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Cut homeodomain transcription factor is a novel regulator of growth and morphogenesis of cortex glia niche around neural cells

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Cut homeodomain transcription factor is a novel regulator of cortical glia morphogenesis and niche maintenance around neural stem cells

Dear Dr. Arya:

Two experts in the field have reviewed your manuscript, and I have read it as well. We all agree that the overall idea that Cut regulates cortex glia growth/morphology and proliferation is supported by the data. However, there are a number of major concerns that will need to be addressed. While your manuscript is not currently acceptable for publication in GENETICS, we would welcome a substantially revised manuscript. You can read the reviews at the end of this email.

If you decide to resubmit your manuscript, please include:
1. A clean version of your manuscript;
2. A marked version of your manuscript in which you highlight significant revisions carried out in response to the major points raised by the editor/reviewers (track changes is acceptable if preferred);
3. A detailed response to the editor's/reviewers' feedback and to the concerns listed above. Please reference line numbers in this response to aid the editor and reviewers.

Your paper will likely be sent back out for review.

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Sincerely,

Norbert Perrimon
Associate Editor
GENETICS

Approved by:
Oliver Hobert
Senior Editor
GENETICS

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

The present study by Yadav et al., demonstrates that homeodomain transcription factor Cut regulates growth and development of cortex glial niche that support the neural stem cells. The authors have nicely demonstrated that Cut controls membrane growth and endomitosis of the cortical glial cells. While I believe this study may evoke strong interest in the field, I do have some concerns regarding certain experiments and would also recommend additional staining and imaging to be added before its publication.

1. Figure 1B-1F, the authors need to show individual channel images of Cut and glial markers along with merged image. Figure 1B does not convincingly show Cut is being expressed in all cortex glia as suggested in the graph Figure 1J and results section on Page 5. Hence, a higher magnification of the image would be ideal to look at Cut expression and cortex glia staining patterns along with orthogonal view of a section of the VNC. Also, if one looks at the cortex glia per segment of the VNC, is there a difference in the loss of cortex glia with Cut knockdown in thoracic segments as compared to abdominal segments?
2. Since, most of the cortex glia is lost in Cut knockdown animals at L3, and since cortex glia is important for survival, do the authors observe lethality in these animals?
3. Regarding Figure 1, cortex glia is known to progressively undergo development starting with expansion, encasing and extension of neural stem cells as mentioned by the authors and by Spéder and Brand, 2018. It would be interesting to see if knocking down cut disrupts either of the three stages of cortex glia wrapping around NSCs. It will be helpful to stain the CNS with elav that will mark neurons and look at the disruption of glial membrane around neurons.
4. For Figure 2, like Figure 1, orthogonal and ventral view along with neuronal staining will be helpful as CNS undergoes

significant remodeling at this stage. Glial networks involving different subtypes intertwine and Cut is important for identity of wrapping glia. The authors should look at ensheathing glia, wrapping glia and astrocytic glia that border cortex glia in Cut knockdown animals.
5. For Figure 3, the authors are comparing L2 at 63X and L3 at 20X and I do not understand the point of this comparison. It is well-known that cortex glial numbers do not change until L2 stages but there is a huge increase at L3 stage that coincides with neuronal proliferation. The panels A-C should also be imaged under 20X. Also, if glial nuclei numbers are being counted, isn't using a GFP-NLS, LacZ-NLS or Histone:RFP more appropriate?
6. The authors can use cell death markers to demonstrate cell death in cortex glia in Cut knockdown animals. Since, L3 CNS undergoes remodeling one expects to see cell death. The authors should present a representative image as the image of p35 co-expression in L2 is not convincing enough to say reduction in glial cell numbers although there is some rescue of processes.
7. For Figure 4, the authors should look at proliferation changes using PH3 when overexpressing Cut.
8. For Figure 6, the authors should use Fly-FUCCI to demonstrate defective cortex-glia cell cycling in animals with Cut overexpression.

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

In this paper, Yadav et al. identify the homeodomain transcription factor Cut as essential for the growth and proliferation of the cortex glia (CG) in Drosophila, a glial subtype forming a tight meshwork around neural stem cells and behaving as a niche during larval stage. They first show that Cut is expressed in CG. They then use both knockdown and misexpression strategies to infer Cut's role in CG. First, cut knockdown in CG leads to altered CG morphology, with an overall decrease in membrane density and also results in reduced nuclei number at the population level. Part of the early loss in CG nuclei appears due to apoptosis, as it can be rescued by p35 expression. Interestingly, the loss of cut in CG is also linked to a decrease in the number of neural stem cells in late larval stage. Cut overexpression in CG also modifies CG morphology, this time leading to the formation of globular, compact membrane structures with less however thicker branching. In addition, it creates fewer, albeit larger CG nuclei, with higher DNA content, suggesting that the nuclear division process normally taking place in CG is impaired.
While glial cells are a critical component of the neurogenic niche, how they manage to closely interact with neural stem cells and regulate their functions remain largely unprobed. As such, finding regulators of both their morphology/association with stem cells and their niche function towards stem cell is exciting. In this context, the identification of Cut as a regulator of the morphogenesis and function of the CG will provide ways and means to further probe their function on neural stem cells. In particular, the apparent decrease in stem cell number under cut knockdown, and a rather striking morphology under overexpression are exciting findings.
Although the overall idea that Cut regulates CG growth/morphology and proliferation is supported by the data, I am however concerned by several aspects of this study. First, I do find that some data are overinterpreted, and the specific, related conclusions inaccurate. Some data also lack proper presentation or illustration. Finally, the nomenclature and literature tend to be shaky, with improper quotes and lack of definition of some concepts or features.

Major comments :

1) Data interpretation and conclusion

Part 1. Cut expresses in glial subtypes and maintains niche around neural stem cells
• The authors count nuclei number (using the Repo marker) to estimate the number of Cut+ cells in L3. Or at this stage CG are multinucleated (Fig. 1J). Please refer as number of nuclei, or a clonal labelling technique should be used to assess CG cell number.
• "We (...) noted that NSCs were unusually clumped, irregularly shaped, and were eliminated during development upon cut ablation in cortical glial cells (Fig.1E, F)." I see no data supporting an elimination of NSC during development. First, we have no other timepoints (especially earlier) to assess whether they've been disposed of, or rather not formed from the start. It could also be a loss of the fate determinant rather than a loss of cells. To support this hypothesis, looking at cell death marker along larval stage could be an option, or I would suggest just to stick to the loss without stretching the interpretation The irregular shape of the NSCs is also not defined/shown.

Part 2. Cut is required for the growth of cortical glia membrane, and it's branching
• "Upon Cut knockdown, these glial cells cannot grow the processes; therefore, several glial cell bodies might be eliminated during development". Here also, I do not see the data supporting cell elimination. First, there is no proper counts of cells (versus nuclei, see above Part 1), so it could rather be an issue with proliferation (something mentioned later on). Second, there is no assessment either of cell death. There is some rescue of cut RNAi phenotype by P35 expression in L2 stage, however it is restricted, and also not significant in L3. The authors should perform a timeline of potential CG apoptosis to support this interpretation, or just simply label the phenotype as a loss of CG nuclei.
• Fig. 2A-B': the authors delineate "cell bodies" to show their extension/spread. Again, clonal labelling should be performed to ascertain which membranes belong to one CG cell.

Part 3. Cut defective cortical glial cells are unable to increase their nuclei number during development
• "In order to understand when the above-noted reduction in the tVNC cortical glia number". There is no data showing this (same remark with clonal labelling, and nuclei counts come later).
• "...profound loss of cells is visible (Fig.3D-E, G)." and "It indicates that the cells (...) are eliminated partly by apoptosis and other unknown process/s (Fig.3)." There is no cell count. Nuclei are also not shown (no co-staining with Repo for example).
• "We see the reduced nuclei and defective membrane growth, which is only partially restored by P35 expression; thus, we conclude that the loss of Cut in cortical glial cells inflicts growth defects likely by interfering with endomitosis." To discriminate between apoptosis or altered proliferation the authors could check cell death overtime (see Part 2) and determine the number of nuclei per cells or the nuclei volume/DNA content (as they do later for the overexpression experiment).

Part 5. Constitutive activation of Cut increases DNA content in cortical glial cells
• "Together these observations indicate that constantly high Cut levels inhibit nuclei splitting during endomitosis, and these nuclei remain endoreplicated instead of separating. Therefore, we propose that the homeodomain protein Cut is required for the cortical glial cells to increase the DNA content. Conversely, the nuclei divide when its level goes down, and cells undergo endomitosis." This interpretation can be proposed but there is no strong data to support it. Especially, I do not understand "the homeodomain protein Cut is required for the cortical glial cells to increase the DNA content", as here we are in an overexpression context, and not a loss of function. Cut overexpression might force endoreplication, but that does not support a requirement in normal context. Is there any data on Cut level fluctuation that could explain a switch between endoreplication and endomitosis in normal context? What actually happens to nuclear size/DNA content under cut RNAi?

