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Intrinsic regulation of enteroendocrine fate by Numb

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 10 October 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you can see from the comments, all three referees appreciate the novelty of the proposed Numb-dependent mechanism of enteroendocrine cell specification. However, they also raise significant concerns with the analysis that would have to be addressed in order to consider publication here. In particular, the reviewers point out the need for more detailed analysis of the role of polarised cell division and Numb distribution in daughter cell differentiation, and the role of Numb in Notch activation. They also indicate that more work is needed to convincingly exclude the function of Slit in enteroendocrine cell specification. Given the referees' comments, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single major round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you

foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

Intrinsic regulation of enteroendocrine fate by Numb
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Summary of the data:
This paper by Sallé et al., addresses the molecular mechanism of terminal cell fate determination of enteroendocrine cells (EEs) in the Drosophila midgut. By using the MARCM system the authors achieve local disruption of ee cells to analyse the impact on overall ee cell specification. Through multiple mutant, knock-down and protein overexpressing MARCM assays, they come up with a model, in which cell intrinsic regulation of Notch signalling by Numb promotes ee cell specification. This is contrary to a previously proposed model of ee cell specification through Slit/Robo signalling between ISCs and newly specified ee cells. In their model, the authors propose that symmetric distribution of Numb during late ISC proliferation results in one of the cells taking up the EE fate through the inhibition of Notch signalling by Numb in an AP-2 -mediated endocytosis dependent manner.

Overall, I find that various aspects of this work are very valuable. Most importantly, the generation of clones to induce local manipulation of ee cells is a very clever approach, which leads to a much 'cleaner' way of analysing functional aspects of these cells than previous studies, given that all gal4 lines expressed in ee cells are also expressed in the brain. As such, this paper makes an important contribution to the field. Overall the study is rigorous. However, there are some key experiments that, in my opinion, should be done to fully support the proposed model. Namely, global ee specification is not achieved by stem/ee cell feedback by though a cell intrinsic mechanism.

Major comments:
1- Given that previous reports suggest a local role for ee cells in ISC homeostasis, the authors should assess, as part of their clonal analysis, what is the status of stem and progenitor cells in the tissue area surrounding clones with gain and loss of ee cells. An assessment of ISC proliferation in those contexts would be useful. For example, if disruption of ee numbers such as in Figure 1 C and E impacts on the number of stem cells, that may indirectly impact the potential ISC/ee feedback proposed by another group, hence the lack of effect on ee cell specification. This may be an unlikely possibility but one the authors should test to support their model and strong statements. This applies to Figure 2 as well.

As I said in my summary, the use of clones to disrupt ee cells locally is very valuable. However, one caveat of the system is its inability to account for non-cell autonomous effects when secreted factors are being analysed. Hence, in the experiments of Figure 2, the authors cannot discard non-cell autonomous effect of Slit produced by ee cells outside of the clone. To fully support their conclusions, the authors should knockdown Slit throughout the gut. For example by using voila-gal4ts and nSyb-gal4ts. The former would target ee cells and neurons so it could be a problem but nSyb-gal4 should help to substract potential role of brain derived Slit in the system. An alternative, could be to use the esg flip out system and look at phenotypes ones there has been complete self-renewal of the intestinal epithelium to make sure all ee cells are targeted by the system.

Additional comments:
- In Figure 1 and 3: what happens when ee cells are lost or overrepresented? What is the trade off within the clones? Are there more or less ISCs or ECs. In other words: are ee cells replaced by a different cell type or made at the expense of another cell type? A clarification including quantification of different cell types within the clones would be useful.
- Data in Figure 4 is difficult to see. Image quality improvement would help.

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- Supplementary figure 2I misses its control figure?
-Please provide quantification in Figure 6 A, B
- Please provide controls in Figure 7 including clones carrying single mutations and transgene expression. Quantifications would be important as well.
- Please provide controls in Figure 8
- The quantifications provided in Figure 8 F do not make sense to me. How is it possible to have effect in percentages when neither the total number of cells nor the average number of ee cells in clones is not changed?
- Overall authors should provide quantification for a higher number of guts and clones.

Referee #2:

In this manuscript, Salle et al explored the mechanism underlying EE vs EC fate determination in adult Drosophila midgut. The authors provided several lines of evidence arguing against the involvement of a negative feedback mechanism proposed by others, i.e., EE-derived Slit acts on Robo2 present on ISCs to limit EE production. They provided convincing data to show that Numb functions as a cell intrinsic factor essential for EE fate determination and Numb promotes EE fate by inhibiting N. Consistent with this notion, the authors showed that Numb acts upstream or in parallel with several transcription factors known to regulate EE fate. Using a Numb-GFP reporter, the authors found that Numb exhibited both asymmetric (~80%) and symmetric (~20%) segregation during ISC division. In addition, altering the activity of cell polarity genes such as aPKC and lgl influenced both the dynamics of Numb segregation and the frequency of EE production. The authors also showed that continuing production of Numb is required for EE differentiation. Based on these and other observations, the author proposed that symmetric Numb segregation during ISC division and de novo synthesis of Numb in ISC daughter cells are essential for EE fate choice and this cell-intrinsic mechanism can lead to a balanced and tissue-wide production of EEs/ECs in the absence of a feedback mechanism. For the most part (except for the effect of polarity genes on EE determination), the experiments were well designed and executed, and the data are of good quality. The findings of symmetric and asymmetric segregation of Numb and its potential role in EE fate determination are novel and such mechanism is likely to be conserved in mammalian intestines. However, there are a number of issues needed to be addressed by additional experiments/discussion before acceptance for publication is recommended.

Major concerns:

1. It is clear that Numb is required for EE fate determination but it is not clear to me whether or why symmetric division is the driver for EE fate determination. The authors attempted to address this issue by altering the activity of polarity genes such as aPKC and lgl, which leads to symmetric Numb segregation. However, the effect on EE fate was very mild. In addition, altering the activity of aPKC or lgl could change the ISC division angle and/or affect several other cellular processes, which may contribute to the observed subtle change in EE fate determination. Indeed, it is not clear to me why overexpression of aPKC-CAAX and loss-of-lgl produced the opposite phenotypes with respect to EE formation while both caused symmetric Numb segregation. Did the authors confirmed Numb segregation is altered in lgl mutant cells? How does loss-of-aPKC affect Numb segregation and EE fate? Is there a good correlation between symmetric Numb-GFP segregation and Pros expression? Perhaps a better experiment would be to engineer a Numb that can no longer undergo asymmetric segregation but retain its ability to inhibit N and then determine whether expression of such Numb variant will alter EE fate.

2. In Fig3C, how did the authors mark the early mitotic cells? What fraction of early mitotic cells contained basal Numb-GFP. Fig. 3D-E, it would be informative to show side views of dividing cells to determine the relationship between symmetric/asymmetric Numb-GFP segregation and cell division angle. For example, is Numb-GFP asymmetrically segregated in ISCs that divide apical-basally but symmetrically segregated in ISCs that divide along the basement membrane? It would also be interesting to determine whether altering the cell division angel (for example in Par6 or aPKC RNAi background) affects symmetric vs asymmetric Numb-GFP segregation.

3. A major conclusion of this study is that feedback mechanism is not involved in balancing the EE/EC production. How do the authors explain a previous study showing that RNAi of Slit in EEs increased EE number? In Fig. 1C, the authors showed that local depletion of EEs did not affect the frequency of EE formation in surround tissues. Is it possible that the remaining EEs produced enough secreted signals to maintain the negative feedback? Does killing all the mature EEs using a pan EE-gal4 driver affect EE formation? In Fig. 1E, have the authors verified that EEs in N RNAi still express Slit?

Other concerns:

1. While it is interesting to observe that Numb is both asymmetric and symmetric segregated during ISC division and their respective frequencies correlate with those of EC and EE lineages. It is counterintuitive and puzzling that the asymmetric segregation of Numb into one the two ISC daughters doesn't seem to play a critical role in ISC/EB specification because loss of Numb did not seem to affect ISC self-renewal. In neuroblasts, asymmetric segregation of Numb is critical for asymmetric N signaling and subsequent fate determination of their daughter cells. How then is the asymmetric N activity established/maintained in ISC/EB lineage in the absence of Numb? In light of a recent finding that asymmetric BMP signaling can influence ISC/EB fate choice (Tian 2014), it is likely that BMP signaling may replace the function of Numb to inhibit N in ISCs. The authors should discuss this issue to erase this "puzzle" from readers' minds.

