The GTPase Rab8 differentially controls the long- and short-range activity of the Hedgehog morphogen gradient by regulating Hedgehog apico-basal distribution

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MS TITLE: Rab8 GTPase differentially controls the long and short range activity of the Hedgehog morphogen gradient by regulating Hedgehog apico-basal release

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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 will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. 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 <u fr-original-style=3D"background: none; margin: 0px; padding: 0px; outline: 0px; border: 0px currentColor; border-image: none; font-size: 100%; vertical-align: baseline;" style=3D"background: none; margin: 0px; padding: 0px; outline: 0px; border: 0px currentcolor; font-size: 100%; vertical-align: baseline;">one</u>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.

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

Summary
There are two distinctive models for the secretion and distribution of the Hedgehog (Hh) protein in the Drosophila imaginal wing disc. In the first model, there is a singular pool of secreted Hh that travels to the basolateral surface of the cell and is transported via exosomes and cytonemes. In the second model, there are two secreted pools of Hh (apical and basolateral), Hh is initially endocytosed from the apical membrane and re-internalized, the apically released pool of Hh will activate long-range targets (dpp) and the basolaterally released pool activates short range targets (engrailed). This study provides evidence supporting the second model.
Rab8, a known regulator of apical-basal trafficking, was found to be required for maintaining the proper Hh gradient; loss of Rab8 from Hh producing cells promoted the expansion of Engrailed and reduction of dpp expression in Drosophila wing discs. The authors show that Rab8 is not important for the secretion of the Hh protein but is essential for proper apico-basal release of Hh. Loss of Rab8 decreases the apical release of Hh by positioning early endosomes (important for long-range expression) closer to the basolateral membrane.
Rab8 was also found to be important for the apico-basal positioning of the Hh co-receptor, Ihog. Ihog and Rab8 interact independent of Hh and loss of Rab8 prevented efficient Ihog endocytosis. Loss of Ihog rescued the gradient defects shown in the Rab8 mutant, suggesting that Rab8 also regulates the Hh gradient through the actions of Ihog. In summary the authors found that loss of Rab8 1) decreased apical release of Hh (long-range targets), favoring lateral release (short-range targets) and 2) stimulated the accumulation of Ihog at the basolateral membrane, further promoting the lateral release of Hh.

Comments for the author

Major Concerns
1) Reduction of dpp expression was mostly shown in rab8ui wing discs, this phenotype was rescued by over expressing Rab8 in the posterior but not the anterior, suggesting that Rab8 functions in Hh producing cells and not Hh receiving cells. Since this is a very important point in this paper, there needs to be more evidence to eliminate the possibility that Rab8 is not functioning in anterior cells. One experiment that should be done is to use UAS-RNAi against Rab8 in the posterior (hh-Gal4) as well as in the anterior (ptc-Gal4/ci-Gal4). If Rab8 is affecting Hh secretion, there should only be an effect on dpp/engrailed expression with hh-gal4 driver and not the ptc/ci-gal4 drivers. (repeated for experiments in Figure 2, 4)
2) The text asserts that the apico-basal distribution of Ihog was affected and the level of Ihog was slightly increased at the lateral plasma membrane. However it appears that there is an increase in Ihog expression in the apical, lateral, and basal z-planes (Figure5 A-H). Instead of affecting the distribution Ihog, it appears that loss of Rab8 stabilizes Ihog throughout. To prove that Rab8 is affecting distribution, each plane should be quantified for Ihog expression in wildtype and Rab8 mutant discs.
3) Figure 6 and 7, it is not clear what is apical and what is lateral in the z-projections, thus the shift in endosome markers from apical to lateral is not convincing. There should be a quantification/measurement of these endosome from the apical plasma membrane. Additionally, Ihog, UAS-Rab5 should be co-stained with known apical/lateral markers in Wt and rab8 mutants.
4) Even though the loss of Ihog perfectly rescues the Rab8 phenotype, the other data suggests that Hh could still have impaired apical release. To identify the mechanism, the Ihog/Rab8 double mutant should be analyzed with ptc-Gal4/UAS-ptc1130X from Figure 4 and should be tested to see if early endosomal structures are mispositioned with UAS-Rab5CA-YFP (Figure 6 and 7).

Minor Concerns
1) The Engrailed and Patched expansion is not convincing from the images, particularly since the wild-type images have weak engrailed (Fig2A). This point would be strengthened by using an internal control with an apterous-Gal4 driver targeting the UAS-RNAi of Rab8.
2) The title for Figures S3 is: Rab8 loss of function induces decrease of long-range Hh target gene expression. However the content of the Figure shows the effect of Rab8 depletion on Hh secretion.
3) Figure 5D, H Ihog staining is unclear.
4) "Rab8 is required for Ihog endocytosis through a direct binding"- If this is true, then the UAS-IhogRFP experiments (Figure 5M-P) should be repeated with the Ihog variant with impaired Rab8 binding (6N).
Otherwise the Figures show that Rab8 is required for Ihog protein stabilization indirectly.
5) Rab8 and Ihog are expressed throughout the anterior and posterior. Loss of Rab8 stabilizes Ihog in the anterior and the posterior. Is there a function for Rab8/Ihog interactions in the anterior?
6) The Rab8 antibody in Figure 6 is very weak and not very informative.

Reviewer 2

Advance summary and potential significance to field

In this manuscript the authors describe effects of mutations in Rab8 on Hedgehog signaling processes in the Drosophila wing disc. They observe changes in the range of expression of target genes which are relatively minor, but seem to indicate that Rab8 on the one had plays a positive role in the expression of long range targets, as measured by expression of one reporter construct, and on the other hand, seems to limit expression of short range targets. In the mutant situation Â- which they create either by using RNAi knockdown or a newly induced Rab8 allele, they see a small expansion of short range targets and a reduction in the long range targets. The authors also look at the accumulation of a tagged Hh construct and at the distribution of Ihog. As they observe a small increase in laterally positioned endosomes containing Hh in the Rab8 mutant, they conclude that in the mutant lateral production of Hh is increased and that this is the cause of the increased short range target expression. Conversely, as they seem to observe a reduction in apically localized Hh, they propose that this is the cause of the reduction in long range target expression. Unfortunately the data are sometimes not entirely clear and while the proposed mechanism may explain the partial phenotypes, it is not clear that this is the correct interpretation of the phenotypes.

Comments for the author

Overall, while the question of Hh signaling is interesting, I was not convinced that the very small, and hard to appreciate changes that the authors report in the protein distribution are the cause of the observed effect of changes in target gene expression. All of the changes are small and sometimes hard to see on the Figures that were provided (see specific comments below). And the authors themselves end the manuscript (p.19) with a lot of suggestions ".. could possibly " -".. is likely " - "may be more recycled" etc. In addition, the authors have previously published about the differences in apical vs lateral pools of Hh and their effects. The present manuscript does not really add much to those findings.
Therefore, there is, in my opinion, not enough hard data provided for the conclusions about apical vs lateral secretion being regulated by Rab8 to warrant publication at this time. Rab8 does seem to play a minor role, but whether it is by the proposed recycling and release mechanism or not, seems not firmly answered. And since the authors already published about differences in apical versus lateral pools of Hh, there is not substantial conceptual advance provided.

