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Review COMMONS

Hherisomes, Hedgehog specialized recycling endosomes, are required for high level Hedgehog signaling and tissue growth

Sandrine Pizette, Tamás Matusek, Bram Herpers, Pascal P Thérond and Catherine Rabouille
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Reviewer 1

Evidence, reproducibility and clarity

In this manuscript Pizette et al., investigate the detailed localization of the morphogen reproducibility Hedgehog (Hh). Using immunogold labelling on ultrathin cryosections on Drosophila wing and clarityimaginal discs, they report that 90 % of Hh localizes to a an endocytic compartment that can be assimilated to recycling endosomes because of its dependence on the small GTPase Rab11 known to be associated with late recycling endosomes (on the opposition to Rab4 which is generally rather associated with early recycling). The authors called this organelle as Hherisomes. Hherisomes appear to emanate from sorting endosomes as generally bona fide recycling tubules in most cells (Klumperman and Raposo, CSH reviews, 2014).
The results are interesting, of high quality, and mostly support the conclusions of the authors.

I have nevertheless some comments and questions that could be addressed by the authors.
- As there are other GTPases involved in recycling can the authors exclude any role of Rab4?
- If I am not wrong Rab5 and Rab4 have been involved in internalization /recycling of Hh (D'Angelo et al. Dev Cell , 2015). Maybe this should be discussed to avoid misunderstanding by the readers?
- The Hherisomes are likely to represent a very specialized recycling endosome. I am wondering whether bona fide recycling endosomes exist in these cells committed for normal recycling of other cargo? Or there these very same recycling endosomes that are also exploited for general recycling?
- One point that is unclear for me is whether Hherisomes are only observed after boosting by overexpression.?
- These compartments are in my opinion quite analogous to Birbeck granules of Langerhans Cells that are also specialized recycling endosomes (Mc Dermott R, Mol Biol Cell. 2002 Jan;13(1):317-35). Maybe it would be of interest to make an analogy? If there are some?
-The density of the content of these recycling endosomes and actually also their size puzzles me. In some micrographs one can also appreciate a particular structure with some striations? Is this common? Do the authors have any hypothesis on such density? (Fig. 4A right panel)

Significance

-In any case the arguments of the authors explaining why they were not observed before are perfectly pertinent. The contribution in the field is significant . Maybe the authors should somehow

explain how these results are integrated with the former reports indicating the role for other Rabs such as Rab5 and Rab4

My expertise is on intracellular trafficking, endosomes, Lysosomes, Related Organelles and Electron Microscopy

Reviewer 2

Evidence, reproducibility and clarity

Hedgehog is a secreted signalling molecule that spreads over multiple cell diameters to regulate tissue growth and patterning. Hedgehog proteins are dually lipid modified. These lipid modifications play an important role in receptor binding and signal transduction; however, they also make Hedgehog proteins hydrophobic, raising the question of how these lipid moieties are dealt with to allow secretion and transport to receiving cells. Many models have been proposed to explain how the lipid moieties are shielded to allow diffusion, including exosomes, cytonemes and lipophorin particles. Indeed, each of these systems may be at play and the range and level of Hedgehog signalling could be determined by the amount of Hedgehog directed to a specific carrier.

Using the Drosophila imaginal wing disc, a well characterised system for investigating Hedgehog transport and diffusion, the authors explore the nature of the intracellular compartments involved in Hedgehog secretion. Using immune-electron microscopy they visualise Hedgehog at the ultrastructural level and identify an endocytic compartment in which Hedgehog accumulates or traverses prior to secretion, which the authors term a Hherisome. The authors claim that Hedgehog endocytosis is required for delivery of Hedgehog to, and formation of, Hherisomes. They demonstrate that the compartment is not degradative in nature but rather represents tubular structures emanating from multivesicular bodies. Overexpressed Hedgehog or Rab11, both a marker of and an essential component of recycling endosomes, boosts Hherisome formation. Conversely, overexpression of the transporter Dispatched, which has a poorly understood but essential role in controlling Hedgehog secretion, inhibits Hherisome formation. This leads the authors to propose a model in which two pools of Hedgehog, which trigger signalling to different intensities, exist and that Dispatched and Rab11 may control the trafficking of Hedgehog to these distinct pools, with the Rab11/Hherisome pool required to sustain physiological Hedgehog activity.

Major comments:

These data are of high quality, in particular the EM and, as a whole, well quantified with good controls in place. However, I am not convinced these data are sufficient to support the authors claims. The authors propose to have identified a novel endocytic structure called the Hherisome, the formation of which can be driven by the overexpression of Hedgehog and Rab11 and inhibited by Dispatched. Yet they do not back up this claim with any hard quantification. The authors claim Hedgehog overexpression results in Hherisomes appearing 1.5 times more abundant at the apical side but then do not show the data. Likewise, the authors suggest Rab11 overexpression boosts biogenesis of Hherisomes but without the quantification to back up such a claim. The authors do convincingly show both manipulations lead to an increase in Hedgehog labelling of endocytic tubules but this can be explained by overexpressed Hedgehog saturating the endocytic system and the fact that overexpression of Rab proteins, including Rab11 (Wilcke, M et al. JCB 2000), perturbs endosome morphology and function with consequences for the distribution and transit of endosomal cargo. As such the tubular structures may not represent a novel specialised compartment like the authors propose but rather a classical endosomal recycling tubule through which Hedgehog is transiting. Hydrophobic regions of endosomal proteins have been shown to insert directly into the hydrophobic lipid tail region of the membrane to drive membrane curvature by expanding the area of one lipid monolayer leaflet relative to the opposite leaflet, forcing the bilayer to take on curvature and drive the formation of tubules (such is seen with the clathrin accessory protein epsin). It is conceivable that Hedgehog, via its lipid moieties, could function similarly to drive tubule (Hherisome) formation but it is also conceivable that this could be an artefact of Hedgehog overexpression.

The authors findings that Dispatched and Rab11 overexpression have opposite effects on high level Hedgehog signalling is clear. The rescue of growth in Dispatched mutants by Rab11 overexpression is particularly impressive. However, these findings are largely observational with little mechanistic insight. In order to make sense of these findings and make a significant advance in the field it would require identifying how these manipulations affect Hedgehog targeting i.e. an increase in Hedgehog targeting to exosomes versus cytonemes etc...

Minor Comments:

The authors findings that Hedgehog first needs to be endocytosed to reach these endocytic structures and that these structures are not degradative in nature is very convincing and fits with what is already known about Hedgehog secretion. However, this seems at odds with a previous finding that Dispatched is necessary for Hedgehog endocytosis (D'Angelo, G et al. Dev Cell 2015) as the authors show Dispatched is not required for Hherisome formation.
How do the authors reconcile these two findings?

The authors also observe Hedgehog in tubular Hherisome structures in the anterior compartment. Do they believe this pool of Hedgehog is capable of another round of secretion?

The loss of Dispatched has been shown to cause a dramatic accumulation of Hedgehog in secreting cells (Callejo, A et al. PNAS 2011). The authors make no mention as to whether they see such an accumulation in their EM images and if so where does Hedgehog accumulate? They state Hedgehog in tubular endocytic structures is unaffected, does this mean Hedgehog is accumulating elsewhere such as the plasma membrane?

In conclusion, without further evidence I think the authors claims to have identified a novel endocytic structure the Hherisome are speculative and that the structure likely represents a Rab11 recycling tubule through which Hedgehog transits through following endocytosis and prior to resecretion. The data and the methods are presented in such a way that they can be reproduced and the experiments are adequately analysed.

Significance

I think the authors findings do not represent a significant advance for the field. It has already been demonstrated that Hedgehog undergoes endocytosis prior to resecretion (D'Angelo, G et al. Dev Cell 2015 and Callejo, A et al. PNAS 2011). The authors propose that formation of a specialized Hedgehog containing endocytic structure is required for high level Hedgehog signalling but I find a lack of evidence to prove this novel structure is not merely a normal recycling tubule. As such the work builds upon and confirms previous findings on the need for Hedgehog endocytosis prior to secretion but does not represent a big advancement.

I would suggest the Hedgehog signalling community and developmental biology community are the main target audience. They will find the observation that Rab11 and Dispatched overexpression can modify the intensity of Hedgehog signalling and that Rab11 overexpression can rescue the loss of growth seen in Dispatched mutant wing discs the most interesting findings. These findings suggest the existence of multiple Hedgehog pools with different signalling intensities. However, the findings lack real mechanistic insight.