2. Previous studies indicate that symmetric ISC division can also produce two ISCs. What then determines EE vs ISC fate choice after a symmetric division?

3. A previous study showed that overexpression of aPKC-CAAX inhibited ISC fate and promoted EC fate likely by activating N (Goulas et al., 2012), whereas in this study, the authors showed that aPKC-CAAX overexpression promoted EE fate (Fig. 8) through promoting Numb symmetric segregation and thus inhibiting N. How do they reconcile these seemingly contradictory results? Does aPKC-CAAX overexpression promoted EE fate depending on Numb?

4. Some data are missing:
---P14, "Furthermore, as 59% of these cells also co-expressed Dl, they likely represent newly differentiated EEs recently made from ISCs and which still have Dl protein".
---P21, "We found no obvious correlations between Numb and Pros segregation in adult ISCs. While, we observed Pros expression in 13.5% of dividing cells (10/74 cells); asymmetric Pros segregation occurred infrequently in 6.8% (5/74 cells) of dividing cells. Pros and Numb-GFP colocalized asymmetrically infrequently in 5.4% (4/74 cells) of dividing cells. 5/74 of the dividing cells also had asymmetric Numb-GFP with symmetric Pros in the future daughter cells". (These data should be included in supplementary figures).

5. P10, "NRE-Gal4" needs a reference.

Referee #3:

In this manuscript, Bardin and colleagues studied the mechanisms of enteroendocrine cell specification in adult Drosophila intestine. They first provided evidence to argue against the existence of a local feedback mechanism in regulating EE differentiation, and then proposed instead an intrinsic mechanism by Numb in the process. They found that Numb was required for EE differentiation and overexpression of Numb produced more EE cells. Interestingly, Numb protein showed both asymmetric and symmetric segregation in dividing cells. Using subcellular distribution of Notch-GFP as a Notch activity indicator, the authors found that the percentage of dividing cells with symmetric Numb segregation was consistent with the overall ratio of EE cell population and the percentage of precursor cells that adopts EE cell fate. In addition, disrupting the function of cell polarity genes caused altered production of EEs, possibly due to altered symmetric or asymmetric Numb segregation during stem cell division. These observations let the authors to propose that Numb act as an intrinsic factor to regulate EE fate decision during asymmetric stem cell division by inhibiting Notch.

Numb is best known as a critical cell fate regulator during asymmetric cell division of neuronal stem cells during Development. The identification of Numb as an important regulator for EE cell specification in intestine is novel and should be of interest to the field. But one major concern is that many results are only correlational observations, which are not sufficient to draw the conclusion that different modes of numb segregation lead to distinct progenitor cell fate.

Specific comments:

Direct evidence is needed to support a role for Numb in regulating Notch during EE specification. Does Numb segregation correlate with reduced Notch activity in dividing cells? Does loss or gain of function of Numb cause increased or decreased Notch activity, respectively, in progenitor cells?

The genetic results do not seem to be strong in supporting an instructive role for Numb in EE specification. Pros+ cells are significantly reduced but not eliminated numb mutant clones. Do these mutant Pros+ cells express EE-peptides similar to normal EE cells? On the other hand, overexpression of numb only mildly increases EE production, and does not seem to block EC differentiation. Does it mean that Numb is not sufficient in inducing EE cell fate?

Because the phenotypes associated with altered numb function are relatively week, it might not be suitable to consider Numb in a linear pathway with Ttk or Sc. Is the segregation pattern of Numb during stem cell division altered upon sc overexpression or ttk RNAi?

Figure 8: aPKC-CAAX over-expression leads to 92% symmetric distribution of Numb, but this only causes a mild increase of EE cells. The result seems to argue against the hypothesis that symmetric distribution of Numb is related to EE specification.

Fig.1 and 2 showed some interesting data, but they are not directly linked to the rest of the story and therefore appear unnecessary.

1st Revision - authors' response 14 March 2017

(Please see next page)

Referee #1:

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Overall, I find that various aspects of this work are very valuable. Most importantly, the generation of clones to induce local manipulation of ee cells is a very clever approach, which leads to a much 'cleaner' way of analysing functional aspects of these cells than previous studies, given that all gal4 lines expressed in ee cells are also expressed in the brain. As such, this paper makes an important contribution to the field. Overall the study is rigorous. However, there are some key experiments that, in my opinion, should be done to fully support the proposed model. Namely, global ee specification is not achieved by stem/ee cell feedback by though a cell intrinsic mechanism.

We would like to thank this reviewer for his/her general appreciation of our study and of the technique that we developed for the local ablation of EE cells. We have addressed his/her comments with numerous new experiments to improve the manuscript.

Major comments:
1- Given that previous reports suggest a local role for ee cells in ISC homeostasis, the authors should assess, as part of their clonal analysis, what is the status of stem and progenitor cells in the tissue area surrounding clones with gain and loss of ee cells. An assessment of ISC proliferation in those contexts would be useful. For example, if disruption of ee numbers such as in Figure 1 C and E impacts on the number of stem cells, that may indirectly impact the potential ISC/ee feedback proposed by another group, hence the lack of effect on ee cell specification. This may be an unlikely possibility but one the authors should test to support their model and strong statements. This applies to Figure 2 as well.

The reviewer raises a good point that we now have addressed in a number of ways and found no evidence that our clonal alterations impact ISC numbers or proliferation in surrounding tissue:

First, as suggested by the reviewer, we performed PH3 staining to assess whether EE loss or EE surplus affect ISC proliferation of surrounding tissue compared to controls and found ISC proliferation rates were unchanged in these conditions (new Expanded View 1). Secondly, as suggested by the reviewer, we also assessed ISC numbers per se by staining for two ISC markers Dl and Spdo in posterior midguts of controls, EE killing, or ectopic EEs. While we did not observe a difference between these conditions, the staining was somewhat variable and difficult to assess at the whole gut level in low magnification images needed for this analysis. We therefore considered the above experiment on PH3 staining as more quantitative and more to the point of the reviewer as to whether proliferation was affected. Of note, in response to Reviewer 2, we verified that the ectopic EE cells induced using Notch RNAi produce large quantities of Slit (see new Appendix Fig S2D,D'). Therefore, since in the EE killing experiment EE cells are lost (and any EE derived signal including Slit) and upon induction of extra EE cells, Slit is overproduced, we felt that performing this analysis for Fig 2 was redundant with this new data and, in the interest of time, we did not repeat these for Fig2. If the Reviewer felt that this is critical, we could repeat this analysis for these genotypes. However, altogether our new data provide strong evidence against alteration of EE density leading to indirect effect on ISC proliferation in surrounding tissue.

As I said in my summary, the use of clones to disrupt ee cells locally is very valuable. However, one caveat of the system is its inability to account for non-cell autonomous effects when secreted factors are being analysed. Hence, in the experiments of Figure 2, the authors cannot discard non-cell autonomous effect of Slit produced by ee cells outside of the clone. To fully support their conclusions, the authors should knockdown Slit throughout the gut. For example by using voila-gal4ts and nSyb-gal4ts. The former would target ee cells and neurons so it could be a problem but nSyb-gal4 should help to substract potential role of brain derived Slit in the system. An alternative, could be to use the esg flip out system and look at phenotypes ones there has been complete self-renewal of the intestinal epithelium to make sure all ee cells are targeted by the system.

The current model for EE fate control proposed in both Biteau and Jasper, Cell Reports, 2014 as well as Zeng, Han...Lin, Hou, Cell Reports, 2015 is that local Slit signals controls the proportion of EE cells in the tissue through signaling to ISCs and inhibiting their cell fate commitment. Indeed, Biteau and Jasper state "The origin of this signal is the EEs themselves, establishing a negative feedback loop and suggesting that ISCs constantly assess their immediate environment to control the destiny of their progeny and specifically replace missing EEs in the absence of a Slit signal." Zeng et al conclude: "In summary, these data suggest that extrinsic Slit-Robo2 signaling from EE cells to ISCs regulates the proportion of EE cells through a negative feedback mechanism to keep the right balance of differentiated cells in the posterior midgut epithelium." Neither study, however, directly tested whether indeed this was due to local feedback, both instead assessing a global EE cell knockdown of Slit on EE fate. The strength of our study is that we have tested this

concept experimentally, not only for Slit, but for any potentially EE derived through our use of modulation of EE densities locally. Our data strongly indicate that local EE modification has no ability to control surrounding EE density. We believe that our finding that lineage intrinsic mechanisms control EE fate production and not EE-ISC feedback is an important conceptual advance for the field.