Specific comments:

1) In Figure 1 and 2 (and other Figures) the authors indicate the compartment boundary with a white dotted line. How was this defined? I was under the impression that the expression of Engrailed defined the posterior compartment. So here this is not the case? But then how do the authors decide where to draw the line? This needs to be much more carefully explained!

2) Figure 4 A and B: I am not sure what I should be seeing in these Figures. The authors say there is accumulation of trapped Hh at the plasma membrane? I don't know where the membrane is, all I see is a "patch of white", and the supplementary Figure 4 doesn't help either. The authors need at the very least to supply a schematic drawing next to these pictures that tell us where apical is in these cells, where basal is, etc. Also, why are we seeing two stripes crossing the disc field in C'?. What are we looking at? It is very difficult for the non-expert reader to see any difference between S4A and C, or S4 B and D. And the E and F series are also seemingly not very different at all.

Since the authors base a lot of their conclusions on these results, it must be possible to make them more visible and better understandable.

3) Authors state p.11 : they see apical and lateral trapping of extracellular Hh in wildtype, but in the rab8 mutant, they see only an increase of Hh in the producing P cells, but less apically trapped Hh in A cells, and an increase laterally. Why do they know that this change in accumulation reflects a RELEASE problems by the P cells? (rather than, for instance, changes in turn over).

4) In previous publications by the authors the difference in apical vs lateral Hh was shown to be due to Dally and Notum activity. How does this fit into the model?

Minor comments:
p. 7 bottom: "we assessed the function of Rab8 specifically in the hh expressing posterior producing cells.."
What are "posterior producing cells"? Do the authors mean: in posterior cells that produce Hh? (Hh to my knowledge does not induce – produce – posterior cell fates?)

Figure 5: One can see an increase in staining of Ihog in E as compared to A (apical) but there is no convincing increase in F, or H. The panels of I to L are more convincing. I would suggest deleting panels A to H, if they cannot be made clearer, and just show the second half of the Figure.

p. 14: the authors write: "Given the strong effect of rab8 loss of function on Ihog dynamics…" What are these "strong effects"? (29% vs 14%?) Or what are the authors referring to?

Reviewer 3

Advance summary and potential significance to field

This work concerns how the apicobasal distribution of the Hedgehog (Hh) morphogen is controlled. This issue is an important one in the Hedgehog field and is also of interest for scientists in the fields of development and cell biology, as it raises the issue of how cells respond to different doses of morphogen and of how cell trafficking controls a morphogen gradient. More specifically, this manuscript shows that the Rab8 protein, which is known to control apicobasal trafficking, controls the apicobasal distribution of Hh and therefore the expression of its target genes in the responding cells. It seems to do so by two means: it prevents the endocytosis of Ihog, a Hh co-receptor that normally prevents HH spreading from posterior cells and it controls the apical distribution of the Rab5 endosomes in which HH is endocytosed.

Comments for the author

Overall this work is very interesting both for the questions that it addresses and for the mechanisms that it reveals. However, the quantitative analysis is insufficient to be totally convincing, especially since some results are quite subtle and there is almost no statistical analysis (see details below). Moreover, there are a few internal contradictions that need to be clarified and a few controls that have to be done, especially to ensure that some of the tools that they use are not a cause of artifacts (see the specific points 8 and 12). Finally, the author have to carefully read their manuscript to improve the references to the different panels of the Sup figures and to ensure that the order of the figures panels in the text corresponds to the order of the panels in the figures (see examples below).

MAJOR POINTS
Quantification of the images This is clearly the weakest point of this work.
On average only 4 to 5 discs (up to 6-8 at best) were analyzed for each genotype. Moreover, there is no information on the reproducibility or variability of the experiments. Could the authors provide clear information on how many discs they observed and how many times each experiment was independently performed. Note that the weakness of some effects should not a problem if the authors improve the quantitative analysis.
I also recommend to quantify more discs and to perform a statistical analysis, especially when the effects are weak or subtle. I must express my surprise that in Fig 3 P and Q, a p-value was calculated with n=4 for some samples. Could the authors explain what test they used for such a small sample?
The quantification graphs are a bit confusing: they are sometimes in pixels, sometimes in microns and there is no information on how the position 0 is defined. Could the authors also explain how they take into account variation in the size of the discs? This is especially important as rab8 affects dpp expression.
Could the author also indicate how many times the Western blots were performed and indicate the molecular weights on all the blots?

Specific points
1. In figure 1, the authors clearly show that a reduction in Rab8 suppresses the dpp-dependent outgrowth induced by posterior Hh overexpression but has no effect on En and Ptc. Then, in figure 2, the authors show that the loss of Rab8 depletion in a wt context affects both dppZ and En. The authors should confirm that Rab8 reduction reduces the increase of dppZ induced by posterior Hh overexpression. How do they explain the different effects on En?
2. Concerning the connection between En broadening and dppZ reduction, there is a clear logic issue (p8). The data in the figure 3 indeed allow one to reject that “the effects of Rabs8 reduction on dpp are “solely” due to the broadening of En expression domain, but, it does not absolutely mean that that the “dpp loss is independent of anterior En expression”, it just mean that it is not solely due to En.
3. The data that led the authors to conclude that the lack of effect of rab8-RNAi on HH secretion in S2 cells (Fig S3C) have to be interpreted more cautiously as Rab8 levels do not seem to be dramatically decreased (40% still present). Did the authors try different interfering dsRNA (alone or in combination)? Moreover, it seems that only one blot was quantified in 3C (no error bar) while in 3B the amounts of Renilla probably correspond to a mean of replicates (not indicated, but suggested by the presence of the error bars)…
4. To validate the experiment with ptc 1130X (Fig4), it is critical to validate this tool by checking that it has the same apicobasal localization than wild-type PTC overexpressed in the same condition.
5. Figure 4: Why do extracellular HH levels drop at the AP border in the “quantification curves”? It should stay high in the posterior region? Why are the distances now in micron and no longer in pixels?
6. I do not understand why the authors conclude on p 11 that there is an increase of the release of lateral HH from the producing (P cell) while they also say (and show) a few lines before (last paragraph, page 10) that “there is an increase of extracellular HH at the apical side of producing P cells”. Is there a decrease in the lateral region in these P cells? I also recommend that the author carefully quantify the distribution of HH along the apicobasal axis.
7. In Fig 4S, it seems that rab8U1 has an effect on actin accumulation, especially in the apical region? If the case, this should be taken into consideration. If not the case, such variation is a good illustration of why the author should absolutely analyses and quantify more discs, and apply a statistical test that takes these variations into account (see above).
8. The study of the effect of Rab8 on Rab5CA in figure 6 make sense only if the apicobasal distribution of wild-type Rab5 has the same distribution. Could the author show it or refer to any published work that shows it?
9. It would also be interesting (and important) to understand whether a Rab8 depletion acts on all Rba5 endosomes or specifically on those that carry Hh. Could the authors analyze whether the pool of Rab5 vesicles that are co-labelled with HH are more mislocalised than the HH negative Rab5 endosomes.
10. In Figure 6N, the blot showing the immunoprecipated proteins is of very poor quality compared to the other blots and the absence of Rba8 coimmuprecipitation is not convincing as Ihog itself is barely detected in the immunoprecipitated fraction. Given this and the fact that this

mapping does not add to the story, I suggest to remove it. Surprisingly, it seems that in S2 cells Rab8 overexpression increases the levels of Ihog, while its absence in vivo also increase Ihog levels. How do the authors explain this?

