Extensive crosstalk of G protein-coupled receptors with the Hedgehog signalling pathway

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DOI: 10.1242/dev.189258

Editor: Thomas Lecuit

Review timeline

Original submission:	7 February 2020
Editorial decision:	9 April 2020
First revision received:	16 November 2020
Editorial decision:	13 January 2021
Second revision received:	12 February 2021
Accepted:	19 February 2021

Original submission

First decision letter

MS ID#: DEVELOP/2020/189258

MS TITLE: Extensive crosstalk of G protein-coupled receptors with the Hedgehog signalling pathway

AUTHORS: Farah Saad and David Hipfner

I sincerely apologise for the long time before being able to come back to you. This is due to delays in the review process and my own difficulties following confinement 3 weeks ago. I am sorry about this.
I have received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees do express interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. I think that point 1 of Rev1 and point 2 of Rev 3 on the specific regulation of Dpp by Hh address a common issue that ought to be taken care of one way or another. I would primarily encourage you to see how this can be addressed experimentally though I do understand that you may not be able to do this at the moment, unless you already have data that deal with this. You may also amend the interpretation to be more cautious about the interpretation. We are happy to give you extra time in order to be able to revise your manuscript by additional experiments.
Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors investigated whether G-Protein Coupled Receptors (GPCRs) regulate Hh signaling in flies. In mammals, there was evidence that a few select GPCRs signaled to heterotrimeric G-proteins Gas and Gai to modify cAMP synthesis and alter Shh target gene expression. The authors hypothesized that this could be a generalized role for many GPCRs. To investigate this, Drosophila was used as the model system since flies have a smaller number of GPCRs and there are readily available tools to monitor Hh signaling in the wing disc. Initially the authors found that loss of Gas decreased dpp-lacZ (a downstream Hh target gene) and loss of Gai increased dpp-lacZ. RNAseq was then used to investigate which of the 116 GPCRs in Drosophila were expressed in late third instar larval wing discs. Of the 22 GPCRs that were identified seven GPCRs had a phenotype similar to Gas and four had a similar phenotype to Gai, suggesting that multiple GPCRs can influence Hh signaling. Lastly the authors focused on a Mthl5 and suggested that this GPCR stimulates Gai to decrease cAMP/PKA levels to modulate Hh activity.

Major Concerns:

Dpp isn’t exclusive to the Hh Signaling pathway The authors routinely monitor GPCRs on Hh signaling by looking at dpp-lacZ, wing growth, and ptc-lacZ.
Of these reporters, only ptc-lacZ is exclusive to the Hh pathway. Furthermore when there is decrease in Hh activity (loss of Gas or increased Mthl expression Fig3, 8), Engrailed (which wasn’t monitored) would be the first to be eliminated, then ptc would be reduced, and finally dpp may also be reduced. Thus it was suspicious in many of these experiments, there were changes to dpp but not ptc-lacZ suggesting that the effects of GPCRs are not Hh-dependent. To link the role of GPCRs to Hh, it would be essential to always monitor ptc-lacZ and Engrailed staining . If there isn’t a correlation with ptc and Engrailed, then the authors should consider that the effects they are seeing from the GPCRs are related to another regulator of dpp.

Limited Increase in Activity It is also challenging to see a significant increase in dpp/ptc-lacZ (Fig 2,6) intensity at the AP border in a wild-type background. It would be more convincing if loss of these GPCRs (Gai) are increasing Hh activity and are specific to Hh if they could rescue ptc activity in a Fused mutant background.

Mthl is expressed only in the ventral region but most experiments were analyzed in the dorsal region In Figure 5, the authors stain for the expression of Mthl5 and found that it is endogenous to the ventral region of the wing disc and absent in the dorsal region. Most of the experiments done however are with a dorsal Gal-4 driver. If the experiments were repeated with a driver that was expressed also in the ventral region, the results would be more representative of physiological conditions.

Minor Concerns:

Measurements of Wing Vein Spaces: Increase in L3-L4 vein spacing is difficult to see by eye. Measurement of the L3-L4 to the whole wing would be more convincing if there was a control of other wing vein spacing (L1-L2, L2-L3, L4-L5) shrinking or staying the same by comparison.

Cell Death: Figure 8I loss of activity appears to be due to cell death

Figure 9 I-N: Only showing disc size, but should also show target gene staining.

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General measurements: To reduce variability, the flies should have had a short laying time and wings/wing discs collected from same developmental stage. (It wasn't clear in the methods and materials

Smoothened staining: Difficult to monitor anterior Smoothened staining, better to be co-stained with ptc instead of Ci-155 because of the down regulation of Ci at the most posterior portion of the AP border. Also would be useful if could see all three panels and where the AP border is marked.

Conclusion:
My recommendation is to reject the paper because the connection to Hh signaling is too weak.

Comments for the author

Conclusion:
My recommendation is to reject the paper because the connection to Hh signaling is too weak.

<u>Reviewer 2</u>

Advance summary and potential significance to field

In this paper Saad and Hipfner describe a series of experiments designed to investigate the influence of G-protein coupled receptor (GPCR) activity on the responsiveness of cells to Hh signaling activity. The study is predicated upon the proposition that modulation of cAMP levels influences the sensitivity of cells to Hh signaling by modulating the phosphorylation of Smo by PKA. In support of this proposal, they first show that depletion of Gas – predicted to decrease cAMP levels – in the wing imaginal discs, attenuates the response of anterior compartment cells to Hh activity leading to a reduction in wing size; conversely , depletion of Gai causes expansion of the dpp expression domain and a concomitant increase in wing size and patterning defects. Based on expression analysis, they next identified GPCRs expressed in the wing imaginal disc and used the same strategy to modulate their expression and analyse the effects. This led to identification of 7 GPCRs, which they conclude potentiate Smo activity and 4 which they conclude attenuate Smo activity.