*The referee specifically mentions that: "*in the experiments of Figure 2, the authors cannot discard non-cell autonomous effect of Slit produced by ee cells outside of the clone." *If EEs outside the clone are controlling the ISCs outside the clone, one would still expect perturbation of this signal at the clone boundary, akin to classic observations at clone boundaries during lateral inhibition of Notch-Dl (Heitzler, et al., 1996). Our analysis of the 30 micron area surrounding clones (Fig 1H, I and Fig 2E, F) shows no effect in the boundary region, arguing against said feedback signal. We were very careful in our text to restrict our conclusions to a local function, stating that our data argue against "local compensatory feedback signals", which is what we had designed the "EE killing" experiment to test. Furthermore, if, as the reviewer mentioned, there is a buffering effect non-autonomously by Slit producing EE cells outside of the Slit RNAi clones, we find it difficult to understand how this feedback could precisely tune EE fate production by the ISCs in a localize manner. In other words, our data demonstrate that loss of numerous EEs in a region of tissue does not trigger a compensatory response, indicating that a decrease in local Slit levels (or other EE derived signal) is not sufficient to trigger EE production. In addition, our experiments increasing EE cells and assess a potential effect non-autonomously (Fig 1E-I), also argue that compensation does not occur in the wild-type tissue to reduce the number of EE cells. Furthermore, our data demonstrate that modulation of Slit levels in a clone does not lead to alteration of adjacent EE cells (Fig 2A-I), strongly arguing against a feedback signalling model.*

While our data, experiments, and conclusions are regarding local feedback, we have performed a number of additional experiments to further address a potential global gut function of Slit signaling.

-First, as suggested both by Reviewer 1 and Reviewer 3, we tried to kill all EE cells throughout the intestine using both ProsGal4, GAL80ts and Rab3Gal4, Gal80ts. Unfortunately, with the induction times that we tried (2 and 4 days) we could not successfully kill a large portion of EEs using this method.

-Secondly, as suggested by the reviewer, we tried to use the esg-Flip out system to general clonally derived guts expressing UAS-Slit RNAi throughout. However, in our hands, while we had some patches of tissue marked, this method did not result in all gut EE cells expressing RNAi, a requirement for this experiment.

-We then used Pros^{Voila}-Gal4, UAS-Slit RNAi to knock down Slit in both the brain and in the intestine. Consistent with the previous data knocking-down Slit using amonGal4 (showing a 1.3-1.5X increase in EE cells, Biteau and Jasper, Cell Rep, 2014 Fig2G and Zeng, Han…Lin, Hou, Cell Rep, 2015, Fig S7A) and actin-Gal4 (showing a 1.6X increase in EE Cells, Biteau and Jasper, Cell Rep, 2014, Fig2F), we found a similar 1.3X increase in the density of EE/total cells with Pros^{Voila}-Gal4 (see new Appendix Fig S2). Thus, there is a mild effect on EE cells upon Slit knockdown. This could support either some sort of global requirement in EE cells for Slit or a role of neural derived

Slit which would also be targeted by the Pros^{Voila}-Gal4. It should be mentioned that a 1.3-1.6X alteration in EE density is quite small and therefore is not likely to be a major contributor to EE versus EC fate choice. In addition, as a 1.5X difference is seen between the amon-Gal4 and actin-Gal4 control lines (compare Biteau and Jasper, Fig 2F and G), this level of variation also occurs due to genotypic variation.

-The reviewer suggested using an nSybGal4 driver to target neurons, however this driver also appears to be expressed in some EE cells (as suggested by the reviewer and as document in Bost, thesis, Columbia University Press, 2014), which we felt would confound any interpretation of knocking down Slit with this driver.

-Nevertheless, we did use nSybGAL80, ProsVoila-GAL4 line (nSyb-Gal80 should block brain expression and some EEs, while Pros-Voila should drive in gut EE cells) to express Slit RNAi throughout development and adult life, with the idea that a positive result would have provided evidence for EE-derived Slit controlling ISCs. However, in this experiment, we found no difference between nSybGAL80, ProsVoila-GAL4> control or UAS-Slit RNAi. Therefore, while these data might suggest that neuronal derived Slit plays a role. However, the caveat mentioned above is that the nSyb might also block expression in EE and therefore a role for EE derived Slit cannot be definitely ruled out with these available drivers. In addition, we have noted a weak brain expression in this line, suggesting that nSyb-GAL80 cannot completely block brain expression.

Our data would not exclude some sort of compensatory effect when all EE cells are killed, however, our data do argue strongly against such a feedback precisely tuning EE fate during routine homeostasis and which is on what we conclude in this manuscript. In addition, what type of physiological stress could trigger such general EE loss in the normal life of a fly is unclear.

Therefore, we feel that our data argue against a local compensatory feedback signaling and demonstrate that Slit does not act as a very precise or robust feedback mechanism at a local level.

We believe that there are several factors that could explain why global knock-down of Slit elicits an effect whereas local Slit loss does not:

A) *It could be that Slit does not act locally, but acts at a more global level throughout the entire gut (as suggested by the reviewer) or in the amon+ cells of the brain to buffer EE cells.*
B) *It should also be noted in Biteau, 2014 (Fig2F and G), alterations in EE density also varied between controls from 8% in the amonGal4 line to around 12.5% in the actin-Gal4 line, suggesting that genetic differences can impact EE density. The effect of ProsGal4ts>Slit RNAi is a 1.3X increase in EE density, raising the possible that subtle alteration can be due to genotype differences.*
C) *Recent work from the Igaki lab (Vaughen, Dev Cell, 2016) has demonstrated that Slit-Robo2 signaling functions in delamination and cell death of tumorous cells. It is therefore possible that the phenotype of a mild increase in EE cells upon Slit/Robo2 alteration is due to this function leading to a block of delamination and turnover of EE cells.*

We have now added a potential explanation to our text:
" It is likely that the observed increase of 1.3-1.5X of EE cell density in Slit knockdown by ProsVoila-Gal4 and amonGal4 require a loss of Slit throughout EE cells of the gut, perhaps suggestive of some more global level control of EE cells. It is also possible that ISCs could be controlled through neuronal Slit ligand production rather than local EE-produced Slit. Alternatively, Slit/Robo2 signaling may be promoting EE cell delamination and death as recently described for tumorous cells of the wing disc (Vaughen et al., 2016)." And in the Discussion:
"Different scenarios could account for the lack of local effect of clonal alteration of Slit levels on EE cells and the mild 1.3-1.5X increase in EE cell density when Slit signaling is knocked down globally in EE cells with ProsVoilaGal4 and amonGal4. It is possible that Slit has a more global function to buffer EE levels without controlling cell fate decisions locally. Alternatively, neuronal derived Slit also targeted by EE Gal4 drivers could subtly regulate EE numbers. Finally, Slit/Robo2 signaling has recently been shown to play a role in cell delamination and death (Vaughen et al., 2016), which may contribute to the altered EE numbers.."

Additional comments:
- In Figure 1 and 3: what happens when ee cells are lost or overrepresented? What is the trade off within the clones? Are there more or less ISCs or ECs. In other words: are ee cells replaced by a different cell type or made at the expense of another cell type? A clarification including quantification of different cell types within the clones would be useful.

This is an interesting point: to address this, as suggested by the Reviewer we have now added new Appendix Fig S4 where we show the quantification of ISC and EC number and percentage per clone in wild-type compared to numb^{15} mutant clones, UAS-Numb. In addition, we have added new Fig8N, a quantification of our transient clone data including ISC/EB cells. While at the clone level, there is a trend to have more ECs in numb mutants and less in UAS-Numb clones, compared to wild-type, neither is statistically significant. Indeed, as the EE decision is made once every 4-5 divisions on average, detecting a slight increase will be difficult. Importantly, however, by analyzing our transient clone data of the single cell clones arising from wild-type or numb mutants, we find that in numb mutants, while the combined EB/ISC population remains constant, the EC population increases while the EE population is lost. This, indeed, supports the idea that the cells that would normally become EE, now take EC fate. We have now included this in (Fig 8N) and stated: "In addition, analysis of single cell numb^{15} mutant clone indicated that there was a relative increase in EC cells, suggesting that EC cells are produced at the expense of EE cells in absence of numb activity (see below and Fig 8N)."