Discussion
• "Since Cut defective cortical glial cells show severely hampered growth of cytoplasmic extension even in early L2, we think that Cut might act upstream of the PI3K/Akt signaling." I understand this is a discussion point, but I do not get how the authors proposes such epistasis (which thy actually could perform if they want to support their point). PI3K/Akt signalling is active very early in L1, after larvae start feeding.

General comments/questions
• Considering the globular, compact morphology of the CG membrane and a lower count of CG nuclei (see Part 3) under cut RNAi in CG, I would think that original CG cells (mostly mononucleated at early stage) have not grown/proliferated properly, and stay as individual, single-nuclei cells (rather than death). The authors could check this by looking at proliferative (PH3, EDU, FUCCI) or nuclear (volume, DNA content) markers. Also counting the number of nuclei per cell over time would be a strong support.
• Both in cut RNAi and cut overexpression, CG nuclei numbers go down. For cut overexpression, the authors propose that cut favors endoreplication versus proper nuclear division, and that lower cut levels would thus rather favor, or be permissive to, proper nuclear division. How do they reconcile this hypothesis with lower counts in cut RNAi?
• Other studies have shown a dramatic, globular phenotype for CG under various loss of functions (Coutinho-Budd at al, 2017 with members of the membrane fusion machinery; Rujano et al, 2022 with dup RNAi and members of the cell-cell fusion machinery). That should be discussed and compared with rather than with FGF signaling.
• A previous study has shown that a dramatic alteration of CG morphology do not lead to NSC loss (Spéder and Brand, 2018). This should be discussed with respect to the findings with cut RNAi. Is it rather a specific function of cut in CG than linked to a change in morphology?

2) Data analysis and representation

• Please show individual colour channels to support the colocalization in Fig. 1B-F.
• A nuclear (Repo) staining should be shown in Fig. 2 to 5.
• Chamber size (Fig. 4H): I do not understand what is measured there (I could not find it in the methods). What is the unit? Also, if a chamber is defined by containing one NSC (see comment below in 3), then a staining should be added to understand where we are, especially regarding the z-depth.
• Membrane volume (Fig. 3H and 4I): I am not sure I understood how the membrane volume has been measured. I find the Methods hard to follow. For example: "To measure the cell size of cortical glial cells (GFP+, Repo+) multipoint selection tool was used to outline the area. The area outlining was done using the freehand selection tool around repo staining, followed by the analyze-measure option." How is the outlined area defined (One cell? How knowing where to stop?)? The control values are actually quite different between Fig. 3H and 4I.

3) Nomenclature

• Endomitosis. Could the author exactly explain what they mean here? The endoreplicative/proliferation field itself has fluctuating definitions. Considering the previous literature for the Drosophila glia, it seems important to me to define whether they consider endomitosis as leading to full nuclear (but not cytoplasmic) separation (the term used in other glial cells like subperineurial glia; what I think they choose here) or if they use a stricter definition in which endomitosis cannot reach this state and only exhibit

partial mitotic traits (in which case mitosis with incomplete cytokinesis should be used, as in Rujano et al). It is needed to ensure the readers knows what is described here and how it compares to/matches with cellular events described in previous studies.
• CG chamber: it needs to be properly defined (cellular landmarks?)
• Main/side branches: the idea is interesting and novel in the context of the CG (and would fit with other known roles of Cut). How do the authors define it, as CG are big multinucleated cells with many branching? Is it when radiating from one nucleus to another from the same cell?
• I think cortex glia is more usual than cortical glia.

4) Literature

• The authors state that "the glial cells also form the blood-brain barrier", using a general reference and seemingly not specifying the species. As such it seems it imply it is a generic trait of the blood-brain barrier, whereas most species have an endothelial barrier. It needs to be corrected or precised.
• "In the ventral nerve cord (VNC) of the Drosophila larval nervous system, cortical glial cells are mostly found at the ventral and lateral sides and remain closely associated with neural cells and also regulate their fate (Ito et al., 1995). Similar to cortical glia, mammalian astrocytes also extend the membrane extensions towards the outer surface of synaptic neuropils (Awasaki et al., 2008)."
To my knowledge, Ito et al does not show that CG regulate NSC fate. The Awasaki et al paper does not discuss the organization of mammalian astrocytes and its comparison to CG.
• "Growth of the glial cell membrane requires nutrient-dependent activation of Insulin and PI3K signaling (Yuan et al., 2020)." The firs reference to show this is Spéder and Brand, 2018.
• "Cortical glia continues to expand their cytoplasmic extensions from the late embryonic stage (Coutinho-Budd et al., 2017; Ito et al., 1995). The following reference should be added: Pereanu et al, 2005; Spéder and Brand, 2018; Rujano et al., 2022. The Ito et al, 1995 reference should be removed.
• "Therefore, as a first step, we checked the expression profile of the cortical glia Gal4 driver, cyp4g15-Gal4, and found it, also expressed from the late embryonic stage and can be used for the study (data not shown). » This driver has been described in Spéder and Brand, 2018. Please either quote this paper or add the data in supplemental.
• "Interestingly, the membrane extensions coming out from different cells are well connected and self-tilled on one another (Coutinho-Budd et al., 2017; Rujano et al., 2022)." I do not think that any of these papers showed that different CG cells are connected through membrane extensions. Also, I do not think this is accurate: "self-tilled on one another". What does "self-til(l)ed" and "on one another" mean? CG do not tile on top of each other.
• "Thus, we conclude that Cut is required and sufficient for the growth of cortical glial membrane processes (Coutinho-Budd et al., 2017)." Why this reference ? These are the own data of this study. Or if the purpose was to reference just the "growth of cortical glial membrane processes", please complete with other references.
• "Several reports from independent labs strongly indicate that cortical glial cells increase their nuclei number by undergoing endomitosis (Coutinho-Budd et al., 2017; Rujano et al., 2022; Unhavaithaya and Orr-Weaver, 2012; Yuan et al., 2020)." Unhavaithaya and Orr-Weaver, 2012 do not look at CG, but subperineurial glia. It should be removed.

Minor comments :
1. Fig. 1: I am a bit surprised that the scale bar is the same between panels G and I considering the sizes of the Dpn nuclei (or are NSC nuclei bigger under cut RNAi?). Also, while there are fluctuations depending on where the separation between central brain and ventral nerve cord is set, I tend to find the mean number of NSCs in control low. Could the authors describe the region they used?
2. L3 is a long stage. When were the larvae dissected? Also, when were the RNAi/overexpression driven from? The driver onset? Did I miss it?
3. Units are missing on some scale bars and graphs
4. Please check the italics for genes/genotype
5. In the title, I am not sure "maintenance" is correct. I feel this is rather looking at formation of the niche.
6. • "(Avet-Rochex et al., 2012; Dong et al., 2020; Read et al., 2009; Reddy B. V. V. G.and Irvine Kenneth, 2011; Spé Der and Brand, 2018; Witte et al., 2009)." Please correct the format of Reddy B. V. V. G.and Irvine Kenneth, 2011 and the spelling of Spé Der and Brand, 2018
7. I am not fully sure that single surface or line plot add much to the representation of the membrane phenotype, which is clear on the inset.
8. Please add some page/line numbers so it is easier to reference for reviewers.

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

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1. Figure 1B-1F, the authors need to show individual channel images of Cut and glial markers along with merged image. Figure 1B does not convincingly show Cut is being expressed in all cortex glia as suggested in the graph Figure 1J and results section on Page 5. Hence, a higher magnification of the image would be ideal to look at Cut expression and cortex glia staining patterns along with orthogonal view of a section of the VNC.

Response: Based on the reviewer's suggestion, we have made several additions to better visualize the expression of Cut in all cortex glia. These include zoom-in images and individual channel images (Supplementary Fig. 1D-I), as well as ventral and orthogonal sections to show Cut expression (red) in pan glia (*Repo>eGFP*) (Fig. 1B) and cortex glia (*Cyp4g15>eGFP*) (Fig. 1C).

Also, if one looks at the cortex glia per segment of the VNC, is there a difference in the loss of cortex glia with Cut knockdown in thoracic segments as compared to abdominal segments?

Response: Cut Knockdown affects the cortex glia overall in the CNS; including the abdominal region, as well as optic lobes. We have included supplementary data in Fig. S2I,as well as quantification of nuclei (Fig.S2J).

2. Since, most of the cortex glia is lost in Cut knockdown animals at L3, and since cortex glia is important for survival, do the authors observe lethality in these animals?

