Hedgehog produced by the Drosophila wing imaginal disc induces distinct responses in three target tissues

Ryo Hatori and Thomas B. Kornberg
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Reviewer 1

Evidence, reproducibility and clarity

The paper by Hatori and Kornberg explores the role of Hh secreted from the wing disc notum region, in the development of three different target tissues: the anterior compartment of the notum, the air sac primordium (ASP) and the muscle-precursor cells. In all three cases, they demonstrate that the Hh source is emanating from the wing disc, while the effects are paracrine on the target tissues where the cell-autonomous elements and transcriptional responses to the Hh signaling pathway are activated. By various criteria developed in the Kornberg lab, Hh appears to be transmitted to the target tissues by cytonemes. Upon compromising Hh signaling the ASP is smaller and misshapen, the depth of the myoblast layer is increased, and Dpp expression in the anterior region of the notum is reduced. When comparing the transcriptional responses of the three target tissues, some of the induced genes are common while others are specific to a given target tissue.

This work employs a very rich and impressive arsenal of tools developed in the Kornberg lab to study Hh signaling and cytonemes. Thus, all their conclusions regarding the source of Hh and the identification of the responding tissues and the range of Hh diffusion are highly convincing. Regarding the involvement of cytonemes, since the Kornberg lab has established the gold standard to identify them, define the contact sites with the target tissue by GRASP, and disrupt cytonemes as specifically as possible, the conclusions are as good as one may hope for.

Significance

However, I find the paper unimpressive because it has no clear message or focus, and thus ends up being quite descriptive. It does not provide novel insights regarding cytonemes or ASP development. The fact that the tissue context determines the transcriptional output to a common signaling source is well established, and this paper provides just another example for this very basic and established developmental paradigm. The place where this paper could have provided novel insights relates to the actual biological role of Hh in each of the three target tissues, but in this context, the authors provide a very cursory description of the phenotype without going into deeper mechanistic details. For example: Is it important to express the Dpp stripe in the notum? How does Hh signaling intersect with Wg signaling in the muscle precursor cells, and does it change the ratio between transit-amplifying cells and differentiated myoblasts? Does it impinge on the propensity of

the transit-amplifying step? Is this the reason for the thicker layer of muscle precursor cells when Hh signaling is compromised?

Reviewer 3

Evidence, reproducibility and clarity

Ryo Hatori and Thomas Kornberg´s manuscript presents a very nice example of how Hedgehog produced in one tissue (eg. posterior compartment of the wing disc, in particular in the notum area) can activate target gene expression in three different cell populations (A compartment of the notum, myoblasts and the tracheal ASP). They conclude that "The key findings are that Hh signaling in the ASP is cytoneme-mediated and gene targets of Hh signaling differ in the disc, ASP, and myoblasts." The ms is clearly written, thoroughly discussed and figures are very well organized, self-explanatory and confocal images outstanding. I congratulate authors for all of this. The balance between descriptive (analysis of gene expression patterns of potential Hh targets in the three different cell populations) and functional analysis (genetic tests to validate Hh coming from the P compartment as responsible for the expression of its targets in the three populations) is just thorough and all these data clearly demonstrate the role of Hh in regulating these genes in the three cell populations. At the very end of the paper, authors present clear evidence that Hh coming from the P compartment is located in cytonemes and that these interact with other cytonemes emerging from the ASP. Functional experiments depleting elements involved in cytoneme formation validate their proposal. In order to complete their story, I would expect some functional experiments concerning the role of cytonemes in the regullation of Hh target genes in the ASP. In other terms, whether it is the Hh coming from the P compartment and traveling through cytonemes the only one that activates Hh target genes in the ASP is something that authors should validate by providing functional data (eg. depleting elements involved in cytoneme formation and see how target gene expression is affected.

Minor comments:

Panels 1J and 1K: labels should be bigger

Panels 1D, E, L, M, N, O should be shown at the same size and identical scale bar, in order to facilitate size comparison.

Panels 2A-H: any way of quantifying the signal along the length of the ASP in the different experimental conditions?

Pg7: "ectopic overexpresion of hh-RNAi" hhRNAi is neither expressed ectopically nor overexpressed...

Panel 3C: Cut signal is weak

Pg7: "myoblast overexpression of Ptc or smo-RNAi" smoRNAi is not overexpressed either.... The sentence should be reformatted.