-Data in Figure 4 is difficult to see. Image quality improvement would help.

We agree that in this tissue, the crescents do not project well and are sometimes

difficult to see in a 2D image. To address this, we have now added three new "Expanded view" images of rotating 3D reconstructions (new Movies EV2-4).

-Supplementary figure 2I misses its control figure?
We have now added this in new Appendix Fig S3

-Please provide quantification in Figure 6 A, B
We have now added this in new Fig 6

-Please provide controls in Figure 7 including clones carrying single mutations and transgene expression. Quantifications would be important as well.

We have now added this in new Fig 7; the UAS-Numb alone control is shown in Fig 3 which we explicitly make reference to in the text.

- Please provide controls in Figure 8
We have now added this in new Fig 8

- The quantifications provided in Figure 8 F do not make sense to me. How is it possible to have effect in percentages when neither the total number of cells nor the average number of ee cells in clones is not changed?

This is simply because there was a trend towards an increase in the average number of EEs per clone of the UAS-aPKC caax and a trend towards the decrease in the average number of cells per clone of the UAS-aPKC caax, though neither of these were statistically significant. However, the percentage of EEs per clone in the UAS-aPKC caax condition now becomes statistically significant due to these two trends. We have now increased our N for this experiment, and still find the same results (new Fig 8 G-J).

- Overall authors should provide quantification for a higher number of guts and clones.
We have now increased our numbers for wild-type (n=60), UAS-aPKC caax (n=57), lgl^4 (n=106) mutant clones, which still shows the same finding as our original numbers.

Referee #2:

In this manuscript, Salle et al explored the mechanism underlying EE vs EC fate determination in adult Drosophila midgut. The authors provided several lines of evidence arguing against the involvement of a negative feedback mechanism proposed by others, i.e., EE-derived Slit acts on Robo2 present on ISCs to limit EE production. They provided convincing data to show that Numb functions as a cell intrinsic factor essential for EE fate determination and Numb promotes EE fate by inhibiting N. Consistent with this notion, the authors showed that Numb acts upstream or in parallel with several transcription factors known to regulate EE fate. Using a Numb-GFP reporter, the authors found that Numb exhibited both

asymmetric (~80%) and symmetric (~20%) segregation during ISC division. In addition, altering the activity of cell polarity genes such as aPKC and lgl influenced both the dynamics of Numb segregation and the frequency of EE production. The authors also showed that continuing production of Numb is required for EE differentiation. Based on these and other observations, the author proposed that symmetric Numb segregation during ISC division and de novo synthesis of Numb in ISC daughter cells are essential for EE fate choice and this cell-intrinsic mechanism can lead to a balanced and tissue-wide production of EEs/ECs in the absence of a feedback mechanism. For the most part (except for the effect of polarity genes on EE determination), the experiments were well designed and executed, and the data are of good quality. The findings of symmetric and asymmetric segregation of Numb and its potential role in EE fate determination are novel and such mechanism is likely to be conserved in mammalian intestines. However, there are a number of issues needed to be addressed by additional experiments/discussion before acceptance for publication is recommended.

We would like to thank this reviewer for his/her general opinion of our manuscript and have now done a large number of experiments to address the specific points raised by this reviewer.

Major concerns:

1. It is clear that Numb is required for EE fate determination but it is not clear to me whether or why symmetric division is the driver for EE fate determination. The authors attempted to address this issue by altering the activity of polarity genes such as aPKC and lgl, which leads to symmetric Numb segregation. However, the effect on EE fate was very mild. In addition, altering the activity of aPKC or lgl could change the ISC division angle and/or affect several other cellular processes, which may contribute to the observed subtle change in EE fate determination. Indeed, it is not clear to me why overexpression of aPKC-CAAX and loss-of-lgl produced the opposite phenotypes with respect to EE formation while both caused symmetric Numb segregation. Did the authors confirmed Numb segregation is altered in lgl mutant cells? How does loss-of-aPKC affect Numb segregation and EE fate?

The role of polarity on Numb localization and EE fate is an important point that the reviewer raises and we have now conducted additional experiments (incorporated into new Fig 8E-I) as well as added a better discussion of this in the manuscript.

We believe that there are several levels of control on Numb at play here: regulation of Numb localization which may initiate EE fate acquisition, but as we had found that de novo protein synthesis of Numb is also required (Fig 8K-N), this indicates that localization is not sufficient. In addition, the cell polarity regulators lgl and aPKC, which control phosphorylation status of Numb, also likely have critical roles in controlling the activity of Numb protein as previously discussed (Langevin, CB, 2005; see Discussion of Wirtz-Peitz, Cell, 2008). We believe that it is for this reason that aPKCcaax has a different phenotype that lgl: Numb being active in aPKCcaax and inactive in lgl mutants.

To summarize our data:

1. In lgl mutants, we see that EE cells are lost, like in numb mutants, as in other developmental contexts. We believe that this effect is through regulation of Numb activity as has been suggested in the SOP (Langevin, CB, 2005). Indeed, upon the reviewer's suggestion we analyzed the localization of Nb-GFP in lgl mutants: While technically a very challenging experiment, we were able to assess Nb-GFP in 2 prometa/metaphase and in 4 telophase cells. We observed a lack of basal crescent formation in the prometa/metaphase (in contrast to wild-type where this crescent is always seen). This is reminiscent to the delocalization occurring during prometa/metaphase in lgl mutants in the nervous system. In telophase, however, we observed Nb-GFP present in crescents. Of note, this "telophase rescue" effect is well described in the literature and is detected in lgl mutants in neuroblasts (Peng, Nature, 2000; Lee, Nature, 2006) and sensory organ precursors (Langevin, Current Biology, 2005). In the SOP, lgl is essential for Numb activity and early localization, but not final telophase segregation. We have now changed the text accordingly to reflect the fact that lgl affects only alteration of Numb during early mitosis: "Of note, in sensory organ precursor cells and neuroblasts, while asymmetric Numb localization in early Mitosis does not occur, asymmetric telophase segregation is unaffected. However, lgl and numb both similarly affect cell fate decisions, indicating that the activity of Numb is abrogated (Langevin et al., 2005, Nishimura et al., 2007, Wirtz-Peitz et al., 2008). Consistent with Numb being inactive, we find that lgl4 had greatly reduced average EE number per clone with 1.1 compared to 3.3 in wild-type, and also a reduction in the percentage of EE cells per clone with only 1.8% compared to 10.2% in wild-type (Fig 8C,C';G-I)."
Thus, the published data in the SOP lineage indicate that lgl mutants have the same phenotype on cell fate as numb, indicating that it is required for Numb activity. Since, the telophase segregation of Numb occurs asymmetrically as in wild-type and only the early prometaphase localization is defective, this suggests that 1.) either early localization of Numb during the cell cycle is essential for its ability to inhibit Notch in the following cell cycle or 2.) there are additional activities controlling Numb activity linked to the phosphorylation status as previously noted (Langevin, CB, 2005; Wirtz-Peitz, Cell, 2008). We had previously discussed this in the text and have now added the above text, which is more clear.

2. aPKC mutation (new Figure 8E-I), EE cells are lost, as in numb mutants, the opposite of aPKCcaax, implying that Numb is not active in aPKC mutant conditions. We were unable to look at Numb-GFP in the mutant context since we were unable to find a fly line to make RED marked clones or negatively marked clones on this chromosome arm. The construction of such a line would have taken a long time. As aPKC mutation does not have telophase rescue in the nervous system, it would be predicted that Numb would be symmetric yet not phosphorylated and presumably inactive. However, the role of aPKC on early and late metaphase asymmetric localization of Numb is well described in the neuroblast system (Lee, Nature, 2006).

