JCB

Tissue-wide coordination of Epithelium-to-Neural Stem Cell transition requires Neuralized

Chloe Shard, Juan Luna-Escalante, and Francois Schweisguth

Corresponding Author(s): Francois Schweisguth, Institut Pasteur

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June 4, 2020

Re: JCB manuscript #202005035

Dr. Francois Schweisguth
Institut Pasteur
BDCS
CNRS UMR3738
25 rue du Dr Roux
Paris 75015
France

Dear François,

Thank you for submitting your manuscript entitled "Neuralized regulates a travelling wave of Epithelium-to-Neural Stem Cell morphogenesis in Drosophila". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

As you will see, all three reviewers were enthusiastic about the work presented, considering it a significant advance in the field. However, all three felt that it would benefit from some revisions that would increase the clarity of the results and their interpretation. Most of these can be accomplished with text modifications and clarifications, or some additional analysis of data in hand. Reviewer #2 points out that your conclusion about mechanical coupling is over-stated, with the current data set. They suggest either some seemingly plausible additional experiments to strengthen this point, or some more substantial revisions to the text to make it clear that this point is speculative.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Mark Peifer, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript, the authors study an EMT-like process, the transition of the epithelium of the optic lobe of third instar Drosophila larvae to non-epithelial neural stem cells (NSC; neuroblasts). They found that neuralized (neur), which encodes a conserved E3 ubiquitin ligase, regulates apical constriction of epithelial cells, which define a pre-NSC, which still harbors epithelial characteristics. The authors suggest that the function of Neur is mediated by the downregulation of Crb via the degradation of specific isoforms of Sdt, which are targets of Neur, as previously shown by the Schweisguth lab. Apical constriction is mediated via accumulation of MyoII and the formation of an actin cable, which assures a coordinated constriction of a row of cells and hence ensures collective morphogenetic behavior.

This manuscript confirms that mechanisms described previously to act in other contexts are also involved in the process analyzed here: i) regulation of Crb by Neur via Sdt (previously shown in the in the developing gut of the Drosophila embryo); ii) a role of Neur in collective apical constriction of epithelial cells (previously shown during mesoderm invagination in the fly embryo); iii) Pulsing of MyoII prior to apical constriction (shown in different other epithelia); iv) formation of an actomyosin cable

at the contact site of cells expressing high and low levels of Crb (shown previously in the developing salivary gland primordium of the fly embryo). Putting all these observations together, the manuscript adds an interesting aspect with respect to collective behavior of cells in a tissue and is certainly interesting for scientists working in this field.

I have several questions/comments on the manuscript:
1.Fig. 1F: do really ALL Neur-GFP positive cells express Wor?

2.Fig. 1I /page 7: They write: "Given that Neur cells co-express Hth, ...". However, on page 6 they write: "... whereas ... Hth display only partial overlap with Neur-GFP". The latter does not fit the former.

3.From the data presented in Fig. 3, it is difficult to follow the conclusion on the role of Crb. They write (page 9): "We found that Crb localized at apical junctions in NE and lateral TZ cells but was lost in medial epi-NSCs". Since Neur expression is not shown, this conclusion is difficult to follow. I guess the authors used the most medial E-Cad positive cells (negative for Crb) as marker for the epic-NSCs. But they should write that. Does Crb (which should be Crb-GFP) really localize at junctions? In many epithelia, it localizes apical to E-Cadherin. At the bottom of page 9 they come with a model, which at this stage is not really justified by the data.

4.Page 10/Fig. 4C,E: they write: ".. we found that Neur-regulated isoforms of Sdt are expressed in the NE and TZ". In Fig. 4E, there seems to be more than one cell row, which is positive for E-Cad but negative for Sdt-GFP3, whereas Fig. 3E shows only one cell row. Does Sdt-GFP3 has a different expression pattern than other Sdt isoforms?

5.In Fig. 5F-H, the supra-cellular myosin cable is not really obvious in the wild-type control, so the conclusion that it is affected upon excision of exon 3 (Fig. 6G) is difficult to follow.

6.On page 13 they write: "... suggest a model whereby fate transition is mechanically coupled with cellular rearrangement to promote smooth progression of the differentiation front". Do they imply that fate transition is mediated by mechanical processes? In other words: what is cause, and what is consequence?

7. The last sentence of the Results needs smore explanation. They argue that the down regulation of Crb by Neur results in mechanical changes at apical junctions, which results in the adoption of NSC fate. However, while members of the Crb complex are downregulated in epi-NSCs, E-Cadherin is not. So why should there be "mechanical changes at apical junctions", if one assumes, that the presence of E-cadherin indicates the presence of junctions?