Significance

With the addition of new functional data (see above), I believe this manuscript will be another nice example of how a signaling molecule can signal to a nearby tissue through cytonemes. This work, one it is finished, will contribute to the better understanding of the developmental biology of Drosophila, in particular of the wing primordium and associated cell populations. Previous publications from the Kornberg and Isabel Guerrero's lab have provided evidence of the existence and functional role of cytonemes in Hh signaling.

I am a Drosophilist and a fan of Kornberg's science.

Reviewer 4

Evidence, reproducibility and clarity

The authors of this paper analyse the potential role of Hedgehog (Hh) signalling in the wing imaginal disc and its associated structures in drosophila larvae. Tom Kornberg and his lab have pioneered the field of hedgehog signalling many years ago, and his lab has characterized many of the basic features of Hh signalling, especially during wing imaginal disc development. His lab has also pioneered the characterization of signalling filopodia, or cytonemes, as carriers for many signalling molecules active during development. In this manuscript, Hatori and Kornberg report that Hh secreted from the wing imaginal disc affect gene expression in three different cell populations, in the anterior wing disc cells (well known and described many years ago), in the air sac primordium and in a subset of myoblasts associated with the disc. They report here that some of the target genes are the same as those reported in the disc, while others are different among the three tissues.

Furthermore, the authors provide evidence that in all three tissues, Hh signalling is cytoneme-mediated and cytoneme-dependent.

Major comments

1. Although the signalling response to the different tissues has been investigated, there is no information as to what cellular behaviour Hh signalling might influence. Are cell numbers affected in the ASP in the absence of Hh signalling? Does Hh control cell division, or other parameters? The phenotypes are described at a rather superficial level (growth is reduced and morphogenesis abnormal...). The same shortcoming for the myoblasts; the author just say that Hh signalling might have a formative role for the myoblasts.

2. The main claim of the authors is that the disc proper is producing instructive Hh to guide the development of myoblast, ASP and notum. The direct test of such a hypothesis is to remove Hh from the disc proper. However, the experiments shown always disrupt all Hh sources. Is it possible to perturb Hh using a disc proper driver?

3. The authors argue that Hh signalling is mediated and dependent of cytonemes. Figure 5 supports that Hh is transported along this filopodia and that perturbing filopodia reduces Hh amounts in the receiving tissue. However, to claim dependency, it is critical to look at Hh target gene expression during disruption of filopodia. The authors mention an En reduction in Figure 5R without any image. Such images would be very supportive to make that claim.

4. Is it possible to look at target gene expression upon expression of Hh in the ASP? That would certainly make the point about target genes much stronger; in this case, target genes would not be expressed in a graded manner anymore.

Minor comments

The images displayed in the figures are generally very appealing (specially the cytoneme ones). However, the figures itself are mostly overcrowded and would profit from some restructuration. The inclusion of the data of each part of the experiment, all in one figure makes, them very close to finish the alphabet when naming panels! The addition of some Supplementary figures would be a good option. Also, the separation of panels that represent different sets of experiments would be desirable across the whole manuscript (as it is done in figure 6). This comment applies for figure 1 to 5.

Some specific comments for each figure: Figure 1:

-A direct comparison between panels D and E is essential. Also, the E panel (expression of Hh in ASP) should be compared to B; how close to the P compartment of the wing disc proper does the ASP in E come?
-Panel F does not seem to be representative of the quantification made in panel J.
-Panel F is the control for panel I, it would be good to display them side by side.

-A control under similar conditions for panels L to O should be shown.
-Taking into account the variability in size of the whole disc (and the ASP with it, based on this and other papers) and the absence to any mention of fine staging in the manuscript, it can not be excluded that the very low n used in the quantification of ASP size (panel K) might result in errors of interpretation.

Figure 2:
-The figure would profit from some more information about the genotype in the labels. e.g. the labels in the left side of panels Q do not include where or what transgene is been expressed.
-In the text it is claimed that Aop distribution is graded. Could the authors choose a better image for panel T?

Figure 3:
-The disc shown in A is somewhat irritating. Could the authors show a lower magnification in an inset, like in figure 4 A?
-Panels G to M can be placed in Supplementary.
-On line 21 of page 9, the authors direct the reader to "Figure 3". It is not clear what they mean by that.