3. Our previous results and new data suggest that forcing more Numb symmetric in the contexts of overexpression of aPKCcaax, UAS-Numb or UAS-Numb-5A (a phosho mutant at aPKC sites that promotes symmetric segregation) is sufficient to produce extra EE cells. We believe that this effect is not stronger for several reasons: First, Numb symmetric segregation would likely only impact EE cells that arise from ISC cell division. Recent lineage tracing data from Zeng and Hou (see Fig 5, Zeng, Dev, 2015) indicate that many EEs arise from the differentiation of an ISC directly into an EE cell. Second, Numb requires partner proteins like alpha-adaptin, which may be limiting. Our overexpression data are consistent also with the partial cell fate transformations that are detected in the nervous system upon UAS-Numb overexpression (Frise, PNAS, 1996). Finally, our transient clone data indicate de novo synthesis and numb gene activity are required in differentiating cells (Fig 8K-M). Therefore, we believe that polarity likely plays an initial role in helping to keep Notch off, but this must be sustained by continual expression and activity of Numb. Of note, the temporal functions of Numb polarized segregation have not yet been explored in the nervous system, though our data would suggest that numb gene activity may also be required after Numb segregation.

The reviewer also correctly points out that alteration of aPKC and lgl may have many cellular effects. We do point out this caveat on p.20 " Lgl and aPKC have many cellular functions and additional roles on factors other than Numb cannot be excluded." Overall, our combined phenotypic data as well as data on Numb-GFP and NotchiGFP localization strongly support our model proposing that symmetric distribution of Numb establishes an initial bias that is then reinforced with newly synthesized Numb acting with its partners of the AP-2 complex.

Is there a good correlation between symmetric Numb-GFP segregation and Pros expression?

We had initially mentioned this in our Discussion, but given the reviewer's comment, we have now moved this data into the results section (New Appendix Fig S5). "While we observed Pros expression in 13.5% of dividing cells (10/74 cells); asymmetric Pros segregation occurred in 6.8% (5/74 cells) of dividing cells. Pros and Numb-GFP colocalized asymmetrically in 5.4% (4/74 cells) of the dividing cells. In addition, 5/74 of the dividing cells also had asymmetric Numb-GFP with symmetric Pros in the future daughter cells. This lack of correlation would be expected if Numb and Pros acted at different times in subsequent cell cycles, for example." We had extensively looked at Pros protein and Numb-GFP and found no general correlation. This would be expected if Numb acts in a first cell cycle to inhibit Notch, which in the following cell cycle would derepress Scute and drive Pros expression at later time or in the following cell cycle.

Perhaps a better experiment would be to engineer a Numb that can no longer undergo asymmetric segregation but retain its ability to inhibit N and then determine whether expression of such Numb variant will alter EE fate.

Thanks for this suggestion. We have tested the Numb-5A mutant, an alternative aPKC phosphorylation site mutant rending Numb more symmetric (Smith, EmboJ, 2007). We find that this also promotes extra EE cell fate. We have now added this data to new Fig 8D; D', G-I. In addition, the overexpression of wild-type Numb, which presumably leads to more symmetric Numb inheritance (Fig 3C) also drives extra EE cell fate. Another set of fly lines harboring different aPKC-phospho-mutants in Numb developed by Cheng-Yu Lee's lab (U. Michigan, personal communication) were no longer available.

2. In Fig3C, how did the authors mark the early mitotic cells? What fraction of early mitotic cells contained basal Numb-GFP. Fig. 3D-E, it would be informative to show side views of dividing cells to determine the relationship between symmetric/asymmetric Numb-GFP segregation and cell division angle. For example, is Numb-GFP asymmetrically segregated in ISCs that divide apical-basally but symmetrically segregated in ISCs that divide along the basement membrane? It would also be interesting to determine whether altering the cell division angel (for example in Par6 or aPKC RNAi background) affects symmetric vs asymmetric Numb-GFP segregation.

Presumably the reviewer is referring to Fig 4 and not Fig 3: In this experiment, early mitotic cells were detected based on chromatin condensation or PH3 staining. 100% of the early mitotic cells that we assessed (n=119) displayed this basal "patch" of Numb-GFP localization. We had previously mentioned "Interestingly, this early asymmetric polarization was observed during alldivisions." We have now stated: "Interestingly, this early asymmetric polarization was observed during in 100% of cells in prophase, n=119.", to further clarify.

We have now included expanded views of 3 movies of rotating images of the basal "patch", asymmetric, and symmetrically segregating Numb crescents (Movies EV2-4). In addition, upon suggestion of the reviewer, we reanalyzed our data for potential correlations with Numb segregation and division angle. We find that there are more divisions parallel to the epithelial plane having symmetric Numb segregation than with a tilted angle. We have now added this observation to new Fig 4E.

It would also be interesting to determine whether altering the cell division angel (for example in Par6 or aPKC RNAi background) affects symmetric vs asymmetric Numb-GFP segregation.

This experiment has been previously done for the PTB-domain of Numb in Goulas, Cell Stem Cell, 2012 who demonstrated that Par6 and aPKC indeed alter both angle and inheritance of the PTB-domain of Numb. Therefore, we felt that it would not be possible with these experiments to uncouple the division angle per se from the symmetric vs asymmetric effects.

3. A major conclusion of this study is that feedback mechanism is not involved in balancing the EE/EC production. How do the authors explain a previous study

showing that RNAi of Slit in EEs increased EE number? In Fig. 1C, the authors showed that local depletion of EEs did not affect the frequency of EE formation in surround tissues. Is it possible that the remaining EEs produced enough secreted signals to maintain the negative feedback?

We would first like to clarify our conclusions relative to those previously made by Biteau and Jasper, Cell Reports, 2014 and Zeng, Han...Lin, Hou, Cell Reports, 2015: These studies indeed both proposed that local Slit signals control the proportion of EEs in the tissue via signaling to ISCs to inhibit EE cell fate commitment. While both studies provided evidence that the knockdown of Slit using amonGal4 (EE and neurons) or actinGal4 (ubiquitous) results in a mild 1.3-1.6X increase in EE density, neither study tested whether, indeed, this signal could act at over local distances. One of the strengths of our study is that we test precisely this. We find that neither increasing nor decreasing EE cell numbers, nor increasing or decreasing Slit levels leads to any effect locally on EE density. The conclusions that we make are regarding a lack of local feedback control on cell fate that our data strongly support. We believe that our findings provide an important conceptual shift in thinking about this cell fate decision not being controlled by local feedback, but instead being a lineage intrinsic decision.

We have now also repeated the knock-down of Slit in adults using ProsVoila>UAS-Slit RNAi. Consistent with the published reports using amonGAL4 lines, we find a subtle, but statistically significant alteration in EE cell density of 1.3X more upon Slit knock-down. It should be noted that genotype also can similarly influence EE density as the amonGal4 and actinGal4 controls presented in Biteau and Jasper, Cell Reports, 2014 (see their Fig2F and G) are approximately 8% and 12.5%, respectively, for a 1.5X difference.
In addition, we believe that the differences in local versus global effect could be explained by the following possibilities:
1. Slit may be derived by neurons, which are also targeted by the ProsGal4 line.
2. Slit may act more globally throughout the tissue to buffer EE density in a subtle manner to 1.3X, without having the ability to fine-tune local ISC-EE signals.
3. The role of Slit/Robo2 signaling may be indirect and via the recently characterized function of this pathway in cell delamination and cell death (Vaughen, Dev Cell, 2016) perhaps having a differential impact on EEs and ECs.

Irrespective, we feel that experiments directly test the role of local feedback and provide strong evidence that an EE emitted signal does not block EE specification during routine tissue homeostasis.

Does killing all the mature EEs using a pan EE-gal4 driver affect EE formation?
In Fig. 1E, have the authors verified that EEs in N RNAi still express Slit?

As suggested by the reviewer, we attempted to kill all EE cells with both Pros^{Voila}-Gal4 Gal80ts UAS-Reaper and Rab3-Gal4 Gal80ts UAS-Reaper, but could not successfully kill a majority of EE cells perhaps due to timing of induction (2-4d). We also now

verified that the ectopic EE produced in Notch RNAi conditions express the proposed Slit (new Appendix Fig S2D).