Response: As per the reviewer's suggestion, we conducted a survival assay to determine how a decrease in Cut in the cortex glia affects an organism's survival. We observed some lethality during development, but it was not significant until adulthood. However, we did notice a developmental delay progressing through different stages (see supplementary Fig. 3A). In adult organisms, a decrease in Cut levels resulted in a significant reduction in average life span compared to control animals (supplementary Fig. 3B). Since Cut knockdown does not show a severe consequence on the overall life span of the organism it may be due to a late and progressive effect on the cortex glia morphogenesis in larval life. In contrast, to Coutinho-Budd et al. (2017), where severe organismal lethality was observed due to the loss of cortex glia at the L1 stage by expression of strong apoptotic inducers like hid. The data is added to the text and figures are included as a supplementary Fig. 3B.

3. Regarding Figure 1, cortex glia is known to progressively undergo development starting with expansion, encasing and extension of neural stem cells as mentioned by the authors and by Spéder and Brand, 2018. It would be interesting to see if knocking down cut disrupts either of the three stages of cortex glia wrapping around NSCs. It will be helpful to stain the CNS with elav that will mark neurons and look at the disruption of glial membrane around neurons.

Response: on the suggestions we have conducted the timed experiments and included the data in Fig. 3. We have compared how the nuclei number and processes grow in control and Cut knockdown tVNC at 0-3hr, 24-28hr, and 48-54hr and in the wandering third instar. Briefly, the knockdown of Cut in cortex glia shows an impact on the growth and development of network and nuclei number as early as ALH 24-28hr. Even though the number of cortex glial nuclei does not significantly decrease till L2 upon Cut knockdown, the membrane connections are severely affected, and fine networks are mostly missing even at ALH 24hr. The revised data is added in the text and Fig 3.
We have modified Fig. 2 for clarity and added DAPI staining along with GFP in cortex glia to show how disruption of Cut expression in cortex glia affects overall glial morphogenesis and how neural cells are covered with cortex glia processes (Fig. 2A-B', graph 3J,K).

4. For Figure 2, like Figure 1, orthogonal and ventral view along with neuronal staining will be helpful as CNS undergoes significant remodelling at this stage. Glial networks involving different subtypes intertwine and Cut is important for identity of wrapping glia. The authors should look at ensheathing glia, wrapping glia and astrocytic glia that border cortex glia in Cut knockdown animals.

Response: The ventral view along with DAPI staining (Fig. 2A-B", graph 3J,K) is added for clarity as suggested by the reviewer. Due to the current unavailability of type-specific antibodies and Gal4 lines with us, it is not possible to immediately check the role of Cut in other glial types. The experiment is certainly important and is on the to-do list of future experiments.

5. For Figure 3, the authors are comparing L2 at 63X and L3 at 20X and I do not understand the point of this comparison. It is well-known that cortex glial numbers do not change until L2 stages but there is a huge increase at L3 stage that coincides with neuronal proliferation. The panels A-C should also be imaged under 20X.

Response: Figure 3 has been revised thoroughly for clarity. It is modified to illustrate the development of cortex glia at different time points, including how Cut loss affects the growth of cellular processes and nuclei number. Additionally, we have included DNA data to better analyze defects.

Also, if glial nuclei numbers are being counted, isn't using a GFP-NLS, LacZ-NLS or Histone:RFP more appropriate?

Response: Since the UAS-eGFP (BL-5431) is expressed in the nucleus as well as in the cytoplasm, it is very useful in marking both the cytoplasm and nucleus simultaneously, thus we have used this line. To count the glia nuclei, we have used the Repo+ GFP+ (cortex Glia) combination. We have quantified only the cortex glia nuclei that are Repo+ve to avoid any confusion.

6. The authors can use cell death markers to demonstrate cell death in cortex glia in Cut knockdown animals. Since, L3 CNS undergoes remodeling one expects to see cell death. The authors should present a representative image as the image of p35 co-expression in L2 is not convincing enough to say reduction in glial cell numbers although there is some rescue of processes.

Response: We apologize for any confusion regarding the counting of glia cells/nuclei in the cortex glia. It is difficult to accurately count cell numbers due to the extensions and fusion of multinucleated glia. Therefore, researchers have been counting nuclei numbers instead and have observed an increase in nuclei numbers during development. However, it is still unclear if the actual number of cortex glia cells increases during development. Our experiment involving the UAS-P35 rescue showed only marginal delays in the loss of nuclei upon Cut knockdown. Now, with the new loss of function data, we have learned that Cut regulates the growth of cortex glia cells by affecting endocycle/endomitosis, and nuclei numbers do not increase due to a failure in timely DNA synthesis. We now believe that the P35 rescue experiment is not the correct method for addressing the question. Therefore, we have revised our text and figure to include DAPI measurement data that supports our findings that the loss of Cut interferes with the endocycling process in cortex glia, preventing nuclei from increasing their DNA content to enter endomitosis Since reviewers 2 also have several questions regarding the P35 data presented in this figure we have thoroughly revised the text and Figure 3.

7. For Figure 4, the authors should look at proliferation changes using PH3 when overexpressing Cut.

Response: Since cortex glia do not undergo conventional mitosis, they do not show labeling for Ph3. During the initial phase of the project, we conducted Ph3 staining. However, we did not find much evidence of Ph3-positive nuclei. We personally discussed this with Coutinho-Budd Jaeda and Mark Freeman, who works extensively on glia development, and they had similar observations. It is now clear that cortex glia increases their nuclear size by endocycling and number by endomitosis (Rujano et al., 2022).

8. For Figure 6, the authors should use Fly-FUCCI to demonstrate defective cortex-glia cell cycling in animals with Cut overexpression.

Response: Thank you for this suggestion. We have incorporated FUCCI data into Fig. 6K-O as suggested by the reviewer for greater clarity on the cell cycle. Our findings indicate that Cut overexpression leads to more cells (51.81%) being stuck in

the G2/M phase of ALH 96hr compared to control, which corresponds to increased DNA content and cell size.

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

In this paper, Yadav et al. identify the homeodomain transcription factor Cut as essential for the growth and proliferation of the cortex glia (CG) in Drosophila, a glial subtype forming a tight meshwork around neural stem cells and behaving as a niche during larval stage. They first show that Cut is expressed in CG. They then use both knockdown and misexpression strategies to infer Cut's role in CG. First, cut knockdown in CG leads to altered CG morphology, with an overall decrease in membrane density and also results in reduced nuclei number at the population level. Part of the early loss in CG nuclei appears due to apoptosis, as it can be rescued by p35 expression. Interestingly, the loss of cut in CG is also linked to a decrease in the number of neural stem cells in late larval stage. Cut overexpression in CG also modifies CG morphology, this time leading to the formation of globular, compact membrane structures with less however thicker branching. In addition, it creates fewer, albeit larger CG nuclei, with higher DNA content, suggesting that the nuclear division process normally taking place in CG is impaired.
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Although the overall idea that Cut regulates CG growth/morphology and proliferation is supported by the data, I am however concerned by several aspects of this study. First, I do find that some data are overinterpreted, and the specific, related conclusions inaccurate. Some data also lack proper presentation or illustration. Finally, the nomenclature and literature tend to be shaky, with improper quotes and lack of definition of some concepts or features.

Majorcomments :

1) Data interpretation and conclusion

Part 1. Cut expresses in glial subtypes and maintains niche around neural stem cells
• The authors count nuclei number (using the Repo marker) to estimate the number of Cut+ cells in L3. Or at this stage CG are multinucleated (Fig. 1J). Please refer as number of nuclei, or a clonal labelling technique should be used to assess CG cell number.

Response: The nuclei/cell count error was rectified by specifying that only CG nuclei (GFP+, Repo+) were counted, revealing a defect in increased nuclei number. Cortex glial cells are multinucleated and make syncytium. Due to fusion it is difficult to count them with clonal tools. Several leading labs have already tried to make clone/MARCM in Cortex glia and but did not succeed (Awasaki et al. 2008, Coutinho-Budd et al. 2008, Stork et al. 2014). Still, a recent study by Rujano et al.

(2022) demonstrated that individual cortex glia are multinucleated and that one clone could have a variable number of numerous nuclei using a recently developed RAEPPLI tool.

• *"We (...) noted that NSCs were unusually clumped, irregularly shaped, and were eliminated during development upon cut ablation in cortical glial cells (Fig.1E, F)." I see no data supporting an elimination of NSC during development. First, we have no other timepoints (especially earlier) to assess whether they've been disposed of, or rather not formed from the start. It could also be a loss of the fate determinant rather than a loss of cells. To support this hypothesis, looking at cell death marker along larval stage could be an option, or I would suggest just to stick to the loss without stretching the interpretation The irregular shape of the NSCs is also not defined/shown.*

Response: Thank you for the suggestion. We added the data in the revised manuscript (Fig:1H-J, Graph 1K,L) . To confirm the identity of neural stem cells (NSCs) and check if loss of Cut affected them, we profiled NSCs from early to late larval stages and counted their numbers using dpn antibody. In ALH 48-54hr, the numbers of NSCs in control and upon Cut knockdown were the same. The data is added as (Graph 1L). We removed the comment on irregular shape and size and how cortex glia influences NSC fate non-autonomously, which is part of ongoing research.