Figure 4:
-It would be instructive to have an overall view of the entire disc for the left part of the Figure (D-H), as shown in A.
-There is weird genotype bar on top of panels F to J bearing no text. Figure 6:

- Regarding their positive feedback hytopthesis, Hh:CD2 has been used in the past to stabilize filopodia (Gonzalez-Mendez 2017). As an alternative interpretation to the authors "positive-feedback hypothesis", it is possible that cytonemes are being stabilized by interactions with the anterior compartment of the disc proper. To test their hypothesis, the authors should use another way of activating the pathway (maybe expression of constitutively active Smoothened is a good option).
-All the images shown in this and previous papers suggest that the number of cytoneme per ASP can be variable between samples with the same genotype. Therefore, the quantification of cytoneme number in 4 ASP per genotype presented in figure 6A seems extremely fragile.
-The panel organization up-down, up-down, up-down, is somewhat difficult to follow.
-In a previous study from the same lab (Roy et al 2011), the authors conclude "that cells can make several types of cytonemes, each of which responds specifically to a signaling pathway". It would be very good to discuss the implications of the result in Figure 6B and those of the cited paper.

Figure 7:
-This figure does not introduce new results and it is mostly a discussion. It would be ideal to integrate both the figure and the text into the discussion.
-Is it possible that in the region where ASP, myoblasts and the anterior Notum cells "respond to Hh, the response is lower because three tissues compete for making filopodia contacts, instead of just one tissue in the wing blade for example?
-Line 23 page 14. Saying that Drosophila may only have four signalling proteins is wrong.

Significance

The concept that signalling from a distinct source affects target gene response(s) differently in distinct, responding tissues, is not novel. In most known cases, signal interpretation is tissue-specific and relies on a cooperation between different tissue-specific factors and signalling mediators. However, in order to further study the role of different signalling pathways in the context of the wing imaginal disc and its associated structures, the study by Hatori and Kornberg is important, as it sets the scene for such additional studies.

First revision

Author response to reviewers' comments

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

The paper by Hatori and Kornberg explores the role of Hh secreted from the wing disc notum region, in the development of three different target tissues: the anterior compartment of the notum, the air sac primordium (ASP) and the muscle-precursor cells. In all three cases, they demonstrate that the Hh source is emanating from the wing disc, while the effects are paracrine on the target tissues where the cell-autonomous elements and transcriptional responses to the Hh signaling pathway are activated. By various criteria developed in the Kornberg lab, Hh appears to be transmitted to the target tissues by cytonemes. Upon compromising Hh signaling the ASP is smaller and misshapen, the depth of the myoblast layer is increased, and Dpp expression in the anterior region of the notum is reduced. When comparing the transcriptional responses of the three target tissues, some of the induced genes are common while others are specific to a given target tissue.

This work employs a very rich and impressive arsenal of tools developed in the Kornberg lab to study Hh signaling and cytonemes. Thus, all their conclusions regarding the source of Hh and the identification of the responding tissues and the range of Hh diffusion are highly convincing. Regarding the involvement of cytonemes, since the Kornberg lab has established the gold standard to identify them, define the contact sites with the target tissue by GRASP, and disrupt cytonemes as specifically as possible, the conclusions are as good as one may hope for.

Reviewer #1 (Significance (Required)):

However, I find the paper unimpressive because it has no clear message or focus, and thus ends up being quite descriptive. It does not provide novel insights regarding cytonemes or ASP development. The fact that the tissue context determines the transcriptional output to a common signaling source is well established, and this paper provides just another example for this very basic and established developmental paradigm. The place where this paper could have provided novel insights relates to the actual biological role of Hh in each of the three target tissues, but in this context, the authors provide a very cursory description of the phenotype without going into deeper mechanistic details. For example: Is it important to express the Dpp stripe in the notum? How does Hh signaling intersect with Wg signaling in the muscle precursor cells, and does it change the ratio between transit-amplifying cells and differentiated myoblasts? Does it impinge on the propensity of the transit-amplifying step? Is this the reason for the thicker layer of muscle precursor cells when Hh signaling is compromised?