These conclusions are based on analysis of wing size and hh target gene expression.
One of the latter group of GPCRs, Mthl5, is then subjected to more in depth analysis. The same assays – L3-L4 wing area, dpp and ptc expression and ci155 levels are employed to explore the effects of an mlth5 loss of function mutant allele on wing development. The data are consistent with Mthl5 attenuating Hh pathway activity; and in support of this, they show that overexpression of Mthl5 causes a reduction in hh target gene expression and wing size. Using genetic approaches to attenuate the dpp pathway, the authors provide data to support the proposal that the wing defects seen in the mutant flies are caused by ectopic dpp expression.
While all of these finding are consistent with Mthl5 modulating Hh activity by lowering cAMP levels, they do not demonstrate such an effect. To address this more directly, the authors employed S2 cells expressing a cAMP sensor and used genetic approaches to increase Gai activity or reduce cAMP levels in the mlth5 mutant. Finally, they examined the levels of Smo as a proxy for phosphorylation – in wing discs mutant for or overexpressing mthl5.

The experiments reported here have been carefully designed and the data well documented. Some of the effects are quite subtle, but in general the authors interpretations are well founded. Given the pleiotropic effects of PKA and the multiple inputs into its regulation by GPCRs, it is perhaps not so surprising that many GPCRs can modulate hh pathway activity as revealed by this analysis. Nevertheless, these are interesting findings that add to our understanding of the complex influences on cell fate specification.

Comments for the author

My main reservation with this analysis is that while the underlying proposition is that GPCRs influence Hh pathway activity by modulating the PKA-dependent phosphorylation of Smo, none of

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the experiments actually measure Smo phosphorylation.Such assays would enhance the analysis significantly.

I also wonder why there is apparently no effect of modulating cAMP levels on Smo in the posterior compartment – for instance, in response to Mthl5 overexpression driven by ap-GAL4 (Fig. 9 Q-R). Is the enhanced stability of Smo in the posterior compartment independent of PKA-dependent phosphorylation?

Reviewer 3

Advance summary and potential significance to field

This works addresses the role of GPCR in Hedgehog (HH) signaling during development, using the fly wing as a model. Both the role of HH in the wing development and the core components of the HH pathway are fairly well characterize. The implication of GPCRs in the wing development has not been addressed yet and their relationship with HH signaling is not well documented. Moreover, despite the central role of PKA in fly HH signaling how cAMP is regulated in this process is poorly understood. In the first half of the paper the authors performed a mini RNAi screen on two dozen on GPCRs highly expressed in the wing imaginal disc and show that the downregulation of a large subset of them leads to wing growth and/or wing patterning effects and modulates the responses to HH. The second half of the paper is devoted to a genetic study of the role of Mth15 in wing development in relationship with HH signaling. Their data supports a model in which Mthl15 would negatively modulate HH signaling by reducing the levels of cAMP (via Galphai).

Comments for the author

General recommendations

Overall, the data are of good quality and the manuscript is clearly written. However, while the data globally support the conclusions drawn by the author, they are not always sufficiently demonstrative. A few extra experiments should be sufficient to fill the bigger gaps but I also recommend that the authors make significant changes in text to include alternate interpretation of the data (see main issue 2) and better explanations to correlate what happens in the disc and in the wing (see main issue 3). Finally, the figures requires some extensive reorganization and even in several cases, an improvement of their visual presentation (see details below).

Main issues
1. I do not understand where is the novelty in the Figure 1 which seems to exactly correspond to the Fig 7 of Praktiknjo et al. (from the same lab). I might misunderstand something but if not, this figure does not have its place here and should be removed. However, as it is important to have independent confirmation of data and as these results are important to understand the following experiments, I suggest to (i) add these data (without the effects on CI) in the other Figures: A-C in Fig 2, F in Fig 3 and H in Fig 4 and (ii) clearly mention that it confirms what was already shown.

2. Effects of the mth15 mutant on dpp.

a) To better understand the comparison between the wild-type and mutants situations (fig 6, 7 and 9) it would be useful to have more information on how the discs of the different genotypes that they directly compare were labeled and imaged: labelled in parallel or not, imaged the same day with the same microscope setting or by independent parallel batches that include the same number of discs of the different genotypes etc
b) To strengthen the demonstration of the effects of mtl15, the rescue experiment (Fig 8 E-I) could be associated to this Figure (see below).
c) The authors should clarify (based on what they see for CI and ptc) their interpretation of the changes in dpp expression. I do not dispute that fact that mlh15 MB affects dpp (and to a lesser degree ptc), and the size of wing disc in a Dpp signaling dependent manner. However, it seems important to strengthen the demonstration that the increase in dpp expression is a direct result of the activation of HH signaling and not an indirect one via another mechanism. First, the enlargement of the dpp domain could also be due to (i) a suppression of late en expression,

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reflecting a lower response to high HH levels and leading to an extension of dpp expression toward the A/P boundary, and/or (ii) an increase in HH production. I may not be possible to fully exclude all these alternate explanations (despite the fact that analysis of the pattern of CI accumulation near the AP could help to reject the ?late en? possibility), but they should not be omitted.
More importantly, the effect on dpp and on overgrowth could -at least partially- be due to increased apoptosis which is known to induce both dpp expression and dpp and wg dependent proliferation (see Ryoo et al. Dev Cell. 2004 or Pérez-Garijo et al. Development. 2004). The authors therefore need to check whether the effects of mthl5MB on dpp expression and on disc growth are still visible when apoptosis is suppressed by genetic means.

Finally, the author have no direct proof mth15 acts on dpp expression though HH/SMO signaling and not in parallel (to enhance the effects of HH/SMO). I have no simple solution to offer as affecting HH signaling would totally suppress dpp expression but this possibility should be clearly mentioned.

3. Effects of the mth15 mutants on the wing and disc size. What happens with the effects on growth seems to be very complex and is very confusing. In brief, the mthl5MB mutant leads to a enlargement of the wing disc but have no effect on the wing pouch which leads to the wing blade, meaning that the overgrowth concerns the regions outside the wing pouch as the notum and pleura, which give adult structures outside the wing blade. However, in the adult, the wing itself is enlarged but the author give no information on the notum and pleura-derived structures. Moreover, there is an increase of ddpZ expression in the wing pouch but do not show the other regions. Then, how do the author explain the larger wing blade if the size of the wing pouch (which is the future wing blade) is not affected? Why the increase of dpp in the wing pouch does not affect the size of the pouch but affects the size of the wing? I find also difficult to correlate the effects on the enlargement of the wing disc outside the wing pouch with Dpp signaling as the dppZ images are centered on the wing pouch. It would help to understand the link between both effects if the authors could show the effect of the pka simple and double mutants on dppZ or the effect of Galphai or Dnc overexpression on the growth of the wild-type and mutant discs. I also suggest to add more explanations to clarify all these questions and to add a model to clearly explain what happens within the developing disc.

