Competition between two phosphatases finetunes Hedgehog signaling

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RE: JCB Manuscript #202010078

Dr. Alan Jian Zhu
Peking University
School of Life Sciences
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China

Dear Dr. Zhu,

Thank you for submitting your manuscript entitled "A competition between two phosphatases maintains Hedgehog signaling homeostasis". The manuscript was assessed by three expert referees, whose comments are appended below.

As you will see, all three reviewers were quite enthusiastic about your work. They all have minor suggestions to provide clarity -- the only point that involves experimental support is addressing the issue of controls done at the same temperature as experiments. The issue of whether the PpV-Wdb complex is catalytically active and, if so, whether this activity serves a biological purpose could be addressed in the Discussion without additional experimentation. We would like to see a revised version with a detailed point-by-point response addressing all of the issues raised by the reviewers as well as highlighted changes in the manuscript text, and we will aim to assess the changes editorially, without re-review, if possible.

We would be happy to publish your paper in JCB pending these changes and final revisions necessary to meet our formatting guidelines (see details below).

1) Text and figure limits: Character count for Articles and Tools 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.
   JCB Articles may have up to 10 main and 5 supplementary figures, as well as up to 10 supplementary video or flash animation files.

2) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: Competition between two phosphatases finetunes hedgehog signaling

Running title (we can accommodate an extension of the character count): phosphatase PpV as a homeostatic regulator of Hedgehog signaling

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 a summary statement on the title page of the 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 3BB'CC' right-side views, 7B, 7A'A'A'A' (also 7C' panels), S4 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: 6C

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: 1F

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.
- For all cell lines, vectors, fly lines, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features *even if described in other published work or gifted to you by other investigators*
- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.
- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.
- 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 reconstructions, surface or volume rendering, gamma adjustments, etc.).

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.**

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, 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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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 14 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,

Mark Peifer, PhD
Monitoring Editor, Journal of Cell Biology

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

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

This manuscript by Liu et al reports that the PpV/PP6 phosphatase and Hh signaling regulate each other during Drosophila wing development by a surprising mechanism. PpV binds and inactivates the PP2A regulatory subunit Wdb in a manner that is independent from PpV phosphatase activity. This impacts Hh signaling because PP2A-Wdb is required for the dephosphorylation of Smo, negatively regulating its plasma membrane expression. In turn, Hh signaling regulates PpV expression, providing a regulatory circuit.
This study constitutes an impressive amount of work. The experiments are logical and mostly very well conducted (with one minor exception mentioned below). The results are clear and correctly interpreted. The molecular mechanism of interplay between phosphatases demonstrated here is novel and may have implications for other biological processes where PP2A-Wdb/B56 plays essential roles, such as mitosis. I recommend publication after minor revisions.

Fig 1 A-F and S1: All conditions are not at the same temperature. This makes comparisons problematic because wing development is influenced by temperature. For example, the comparison between 1A and 1C, which is quantified and analyzed statistically in 1F is flawed for this reason. Proper controls at the same temperature should be included.

Some figure panels are not described in the Results text. For example, Fig 2I-J. Although I could make sense of them, these results should be described in the text, with references to all figure panels.

Specify what Smo and mSmo refer to in the legends of Figs 2 and 4.

Line 162: The phrase "...was only be inhibited..." needs correction.

Ln 184-5: This sentence needs work: "Endogenous as well as Wdb protein overexpressed in S2 cells migrated as a doublet...".

Line 234: Replace "We recently found that..." with "We found that..." as the former phrase typically refers to published work.

In the Discussion: "Unfortunately, regulation of Wdb by Hh is indirect." Why unfortunately?

Although this may be outside the scope of the current study, it would be interesting to know if the PpV-Wdb complex is catalytically active and, if so, whether this activity serves a biological function. I think this point should be at least discussed if not explored experimentally.

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

This manuscript describes an outstanding example of well-reasoned, high quality work. The fact that the results led to unexpected outcomes give confidence that the data has not been made to conform to preconceived models. The discovery of this PpV system that regulates Smo phosphorylation and Hh signaling is important for our basic understanding of the Hh pathway and possibly for translational goals. Publication is strongly recommended.

The following minor suggestions are intended as examples that are provided to encourage the authors to find more direct and simpler (less stilted) ways to present their findings and ideas.

Ln 17 I do not understand the term "robustly controlled": what is the intended meaning in the context of graded distribution and response?