We performed additional experiments that will hopefully satisfy this request for a stronger biological message. To understand how Hh signaling controls the size of the ASP, we monitored the number of cells, frequency of dividing cells, and frequency of cell death in genotypes that perturb Hh signaling. As described in lines 147-160 and data shown in new Figure 1 S1, the new results indicate that Hh signaling controls ASP size by regulating cell division in the ASP. To investigate the mechanism of Hh dissemination in the ASP, we determined that the Hh and Bnl pathways are linked in fundamental ways that are manifested not only in shared control of transcriptional outputs of target cells (page 17 lines 471-488, Fig 8), but also in cytoneme production/behavior (page 12,13 lines 333-364, Fig 7). This finding has fascinating and profound implications for the cytoneme mechanism of morphogen dispersion.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Ryo Hatori and Thomas Kornberg´s manuscript presents a very nice example of how Hedgehog produced in one tissue (eg. posterior compartment of the wing disc, in particular in the notum area) can activate target gene expression in three different cell populations (A compartment of the notum, myoblasts and the tracheal ASP). They conclude that "The key findings are that Hh signaling in the ASP is cytoneme-mediated and gene targets of Hh signaling differ in the disc,

ASP, and myoblasts." The ms is clearly written, thoroughly discussed and figures are very well organized, self-explanatory and confocal images outstanding. I congratulate authors for all of this. The balance between descriptive (analysis of gene expression patterns of potential Hh targets in the three different cell populations) and functional analysis (genetic tests to validate Hh coming from the P compartment as responsible for the expression of its targets in the three populations) is just thorough and all these data clearly demonstrate the role of Hh in regulating these genes in the three cell populations. At the very end of the paper, authors present clear evidence that Hh coming from the P compartment is located in cytonemes and that these interact with other cytonemes emerging from the ASP. Functional experiments depleting elements involved in cytoneme formation validate their proposal. In order to complete their story, I would expect some functional experiments concerning the role of cytonemes in the regullation of Hh target genes in the ASP. In other terms, whether it is the Hh coming from the P compartment and traveling through cytonemes the only one that activates Hh target genes in the ASP is something that authors should validate by providing functional data (eg. depleting elements involved in cytoneme formation and see how target gene expression is affected.

We performed additional experiments to address this point. We knocked down genes that have been shown to be involved in cytoneme formation/function - *dia*, *nrg*, *syt4* - and assayed the effects on the expression of the downstream target gene Ptc. The amount of Ptc in the ASP decreased compared to control, suggesting that cytonemes are indeed required for Hh signaling. Although the reduction was not 100%, the experiments involved time-limited expression of RNAi transgenes because continuous expression is not compatible with normal development. Nonetheless, the results are consistent with our studies with these transgenes and support the conclusion that ASP cytonemes are essential for Hh signaling in the ASP. A new Figure 6 with panels F-J shows these new results that are described in lines 328-331.

Minor comments:

Panels 1J and 1K: labels should be bigger
Done

Panels 1D, E, L, M, N, O should be shown at the same size and identical scale bar, in order to facilitate size comparison.
Done

Panels 2A-H: any way of quantifying the signal along the length of the ASP in the different experimental conditions?
Because the shape of the ASP is not the same between control and mutant conditions, it would be hard to make a comparable quantification between them.

Pg7: "ectopic overexpresion of hh-RNAi" hhRNAi is neither expressed ectopically nor overexpressed...
Phrase changed to: “expression of *hhRNAi*”

Panel 3C: Cut signal is weak
Cut is expressed in the myoblasts and there are no myoblasts in the optical section shown in panel 3C.

Pg7: "myoblast overexpression of Ptc or smo-RNAi" smoRNAi is not overexpressed either.... The sentence should be reformatted.
Sentence changed to: “myoblast expression of Ptc or *smoRNAi* under *1151-gal4”*

Reviewer #2 (Significance (Required)):

With the addition of new functional data (see above), I believe this manuscript will be another nice example of how a signaling molecule can signal to a nearby tissue through cytonemes. This work, one it is finished, will contribute to the better understanding of the developmental biology of Drosophila, in particular of the wing primordium and associated cell populations. Previous publications from the Kornberg and Isabel Guerrero's lab have provided evidence of the existence and functional role of cytonemes in Hh signaling.

I am a Drosophilist and a fan of Kornberg's science.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

The authors of this paper analyse the potential role of Hedgehog (Hh) signalling in the wing imaginal disc and its associated structures in drosophila larvae. Tom Kornberg and his lab have pioneered the field of hedgehog signalling many years ago, and his lab has characterized many of the basic features of Hh signalling, especially during wing imaginal disc development. His lab has also pioneered the characterization of signalling filopodia, or cytonemes, as carriers for many signalling molecules active during development.
In this manuscript, Hatori and Kornberg report that Hh secreted from the wing imaginal disc affect gene expression in three different cell populations, in the anterior wing disc cells (well known and described many years ago), in the air sac primordium and in a subset of myoblasts associated with the disc. They report here that some of the target genes are the same as those reported in the disc, while others are different among the three tissues. Furthermore, the authors provide evidence that in all three tissues, Hh signalling is cytoneme-mediated and cytoneme- dependent.