MINOR POINTS
1. Could the authors indicate how they classify the discs according to their size in Fig 1? Automatic quantification of the surface? Double-blind sorting by eye?
2. The authors have to carefully check their references to the different panels of the Figures, including the S figures. For instance and among many others: in the first paragraph of the p 7, the authors refer to figure S2A-D, while this part concerns only the panels S2A and B…; Fig 2C comes before 2A and 2B; S6D before S6A…; on the last lane of p19, 4G is probably 4G-H; it also seems that there are not references in the main text to some panels form the Sup figures (as figure S5A-F with the a PKC …) …
3. Brackets are missing for "Figure S1H-Q" p 8 4. P 12 A reference should be added at the end of the first paragraph (on Ihog and HH release)
5. Figure 5: Could the authors explain what the large fold seen in the z sections compared to the z sections shown in the other figures. Could the authors also shown xy sections for ex Ihog?
6. RNAi is directed against mRNA, and should therefore be written with the name of the gene (in tialics) not of the protein

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First revision

Author response to reviewers' comments

Rebuttal:

Reviewer1:
1) Reduction of dpp expression was mostly shown in rab8ui wing discs, this phenotype was rescued by over expressing Rab8 in the posterior but not the anterior, suggesting that Rab8 functions in Hh producing cells and not Hh receiving cells. Since this is a very important point in this paper, there needs to be more evidence to eliminate the possibility that Rab8 is not functioning in anterior cells. One experiment that should be done is to use UAS-RNAi against Rab8 in the posterior (hh-Gal4) as well as in the anterior (ptc-Gal4/ci- Gal4). If Rab8 is affecting Hh secretion, there should only be an effect on dpp/engrailed expression with hh- gal4 driver and not the ptc/ci-gal4 drivers. (repeated for experiments in Figure 2, 4)

We thank the reviewer for this comment. Indeed, the possible role of rab8 in anterior cells cannot be entirely excluded based on the experiments we presented. Nevertheless, we have already provided examples in which depleting rab8 specifically in the posterior cells leads to non-autonomous defects in the anterior in the original manuscript (for ex. Fig. 2L-Q; 3A-L, which correspond to NEW Figure 2F-H and NEW Figure 3A-F) similar to the rab8^{U1} mutant. The rescue experiment provided further confirmation that Rab8 regulates Hh activity in producing and not in receiving cells (NEW Sup Fig1 J-L'').
We also have two other independent conditions in which rab8 was removed specifically from posterior which led to such an effect:
1. In the NEW Fig. 1 we provide new quantification of dpp expression in the Hh overexpression tester line compared to discs depleted specifically for posterior expression of rab8. Distal dpp is clearly reduced in the absence of rab8 in the posterior compartment (compare NEW Figure 1B'' and 1C'', and quantification in 1F) in this sensitized background.
2. In Figure 3 we used a strong hh hypomorphic background (hhGal4/hh^{ts2}) and depleted rab8 exclusively in the posterior compartment. Anterior En expression was not (or very weakly) present in these hh hypomorph discs, allowing us to directly measure the effect of rab8 depletion on dpp expression, independently of its regulation by En. We found a strong reduction of dpp expression when we depleted rab8 in this context (compare NEW Figure 3B' to C'). Of note, we also updated the dpp range measurement with more discs included in the quantification (NEW Figure 3E).

In order to answer reviewer's comment, we performed an additional set of experiments which have been added in NEW sup. Fig. 1. We now provide a quantification of En and dpp from discs in which the expression of UAS-RNAi against rab8 is driven by ptcgal4. Depletion of rab8 in the anterior cells (ptcgal4) did not show a change in En nor in dpp pattern (NEW Supplementary Figure 1I).

Altogether, these experiments strongly suggest that the effect we see on Hh targets in the rab8 mutant is specific to the posterior cells. We would like to note however, that the general effect of rab8 removal on both early endosomal positioning and Ihog stability could potentially influence Hh signaling in anterior cells in a sensitized background, although in our hands these effects are not sufficient to produce an anterior phenotype. For this manuscript we focused on Hh producing cells.

2) The text asserts that the apico-basal distribution of Ihog was affected and the level of Ihog was slightly increased at the lateral plasma membrane. However it appears that there is an increase in Ihog expression in the apical, lateral, and basal z-planes (Figure5 A-H). Instead of affecting the distribution Ihog, it appears that loss of Rab8 stabilizes Ihog throughout. To prove that Rab8 is affecting distribution, each plane should be quantified for Ihog expression in wildtype and Rab8 mutant discs.

We thank the reviewer for this remark. Based on the Ihog labeling as well as the IhogRFP stability experiments, indeed it is not obvious whether there is a general stabilization of Ihog, or whether there is also a change in its subcellular distribution. We followed the reviewer's advice and performed plane by plane quantification of Ihog. We carried out these measurements in discs in which we could compare the distribution and level of Ihog to an internal control (using apGal4 and the degradFP system described in the manuscript). This allowed us to compare the apicobasal intensity and distribution of Ihog in wild type or rab8 depleted cells within the same disc. A representative example of this quantification is presented in the NEW Figure 5B. We found that lateral Ihog increases at most by 40% (repeated 6 times). This indicates that in the absence of rab8, Ihog stability is predominantly increased on the lateral side. In conclusion, a lack of rab8 function leads to increased lateral Ihog stability, but also a change in the apicobasal distribution of Ihog.

3) Figure 6 and 7, it is not clear what is apical and what is lateral in the z-projections, thus the shift in endosome markers from apical to lateral is not convincing. There should be a quantification/measurement of these endosome from the apical plasma membrane. Additionally, Ihog, UAS-Rab5 should be co-stained with known apical/lateral markers in Wt and rab8 mutants.

We thank the reviewer for this comment and have now provided the distribution of endosomes relative to an apical plasma membrane marker (NEW Supplementary Figure 6). We have used Cadherin to label the apical and subapical membrane.
We first analyzed the distribution of both the Rab5 knock-in ($Rab5^{KI}$) and UAS-$Rab5^{CA}$ variants. Both show a similar strong enrichment at the apical and subapical domains (between the 0 and 5 micron mark) close to the Cadherin staining (NEW Supplementary Figure 6A-C).