Ln 20 "independent of its phosphatase activity" - what does "it" refer to?

Ln 23: "PpV is also a direct target of Hh signaling, whose expression itself is regulated by differential
Hh signaling. Suggest simpler: PpV expression is regulated by Hh signaling.

Ln 26 "robust and reliable Hh signaling" These descriptors do not have clear meaning or relevance.

Ln 79 Suggest more direct and simpler: Hh signaling is one of the major signaling systems that patterns the Drosophila wing, and plays a key role...

Ln 82 "stereotypical" is not a good word choice; suggest instead: "which has been previously observed in several conditions of reduced Hh signaling."

Ln 84 "Loss of the anterior cross vein (ACV), but not reduced distance between L3 and L4 veins, in PpV RNAi adult wings was partially suppressed by increased activity of puckered (Fig. 1, D and E), a negative regulator of c-Jun N-terminal kinase (JNK) signaling, suggesting that PpV plays a specific role in Hh signaling, in addition to its previously reported role in inhibiting JNK-dependent tumor progression (Ma et al., 2017)."

This sentence is confusing and leaves open the issue of restoration of the ACV by JNK. Perhaps it might be better to describe evidence for influence of Hh components and include the JNK data after?

Ln 86 Please provide reasoning for introducing puk/JNK into this analysis

Fig S1 salm should be introduced and defined. ACV phenotypes can have variable expressivity - can the authors provide some measure of variation and reproducibility?

Ln 95 "Together, the above results establish an important requirement of PpV activity in mediating Hh signaling during Drosophila wing development." This seems somewhat overblown given the results show partial loss of function from partial knock down conditions.

Ln 106 The terms high, intermediate, and low threshold signaling are confusing - referring directly to Hh signaling levels might be clearer?

Ln 146 It is important to counterstain with an apical marker to justify this statement. Fig 3 legend does not identify color scheme or adequately describe the panels. I do understand the (a) and (b) images. In general, the Figure legends lack adequate descriptions of figure panel details.

Ln 191 suggest replacing "migratory patterns" with "electrophoretic migration"

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

Although the main components of the Hh signalling pathway in flies but also other systems are well known, fine tuning by feed forward and feedback mechanisms have been studied less and new components are still being revealed. The fine tuning mechanisms are important and especially interesting in pathology and disease/tumor context, where a modulation of the Hh signal and less
so loss-of-function/signal situations is relevant. In this study, a feed back mechanism for Hh signalling is identified which is important for a down-regulation of the Hh signal to avoid overshooting signals. The protein phosphatase PpV is identified as a component of that feed-back mechanism. On the one side the authors present convincing data showing that PpV is a direct transcriptional target of Hh signalling, including promoter analysis. On the other side, the study shows that PpV binds to a regulatory subunit of PP2A and in this manner antagonises PP2A. PP2A is known to keep the Hh receptor Smoothened in an active (dephosphorylated) state. The authors present good arguments for a novel and surprising mechanism for the PpV - PP2A antagonism, in that PpV binds and then targets the PP2A regulatory subunit widerborst to proteolysis by the proteasome.

In summary, the study reveals a novel feed-back mechanism in Hh signalling, which is likely to be conserved, given the conservation of PpV/Pp6. The study also characterises in detail the mechanisms underlying the interactions of PpV with the classical components of Hh signalling, including the promoter analysis on the downstream side and PP2A as target on the upstream part. Given the high quality and conclusiveness of the data, I strongly recommend publication of the manuscript in JCB.

In the following I will list some criticism and suggestions, which will improve the manuscript.

* Some experiments were conducted in cultured cells (Schneider cells) in an appropriate way. These experiments are „out-of-context“ and components are introduced into a wild type background resulting in a mixture of constructs and endogenous components, which may interact in unforeseeable ways. For these reason the conclusions have to be carefully stated. Generalizations of these data to the physiological situation may not be possible. The authors tend to directly conclude from the cultured cells to the situation in the larvae/pupae. I would recommend more careful statements for data with cultured cells which have not been confirmed in vivo. For example the conclusion of proteosomal degradation is solely based on cell culture data (Fig. 3), the direct binding of PpV to widerborst is solely based on cell culture study. Given the clearly described genetic interaction of PpV and PP2A there is no doubt about the negative interaction. I am not sure however, whether the possibility of widerborst binding to PpV as shown in Fig. 5 is the relevant mechanism also in wing discs. I am also not sure about the role of Pase activity of PpV. The authors present convincing arguments based on cell culture data and overexpression of the mutated PpV. However it is conceivable that the mutated PpV interacts in some way with the endogenous catalytically active PpV. The authors did not test the inactive allele in the background lacking endogenous PpV. I do not ask to do such experiments but recommend to consider these conceivable possibilities in the conclusions.