Major comments

1. Although the signalling response to the different tissues has been investigated, there is no information as to what cellular behaviour Hh signalling might influence. Are cell numbers affected in the ASP in the absence of Hh signalling? Does Hh control cell division, or other parameters? The phenotypes are described at a rather superficial level (growth is reduced and morphogenesis abnormal...). The same shortcoming for the myoblasts; the author just say that Hh signalling might have a formative role for the myoblasts.

Addressed in response to Reviewer #1 above and repeated here:
To understand how Hh signaling controls the size of the ASP, we monitored the number of ASP cells, frequency of dividing cells, and frequency of cell death in genotypes that perturb Hh signaling in the ASP. As described in lines 147-160 and data shown in new Figure 1 S1, the new results indicate that Hh signaling controls ASP size by regulating cell division in the ASP. We also performed additional experiments that explore the coordination of Bnl and Hh signaling, and the results have a stronger biological message that we think has fascinating implications for the cytoneme mechanism of morphogen dispersion.

2. The main claim of the authors is that the disc proper is producing instructive Hh to guide the development of myoblast, ASP and notum. The direct test of such a hypothesis is to remove Hh from the disc proper. However, the experiments shown always disrupt all Hh sources. Is it possible to perturb Hh using a disc proper driver?

We may not fully understand the question but are unaware of a driver that is specific to the notum region of interest.

3. The authors argue that Hh signalling is mediated and dependent of cytonemes. Figure 5 supports that Hh is transported along this filopodia and that perturbing filopodia reduces Hh amounts in the receiving tissue. However, to claim dependency, it is critical to look at Hh target gene expression during disruption of filopodia. The authors mention an En reduction in Figure 5R without any image. Such images would be very supportive to make that claim.

To address this point, we determined that Ptc is a more sensitive downstream target than En and found that knocking down genes required for cytoneme formation and function reduced the amount of Ptc in the ASP compared to control. A revised Figure 6 with new panels F-J and revised text (lines 32-331) present these new results.

4. Is it possible to look at target gene expression upon expression of Hh in the ASP? That would certainly make the point about target genes much stronger; in this case, target genes would not be expressed in a graded manner anymore.

To address this point, we have analyzed the expression of the target genes upon the expression of Hh:CD2 in the ASP. In control, expression contours of the targets are graded and are specific to the bulb or the stalk of the ASP. However, we found that upon expression of Hh in the ASP, the expression of the targets became uniform throughout the ASP or altogether diminished. We believe that these data further strengthen the case that these genes are targets of Hh signaling in the ASP. New panels Fig. 2I''', J''', K''', L''', M''' and revised text (lines 200-201) present these results.

Minor comments

The images displayed in the figures are generally very appealing (specially the cytoneme ones). However, the figures itself are mostly overcrowded and would profit from some restructuration. The inclusion of the data of each part of the experiment, all in one figure makes, them very close to finish the alphabet when naming panels! The addition of some Supplementary figures would be a good option. Also, the separation of panels that represent different sets of experiments would be desirable across the whole manuscript (as it is done in figure 6). This comment applies for figure 1 to 5.

We revised the figures by transferring some panels to supplemental figures. The following panels were put in new supplemental figures or a new main figure:
Fig1. Q-R >> Fig1. S2
Fig2. M-N' >> Fig2 S2
Fig2. O-P >> Fig2 S3
Fig3. H-M>> Fig3S
Fig4. F-J >> Fig4S
Fig5. M-R>> Fig6

Some specific comments for each figure:

Figure 1:
-A direct comparison between panels D and E is essential. Also, the E panel (expression of Hh in ASP) should be compared to B; how close to the P compartment of the wing disc proper does the ASP in E come?
Panels D and E are now the same size with the same scale bar. We did not understand the purpose of comparing E and B.

-Panel F does not seem to be representative of the quantification made in panel J.
We increased the brightness of the image so that Hh:GFP puncta can be seen better and so that it better represents the data in panel J.

-Panel F is the control for panel I, it would be good to display them side by side.
Done

-A control under similar conditions for panels L to O should be shown.