8. Throughout the text they conclude that the function of Neur is to downregulate Crb. However, Neur also down-regulates a subset of Sdt isoforms and PATJ. And PATJ has been shown to regulate AJ stability by regulating Myosin localization (Sen et al., 2012). What is the proof that the effects described are due to loss of Crb? They are more careful in the conclusion on page 12 (Fig. 6), where they write that "... RhoGEF3 appeared to contribute to the apical constriction ... independently of the downregulation of the Crb complex".

Minor points:

a.Page 11, 3rd row from the bottom: it should be Fig 5, not Fig. S5

b.Fig. 7I, J: A cartoon showing what they measured would be helpful.

c. I think that the title does not really point to the major findings.

Reviewer #2 (Comments to the Authors (Required)):

This manuscript investigates the cell biological mechanisms involved in the progression of neuroepithelial cells in the Drosophila optic lobe to a stem cell identity. First, the authors define a cell type that is epithelial, but associated with stem cell markers, which they define as epi-NSC. The authors find that this epi-NSC identity is associated with Neuralized-dependent apical constriction. The authors demonstrate pulsatile myosin contractions during stem cell emergence, reminiscent of the work of Simoes et al., 2017 and An et al., 2017 in the embryonic neuroectoderm. An actomyosin cable is also assembled at the border between epi-NSC and other transition zone cells, causing a straight interface. They show that apical constriction is promoted by Neuralized down-regulating Crb through Sdt and by RhoGEF3 expression.

The authors have done a beautiful job characterizing the cell biology of this system, which is fascinating. However, they fall short of demonstrating that mechanics is playing a substantial role in cell fate (see below). Thus, the conclusion that the Neuralized gene can "mechanically couple NSC fate acquisition with cell-cell rearrangement" is overstated. I suggest rephrasing this or having the authors analyze cell fate acquisition in mutants where apical constriction/actomyosin cable assembly is defective.

Major comments:

1. The authors propose that, "Neuralized therefore appears to mechanically couple NSC fate acquisition with cell-cell rearrangement to promote smooth progression of the differentiation front". However, the authors do not examine NSC fate in mutants that disrupt cell rearrangements, leading us to question whether it is important for the developmental process. Examining the consequence of disrupting this process, such as by using the NSC markers shown in Fig. 1, would enhance the clarity and significance of the work. Is the subsequent EMT-like transition inhibited or delayed? Are there significant differences in resulting NSC number?

2. The function of apical constriction in the integration of NSCs across the compartment boundary (as modeled in Fig. 7F) is confusing. Either bolstering this with further evidence or revising the model will benefit the paper. The authors showed that knocking-out RhoGEF3 inhibited apical constriction and that this was independent of Sdt/Crb regulation. However, in the RhoGEF3 knock-out condition the boundary between the epiNSCs and the rest of the transitional zone (Neur-GFP negative) appears to be straight, which would suggest that epiNSCs are still integrating into the NSC compartment. Thus, it appears that apical constriction is not important for NSC integration. A possible interpretation is that the supracellular myosin cable is important for boundary formation, but that apical constriction in the epiNSCs is not. Perhaps the myosin cable is important for the straightness of the boundary, while apical constriction is more important for the rate of integration?

Clarifying what the authors think the relative contributions of the actomyosin cable and apical constriction are in the context of NSC integration would help readers understand the importance of the cell biological effects that the authors described.

General minor comments -

1. Presenting larger views of smaller numbers of cells would help readers see and interpret the data. For most of the points made in this paper, a view containing one or two horizontal rows of cells (i.e., a couple of NSCs, an epiNSC, and a couple of Neur-negative TZ cells) would be sufficient, and perhaps more clear, than the lower resolution tissue-scale views.
2. I would suggest cropping and enlarging many of the figures so that a similar resolution to the schematic presented in Fig. 3B is shown (except of course in cases where tissue-scale observations are necessary, such as demonstrating the supracellular actomyosin)
3. Using multiple arrows in images to indicate features is distracting and in some cases interferes with visualizing the data. For instance, in Fig. 3A, it is difficult for a reader to judge for themselves the intensity of myosin along junction 'c' because it is covered by arrows in many of the cells in the image. If the authors were to show fewer cells, and then indicate the junctions of interest at the edges of the image, rather than on top of the data, this would be improved.
4. Showing less data (when redundant) in the primary figures would help make them more concise. For example, in Fig. 1, the data on Ase and Hth are essentially redundant with the Dpn data (at least in terms of demonstrating the intermediate state of the epiNSCs), and could be moved to the supplement in order to consolidate the message of the main figure.
5. The model for how the authors think apical constriction in the epiNSC cells is coordinated is a little unclear - Fig. 2 argues that apical-medial myosin pulsing is correlated with apical constriction, but from then on only junctional myosin levels are quantified. For instance, in Fig. 6 they demonstrate that RhoGEF3 knock-out does not perturb junctional myosin, but there is no analysis of medial myosin.
6. Fig. 6: There should be a quantification of mean angle, as in Fig. 7, for the RhoGEF3 knock-out. If there were, it might show that the disruption of apical constriction is not as important as the maintenance of the myosin cable at the compartment boundary for integrating epiNSCs into the NSC zone, as the compartment boundary seems quite straight in this manipulation (e.g., Fig. 6C + 6E), and while apical constriction is inhibited and total myosin levels are a bit lower, the relative differences in junctional myosin between compartments / columns of cells appears to be preserved.
7. Fig. 7: G-J: A direct measurement of the straightness of the Neur-GFP front would be more meaningful than measuring the angle between cell centroids because this would reflect a tissue-level feature.