Referees cross-commenting

I agree with all the comments of the other reviewers.

Reviewer 1 raises a valid question. How do these findings relate to previous findings from the authors in which they show a role for Rab4 in recycling Hedgehog after its internalisation? Rab4 RNAi has been shown to affect the long-range activity of Hh. It would be interesting to known what impact it has on Hherisome formation or at least add a section to the discussion regarding Rab4 and how it could relate to Rab11 and Hherisomes.

Reviewer 3 appears to share my main concern that, in my opinion, the authors have not done enough to prove that these Hherisomes are a novel specialised compartment, as opposed to a normal recycling tubule emanating from an endosome.

Reviewer 3

Evidence, reproducibility and clarity

In the article “Hherisomes, a new Hedgehog-containing endocytic compartment required for high level Hedgehog signaling and tissue growth" the authors describe how spreading and secondary secretion of Hh occurs from a specialized recycling compartment and that Hh sorting can be modulated by positive regulator disp and rab11 with effects on Hh signaling and wing pouche growth. Using the Drosophila wing imaginal disc as a well-studied morphogen tissue, the authors use genetic manipulation with the UAS-Gal4 system and immuno-electron microscopy to adress via which subcellular compartment Hh is trafficking near the apical domain of secreting and receving cells. Interestingly, the identified membrane-bound compartment contains 90% of intracellular Hh, according to EM quantifications. This compartment only weakly colocalises with LAMP1 or internalized BSA, but with rab11, thus corresponding to recycling endosomes. Overexpression of Hh as well as Rab11, increases their formation and Hh content. In contrast, providing excess of the Hh transporter Dispatched (Disp), significantly reduces their formation.
Moreover, sorting of Hh into this compartment is coupled to high Hh signaling activity and tissue growth. As Rab11 overexpression stimulates both high level Hh signaling and disc pouch growth whereas Disp overexpression decreases both.

Major comments:

In general, it is hard to understand how cytoneme and apical secretion are trafficking routes of Hh, but both end up in the same (90%) endocytic/recylcing compartment. I also disagree with the authors to call it a different name, when instead it is a recycling endosome compartment. It is worth showing these very nice EM analyses but I would prefer the authors would not oversell it. EM quantifications should be present in the main figures to convince, as they look rather descriptive.
The paragraph about the identity of Hherisomes is rather weak, as the conclusion suggests. They are not lysosomes...but recycling endosomes. This has been demonstrated by the authors before that Hh is secreted a second time via rab4 endosomes to the apical side (D'Angelo Dev Cell 2015). How is this study expanding the previous findings and connects to them?
The authors mention that live imaging of Hherisomes with fluorescently tagged Hh is not possible, but would pulse-chase immunofluorencence co-stainings with fixation methods preserving the endosomal tubules be a possibility to back up the EM findings? In that respect, how does the story relate to rab4 recycling compartment? How big is the overlap of rab11/rab4/Hh?
Besides BSA internalization can the authors perform recycling/pulse-chase assays of Hh to show that it is endocytosed Hh accumulating in rab 11 endosomes and secondly that this endocytosed Hh gets resecreted via the „Hherisomes".

Minor comments:
Language can be improved. Especially some more explanation and details of the experimental model system in each section to help people from other areas of cell and developmental biology understand.
Regarding Wg staining of those recycling endosomes, is it within the expression domain of Wg or outside? Was quantification done as with Hh?

Significance

The diversity of morphogen secretion and trafficking models, for Hh as well as Wg, suggests that aspects of morphogen trafficking and regulation of gradient formation remain to be discovered. Thus, in the light of different models of Hh release it is a valuable to approach Hh intracellular localisation by immuno-electron microscopy analysis. But I feel the study is lacking some functional

testing of their hypothesis and complementary methods to show, what is the reason for this specialized compartment, what is ensuring its specificity?
It is important to understand why endocytosis is so important for secretion of morphogens and gradient formation. This study shows that the majority of Hh is present in recycling endosomes in secreting cells, and modulation of recycling endosomes formation modulates Hh signaling in the receiving compartment.
This work is important for the field of morphogen signaling.
I work on morphogen secretion and signaling in Drosophila.

Referees cross-commenting

Yes I fully agree.
I would also like to know about the relation to rab4 sorting.

Author response to reviewers' comments

Dear Editor at the *Journal of Science*

Please find our rebuttal following the reviews we got after submitting to *Review commons*. There are two parts

-General response to reviewers.
-Point-by-point responses to reviewers.

GENERAL RESPONSES TO REVIEWERS.

We would like to thank the reviewers for their valuable comments and constructive criticisms of the manuscript. Although their reviews are extensive and detailed, many of their concerns overlap and are mostly conceptual.

After careful reading of their reviews, we outline the following recurring points and provide a response that will be incorporated in a revised manuscript when invited to do so.
In a nutshell:

Point 1: All reviewers question whether Hherisomes exist in the absence of Hedgehog (Hh) overexpression, that is, with endogenous Hh, or ask for quantifications.

>> In the first part of the results (Figure 2A, A'), we have clearly stated that Hherisomes exist in wild type discs, albeit at smaller size, numbers and Hh density than when full length Hh is overexpressed (see Table 1). To clarify the matter, we will incorporate one typical quantification of the Hherisome features in both backgrounds (see additional new Table) below.

New Table : Hherisomes features in the Drosophila disc posterior compartment in wildtype and upon Hh overexpression.

Genotype	Numbers of Hherisomes profiles/ per disc cell section.	Hh Labeling density in Hherisomes/um2 (as in Table 1)	Size nm
OreR (wildtype_	1	73.5±50	125±78nm
UAS HhM4, hhGal4	4.2	520±30	205±130nm
Fold increase	4.2	7	2

The quantification of the numbers of Hherisomes was performed by scoring disc sections in each

background directly at the microscope. 144-243 cells profiles were examined for their number of Hherisomes that were directly recorded as “Number of Hherisomes/cells”. All the data were added, hence the lack of SEM. This will be added to Materials and Methods. Representative pictures are shown in (Figure 2B, B’’, arrows, of the original version).
For the labeling density, those are the results already presented in Table 1 and the method is presented in the original manuscript.

Point 2: Reviewers 2 and 3 challenge us as to whether the specialized Hh enriched recycling tubules that we have identified, characterized and named Hherisomes, are a novel compartment, or whether they are classical recycling endosomes through which Hh transits along with other cargos. Reviewer 3 is even not favorable to the introduction of a new name.
>> What is correct in the reviewers’ claim is that Hherisomes is not a new compartment. They are recycling tubules emanating from endosomes, likely to correspond as to what is generally referred to, as “recycling tubular endosomes”. What is important here, is that:

-These Hh enriched tubules are often close to the vacuole of an endosome. In fly discs, the endosomal profiles are all similar as they all contain internal vesicles and correspond to broad definition of Multi Vesicular Bodies (MVBs). MVBs are the only structures observed in disc cells that are compatible with the endosomal pathway. Typical morphology corresponding to early endosomes and lysosomes are not observed. Therefore, the observed MVBs, although morphologically homogeneous, likely correspond to functionally different endosomes. Of note, MVBs also contain Hh, but at a much lower density (see Table 1).

-Importantly, the Hh enriched tubules are morphologically different from these endosomes.

-The Hh loading of these tubules is boosted by Rab11.

Therefore, we propose that Hherisomes are “Rab11 dependent recycling tubular endosomes”. Although it is correct that recycling endosomes have been described before, it has not been done in the context of Hh.

The question is whether they are classical or specialized/Hh-dedicated recycling tubular endosomes. Are they used by other cargo?
To our knowledge, other cargos have not been studied in details in these cells, so it is therefore difficult to adequately comment on this point. What we can say is that the overexpression of neither the lipidated Wg protein, nor a secreted form of GFP, nor the Hedgehog-N peptide fused with GFP, and not even the dually lipidated Hh tagged with GFP (see point 17), do induce their formation and are enriched within the Hherisomes. Thus, the overexpression of a potential cargo, even one that is covalently bound to a lipid moiety does not trigger the biogenesis of this compartment, or its enrichment in this compartment.