Minor comments:

I 1 Delete „A“ Competition between....
I 16 Delete „coordinated“ The study reveals a feed back mechanism within the Hh pathway. I do not see what is coordinated with hh signalling here.
I 25 Deleted „graded“. It is clearly shown that the PpV mechanism is important for high level signalling, but there is no data about a graded response or involvement.
I 38 „Among“ instead of „Of“.....
I 88 In addition to the role in JNK signalling also mention and cite here the role of PpV in controlling Cdc25/Twine levels (Liu et al G3 2019, Liu et al PlosGen 2020).
I 105 „Consistent with“ instead of „it is not surprising"
the authors state that few mutant males survived to pupal stage. the authors should give the order of magnitude, if possible. Was it 1 out of 1000 or 1 out of 10? Did the authors test how much of the maternal PpV was left in these larvae/pupae by western blot?
l131 use past tense provided
l133 consistent with instead of „As"
l149 „a“ not „an”
l162 delete „be”
l166 Please briefly explain why Lys was mutated for the non-experts
l195 please mention here that the mutant PpV is introduced into a wild type background containing an active allele.
L239 the specificity of the PpV antibody in immunostainings should be demonstrated by staining of mutant larvae/pupae.

Figures: The data rely mainly on single representative images and photographs of western blots. There is little image quantification to describe the variance of the data. I do not ask to include such an analysis of the images, since the phenotypes and effects are qualitative in nature and do not require proper quantification. To systematically revise the manuscript with complementing all qualitative data with quantification would not improve much the data and would be out of scale.

Fig. 2CD. An additional cross vein is visible. But the positioning is different between C and D. Why?
Fig. 2K-M The western blot shows a depletion of Smo after PpV-Myc expression. A similar reduction is not really observed in the images of immunostainings. Why? What is mSmo?
Fig. 2J is not cited in the text
Fig. 3C This panel is cited in the text as PpV mutant not PpV RNAi.

* Please provide proper literature reference for using Flybase, Hybridoma bank, Bloomington and other stock centres. Please check at these web sites how these services should be cited. Stock numbers and catalogue number are not the proper way to refer to these materials, as these numbers are likely to change over time.
Point-by-point response to editor and reviewers’ critiques

1) Text and figure limits: Character count for Articles and Tools 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.

In the revised manuscript, character count for Articles and Tools is 40895 in total. However, character count for title page, abstract, introduction, results and discussion is only 27693. As reviewer #2 asked for more detailed descriptions in figure legends and reviewer #3 for detailed acknowledgement for stock centers and collections, it is difficult to further reduce the number of words in figure legends and acknowledgement.

2) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: Competition between two phosphatases finetunes hedgehog signaling

Running title (we can accommodate an extension of the character count): phosphatase PpV as a homeostatic regulator of Hedgehog signaling.

We changed the title and running title as suggested.

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 a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.

An eTOC summary has been added in the title page section.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 3BB'CC' right-side views, 7B, 7A'A'A'A' (also 7C' panels), S4

Scale bars are now present on all microscopy images.

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: 6C

Nucleic acid size marker has been incorporated in the panel 6C.

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
The numbers of adult wings with corresponding genotypes used for statistical analysis shown in panel 1F are listed in the figure legends (lines 800-801).

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.

We added experimental details for in situ hybridization, adult fly wing imaging and HEK293T cell culture to the Materials and methods (lines 431-444 and 449-451).

- For all cell lines, vectors, fly lines, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features *even if described in other published work or gifted to you by other investigators*

Database / vendor ID of all cell lines, vectors, fly strains and constructs/cDNAs are now listed in Materials and methods.

- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.

Complete information of all antibodies used in the study are listed in Materials and methods.

- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.

Table S2 lists all the oligos used in the study.

- 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 reconstructions, surface or volume rendering, gamma adjustments, etc.).

Details for microscope image acquisition and manipulation are provided in Materials and methods.
Responses to reviewer #1’s critiques:

Fig 1 A-F and S1: All conditions are not at the same temperature. This makes comparisons problematic because wing development is influenced by temperature. For example, the comparison between 1A and 1C, which is quantified and analyzed statistically in 1F is flawed for this reason. Proper controls at the same temperature should be included.