Typo:

p. 7: 'NCS' → 'NSC'

Reviewer #3 (Comments to the Authors (Required)):

This is a beautiful paper analysing the transition from the epithelial state to the neural stem cell state within the optic lobe neuroepithelium in the fly larval (L3) brain. The authors identify an intermediary cell state whereby the neuroepithelial cells retain certain epithelial features such as E-Cad and apical actomyosin but lose components such as Crumbs and adopt what the authors term an epi-NSCs. They describe these cell undergoing apical constrictions driven by a pulsatile apical-medial actomyosin cytoskeleton. These oscillations depend on Neur and RhoGEF3. Furthermore an actomyosin cable forms at the differentiation front where the epiNSCs have downregulated Crumbs and thus an anisotropy border forms, leading to junctional myosin accumulation.

Using in depth immunofluorescence analyses of fixed and live samples combined with clever genetics such as clonal mutant or overexpression studies, the authors support the conclusion above with very high quality data. The figures are very well laid out and explained, making it easy for a non-expert in this tissue to understand what is shown and what the data imply. The manuscript itself is very clearly written.

I have no major concerns regarding any of the data or interpretations, only a few suggestions or question where the authors could maybe be a bit clearer in spelling out their conclusions.

With regards to the myosin fluctuations observed in both NE and epiNSCs: did the authors quantify the myosin cycle length in these two populations? In many instances such as the mesoderm or amnioserosa differences in cycle length have been linked to differences in productive versus unproductive area fluctuations, with longer cycle length being less productive, and in both cases a switch in cycle length turning unproductive into productive over time. Is something like this at work here?

It would be good if the authors could spell out more clearly in the paper what they deduce the role of the apical constrictions to be: are these driving area shrinkage to be able to accommodate more cells within a single line of epiNSCs? Or are they required to drive the cell intercalation that the authors presume must happen to integrate cells starting to express neur into a single line of cells? It would be too much for this paper to address this experimentally, but it would be good to understand the authors' thinking on these different possibilities.
Experimentally related to this: do the authors know whether in the absence of RhoGEF3 apical area oscillations are still happening? Are the fluctuations or the ratcheting perturbed? The authors only show that the apical area in the first row of cells is larger than in the wild-type.

In the discussion, the authors conclude the first paragraph by stating that 'Thus, mechanical coupling by Neur is proposed...'. I am not sure this can be called mechanical coupling. To me, such a term would suggest some mechanosensing is involved, and there is no evidence that this is the case. The authors need to be more specific in what they mean here.

Figure 4G'G', sdt[GFP3] flip-out experiments: The authors show Patj staining here, but I would be curious to know what this alteration does to Crumbs levels and localisation? Does the change in Patj reflect an identical change in Crumbs?

Minor comments:

Page 11, beginning of new paragraph:
The sentence describing the Brd[R] protein says 'where the lysine(K) residues have been mutated', which lysine residues are referred to? All? Or specific ones?

Page 13, beginning of new paragraph:
The authors state:
'The dynamics of the NE-NSCs fate transition has so far mostly been considered in one dimension, with the OL epithelium viewed as a cross-section and with a particular focus on temporal dynamics (Egger et al., 2010; Weng et al., 2012; Ngo et al., 2010; Wang et al., 2011; Orihara-Ono et al., 2011). In contrast, how fate dynamics is coordinated in a two dimensional epithelium has not been examined.'

I would consider what they describe in the first sentence as two dimensions, looking at a cut plane of an epithelium in xz, and their approach presented here mostly also looking in two dimensions, though a different orientation in xy (though combined with some images in xz as well). One dimension, as in the first sentence, would refer to a line!

Page 13, 8 lines from the bottom of the page:
There is a word missing here or an 'in' too many, the sentence says '...the up-regulation of Neur in induces...'