On the contrary, overexpression of full length untagged Hh led to a 4.2-fold increase in Hherisome number accompanied with a 4.5- to 7-fold increase of Hh density therein (see point 1 and original Table 1), when compared to endogenous Hherisomes that are present in these wildtype cells. In this regard, we claim that Hherisomes is a new specialized compartment and we wish to keep this name. It is likely that the biochemical properties of Hh enables it to be preferentially captured and enriched in recycling tubules, perhaps by what Reviewer 2 calls “hijacking”. Therefore, giving these Hh positive recycling tubules a specific name that is linked to Hh seems justified. As Hherisomes represent recycling tubular endosomes (see above), we could name these compartments “Hh specialized recycling tubular endosomes”. We rather name them “Hherisomes” that is less of a mouthful, even though we also agree that the multiplication of names does not always clarify things. We will try our best that readers understand what Hherisomes are. In this regard, we have slightly modified the title.

In fact, our naming of the Hh compartment is very similar to “MHII compartments” that are lysosomes specialized in antigen presentation by class II molecules, and to Birbeck granules of Langherhans cells that are also specialized recycling endosomes enriched in Langherin.

a. All reviewers ask about the relationship between Rab4 (Rab5), Rab11 and Hh trafficking in the Hh producing cells.

>> It is correct that the Rab4 data reported by D'angelo et al (2015) suggested that a pool of Hh follows a rapid recycling route from early endosomes after its endocytosis. Here, we report the Hh trafficks through recycling tubular endosomes where it is stored (Hherisomes), and we clearly show with our detailed ultrastructural analysis that Hherisomes are not classical vacuolar early endosomes. This has not yet been reported before and is therefore a new piece of Hh biology.
We cannot formally exclude that Rab4 may have a modulating function in the formation of Hherisomes. However, D'Angelo at al (2015) has shown that silencing Rab4 decreases Hh **long range/low level signaling,** but not short range. If the Rab4 pathway were to be competing with the Rab11 pathway, the absence of Rab4 should lead to a pool of Hh more accessible to Rab11. Therefore, the overexpression of Rab11 should mimic a Rab4 loss of function phenotype. However, in the present manuscript, Rab11 overexpression led to an increase of Hh **short range/high level activity** without change in long range. Therefore, we propose that Rab4 likely works in parallel to Hherisome formation. We will discuss this at more length in a revised version.
Of note, several other Rabs are now linked to Hh. However, it is not possible at this stage to test them all with this approach. We argue that we provide a novel piece of the Hh puzzle, not the completion of the full understanding of how Hh is and trafficked intracellularly, spread and signals.

Point 4: Reviewers 2 and 3 also found that the IEM studies should be expanded to mechanistic data including what makes Hh more competent in short range signaling, how loss of *dispatched* antagonizes this process, and what ensures the compartment specificity for Hh.
>>In this manuscript, we have performed experiments showing a function for Hherisomes. We provide clear evidences that they are necessary for efficient Hh short range signaling and tissue growth.
We agree that understanding what happens to Hh during its trafficking in Hherisomes and how it acquires this signaling and growth property is an interesting, perhaps critical, outcome of this study. Is it oligomerisation, interaction with different proteins, such as proteoglycans and lipid molecules, further modifications? This will be for the future. Digging into the specifics of these is at present beyond the scope of this study as it would require years of new research.
With regard to Disp, as its overexpression prevents Hherisome formation, we argue that in these conditions Hh can no longer acquire the specific properties provided by trafficking through Hherisomes and is therefore less competent in short range/high level signaling.
As for the specificity of this compartment for Hh, it might be due to wild type Hh properties and not only to its covalent links to specific lipids. Indeed, only full length untagged Hh, but neither a form devoid of cholesterol, nor a tagged lipidated form of Hh, can boost Hherisome formation (see point 17).

Point 5: Reviewers 2 and 3 also ask what is the relationship between Hherisomes, cytonemes and exosomes?
>>Regarding how Hh trafficking through Hherisomes could specifically target it to cytonemes and exosomes, we agree that the link is hypothetical. Please note that our study was not specifically aimed to provide this link. At the start, the notion was to look in an unbiased manner at Hh trafficking in producing cells. Either the observations would fit with existing observations or it would open new avenues, which appears to be the case.
Furthermore, for the first time, the resolution of intracellular Hh distribution indicates that it is mostly in Hherisomes. However, a small portion is also present in the vacuolar part of endosomes displaying internal vesicles (MVBs), the ones potentially leading to the release of exosomes.

Point 6: Reviewer 2 also raised the argument that our finding is not significantly new. According to him/her, this manuscript only confirms at the ultrastructural level some of those observations that were previously made by immunofluorescence.
>>We respectfully disagree. First, this present study goes way beyond only confirming Hh endocytosis. Our high resolution ultrastructural analysis identifies that following endocytosis, Hh is stored in recycling tubular endosomes, the Hherisomes. This was not proposed after immunofluorescence studies of D'angelo et al (2015) (see point 3). Second, this ultrastructural resolution of Hh distribution has never been achieved before, which in itself, is valuable. Third, we identify that the process of Hherisome formation is antagonistic to, and likely independent of Dispatched function, a point clearly exciting for the Hh community. Fourth, we show that Hh trafficking through recycling tubules appears to be essential for signaling and disc growth. The latter aspect is highly original. Reviewer 2 kindly remarks that it is very impressive.

In this regard, we reiterate here the several advances that our manuscript represents for the Hh field
a. What is original in this manuscript is that most of its data are generated from ultrastructural analysis of Hh distribution in many different genetic backgrounds in a quantified manner. This gives our manuscript an **unprecedented cell biological** edge in the Hh field which has been lacking until now.

b. We have not stopped at the description of the Hh containing recycling tubular endosomes (the Hherisomes) in these backgrounds. Instead, we experimentally assign a function to these compartments through detailed known assays in **developmental biology**. We establish a strong correlation between the presence of Hherisomes (and Hh therein) and signaling and tissue growth. Interestingly, these two functions are modulated by Disp and Rab11 expression. This has so far not been shown.

c. Hh can be endocytosed and reach the recycling compartment in a *dispatched* mutant disc (not only in the posterior but also in the anterior compartment), arguing that at least a portion of Hh endocytosis is Dispatched independent.

d. We acknowledge that the *in vivo* manipulations we present do not clarify the functional link between Hh recycling routes and carrier loading (cytonemes, exovesicles, see **point 5**), but this was not our intention. These experiments were not meant to prove or disprove existing hypotheses. Instead, they provide **new avenues for the Hh community**.
These advances will be discussed in more detail in a revised manuscript.

POINT-BY-POINT RESPONSE TO REVIEWERS
Reviewer #1
Evidence, reproducibility and clarity.
The results are interesting, of high quality, and mostly support the conclusions of the authors.
>>We thank Reviewer 1 for their insightful comments, we hope our answers provide more context, and clarify their concerns.

I have nevertheless some comments and questions that could be addressed by the authors.

7. As there are other GTPases involved in recycling, can the authors exclude any role of Rab4?
>>See point 3**.**

8. If I am not wrong, Rab5 and Rab4 have been involved in internalization /recycling of Hh (D'Angelo et al. Dev Cell, 2015). Maybe this should be discussed to avoid misunderstanding by the readers?
>>See point 3**.**

9. The Hherisomes are likely to represent a very specialized recycling endosome. I am wondering whether bona fide recycling endosomes exist in these cells committed for « normal « recycling of other cargo? Or there these very same recycling endosomes that are also exploited for general recycling?
>>See point 2 **and our original discussion:**

10. One point that is unclear for me is whether Hherisomes are only observed after boosting by overexpression.?
>>See point 1.

11. These compartments are in my opinion quite analogous to Birbeck granules of Langerhans Cells that are also specialized recycling endosomes (Mc Dermott R, Mol Biol Cell. 2002 Jan;13(1):317-35). Maybe it would be of interest to make an analogy? If there are some?
>>We thank the reviewer for pointing to this paper that we were unaware of (PMID: 11809842). In fact, it appears that Birbeck granule biogenesis follows a route that is very similar to what we propose for Hherisomes. Their structure is similar, although the Birbeck granules appear more rod-like than Hherisomes. Their formation is downstream of endocytosis from the plasma membrane and depends on Rab11. And both represent the recycling tubules emanating from recycling endosomes and store specific proteins. We will add this analogy in the discussion together with the GLUT4 compartment.

We will mention these granules in the discussion.