Other concerns:

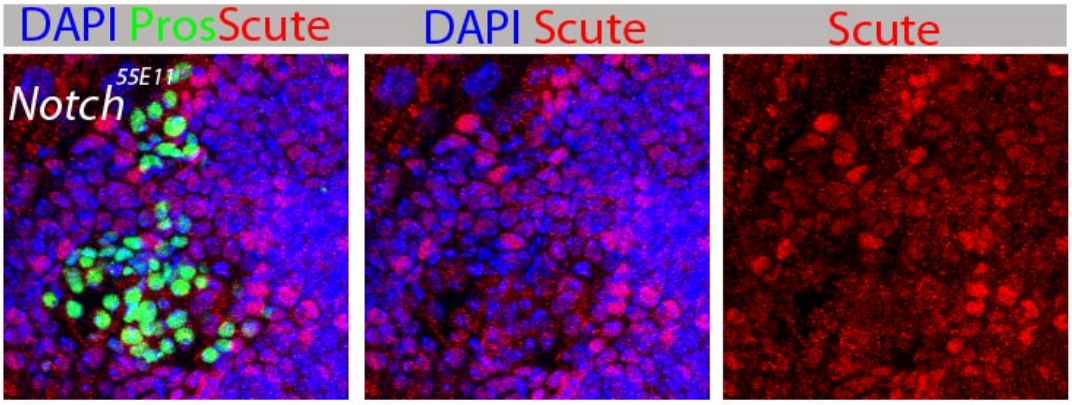
1. While it is interesting to observe that Numb is both asymmetric and symmetric segregated during ISC division and their respective frequencies correlate with those of EC and EE lineages. It is counterintuitive and puzzling that the asymmetric segregation of Numb into one the two ISC daughters doesn't seem to play a critical role in ISC/EB specification because loss of Numb did not seem to affect ISC self-renewal. In neuroblasts, asymmetric segregation of Numb is critical for asymmetric N signaling and subsequent fate determination of their daughter cells. How then is the asymmetric N activity established/maintained in ISC/EB lineage in the absence of Numb? In light of a recent finding that asymmetric BMP signaling can influence ISC/EB fate choice (Tian 2014), it is likely that BMP signaling may replace the function of Numb to inhibit N in ISCs. The authors should discuss this issue to erase this "puzzle" from readers' minds.

We thank the reviewer for this point. We understand and agree that it is a bit counterintuitive that the asymmetric segregation of Numb is not essential for ISC fate. Nevertheless, this is what the genetics indicates (see Bardin, Development, 2010 and new Appendix Fig S4A-D). It may well be that in wild-type contexts, when Numb is present, it does have a major role in biasing the decision, but that additional backup mechanisms exist to allow for ISCs to be correctly maintained in absence of Numb. It would not be surprising that multiple mechanisms ensured the robustness of ISC maintenance. Certainly, the data of Tian, 2014 suggesting that Bmp signaling can impact Notch to control fate is very relevant and we have now added a better description of this work and discussion of this point including additional concepts such as lateral inhibition and control via Sara endosomes.

"Interestingly, our data here and our previous data (Bardin et al., 2010) indicate that numb is not essential for ISC cell fate establishment or maintenance despite being asymmetrically segregated in a majority of ISC divisions. How then is Notch signaling kept off or low in the ISC to prevent its differentiation and how is a biased asymmetric fate outcome established? It is possible that Numb might normally bias this decision, while additional mechanisms may promote ISC in numb mutant clones. However, clearly additional mechanisms exist to regulate this important fate decision. One such mechanism is the asymmetric secretion of Bmp ligands on the basal side of the EC cells has been shown to inhibit Notch signaling in the most basal daughter cell (Tian et al., 2014). It is also likely that feedback mechanisms described during lateral inhibition in the nervous system act to amplify Notch signaling differences between the two ISC daughter cells. Consistent with this, we have previously shown that inactivation of Notch target genes of the E(spl) family are critical to limit ISC fate specification (Bardin et al., 2010). In addition, control of trafficking and activity of Delta and Notch through sara endosomes (Montagne et al., 2014) as well as addition mechanisms probably act redundantly to ensure ISC maintenance."

2. Previous studies indicate that symmetric ISC division can also produce two ISCs. What then determines EE vs ISC fate choice after a symmetric division?

How ISC symmetric division leading to two ISCs occur and what determines EE vs ISC fate after an ISC division are clearly very important questions. We feel, however, that these are beyond the scope of our work here. Regarding ISC/EE fate after division, our current hypothesis is that the precise levels of Scute in the nucleus dictate EE versus ISC choice and likely relies on complex feedback loops controlling Scute expression and protein activity (known for bHLH factors, see Quan XJ, Cell, 2016). Our previous work and that of others suggests that Scute plays a crucial role in EE fate. We have recently found that when Notch is inhibited (N RNAi), Scute accumulates to high levels in the nuclei of ISCs (see unpublished data below). This suggests, 1. That Notch signaling normally inhibits Scute accumulation, 2. In absence of Notch (or in low Notch, presumably similar to when Numb is acting) ISCs with high Scute and EEs without Scute can both be specified and may depend on levels of Scute. Of note, this hypothesis is consistent with genetic data of (Guo, Science, 2015, Fig 6D-H).



In *Notch* mutant clones Pros+ EE cells (Green) have low and more cytoplasmic Scute (red). Pros negative cells (ISC cells) have higher levels and nuclear Scute protein (red).

Regarding a symmetric ISC division leading to two ISCs: this is indeed a major outstanding question in the field. There is some evidence that cell cycle kinetics can impact this (Kohlmaier, Oncogene, 2015), and it certainly merits further investigation. Again, we feel that this question is a study of its own and outside of the scope of our work here.

3. A previous study showed that overexpression of aPKC-CAAX inhibited ISC fate and promoted EC fate likely by activating N (Goulas et al., 2012), whereas in this study, the authors showed that aPKC-CAAX overexpression promoted EE fate (Fig 8) through promoting Numb symmetric segregation and thus inhibiting N. How do they reconcile these seemingly contradictory results? Does aPKC-CAAX overexpression

promoted EE fate depending on Numb?

Goulas et al. using an actGal4 flip-out clone induction method at 4-5d post heat shock, they found that overexpression of aPKC-CAAX produced smaller clones, though larger clones were also detected. Of the small clones in the 1-4 cell bin, they show that 46% have lost stem cells. Whether the stem cells died or differentiated under these conditions is not clear. In addition, the actGal4 flip-out induction method can also target non-stem cells and therefore it is not entirely clear that clones derived from single stem cells are being analyzed. Our analysis focuses on MARCM clones at a later time point, which may correspond to the larger clones that were seen in the Goulas analysis. We did note in the course of our experiments that clones were less frequent than controls, which could be due to ISC loss as previously detected. We did obtain clones however. We quantified EE numbers in these clones whereas this analysis was not performed in Goulas, so an effect on EE cells in their data cannot be determined.
Because there is no discrepancy between our data and that of Goulas and colleagues and in the interest of time, we did not directly test whether numb was essential in the aPKCcaax context (as these are both on the 2nd chromosomes and construction of this line and the experiments would have taken significantly longer than our allocated time.)

4. Some data are missing:
---P14, "Furthermore, as 59% of these cells also co-expressed Dl, they likely represent newly differentiated EEs recently made from ISCs and which still have Dl protein".
Thanks- we have now added the reference in the text to Fig 5C-C"': "Furthermore, as 59% of these cells also co-expressed Dl (Fig 5C-C'), they likely represent newly differentiated EEs recently made from ISCs and which still have Dl protein."

---P21, "We found no obvious correlations between Numb and Pros segregation in adult ISCs. While, we observed Pros expression in 13.5% of dividing cells (10/74 cells); asymmetric Pros segregation occurred in 6.8% (5/74 cells) of dividing cells. Pros and Numb-GFP colocalized asymmetrically infrequently in 5.4% (4/74 cells) of dividing cells. 5/74 of the dividing cells also had asymmetric Numb-GFP with symmetric Pros in the future daughter cells". (These data should be included in supplementary figures).
These have now been included as new Appendix Fig S5A-B" and based on Reviewer 1's comments, we have now included the numbers in the main text, bottom of p.13: "We found no obvious correlations between Numb and Pros segregation in adult ISCs (Appendix Fig S5A-B'). While we observed Pros expression in 13.5% of dividing cells (10/74 cells); asymmetric Pros segregation occurred in 6.8% (5/74 cells) of dividing cells. Pros and Numb-GFP colocalized asymmetrically in 5.4% (4/74 cells) of the dividing cells. In addition, 5/74 of the dividing cells also had asymmetric Numb-GFP with symmetric Pros in the future daughter cells."

