E3 Ubiquitin Ligase Wwp1 Regulates Ciliary Dynamics of the Hedgehog Receptor Smoothened

Bo Lv, Michael Stuck, Paurav Desai, Oscar Cabrera, and Gregory Pazour

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December 11, 2020

Re: JCB manuscript #202010177

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 "The E3 Ubiquitin Ligase Wwp1 Binds Ptch1 and Regulates Smoothened's Ciliary Localization". 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.

As you will see, while the reviewers find your study a potentially important follow-up to your recent paper showing that Smo ubiquitination regulates its ciliary localization, they all question the importance of WWP1 given the low frequency at which it is detected. Furthermore, they have provided additional constructive feedback which I hope you agree addressing will further improve your study. In particular:

Points 1, 4, 6, 7 of Reviewer #1 can be addressed in your text.
Point 2 of Reviewer #1: while it is important to look at endogenous target genes, it is not critical to look in different cell lines for resubmission to JCB.
Points 3 and 5 of Reviewer #1 are critical to address.

In addition, we hope that you will be able to address all of the remaining reviewer comments in your revised manuscript.

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, https://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.

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

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

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

Sincerely,

Maureen Barr, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

This paper from Pazour and colleagues is a follow-up of a previous paper published by the same group (JCB 2020) showing that SMO ubiquitination regulates its ciliary accumulation and is also related to a recent paper from the Nachury group showing that K63-linked ubiquitination of GPCRs (including SMO) mark them for ciliary exit facilitated by the BBSome. In this manuscript, Lv et al. collect a list of 147 ubiquitin-related genes previously connected to cilia or hedgehog signaling and conduct a focused screen to systematically identify ubiquitin-related genes that suppress levels of ciliary Smoothened at the basal state. Although Lv et al. identify nine genes (Arih2, Mgrn1, Maea,

Wwp1, Ube2l3, Bap1, Kctd5, Skp1a, and Skp2), the paper largely focuses on two: Wwp1 (the only E3 ligase identified in their screen that localizes to the primary cilium) and Ube2l3 (the only E2 conjugating enzyme that increased ciliary SMO at basal levels).

The goal of this paper is to identify the machinery that transfers ubiquitin to SMO. The most in-depth analysis that advances the field is of the ligase WWP1. The model presented by the authors is that WWP1 bound to PTCH1 catalyzes SMO ubiquitination and drives its exit from cilia. When SHH clears PTCH1 from cilia, the associated WWP1 is also cleared. This prevents SMO ubiquitination and thus allows it to accumulate in cilia. While this model is new and may advance the field, the following issues need to be resolved:

1/ The Salic group has shown (PNAS 2016 and Dev Cell 2020) that PTCH1 clearance from cilia is not required for SMO accumulation and Hedgehog pathway activation. Biochemical inactivation of PTCH1 (without ciliary clearance) is sufficient. This data is not consistent with the authors' model that WWP1-mediated clearance from cilia is what drives SMO accumulation and Hedgehog pathway activation. Also, the authors' model suggests that the phenotype of WWP1 loss should be the same as the phenotype of PTCH1 loss, but WWP1 loss has a much milder Hedgehog phenotype. Does PTCH1 inactivation (without clearance) also lead to the loss of WWP1 from cilia? Clearance of WWP1 from cilia does not seem to be the major regulatory step that leads to Hedgehog pathway activation in response to Hedgehog ligands-this should be made clear and addressed. Does the WWP1 KO phenotype look like the classic PTCH KO phenotype?

2/ For WWP1 and each of the proteins tested in the paper, the authors should measure the effect of gene inactivation on endogenous Hedgehog target genes (like Gli1) by quantitative PCR in a different cell line to test if these genes are relevant for hedgehog signaling outside of the context of the one reporter-based assay used in the paper. This is the only way to compare the magnitude of the effect of loss of these genes to ablation of core components like patched and smoothened and determine their physiological relevance to in vivo Hedgehog signaling.

3/ In assays with WWP1 at cilia, <10% of cells have detectable WWP1. How can WWP1 be a key regulator of Hedgehog signaling if only a small fraction of cells express it in cilia? The authors should use a detection method or protocol that allows assessment of WWP1 in a majority of cells.