12. The density of the content of these recycling endosomes and actually also their size puzzles me. In some micrographs one can also appreciate a particular structure with some striations? Is this common? Do the authors have any hypothesis on such density? (Fig. 4A right panel).
>>The density in IEM can be puzzling and can in fact be artificially created by the antibody/gold complexes used for detection that concentrate the uranyl acetate used for contrasting the sections. It can also be created by the aldehyde crosslinking of the proteins within a structure, especially when the density is high, as it is the case for the secretory granules of the exocrine pancreas for instance. The density also appears to be linked to lipid density and packing.
About the striations, they were not very common and we are not sure what they represent.

Significance:
13. In any case the arguments of the authors explaining why they were not observed before are perfectly pertinent. The contribution in the field is significant. Maybe the authors should somehow explain how these results are integrated with the former reports indicating the role for other Rabs such as Rab5 and Rab4.
>> Thank you and see point 3.

Reviewer #2
Evidence, reproducibility and clarity:
Major comments:
14. These data are of high quality, in particular the EM and, as a whole, well quantified with good controls in place. However, I am not convinced these data are sufficient to support the authors claims. The authors propose to have identified a novel endocytic structure called the Hherisome, the formation of which can be driven by the overexpression of Hedgehog and Rab11 and inhibited by Dispatched. Yet they do not back up this claim with any hard quantification. The authors claim Hedgehog overexpression results in Hherisomes appearing 1.5 times more abundant at the apical side but then do not show the data.
>> Regarding the boosting of Hherisome biogenesis upon Hh overexpression, we first wish to draw the attention of the reviewer to Table 1 of the original manuscript. There, it is clearly shown that the **labeling density** of Hh in Hherisomes upon Hh expression in the posterior compartment increases **7**-fold for UAS-Hh M4 and **4.5** fold for UAS-Hh M1 when compared to wild type discs (compare line 1 in OreR and lines 2 and 5 for Hh overexpression). However, we also want to draw the attention to the important fact that Hherisomes are visualized and exist in wildtype discs, albeit at lower numbers, size and Hh density.

-Regarding the number of Hherisomes: it increases about 4 times upon Hh overexpression compared to wild type, see **point 1**.

-Regarding the positioning of the Hherisomes: The apical versus basolateral distribution of Hherisomes has a ratio of 1:1 in wildtype discs and 1.5-2.2:1 in discs overexpressing Hh. Therefore, the apical site appears slightly enriched with Hherisomes upon overexpression. The quantification was performed as in point 1, but with distinguishing the apical from the baso-lateral Hherisomes. However, this will not be further detailed in the manuscript.

15. …..the formation of which can be driven by the overexpression of Hedgehog and Rab11 and inhibited by Dispatched
Likewise, the authors suggest Rab11 overexpression boosts biogenesis of Hherisomes but without the quantification to back up such a claim.
>>When Rab11 is co-expressed with Hh, the overall number of Hherisomes in the disc posterior compartment does not significantly change when compared to Hh overexpression alone. 70 such fields were taken into account. We will add this data to the manuscript. What changes is the Hh labeling density of Hh in Hherisomes which increases by more than 2 fold (see original Table 1, compare line 5 to line 6).

New Suppl Table: Number of Hherisomes and their Hh concentration upon Hh, Rab11 and Disp overexpression in the disc posterior compartment

Genotype	Numbers of Hherisomes profiles/ per **10um2 (of positive fields)**	Hh Labeling density in Hherisomes/um2 (as in Table 1)
hhGal4, UAS Hh**M1**,	Post: 2.75	328
UAS Hh**M4**, Rab11YFP, hhGal4	Post: 2.35	721
UAS Hh**M4**, DispHA, hhGal4	Post: 0.15*	Nd

Furthermore, in discs overexpressing both UAS-Hh and UAS-Rab11, some of the Hherisomes became very large. We will adjust the discussion on the latter.

-Upon DispHA overexpression (with UAS-Hh), the number of Hherisomes drops dramatically when compared to UAS-Hh alone (see New Suppl Table above). This is why we refer to a 93% reduction in the manuscript. We will add this to the manuscript.

16. The authors do convincingly show both manipulations lead to an increase in Hedgehog labelling of endocytic tubules but this can be explained by overexpressed Hedgehog saturating the endocytic system and the fact that overexpression of Rab proteins, including Rab11 (Wilcke, M et al. JCB 2000), perturbs endosome morphology and function with consequences for the distribution and transit of endosomal cargo. As such the tubular structures may not represent a novel specialised compartment like the authors propose but rather a classical endosomal recycling tubule through which Hedgehog is transiting.
>> As to whether Hherisomes are a novel specialized compartment, see point 2.
We also point out that Hherisomes are observed in a wild-type context as mentioned above (point 1), so they are formed without saturating the endocytic system. In addition, overexpressing other molecules (such as Lamp1 and Wg) that traffic through endosomes and could potentially saturate the system, does not lead to their enrichment in Hherisomes nor in their formation. Not even dually lipidated Hh tagged with GFP (points 2 and 17).

17. Hydrophobic regions of endosomal proteins have been shown to insert directly into the hydrophobic lipid tail region of the membrane to drive membrane curvature by expanding the area of one lipid monolayer leaflet relative to the opposite leaflet, forcing the bilayer to take on curvature and drive the formation of tubules (such is seen with the clathrin accessory protein epsin). It is conceivable that Hedgehog, via its lipid moieties, could function similarly to drive tubule (Hherisome) formation but it is also conceivable that this could be an artefact of Hedgehog overexpression.
>>We respectfully disagree. There are three arguments indicating that Hh overexpression *per se* does not create an artifact that leads to the formation of Hherisomes. First, Hherisomes exist in wildtype discs not overexpressing Hh (point 1). Second, their formation is not boosted by the overexpression of lipidated Wg (a palmytoylate-modified morphogen, point 2). Third, Hherisome formation is also not boosted by overexpressing dually lipid-modified HhGFP (as mentioned on p18 of the manuscript).

New Table:

Genotype	Numbers of Hherisomes profiles/ per disc **cell** section.	Hh Labeling density in Hherisomes/um2 (as in Table 1)	Size nm
OreR	1 (144 cells over 5 sections)	73.5±50	125±78nm
UAS Hh**M4**, hhGal4	4.2 (243 cells over 8 sections)	520±30	205±130nm
Fold increase	4.2	7	2
UAS Hh**GFP**, hhGal4	1.25 (30 cells)	94±10 (Anti GFP abcam) 88±20 (anti TK)	
Fold increase	1.25	1.1-1.28	

One would expect similar effects if Hherisome formation was solely dependent on lipids and not on wild-type Hedgehog as a whole (point 4). This is not the case and it will be discussed in more details in the revised manuscript.

18. The authors findings that Dispatched and Rab11 overexpression have opposite effects on high level Hedgehog signalling is clear. The rescue of growth in Dispatched mutants by Rab11 overexpression is particularly impressive.
>> We thank the reviewer for this positive comment. We fully agree with this.

19. However, these findings are largely observational with little mechanistic insight. In order to make sense of these findings and make a significant advance in the field it would require identifying how these manipulations affect Hedgehog targeting i.e. an increase in Hedgehog targeting to exosomes versus cytonemes etc...
>>See points 4, 5 and 6**.**

Minor Comments:
20. The authors findings that Hedgehog first needs to be endocytosed to reach these endocytic structures and that these structures are not degradative in nature is very convincing and fits with what is already known about Hedgehog secretion.
However, this seems at odds with a previous finding that Dispatched is necessary for Hedgehog endocytosis (D'Angelo, G et al. Dev Cell 2015) as the authors show Dispatched is not required for Hherisome formation. How do the authors reconcile these two findings?
>> The study presented in D'Angelo et al (2015) used HhGFP and followed its trafficking by fluorescence imaging. As mentioned above, this transgene does not boost the formation of Hherisomes (see point 17) and might not faithfully reproduce all the functions and trafficking routes of full length Hh. In addition, as discussed on page 17 of the manuscript, the IEM analysis reveals a pool of Hh which is not detected by immunofluorescence, likely due to the detergent used for preparation.
Regarding the discrepancy over the role of Dispatched in Hh endocytosis, we find here that in the absence of Dispatched function, a pool of Hh is endocytosed independently of Disp, a fact that was missed in previous studies. These two points will be added to our discussion.

21. The authors also observe Hedgehog in tubular Hherisome structures in the anterior compartment. Do they believe this pool of Hedgehog is capable of another round of secretion?
>>This is a very good question. As we observe Hh positive Hherisomes very distally in the anterior compartment far away from the Hh source, we would argue that it is indeed possible.