Then we also analyzed the distribution of $Rab5^{KI}$ and $Rab5^{CA}$ variants in absence of rab8. The quantification of $Rab5^{KI}$ positive endosomes showed a shift in their distribution, from apical to more lateral (NEW Supplementary Figure 6J-K). $Rab5^{KI}$ went from 70% apical/subapical to 40%, with 60 % of endosomes now located below 5 micron mark from the apical marker. As described in the original manuscript, we saw the same trend with $Rab5^{CA}$. Although this lateral shift is less pronounced compared to $Rab5^{KI}$, the proportion of apically localised endosomes was reduced from 53 to 30% (NEW Figure 7G). Additionally, when we scored only the Hh containing $Rab5^{CA}$ endosomes, we saw a more pronounced change. Namely, the percentage of most apical Hh $Rab5^{CA}$ endosomes decreased from 43% in the control to 22% in the $rab8^{U1}$ mutant (NEW Figure 7H).

Concerning the reviewer's comment on Ihog containing Rab5 endosome distribution, we do not think that measuring this would bring substantial new data to the study. The fact that only 14% of $Rab5^{CA}$-YFP positive endosomes were positive for Ihog in rab8 mutant background (versus 29% in the wild-type control, NEW Supplementary Figure 6L), suggests that the loss of Rab8 impairs Ihog trafficking to these early endosomes. The point we wanted to make is that in a wild-type context, endogenous Ihog is present at the plasma membrane and is efficiently endocytosed. However, in rab8 loss of

function, the level of Ihog at the plasma membrane increases because the protein is not efficiently endocytosed and therefore accumulates.

4) Even though the loss of Ihog perfectly rescues the Rab8 phenotype, the other data suggests that Hh could still have impaired apical release. To identify the mechanism, the Ihog/Rab8 double mutant should be analyzed with ptc-Gal4/UAS-ptc1130X from Figure 4 and should be tested to see if early endosomal *structures are mispositioned with UAS-Rab5CA-YFP (Figure 6 and 7).*

We thank the reviewer for this suggestion. Inducing Ihog RNAi in the posterior compartment in a rab8^{U1} mutant context was sufficient to restore the expression of proximal targets close to wild-type, with the decrease of En and Ptc expansion and increase of proximal dpp, but crucially not of distal dpp which depends on apical Hh release (Figure 8A-F).
We are not sure what the reviewer meant with the 'other data', we think the reviewer is probably referring to the change in the distribution of Rab5 endosomes and Hh when we remove Ihog in the rab8 mutant. We agree with this reviewer that the apical Hh release and Rab5 mislocalization might not be rescued when removing Ihog and it is important to test.
Based on this, although the experiment outlined by this reviewer would have been an ideal condition in which to analyze Hh in both Hh producing and receiving cells, we could not technically carry it out as it is suggested. The combination of rab8^{U1}+Ihog- double mutant with ptcGal4 and ptc^{1130X} is genetically difficult to achieve. We would need to generate a ptc^{1130X}<Ihog- mutant recombinant chromosome, and a ptcGal4<tubGal80ts<Ihog- triple mutant recombinant chromosome, and introduce them into a rab8 mutant background. Also, we cannot express Rab5^{CA} in the posterior and simultaneously overexpress ptc1130X in the anterior compartment with the available tools.

Nevertheless, to further analyze this, we have quantified the distribution of extracellular Hh in Hh producing cells when both Ihog and rab8 are removed. Again, we chose to carry out this experiment in discs where we had an internal control (removing Ihog in the dorsal compartment of rab8^{U1} mutant discs, and quantify extracellular Hh in both the dorsal and the ventral compartments section by section). We observed a reduction in the level of both apical and lateral extracellular distribution, with the lateral extracellular Hh showing the strongest reduction (reduced by almost 50%, NEW Figure 8G-H). This correlates nicely with the rescue of the short range target En in the anterior cells.
Importantly, in this context Rab5 endosomes are still mislocalized (NEW Figure 8I-I''') suggesting that apical Hh endocytosis and subsequently apical release is impaired. Altogether, we think that the mislocalization of Rab5 endosomes observed in the rab8^{U1} mutant is an Ihog independent component of the rab8^{U1} phenotype, that affects Hh distribution independently of the presence of Ihog.

Minor Concerns:
1) The Engrailed and Patched expansion is not convincing from the images, particularly since the wild-type images have weak engrailed (Fig2A). This point would be strengthened by using an internal control with an apterous-Gal4 driver targeting the UAS-RNAi of Rab8.
We have performed the experiment suggested by the reviewer in order to strengthen our findings. In the NEW Supplementary Figure 1G-H, rab8 depletion was driven by the apterous gal4 driver. Comparison between ventral and dorsal compartment clearly showed an expansion of both En and Ptc upon depletion of rab8.

2) The title for Figures S3 is: Rab8 loss of function induces decrease of long-range Hh target gene expression. However the content of the Figure shows the effect of Rab8 depletion on Hh secretion.

This has been corrected

3) Figure 5D, H Ihog staining is unclear.

Figure 5A-H have been removed. Instead we are only showing discs in which we had internal control tissue, and have provided a quantification of Ihog in each plane of apterous RNAi rab8 discs. (NEW Figure 5B).

4) "Rab8 is required for Ihog endocytosis through a direct binding"- If this is true, then the UAS-IhogRFP experiments (Figure 5M-P) should be repeated with the Ihog variant with impaired Rab8

binding (6N). Otherwise the Figures show that Rab8 is required for Ihog protein stabilization indirectly.

We apologize for this mistake, which has been corrected in the text. We do not have evidence for a direct binding

5) Rab8 and Ihog are expressed throughout the anterior and posterior. Loss of Rab8 stabilizes Ihog in the anterior and the posterior. Is there a function for Rab8/Ihog interactions in the anterior?

As UAS-RNAi rab8 X ptcgal4 has no effect on Hh targets (New Sup Fig 1I), there is no apparent role of rab8 in the anterior compartment in the context of Hh signalling.

6) The Rab8 antibody in Figure 6 is very weak and not very informative.

We have presented Rab8 staining in order to know whether Rab8 accumulates in Rab5 endosomes. We have chosen to keep this panel.

Reviewer 2

Overall, while the question of Hh signaling is interesting, I was not convinced that the very small, and hard to appreciate changes that the authors report in the protein distribution are the cause of the observed effect of changes in target gene expression. All of the changes are small and sometimes hard to see on the Figures that were provided (see specific comments below). And the authors themselves end the manuscript (p.19) with a lot of suggestions ".. could possibly " –".. is likely " – "may be more recycled" etc. In addition, the authors have previously published about the differences in apical vs lateral pools of Hh and their effects. The present manuscript does not really add much to those findings.

Therefore, there is, in my opinion, not enough hard data provided for the conclusions about apical vs lateral secretion being regulated by Rab8 to warrant publication at this time. Rab8 does seem to play a minor role, but whether it is by the proposed recycling and release mechanism or not, seems not firmly answered.
And since the authors already published about differences in apical versus lateral pools of Hh, there is not substantial conceptual advance provided.