4/ In Figure 4, a mutant of PTCH1 is shown that loses its interaction with WWP1. However, this mutant in I is still able to partially rescue Hedgehog signaling in Ptch1-/- cells. This suggests that the WWP1 interaction is not the reason why this mutant is less active at suppression of Hedgehog signaling.

5/ Ubiquitination of SMO by WWP1 at the lysine residues identified in the authors' previous JCB paper should be demonstrated since it is a central component of the model. Also, loss of SMO ubiquitination in response to WWP1 ablation should be tested. In this current state, one cannot tell if the effect of WWP1 on SMO ciliary levels is direct or indirect. A direct SMO ubiquitination assay is essential.

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7/ There are apparent discrepancies with the Kim et al. Sci Signal 2016 paper. In a similar series of experiments, in the Kim et al. paper they addback Ptch1PY (with both PPXY motifs mutated) into

Ptch1-/- pMEFs, but observe no increase in ciliary Smo or elevation of pathway activity at basal levels (when compared to Ptch1WT addbacks). In the Kim et al. paper, they speculate that Wwp1 is one of eight Nedd4 family HECT E3 ubiquitin ligases that target Ptch1 and regulate its ciliary exit. In fact, the UbiBrowser cited in this paper (Li et al., Nat Comm 2017) predicts that Wwp1 ubiquitinates Ptch1. Thus, is it possible that Wwp1 ubiquitinates Ptch1 and that the reason the authors did not see a Ptch1 localization phenotype in Wwp1-/- cells is due to functional redundancy with one of the other seven Nedd4 family HECT E3 ubiquitin ligases identified in the Kim et al. paper?

Minor Points:

1/ Line 29: Should be "levels of SMO" instead of "levels of Arih2"?
2/ In Figure 2A, are the colors for -SAG and +SAG switched?
3/ Given similarities in approach and findings, I think it's important to cite the recently published paper "Ubiquitin chains earmark GPCRs for BBSome-mediated removal from cilia" (Shinde et al., JCB 2020).

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

In this manuscript Lv and colleagues elegantly demonstrate that the E3 ubiquitin ligase Wwp1 binds Ptch1 and cooperates with the E2 ubiquitin ligase Ube213 to promote ubiquitination of Smo in cilia when the Hedgehog pathway is off, thereby keeping Smo ciliary levels low in the absence of Hedgehog ligand. In a recent study (Desai et al., JCB, 2020) the authors showed that Smo is ubiquitinated when the Hedgehog pathway is off, which causes Smo to be removed from cilia by the IFT machinery. However, the enzymes responsible for ubiquitinating Smo in cilia under these conditions were not identified. Here the authors developed and characterized a reporter line, GreenBomb, in mouse embryonic fibroblasts, which allows for robust simultaneous detection of ciliary FLAG-Smo localisation and Hedgehog pathway activity via expression of a GFP-tagged target gene. By using the GreenBomb cells the authors then carry out a comprehensive CRISPR-based screen to identify genes of the ubiquitin proteasome system that regulate ciliary Smo accumulation and target gene expression. After secondary screening and careful validation, the authors end up with a handful of genes that specifically affect Smo ciliary localisation and target gene expression in the basal state, amongst which the E3 ubiquitin ligase Wwp1 and the E2 ubiquitin ligase Ube213 are the most interesting as they localize to cilia. By using a variety of molecular and cell-based approaches the authors dissect the molecular mechanism by which Wwp1 and Ube213 controls ubiquitination and ciliary presence of Smo, and they further reveal that ubiquitinated Smo can be deubiquitinated by AMSH*, indicating the Smo has K63-linked ubiquitin chains.

Overall this is an impressive and solid piece of work that will be of great interest to the JCB readership and cell- and developmental biologists in general. The experimental approaches are very elegant and the data of very high quality. I applaud the authors for this very nice piece of work and recommend publication provided that the authors can adequately address the following issues.

Main comment

Figure 4F, G: why is the proportion of cell displaying WWP1 at cilia so low (<10%) if WWP1 is a central regulator of the Shh pathway?

Minor comments

Figure 2A: Why is the GFP MFI lower in the SAG-stimulated cells compared to unstimulated cells? Did the authors accidentally mislabel the figure or am I missing something? In Fig. 2C, D it seems the SAG-stimulated cells overall have more SMO in cilia as expected so I am confused about the data in Figure 2A.

Figure 1D: the blue Arl13B text and cilium labelling is very hard to see, can the authors enhance contrast to make it more visible to the reader?