Page 15, line 6 from bottom: typo 'isofomrs

Rebuttal

Reviewer #1

In this manuscript, the authors study an EMT-like process, the transition of the epithelium of the optic lobe of third instar Drosophila larvae to non-epithelial neural stem cells (NSC; neuroblasts). They found that neuralized (neur), which encodes a conserved E3 ubiquitin ligase, regulates apical constriction of epithelial cells, which define a pre-NSC, which still harbors epithelial characteristics. The authors suggest that the function of Neur is mediated by the downregulation of Crb via the degradation of specific isoforms of Sdt, which are targets of Neur, as previously shown by the Schweisguth lab. Apical constriction is mediated via accumulation of MyoII and the formation of an actin cable, which assures a coordinated constriction of a row of cells and hence ensures collective morphogenetic behavior.

This manuscript confirms that mechanisms described previously to act in other contexts are also involved in the process analyzed here: i) regulation of Crb by Neur via Sdt (previously shown in the in the developing gut of the Drosophila embryo); ii) a role of Neur in collective apical constriction of epithelial cells (previously shown during mesoderm invagination in the fly embryo); iii) Pulsing of MyoII prior to apical constriction (shown in different other epithelia); iv) formation of an actomyosin cable at the contact site of cells expressing high and low levels of Crb (shown previously in the developing salivary gland primordium of the fly embryo). Putting all these observations together, the manuscript adds an interesting aspect with respect to collective behavior of cells in a tissue and is certainly interesting for scientists working in this field.

We thank the referee for her/his overall positive evaluation of our work

I have several questions/comments on the manuscript:
1.Fig. 1F: do really ALL Neur-GFP positive cells express Wor?

The referee is correct, some Neur-GFP positive epi-NSCs do not show detectable Wor, presumably because Neur is expressed slightly before Wor. We now write: '...most Neur-positive TZ cells expressed Worniu (Wor)...'

2.Fig. 1I /page 7: They write: "Given that Neur cells co-express Hth, ...". However, on page 6 they write: "... whereas ... Hth display only partial overlap with Neur-GFP". The latter does not fit the former.

Hth displayed only partial overlap with Neur-GFP. We therefore modified the first sentence and now state: "Since Neur-positive TZ cells begin to express Hth..."

3.From the data presented in Fig. 3, it is difficult to follow the conclusion on the role of Crb. They write (page 9): "We found that Crb localized at apical junctions in NE and lateral TZ cells but was lost in medial epi-NSCs". Since Neur expression is not shown, this conclusion is difficult to follow. I guess the authors used the most medial E-Cad positive cells (negative for Crb) as marker for the epic-NSCs. But they should write that. Does Crb (which should be Crb-GFP) really localize at junctions? In many epithelia, it localizes apical to E-Cadherin. At the bottom of page 9 they come with a model, which at this stage is not really justified by the data.

The referee is correct. As observed in many other epithelia in *Drosophila*, Crb is located just apical to the AJs in the NE. This is now shown in Fig S2. Otherwise we are now more precisely describing our results. We now refer to Crb-GFP (instead of Crb) and explain how epi-NSCs were identified based on shape and position when Neur-GFP could not be used as a marker: "Crb-GFP localized apical to the AJs in NE and lateral TZ cells but was lost in epi-NSCs, identified as apically constricted medial TZ cells..."
We agree with the referee that reference to our model came too early and have modified this sentence.

4.Page 10/Fig. 4C,E: they write: ".. we found that Neur-regulated isoforms of Sdt are expressed in the NE and TZ". In Fig. 4E, there seems to be more than one cell row, which is positive for E-Cad but negative for Sdt-GFP3, whereas Fig. 3E shows only one cell row. Does Sdt-GFP3 has a different expression pattern than other Sdt isoforms?

Since the loss of E-Cad is relatively gradual in epi-NSCs and early NSCs, it is possible to occasionally have more than one cell row with down-regulated Crb/Sdt and persistent E-cad. Nevertheless, to avoid confusion, we have replaced this panel.
Otherwise, the distribution pattern of Sdt-GFP3 (NBM-containing isoforms) is very similar to those seen for Sdt-GFP (all isoforms) relative to Patj, arguing that NBM-containing isoforms accumulate like total Sdt isoforms in the OL (note that the short Sdt isoforms cannot be detected independently of the long ones in double staining experiment)

5.In Fig. 5F-H, the supra-cellular myosin cable is not really obvious in the wild-type control, so the conclusion that it is affected upon excision of exon 3 (Fig. 6G) is difficult to follow.

We are now showing a high mag view of the wild-type Neur-GFP MyoII-TagRFP control to better show junctional MyoII. Also, the quantification in I reveals a clear difference of MyoII accumulaiton the 'b' junctions relative to the wild-type control (see Fig. 3C). We understand that it would be nicer to measure the effect of deleting exon 3 (in the Optix domain) using the Vsx1 as an internal wild-type control. However, because Neur-GFP is in the same channel as Sdt-GFP3, Neur cannot be used as an epi-NSC marker and it is difficult to assign junction identities.

6.On page 13 they write: "... suggest a model whereby fate transition is mechanically coupled with cellular rearrangement to promote smooth progression of the differentiation front". Do they imply that fate transition is mediated by mechanical processes? In other words: what is cause, and what is consequence?