Images in panels 1A and 1B showing adult fly wings of wild type as well as dpp>ptc maintained at 29°C replaced those maintained at 25°C. Accordingly, a new set of quantification and statistical analysis was performed and now shown in panel 1F.

Some figure panels are not described in the Results text. For example, Fig 2I-J. Although I could make sense of them, these results should be described in the text, with references to all figure panels.

Panels of Fig. 2I-J” are now cited in the text (line 137).

Specify what Smo and mSmo refer to in the legends of Figs 2 and 4.

The definition of “Smo” and “mSmo” is stated in figure legends (lines 823-824 and 863-864).

Line 162: The phrase "...was only be inhibited..." needs correction.

This has been corrected (line 162).

Line 184-5: This sentence needs work: "Endogenous as well as Wdb protein overexpressed in S2 cells migrated as a doublet...".

This sentence has been rephrased as “Endogenous as well as overexpressed Wdb protein in S2 cells migrated as a doublet...” (lines 183-184).

Line 234: Replace "We recently found that..." with "We found that..." as the former phrase typically refers to published work.

“recently” has been deleted from this sentence (line 233).

In the Discussion: "Unfortunately, regulation of Wdb by Hh is indirect." Why unfortunately? “Unfortunately” has been replaced by “However” (line 314).

Although this may be outside the scope of the current study, it would be interesting to know if the PpV-Wdb complex is catalytically active and, if so, whether this activity serves a biological function. I think this point should be at least discussed if not explored experimentally.

A sentence describing potential effect of PpV-Wdb complex has been added in Discussion (lines 375-376).
Responses to reviewer #2's critiques:

Ln 17 I do not understand the term "robustly controlled": what is the intended meaning in the context of graded distribution and response?
We replaced this phrase with the sentence “How its signaling activity is finetuned in response to fluctuated Hh is less understood” (lines 20-21).

Ln 20 "independent of its phosphatase activity" - what does "it" refer to?
This sentence has been rephrased (line 25).

Ln 23: "PpV is also a direct target of Hh signaling, whose expression itself is regulated by differential Hh signaling." Suggest simpler: PpV expression is regulated by Hh signaling.
We changed the sentence as suggested (line 28).

Ln 26 "robust and reliable Hh signaling" These descriptors do not have clear meaning or relevance.
This sentence has been rephrased (line 30).

Ln 79 Suggest more direct and simpler: Hh signaling is one of the major signaling systems that patterns the Drosophila wing, and plays a key role...
We changed the sentence as suggested (line 80).

Ln 82 "stereotypical" is not a good word choice; suggest instead: "which has been previously observed in several conditions of reduced Hh signaling:
We changed the sentence as suggested (lines 83-84).

Ln 84 "Loss of the anterior cross vein (ACV), but not reduced distance between L3 and L4 veins, in PpV RNAi adult wings was partially suppressed by increased activity of puckered (Fig. 1, D and E), a negative regulator of c-Jun N-terminal kinase (JNK) signaling, suggesting that PpV plays a specific role in Hh signaling, in addition to its previously reported role in inhibiting JNK-dependent tumor progression (Ma et al., 2017)."
This sentence is confusing and leaves open the issue of restoration of the ACV by JNK. Perhaps it might be better to describe evidence for influence of Hh components and include the JNK data after?

Ln 86 Please provide reasoning for introducing Puc/JNK into this analysis
Thanks for the suggestion. We reorganized the description of the data to make it more clearly presented (lines 85-87).

Fig S1 salm should be introduced and defined. ACV phenotypes can have variable expressivity -
can the authors provide some measure of variation and reproducibility?
This issue has been addressed in Figure S1 figure legends (Supplemental legends lines 3-4).

Ln 95 "Together, the above results establish an important requirement of PpV activity in mediating Hh signaling during Drosophila wing development."

This seems somewhat overblown given the results show partial loss of function from partial knock down conditions.
We removed “important” from the sentence (line 96).

Ln 106 The terms high, intermediate, and low threshold signaling are confusing - referring directly to Hh signaling levels might be clearer?
Thanks for the suggestion. We replaced “threshold” with “level” in the text.