Figure 1G: they grey dots in the middle of the graph are really difficult to see, is it possible to color them a bit darker? The authors might also want to chose a different color for the stipled lines in the same figure (e.g. yellow or orange) as it is quite hard to see the stipled lines, especially the vertical ones, as they appear now.

Figure 2E, Figure S3 and page 7 top: the unspecific 150 kDa band is not only present in the Arih2-/- cells but also the Mgrn1-/- cells (perhaps also the Maea-/- cells). Although I do not think the authors need to characterize the nature of this unspecific band further, they should refer to Figure S3 on top of page 7 and clearly indicate here and in Figure 2E that the 150 kDa band is a protein that binds non-specifically to the FLAG antibody and appears to be upregulated in the Arih2-/- and Mgrn1-/- cells.

Figure 2E, Figure 7D and page 7 lines 10+11: can the authors formally exclude that loss of Arih2-/- leads to upregulation of SMO expression? They show qRT-PCR data for the Ube213-/- cells in Figure S7 but I could not find similar data for Arih2.

Page 8 bottom: "AMSH*", what does "*" mean?

Page 10 line 5: please insert the Desai et al. 2020 reference at the beginning of line 5.

Page 11 line 3: "degradationof", a space is missing.

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

This paper by Lv et al builds on another recent paper from the same group showing that ubiquitination of Smo leads to its removal from the cilium under basal conditions. This work provides mechanistic insight into those observations by identifying the specific components of the ubiquitin machinery involved in this process. Lv et al perform a CRISPR screen for Ub-related genes that regulate the removal of Smo to cilia together with Ptch and the IFT/BBS machinery.
The authors show that the E3 ligase Wwp1 as important for preventing accumulation of Smo in cells not stimulated by Hh, and show that it localizes to cilia.
They show that Wwp1 interacts with Ptch1 and that this interaction is important for Wwp1 localization to cilia and for removal of Smo from the cilium. The authors further show that targeting a DUB that removes K63 polyubiquitination to the cilium also results in Smo accumulation to the cilium.
The authors have also identified an E2 that acts with Wwp1 and Arih2 (which regulates overall SMO levels) and show that it also localizes to cilia and is important for removal of Smo under basal conditions.

This is a careful, well-performed, mechanistic study that provides insight on a key step in ciliary regulation of Hh signaling. I think it could be published with some fairly minor changes.

One point is that I wonder why Wwp1 is only seen in cilia with such low frequency? Is it just at endogenous levels that are too low to detect by antibody? Is it only expressed at low levels generally? Is it perhaps dynamic in some way? It seems slightly out of line with the effect that is seen which is SMO accumulating in nearly all cilia. I wonder if the authors could comment on this more extensively.

I also thought the introduction could do with more focus. The first paragraph is long and goes into more detail about IFT than is necessary. The authors could focus more on the role of IFT and the Bbsome in Smo trafficking.

Finally, in the model figure I thought a bit more detail would be nice. While there is something to be said for simplicity, the work has quite a bit of mechanistic detail that it might be helpful to incorporate into the model- like how this Ub system is interfacing with IFT, for example.

Dear Dr. Pazour,

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Monitoring Editor

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this should be made clear and addressed. Does the WWP1 KO phenotype look like the classic PTCH KO phenotype?

Our model does not predict that the loss of Wwp1 would phenocopy the loss of Ptch1 and we did not suggest this in our manuscript. The idea is not logical based on extensive evidence from many investigators. The oldest being the fact that overexpression of Smo is sufficient to cause ciliary accumulation but not activation of the pathway. Clearly, Ptch1 regulates Smo activity through other mechanisms besides regulating its ciliary localization. Our model is that Wwp1 regulates ciliary levels of Smo. Activation of the pathway allows ciliary accumulation of Smo where it can be activated by other mechanisms. To strengthen this point we added "and become activated by other mechanisms" to the discussion of our model.

"Pathway activation promotes the removal of Wwp1 from cilia thus allowing any Smo that enters the cilium to remain and become activated by other mechanisms (Fig. 8)"

2/ For WWP1 and each of the proteins tested in the paper, the authors should measure the effect of gene inactivation on endogenous Hedgehog target genes (like Gli1) by quantitative PCR in a different cell line to test if these genes are relevant for hedgehog signaling outside of the context of the one reporter-based assay used in the paper. This is the only way to compare the magnitude of the effect of loss of these genes to ablation of core components like patched and smoothened and determine their physiological relevance to in vivo Hedgehog signaling.