No, fate transition is mediated by transcription factors (L'sc) downstream of signaling (EGFR, Notch) and mechanical processes (involving Neur) is downstream. In our view, mechanical coupling organize cells in space. We now write p.13: "Our observations therefore suggest a model whereby epithelial cortex remodeling in individual cells promotes smooth progression of the differentiation front at the tissue level by facilitating the organization of cells in space that are at a similar stage of fate transitions."

7. The last sentence of the Results needs smore explanation. They argue that the down regulation of Crb by Neur results in mechanical changes at apical junctions, which results in the adoption of NSC fate. However, while members of the Crb complex are downregulated in epi-NSCs, E-Cadherin is not. So why should there be "mechanical changes at apical junctions", if one assumes, that the presence of E-cadherin indicates the presence of junctions?

We assume (but do not show, i.e. we clearly state that we did not measure tension) that junctions along stretches of straight epi-NSCs/TZ interface are under increased tension: this was what we meant by "mechanical changes at apical junctions". We understand that this statement may be more appropriate in the discussion section. The end of the results section now reads: "We therefore suggest that the Crb-regulated accumulation of junctional MyoII downstream of Neur promotes the formation of a precisely lined-up single-cell row of epi-NSCs."

8. Throughout the text they conclude that the function of Neur is to downregulate Crb. However,

Neur also down-regulates a subset of Sdt isoforms and PATJ. And PATJ has been shown to regulate AJ stability by regulating Myosin localization (Sen et al., 2012). What is the proof that the effects described are due to loss of Crb? They are more careful in the conclusion on page 12 (Fig. 6), where they write that "... RhoGEF3 appeared to contribute to the apical constriction ... independently of the downregulation of the Crb complex".

The referee is correct. We now more clearly indicate throughout the text that Neur down-regulates the Crb complex (and not merely Crb).

Minor points:

a.Page 11, 3rd row from the bottom: it should be Fig 5, not Fig. S5
corrected

b.Fig. 7I, J: A cartoon showing what they measured would be helpful.
done
note that we are now using a different assay, and are still reaching the same conclusion

c. I think that the title does not really point to the major findings.
we failed to come up with a better title given the constraints if the journal

Reviewer #2

This manuscript investigates the cell biological mechanisms involved in the progression of neuroepithelial cells in the Drosophila optic lobe to a stem cell identity. First, the authors define a cell type that is epithelial, but associated with stem cell markers, which they define as epi-NSC. The authors find that this epi-NSC identity is associated with Neuralized-dependent apical constriction. The authors demonstrate pulsatile myosin contractions during stem cell emergence, reminiscent of the work of Simoes et al., 2017 and An et al., 2017 in the embryonic neuroectoderm. An actomyosin cable is also assembled at the border between epi-NSC and other transition zone cells, causing a straight interface. They show that apical constriction is promoted by Neuralized down-regulating Crb through Sdt and by RhoGEF3 expression.

The authors have done a beautiful job characterizing the cell biology of this system, which is fascinating. However, they fall short of demonstrating that mechanics is playing a substantial role in cell fate (see below). Thus, the conclusion that the Neuralized gene can "mechanically couple NSC fate acquisition with cell-cell rearrangement" is overstated. I suggest rephrasing this or having the authors analyze cell fate acquisition in mutants where apical constriction/actomyosin cable assembly is defective.

We thank the referee for her/his positive evaluation of our work. While our data provide no evidence that mechanics regulate cell fate (see also below), our analysis indicates that the precise positioning of emerging epi-NSC into a single cell row involves mechanics downstream of cell fate via Neur. We hope that we are now rephrasing this idea correctly.

Major comments:

1. The authors propose that, "Neuralized therefore appears to mechanically couple NSC fate acquisition with cell-cell rearrangement to promote smooth progression of the differentiation front". However, the authors do not examine NSC fate in mutants that disrupt cell rearrangements, leading us to question whether it is important for the developmental process. Examining the consequence of disrupting this process, such as by using the NSC markers shown in Fig. 1, would enhance the clarity and significance of the work. Is the subsequent EMT-like transition inhibited or delayed? Are there significant differences in resulting NSC number?