Part 2. Cut is required for the growth of cortical glia membrane, and it's branching
• *"Upon Cut knockdown, these glial cells cannot grow the processes; therefore, several glial cell bodies might be eliminated during development". Here also, I do not see the data supporting cell elimination. First, there is no proper counts of cells (versus nuclei, see above Part 1), so it could rather be an issue with proliferation (something mentioned later on). Second, there is no assessment either of cell death. There is some rescue of cut RNAi phenotype by P35 expression in L2 stage, however it is restricted, and also not significant in L3. The authors should perform a timeline of potential CG apoptosis to support this interpretation, or just simply label the phenotype as a loss of CG nuclei.*

Response: We appreciate this very thoughtful remark. We have rewritten this section for clarity. We only count nuclei rather than cells due to multinucleated cortex glia and difficulty in cell counting (as discussed above). It is unclear if cortex cell number increases during development. Now, with the new loss of function data, we have learned that Cut regulates the growth of cortex glia cells by affecting endocycle/endomitosis, and nuclei numbers do not increase due to a failure in timely DNA synthesis. Thus, a rescue with P35 is meaningless at this point. We revised the text and Figure 3 and added DAPI measurement data to show that loss of cut interferes with endocycling in cortex glia.

• *Fig. 2A-B': the authors delineate "cell bodies" to show their extension/spread. Again, clonal labelling should be performed to ascertain which membranes belong to one CG cell.*

Response: Due to fusion and formation of syncytium it is difficult to count them with clonal tools. Several leading labs have already tried to make clone/MARCM in Cortex glia but did not succeed (Awasaki et al. 2008, Coutinho-Budd et al. 2008, Stork et al. 2014). Changes are made in Figure 2 as reviewer one also suggested, and the related text has been modified for clarity.

Part 3. Cut defective cortical glial cells are unable to increase their nuclei number during development

• *"In order to understand when the above-noted reduction in the tVNC cortical glia number". There is no data showing this (same remark with clonal labelling, and nuclei counts come later).*

Response: We have rewritten this section to improve clarity. We only count nuclei as it is difficult to count cell numbers due to cytoplasmic extensions and syncytium formation. We are still determining if cortex cell numbers increase during development. We found that the defect in nuclei upon cut knockdown can only be marginally delayed by P35, and so an experiment to show the rescue of "cells" with P35 is meaningless. We are only looking at nuclei. We have revised the text and figure thoroughly, adding several new experiments for more clarity. We have also included DAPI measurement data to support that cut knockdown interferes with the endocycling process in cortex glia.

• *"...profound loss of cells is visible (Fig.3D-E, G)." and "It indicates that the cells (...) are eliminated partly by apoptosis and other unknown process/s (Fig.3)." There is no cell count. Nuclei are also not shown (no co-staining with Repo for example).*

Response: As mentioned earlier, this section has undergone significant revisions, including changes to both the text and figure. We have always classified GFP+ and Repo+ nuclei as cortex glial nuclei, not cells, this error is now corrected.

• *"We see the reduced nuclei and defective membrane growth, which is only partially restored by P35 expression; thus, we conclude that the loss of Cut in cortical glial cells inflicts growth defects likely by interfering with endomitosis." To discriminate between apoptosis or altered proliferation the authors could check cell death overtime (see Part 2) and determine the number of nuclei per cells or the nuclei volume/DNA content (as they do later for the overexpression experiment).*

Response: As mentioned, we revised the figure and removed cell death data because we could not count cell numbers due to complex morphology, fusion and syncytium formation. We counted nuclei. As per the reviewer's suggestion, we quantified DNA content by measuring DAPI's integrated density at different larval time points ALH 24-28hr and ALH 48-54hr, data is shown in figure 3, and the text is modified. We found that loss of Cut in cortex glia inflicts growth defects and slows DNA increase, which may affect their entry into endomitosis.

Part 5. Constitutive activation of Cut increases DNA content in cortical glial cells
• "Together these observations indicate that constantly high Cut levels inhibit nuclei splitting during endomitosis, and these nuclei remain endoreplicated instead of separating. Therefore, we propose that the homeodomain protein Cut is required for the cortical glial cells to increase the DNA content. Conversely, the nuclei divide when its level goes down, and cells undergo endomitosis." This interpretation can be proposed but there is no strong data to support it. Especially, I do not understand "the homeodomain protein Cut is required for the cortical glial cells to increase the DNA content", as here we are in an overexpression context, and not a loss of function. Cut overexpression might force endoreplication, but that does not support a requirement in normal context. Is there any data on Cut level fluctuation that could explain a switch between endoreplication and endomitosis in normal context? What actually happens to nuclear size/DNA content under cut RNAi?
Response: Thank you for this question. Several new experiments were done to understand the role of Cut in normal vs ectopic situations and summarized in Figure 3. When Cut is knocked down, the DNA content of the cortex glia cannot increase at the correct time, and the nuclei number does not increase. Thus, Cut is required to increase the cortex glia's DNA content. It is shown that in the process of Drosophila oogenesis, follicular cells undergo endocycle to increase their DNA content. To ensure proper development, the downregulation of Cut is necessary to switch from mitosis to endocycle (Sun & Wu-Min Deng, 2005). Interestingly, the role of Cut in cortex glia cells is different, as high levels of Cut promote DNA increase per nuclei. This suggests that Cut may have diverse and tissue-specific effects on the cell cycle. The section is added in the revised version.

Discussion
• "Since Cut defective cortical glial cells show severely hampered growth of cytoplasmic extension even in early L2, we think that Cut might act upstream of the PI3K/Akt signaling." I understand this is a discussion point, but I do not get how the authors proposes such epistasis (which thy actually could perform if they want to support their point). PI3K/Akt signalling is active very early in L1, after larvae start feeding.

Response: The section is modified, and a reference is added “As Cut defective cortex glia show severely hampered the growth of cytoplasmic extension even in early L2, we think that Cut may function within the PI3K/Akt signaling pathway in glial cells in a manner analogous to what has been observed in pancreatic cancer cells (Ripka et al 2010)”

General-comments/questions
• Considering the globular, compact morphology of the CG membrane and a lower count of CG nuclei (see Part 3) under cut RNAi in CG, I would think that original CG cells (mostly mononucleated at early stage) have not grown/proliferated properly, and stay as individual, single-nuclei cells (rather than death). The authors could check this by looking at proliferative (PH3, EDU, FUCCI) or nuclear (volume, DNA content) markers. Also counting the number of nuclei per cell over time would be a strong support.

Response: Thank you for requesting this data and excellent suggestion. We conducted additional experiments and included the results in Figure 3. To address

the issue, we monitored the DNA content in the Cortex Glia after Cut knockdown and compared it with the control. The data is shown in Figure 3J,K , which clearly indicates that the increase in DNA content is impeded upon Cut knockdown.

• *Both in cut RNAi and cut overexpression, CG nuclei numbers go down. For cut overexpression, the authors propose that cut favors endoreplication versus proper nuclear division, and that lower cut levels would thus rather favor, or be permissive to, proper nuclear division. How do they reconcile this hypothesis with lower counts in cut RNAi?*

Response: This explanation follows up on the previous comment. We have measured the DNA content in Cut knockdown and gained insight into how it affects the cells. The new data is presented in Figure 3 (page number and line number). We observed the DNA content in Cortex Glia after Cut knockdown and compared it to the control. The results show that DNA content increase is hindered when Cut is knocked down. This suggests that Cut is necessary for timely DNA synthesis, and without it, the cells are unable to increase their DNA content to a level where their nuclei can divide. On the other hand, constitutive overexpression of Cut leads to an apparent gain of DNA, even higher than the control (figure 6J). However, nuclei numbers still do not increase in cases of Cut overexpression, even though there is more DNA. We currently hypothesize that the levels of Cut in cortex glia may be dynamic and need to decrease for mitosis to occur.

• *Other studies have shown a dramatic, globular phenotype for CG under various loss of functions (Coutinho-Budd at al, 2017 with members of the membrane fusion machinery; Rujano et al, 2022 with dup RNAi and members of the cell-cell fusion machinery). That should be discussed and compared with rather than with FGF signaling.*

Response: The discussion focused on FGF activation, which induces membrane growth and increases the number of cortex glia nuclei. Comparing this phenotype with Cut overexpression highlights different mechanisms behind cortex glia growth that may act synergistically. Future studies will address their interaction.