22. The loss of Dispatched has been shown to cause a dramatic accumulation of Hedgehog in secreting cells (Callejo, A et al. PNAS 2011). The authors make no mention as to whether they see such an accumulation in their EM images and if so where does Hedgehog accumulate? They state Hedgehog in tubular endocytic structures is unaffected, does this mean Hedgehog is accumulating elsewhere such as the plasma membrane?
>> In dispatched mutant, we did not observe any accumulation in other intracellular compartments. Regarding the plasma membrane, as explained in the discussion of the manuscript, the IEM technique we used can visualize the intracellular Hh but does not allow a quantitative assessment of the extracellular pool. Therefore, we cannot determine the intracellular/extracellular Hh ratio.

23. In conclusion, without further evidence I think the authors claims to have identified a novel endocytic structure the Hherisome are speculative and that the structure likely represents a Rab11 recycling tubule through which Hedgehog transits through following endocytosis and prior to resecretion.
>>See point 2**.**

24. The data and the methods are presented in such a way that they can be reproduced and the experiments are adequately analysed.
>>Thank you.

Significance
25. I think the authors findings do not represent a significant advance for the field. It has already

been demonstrated that Hedgehog undergoes endocytosis prior to resecretion (D'Angelo, G et al. Dev Cell 2015 and Callejo, A et al. PNAS 2011).
>>We respectfully disagree. See point 6.

26. The authors propose that formation of a specialized Hedgehog containing endocytic structure is required for high level Hedgehog signaling but I find a lack of evidence to prove this novel structure is not merely a normal recycling tubule. As such the work builds upon and confirms previous findings on the need for Hedgehog endocytosis prior to secretion but does not represent a big advancement.
>>See point 2.

27. I would suggest the Hedgehog signaling community and developmental biology community are the main target audience. They will find the observation that Rab11 and Dispatched overexpression can modify the intensity of Hedgehog signaling and that Rab11 overexpression can rescue the loss of growth seen in Dispatched mutant wing discs the most interesting findings. These findings suggest the existence of multiple Hedgehog pools with different signaling intensities. However, the findings lack real mechanistic insight.
>>See point 4.

Referees cross-commenting
28. I agree with all the comments of the other reviewers.
Reviewer 1 raises a valid question. How do these findings relate to previous findings from the authors in which they show a role for Rab4 in recycling Hedgehog after its internalisation? Rab4 RNAi has been shown to affect the long-range activity of Hh. It would be interesting to known what impact it has on Hherisome formation or at least add a section to the discussion regarding Rab4 and how it could relate to Rab11 and Hherisomes
>>See point 3.

29. Reviewer 3 appears to share my main concern that, in my opinion, the authors have not done enough to prove that these Hherisomes are a novel specialised compartment, as opposed to a normal recycling tubule emanating from an endosome.
>>See point 2.

Reviewer #3
Evidence, reproducibility and clarity
>>We thank the reviewer for their critical assessment.

Major comments
30. In general, it is hard to understand how cytoneme and apical secretion are trafficking routes of Hh, but both end up in the same (90%) endocytic/recycling compartment.
>> See point 5.

31. I also disagree with the authors to call it a different name, when instead it is a recycling endosome compartment. It is worth showing these very nice EM analyses but I would prefer the authors would not oversell it.
>>See point 2.

32. EM quantifications should be present in the main figures to convince, as they look rather descriptive.
>>EM studies are descriptive by nature. However, they were carefully quantified in the Table 1 that is a main figure and an integral and critical part of the data. It will be printed along the other figures in the published article and be readily available for easy viewing.

33. The paragraph about the identity of Hherisomes is rather weak, as the conclusion suggests. They are not lysosomes...but recycling endosomes. This has been demonstrated by the authors before that Hh is secreted a second time via rab4 endosomes to the apical side (D'Angelo Dev Cell 2015). How is this study expanding the previous findings and connects to them?
>>We identified Hherisomes as recycling tubular endosomes through their link to Rab11. As for the link with Rab4, see point 3.

34. The authors mention that live imaging of Hherisomes with fluorescently tagged Hh is not

possible, but would pulse-chase immunofluorencence co-stainings with fixation methods preserving the endosomal tubules be a possibility to back up the EM findings? In that respect, how does the story relate to rab4 recycling compartment? How big is the overlap of rab11/rab4/Hh?
Besides BSA internalization can the authors perform recycling/pulse-chase assays of Hh to show that it is endocytosed Hh accumulating in Rab11 endosomes and secondly that this endocytosed Hh gets resecreted via the „Hherisomes".
>>We thank the reviewer for these excellent experimental suggestions. We considered these before. The only prerequisite would be that we should see a correlation between the IEM studies and the immunofluorescence. Unfortunately, this is not the case, at least with the permeabilization that we have done. Indeed, we looked at Hh distribution in Rab11 overexpressing discs similar to what we present on Figure6-Suppl1 where we analyzed several parameters such as Hh puncta number, size and intensity. We have not observed any significant change in any of these parameters when Rab11 was overexpressed, when compared to the control. As mentioned above, the discrepancy might solely come from the techniques used, and other milder detergents could be tried. This is the target for a further study.
We have also tried to design a pulse chase experiment with a Hh antibody and develop an endocytic/recycling assay for endogenous Hh, but unfortunately, this was not successful. Last, we are now also reluctant to use HhGFP as we found that expression of this form does not induce HHerisome formation.
Regarding Rab4, see point 3.

Minor comments

34. Language can be improved. Especially some more explanation and details of the experimental model system in each section to help people from other areas of cell and developmental biology understand. Regarding Wg staining of those recycling endosomes, is it within the expression domain of Wg or outside? Was quantification done as with Hh?
>>WgHA was expressed using the dpp>gal4 driver, therefore leading to its expression within the Dpp expression domain that partly overlaps with part of the endogenous Wg domain. As we could not mark both domains simultaneously in this setting by IEM, we could not assess whether Wg reaches recycling endosomes within wg producing cells or as a result of its internalization by Wg receiving cells.

Significance

35. The diversity of morphogen secretion and trafficking models, for Hh as well as Wg, suggests that aspects of morphogen trafficking and regulation of gradient formation remain to be discovered. Thus, in the light of different models of Hh release it is a valuable to approach Hh intracellular localisation by immuno-electron microscopy analysis. But I feel the study is lacking some functional testing of their hypothesis and complementary methods to show, what is the reason for this specialized compartment, what is ensuring its specificity?
>>See point 4**.**

36. It is important to understand why endocytosis is so important for secretion of morphogens and gradient formation. This study shows that the majority of Hh is present in recycling endosomes in secreting cells, and modulation of recycling endosomes formation modulates Hh signaling in the receiving compartment. This work is important for the field of morphogen signaling.
>>We thank the reviewer for their insightful comments, and appreciate their assessment of significance.

37. I would also like to know about the relation to rab4 sorting.
>>See point 3.

First decision letter

MS ID#: JOCES/2021/258603

MS TITLE: Hherisomes, a new Hedgehog-containing endocytic compartment required for high level Hedgehog signaling and tissue growth

AUTHORS: Sandrine Pizette, Tamas Matusek, Bram Herpers, Pascal P Therond, and Catherine Rabouille
ARTICLE TYPE: Research Article

Thank you for transferring your manuscript to Journal of Cell Science. Please revise your manuscript as you have outlined in the cover letter. I look forward to seeing the revised version of the manuscript.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. 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 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. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

First revision

Author response to reviewers' comments

Dear Dr Jennifer Lippincott-Schwarz, Editor at the *Journal of Science*

Please find our rebuttal following the reviews we got after submitting to *Review common*s. There are two parts
-General response to reviewers.
-Point-by-point responses to reviewers.

GENERAL RESPONSES TO REVIEWERS.
We would like to thank the reviewers for their valuable comments and constructive criticisms of the manuscript. Although their reviews are extensive and detailed, many of their concerns overlap and are mostly conceptual.
After careful reading of their reviews, we outline the following recurring points and provide a response that have been incorporated in a revised manuscript.
In a nutshell:

Point 1: All reviewers question whether Hherisomes exist in the absence of Hh overexpression, that is, with endogenous Hh, or ask for quantifications.
>> In the first part of the results (Figure 2A, A'), we have clearly stated that Hherisomes exist in wild type discs, albeit at smaller size, numbers and Hh density than when full length Hh is overexpressed. To clarify the matter, we will incorporate one typical quantification of the

Hherisome features in both backgrounds (see new Suppl Table 1). The quantification method is indicated in the Materials and Methods of the revised manuscript.