Ln 146 It is important to counterstain with an apical marker to justify this statement. Fig 3 legend does not identify color scheme or adequately describe the panels. I do understand the (a) and (b) images.
Thanks for the suggestion. As the polarized localization of Wdb can be easily recognized by the outline of the wing disc shape, we did not include an apical marker staining in the disc.

In general, the Figure legends lack adequate descriptions of figure panel details.
Figure legends have been rewritten to improve its readability.

Ln 191 suggest replacing "migratory patterns" with "electrophoretic migration"
We replaced "migratory patterns" with "electrophoretic migration" as suggested (line 190).
Responses to reviewer #3's critiques:

I 1 Delete "A" Competition between....
We changed the title as editor and reviewer suggested.

I 16 Delete "coordinated" The study reveals a feed back mechanism within the Hh pathway. I do not see what is coordinated with hh signalling here.
We deleted "coordinated" (line 20).

I 25 Deleted "graded". It is clearly shown that the PpV mechanism is important for high level signalling, but there is no data about a graded response or involvement.
We changed the sentence as reviewers #2 and #3 suggested.

I 38 "Among" instead of "Of".....
We changed the phrase as suggested (line 41).

I 88 In addition to the role in JNK signalling also mention and cite here the role of PpV in controlling Cdc25/Twine levels (Liu et al G3 2019, Liu et al PlosGen 2020).
As loss of ACV is a common phenotype shared by reduced Hh signaling and enhanced JNK activation, the experiments described in Figure 1 aimed to distinguish the specificity of PpV in Hh signaling. We cited the G3 and Plos Genetics papers (Liu et al., 2019; Liu et al., 2020) when introducing the known function of PpV (line 178).

I 105 "Consistent with" instead of "it is not surprising"
We prefer ".it is not surprising" as the the sentence does not flow well with ""Consistent with".

I 112 the authors state that few mutant males survived to pupal stage. the authors should give the order of magnitude, if possible. Was it 1 out of 1000 or 1 out of 10? Did the authors test how much of the maternal PpV was left in these larvae/pupae by western blot?
The survived rate has been specified in the text (line 114). It is difficult to access the contribution of maternal PpV because no homozygous PpV^KO females survive to larval stage. Nevertheless, as shown in Fig. 3A, no PpV protein can be detected in PpV^KO hemizygous males.

I 131 use past tense provided
This has been corrected (line 132).

I 133 consistent with instead of "As"
We replaced "As" with "Consistent with" (line 134).

I 149 "a" not "an"
This has been corrected (line 149).
Please briefly explain why Lys was mutated for the non-experts
The explanation has been added in the text (line 167).

Please mention here that the mutant PpV is introduced into a wild type background containing an active allele.
Thanks for the suggestion. This point has been added in the text (line 195).

The specificity of the PpV antibody in immunostainings should be demonstrated by staining of mutant larvae/pupae.
The specificity of the PpV antibody has been tested in PpV RNAi (immunostaining) and in PpVKO males (Western blotting).

Figures: The data rely mainly on single representative images and photographs of western blots. There is little image quantification to describe the variance of the data. I do not ask to include such an analysis of the images, since the phenotypes and effects are qualitative in nature and do not require proper quantification. To systematically revise the manuscript with complementing all qualitative data with quantification would not improve much the data and would be out of scale.

Fig. 2CD. An additional cross vein is visible. But the positioning is different between C and D. Why?
When Hh signaling is enhanced by knocking down wdb, additional cross veins appear in variable places between L2 and L3.

Fig. 2K-M The western blot shows a depletion of Smo after PpV-Myc expression. A similar reduction is not really observed in the images of immunostainings. Why? What is mSmo?
Activation of Hh signaling promoted Smo phosphorylation, resulting in a weaker but more spread out Western blot signal. PpV overexpression had a similar effect. However, wdb RNAi treatment not only altered Smo phosphorylation pattern but also increased Smo protein amount. We also specified mSmo in the figure legends.

Fig. 2J is not cited in the text
This has been addressed per response to reviewer #1’s critiques.

Fig. 3C This panel is cited in the text as PpV mutant not PpV RNAi.
Fig. 3C described the effect of PpV RNAi, which we referred in the text as “PpV knockdown” (line 148).

* Please provide proper literature reference for using Flybase, Hybridoma bank, Bloomington and
other stock centres. Please check at these web sites how these services should be cited. Stock numbers and catalogue number are not the proper way to refer to these materials, as these numbers are likely to change over time. Proper literature references and acknowledgements for using materials from stock centers and collections have been added.