4. Scale bars are missing in all figures.

Other points

1. Could the authors provide the list of the 116 most expressed GPCR (as sup data) and indicate their class?

2. The authors should enhance the presentation of their results by working on the figures (and their legends) to make them easier to read and interpret without the main text.

a) Although the quality of the data is very good and systematically quantified, the authors should better explain what they have done to help interpreting them. As the number of discs used for quantification is quite low (n=5), the authors could also indicate how many discs were imaged and the number of discs or wings showing the reported effects.
b) The orientation of the discs with the posterior region toward the left is quite unconventional in animal biology. As it is clearly indicated, there is nothing wrong about it, but it makes things a more difficult for the readers c) On the same line, adding a thin spacing line between the different images of a same disc would help to ?read? the figures.
d) Within a figure, the author should check that the spacing between the horizontal panel is always the same (for instance in Fig 4 compare the spacing between A and C to the spacing between C and E or E and G).
e) The letters of the panels are unusually big and sometimes on the image, sometimes next to it which give an overall feeling of partial achievement.
f) The text in blue is barely visible on the back background

3. Specific points
a) Figure 2: The panel B is very hard to understand. As it is not mentioned in the legends that it show Z sections. Adding a thin white line between the images would also help b) Figure 7: could the

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authors should Ci alone, not only in the merge? The asymmetric pattern of salmZ overexpression should be explained.

c) Figure 8 : this figure lacks focus and strength and I suggest to remove it. A-B and E-G could be added to fig 6 (or as sup data to fig 6) to validate that the effects of the MB mutant are indeed due to a defect in mlh15 activity. C-D which is the most important part of this figure is related to Fig 9 and could be inserted as the beginning of fig 9 (or as a sup fig to fig 9).

First revision

Author response to reviewers' comments

We thank the reviewers for their thoughtful comments on our manuscript. They raised a number of valid concerns that needed addressing, and there were also suggested modifications to the organization of figures to improve the flow of the manuscript.
Despite the challenges in the current COVID situation, we managed to complete experiments to address many of the issues raised. Our responses to the comments are outlined below, followed by a summary of changes we made to the manuscript.

Reviewer 1:
Major
Concerns:

1. Dpp isn't exclusive to the Hh Signaling pathway
The authors routinely monitor GPCRs on Hh signaling by looking at dpp-lacZ, wing growth, and ptc-lacZ. Of these reporters, only ptc-lacZ is exclusive to the Hh pathway. Furthermore when there is decrease in Hh activity (loss of Gas or increased Mthl expression Fig3, 8), Engrailed (which wasn't monitored) would be the first to be eliminated, then ptc would be reduced, and finally dpp may also be reduced. Thus it was suspicious in many of these experiments, there were changes to dpp but not ptc- lacZ suggesting that the effects of GPCRs are not Hh-dependent. To link the role of GPCRs to Hh, it would be essential to always monitor ptc-lacZ and Engrailed staining . If there isn't a correlation with ptc and Engrailed, then the authors should consider that the effects they are seeing from the GPCRs are related to another regulator of dpp.

The reviewer raises a good point that we did not carefully consider, namely that *dpp*- LacZ is not only regulated by the Hh pathway. We address this point further in our response to point 2d) of Reviewer 3's comments.

We would make three points to the reviewer's other comments. First, we used more than just *dpp*-LacZ and Ptc as readouts. For most of the Gαi-like GPCRs, we also saw substantial changes in stabilization of Ci^{155}, which is another specific readout of Hh signaling, and one that occurs in direct response to pathway activation. The observed expansion of the domain of Ci stabilization in most cases matched the expansion of *dpp* expression. As stabilization of Ci is sufficient to de-repress *dpp* expression (Methot and Basler, 1999), we think this correlation is strong evidence that it is actually Hh signaling that is responsible for the effects on *dpp*. This is further supported by the effects on Smo in gain- and loss-of-function experiments with Mthl5. Like Ci, Smo stabilization is a direct readout of Hh pathway activation in cells, and we observed changes in Smo levels that go in the same direction as the effects on Hh target gene expression.

Second, we also used changes in classical Hh-dependent patterned growth phenotypes in GPCR depleted/mutant wings to support our conclusions. Specifically, because Hh target gene expression is activated in anterior but not posterior compartment cells, changes in the ratio of anterior to posterior wing area (as we used in analyzing the *mthl5* mutants) upon depletion of a regulator in both compartments can be a good indicator of Hh pathway activity. We provided experimental support for this in what is now modified Fig. 5H, showing that low-level Hh pathway

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activation throughout both anterior and posterior compartments in wild-type Smo-overexpressing discs produces precisely this anterior-biased effect. We also analyzed the ratio of L3-L4:total wing area, which is a sensitive and widely-used readout of Hh pathway activity in wings. This also changed in the Gαi-like GPCR depleted wings, in a way that fit well with the effects we observed on Hh target gene expression. These matching effects on Hh-dependent patterning of adult wings and changes in (mostly) Hh pathway-specific readouts in discs strongly suggest that the Gαi-like GPCRs are affecting Hh signaling. This is further supported by our observation of attenuation of Hh-induced *ptc*-reporter activity by Mthl5 in S2 cells, as well as by analyses of GPCR function in mammals, particularly Gpr161, that have led to largely the same conclusion.

The argument in our study is less clear for the Gαs-like GPCRs, whose depletion mainly affected (narrowed) *dpp* expression and didn't cause the same sort of classical Hh pathway adult wing phenotypes. We would point out that Mthl5 overexpression also narrowed *dpp* expression. If we accept that the expansion of *dpp* expression in the *mthl5* loss-of-function situation is due to increased Hh pathway activity for the reasons outlined above, then the fact that we see the opposite effect on *dpp* expression in the gain-of-function situation is most simply explained by reduced Hh pathway activity.