This has been done for Wwp1 and Ube2l3. The data is consistent with the GFP reporter with Gli1 mRNA in Wwp1 mutant cells showing a 4 fold increase at the basal state and a slight increase with stimulation. Gli1 mRNA in Ube2l3 mutant cells show a 2 fold increase in Gli1 basal expression and a reduction in induced Gli1 expression to about half of controls. This is now included in figures 7I and S3.

3/ In assays with WWP1 at cilia, <10% of cells have detectable WWP1. How can WWP1 be a key regulator of Hedgehog signaling if only a small fraction of cells express it in cilia? The authors should use a detection method or protocol that allows assessment of WWP1 in a majority of cells.

As this was a major concern of all three reviewers, we put considerable effort into addressing this question. We feel that the finding of Wwp1 in only about 10% of cilia is due to low levels in the cilia and high levels in the cell body making it hard to detect the ciliary pool. To circumvent this problem, we targeted biotin ligase to cilia by fusing it to Sstr3 and co-expressed it with Wwp1-Avi. The Avi tag is a short peptide that can be biotinylated by the ciliary-targeted biotin ligase. Note that the biotin ligase does not promiscuously biotinylate nearby lysines but requires the lysine to be in the context of the 15 amino acid Avi tag. This approach increased our sensitivity and decreased the amount of cell body label. Under these conditions, about 75% of cilia have detectable Wwp1 and this drops to about 10% upon SHH stimulation. Controls were carried out to show this is Ptch1 dependent. This data is now included as Figure 3C, D.

4/ In Figure 4, a mutant of PTCH1 is shown that loses its interaction with WWP1. However, this mutant in I is still able to partially rescue Hedgehog signaling in Ptch1-/- cells. This suggests that the WWP1 interaction is not the reason why this mutant is less active at suppression of Hedgehog signaling.

This relates back to point 1 discussed above where one needs to distinguish ciliary localization from activation. We are not proposing that ciliary localization of Smo is sufficient to activate it. Our data indicates that that Ptch1 PY motif mutants are unable to bind Wwp1 and are unable to keep Smo out of

cilia at the basal state. The observation that these Ptch1 PY motif mutants can still suppress Smo is expected and consistent with our model.

5/ Ubiquitination of SMO by WWP1 at the lysine residues identified in the authors' previous JCB paper should be demonstrated since it is a central component of the model. Also, loss of SMO ubiquitination in response to WWP1 ablation should be tested. In this current state, one cannot tell if the effect of WWP1 on SMO ciliary levels is direct or indirect. A direct SMO ubiquitination assay is essential.

To address this point we added an experiment where we co-expressed Smo and Wwp1 in HEK293 cells along with tagged Ub and then measured the amount of Ub ligated to Smo. Wild-type Smo co-expressed with wild-type Wwp1 incorporated a relatively large amount of Ub compared to wild-type Smo co-expressed with enzymatically dead Wwp1 or wild-type Wwp1 expressed with mutant versions of Smo that contained no intracellular lysines or had the critical lysines in loop 3 mutated to arginines. While not proving that Wwp1 directly ubiquitinates Smo, it is good support for this hypothesis.

This experiment is included in Figure 6G.

6/ Ube2l3 is proposed to be a ciliary E2 in Figure 7. It is not clear how this is related to the main story on WWP1. Is Ube2l3 an E2 for WWP1 in SMO ubiquitination? If so, this is not experimentally demonstrated in the paper.

The observation that Ube2l3 functions as an E2 for Wwp1 was previously published (French et al., 2017; Marteijn et al., 2009; Marteijn et al., 2005) and cited in our paper.

7/ There are apparent discrepancies with the Kim et al. Sci Signal 2016 paper. In a similar series of experiments, in the Kim et al. paper they addback Ptch1PY (with both PPXY motifs mutated) into Ptch1-/- pMEFs, but observe no increase in ciliary Smo or elevation of pathway activity at basal levels (when compared to Ptch1WT addbacks). In the Kim et al. paper, they speculate that Wwp1 is one of eight Nedd4 family HECT E3 ubiquitin ligases that target Ptch1 and regulate its ciliary exit. In fact, the UbiBrowser cited in this paper (Li et al., Nat Comm 2017) predicts that Wwp1 ubiquitinates Ptch1. Thus, is it possible that Wwp1 ubiquitinates Ptch1 and that the reason the authors did not see a Ptch1 localization phenotype in Wwp1-/- cells is due to functional redundancy with one of the other seven Nedd4 family HECT E3 ubiquitin ligases identified in the Kim et al. paper?