• A previous study has shown that a dramatic alteration of CG morphology do not lead to NSC loss (Spéder and Brand, 2018). This should be discussed with respect to the findings with cut RNAi. Is it rather a specific function of cut in CG than linked to a change in morphology?

Response: According to the research paper, when insulin signaling is impaired, the cortex glia cannot create larger chambers. However, the study found that glial networks still exist in the central nervous system. This is evident in their Figure 2B and B', which clearly show the presence of cortex glia after ALH 72 hours. Additionally, their Figure 3 indicates an incomplete casing but still visible cortex glia around neural cells. Thus, the reaming glial networks appear to be sufficient to support the NSCs. In contrast, our data shows that when there is a Cut knockdown, cytoplasmic processes are eliminated, and the cortex glia cannot grow. As a result, the cortex glia can likely not provide essential nutrients to the NSC or protect it, or NSCs die for a yet unknown reason.

2) Data analysis and representation

• *Please show individual colour channels to support the colocalization in Fig. 1B-F.*

Response: added the colocalized images with Repo Fig. 3A-H

• *A nuclear (Repo) staining should be shown in Fig. 2 to 5.*

Response: in the revised manuscript Repo staining is added. Fig.S2,3,S3

• *Chamber size (Fig. 4H): I do not understand what is measured there (I could not find it in the methods). What is the unit? Also, if a chamber is defined by containing one NSC (see comment below in 3), then a staining should be added to understand where we are, especially regarding the z-depth.*

Response: We are referring a chamber where they are largely surrounded by thick extensions coming out from one nucleus/cytoplasmic body within which fine extensions cover neural cell bodies. Since we are measuring the size of a chamber its unit is um2. The details are also updated in the text.

• Membrane volume (Fig. 3H and 4I): I am not sure I understood how the membrane volume has been measured. I find the Methods hard to follow. For example: "To measure the cell size of cortical glial cells (GFP+, Repo+) multipoint selection tool was used to outline the area. The area outlining was done using the freehand selection tool around repo staining, followed by the analyze-measure option." How is the outlined area defined (One cell? How knowing where to stop?)? The control values are actually quite different between Fig. 3H and 4I.

Response: Figure 3 has been changed and the old Fig 3H is now 2G. Here we are measuring the volume of the whole tVNC not just one cell/ nuclei. We have outlined the whole tVNC of larval CNS and thereafter thresholding was done using image J/Fiji software. The thresholding procedure is used in image processing to select pixels of interest based on the GFP intensity of the pixel values. Thereafter Measure stack plugin was used to find the fluorescent area of the cortex glia section of tVNC (marked with eGFP in *Cyp4g15-GAL4*). The area obtained was thus multiplied by the number of stack intervals (=2) to find out the volume of cortex glia.
Fig. 2G and 4H belong to different data sets and for each data set one specific threshold was chosen that fits best for the actual staining and was kept constant throughout the analysis of that set. This is also updated in the method section.

3)Nomenclature

• *Endomitosis. Could the author exactly explain what they mean here? The endoreplicative/proliferation field itself has fluctuating definitions. Considering the previous literature for the Drosophila glia, it seems important to me to define whether they consider endomitosis as leading to full nuclear (but not cytoplasmic) separation (the term used in other glial cells like subperineurial glia; what I think they choose here) or if they use a stricter definition in which endomitosis cannot reach this state*

and only exhibit partial mitotic traits (in which case mitosis with incomplete cytokinesis should be used, as in Rujano et al). It is needed to ensure the readers knows what is described here and how it compares to/matches with cellular events described in previous studies.

Response: In the subpereineural glial, the nuclei division is complete, and there is no cytokinesis; thus, the cells clearly appear multinucleated. However, in the case of cortex glia, the pattern is different. The nuclei are completely separated, but due to incomplete cytokinesis, the cell never divides and separates; instead, they remain connected by thin processes. The exact process of endomitosis at the molecular level remains unclear. However, as we gain more knowledge about these unique forms of mitosis, the definitions may become clearer.

• CG chamber: it needs to be properly defined (cellular landmarks?)

Response: We're referring to a chamber with thick extensions from a single cortex glial nucleus body. Moreover, the fine extensions that emerge from these thick ones surround neural cell bodies from the inside. The details are also updated in the text.

• Main/side branches: the idea is interesting and novel in the context of the CG (and would fit with other known roles of Cut). How do the authors define it, as CG are big multinucleated cells with many branching? Is it when radiating from one nucleus to another from the same cell?

Response: We are considering main branches as those that are directly coming out of one nucleus/cell body. The side ones are those that are coming out of these branches. In Figure 2 C, D individual cortex glia cell body extends thick (yellow arrowheads) and lean processes (red arrows) in multiple directions.

• *I think cortex glia is more usual than cortical glia.*

Response: Replaced throughout the text as suggested.

4)Literature

• *The authors state that "the glial cells also form the blood-brain barrier", using a general reference and seemingly not specifying the species. As such it seems it imply it is a generic trait of the blood-brain barrier, whereas most species have an endothelial barrier. It needs to be corrected or precised.*

Response: The sentence is modified by adding details as “In addition, the glial cells also form the blood-brain barrier and compartmentalize CNS into specialized domains in several invertebrates and vertebrates such as elasmobranch fishes(Abbott, 2005; Awasaki et al., 2008; Oland & Tolbert, 2003)(Abbott, 2005; Awasaki et al., 2008; Oland and Tolbert, 2003).”

• "In the ventral nerve cord (VNC) of the Drosophila larval nervous system, cortical glial cells are mostly found at the ventral and lateral sides and remain closely

associated with neural cells and also regulate their fate (Ito et al., 1995). To my knowledge, Ito et al does not show that CG regulate NSC fate. The

Response: Ito et al included for cortex glia distribution. The missing references for the association of cortex glia and NSC are added (Dumstrei et al 2003, Coutinho-Budd et al., 2017, Read, 2018, Dong, Q et al 2020).

Similar to cortical glia, mammalian astrocytes also extend the membrane extensions towards the outer surface of synaptic neuropils (Awasaki et al., 2008)." Awasaki et al paper does not discuss the organization of mammalian astrocytes and its comparison to CG.

Response: replace with a review reference Zhou et al. 2019

• *"Growth of the glial cell membrane requires nutrient-dependent activation of Insulin and PI3K signaling (Yuan et al., 2020)." The first reference to show this is Spéder and Brand, 2018. Add references*

Response: Added.

• *"Cortical glia continues to expand their cytoplasmic extensions from the late embryonic stage (Coutinho-Budd et al., 2017; Ito et al., 1995). The following reference should be added: Pereanu et al, 2005; Spéder and Brand, 2018; Rujano et al., 2022. The Ito et al, 1995 reference should be removed.*

Response: Thank you, the references are added. Spéder and Brand, 2018 is not include, their data shows cortex glia expansion from ALH 0. We have included the embryonic expression in Figure S2 I,I'

• *"Therefore, as a first step, we checked the expression profile of the cortical glia Gal4 driver, cyp4g15-Gal4, and found it, also expressed from the late embryonic stage and can be used for the study (data not shown). Data available with us*
» This driver has been described in Spéder and Brand, 2018. Please either quote this paper or add the data in supplemental.

Response: Spéder and Brand, 2018 have used the Gal4 for the first time and shown its expression from ALH0hr. We have checked that the Gal4 also expresses in the embryo. Data is added in the supplementary Fig S2I.
We have added the Spéder and Brand 2018 reference where we have first mentioned the gal4 "We used the UAS-Gal4 system to mark the glia with GFP using *Repo-Gal4* (pan glia) and *Cyp4g15-Gal4* (cortex glia) drivers (Fig. 1B,C) (Spéder and Brand 2018, Gonzalez-Gutierrez et al., 2019; Rujano et al., 2022)".

• "Interestingly, the membrane extensions coming out from different cells are well connected and self-tilled on one another (Coutinho-Budd et al., 2017; Rujano et al., 2022)." I do not think that any of these papers showed that different CG cells are connected through membrane extensions. Also, I do not think this is accurate: "self-

tilled on one another". What does "self-til(l)ed" and "on one another" mean? CG do not tile on top of each other. Need to write in a better way

Response: The self-tiling was shown in Coutinho-Budd et al., 2017, Fig 1E by using colored markers and a Flip Out technique. As per my understanding, this is the first evidence showing the glia clones are arranged very closely. They have used term self-tilled there as well.
The sentence is modified as “Interestingly, the cytoplasmic processes coming out from different cells are well connected and self-tilled **to** one another”

• *"Thus, we conclude that Cut is required and sufficient for the growth of cortical glial membrane processes (Coutinho-Budd et al., 2017)." Why this reference? These are the own data of this study. Or if the purpose was to reference just the "growth of cortical glial membrane processes", please complete with other references.*

Response: Thank you for pointing out, the reference was mistakenly inserted. It is removed.