The control for L-O is panel D. All are now the same size with the same scale bar.

-Taking into account the variability in size of the whole disc (and the ASP with it, based on this and other papers) and the absence to any mention of fine staging in the manuscript, it can not be excluded that the very low n used in the quantification of ASP size (panel K) might result in errors of interpretation.
We have increased the sample numbers for this analysis to more than 12 for each genotype.

Figure 2:
-The figure would profit from some more information about the genotype in the labels. e.g. the labels in the left side of panels Q do not include where or what transgene is been expressed.
We have added the genotypes on the left side of the panels K-M'''.

-In the text it is claimed that Aop distribution is graded. Could the authors choose a better image for panel T?

We have replaced this panel (new Fig2 M) with a better image that justifies our description.

Figure 3:
-The disc shown in A is somewhat irritating. Could the authors show a lower magnification in an inset, like in figure 4 A?
Because we are focusing only on the notum region of the wing disc, a lower magnification image that includes the entire disc would not help clarify the regions we are focusing in this study. The schematic in Fig1A shows the entire disc and the myoblasts.

-Panels G to M can be placed in Supplementary.

Done; panels G-M are in a new supplementary Figure 3S.

-On line 21 of page 9, the authors direct the reader to "Figure 3". It is not clear what they mean by that.

We corrected this to Figure 4 which refers to the expression of targets in the Notum.

Figure 4:
-It would be instructive to have an overall view of the entire disc for the left part of the Figure (D-H), as shown in A.
Since we are only focusing on the notum primordium portion of the wing disc, we felt that adding an image of the entire disc would not be instructive.

-There is weird genotype bar on top of panels F to J bearing no text.
We have added the letters “wild-type” to the box.

Figure 6:
-Regarding their positive feedback hytopthesis, Hh:CD2 has been used in the past to stabilize filopodia (Gonzalez-Mendez 2017). As an alternative interpretation to the authors "positive-feedback hypothesis", it is possible that cytonemes are being stabilized by interactions with the anterior compartment of the disc proper. To test their hypothesis, the authors should use another way of activating the pathway (maybe expression of constitutively active Smoothened is a good option).
We performed additional experiments to analyze ASP cytonemes in a genotype that expresses constitutively active Smoothened (SmoM2) in the ASP. This genotype gave a result similar to expressing Hh:CD2: the number of cytonemes did not change but the length of the tip cytonemes increased. The new data are incorporated into Fig. 7 and are described in text lines 348-364.

-All the images shown in this and previous papers suggest that the number of cytoneme per ASP can be variable between samples with the same genotype. Therefore, the quantification of cytoneme number in 4 ASP per genotype presented in figure 6A seems extremely fragile.

To address the low number of sample number, we have also increased the sample size to 10 for each sample.

-The panel organization up-down, up-down, up-down, is somewhat difficult to follow.
Panels have been rearranged where possible.

-In a previous study from the same lab (Roy et al 2011), the authors conclude "that cells can make several types of cytonemes, each of which responds specifically to a signaling pathway". It would be very good to discuss the implications of the result in Figure 6B and those of the cited paper.
This issue is relevant to Fig. 7A,B and is discussed in the revised text in the Discussion (lines 457-467).

Figure 7:
-This figure does not introduce new results and it is mostly a discussion. It would be ideal to integrate both the figure and the text into the discussion.
Although Fig. 8B introduces new results, we understand that the other panels in the figure

summarize our findings and are included to introduce the experiments that yielded the results in Fig. 8B and to frame the Discussion. If deemed more appropriate, panels C,D and E could be a separate figure.

-Is it possible that in the region where ASP, myoblasts and the anterior Notum cells "respond to Hh, the response is lower because three tissues compete for making filopodia contacts, instead of just one tissue in the wing blade for example?
This point was not accurately presented in the previous version of the manuscript and it has been deleted.

-Line 23 page 14. Saying that Drosophila may only have four signalling proteins is wrong.
Phrase deleted.

Reviewer #3 (Significance (Required)):

The concept that signalling from a distinct source affects target gene response(s) differently in distinct, responding tissues, is not novel. In most known cases, signal interpretation is tissue-specific and relies on a cooperation between different tissue-specific factors and signalling mediators. However, in order to further study the role of different signalling pathways in the context of the wing imaginal disc and its associated structures, the study by Hatori and Kornberg is important, as it sets the scene for such additional studies.