We do not agree with the opinion of this reviewer that the effects observed in the rab8 mutant are small.
For example, in Figure 1, depleting rab8 by RNAi led to a significant rescue of dpp-dependent anterior outgrowth (a decrease from 95% to 20% of discs showing severe outgrowth is observed upon RNAi against rab8 in Hh producing cells). To our knowledge, only the depletion of Disp or Rasp, direct regulators of Hh, are able to give a stronger rescue.

Moreover, the reduction of dpp expression is not small; the range of Dpp expression was reduced by 50%, from a range of 6-8 cells in wild-type to 3-4 cells in the rab8^{U1} mutant (Figure 2). We observed a similar 50% reduction of Dpp expression in Figure 3 (of note, we updated our quantification with more samples for dpp-lacZ, NEW Figure 3E). Again, few mutants affecting Hh production show such a strong effect on Dpp. Regarding the increase in Ihog protein, we observed a 40% increase of lateral Ihog level in cells depleted for Rab8 (NEW Figure 5B).

We also do not agree with reviewer's comment regarding the lack of substantial conceptual advance.

We previously suggested that the Hh gradient is composed of two pools, one apical and one basolateral, both linked to the range of Hh activity. We proposed that the sum of these pools is required for the precise patterning of the wing imaginal disc (Ayers et al., Developmental Cell 2010). At the time we did not have any means to manipulate the equilibrium of these two pools in the producing cells. Moreover, other studies have suggested that the Hh gradient is composed of a single pool of Hh which is supplied by Hh basolateral release from where it is transported to the receiving tissue via cytonemes to activate short and long-range targets (Bilioni et al., Developmental Biology 2013; Chen et al., Development 2017). This illustrates the current contention regarding the composition of the Hh morphogen gradient and the relative contribution of apico-basal trafficking

in the regulation of polarized Hh secretion.

In this current study, we could change the equilibrium of these two pools in the producing cells. We show that interfering with rab8 function in Hh producing cells impedes Hh distribution and subsequent target gene expression in receiving cells, inducing an imbalance between the apically and basolaterally released Hh when compared to wild-type. Strikingly, this results in a differential effect on Hh target gene expression, with a reduction in long-range and increase in short-range targets. To our knowledge, this is the first report to identify differential regulation of short- and long-range morphogen activity through the regulation of polarized trafficking and morphogen distribution by a Rab protein.

These results also confirm two main points: first, that there is more than one pool of Hh, one basal and one apical, and second, that the basolateral pool clearly induces short range targets whereas the apical pool induces the long- range ones. At the mechanistic level, we demonstrate that Rab8 is critical in the establishment of the Hh gradient, and the correct apico/basal distribution of Hh. This is achieved through its regulation of the apico-basal distribution of Ihog, a Hh binding protein, as well as the position of early and recycling endosomes in Hh-producing cells.

These findings uncover a novel function for Rab proteins in controlling the differential activities of a morphogen. It also provides new conceptual findings for morphogen gradients, whereby morphogen pools follow different routes in producing cells, and contribute differently to the activity of the morphogen gradient. Importantly, we provide new evidence that Hh activity is divided between two functionally distinct Hh pools which collectively comprise the morphogen gradient.

Specific comments:

1) In Figure 1 and 2 (and other Figures) the authors indicate the compartment boundary with a white dotted line.
How was this defined? I was under the impression that the expression of Engrailed defined the posterior compartment. So here this is not the case? But then how do the authors decide where to draw the line? This needs to be much more carefully explained!

We apologize if this is not made clear in the manuscript. On page 2 of the manuscript text we describe "A cells close to the source respond to high levels of Hh by expressing the short-range target transcription factor Engrailed (En) and the Hh receptor Patched (Ptc)". Therefore, as En is also expressed in the posterior compartment, it cannot be used to determine compartment boundary. Ptc however is only expressed in the anterior cells, so consequently we used Ptc staining to define the A/P compartment boundary. This is now described in the legend of Figure 1. In panels where Ptc staining was not available (like the NEW Supplementary Figure 1K-L'') we defined the compartment boundary using the Gal4 expression domains, namely the lack of expression of Rab8, GFP, etc. Whenever it is applicable we have now included this information in the figure legends.

2) Figure 4 A and B: I am not sure what I should be seeing in these Figures. The authors say there is accumulation of trapped Hh at the plasma membrane? I don't know where the membrane is, all I see is a "patch of white", and the supplementary Figure 4 doesn't help either. The authors need at the very least to supply a schematic drawing next to these pictures that tell us where apical is in these cells, where basal is, etc. Also, why are we seeing two stripes crossing the disc field in C'?. What are we looking at? It is very difficult for the non- expert reader to see any difference between S4A and C, or S4 B and D. And the E and F series are also seemingly not very different at all.
Since the authors base a lot of their conclusions on these results, it must be possible to make them more visible and better understandable.

We apologize for the confusion. The aim of these experiments were to 'catch' Hh in the anterior cells in both control and the rab8^{U1} mutant discs. To do so we used a mutant form of the Ptc receptor (Ptc^{1130X}), which is able to bind Hh but cannot be internalized. We expressed it only in anterior cells, in order to trap released Hh at the surface of Hh receiving cells. This trapping results in a 'patch of white' in the anterior cells, which is indeed surface bound Hh. We provide now an explanatory scheme next to the main figure panels on NEW Figure 4.

Because Ptc^{1130X} sequesters extracellular Hh (recognized in non-detergent conditions), we believe that the Hh pattern we observed is due to accumulation of Hh at the plasma membrane.

Concerning Supplementary Figure 4C', the 'two stripes' are a result of cell compaction at the dorso-ventral boundary. This is readily visible with phalloidin labeling as well as any apical plasma membrane marker. This is now also indicated in the figure legend.

In S4A,C and S4B,D we showed the additional channels presented on the main figure (NEW Figure 4 C-F). The fact that the reviewer cannot see a difference is actually the point we wanted to make: the expression pattern of Ptc^{1130X} is not different between control and $rab8^{U1}$ mutant discs. This shows that the variation in Hh accumulation along the apico-basal poles in anterior cells is not due to a change in the Ptc^{1130X} distribution in the rab8 mutant. This allowed us to directly compare the amount of surface bound Hh in between the genotypes.

Concerning old S4E and S4F we agree with the reviewer that the change in Hh levels in the anterior cells is hard to appreciate on single sections. That is why we originally presented stacks on the main figure (NEW Figure 4H-I'''). As S4E and S4F do not provide additional information we therefore decided to remove them. Instead we now include the quantification protocol that allowed us to quantify extracellular Hh in Ptc^{1130X} discs (NEW Supplementary Figure 4E).

Importantly, we also carefully quantified extracellular Hh on XY sections on more discs both in the posterior compartment and also on the surface of the anterior Ptc^{1130X} expressing cells (NEW Figure 4C-G') in the revised manuscript. We now show that the distribution of extracellular Hh in Hh producing cells of the $rab8_{U1}$ mutant is shifted towards the lateral disc regions. This correlates well with the increase of released Hh found laterally in the receiving cells of mutant discs. We also present crops of the A/P boundary on the NEW Figure 4C-F panels to more clearly illustrate the differences.