5. P10, "NRE-Gal4" needs a reference.

Thanks for pointing this out- it has now been included.

Referee #3:

In this manuscript, Bardin and colleagues studied the mechanisms of enteroendocrine cell specification in adult Drosophila intestine. They first provided evidence to argue against the existence of a local feedback mechanism in regulating EE differentiation, and then proposed instead an intrinsic mechanism by Numb in the process. They found that Numb was required for EE differentiation and overexpression of Numb produced more EE cells. Interestingly, Numb protein showed both asymmetric and symmetric segregation in dividing cells. Using subcellular distribution of Notch-GFP as a Notch activity indicator, the authors found that the percentage of dividing cells with symmetric Numb segregation was consistent with the overall ratio of EE cell population and the percentage of precursor cells that adopts EE cell fate. In addition, disrupting the function of cell polarity genes caused altered production of EEs, possibly due to altered symmetric or asymmetric Numb segregation during stem cell division. These observations let the authors to propose that Numb act as an intrinsic factor to regulate EE fate decision during asymmetric stem cell division by inhibiting Notch.

Numb is best known as a critical cell fate regulator during asymmetric cell division of neuronal stem cells during Development. The identification of Numb as an important regulator for EE cell specification in intestine is novel and should be of interest to the field. But one major concern is that many results are only correlational observations, which are not sufficient to draw the conclusion that different modes of numb segregation lead to distinct progenitor cell fate.

We would like to thank this reviewer for his/her comments on the manuscript. We have now done a large number of additional experiments to address this reviewer's and the other two reviewers points, which we detail below. Overall, we feel that our manuscript provides solid and compelling data showing that EE cells are NOT strongly controlled through local compensatory feedback, that Numb is a critical factor to specifically control EE fate, that it segregates both symmetrically and asymmetrically, and that polarity factors regulating asymmetric distribution and also like activity of Numb also have important roles in EE fate. We believe that together these data go beyond correlation, provide novel insight into how cell fate decisions are made in an adult homeostatic tissue, and lead to a new model for EE fate acquisition based on a lineage intrinsic instead of feedback signaling from EE cells.

Specific comments:

Direct evidence is needed to support a role for Numb in regulating Notch during EE specification. Does Numb segregation correlate with reduced Notch activity in dividing cells? Does loss or gain of function of Numb cause increased or decreased

Notch activity, respectively, in progenitor cells?

To our knowledge, the only known function of Numb in Drosophila is in inhibiting Notch signaling during binary cell fate decisions. This has been extremely well characterized in the central nervous system, peripheral nervous system, and muscle precursor cells. While this does not exclude the possibility that in the EE cell fate decision, Numb has some other relevant target, we feel this is extremely unlikely for the following reasons:

1. *There is an abundance of data indicating that no or low levels of Notch signaling are required for EE fate (Michelli and Perrimon, Nature, 2006; Ohlstein and Spradling, Nature, 2006; Ohlstein and Spradling, Science, 2007; Biteau and Jasper, Cell Reports, 2014; Beehler-Evans and Micchelli, Dev, 2015; Wang…Xi, Dev., 2015) and our data (Appendix Fig S3A-A""). Thus, a state of low Notch is required for EE fate.*
2. *We find that mutation of either numb or alpha-adaptin, genes known to act together to regulate Notch activity during asymmetric cell divisions of the SOP, leads to loss of EE cells (Fig 3 and Fig 6F, F' H-K). Furthermore, our data indicate that Numb requires this interaction with alpha-adaptin (Fig 6G, G', H-K). The known function of this complex is to regulate Notch trafficking and signaling activity.*
3. *Notch inactivation in numb, is sufficient to produce EE cells. This suggests that in the numb mutant, high levels of Notch prevent EE fate and that, when Notch signaling is lowered through genetic means, numb is not required.*
4. *Importantly here, gain and loss of Numb can phenocopy the loss and gain of Notch phenotypes on EE cells, respectively.*

The reviewer has also proposed an experiment to monitor Notch activity upon symmetric segregation of Numb, upon Numb over expression or loss of function. However, the predicted results of this experiment are not so straightforward:

First, it is known that there is no detectible Notch activity in dividing (anaphase and telophase) ISCs as detected by the available Su(H)GBE reporter of Notch activity (see Perdigoto…Bardin, Dev, 2011 Fig 6H). Numb blocks ligand dependent Notch signaling and would be predicted to act at cytokinesis or in the following cell cycle to inhibit Notch signaling between the two sister cells. Therefore, we would not expect an increase in Notch reporter activity during anaphase-telophase. Consistent with this, ISCs are not lost in numb mutants (new Appendix Fig S4)

Secondly, given the data in the nervous system, upon numb loss of function, one would not expect high aberrant levels of Notch all over, but instead the prediction would be that the 20% of divisions that would normally result in Numb-inhibited Notch leading to EE fate acquisition would be lost. Trying to directly visualization a change from 80% of divisions leading to nuclear Notch in one sister to the predicted 100% in numb mutants, is extremely difficult. We, nonetheless, tried to examine NiGFP in numb mutant clones, but the occurrence of pairs of daughter cells in the correct stage to assess nuclear Notch is rare and we did not get a large enough dataset to quantitatively compare mutant and

wild-type. However, it is clear from our assessment of downstream cell fate markers, that we have a relative increase in EC fate producing divisions over EE fate producing divisions (new Fig S8N). Though we do not visualize Notch activity in this experiment, it is known that strong Notch is necessary and sufficient for EC fate, and is therefore indirect evidence of extra Notch activity in the numb mutant context.

The genetic results do not seem to be strong in supporting an instructive role for Numb in EE specification. Pros+ cells are significantly reduced but not eliminated numb mutant clones. Do these mutant Pros+ cells express EE-peptides similar to normal EE cells? On the other hand, overexpression of numb only mildly increases EE production, and does not seem to block EC differentiation. Does it mean that Numb is not sufficient in inducing EE cell fate? Because the phenotypes associated with altered numb function are relatively week, it might not be suitable to consider Numb in a linear pathway with Ttk or Sc.

We would like to first clarify a point: The mutant phenotype of numb^{15} as well as a newly generated deletion mutant of numb we have generated in the lab by CRISPR-mediated deletion (new Appendix Fig S3L-N) are extremely strong with very few EEs in any clones. There are so few EE cells in the numb^{15} mutant clones that staining for peptides in a quantitative manner to know how the numb mutant EEs compare to wt is not feasible. As the peptide hormones are regionally expressed and often only in 1 EE in a pair, we would need very high numbers of numb mutant clones (in which finding and EE cell is extremely rare) in order to determine if the numb mutants differed from wild-type in this regard. In addition, it is likely that the EE cells occasionally present in numb mutant clones are due to a cell that for some reason has maintained some Numb protein and therefore may not represent a mutant cell per se.

With respect to the overexpression, the reviewer's conclusion is that numb is not sufficient to drive EE fate or block EC differentiation. In contrast, we would interpret this differently: Numb is sufficient to drive EE fate, just not in 100% of divisions. The ability of UAS-Numb transform some but not all cell fate decisions has also been described the peripheral nervous system (see Frise, PNAS, 1996). Numb must interact with other endocytic trafficking components such as alpha-adaptin, which could be limiting in these cells. We did leave open the possibility for parallel pathways, which are always difficult to exclude, as we concluded: "Altogether, our data indicate that numb acts upstream of or in parallel to previously described regulators of EE fate." We now explicitly state this as well in our Fig 9 legend:
"Our genetic data suggest that Numb inhibits Notch through its interaction with the AP-2 complex. Inactivation of Notch drives expression of the proneural genes Scute and Asense, which, in turn, promote Pros expression and EE fate acquisition. Parallel action of these genes cannot be ruled out. Ttk69 is also required to block EE fate (not shown) (Wang et al, 2015)."

Is the segregation pattern of Numb during stem cell division altered upon sc overexpression or ttk RNAi?