Finally, it is important to note, as explained in Pusapati et al. (2018), that GPCRs do not necessarily behave as classical activators and inhibitors of Hh signaling, but rather as enhancers and attenuators. If they were obligate signal transducers like Fu, I agree we would expect to see the effects the reviewer describes - loss of high threshold responses first, followed by loss of low-threshold responses. But GPCRs are thought to work differently, enhancing or attenuating ongoing signaling by changing the sensitivity of cells to ligand - essentially turning up or down the gain on signaling - to shape the responses in tissues. There is no "loss" of target gene expression in either Gαs- or Gαi- like GPCR-depleted discs - low, medium, and high threshold responses are all activated. But there is a shift up or down the Hh gradient in cells' ability to respond that correlates with whether Gαs- or Gαi-like GPCRs are depleted. We expected that a change in ligand sensitivity would have the greatest effect in cells exposed to low and limiting levels of Hh. In fact, this is what we previously observed in *gas*-depleted S2 cells (Praktiknjo et al. 2018). We think this explains why the effects on target gene expression that we see most clearly in discs are in cells away from the A-P boundary where the low threshold responses like *dpp* (and Ci) occur. We have more thoroughly summarized all of these points in the revised Discussion.

2. Limited Increase in Activity

It is also challenging to see a significant increase in dpp/ptc-lacZ (Fig 2,6) intensity at the AP border in a wild-type background. It would be more convincing if loss of these GPCRs (Gai) are increasing Hh activity and are specific to Hh if they could rescue ptc activity in a Fused mutant background.

I assume the reference is to the original Figure 4, not Figure 2 (which had no staining). It's not necessarily an increase in intensity, but an increase in width of the stripe in the dorsal compartment of GPCR-depleted discs that we observed. While some GPCRs have stronger effects than others, it seems fairly clear in many panels - e.g. *dpp* in modified Fig. 4A, 4C, 4G, and 4I, Ptc in 4C and E. We also added new stainings in modified Fig. 5 that show the expansion more clearly, particularly for Ptc in wild-type and *mthl5^{MB03076}/Df(3R)BSC514* discs that were dissected, processed, and imaged in parallel. We also went to great lengths throughout to quantify fluorescence in a statistically meaningful way and have added in indicators of statistical significance on several graphs where it is not obvious. Finally, the observations on target genes in discs match the phenotypes we see in adult wings for both the Gαi-like GPCR-depleted discs and the *mthl5* mutants, which is a classical Hh gain-of-function phenotype.

As mentioned above, GPCRs like Gpr161 appear to function by shaping the sensitivity of cells to Hh rather than by participating in signal propagation *per se*. The epistasis analysis in Pusapati et al. (2018) clearly places Gpr161 upstream of or parallel to Smo, and we see effects on Smo in *mthl5* mutants that suggest this GPCR also acts upstream. Modifying GPCR activity would therefore not be expected to rescue signaling in conditions where signal transduction is blocked at a point downstream of Smo.

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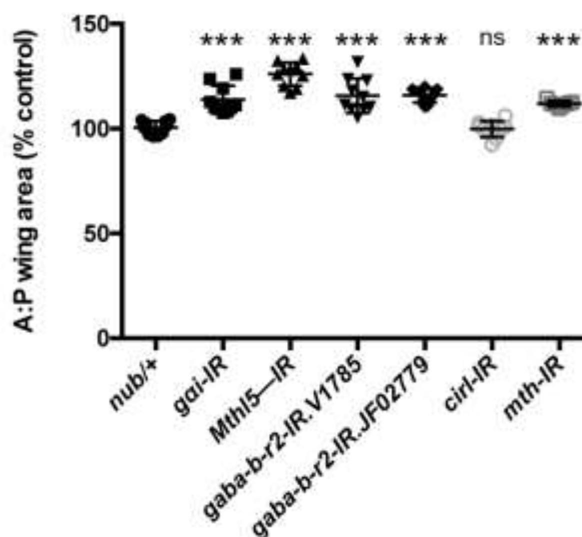
*3. **Mthl is expressed only in the ventral region** but most experiments were analyzed in the dorsal region In Figure 5, the authors stain for the expression of Mthl5 and found that it is endogenous target gene to the ventral region of the wing disc and absent in the dorsal region. Most of the experiments done however are with a dorsal Gal-4 driver. If the experiments were repeated with a driver that was expressed also in the ventral region, the results would be more representative of physiological conditions.*

This is was a problem resulting from poor figure design on our part - we didn't include a wild-type disc staining. We have now added two new panels (**modified Figure 4A and B**) showing that Mthl5 is ubiquitously expressed in discs. We moved the panel showing that dorsal Mthl5 levels are strongly reduced when the RNAi transgene is expressed throughout the dorsal compartment to the new Supplemental Figure 2. This is entirely consistent with our experimental results.

Minor Concerns:

- Measurements of Wing Vein Spaces: Increase in L3-L4 vein spacing is difficult to see by eye. Measurement of the L3-L4 to the whole wing would be more convincing if there was a control of other wing vein spacing (L1-L2, L2-L3, L4-L5) shrinking or staying the same by comparison.

This phenotype is how we were able to identify these GPCRs at the microscope as screen hits in the first place. We see highly significant changes (*p*<.0001) from our measurements of adult wings, with no overlap with the range of wild-type measurements for all but Cirl. As a separate readout of Hh signaling activity, we measured the anterior:posterior compartment area of these wings, expecting that it should be higher if Hh signaling was impacted (as discussed above). The results of this analysis were almost identical, with the exception of Cirl which was not significantly different from controls - see accompanying graph. (Flipped around, this means by definition that the area from L4 to posterior margin shrunk relative to L1-L4 and to total wing area.) As this does not much change the conclusion of the experiment and would require some explanation in the text, we did not include this data in the revised manuscript.