3) Authors state p.11 : they see apical and lateral trapping of extracellular Hh in wildtype, but in the rab8 mutant, they see only an increase of Hh in the producing P cells, but less apically trapped Hh in A cells, and an increase laterally. Why do they know that this change in accumulation reflects a RELEASE problems by the P cells? (rather than, for instance, changes in turn over).

Since we cannot directly measure the amount of Hh secreted at the specific poles of P cells, i.e. apical, or basal, we used the distribution of accumulated Hh on receiving A cells as a proxy. Figure 4 shows that in the absence of rab8, more released Hh is found at the lateral pole at the expense of the apical one of the receiving cells (with the use of expression of the Ptc^{1130X} receptor, deficient for endocytosis). We agree with this reviewer's comments that indeed, the accumulation of trapped Hh laterally could be due to a local change in the rate of Hh turn over in producing cells.

However, our analysis of Hh protein levels in $rab8^{U1}$ homozygote discs by WB did not reveal any change when compared to the control, suggesting that Rab8 does not regulate the overall quantity of Hh at steady state, and that Hh turnover is likely similar in both backgrounds. This result is corroborated by our analysis of Hh levels in the extracellular medium of cultured cells depleted for rab8. Again, the level of Hh in both conditions is similar, suggesting that the total amount of secreted Hh is not affected in the absence of Rab8.

Taken together these results show that in absence of rab8:
1. *The overall Hh level is unchanged,*
2. *The level of secreted Hh is unchanged and*
3. *The change in the distribution of Hh at the surface of receiving cells suggests that rab8 loss of function modifies Hh (or Hh regulators) intracellular routing in producing cells, leading to an increased accumulation to basal side and less on the apical surface of Hh receiving cells.*

As this reviewer has indicated, we cannot exclude the possibility that this observation is a consequence of a local change in the rate of Hh turn over, resulting in an increase in laterally released Hh. We have modified our text to address this point. Instead of using secretion, we now talk about final distribution.

4) In previous publications by the authors the difference in apical vs lateral Hh was shown to be due to Dally and Notum activity. How does this fit into the model?
We have previously found evidence for the involvement of the glypican Dally and the hydrolase Notum in the long-range apical spreading of Hh. Nevertheless, reduction of either of these proteins in Hh-producing cells reduces the range within which dpp is expressed, whereas short-range target En is untouched, which is different from the rab8 mutant phenotype.
Also, Dally distribution is not affected in rab8 mutant (data not shown). We believe that rab8 is involved in the regulation of Hh intracellular routing, while Dally is involved more downstream once Hh is present at the apical surface of producing cells.

Minor comments:
p. 7 bottom: "we assessed the function of Rab8 specifically in the hh expressing posterior producing cells.." What are "posterior producing cells"? Do the authors mean: in posterior cells that produce Hh? (Hh to my knowledge does not induce – produce – posterior cell fates?).

This has been corrected.

Figure 5: One can see an increase in staining of Ihog in E as compared to A (apical) but there is no convincing increase in F, or H. The panels of I to L are more convincing. I would suggest deleting panels A to H, if they cannot be made clearer, and just show the second half of the Figure.

We thank the reviewer for this comment. We agree, and removed panels A-H. We directly start now with the analysis of Ihog level section by section (see NEW figure 5).

p. 14: the authors write: "Given the strong effect of rab8 loss of function on Ihog dynamics…" What are these "strong effects"? (29% vs 14%?) Or what are the authors referring to?

In the original manuscript we referred to both Ihog stability and level distribution change in the rab8^{U1} mutant context. We have tuned down this part of the text in the revised manuscript.

Reviewer 3

MAJOR POINTS
Quantification of the images
This is clearly the weakest point of this work.
On average only 4 to 5 discs (up to 6-8 at best) were analyzed for each genotype. Moreover, there is no information on the reproducibility or variability of the experiments.
Could the authors provide clear information on how many discs they observed and how many times each experiment was independently performed. Note that the weakness of some effects should not a problem if the authors improve the quantitative analysis.
I also recommend to quantify more discs and to perform a statistical analysis, especially when the effects are weak or subtle. I must express my surprise that in Fig 3 P and Q, a p-value was calculated with n=4 for some samples. Could the authors explain what test they used for such a small sample?

We have increased the sample size included in the quantifications wherever it was possible. Additionally, all of our experiments were repeated at least 3 times. We indicate this in the Materials and Methods section of the revised manuscript.

We apologize for the original Fig 3P and O panels. We used Student's t-test, for which the minimal sample size to our knowledge is 4. Also, the low sample size included here was the consequence of the genotype used in which it was difficult to obtain En and Ptc staining distinguishable from the background.
We have now repeated this experiment and present a new graph for both En and Ptc quantification. We found the same effect as before which confirmed that loss of rab8 affect dpp-lacZ expression independently of Engrailed.

We agree with the reviewer that we needed to analyze more discs, and have provided an independent quantification with a higher sample number, 8 to 10 discs per genotype for most

panels. New figure panels with updated n numbers (indicated in the figure legends or panels) and new quantifications are as follows:
NEW Figure 1D-F NEW Figure 3D-H, J,K
NEW Figure 4G NEW Figure 7G-I
NEW Supplementary Figure 1H,I NEW Supplementary Figure 6C,K

The quantification graphs are a bit confusing: they are sometimes in pixels, sometimes in microns and there is no information on how the position 0 is defined.

We apologize for this and have converted the pixel values to micron in order to unify the presentation.

Could the authors also explain how they take into account variation in the size of the discs? This is especially important as rab8 affects dpp expression.

Our crosses were synchronized and time of egg lays were controlled. rab8^{U1} mutant discs are not smaller compared to wild type discs. Several direct comparisons between wt and rab8 mutant disc can be found in the manuscript. For example, the Supplementary Figure 1B panel presents a complete rab8 mutant disc next to a wt disc. Also in figure 2 A and B, similar size wt and rab8 mutant are presented. We also compared cell sizes of both ventral and dorsal compartments in apterous>UAS-RNAi Rab8 discs. This did not reveal any cell size difference (data not shown). Altogether, we have no evidence that the restricted dpp expression we observed in rab8 mutant could correlate with a smaller disc size.

Could the author also indicate how many times the Western blots were performed and indicate the molecular weights on all the blots?

Western blots were performed three times from three different experiments. We also indicated the molecular weights next to the blots.

Specific points
1. In figure 1, the authors clearly show that a reduction in Rab8 suppresses the dpp-dependent outgrowth induced by posterior Hh overexpression but has no effect on En and Ptc. Then, in figure 2, the authors show that the loss of Rab8 depletion in a wt context affects both dppZ and En. The authors should confirm that Rab8 reduction reduces the increase of dppZ induced by posterior Hh overexpression.