Point 2: Reviewers 2 and 3 challenge us as to whether the specialized Hh enriched recycling tubules that we have identified, characterized and named Hherisomes, are a novel compartment, or whether they are classical recycling endosomes through which Hh transits along with other cargos. Reviewer 3 is even not favorable to the introduction of a new name.
>> What is correct in the reviewers' claim is that Hherisomes is not a new compartment. They are recycling tubules emanating from endosomes, likely to correspond as to what is generally referred to, as "recycling tubular endosomes". What is important here, is that
-These Hh enriched tubules are often close to the vacuole of an endosome. In fly discs, the endosomal profiles are all similar as they all contain internal vesicles and correspond to broad definition of Multi Vesicular Bodies (MVBs). MVBs are the only structures observed in disc cells that are compatible with the endosomal pathway. Typical morphology corresponding to early endosomes and lysosomes are not observed. Therefore, the observed MVBs, although morphologically homogeneous, likely correspond to functionally different endosomes. Of note, MVBs also contain Hh, but at a much lower density (see Table 1).
-Importantly, these tubules are morphologically different from these endosomes.
-The Hh loading of these tubules is boosted by Rab11.

Therefore, we propose that Hherisomes are "Rab11 dependent recycling tubular endosomes". Although it is correct that recycling endosomes have been described before, it has not been done in the context of Hh.

The question is whether they are classical or specialized/Hh-dedicated recycling tubular endosomes. Are they used by other cargo?
To our knowledge, other cargos have not been studied in details in these cells, so it is therefore difficult to adequately comment on this point. What we can say is that the overexpression of neither the lipidated Wg protein, nor a secreted form of GFP, nor the Hedgehog-N peptide fused with GFP, and not even the dually lipidated Hh tagged with GFP (see point 17), do induce their formation and are enriched within the Hherisomes. Thus, the overexpression of a potential cargo, even one that is covalently bound to a lipid moiety does not trigger the biogenesis of this compartment, or its enrichment in this compartment.
On the contrary, overexpression of full length untagged Hh led to a 4.2-fold increase in Hherisome number accompanied with a 4.5- to 7-fold increase of Hh density therein (see point 1 and Table 1), when compared to endogenous Hherisomes that are present in these wildtype cells. In this regard, we claim that Hherisomes is a new specialized compartment and we wish to keep this name.
It is likely that the biochemical properties of Hh enables it to be preferentially captured and enriched in recycling tubules, perhaps by what Reviewer 2 calls "hijacking". Therefore, giving these Hh positive recycling tubules a specific name that is linked to Hh seems justified. As Hherisomes represent tubular recycling endosomes (see above), we could name these compartments "Hh specialized recycling tubular endosomes". We rather name them "Hherisomes" that is less of a mouthful, even though we also agree that the multiplication of names does not always clarify things. We will try our best that readers understand what Hherisomes are. In this regard, we have slightly modified the title.
In fact, our naming of the Hh compartment is very similar to "MHII compartments" that are lysosomes specialized in antigen presentation by class II molecules, and to Birbeck granules of Langherhans cells that are also specialized recycling endosomes enriched in Langherin.

Point 3. All reviewers ask about the relationship between Rab4 (Rab5), Rab11 and Hh trafficking in the Hh producing cells.
>> It is correct that the Rab4 data reported by D'angelo et al (2015) suggested that a pool of Hh follows a rapid recycling route from early endosomes after its endocytosis. Here, we report the Hh traffics through recycling tubular endosomes where it is stored (Hherisomes), and we clearly show with our detailed ultrastructural analysis that Hherisomes are not classical vacuolar early endosomes. This has not yet been reported before and is therefore a new piece of Hh biology.
We cannot formally exclude that Rab4 may have a modulating function in the formation of Hherisomes. However, D'Angelo at al (2015) has shown that silencing Rab4 decreases **Hh long range/low level signaling**, but not short range. If the Rab4 pathway were to be competing with the Rab11 pathway, the absence of Rab4 should lead to a pool of Hh more accessible to Rab11.

Therefore, the overexpression of Rab11 should mimic a Rab4 loss of function phenotype. However, in the present manuscript, Rab11 overexpression led to an increase of Hh **short range/high level activity** without change in long range. Therefore, we propose that Rab4 likely works in parallel to Hherisome formation. We discuss this in the revised version.

Of note, several other Rabs are now linked to Hh. However, it is not possible at this stage to test them all with this approach. We argue that we provide a novel piece of the Hh puzzle, not the completion of the full understanding of how Hh is and trafficked intracellularly, spread and signals.

Point 4: Reviewers 2 and 3 also found that the IEM studies should be expanded to mechanistic data including what makes Hh more competent in short range signaling, how loss of *dispatched* antagonizes this process, and what ensures the compartment specificity for Hh.
>>In this manuscript, we have performed experiments showing a function for Hherisomes. We provide clear evidences that they are necessary for efficient Hh short range signaling and tissue growth.
We agree that understanding what happens to Hh during its trafficking in Hherisomes and how it acquires this signaling and growth property is an interesting, perhaps critical, outcome of this study. Is it oligomerisation, interaction with different proteins, such as proteoglycans and lipid molecules, further modifications? This will be for the future. Digging into the specifics of these is at present beyond the scope of this study as it would require years of new research.
With regard to Disp, as its overexpression prevents Hherisome formation, we argue that in these conditions Hh can no longer acquire the specific properties provided by trafficking through Hherisomes and is therefore less competent in short range/high level signaling.
As for the specificity of this compartment for Hh, it might be due to wild type Hh properties and not only to its covalent links to specific lipids. Indeed, only full length untagged Hh, but neither a form devoid of cholesterol, nor a tagged lipidated form of Hh, can boost Hherisome formation (see point 17).

Point 5: Reviewers 2 and 3 also ask what is the relationship between Hherisomes, cytonemes and exosomes?
>>Regarding how Hh trafficking through Hherisomes could specifically target it to cytonemes and exosomes, we agree that the link is hypothetical. Please note that our study was not specifically aimed to provide this link. At the start, the notion was to look in an unbiased manner at Hh trafficking in producing cells. Either the observations would fit with existing observations or it would open new avenues, which appears to be the case.
Furthermore, for the first time, the resolution of intracellular Hh distribution indicates that it is mostly in Hherisomes. However, a small portion is also present in the vacuolar part of endosomes displaying internal vesicles (MVBs), the ones potentially leading to the release of exosomes.

Point 6: Reviewer 2 also raised the argument that our finding is not significantly new. According to him/her, this manuscript only confirms at the ultrastructural level some of those observations that were previously made by immunofluorescence.
>>We respectfully disagree. First, this present study goes way beyond only confirming Hh endocytosis. Our high resolution ultrastructural analysis identifies that following endocytosis, Hh is stored in recycling tubular endosomes, the Hherisomes. This was not proposed after immunofluorescence studies of D'angelo et al (2015) (see point 3). Second, this ultrastructural resolution of Hh distribution has never been achieved before, which in itself, is valuable. Third, we identify that the process of Hherisome formation is antagonistic to, and likely independent of Dispatched function, a point clearly exciting for the Hh community. Fourth, we show that Hh trafficking through recycling tubules appears to be essential for signaling and disc growth. The latter aspect is highly original. Reviewer 2 kindly remarks that it is very impressive.
In this regard, we reiterate here the several advances that our manuscript represents for the Hh field
a. What is original in this manuscript is that most of its data are generated from ultrastructural analysis of Hh distribution in many different genetic backgrounds in a quantified manner. This gives our manuscript an **unprecedented cell biological** edge in the Hh field which has been lacking until now. We identified a compartment storing the vast majority of intracellular Hh that have not, to our knowledge, been described before.

b. We have not stopped at the description of the Hh containing recycling tubular endosomes (the

Hherisomes) in these backgrounds. Instead, we experimentally assign a function to these compartments through detailed known assays in **developmental biology**. We establish a strong correlation between the presence of Hherisomes (and Hh therein) and signaling and tissue growth. Interestingly, these two functions are modulated by Disp and Rab11 expression. This has so far not been shown.

c. Hh can be endocytosed and reach the recycling compartment in a *dispatched* mutant disc (not only in the posterior but also in the anterior compartment), arguing that at least a portion of Hh endocytosis is Dispatched independent.