Redundancy could be the reason that we did not see a Ptch1 localization phenotype but it could also be that Ptch1 removal uses another E3 ligase. We do not know the reason for the discrepancy between our data and the Kim et al work. We have added a statement to the text alerting the reader to the differences so they can compare both works.

Minor Points:

1/ Line 29: Should be "levels of SMO" instead of "levels of Arih2"?

Yes, thank you for finding this.

2/ In Figure 2A, are the colors for -SAG and +SAG switched?

No the colors are not switched. However, we can see why this is confusing and have replotted this data using a scatter plot similar to what we originally used in Figure 1G.

3/ Given similarities in approach and findings, I think it's important to cite the recently published paper "Ubiquitin chains earmark GPCRs for BBSome-mediated removal from cilia" (Shinde et al., JCB 2020).

This has been cited on page 11

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

In this manuscript Lv and colleagues elegantly demonstrate that the E3 ubiquitin ligase Wwp1 binds Ptch1 and cooperates with the E2 ubiquitin ligase Ube213 to promote ubiquitination of Smo in cilia when the Hedgehog pathway is off, thereby keeping Smo ciliary levels low in the absence of Hedgehog ligand. In a recent study (Desai et al., JCB, 2020) the authors showed that Smo is ubiquitinated when the Hedgehog pathway is off, which causes Smo to be removed from cilia by the IFT machinery. However, the enzymes responsible for ubiquitinating Smo in cilia under these conditions were not identified. Here the authors developed and characterized a reporter line, GreenBomb, in mouse embryonic fibroblasts, which allows for robust simultaneous detection of ciliary FLAG-Smo localisation and Hedgehog pathway activity via expression of a GFP-tagged target gene. By using the GreenBomb cells the authors then carry out a comprehensive CRISPR-based screen to identify genes of the ubiquitin proteasome system that regulate ciliary Smo accumulation and target gene expression. After secondary screening and careful validation, the authors end up with a handful of genes that specifically affect Smo ciliary localisation and target gene expression in the basal state, amongst which the E3 ubiquitin ligase Wwp1 and the E2 ubiquitin ligase Ube213 are the most interesting as they localize to cilia. By using a variety of molecular and cell-based approaches the authors dissect the molecular mechanism by which Wwp1 and Ube213 controls ubiquitination and ciliary presence of Smo, and they further reveal that ubiquitinated Smo can be deubiquitinated by AMSH*, indicating the Smo has K63-linked ubiquitin chains.

Overall this is an impressive and solid piece of work that will be of great interest to the JCB readership and cell- and developmental biologists in general. The experimental approaches are very elegant and the data of very high quality. I applaud the authors for this very nice piece of work and recommend publication provided that the authors can adequately address the following issues.

Thank you!

Main comment

Figure 4F, G: why is the proportion of cell displaying WWP1 at cilia so low (<10%) if WWP1 is a central regulator of the Shh pathway?

Please see response to Reviewer 1 comment 3.

Minor comments

Figure 2A: Why is the GFP MFI lower in the SAG-stimulated cells compared to unstimulated cells? Did the authors accidentally mislabel the figure or am I missing something? In Fig. 2C, D it seems the SAG-stimulated cells overall have more SMO in cilia as expected so I am confused about the data in Figure 2A.

The data was plotted relative to controls. We realize that this was confusing and replotted the data using a scatter plot similar to what was used in Figure 1G.

Figure 1D: the blue Arl13B text and cilium labelling is very hard to see, can the authors enhance contrast to make it more visible to the reader?

It has been enhanced.

Figure 1G: they grey dots in the middle of the graph are really difficult to see, is it possible to color them a bit darker? The authors might also want to chose a different color for the stipled lines in the same figure (e.g. yellow or orange) as it is quite hard to see the stipled lines, especially the vertical ones, as they appear now.

We have made the dots darker and smaller. The stippled lines were changed from gray to black.