• *"Several reports from independent labs strongly indicate that cortical glial cells increase their nuclei number by undergoing endomitosis (Coutinho-Budd et al., 2017; Rujano et al., 2022; Unhavaithaya and Orr-Weaver, 2012; Yuan et al., 2020)." Unhavaithaya and Orr-Weaver, 2012 do not look at CG, but subperineurial glia. It should be removed.*

Response: Unhavaithaya and Orr-Weaver, 2012 reference has been Removed.

Minor-comments:
1. Fig. 1: I am a bit surprised that the scale bar is the same between panels G and I considering the sizes of the Dpn nuclei (or are NSC nuclei bigger under cut RNAi?). Also, while there are fluctuations depending on where the separation between central brain and ventral nerve cord is set, I tend to find the mean number of NSCs in control low. Could the authors describe the region they used?

Response: The images has been changed to avoid confusion, upon Cut-knockdown in cortex glial cells NSCs are unusually clumped and were eliminated during development. Only the tVNC area was selected for NSC quantification. Graph 1G has been modified with more data sets.

2. L3 is a long stage. When were the larvae dissected? Also, when were the RNAi/overexpression driven from? The driver onset? Did I miss it?

Response: The larvae were dissected in the late 3rd wandering stage in most of the experiment wherever we are referring late L3. This is now modified to LL3 (late larval 3) throughout the text. Also we checked the expression profile of the cortex glia-Gal4 driver, *Cyp4g15-Gal4*, and found that it expresses from late embryonic stage Figure S2 I,I’ and continues its expression through different larval stages.

3. Units are missing on some scale bars and graphs

Response: Scale bars are revised. Details are also added in figure legends.

4. Please check the italics for genes/genotype
Response: Genes and genotypes are made italics thought the manuscript

5. In the title, I am not sure "maintenance" is correct. I feel this is rather looking at formation of the niche.

Response: We are using the term "maintenance" because we have noticed that when Cut is knocked down from embryonic stages, it does not significantly impact the cytoplasmic extensions of cortex glia until L1(compare Fig.3A with 3B). Additionally, the number of nuclei representing individual cells is similar to the control in L1. However, these cells begin to lose their extensions over time, later in L1 and worsening as the developmental stage progresses towards LL3 (Fig.3H,H').

6. • "(Avet-Rochex et al., 2012; Dong et al., 2020; Read et al., 2009; Reddy B. V. V. G.and Irvine Kenneth, 2011; Spé Der and Brand, 2018; Witte et al., 2009)." Please correct the format of Reddy B. V. V. G.and Irvine Kenneth, 2011 and the spelling of Spé Der and Brand, 2018

Response: All the references are reformatted.

7. I am not fully sure that single surface or line plot add much to the representation of the membrane phenotype, which is clear on the inset.

Response: To provide a clearer understanding of the extension, plots are utilized. The thick extensions create strong peaks while the finer and closer ones create short and condensed peaks in control (refer to figure number and page number). In Cut knockdown cortex glia, the number of peaks is significantly lower, farther than the control, and the intensities vary, indicating a reduction in cytoplasmic extensions (refer to Fig 2E, F). Additionally, the plot profile along a line (along the white line in Supplementary Fig 2A, B) in the tVNC shows an apparent loss of the glial network upon Cut knockdown.

8. Please add some page/line numbers so it is easier to reference for reviewers.

Response: As per suggestion we have added page/line number in new version of the manuscript.

August 14, 2023
RE: GENETICS-2023-306277

Dear Dr. Arya:

I am pleased to accept your manuscript entitled "**Cut homeodomain transcription factor is a novel regulator of cortex glia morphogenesis and maintenance of neural niche** " for publication in GENETICS, pending minor revision. I expect you should be able to submit a revised manuscript within 30 days. A suitably revised manuscript will be acceptable for publication; I don't expect to send it out for review.

Please ensure that you have included a Data Availability Statement at the end of the Materials and Methods section. Details available at https://https://academic.oup.com/genetics/content/prep-manuscript. The DAS should include the accession numbers or DOIs of any data you have placed in public repositories, describe supplemental material, include applicable IRB numbers, and may include specifications for how to properly acknowledge or cite the data.

When revising the ms., please make an effort to shorten it, because that almost always improves a manuscript. We urge authors to heed the advice of Strunk and White: "omit needless words"1. Follow this link to submit the revised manuscript: Link Not Available

Thank you for submitting this story to Genetics.

Sincerely,

Norbert Perrimon
Associate Editor
GENETICS

Approved by:
Oliver Hobert
Senior Editor
GENETICS

Reviewer comments:

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

Yadav et al., have made significant changes to their manuscript and have addressed all the concerns I had with the previous submission. I am satisfied with the response and the new version of the manuscript, and I would highly recommend this version of the manuscript for acceptance.

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

I thank the authors for having taken the time and made the effort to assess and respond to my comments. I am glad that some of my (and the other reviewer's) suggestions have allowed to identify a clear role of Cut in cortex glia for regulating proliferative strategies (and especially endoreplication versus mitosis) rather than apoptosis. These are very nice results that will be of interest to the field.

In overall, I think the experimental data provided in this revised version support the central message that Cut is required for cortex glia growth and proliferation. The authors have also clarified the difference between cell and nuclei in this cortex glia context, and made a substantial effort of quantification of different proliferative parameters (nuclei number; nuclei volume; DAPI intensity; Fly-FUCCI).

While I do not think that more experiments are required, I strongly feel that the paper need additional revisions at the writing

levels, on definition, interpretation/logical links and writing. In particular:

1. Definition of endoreplication/endocycle/endomitosis
I previously asked the authors to clarify their definitions, as the field in general (not only Drosophila or cortex glia) tends to be a bit muddy. Their reply to my comment stated what is known for cortex glia but did not answer this specific issue, and the nomenclature used in the text is still not defined early enough nor within the context of the previous literature in the field.
In particular, endomitosis is defined strictly as DNA replication bearing some mitotic hallmarks but without nuclear division (see Edgar et al., 2014, Nature Reviews). Within this definition, endomitosis does not generate multinucleated cells. Endomitosis has also been used in a larger sense as an aborted mitosis without cell division, yet going until nuclear division and yielding two nuclei, (in Drosophila, this term has been chosen for subperineurial glia for example).
From what I understand, the authors used endomitosis in the latter definition (or is it used as an equivalent for multinucleation?). It is totally fine if the authors want to label nuclear division without cellular division and the production of multinucleated cells as endomitosis, but then they should:
i) state/define it clearly early on;
ii) explain it corresponds to acytokinetic mitosis in the paper which has actually identified/quantified the syncitial character of cortex glial cells (Rujano et al., 2022), so the field can track what corresponds to what across studies;
iii) accurately report the conclusions of other papers; for example:
- Lines 205-206 and Lines 362-363: Rujano et al do not conclude that cortex glia become multinucleated by endomitosis (see above);
- Lines 268-270: Unhavaithaya and Orr-Weaver is on subperineurial glia and not the cortex glia (as already remarked in revision 1); and again Rujano et al is not to reference for endomitosis in cortex glia.
I think it is an important point to fix as this kind of varying nomenclature is what brings confusion for readers.

2. Definition of chamber
Similarly, a cortex glia chamber has been used previously (Spéder and Brand, 2018; Dong et al, 2021) to define a continuous membrane encasing one neuroblast and its secondary progeny. Here the authors employ this term (if I understood correctly) for an enclosure around any cell type, and coming from the membrane around one nucleus within multinucleated cortex glial cells -a very different definition. It is again fine to use it this way, but it should be clearly stated early on in the text (I could not find it there, only in the response to my comments). Also, as chambers will vary in size depending on what it encases (primary neurons; neuroblast and immature secondary neurons; maturing neurons), the authors should ensure and state that they have measured in the same environment/locations/cell types.

3. Intepretation/Logical link
I do not understand some interpretations, which either should be clarified or revised:
• Figure S3A: from this, the authors conclude that there is "developmental delay progressing through different stages" (Lines 223-224). However, there is no statistical difference between control and cut RNAi in both pupariation and eclosion. Did I miss something?
• In the same section, the authors say "We observed significant pupal lethality during development." Where are the supporting data?
• Lines 184-186
"The finding that Cut knockdown prevents cortex glia nuclei from growing extensions and filling gaps, is significant since these cells normally grow efficiently when a few of their neighbors are ablated (Jaeda C. Coutinho-Budd et al. 2017)."
And lines 323-326:
"It is significant that when cortex glia are ablated in a restricted area, the neighboring cortex glia extends their processes and fills the gaps (Jaeda C. Coutinho-Budd et al. 2017; Hirase et al. 2022). However, Cut defective cortex glia does not show such compensatory growth, resulting in visible wide gaps in the glial trophospongium in the larval CNS."
I do not understand the causal significance here. The analysis from Coutinho-Budd et al is done in a mosaic context, where other cortex glial cells are wild-type and thus can respond. The experiments done in this study are through the whole cortex glia population so all experiencing Cut knockdown. Thus I am not sure why we should expect any compensation mechanism. Ablation throughout the population could not be expected to have any compensatory power either.