POINT-BY-POINT RESPONSE TO REVIEWERS
Reviewer #1
Evidence, reproducibility and clarity.
The results are interesting, of high quality, and mostly support the conclusions of the authors.
>>We thank Reviewer 1 for their insightful comments, we hope our answers provide more context, and clarify their concerns.

I have nevertheless some comments and questions that could be addressed by the authors.

7. As there are other GTPases involved in recycling, can the authors exclude any role of Rab4?
>>See point 3**.**

8. If I am not wrong, Rab5 and Rab4 have been involved in internalization /recycling of Hh (D'Angelo et al. Dev Cell, 2015). Maybe this should be discussed to avoid misunderstanding by the readers?
>>See point 3**.**

9. The Hherisomes are likely to represent a very specialized recycling endosome. I am wondering whether bona fide recycling endosomes exist in these cells committed for « normal « recycling of other cargo? Or there these very same recycling endosomes that are also exploited for general recycling?
>>See point 2 **and our original discussion:**

10. One point that is unclear for me is whether Hherisomes are only observed after boosting by overexpression.?
>>See point 1.

11. These compartments are in my opinion quite analogous to Birbeck granules of Langerhans Cells that are also specialized recycling endosomes (Mc Dermott R, Mol Biol Cell. 2002 Jan;13(1):317-35). Maybe it would be of interest to make an analogy? If there are some?
>>We thank the reviewer for pointing to this paper that we were unaware of (PMID: 11809842). In fact, it appears that Birbeck granule biogenesis follows a route that is very similar to what we propose for Hherisomes. Their structure is similar, although the Birbeck granules appear more rod-like than Hherisomes. Their formation is downstream of endocytosis from the plasma membrane and depends on Rab11. And both represent the recycling tubules emanating from recycling endosomes and store specific proteins. We have now added this analogy in the discussion together with the GLUT4 compartment.

12. The density of the content of these recycling endosomes and actually also their size puzzles me. In some micrographs one can also appreciate a particular structure with some striations? Is this common? Do the authors have any hypothesis on such density? (Fig. 4A right panel).
>>The density in IEM can be puzzling and can in fact be artificially created by the antibody/gold complexes used for detection that concentrate the uranyl acetate used for contrasting the sections. It can also be created by the aldehyde crosslinking of the proteins within a structure, especially when the density is high, as it is the case for the secretory granules of the exocrine pancreas for instance. The density also appears to be linked to lipid density and packing.
About the striations, they were not very common and we are not sure what they represent.

Significance:
13. In any case the arguments of the authors explaining why they were not observed before are perfectly pertinent. The contribution in the field is significant. Maybe the authors should somehow

explain how these results are integrated with the former reports indicating the role for other Rabs such as Rab5 and Rab4.
>> Thank you and see point 3.

Reviewer #2
Evidence, reproducibility and clarity:
Major comments:
14. These data are of high quality, in particular the EM and, as a whole, well quantified with good controls in place. However, I am not convinced these data are sufficient to support the authors claims. The authors propose to have identified a novel endocytic structure called the Hherisome, the formation of which can be driven by the overexpression of Hedgehog and Rab11 and inhibited by Dispatched. Yet they do not back up this claim with any hard quantification. The authors claim Hedgehog overexpression results in Hherisomes appearing 1.5 times more abundant at the apical side but then do not show the data.
>> Regarding the boosting of Hherisome biogenesis upon Hh overexpression, we first wish to draw the attention of the reviewer to Table 1 of the original manuscript. There, it is clearly shown that the labeling density of Hh in Hherisomes upon Hh expression in the posterior compartment increases 7-fold for UAS- Hh M4 and 4.5 fold for UAS-Hh M1 when compared to wild type discs (compare line 1 in OreR and lines 2 and 5 for Hh overexpression). However, we also want to draw the attention to the important fact that Hherisomes are visualized and exist in wildtype discs, albeit at lower numbers, size and Hh density.

-Regarding the number of Hherisomes: it increases about 4 times upon Hh overexpression compared to wild type, see point 1. This is now shown in Suppl Table S1.

-Regarding the positioning of the Hherisomes: The apical versus basolateral distribution of Hherisomes has a ratio of 1:1 in wildtype discs and 1.5-2.2:1 in discs overexpressing Hh. Therefore, the apical site appears slightly enriched with Hherisomes upon overexpression. The quantification was performed as in point 1, but with distinguishing the apical from the baso-lateral Hherisomes. However, this will not be further detailed in the manuscript.

15. …..the formation of which can be driven by the overexpression of Hedgehog and Rab11 and inhibited by Dispatched
Likewise, the authors suggest Rab11 overexpression boosts biogenesis of Hherisomes but without the quantification to back up such a claim.
>>When Rab11 is co-expressed with Hh, the overall number of Hherisomes in the disc posterior compartment does not significantly change when compared to Hh overexpression alone. 70 such fields were taken into account. We now mention this more clearly in the manuscript (Suppl Table S3). As a result, we don't claim that Rab11 boosts Hherisome formation.
However, Rab11 overexpression changes the Hh labeling density of Hh in Hherisomes leading to a more than 2-fold increases (see original Table 1, compare line 5 to line 6). Furthermore, in discs overexpressing both UAS-Hh and UAS-Rab11, some of the Hherisomes became very large. We have also mentioned this point in the revised manuscript.

-Upon DispHA overexpression (with UAS-Hh), the number of Hherisomes drops dramatically when compared to UAS-Hh alone (see New Suppl Table 3). This is why we refer to a 93% reduction in the manuscript.

16. The authors do convincingly show both manipulations lead to an increase in Hedgehog labelling of endocytic tubules but this can be explained by overexpressed Hedgehog saturating the endocytic system and the fact that overexpression of Rab proteins, including Rab11 (Wilcke, M et al. JCB 2000), perturbs endosome morphology and function with consequences for the distribution and transit of endosomal cargo. As such the tubular structures may not represent a novel specialised compartment like the authors propose but rather a classical endosomal recycling tubule through which Hedgehog is transiting.
>> As to whether Hherisomes are a novel specialized compartment, see point 2.

We also point out that Hherisomes are observed in a wild-type context as mentioned above (point 1), so they are formed without saturating the endocytic system. In addition, overexpressing other

molecules (such as Lamp1 and Wg) that traffic through endosomes and could potentially saturate the system, does not lead to their enrichment in Hherisomes nor in their formation. Not even dually lipidated Hh tagged with GFP (points 2 and 17).

17. Hydrophobic regions of endosomal proteins have been shown to insert directly into the hydrophobic lipid tail region of the membrane to drive membrane curvature by expanding the area of one lipid monolayer leaflet relative to the opposite leaflet, forcing the bilayer to take on curvature and drive the formation of tubules (such is seen with the clathrin accessory protein epsin). It is conceivable that Hedgehog, via its lipid moieties, could function similarly to drive tubule (Hherisome) formation but it is also conceivable that this could be an artefact of Hedgehog overexpression.
>>We respectfully disagree. There are three arguments indicating that Hh overexpression *per se* does not create an artifact that leads to the formation of Hherisomes. First, Hherisomes exist in wildtype discs not overexpressing Hh (point 1). Second, their formation is not boosted by the overexpression of lipidated Wg (a palmytoylate-modified morphogen, point 2). Third, Hherisome formation is also not boosted by overexpressing dually lipid-modified HhGFP (new data added in the manuscript). This is now presented in Suppl Table S1. One would expect similar effects if Hherisome formation was solely dependent on lipids and not on wild-type Hedgehog as a whole (point 4). This is not the case and it is now discussed in details in the revised manuscript.

18. The authors findings that Dispatched and Rab11 overexpression have opposite effects on high level Hedgehog signalling is clear. The rescue of growth in Dispatched mutants by Rab11 overexpression is particularly impressive.
>> We thank the reviewer for this positive comment. We fully agree with this.