Figure 2E, Figure S3 and page 7 top: the unspecific 150 kDa band is not only present in the Arih2-/- cells but also the Mgrn1-/- cells (perhaps also the Maea-/- cells). Although I do not think the authors need to characterize the nature of this unspecific band further, they should refer to Figure S3 on top of page 7 and clearly indicate here and in Figure 2E that the 150 kDa band is a protein that binds non-specifically to the FLAG antibody and appears to be upregulated in the Arih2-/- and Mgrn1-/- cells.

We have added the requested information to the text on pages 7 and 18.

Figure 2E, Figure 7D and page 7 lines 10+11: can the authors formally exclude that loss of Arih2-/- leads to upregulation of SMO expression? They show qRT-PCR data for the Ube213-/- cells in Figure S7 but I could not find similar data for Arih2.

qRT-PCR data indicates that Smo is not upregulated by the loss of Arih2. A detailed study of Arih2 is in preparation and will include this data.

Page 8 bottom: "AMSH*", what does "*" mean?

AMSH* is a Stam2-AMSH fusion and this is the name given in the published reference. We put (Stam2-AMSH fusion) after AMSH* to clarify the name.

Page 10 line 5: please insert the Desai et al. 2020 reference at the beginning of line 5.

Inserted.

Page 11 line 3: "degradationof", a space is missing.

Thank you

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

This paper by Lv et al builds on another recent paper from the same group showing that ubiquitination of Smo leads to its removal from the cilium under basal conditions. This work provides mechanistic insight into those observations by identifying the specific components of the ubiquitin machinery involved in this process. Lv et al perform a CRISPR screen for Ub-related genes that regulate the removal of Smo to cilia together with Ptch and the IFT/BBS machinery.

The authors show that the E3 ligase Wwp1 as important for preventing accumulation of Smo in cells not stimulated by Hh, and show that it localizes to cilia.

They show that Wwp1 interacts with Ptch1 and that this interaction is important for Wwp1 localization to cilia and for removal of Smo from the cilium. The authors further show that targeting a DUB that removes K63 polyubiquitination to the cilium also results in Smo accumulation to the cilium.

The authors have also identified an E2 that acts with Wwp1 and Arih2 (which regulates overall SMO levels) and show that it also localizes to cilia and is important for removal of Smo under basal conditions.

This is a careful, well-performed, mechanistic study that provides insight on a key step in ciliary regulation of Hh signaling. I think it could be published with some fairly minor changes.

Thank you!

One point is that I wonder why Wwp1 is only seen in cilia with such low frequency? Is it just at endogenous levels that are too low to detect by antibody? Is it only expressed at low levels generally? Is it perhaps dynamic in some way? It seems slightly out of line with the effect that is seen which is SMO accumulating in nearly all cilia. I wonder if the authors could comment on this more extensively.

Please see Reviewer 1 comment 3.

I also thought the introduction could do with more focus. The first paragraph is long and goes into more detail about IFT than is necessary. The authors could focus more on the role of IFT and the Bbsome in Smo trafficking.

We have edited the intro to add detail of IFT and BBSome in Smo trafficking.

Finally, in the model figure I thought a bit more detail would be nice. While there is something to be said for simplicity, the work has quite a bit of mechanistic detail that it might be helpful to incorporate into the model- like how this Ub system is interfacing with IFT, for example.

We have enhanced the model.

May 26, 2021

RE: JCB Manuscript #202010177R

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 "The E3 Ubiquitin Ligase Wwp1 Binds Ptch1 and Regulates Smoothened's Ciliary Localization". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

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

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

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

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

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

The following alternative titles are suggested:

E3 Ubiquitin Ligase Wwp1 regulates the dynamics of Hedgehog receptors Patch1 and Smoothened

Or

E3 Ubiquitin Ligase Wwp1 regulates the ciliary dynamics of Hedgehog receptors

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

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

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

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

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

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

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

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

B. FINAL FILES:

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

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-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://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.

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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, PhD
Monitoring Editor

Andrea L. Marat, PhD
Senior Scientific Editor

Journal of Cell Biology

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

The authors have addressed my comments and I support publication.

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

The authors have adequately addressed all the comments I had for the initial version of the manuscript and I therefore recommend acceptance for publication in JCB.

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

The authors have improved the manuscript from the version originally submitted- They improved readability and addressed the key concerns of myself as well as the other reviewers. In my opinion the manuscript is acceptable for publication.