisfactorily addressed the comments.

A few formatting corrections:

Figure legends list which type of ANOVA was used, but not the posthoc analysis that yields the reported p-values.

Figure 6 legend: Hedgehog media (SHH) should read "Sonic Hedgehog" media.

Formatting for proteins vs mouse genes needs correcting throughout (i.e. Ift88 (italics) encodes IFT88)