Submission to Development

First decision letter

MS ID#: DEVELOP/2020/195974

MS TITLE: Hedgehog produced by the Drosophila wing disc induces distinct expression responses in three tissues

AUTHORS: Thomas Kornberg and Ryo Hatori

Thank you for submitting your paper to Development through Review Commons. I have read your manuscript, the reviewers comments with your response, and I have also discussed the manuscript with another editor. We have also solicited advice from one of the original referees of your manuscript.The referee's 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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referee's comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. This will provide clarifications and improve the accessibilityof your study. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

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.

Reviewer 1

Advance summary and potential significance to field

Authors have satisfactorily addressed almost all my concerns and the ones of Reviewer 3. As such, I support publication of this very nice research article in Development once all minor concerns (see below) are being addressed. I congratulate the authors for the whole work. I really enjoyed reading it.

Comments for the author

- Line 116: Fig. 1F should be Fig. 1F, G
- Line 120: Fig. 1G,H should be Fig G'', H
- Line 141: Although en is a "selector gene" for the posterior compartment (García-Bellido, 1975) where it is a positive regulator of hh (Tabata et al., 1992).. Include more recent references such as Bejarano et al Dev Biol 2009.
- Line 152: be more explicit about which Caspase is monitored (Dcp1 or Dronc) and which Ab.
- Figure 2 is very disorganized and panel sizes very different. I would suggest authors to re-organize this figure (especially I-J) so that reader understands the message more easily.
- Figure 3B, C: authors should play with the focal plane so that Cut labeling myoblasts and hh reporters being expressed in the disc proper can be seen in the same Figure or panel. Similarly, in panel D-G, how sure can I be that the focal plane is finding the myoblasts and not the disc proper. Perhaps, authors might want to say something in the figure legend or add some co-stainings (DAPI, Cut).
- Statistics: authors should explain which error bars are shown in all figures and the number of samples per experiment. A dedicated section should be included in M&M to explain the statistical analysis and the programs being used. Whether histograms or scattered plots to be used in all figures is an issue that has to be decided by authors.

Second Revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
Authors have satisfactorily addressed almost all my concerns and the ones of Reviewer 3. As such, I support publication of this very nice research article in Development once all minor concerns (see below) are being addressed. I congratulate the authors for the whole work. I really enjoyed reading it.

Reviewer 1 Comments for the Author:
-Line 116: Fig. 1F should be Fig. 1F, G
Corrected.

-Line 120: Fig. 1G,H should be Fig G'', H
Corrected.

-Line 141: Although en is a "selector gene" for the posterior compartment (García-Bellido, 1975) where it is a positive regulator of hh (Tabata et al., 1992).. Include more recent references such as Bejarano et al, Dev Biol 2009.
The reference suggested by the reviewer was added.

-Line 152: be more explicit about which Caspase is monitored (Dcp1 or Dronc) and which Ab.
Reference to Dcp1 was added to the results and to the methods to clarify this point.

-Figure 2 is very disorganized and panel sizes very different. I would suggest authors to re-organize this figure (especially I-J) so that reader understands the message more easily.

Figure 2 was split in to new Figure2 and new Figure3 to make the data easier to understand for the readers.

-Figure 3B, C: authors should play with the focal plane so that Cut labeling myoblasts and hh reporters being expressed in the disc proper can be seen in the same Figure or panel. Similarly, in panel D-G, how sure can I be that the focal plane is finding the myoblasts and not the disc proper. Perhaps, authors might want to say something in the figure legend or add some co-stainings (DAPI, Cut).
We added a cross section image (X-Section) image in Figure 4 B'',B'', D-G', which shows the expression of hh reporters in both the myoblasts and disc cells in one panel.

-Statistics: authors should explain which error bars are shown in all figures and the number of samples per experiment. A dedicated section should be included in M&M to explain the statistical analysis and the programs being used. Whether histograms or scattered plots to be used in all figures is an issue that has to be decided by authors.
We added a sentence in the new Statistical analysis section in methods to clarify that all error bars show standard deviation. We added the sample number in figure legends wherever they were missing. In the new Statistical analysis section, we also outline the program we used to statistical analysis.

Second decision letter

MS ID#: DEVELOP/2020/195974

MS TITLE: Hedgehog produced by the Drosophila wing imaginal disc induces distinct responses in three target tissues

AUTHORS: Ryo Hatori and Thomas B Kornberg
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.