This is a good point. We did observe a delay in AJ disassembly due to perturbation of Neur regulation of the Crb complex, such that apical E-cad persisted into NSCs (Fig.4K-L). This delay in the EMT-like transition resulted in a few rows of newly specified NSC appearing to remain transiently adhered to the NE. However, we have not yet observed any clear defects in NSC behavior due to this (the division plane is still re-orientated correctly perpendicular plane of the tissue). Other developmental consequences, in terms of cell fate, or perturbed cell rearrangements appear to be minimal, if not null. As reported in Perez-Mockus *et al.* (2017), the loss of the regulation of the Crb complex by Neur in the $sdt^{\Delta 3}$ mutant (deletion of exon 3) had no detectable major developmental defects and the adults are viable. Here, we examined OL development using various NSC markers, including temporal patterning TFs. Our analysis did not reveal detectable phenotypes in relation to cell fate or temporal patterning of NSCs (note that expression of Ase and Dpn are shown in cross-section views in Fig.4I-L; obviously, we also examined surface views, as well as other markers, including TFs of the temporal series, such as Hth, Eyeless and Dichaete). These results, negative in nature, are not shown in the manuscript. So, we cannot (and did not) argue that precision is important for fate patterning in the OL.

2. The function of apical constriction in the integration of NSCs across the compartment boundary (as modeled in Fig. 7F) is confusing. Either bolstering this with further evidence or revising the model will benefit the paper. The authors showed that knocking-out RhoGEF3 inhibited apical constriction and that this was independent of Sdt/Crb regulation. However, in the RhoGEF3 knock-out condition the boundary between the epiNSCs and the rest of the transitional zone (Neur-GFP negative) appears to be straight, which would suggest that epiNSCs are still integrating into the NSC compartment. Thus, it appears that apical constriction is not important for NSC integration. A possible interpretation is that the supracellular myosin cable is important for boundary formation, but that apical constriction in the epiNSCs is not. Perhaps the myosin cable is important for the straightness of the boundary, while apical constriction is more important for the rate of integration?

Clarifying what the authors think the relative contributions of the actomyosin cable and apical constriction are in the context of NSC integration would help readers understand the importance of the cell biological effects that the authors described.

We thank the referee for his/her excellent questions and remarks. We have tried to clarify in our revised manuscript the roles of MyoII cables and apical constriction in making a precise epi-NSCs front. While apical constriction first revealed, albeit indirectly, the existence of cell-cell re-arrangements (implied by the increase in cell density at the medial edge of the TZ; Fig 2C), it was actually not clear whether medial MyoII contractility and apical constriction promoted the integration of emerging epi-NSCs into the row of pre-existing epi-NSCs. As suggested by the referee (her/his point #6 below), we have now examined the integration of the Neur-positive TZ cells into the epi-NSC row in *rhoGEF3* mutant larvae and found that the Neur-positive/negative boundary within the TZ upon remained unaffected upon loss of RhoGEF3 activity (which appears to affect apical constriction, but not Crb down-regulation nor the formation of MyoII cables), as correctly hinted by the referee. Thus, the $sdt^{\Delta 3}$ mutant phenotype (rougher Neur-positive/negative boundary within the TZ) is best explained by the effect of Crb anisotropy on the formation of the MyoII cables. The results section now ends with: "We therefore suggest that the Crb-regulated accumulation of junctional MyoII downstream of Neur promotes the formation of a precisely lined-up single-cell row of epi-NSCs."

General minor comments -
1. Presenting larger views of smaller numbers of cells would help readers see and interpret the data. For most of the points made in this paper, a view containing one or two horizontal rows of cells (i.e., a couple of NSCs, an epiNSC, and a couple of Neur-negative TZ cells) would be sufficient, and perhaps more clear, than the lower resolution tissue-scale views.

2. I would suggest cropping and enlarging many of the figures so that a similar resolution to the schematic presented in Fig. 3B is shown (except of course in cases where tissue-scale observations are necessary, such as demonstrating the supracellular actomyosin)

Zoomed panels are now provided in Figs 4G,H, 5G-H'', 6A-B'
We feel that low-mag views are also important to show the extent of the localization patterns/phenotypes at the tissue scale, notably to illustrate the differences between the Optix and Vsx1 (internal 'wildtype') domains in the same tissue.

3. Using multiple arrows in images to indicate features is distracting and in some cases interferes with visualizing the data. For instance, in Fig. 3A, it is difficult for a reader to judge for themselves the intensity of myosin along junction 'c' because it is covered by arrows in many of the cells in the image. If the authors were to show fewer cells, and then indicate the junctions of interest at the edges of the image, rather than on top of the data, this would be improved.

We have removed unnecessary arrows.

4. Showing less data (when redundant) in the primary figures would help make them more concise. For example, in Fig. 1, the data on Ase and Hth are essentially redundant with the Dpn data (at least in terms of demonstrating the intermediate state of the epiNSCs), and could be moved to the supplement in order to consolidate the message of the main figure.

These panels were moved to the Fig S1

5. The model for how the authors think apical constriction in the epiNSC cells is coordinated is a little unclear - Fig. 2 argues that apical-medial myosin pulsing is correlated with apical constriction, but from then on only junctional myosin levels are quantified. For instance, in Fig. 6 they demonstrate that RhoGEF3 knock-out does not perturb junctional myosin, but there is no analysis of medial myosin.