- Cell Death: Figure 8I loss of activity appears to be due to cell death

We can't rule this out - it would be difficult to see an increase in cell death in this background, as the levels are already high. However, we saw this phenotype clearly in 8/13 discs examined, and it consistently looks like a downregulation of *dpp*. Also, we did not see apoptosis when we expressed Mthl5-GFP in a wild-type background, so it's not clear why it would do that in the *mthl5* mutant background. This data (now in the new Supplemental Figure 3 for space reasons) is at least not inconsistent with a partial rescue of *dpp* misregulation in the $mthl5^{MB}$ background.

- Figure 9 I-N: Only showing disc size, but should also show target gene staining.

Given that the effects on target genes that we see are, as the reviewer points out, not dramatic compared to something like expression of constitutively active Smo, and that the rescue is only partial, we didn't feel that we would be able to demonstrate a clear difference in target gene expression between the different conditions. We chose to focus our efforts elsewhere.

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- General measurements: To reduce variability, the flies should have had a short laying time and wings/wing discs collected from same developmental stage. (It wasn't clear in the methods and materials).

The crosses comparing wing disc sizes and target gene expression in mutant and wild- type backgrounds were carried out in parallel. We were careful to keep collections short to avoid crowding that could affect disc size, and to collect only wandering L3 larvae.
These details were added to the Materials and Methods.

- Smoothened staining: Difficult to monitor anterior Smoothened staining, better to be co-stained with ptc instead of Ci-155 because of the down regulation of Ci at the most posterior portion of the AP border. Also would be useful if could see all three panels and where the AP border is marked.

We split out the Ci channel (modified Figure 7T and U) to make it easer to interpret. We can unambiguously see where the A-P border is, as well as the down regulation of Ci in cells anterior to it.

Reviewer 2 Comments for the Author:
My main reservation with this analysis is that while the underlying proposition is that GPCRs influence Hh pathway activity by modulating the PKA-dependent phosphorylation of Smo, none of the experiments actually measure Smo phosphorylation.Such assays would enhance the analysis significantly.

Given that we are seeing modulation of signalling rather than on/off effects I'm not sure we would expect to see big changes in Smo phosphorylation. While we agree the exact mechanism remains to be precisely defined, we feel the analysis supports the basic message that many GPCRs cross-talk with the Hh pathway.

I also wonder why there is apparently no effect of modulating cAMP levels on Smo in the posterior compartment - for instance, in response to Mthl5 overexpression driven by ap-GAL4 (Fig. 9 Q-R). Is the enhanced stability of Smo in the posterior compartment independent of PKA-dependent phosphorylation?

We don't have a concrete explanation for this. Overexpressed Smo seems to be phosphorylated by PKA in the posterior compartment (Fan et al., 2012; DOI: 10.1016/j.ydbio.2012.04.007). As mentioned, we suspect that the effects we see are due to relatively moderate effects of individual GPCRs on cAMP levels - for example direct activation of PKA has substantially stronger effects (Jia et al., 2004) - that affect cells at the limit of mounting a response. This may be irrelevant when the pathway is blasting.

Reviewer 3 Comments for the Author:
General recommendations
Overall, the data are of good quality and the manuscript is clearly written. However, while the data globally support the conclusions drawn by the author, they are not always sufficiently demonstrative. A few extra experiments should be sufficient to fill the bigger gaps but I also recommend that the authors make significant changes in text to include alternate interpretation of the data (see main issue 2) and better explanations to correlate what happens in the disc and in the wing (see main issue 3). Finally, the figures requires some extensive reorganization and even in several cases, an improvement of their visual presentation (see details below).

Main issues
***1. I do not understand where is the novelty in the Figure 1** which seems to exactly correspond to the Fig 7 of Praktiknjo et al. (from the same lab). I might misunderstand something but if not, this figure does not have its place here and should be removed. However, as it is important to have independent confirmation of data and as these results are important to understand the following experiments, I suggest to (i) add these data (without the effects on CI) in the other Figures: A-C in Fig 2, F in Fig 3 and H in Fig 4 and (ii) clearly mention that it*

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confirms what was already shown.

The reviewer's suggestion matches how we had originally organized the manuscript. We had switched it for ease of explanation, rather than splitting up Gαs and Gαi experiments in different figures. We have redistributed the data in the original Figure 1 to the modified Figures 1, 2, and 3 as positive and negative controls for other experiments, as suggested.

2. Effects of the mthl5 mutant on dpp.

a) To better understand the comparison between the wild-type and mutants situations (fig 6, 7; and 9) it would be useful to have more information on how the discs of the different genotypes that they directly compare were labeled and imaged: labelled in parallel or not, imaged the same day with the same microscope setting or by independent parallel batches that include the same number of discs of the different genotypes etc

For all experiments where we compared growth or target gene expression in wild-type and mutant discs, we carried out the crosses and dissections in parallel. For target gene expression analysis, samples were processed for immunostaining and imaged in parallel using the same confocal microscope settings. This information has been added to the Materials and Methods. We also added details about the number of discs imaged and number showing phenotypes to the figure legends. For one figure in the original submission (old Fig. 5J-L comparing target gene expression in wild-type and *mthl5*^{MB03076} mutants), we had mixed discs from three independent experiments. We repeated the analysis in the revised manuscript using only discs from the same staining date (new Figure 5J-M), with similar results. We also added new panels showing striking changes in Ptc levels in parallel-processed wild-type and *mthl5*^{MB03076}*/Df(3R)BSC514* discs (new Figure 5O-P).

b) To strengthen the demonstration of the effects of mtl15, the rescue experiment (Fig 8 E-I) could be associated to this Figure (see below).

We agreed that this would make for better organization. We modified the figure by moving the rescue data as suggested. It ended up in a new Supplemental Figure 3 (to save space).

c) The authors should clarify (based on what they see for CI and ptc) their interpretation of the changes in dpp expression. I do not dispute that fact that mlh15 MB affects dpp (and to a lesser degree ptc), and the size of wing disc in a Dpp signaling dependent manner. However, it seems important to strengthen the demonstration that the increase in dpp expression is a direct result of the activation of HH signaling and not an indirect one via another mechanism. First, <u>the enlargement of the dpp domain could also be due to (i) a suppression of late en expression</u>, reflecting a lower response to high HH levels and leading to an extension of dpp expression toward the A/P boundary, and/or (ii) <u>an increase in HH production</u>. I may not be possible to fully exclude all these alternate explanations (despite the fact that analysis of the pattern of CI accumulation near the AP could help to reject the ?late en? possibility), but they should not be omitted.