Indeed, it will be interesting in the future to understand the mechanism and upstream regulators of Numb asymmetric vs symmetric segregation. We were not able to test the effects of scute overexpression due to time constraints, however upon ttk RNAi, Nb-GFP segregates as in wild-type. We have now included this data in new Appendix Fig S5B-B'.

Figure 8: aPKC-CAAX over-expression leads to 92% symmetric distribution of Numb, but this only causes a mild increase of EE cells. The result seems to argue against the hypothesis that symmetric distribution of Numb is related to EE specification.

We will also refer the reviewer to our discussion above on this topic in response to Reviewer 2's point #1.

Briefly, we believe that polarized segregation likely provides an initial bias, but as we describe in the manuscript, regulation of Numb expression and activity after division are also important (see our transient clone experiment, Fig 8K-N).

-In addition, we have now added new data showing the aPKC mutation has the opposite phenotype of aPKCcaax, with a loss of EE cells. Moreover, it is likely that segregation of Numb only impacts those EE cell fate decisions that arise after ISC division and not those that arise from direct differentiation of and ISC into an EE cell as reported by Zeng and Hou, Dev, 2015. We have included a better discussion of these points now in the Discussion:

"Our findings indicate that polarity cues can also impact Numb segregation during cell division and affect EE cell fate determination. The forced expression of aPKCcaax, which renders Numb symmetric, led to a mild increase in EE cells, whereas the inactivation aPKC inactivation has the opposite effect. Moreover, expression of UAS-Numb and UAS-Numb-5A, a form mutant for some aPKC phosphorylation sites that promotes symmetric Numb segregation in the SOP (Smith et al., 2007) resulted in additional EE cells. In addition to affecting Numb segregation, Numb phosphorylation and possibly activity are also altered by aPKC phosphorylation (Betschinger et al., 2003, Smith et al., 2007, Wirtz-Peitz et al., 2008). Interestingly, lgl mutant clones also show a loss of EE cells, consistent with published data that lgl and numb have the same phenotypes in the nervous system and that lgl has additional roles controlling Numb activity (Langevin et al., 2005, Nishimura et al., 2007, Wirtz-Peitz et al., 2008). Thus, we hypothesize that an initial bias in Numb inheritance can initiate inactivation of Notch signaling, however our data also indicate that de novo synthesis of Numb in the differentiating daughter cell is critical for EE cell fate acquisition. Furthermore, polarity cues presumably would act on EE fates that arise after ISC cell division and perhaps not when an ISC directly differentiates into an EE. Thus, alteration of the inherited pool may only produce subtle defects in EE fate specification."

-We also reinforce this data on symmetric Numb segregation in our overexpression experiments of UAS-Numb, which presumably is inherited in both daughters in some cases (see Fig 3C, C', E-H), and UAS-Numb-5A (Fig 8D,D', G-I), a version mutated for some aPKC phosphorylation sites and segregates symmetrically in the SOP (Smith, EmboJ, 2007). In both contexts, extra EE cells are formed.

Fig1 and 2 showed some interesting data, but they are not directly linked to the rest of the story and therefore appear unnecessary.

We have considered this option, however, we would prefer to keep these data in this manuscript that is focused on EE fate determination in general.

2nd Editorial Decision 06 April 2017

Thank you for submitting a revised version of your manuscript. The manuscript has now been seen by the three original referees, who find that all their main concerns have now been addressed. There are just a few minor issues to be dealt with before formal acceptance here. Congratulations on a nice study!

1. Please implement the textual changes requested by referee #2
2. Please add the Author contributions and Conflict of interest sections after the Acknowledgements paragraph.
3. In the Figure EV1 panel C, the image appears to be a composite of two images. Please either provide a different image, or mention in the figure legend.
4. The manuscript currently contains only one EV figure. We can accommodate up to five typeset EV figures, which would render the data more easily accessible for online readers. You might consider transforming up to four of the Appendix figures into EV figures. Please see our author guidelines on details about the content and preparation of Expanded View material (http://emboj.embopress.org/authorguide#expandedview).
5. In the figure legend for Fig 6D, you state that "Ttk69 is also required to block EE fate (not shown) (Wang et al., 2015)." If the data are included in the cited manuscript, "not shown" should be removed. Otherwise, please add the corresponding data.

Please let me know if you have any further questions regarding this or any other points. You can use the link below to upload the revised version.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I am looking forward to seeing the final version.

REFEREE REPORTS

Referee #1:

This is a revised version of the original manuscript by Salle et al.

The authors have provided a detailed and convincing response to my comments, including most of my suggested experiments. I have no further requests. As far as I am concerned the manuscript is suitable for publication.

Referee #2:

In the revision, the authors carried out additional experiments and modified the text to address most of my concerns. I think it is OK to move forward. A few minor things:

P13 "Interestingly, this early asymmetric polarization was observed during in 100% of cells in prophase, n=119.

P19 "In contrast, the inactivation of aPKC had the opposite phenotype with have a decrease of EE cells from 1.6 EEs per clone in controls that represented 9.8% of cells to 0.3 EEs per clone in aPKCK06403 mutant clones that represented 2.9% (Figure 8E-I)".

P24 "Consistent with this, we have previously shown that inactivation of Notch target genes of the E(spl) family are (is) critical to limit (promote) ISC fate specification (Bardin et al., 2010).

P24. While discussing the role of BMP in ISC self-renewal, the authors may want to cite a recently published paper by Tian et al (Tian et al., PNAS 2017) that sustains the authors' previous conclusion that BMP signaling promotes ISC self-renewal, given the confusion in the field about the role of BMP signaling in midgut homeostasis and regeneration.

Referee #3:
The authors have adequately addressed most of the comments. Although direct evidence for a role of numb in asymmetric intestinal stem cell division is still lacking, the findings described in this paper are sufficiently novel and should be of interest to the field.

2nd Revision - authors' response 10 April 2017

Referee #2:
In the revision, the authors carried out additional experiments and modified the text to address most of my concerns. I think it is OK to move forward. A few minor things:

P13 "Interestingly, this early asymmetric polarization was observed during in 100% of cells in prophase, n=119.

Changed to: Interestingly, this early asymmetric polarization was observed in 100% of cells in prophase, n=119.

P19 "In contrast, the inactivation of aPKC had the opposite phenotype with have a decrease of EE cells from 1.6 EEs per clone in controls that represented 9.8% of cells to 0.3 EEs per clone in aPKCK06403 mutant clones that represented 2.9% (Figure 8E-I)".

Changed to: "In contrast, the inactivation of aPKC had the opposite phenotype with a decrease of EE cells from 1.6 EEs per clone in controls representing 9.8% of cells, to 0.3 EEs per clone in aPKCK06403 mutant clones representing 2.9% of cells (Figure 8E-I)."

P24 "Consistent with this, we have previously shown that inactivation of
Notch target genes of the E(spl) family are (is) critical to limit
(promote) ISC fate specification (Bardin et al., 2010).
Changed to:
"Consistent with this, we have previously shown that Notch target genes of the E(spl) family are critical to limit ISC fate specification (Bardin et al., 2010)," which is more clear.

P24. While discussing the role of BMP in ISC self-renewal, the authors may want to cite a recently published paper by Tian et al (Tian et al., PNAS 2017) that sustains the authors' previous conclusion that BMP signaling promotes ISC self-renewal, given the confusion in the field about the role of BMP signaling in midgut homeostasis and regeneration.

Thanks, we added this reference now.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Allison Bardin
Journal Submitted to: EMBO Journal
Manuscript Number: 95622

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We generally aimed to have a minimum sample size >20 for clonal analysis. In some conditions, technical difficulties or thresholding (see below) can lead to smaller sample sizes. For all measurements, sample size is displayed on the graph.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In order to ensure the relevance of EE and PH3 density measurments (Fig1, 2 and EV1), Intestines with a clonal area below 10% of the total area have been excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Normal distribution was not assumed. Consequently, Mann-Whitney tests were applied. For contingency analysis, Chi2 tests were applied.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution was checked using the Saphiro-Wilks test. Most of our data show a non-normal distribution.
Is there an estimate of variation within each group of data?	Variation is represented on graphs as "standard error of the mean" (SEM).
Is the variance similar between the groups that are being statistically compared?	Variances were not compared statistically. Equal variances were not assumed in this study.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies used in this study have been characterized in previous publications. Relevant citations are provided.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No