4. Writing
Some sentences should be rephrased for accuracy and to avoid mis/overinterpretation. It is not exhaustive, but for example:
• Lines 145-146: (NSCs...) "were eliminated during development upon Cut ablation in cortex glia". "eliminated" is somwhat biased, please replace with "lost" for example.
• Lines 149-150: "indicating that their (NSC) number was normal initially and declined only later due to loss of the niche". It is very hard to disentangle the effect on NSC from the loss of Cut in cortex glia per se from its effect on cortex glia morphology. I would suggest to replace "due to loss of the niche" by "due to loss of cut in cortex glia"
• Lines 169-170 : "Interestingly, the cellular processes from different cortex glia are well connected and self-tilled to one another (Jaeda C. Coutinho-Budd et al. 2017; Rujano et al. 2022)." I appreciate the authors have corrected the self-tiled on one another to avoid the confusion between mosaic tiling and tiling on top of each other. I still think "the cellular processes from different cortex glia are well connected" has not been demonstrated by any of these two studies.
• Line 184: "Cut knockdown prevents cortex glia nuclei from growing extensions". The nuclei are not growing extensions, rather the plasma membrane.

• Glia is a plural name, please check the verb spelling throughout the text.
• What is a "neural niche "? Niche is used for stem cells, is it what they imply (and thus it should be stated). Or if the authors means it as a general cellular supporting microenvironment for different neural cell types, I would suggest to use another term.

5. Figures
• Figure 3: please re-use the same way to identify the time windows between pictures (now with exact hours) and graphs (now stages). Also, I am a bit surprised to see very faint Repo staining in panel H'. That suggests that there was a difference in the acquisition with the control (so a better picture should be chosen). Or it means that at this late stage other glial types are affected by cut knockdown, and that should be discussed.

In addition, I have a few remarks on the authors' reply to some of my comments:
• The authors state several times that they could not count cell numbers because of the difficulty to make cortex glia clones. While I think using the overall nuclei count on the entire cell population is sufficient to support their claims, labelling individual cortex glial cells (and their nuclei) is perfectly possible using Raeppli (CAAX + Repo, or NLS, induced early) as they mention themselves (and also Coin-FLP actually). The MARCM-type methods should not be used.
• "5. In the title, I am not sure "maintenance" is correct. I feel this is rather looking at formation of the niche.
Response: We are using the term "maintenance" because we have noticed that when Cut is knocked down from embryonic stages, it does not significantly impact the cytoplasmic extensions of cortex glia until L1(compare Fig.3A with 3B). Additionally, the number of nuclei representing individual cells is similar to the control in L1. However, these cells begin to lose their extensions over time, later in L1 and worsening as the developmental stage progresses towards LL3 (Fig.3H,H')."
I do not understand the reponse, and I also think maintenance is even more inappropriate as the new data now demonstrate clearly that cut is required for building the niche by promoting cortex glia proliferation. If it were maintenance, we would expect some regression from a finalized structure, but the cortex glia niche keeps building and what is seen here is a loss of development/growth for a given time point.
• The fact that the time chosen for the L3 stage is wandering should be indicated in the text or Methods.
• There are still mistakes in the format of the references (sometimes the first name of the author is there, sometimes not; Spé Der and Reddy B. V. V. G are still there). Please check thoroughly.

Reviewer comments:

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

Yadav et al., have made significant changes to their manuscript and have addressed all the concerns I had with the previous submission. I am satisfied with the response and the new version of the manuscript, and I would highly recommend this version of the manuscript for acceptance.

Thank you so much. Indeed, your suggestions have added value to the manuscript. We are grateful.

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

I thank the authors for having taken the time and made the effort to assess and respond to my comments. I am glad that some of my (and the other reviewer's) suggestions have allowed to identify a clear role of Cut in cortex glia for regulating proliferative strategies (and especially endoreplication versus mitosis) rather than apoptosis. These are very nice results that will be of interest to the field.
Indeed. We are very thankful for the constructive critical comments.

In overall, I think the experimental data provided in this revised version support the central message that Cut is required for cortex glia growth and proliferation. The authors have also clarified the difference between cell and nuclei in this cortex glia context, and made a substantial effort of quantification of different proliferative parameters (nuclei number; nuclei volume; DAPI intensity; Fly-FUCCI).

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The existing literature about the diverse endomitotic/endoreplication cycles also has overlapping definitions. Endomitosis can result in the formation of more than two nuclei, depending on how many times the cells undergo cycling and nuclear separation (as discussed in Stetina et al. 2018). In megakaryocytes, Lordier et al. (2008) found that the so-called endomitosis actually fails at cytokinesis, not at anaphase/telophase in these cells, which overlaps with the definition of acytokinetic mitosis.

From what I understand, the authors used endomitosis in the latter definition (or is it used as an equivalent for multinucleation?). It is totally fine if the authors want to label nuclear division without cellular division and the production of multinucleated cells as endomitosis, but then they should:
i) state/define it clearly early on;

We completely understand the confusion surrounding the correct definition while discussing endoreplication/endocycle/endomitosis. We, too, were unclear about this matter and how to discuss it. However, reviewers encouraged us to think more deeply to sort things out. In recent literature,There are two sets of definitions: one discussed by Rujanu (and two other cross-references in the paper), where endoreplication is considered to cover endocycle (G/S) and endomitosis; the other description (Zielke et al 2013, Bruce and Weaver 2001) considers endocycle as a bigger umbrella with variant cell cycles, including endoreplication, endomitosis.
Since the term endoreplication (or endoreduplication) is more frequently used to define polyteny in Drosophila salivary glands, which is known to have alternating G/S cycling and is a more stable definition, we will consider the second definition above to represent the variant cell cycles in the present system. Accordingly, we consider endocycle as a broader term that covers variant cell cycles such as endoreplication and endomitosis. We are certain that the definitions will become more explicit as more molecular details of the process are discovered. Rujanu's paper has also shown the likely presence of aneuploidy, which would require further studies to understand the variant cell cycles in cortex glia.
To avoid confusion we have used endocycle word wherever we refer to increase in DNA content. We have added clarification in text as-
“Normally cortex glia show a significant increase in nuclear volume from L2 onwards and undergo endoreplication (Rujano et al. 2022). In literature there are different overlapping definitions of polyploidization, endoreplication and endocycle. We consider endocycle as a bigger umbrella with variant cell cycles, including endoreplication and endomitosis (Zielke et al 2013, Bruce and Weaver 2001) and will refer to the process as endocycle here onwards. We further investigate if Cut plays a role in increasing the DNA in cortex glia”(currently lines 259-264)

ii) explain it corresponds to acytokinetic mitosis in the paper which has actually identified/quantified the syncitial character of cortex glial cells (Rujano et al., 2022), so the field can track what corresponds to what across studies;

iii) accurately report the conclusions of other papers; for example:
- Lines 205-206 and Lines 362-363: Rujano et al do not conclude that cortex glia become multinucleated by endomitosis (see above);
Considering comments (ii) and (iii) We have expended the sentence in the text as “The number of cortex glia nuclei typically increases from L2 onwards by employing different forms of cell cycle which includes endocycle, endomitosis and acytokinetic mitosis (figure 3I) (Rujano et al. 2022).” (Currently lines 250-251)

- Lines 268-270: Unhavaithaya and Orr-Weaver is on subperineurial glia and not the cortex glia (as already remarked in revision 1); and again Rujano et al is not to reference for endomitosis in cortex glia.

The sentence is modified as “The number of cortex glia nuclei in the tVNC progressively increase from the L1 to LL3 stages (Fig. 5A-C) (Jaeda C Coutinho-Budd et al. 2017; Rujano et al. 2022)” (currently lines- 352-353)

I think it is an important point to fix as this kind of varying nomenclature is what brings confusion for readers.

2. Definition of chamber
Similarly, a cortex glia chamber has been used previously (Spéder and Brand, 2018; Dong et al, 2021) to define a continuous membrane encasing one neuroblast and its secondary progeny. Here the authors employ this term (if I understood correctly) for an enclosure around any cell type, and coming from the membrane around one nucleus within multinucleated cortex glial cells -a very different definition. It is again fine to use it this way, but it should be clearly stated early on in the text (I could not find it there, only in the response to my comments). Also, as chambers will vary in size depending on what it encases (primary neurons; neuroblast and immature secondary neurons; maturing neurons), the authors should ensure and state that they have measured in the same environment/locations/cell types.