We did not study medial MyoII dynamics in the *sd*t and *rhoGEF3* mutants showing defective apical constriction. This is because identification of individual medial TZ cells in our live imaging experiments relies on apical constriction. Given that apical constriction is affected in these mutants, we could not easily identify epi-NSCs and study medial MyoII dynamics in these mutants. Specifically, we cannot use Neur-GFP to mark epi-NSCs since both a membrane marker (RFP) and MyoII (GFP) are needed to analyze the actomyosin ratchet dynamics.

6. Fig. 6: There should be a quantification of mean angle, as in Fig. 7, for the RhoGEF3 knock-out. If there were, it might show that the disruption of apical constriction is not as important as the maintenance of the myosin cable at the compartment boundary for integrating epiNSCs into the NSC zone, as the compartment boundary seems quite straight in this manipulation (e.g., Fig. 6C + 6E), and while apical constriction is inhibited and total myosin levels are a bit lower, the relative differences in junctional myosin between compartments / columns of cells appears to be preserved.
7. Fig. 7: G-J: A direct measurement of the straightness of the Neur-GFP front would be more meaningful than measuring the angle between cell centroids because this would reflect a tissue-level feature.

We thank the referee for both suggestions.
First, we are now more directly measuring the roughness of the boundary using a method used by C. Dahmann and F. Julicher in Rupert et al. (2015) which is described in Fig 7J,J'. This approach confirmed the *sdt* mutant phenotype described initially using cell centroids (see Fig 7K). Since the results obtained using our cell centroid approach are now redundant, they are no longer shown in the revised manuscript.
Second, we studied the *rhoGEF3* mutant brains (Fig 7I) and found no significant difference with the wild-type controls (Fig 7K). Thus, defective apical constriction is not sufficient to cause an epi-NSC integration defect. This argues that enriched junctional MyoII, downstream of Crb anisotropy, may

direct local cell-cell rearrangement to promote integration of emerging epi-NSCs and that defective MyoII accumulation may be responsible for the imprecise boundary in the *sdt* mutants.

Reviewer #3

This is a beautiful paper analysing the transition from the epithelial state to the neural stem cell state within the optic lobe neuroepithelium in the fly larval (L3) brain. The authors identify an intermediary cell state whereby the neuroepithelial cells retain certain epithelial features such as E-Cad and apical actomyosin but lose components such as Crumbs and adopt what the authors term an epi-NSCs. They describe these cell undergoing apical constrictions driven by a pulsatile apical-medial actomyosin cytoskeleton. These oscillations depend on Neur and RhoGEF3. Furthermore an actomyosin cable forms at the differentiation front where the epiNSCs have downregulated Crumbs and thus an anisotropy border forms, leading to junctional myosin accumulation.

Using in depth immunofluorescence analyses of fixed and live samples combined with clever genetics such as clonal mutant or overexpression studies, the authors support the conclusion above with very high quality data. The figures are very well laid out and explained, making it easy for a non-expert in this tissue to understand what is shown and what the data imply. The manuscript itself is very clearly written.

We thank the referee for her/his very positive evaluation of our work

I have no major concerns regarding any of the data or interpretations, only a few suggestions or question where the authors could maybe be a bit clearer in spelling out their conclusions.

With regards to the myosin fluctuations observed in both NE and epiNSCs: did the authors quantify the myosin cycle length in these two populations? In many instances such as the mesoderm or amnioserosa differences in cycle length have been linked to differences in productive versus unproductive area fluctuations, with longer cycle length being less productive, and in both cases a switch in cycle length turning unproductive into productive over time. Is something like this at work here?

We followed the referee's suggestion and found no difference in cycle length. This is now cited as data not shown.

It would be good if the authors could spell out more clearly in the paper what they deduce the role of the apical constrictions to be: are these driving area shrinkage to be able to accommodate more cells within a single line of epiNSCs? Or are they required to drive the cell intercalation that the authors presume must happen to integrate cells starting to express neur into a single line of cells? It would be too much for this paper to address this experimentally, but it would be good to understand the authors' thinking on these different possibilities.

We thank the referee for her/his comment. We have tried to explain better that apical constriction allows for more cells to participate to the epi-NSC row but is not essential for the cell intercalation thought to underlie the formation of a smooth morphogenetic boundary (based on our analysis of the *rhoGEF3* mutant; see our response to referee #2 above).

Experimentally related to this: do the authors know whether in the absence of RhoGEF3 apical area oscillations are still happening? Are the fluctuations or the ratcheting perturbed? The authors only show that the apical area in the first row of cells is larger than in the wild-type.