We are now presenting, in NEW Figure 1, dpp expression in the Hh overexpression tester line compared to discs depleted for rab8 expression specifically posterior. The quantification clearly shows in both cases that proximal dpp is reduced due to an enlargement of the En domain. Moreover, distal dpp is further reduced in the absence of rab8. This confirms that the effect we see on Hh targets in rab8 mutant is specific to the depletion of rab8 in posterior cells. Since the dpp-dependent wing disc outgrowth is suppressed in these discs, it also confirms that the rescue of anterior outgrowth is due to the decrease of ectopic dpp expression.

How do they explain the different effects on En?

We believe that the domain of En expressing cells we observed in the "tester" line cannot be extended further, even in the absence of rab8.

2. Concerning the connection between En broadening and dppZ reduction, there is a clear logic issue (p8). The data in the figure 3 indeed allow one to reject that "the effects of Rabs8 reduction on dpp are "solely" due to the broadening of En expression domain, but, it does not absolutely mean that that the "dpp loss is independent of anterior En expression", it just mean that it is not solely due to En.

We agree, we changed the wording accordingly.

3. The data that led the authors to conclude that the lack of effect of rab8-RNAi on HH secretion in S2 cells (Fig S3C) have to be interpreted more cautiously as Rab8 levels do not seem to be dramatically decreased (40% still present). Did the authors try different interfering dsRNA (alone or in combination)? Moreover, it seems that only one blot was quantified in 3C (no error bar) while in 3B the amounts of Renilla probably correspond to a mean of replicates (not indicated, but suggested by the presence of the error bars)…

The Renilla quantification relates to replicates of experiments done three times.
The western blot present one example showing more than 60% of Rab8. We could not achieve a stronger depletion of Rab8, likely because the protein is quite stable. We have thus concentrated our efforts on the in vivo studies. For this, we have developed several tools in order to increase the efficiency of the Rab8 depletion in vivo, using the DegradFP system (which directly removes the protein) and Dicer2. We believe that the overall Hh secretion is not affected in vivo by the absence of Rab8 for the following reasons:
- *extracellular Hh is present at the surface of producing cells,*
- *we confirmed that Hh is released and can be found at the surface of receiving cells using the Ptc^{1130X} tool.*
- *a decrease in Hh secretion would have resulted in a general decrease in the expression of all target genes, and not the expansion of high level Hh signaling observed in the rab8 mutant.*

4. To validate the experiment with ptc 1130X (Fig4), it is critical to validate this tool by checking that it has the same apicobasal localization than wild-type PTC overexpressed in the same condition.

Ptc^{1130X} is used here as a tool to trap secreted Hh (see also our answer to Reviewer 2). As it is unable to be endocytosed, it cannot display the same apicobasal localization as wild type Ptc, which is not observed at the plasma membrane but only in the endocytic compartment.
Importantly, *we show that the apico-basal distribution of Ptc^{1130X} is similar in presence or absence of rab8 (for example Figure 4H',I').*

5. Figure 4: Why do extracellular HH levels drop at the AP border in the "quantification curves"? It should stay high in the posterior region? Why are the distances now in micron and no longer in pixels?

The signal drops because the measurement is done only in the ptc domain by creating a Ptc^{1130X} mask, and applying it on the Hh channel before quantification. This protocol is now explained with illustrations on the NEW Supplementary Figure 4E. Distances are now also converted using the original measurements and images to display the distance in microns in all figures.

6.I do not understand why the authors conclude on p 11 that there is an increase of the release of lateral HH from the producing (P cell) while they also say (and show) a few lines before (last paragraph, page 10) that "there is an increase of extracellular HH at the apical side of producing P cells". Is there a decrease in the lateral region in these P cells?

We found a correlation between the lateral increase of Hh in the P cells with the lateral increase of released Hh present in A cells. We present an example for this now on NEW Figure 4G-G'. Since the increase in apical Hh found in P cells does not correlate with an increase of apical released Hh in A cells, it suggests that apical Hh is not released from P cells. We propose later that this is potentially due to an endocytic/recycling defect of Hh.

The consequence of the change in Hh staining in the producing cells is difficult to evaluate regarding the gradient of Hh activity. This is why we believe that it is more informative to quantify Hh that can be trapped in receiving cells, which allows us to evaluate the "released" Hh. We have modified our text to explain this point and instead of using secretion, we now talk about final distribution of Hh.

I also recommend that the author carefully quantify the distribution of HH along the apicobasal axis.

We thank the reviewer for this suggestion. We quote here our answer to Reviewer 2 as they raised essentially the same point:
"we also carefully quantified extracellular Hh on XY sections on more discs both in the posterior compartment and also on the surface of the anterior Ptc^{1130X} expressing cells (NEW Figure 4C-G') in the revised manuscript. We now show that the distribution of extracellular Hh in Hh producing cells of the $rab8^{U1}$mutant is shifted towards the lateral disc regions. This correlates well with the increase of released Hh found laterally in the receiving cells of mutant discs. We also present crops of the A/P boundary on the NEW Figure 4C-F panels to more clearly illustrate the differences."

7. In Fig 4S, it seems that rab8U1 has an effect on actin accumulation, especially in the apical region? If the case, this should be taken into consideration. If not the case, such variation is a good illustration of why the author should absolutely analyses and quantify more discs, and apply a statistical test that takes these variations into account (see above).

We are not sure which panel the reviewer is referring to, we assume it is Supplementary Figure 4A' and C'. In general, we used the actin channel to mark the apical membrane domain, and the apical position is where the cortical actin is at the highest level (see for example Z section in NEW Figure 4A''). The level of actin is variable and change in intensity is not observed consistently in all samples when comparing wild-type and rab8 mutant discs. We could present another disc if necessary. We followed the reviewer's suggestion and have quantified more discs (see above). Note that in the original manuscript we also showed that the distribution of apico-basal markers is not affected in the rab8 loss-off-function conditions we used (RNAi, degrad system, or mutant, Supplementary Figure 2), and that cell size is similar to that of the wild type (data not shown). Additionally, we also did not observe increased cell death in the rab8 mutant (Supplementary Figure 1).

8. The study of the effect of Rab8 on Rab5CA in figure 6 make sense only if the apicobasal distribution of wild-type Rab5 has the same distribution. Could the author show it or refer to any published work that shows it?

We agree and thank the Reviewer for this comment. We present now such a comparison in NEW Supplementary Figure 6A-C. As Reviewer 1 also raised this issue we quote our answer to them here: "We first analyzed the distribution of both the Rab5 knock-in ($Rab5^{KI}$) and UAS-$Rab5^{CA}$ variants. Both show a similar strong enrichment at the apical and subapical domains (between the 0 and 5 micron mark) close to the Cadherin staining (NEW Supplementary Figure 6A-C).

9. It would also be interesting (and important) to understand whether a Rab8 depletion acts on all Rba5 endosomes or specifically on those that carry Hh. Could the authors analyze whether the pool of Rab5 vesicles that are co-labelled with HH are more mislocalised than the HH negative Rab5 endosomes.