To address this, we have added an analysis of En expression in discs with depletion of *gas*, *gai,* and three representative GPCRs from both Gαs- and Gαi-like categories (Rk, Mthl5, and Cirl) in the new Supplemental Figure 1. In all cases, we still saw activation of anterior En expression after Gα/GPCR depletion. Also in all cases, the levels of En were sightly lower (by ~20%, after correction for changes in DAPI intensity that reflect loss of cells to apoptosis in the *gai* / *gai*-like GPCR depleted discs). However, this was also true in the posterior compartment where En expression is independent of Hh, for an unknown reason. This confounds the interpretation of the results. What I think we can draw from this is that there is no correlation between the effect on anterior En and the effect on *dpp* expression: although En looks similar in all cases, *dpp* is narrower/lower in two of these conditions and wider in the other three. We conclude in the revised Discussion that "while we don't rule out that a reduction in anterior En levels could contribute to expansion of *dpp* expression in Gαi-like GPCR-depleted discs, it seems unlikely to be the main cause."

An increase in Hh production is harder to address. As we write in the revised Discussion, the fact

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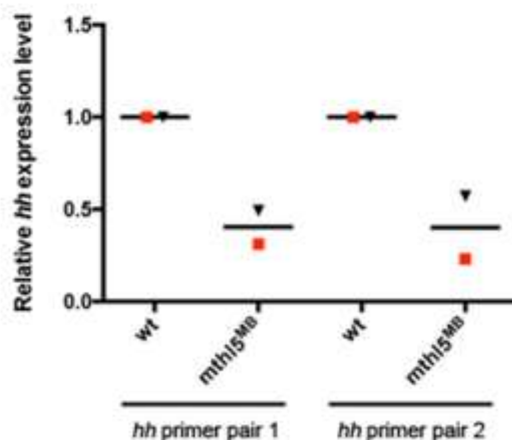
that we see Mthl5-dependent changes in the response to transfected Hh in S2 cell *ptc*-reporter assays is suggestive that this is not the explanation. However, we tried doing qPCR on two pairs of matched RNA samples extracted from batches of ~125 wild-type and *mthl5* mutant discs, using two different *hh* primer pairs and using *rpl32* for normalization. As shown in the graph and table of Ct values below, we actually saw a reproducible decrease (by an average ~60%) in Hh mRNA levels in the *mthl5* mutants (red dots represent one matched RNA pair, black triangles the other). We expected some decrease due to the relative overgrowth of anterior versus *hh*-expressing posterior compartments in the *mthl5* mutants. This result at least suggests there is no dramatic up regulation of *hh* expression in the mutants, but we felt it would require substantial additional investigation for contextualization so we chose not to include it in the revised manuscript.



	hh primers1	hh primers2	rpl32
wild-type_1	28.948	28.128	23.409
mthl5mutant_1	31.002	30.597	23.789
wild-type_2	28.315	27.664	19.608
mthl5mutant_2	30.041	29.156	20.290

d) More importantly, the effect on dpp and on overgrowth could -at least partially- be due to increased apoptosis which is known to induce both dpp expression and dpp and wg dependent proliferation (see Ryoo et al. Dev Cell. 2004 or Pérez-Garijo et al. Development. 2004). The authors therefore need to check whether the effects of mthl5MB on dpp expression and on disc growth are still visible when apoptosis is suppressed by genetic means.

Increased expression of *dpp-LacZ* specifically in apoptotic cells is observed mainly when those cells are prevented from undergoing apoptosis (by co-expressing P35 or in a *thread* mutant background). In this condition, these 'undead' cells can indeed drive dramatic tissue overgrowth. However, without caspase inhibition the cells die so quickly that *dpp* expression is difficult to detect, and discs in which widespread apoptosis is induced are slightly smaller than normal, not larger [Ryoo and Steller, 2004; doi: 10.1016/j.devcel.2004.08.019]. Even when *dpp* is eliminated, discs with high levels of apoptosis are able to compensate by growing to nearly normal size, suggesting that Dpp has little role in driving this compensatory growth in the absence of apoptosis inhibition [Perez-Garijo et al., 2009; doi: 10.1242/dev.034017].

With that in mind, we performed three sets of experiments to address this point. First, as shown in the new Fig. 5Q, widespread apoptosis (activated Caspase 3 positivity) in *mthl5*^{MB03076} discs does not correlate well with the position of apoptotic cells. Most of the apoptotic cells (basal pyknotic nuclei) in which we detect expression of *dpp-LacZ* are located directly below the *dpp* stripe, suggesting that they represent normal *dpp*-expressing cells that died rather than dying cells that

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start expressing *dpp*. Second, in the new Supplemental Figure S4A and B, we show that *dpp* expression is increased in *mthl5* RNAi-depleted discs, even though there is only a moderate increase in apoptotic cells. Finally, in the new Supplemental Figure S4C, we show that inhibiting apoptosis by expressing P35 in the dorsal compartment of *mthl*^{5MB03076}/*Df(3R)BSC514* discs dramatically enhances rather than suppressing disc overgrowth (notice that the image in S4C is at half of the magnification of the images in S4A and B). In these discs, Ci is stabilized over a domain up to three times the normal width, as is *dpp* expression.
Finally, *dpp* expression is mainly restricted to Ci-positive cells in the anterior compartment. All of these observations suggest that there is ongoing Hh pathway hyperactivation in these discs that is independent of any effects of apoptosis.

e) Finally, the author have no direct proof mthl5 acts on dpp expression though HH/ SMO signaling and not in parallel (to enhance the effects of HH/SMO). I have no simple solution to offer as affecting HH signaling would totally suppress dpp expression but this possibility should be clearly mentioned.