We did not study the *rhoGEF3* mutant phenotype using live imaging. Since fluctuations are seen in NE cells of wild-type brains, we would expect apical area to also fluctuate in epi-NSCs. As stated

above in our response to point #5 of referee 2, we did not study medial MyoII dynamics in the *rhoGEF3* mutants because identification of individual medial TZ cells in our live imaging experiments relies on apical constriction, which is defective in this mutant (this is because Neur-GFP cannot be used as an epi-NSC marker when MyoII-GFP is also used).

In the discussion, the authors conclude the first paragraph by stating that 'Thus, mechanical coupling by Neur is proposed...'. I am not sure this can be called mechanical coupling. To me, such a term would suggest some mechanosensing is involved, and there is no evidence that this is the case. The authors need to be more specific in what they mean here.

We did not use the term 'mechanical coupling' to mean force-dependent fate regulation downstream of some mechanosensing process. We now spell out that the Crb-regulated accumulation of junctional MyoII downstream of Neur is proposed to promote the formation of a precisely lined-up single-cell row of epi-NSCs.

Figure 4G'G', sdt[GFP3] flip-out experiments: The authors show Patj staining here, but I would be curious to know what this alteration does to Crumbs levels and localisation? Does the change in Patj reflect an identical change in Crumbs?

We have examined the distribution of Crb-GFP in *sdt*$^{\Delta 3}$ mutant brains and found that the boundary of Crb accumulation is lost upon deletion of sdt exon 3 and that Crb-GFP co-accumulate with E-cad (Fig S3). In other words, the effect seen with Patj is also observed for Crb-GFP. Of note, we did not perform anti-Crb staining in the flip-out experiment because the anti-Crb antibodies we have received do not work well in our hands.

Minor comments:

Page 11, beginning of new paragraph:
The sentence describing the Brd[R] protein says 'where the lysine(K) residues have been mutated', which lysine residues are referred to? All? Or specific ones?
yes, all lysine were mutated (now indicated in the Methods section)

Page 13, beginning of new paragraph:
The authors state:
'The dynamics of the NE-NSCs fate transition has so far mostly been considered in one dimension, with the OL epithelium viewed as a cross-section and with a particular focus on temporal dynamics (Egger et al., 2010; Weng et al., 2012; Ngo et al., 2010; Wang et al., 2011; Orihara-Ono et al., 2011). In contrast, how fate dynamics is coordinated in a two dimensional epithelium has not been examined.'
I would consider what they describe in the first sentence as two dimensions, looking at a cut plane of an epithelium in xz, and their approach presented here mostly also looking in two dimensions, though a different orientation in xy (though combined with some images in xz as well). One dimension, as in the first sentence, would refer to a line!
thanks, corrected

Page 13, 8 lines from the bottom of the page:
There is a word missing here or an 'in' too many, the sentence says '...the up-regulation of Neur in induces...'
thanks, corrected

Page 15, line 6 from bottom: typo 'isofomrs
thanks, corrected

August 7, 2020

RE: JCB Manuscript #202005035R

Dr. Francois Schweisguth
Institut Pasteur
BDCS
25 rue du Dr Roux
CNRS UMR3738
Paris 75015
France

Dear Francois,

Thank you for submitting your revised manuscript entitled "Neuralized regulates a travelling wave of Epithelium-to-Neural Stem Cell morphogenesis in Drosophila". The manuscript was assessed editorially. We feel that you have done a thorough job of addressing all of the issues raised by the reviewers and we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

1) JCB Articles are limited to 10 main and 5 supplementary figures. If you could please try to rearrange the suplementary data to meet this limit, it would be much appreciated (e.g., by combining some of the supplementary data and integrating the one panel from supplementary figure 6 into one of the other supplementary figures). Each figure can span up to one entire page, with all panels fitting on the page.

2) Titles, eTOC: Please consider the following revision suggestions aimed at increasing the accessibility of the work for a broad audience and non-experts.

Title: a title that more clearly explains Neuralized's role would be helpful to increase the discoverability of the work and its appeal.
for instance:
Neuralized couples apical constriction and epithelial-to-neural fate transition at the differentiation front
Neuralized is a key determinant of epithelial-to-neural stem cell morphogenesis
Tissue-wide coordination of epithelial-to-neural fate transition requires Neuralized

Running title: Neuralized controls epithelial-to-neural transition

eTOC summary: A 40-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.
- Please include a summary statement on the title page of the resubmission. It should start with "First author name(s) et al..." to match our preferred style.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Please add scale bars to 2D, 7ABCDGHI, S1CDEI, S2B, S3 all panels, S4AB

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends.
Please indicate n/sample size/how many experiments the data are representative of: 2IJ

5) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.
- Please provide FlyBase or BDSC IDs or other database accession reference for all fly lines. If they are not available, please include a brief descriptions of the basic genetic features of the lines used, even if published in other work or gifted from other investigators.
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b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

6) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section. Please include one brief descriptive sentence per item.

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Sincerely,

Mark Peifer, PhD
Monitoring Editor, Journal of Cell Biology

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