This is a very interesting point. When we looked at rab8 mutant discs with Rab5KI, we saw a mislocalization of rab5 in both the distal anterior (where Hh is not present, data not shown) and posterior compartments. The same is true for the $Rab5^{CA}$ variant when expressed with apGal4 in a $rab8^{U1}$ mutant background. This would clearly imply that rab8 loss-of function has an effect on Rab5 endosome distribution which is independent of their Hh content. But, when we looked specifically at Hh containing $Rab5^{CA}$ endosomes as suggested, we saw that they tend to localize more laterally than the ones not containing Hh in the posterior compartment of $rab8^{U1}$ mutant discs. We present this new data on NEW Figure 7I. Altogether we can conclude, that actually rab8 loss-of- function has a general effect on the early Rab5 endosomes, but this is more pronounced for those containing Hh. We also show that this rab8 phenotype component is likely independent of Ihog levels, as Rab5 endosomes are still mislocalized in a rab8-Ihog conditional double mutant tissue (NEW Figure 8I).

10. In Figure 6N, the blot showing the immunoprecipated proteins is of very poor quality compared to the other blots and the absence of Rba8 coimmuprecipitation is not convincing as Ihog itself is barely detected in the immunoprecipitated fraction. Given this and the fact that this mapping does

not add to the story, I suggest to remove it.
As this was repeated several times we would prefer to keep it in the presentation. Upon over exposure of the blot, the absence of Rab8 is confirmed. Due to the high background, we have chosen this blot exposure.

Surprisingly, it seems that in S2 cells Rab8 overexpression increases the levels of Ihog, while its absence in vivo also increase Ihog levels. How do the authors explain this?
'*This effect was not observed consistently in the different experiments performed on cultured cells. Differential expressivity of the Ihog construct is likely due to the transfection conditions. The analysis of Ihog level in the wing disc is more physiological and is not prone to transfection variation.*

MINOR POINTS
1. Could the authors indicate how they classify the discs according to their size in Fig 1? Automatic quantification of the surface? Double-blind sorting by eye?

We used double blind sorting by the two first authors. We added this to the Materials and Methods.

2. The authors have to carefully check their references to the different panels of the Figures, including the S figures. For instance and among many others: in the first paragraph of the p 7, the authors refer to figure S2A-D, while this part concerns only the panels S2A and B…; Fig 2C comes before 2A and 2B; S6D before S6A…; on the last lane of p19, 4G is probably 4G-H; it also seems that there are not references in the main text to some panels form the Sup figures (as figure S5A-F with the a PKC …)

We thank the reviewer for this comment and made the appropriate changes.

3. Brackets are missing for “Figure S1H-Q” p 8

Lettering Supplementary Figure 1 is simplified now, and we added the brackets to the main text.

4.P 12 A reference should be added at the end of the first paragraph (on Ihog and HH release)

This is now corrected.

5. Figure 5: Could the authors explain what the large fold seen in the z sections compared to the z sections shown in the other figures. Could the authors also shown xy sections for ex Ihog?

This is due to a mounting difference between the different slides. We also present now XY sections on NEW Figure 5C-C'.

6. RNAi is directed against mRNA, and should therefore be written with the name of the gene (in tialics) not of the protein

This is now corrected.

Second decision letter

MS ID#: DEVELOP/2020/191791

MS TITLE: Rab8 GTPase differentially controls the long- and short-range activity of the Hedgehog morphogen gradient by regulating Hedgehog apico-basal distribution

AUTHORS: PASCAL THEROND, Tamas Matusek, Tanvi Gore, Gisela D'Angelo, Cecile Giodano, Catherine Rabouille, and Thomas Tognacci

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.

Reviewer 1

Advance summary and potential significance to field

The authors performed many of the control experiments that were requested, and the manuscript is now sufficient for acceptance in Development.

Comments for the author

Changes to Figures/text:
- The new data shows that the changes to Hh target gene expression are due to Rab8 function in posterior cells and not the anterior cells.
-The short range targets are now clearly extended in a Rab8 knockdown using the ap-Gal4 driver.
-Figure 5B shows a clearer demonstration that Ihog is stabilized on the lateral side.
-The apical-basal z-projections were re-analyzed. Changes to the distribution of the endosomes are difficult to see by eye but were quantified and measured.
- Genetically, it wasn't possible to test whether Hh had impaired apical release in the Ihog/Rab5 double mutant with our suggested experiment but other experiments were done to support this finding.

Reviewer 2

Advance summary and potential significance to field

There is controversy in the field about the different ways extracellular signals are distributed in tissues, and how they form well regulated, stable gradients that dictate developmental outcomes. Here the authors show that even an indirect effect on the Hedgehog signaling factor, which is exerted by Rab8 via the trafficking of a co-factor, Ihog, can make a significant difference in the shape of the final gradient.

Comments for the author

In this revised version of the manuscript the authors have introduced a number of significant changes that make the conclusions of the manuscript easier to evaluate. In particular the many improvements in the Figures now demonstrate the important points of the manuscript in a much more convincing manner.
Specifically:
1) The quantifications in Fig. 1 and elsewhere are very helpful and demonstrate the effect of the rab8 loss in the posterior compartment on anterior cells more convincingly than what was presented before. Figure 3 is also much improved in this respect.
2) quantification of En and dpp in sFig1 is also helpful, since the effects only encompass a few rows of cells.
3) the new quantification of Ihog is also very helpful and convinces me in a more reproducible manner of the conclusions, compared to what was presented in the original manuscript 4) the explanatory scheme next to Fig 4 is very helpful and alleviates my previous confusion.
In addition, the quantification of extracellular Hh is now more convincing with the additional numbers of discs analyzed.
5) the addition of an apical marker (sFig6) was also very helpful, as it was not really clear before what was apical and what was lateral which made it impossible to appreciate the results.
6) similarly the new Fig 5 is much clearer.

Overall the improvements have convinced me that the data substantially support the conclusions provided by the authors. I find that the data now argue strongly for a differential effect of Rab8 on

Ihog trafficking and thus affecting gradient formation by Hedgehog. While clearly not the only mechanism to regulate the Hh gradient in the wing disc, the contribution by Rab8 is of interest to the wider developmental community working on gradients and morphogens.

I would therefore now recommend publication of the manuscript in its revised form without further revisions.

Reviewer 3

Advance summary and potential significance to field

This work concerns how the apicobasal distribution of the Hedgehog (Hh) morphogen is controlled. This issue is an important one in the Hedgehog field and is also of interest for scientists in the fields of development and cell biology, as it raises the issue of how cells respond to different doses of morphogen and of how cell trafficking controls a morphogen gradient. More specifically, this manuscript shows that the Rab8 protein, which is known to control apicobasal trafficking, controls the apicobasal distribution of Hh and therefore the expression of its target genes in the responding cells. It seems to do so by two means: it prevents the endocytosis of Ihog, a Hh co-receptor that normally prevents HH spreading from posterior cells and it controls the apical distribution of the Rab5 endosomes in which HH is endocytosed.

Comments for the author

The authors addressed most of the points that I've address. I still regret however that a quite low number of discs were examined (most of the time around 5).