We agree this is not easy to address experimentally. As we pointed out in the response to Reviewer 1's first comment, as well as in the revised Discussion, we do see upregulation of multiple specific readouts of Hh pathway activity in Gαi / Gαi-like depleted or mutants discs, including Smo itself, as well as Hh pathway-specific phenotypes in adult wings. The sum of these observations suggests that there is misregulation of Hh pathway activity.

However, we did not mean to claim that these GPCRs are core components of the Hh pathway itself. That has clearly been spelled out in mammalian systems - Gpr161 is a SHH pathway attentuator whose activity influences Hh signaling by lowering ligand sensitivity, not an inhibitor of signaling (i.e. not a core component - see response to Reviewer 1's first comment). GPCRs do seem act in parallel to Hh signaling, and we know reasonably well how the GPCR-Gα-cAMP-PKA axis then intersects with Hh signaling. The effects in both mammalian and vertebrate systems are consistent with this. We significantly re-wrote the Discussion to better place our observations in context with what's known about Gpr161 and to more clearly spell out how we (and others) think GPCRs, through cAMP, are able to modulate rather than activate/inhibit Hh pathway activity.

3. *Effects of the mth15 mutants on the wing and disc size. What happens with the effects on growth seems to be very complex and is very confusing. In brief, the mthl5MB mutant leads to a enlargement of the wing disc but have no effect on the wing pouch which leads to the wing blade, meaning that the overgrowth concerns the regions outside the wing pouch as the notum and pleura, which give adult structures outside the wing blade. However, in the adult, the wing itself is enlarged but the author give no information on the notum and pleura-derived structures. Moreover, there is an increase of ddpZ expression in the wing pouch but do not show the other regions. Then, how do the author explain the larger wing blade if the size of the wing pouch (which is the future wing blade) is not affected? Why the increase of dpp in the wing pouch does not affect the size of the pouch but affects the size of the wing? I find also difficult to correlate the effects on the enlargement of the wing disc outside the wing pouch with Dpp signaling as the dppZ images are centered on the wing pouch. It would help to understand the link between both effects if the authors could show the effect of the pka simple and double mutants on dppZ or the effect of Galphai or Dnc overexpression on the growth of the wild-type and mutant discs. I also suggest to add more explanations to clarify all these questions and to add a model to clearly explain what happens within the developing disc.*

The reviewer's comment is based on the premise that *mthl5* mutant adult wings are larger than wild-type, which would not fit with our wing disc data. In fact, although we did not show it, *mthl5* mutant wings are slightly smaller than controls. (This is why we checked in the first place if pouch size was similar in wild-type and *mthl5* mutant discs, despite the clear overgrowth of the mutant discs.) We have added this data to the new supplemental Figure S3A. This is presumably due to the high levels of apoptosis observed in these discs, perhaps even into pupal stages when growth has stopped. We did not intend for Hh and/or Dpp-dependent effects on growth to become a major focus of the paper, as it is likely complex. We wanted to use it only to support the notion that there is an anterior shift in *dpp* expression in the mutants, which is borne out by the anterior expansion of the Dpp target gene *salm*. We attempted to clarify what we think is going on in the

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Discussion. ("The expansion of *dpp* expression towards the anterior compartment in *mthl5* mutants caused a corresponding shift in Dpp signaling activity as evidenced by an increase in the width of the anterior expression domain of *salm*. Cells in both the wing pouch and the pleura are dependent on low-level activation of Dpp signaling for growth (Barrio and Milan, 2017). We imagine that the anterior expansion of *dpp* expression could lead to excessive proliferation of cells in the anterior lateral and hinge region of the discs, which could in part account for overgrowth in *mthl5* mutant discs, though this remains to be confirmed.")

4. Scale bars are missing in all figures.

We added scale bars to all figures.

Other points

1. Could the authors provide the list of the 116 most expressed GPCR (as sup data) and indicate their class?

We added a new Supplemental Table 1. This Excel file lists basic gene information for the 116 GPCRs whose expression we analyzed, based on a review article we cited (Hanlon & Andrew, 2015, doi: 10.1242/jcs.175158).

2. The authors should enhance the presentation of their results by working on the figures (and their legends) to make them easier to read and interpret without the main text.

a) Although the quality of the data is very good and systematically quantified, the authors should better explain what they have done to help interpreting them. As the number of discs used for quantification is quite low (n=5), the authors could also indicate how many discs were imaged and the number of discs or wings showing the reported effects.

We have added information about the numbers of discs analyzed and affected to the figure legends. It should be clearer now, as we alluded to in the original submission, how the *mthl5*^{MB} mutant phenotype varies in discs the same way that it does in adult wings (typically ~40-50% showing obvious defects) We added more detail about how we did the quantification of fluorescence to the Materials & Methods. We also added statistical analyses (*t*-test results) for key graphs where the statistical significance was not obvious from looking at the plots.

b) The orientation of the discs with the posterior region toward the left is quite unconventional in animal biology. As it is clearly indicated, there is nothing wrong about it, but it makes things a more difficult for the readers

We flipped the orientation of all disc images and plots of fluorescence intensity as suggested.

c) On the same line, adding a thin spacing line between the different images of a same disc would help to "read" the figures.

We made this modification to all figures.

d) Within a figure, the author should check that the spacing between the horizontal panel is always the same (for instance in Fig 4 compare the spacing between A and C to the spacing between C and E or E and G).

We tried to correct this in all figures.

e) The letters of the panels are unusually big and sometimes on the image, sometimes next to it which give an overall feeling of partial achievement.

We shrunk the label sizes in all figures.

f) The text in blue is barely visible on the black background

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We switched to a brighter and more visible shade of blue for the labels.

*3. **Specific points***

a) Figure 2: The panel B is very hard to understand. As it is not mentioned in the legends that it show Z sections. Adding a thin white line between the images would also help

We made this modification (and moved the figure to new Supplemental Figure S2 for space reasons).

b) Figure 7: could the authors should Ci alone, not only in the merge? The asymmetric pattern of salmZ overexpression should be explained.