19. However, these findings are largely observational with little mechanistic insight. In order to make sense of these findings and make a significant advance in the field it would require identifying how these manipulations affect Hedgehog targeting i.e. an increase in Hedgehog targeting to exosomes versus cytonemes etc...
>>See points 4, 5 and 6**.**

Minor Comments:
20. The authors findings that Hedgehog first needs to be endocytosed to reach these endocytic structures and that these structures are not degradative in nature is very convincing and fits with what is already known about Hedgehog secretion.
However, this seems at odds with a previous finding that Dispatched is necessary for Hedgehog endocytosis (D'Angelo, G et al. Dev Cell 2015) as the authors show Dispatched is not required for Hherisome formation. How do the authors reconcile these two findings?
>> The study presented in D'Angelo et al (2015) used HhGFP and followed its trafficking by fluorescence imaging. As mentioned above, this transgene does not boost the formation of Hherisomes (see point 17) and might not faithfully reproduce all the functions and trafficking routes of full length Hh. In addition, as discussed in the manuscript, the IEM analysis reveals a pool of Hh which is not detected by immunofluorescence, likely due to the detergent used for preparation. Regarding the discrepancy over the role of Dispatched in Hh endocytosis, we find here that in the absence of Dispatched function, a pool of Hh is endocytosed independently of Disp, a fact that was missed in previous studies. These two points have been added to our discussion.

21. The authors also observe Hedgehog in tubular Hherisome structures in the anterior compartment. Do they believe this pool of Hedgehog is capable of another round of secretion?
>>This is a very good question. As we observe Hh positive Hherisomes very distally in the anterior compartment far away from the Hh source, we would argue that it is indeed possible.

22. The loss of Dispatched has been shown to cause a dramatic accumulation of Hedgehog in secreting cells (Callejo, A et al. PNAS 2011). The authors make no mention as to whether they see such an accumulation in their EM images and if so where does Hedgehog accumulate? They state Hedgehog in tubular endocytic structures is unaffected, does this mean Hedgehog is accumulating elsewhere such as the plasma membrane?
>> In dispatched mutant, we did not observe any accumulation in other intracellular compartments. Regarding the plasma membrane, as explained in the discussion of the manuscript, the IEM technique we used can visualize the intracellular Hh but does not allow a quantitative assessment

of the extracellular pool. Therefore, we cannot determine the intracellular/extracellular Hh ratio.

23. In conclusion, without further evidence I think the authors claims to have identified a novel endocytic structure the Hherisome are speculative and that the structure likely represents a Rab11 recycling tubule through which Hedgehog transits through following endocytosis and prior to resecretion.
>>See point 2.

24. The data and the methods are presented in such a way that they can be reproduced and the experiments are adequately analysed.
>>Thank you.

Significance
25. I think the authors findings do not represent a significant advance for the field. It has already been demonstrated that Hedgehog undergoes endocytosis prior to resecretion (D'Angelo, G et al. Dev Cell 2015 and Callejo, A et al. PNAS 2011).
>>We respectfully disagree. See point 6.

26. The authors propose that formation of a specialized Hedgehog containing endocytic structure is required for high level Hedgehog signaling but I find a lack of evidence to prove this novel structure is not merely a normal recycling tubule. As such the work builds upon and confirms previous findings on the need for Hedgehog endocytosis prior to secretion but does not represent a big advancement.
>>See point 2.

27. I would suggest the Hedgehog signaling community and developmental biology community are the main target audience. They will find the observation that Rab11 and Dispatched overexpression can modify the intensity of Hedgehog signaling and that Rab11 overexpression can rescue the loss of growth seen in Dispatched mutant wing discs the most interesting findings. These findings suggest the existence of multiple Hedgehog pools with different signaling intensities. However, the findings lack real mechanistic insight.
>>See point 4.

Referees cross-commenting
28. I agree with all the comments of the other reviewers.
Reviewer 1 raises a valid question. How do these findings relate to previous findings from the authors in which they show a role for Rab4 in recycling Hedgehog after its internalisation? Rab4 RNAi has been shown to affect the long-range activity of Hh. It would be interesting to known what impact it has on Hherisome formation or at least add a section to the discussion regarding Rab4 and how it could relate to Rab11 and Hherisomes
>>See point 3.

29. Reviewer 3 appears to share my main concern that, in my opinion, the authors have not done enough to prove that these Hherisomes are a novel specialised compartment, as opposed to a normal recycling tubule emanating from an endosome.
>>See point 2.

Reviewer #3
Evidence, reproducibility and clarity
>>We thank the reviewer for their critical assessment.

Major comments
30. In general, it is hard to understand how cytoneme and apical secretion are trafficking routes of Hh, but both end up in the same (90%) endocytic/recycling compartment.
>> See point 5.

31. I also disagree with the authors to call it a different name, when instead it is a recycling endosome compartment. It is worth showing these very nice EM analyses but I would prefer the authors would not oversell it.
>>See point 2.

32. EM quantifications should be present in the main figures to convince, as they look rather descriptive.

>>EM studies are descriptive by nature. However, they were carefully quantified in the Table 1 that is a main figure and an integral and critical part of the data. It will be printed along the other figures in the published article and be readily available for easy viewing.

33. The paragraph about the identity of Hherisomes is rather weak, as the conclusion suggests. They are not lysosomes...but recycling endosomes. This has been demonstrated by the authors before that Hh is secreted a second time via rab4 endosomes to the apical side (D'Angelo Dev Cell 2015). How is this study expanding the previous findings and connects to them?
>>We identified Hherisomes as recycling tubular endosomes through their morphology and their link to Rab11. As for the link with Rab4, see point 3.

34. The authors mention that live imaging of Hherisomes with fluorescently tagged Hh is not possible, but would pulse-chase immunofluorencence co-stainings with fixation methods preserving the endosomal tubules be a possibility to back up the EM findings? In that respect, how does the story relate to rab4 recycling compartment? How big is the overlap of rab11/rab4/Hh?
Besides BSA internalization can the authors perform recycling/pulse-chase assays of Hh to show that it is endocytosed Hh accumulating in Rab11 endosomes and secondly that this endocytosed Hh gets resecreted via the „Hherisomes".
>>We thank the reviewer for these excellent experimental suggestions. We considered these before. The only prerequisite would be that we should see a correlation between the IEM studies and the immunofluorescence. Unfortunately, this is not the case, at least with the permeabilization that we have done. Indeed, we looked at Hh distribution in Rab11 overexpressing discs similar to what we present on Figure6-Suppl1 where we analyzed several parameters such as Hh puncta number, size and intensity. We have not observed any significant change in any of these parameters when Rab11 was overexpressed, when compared to the control. As mentioned above, the discrepancy might solely come from the techniques used, and other milder detergents could be tried. This is the target for a further study.
We have also tried to design a pulse chase experiment with a Hh antibody and develop an endocytic/recycling assay for endogenous Hh, but unfortunately, this was not successful. Last, we are now also reluctant to use HhGFP as we found that expression of this form does not induce HHerisome formation.
Regarding Rab4, see point 3.

Minor comments
35. Language can be improved. Especially some more explanation and details of the experimental model system in each section to help people from other areas of cell and developmental biology understand. Regarding Wg staining of those recycling endosomes, is it within the expression domain of Wg or outside? Was quantification done as with Hh?
>>WgHA was expressed using the dpp>gal4 driver, therefore leading to its expression within the Dpp expression domain that partly overlaps with part of the endogenous Wg domain. As we could not mark both domains simultaneously in this setting by IEM, we could not assess whether Wg reaches recycling endosomes within wg producing cells or as a result of its internalization by Wg receiving cells. This is now added in the legend for Suppl Figure S2A.

Significance
36. The diversity of morphogen secretion and trafficking models, for Hh as well as Wg, suggests that aspects of morphogen trafficking and regulation of gradient formation remain to be discovered. Thus, in the light of different models of Hh release it is a valuable to approach Hh intracellular localisation by immuno-electron microscopy analysis. But I feel the study is lacking some functional testing of their hypothesis and complementary methods to show, what is the reason for this specialized compartment, what is ensuring its specificity?
>>**See** point 4**.**

37. It is important to understand why endocytosis is so important for secretion of morphogens and gradient formation. This study shows that the majority of Hh is present in recycling endosomes in

secreting cells, and modulation of recycling endosomes formation modulates Hh signaling in the receiving compartment. This work is important for the field of morphogen signaling.
>>We thank the reviewer for their insightful comments, and appreciate their assessment of significance.

38. I would also like to know about the relation to rab4 sorting.
>>See point 3.

Second decision letter

MS ID#: JOCES/2021/258603

MS TITLE: Hherisomes, Hedgehog specialized recycling endosomes, are required for high level Hedgehog signaling and tissue growth

AUTHORS: Sandrine Pizette, Tamas Matusek, Bram Herpers, PASCAL P THEROND, and Catherine Rabouille
ARTICLE TYPE: Research Article

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