We have added the clarification of what we are considering as a chamber and how the measurements are done. The text is modified “Since Cut overexpressing glia have more extended processes, the cortex glia chambers are also larger and globular (compare Fig.4 B, C with E, F, graph G). We consider a chamber to be an area that is surrounded by thick extensions coming out from one cytoplasmic body with fine extensions covering neural cell bodies. The chamber size was measured by outlining the cortex glia processes coming out from the cytoplasm around one nucleus and forming an enclosure around progeny” (currently lines 335-340)

3. Intepretation/Logical link
I do not understand some interpretations, which either should be clarified or revised:

• Figure S3A: from this, the authors conclude that there is "developmental delay progressing through different stages" (Lines 223-224). However, there is no statistical difference between control and cut RNAi in both pupariation and eclosion. Did I miss something?
• In the same section, the authors say "We observed significant pupal lethality during development." Where are the supporting data?
• Lines 184-186

Apologies for the confusion. We observed a developmental delay as larvae progressed through various stages, although this delay did not reach statistical significance. We modified the sentence as "There was an indication of delay in progression through different developmental stages although this did not reach statistical significance (Fig. S3A-B)." (currently lines 292-293)

"The finding that Cut knockdown prevents cortex glia nuclei from growing extensions and filling gaps, is significant since these cells normally grow efficiently when a few of their neighbors are ablated (Jaeda C. Coutinho-Budd et al. 2017)."
And lines 323-326:
"It is significant that when cortex glia are ablated in a restricted area, the neighboring cortex glia extends their processes and fills the gaps (Jaeda C. Coutinho-Budd et al. 2017; Hirase et al. 2022). However, Cut defective cortex glia does not show such compensatory growth, resulting in visible wide gaps in the glial trophospongium in the larval CNS."
I do not understand the causal significance here. The analysis from Coutinho-Budd et al is done in a mosaic context, where other cortex glial cells are wild-type and thus can respond. The experiments done in this study are through the whole cortex glia population so all experiencing Cut knockdown. Thus I am not sure why we should expect any compensation mechanism. Ablation throughout the population could not be expected to have any compensatory power either.

Our study demonstrates that the loss of Cut in the entire cortex glia population affects the neural stem cell (NSC) niche. Unlike the mosaic context experiments in Coutinho-Budd et al., where other cortex glial cells were wild-type and could compensate, our study involves knockdown in the entire cortex glia population, making compensatory mechanisms unlikely.
For clarity, the sentence is restructured as " The finding that Cut knockdown prevents cortex glia from growing cytoplasmic extensions and filling gaps, is significant. Although ablation of a few cortical glia causes the unaffected neighboring cortex glia to compensate by growing lamelliform extensions to efficiently to fill the gaps (Coutinho-Budd et al. 2017), a general knockdown of Cut in all the cortex glia affects their growing capability, leading to the presence of spaces all over the tVNC. This clearly indicates that Cut is required for the normal growth and development of lamelliform extensions from the cell body. Upon Cut knockdown, several of these defective glia could not undergo proper

morphogenesis and large spaces can be observed all over the tVNC. (currently 219-241)

4. Writing
Some sentences should be rephrased for accuracy and to avoid mis/overinterpretation. It is not exhaustive, but for example:
• Lines 145-146: (NSCs...) "were eliminated during development upon Cut ablation in cortex glia". "eliminated" is somwhat biased, please replace with "lost" for example.

Suggestion is incorporated. (Currently line-163)

• Lines 149-150: "indicating that their (NSC) number was normal initially and declined only later due to loss of the niche". It is very hard to disentangle the effect on NSC from the loss of Cut in cortex glia per se from its effect on cortex glia morphology. I would suggest to replace "due to loss of the niche" by "due to loss of cut in cortex glia"

The sentence is reframed for clarity “The number of NSCs in tVNC of control and Cut ablated cortex glia did not show any significant difference in early third instar larvae (Fig.1L). This indicates that NSC number was initially normal but declined later, possibly due to loss of the Cut defective cortex glia (Fig.1L).” (currently lines166-168)

• Lines 169-170 : "Interestingly, the cellular processes from different cortex glia are well connected and self-tilled to one another (Jaeda C. Coutinho-Budd et al. 2017; Rujano et al. 2022)." I appreciate the authors have corrected the self-tiled on one another to avoid the confusion between mosaic tiling and tiling on top of each other. I still think "the cellular processes from different cortex glia are well connected" has not been demonstrated by any of these two studies.

The sentence is modified as “Recently, it has been shown that cortex glia are syncytial units and different such units undergo homotypic fusion to allow exchange of cytoplasmic proteins” (currently lines197-198)

• Line 184: "Cut knockdown prevents cortex glia nuclei from growing extensions". The nuclei are not growing extensions, rather the plasma membrane.

Thank you for pointing out this a typo. The sentence is reframed as “The finding that Cut knockdown prevents cortex glia from growing lamelliform extensions and filling gaps, is significant.” (currently lines 212-213)

• Glia is a plural name, please check the verb spelling throughout the text.
Will fix

We have corrected it throughout the text.

• What is a "neural niche "? Niche is used for stem cells, is it what they imply (and thus it should be stated). Or if the authors means it as a general cellular supporting microenvironment for different neural cell types, I would suggest to use another term.

The cortex glia makes specialized microenvironment and support system to regulate the behavior of neural stem cells (NSCs) as well as progenitor cells within the nervous system we term this as neural niche. To avoid confusion, we again change the title as
“Cut homeodomain transcription factor is a novel regulator of growth and morphogenesis of cortex glia niche around neural cells”

5. Figures
• Figure 3: please re-use the same way to identify the time windows between pictures (now with exact hours) and graphs (now stages). Also, I am a bit surprised to see very faint Repo staining in panel H'. That suggests that there was a difference in the acquisition with the control (so a better picture should be chosen). Or it means that at this late stage other glial types are affected by cut knockdown, and that should be discussed.

Thank you for pointing it out. We have changed the figure 3H with a better acquisition, and regarding graph 3I, we have mentioned the stages as this part of the experiment was not synchronized with exact hours.

In addition, I have a few remarks on the authors' reply to some of my comments:
• The authors state several times that they could not count cell numbers because of the difficulty to make cortex glia clones. While I think using the overall nuclei count on the entire cell population is sufficient to support their claims, labelling individual cortex glial cells (and their nuclei) is perfectly possible using Raeppli (CAAX + Repo, or NLS, induced early) as they mention themselves (and also Coin-FLP actually). The MARCM-type methods should not be used.

We agree that labeling individual cortex glia is possible with the very recently available tool, but it's unclear if the tool can provide an exact count. Rujano et al. (2022) extensively used a 4-color Raeppli tool but didn't mention the number of cortex glia cells in the larval CNS. They mentioned that having two same-color clones in close proximity resulted in larger clones. Certainly, future experiments will provide better answers. Currently, we need the tool to address the issue.

• "5. In the title, I am not sure "maintenance" is correct. I feel this is rather looking at formation of the niche.
Response: We are using the term "maintenance" because we have noticed that when Cut is knocked down from embryonic stages, it does not significantly impact the cytoplasmic extensions of cortex glia until L1 (compare Fig.3A with

3B). Additionally, the number of nuclei representing individual cells is similar to the control in L1. However, these cells begin to lose their extensions over time, later in L1 and worsening as the developmental stage progresses towards LL3 (Fig.3H,H')."
I do not understand the reponse, and I also think maintenance is even more inappropriate as the new data now demonstrate clearly that cut is required for building the niche by promoting cortex glia proliferation. If it were maintenance, we would expect some regression from a finalized structure, but the cortex glia niche keeps building and what is seen here is a loss of development/growth for a given time point.

We changed the title as “Cut homeodomain transcription factor is a novel regulator of growth and morphogenesis of cortex glia niche around neural cells”

• The fact that the time chosen for the L3 stage is wandering should be indicated in the text or Methods.

Wandering larvae are referred as LL3 (late larval 3) throughout the text and are also added in the method section.

• There are still mistakes in the format of the references (sometimes the first name of the author is there, sometimes not; Spé Der and Reddy B. V. V. G are still there). Please check thoroughly.

Sorry, we have corrected it throughout the text.

September 13, 2023
RE: GENETICS-2023-306277R1

Dr. RICHA Arya
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Dear Dr. Arya:

Congratulations! We are delighted to inform you that your manuscript entitled "**Cut homeodomain transcription factor is a novel regulator of growth and morphogenesis of cortex glia niche around neural cells**" is acceptable for publication in GENETICS. Many thanks for submitting your research to the journal.

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