We split out the Ci channel in what is now Fig. 6. And as mentioned in the point about Dpp and disc growth, we provide an explanation for the asymmetric *salm* expression.

c) Figure 8 : this figure lacks focus and strength and I suggest to remove it. A-B and E-G could be added to fig 6 (or as sup data to fig 6) to validate that the effects of the MB mutant are indeed due to a defect in mlh15 activity. C-D which is the most important part of this figure is related to Fig 9 and could be inserted as the beginning of fig 9 (or as a sup fig to fig 9).

As detailed in the list of changes to the manuscript, we followed this suggestion, splitting up and thus eliminating the original Figure 8.

<u>**Summary of changes to the manuscript:**</u>

- reduced from 9 figures to 7 (as suggested by reviewer 3); we did this by shuffling data in original Figure 1 into Figures 2, 3, and 4 as controls, creating updated Figures 1, 2 and 3; and from original Figure 8 into the updated Figure 7 and **new Supplemental Figure S3**

- added **new Supplemental Figure S1** showing an analysis of En expression in selected Gα and GPCR depletion conditions

- Moved data from Figure 5B and C to **new Supplemental Figure S2**, and removed Figure 5E (predicted GPCR topology), to save space

- added a graph of total wing area in *mthl5* mutants to the **new Supplemental Figure S3**

- added a new panel to updated Figure 4 (new panels **5A and 5B**) showing Mthl5 staining of a wild-type disc

- re-did the analysis of target gene expression in *mthl5*^{MB03076} mutants versus wild-type using matched discs from animals that were dissected, processed, and imaged in the same batch, in updated Figure 5J-M

- added new panels to updated Figure 5 (**5O and 5P**) of staining showing dramatic upregulation of Ptc in *mthl5*^{MB03076}*/Df(3R)BSC514* mutant discs compared to matched wild-type discs

- added an analysis of apoptosis versus *dpp* expression in *mthl5*^{MB03076} mutants in the **new Figure 5Q**

- added an analysis of apoptosis versus *dpp* expression in *mthl5* RNAi-depleted discs, and of Ci and *dpp* in *mthl5*^{MB03076}*/Df(3R)BSC514* expressing P35 to block apoptosis, in **new Supplemental Figure S4**

- split out the Ci155 channel in updated Figure 7T and U to clearly show the position of the A-P

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boundary

- added supplemental Table 1, an Excel file with gene information about the 116 GPCRs we included in our gene expression profiling experiment

- added information about the numbers of discs examined and showing phenotypes throughout

- added information to the Material and Methods, as requested, to better indicate how certain crosses were handled (in parallel, no crowding, etc.) and how we did the quantification of fluorescence

All of these changes obviously required substantial shuffling and editing of the original text. In particular, we re-wrote much of the Discussion to clarify issues raised by the reviewers and to better place our work in context with what's known in mammalian systems.

Second decision letter

MS ID#: DEVELOP/2020/189258

MS TITLE: Extensive crosstalk of G protein-coupled receptors with the Hedgehog signalling pathway

AUTHORS: Farah Saad and David Hipfner

I apologise for the delay. I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you can see one of the reviewers is very supportive of publication but the other is concerned about alternative interpretations. HHaving discussed with this reviewer, I have decided that we are happy to proceed with publication provided that you revise the manuscript so as to mention alternative interpretations.

Reviewer 1

Advance summary and potential significance to field

While there is clear evidence that a number of GPCRs regulate dpp, the connection to Hh signaling is less convincing. Most of the analysis was done measuring dpp expression, wing vein spacing and wing growth. However, these measurements are not exclusive to the Hh signaling pathway. To address these concerns, the authors have highlighted how Gai and Gas-like GPCRs affect Hh specific components:
Engrailed, Smo, Ci and Ptc.

Comments for the author

Engrailed expression changes were not a valid measurement because there was also reduced expression in the posterior compartment, indicating that engrailed regulation by these GPCRs is likely independent of Hh signaling. Increased expression of smoothened resulted in anterior expansion comparable to Mthl mutants, and Smoothened stability was reduced when Mthl was over-expressed, suggesting that Smoothened is downstream of Mthl. For Gai-like GPCRs, there were substantial changes to Ci stabilization but Ci stabilization is not a good read out of Ci activity: 1) increased Ci-155 levels could indicate deficiencies in processing not activation 2) lower Ci protein levels is correlated with both low and high Hh activity. Modified Gai and Gas-like GPCRs

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consistently caused only limited changes to ptc expression in the wing disc, but there was a stronger effect of Mthl on ptc expression in cell culture.
Taken together, it possible that the identified GPCRs regulate Hh signaling since there are some changes to Hh specific components. The authors claim that the reason dpp is more responsive than ptc to changes in GPCR proteins is because GPCRs more likely act on low threshold responses. However, it is still possible that the dramatic effects on dpp expression are due to another regulator of dpp. The authors present a well written paper with extensive figures and data analysis, but my main concern is that the strong effects on dpp is independent of the Hh signaling pathway.

Reviewer 3

Advance summary and potential significance to field

The authors have thoroughly addressed all my comments. The data are relevant both to the HH and the GPCR fields and to fly development. They are of excellent quality -including their quantification and the numerous carefully conducted control experiments and their rigorous interpretation.

Comments for the author

N/A

Second revision

Author response to reviewers' comments

Reviewer 1 agreed that our data are consistent with GPCRs influencing Hh responsiveness based on changes in several readouts of Hh pathway activity. However, they felt it is still possible that the more dramatic effects on dpp expression we observed are due to another regulator of dpp, rather than Hh signalling. While data we added in the first revision suggested that two known dpp regulators in wing discs (apoptosis, anterior En) are probably not involved, we agree that we cannot rule out that GPCRs affect dpp expression through another mechanism. We have added a statement to this effect in the newly revised Discussion:

“However, given the stronger effects on dpp expression than on other Hh pathway readouts, it remains possible that GPCRs affect dpp through another mechanism.”

Third decision letter

MS ID#: DEVELOP/2020/189258

MS TITLE: Extensive crosstalk of G protein-coupled receptors with the Hedgehog signalling pathway

AUTHORS: Farah Saad and David Hipfner
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

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