Mural Wnt/β-catenin signaling regulates Lama2 expression to promote neurovascular unit assembly

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MS TITLE: Mural norrin/β-catenin signaling regulates Lama2 expression to promote neurovascular unit assembly.

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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. The referees ask for clarifications and additional information. It will be particularly important to provide additional evidence supporting the contention that Apcdd1 is expressed in mural cells. In addition, quantifying lama2 expression in neurovascular cell types in wild type and Apccd1 mutants seems important. Ultrastructural analysis of the retinal blood vessels and surrounding astrocytes would address some of the concerns of Referee 1 and Referee 3.If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The roles for the extracellular matrix (ECM) components in neurovascular development and physiology are understudied. This manuscript makes an important finding by linking canonical Wnt signaling to laminin expression in neurovascular units of the developing retina and cerebellum.

Comments for the author

In this manuscript, Biswas et al. have used in vitro and in vivo strategies to show that Wnt/ß-catenin signaling in mural cells within neurovascular units of the mouse CNS control laminin expression to regulate endothelial barrier integrity. Most of the data are from analysis of angiogenic blood vessels in the developing neonatal retina (at P10 and P14), but some correlative analyses of the cerebellar vasculature are included. The author's data show that lama2/laminin a2 is upregulated by "mural cells" in the neurovascualture after genetic activation of the canonical Wnt pathway. Activation of intracellular Wnt signaling was achieved by deletion of Apcdd1, a Wnt/ß-catenin signaling antagonist. The authors have published previously that Apcdd1-/- mice have enhanced CNS endothelial barrier integrity and the experiments in this manuscript are aimed to further dissect the molecular mechanisms in Apcdd1-/- mice using bulk transcriptome profiling of Apcdd1-/- retinas. Some in vitro data using isolated vascular cells are included. The manuscript is well-written, the data are generally of high quality, and the rationale for conducting many of the experiments is solid. The study will make an important contribution to the field of CNS neurovascular development. However, many of the conclusions about lama2, Norrin/b-catenin, and BRB formation are correlative. There are several concerns that should be addressed experimentally that will strengthen many of the conclusions in the study, particularly related to (i) the premise that Norrin/ß-catenin signaling is primarily deregulated in the Apcdd1-/- mice, and (ii) which cells in the neurovascualture are involved in Lama2 expression and subsequent ECM adhesion. Importantly ultrastructural analysis of the retinal blood vessels and surrounding astrocytes is also needed.

Major comments:
Figure 1: It would be informative if the Lef1 expression were quantified in different retinal endothelial cell sub-populations. In particular, is there differential Lef1 expression in endothelial tip cells versus stalk cells in the primary retinal vascular plexus at P6 and the secondary vascular plexus at P10 and P14? Do differences in Lef1 expression/activation correlate with differences in Lama2 expression?
Figure 3: From the data shown in Figure 3A-D using LacZ as a surrogate marker it seems that multiple neurovascular cell types are expressing Lama2, including pericytes/vSMCs and astrocytes. The relative expression levels should be quantified in the different cell types in the Lama2+/- mice. Similarly, levels of Lama2 should be quantified in the different cell population in the Apcdd1-/- retinas.
Figure 4: The Biocytin-TMR/BRB experiments in Apcdd1-/- mice should be shown alongside the Lam2-/-
results for comparative purposes. The data showing extravasation of Biocytin-TMR in Lama2-/- mice alone are incremental, since abnormal BBB integrity has already been reported in Lama2-/- mice. The authors also cite their own prior work for BRB integrity differences in Apcdd1-/- mice. Combining these results would add more significance to this figure.
Figure 5: The use of the GSK3b inhibitor CHIR for in vitro cell treatments is not a very specific strategy to modulate Wnt/b-catenin signaling activation, since it is a broad agonist of the canonical Wnt pathway and others. The authors should treat the cultured cells with recombinant Wnts (Wnt7a/b) or Norrin followed by quantitation of Lama2 levels. Throughout the manuscript the authors make the general claim that the Norrin/ b-catenin pathway is mainly involved in Apcdd1-/-

mice, presumably because they are studying the retinal vasculature. However, additional Wnt family members (other than Norrin) may be involved in control of Lama2 expression. This should be substantiated by the in vitro experiments suggested above as well as in some in vivo studies to show specific involvement of Norrin over other Wnts.
Figure 6: The authors claim that in Apcdd1-/- mice “retinal astrocytes mature precociously” as evidence by increased levels of GFAP and AQP4 (but not PDGFRa) expression along the retinal vasculature as a result of hyperactive Wnt/b-catenin signaling. An alternative explanation is that the these astrocytes have become secondarily reactive and are responding to vascular and/or neural pathologies in Apcdd1-/- mice.
Indeed, GFAP is marker for reactive astrogliosis. The authors should at least discuss this possibility to clearly explain why secondary reactive gliosis is less likely than primary changes in astrocyte differentiation status or specification.
Figure 7: There may be a modest upregulation of Itga6 in retinal astrocytes (as shown by GFAP co-localization) in Apcdd1-/- mice, but from the data it looks like the vascular endothelial cells and/or pericytes are showing a more robust change in a6 integrin expression. These changes in Itga6 in endothelial cells and/or pericytes should be experimentally quantified and discussed in more detail. Prior studies have shown that Itga6 is expressed in vascular endothelial cells where it serves as a laminin receptor and this should be discussed.
Additional major points: (1) What the study needs is some ultrastructural analyses (transmission EM) of the retinal neurovasculature with a particular focus on the vascular basement membranes as well as perivascular astrocyte end feet in control and in Apcdd1-/- mice. Immuno-EM for Lama5 could also be included using control and Apcdd1-/- retinal or cerebellar samples. (2) Astrocytes from control and Apcdd1-/- mice should be nalayzed in ECM assays to determine if they have adhesion defects. Based on the Itga6 data (Figure 7), Apcdd1-/- astrocytes should show enhanced ECM adhesion. Also, using control and Apcdd1-/- cultured astrocytes, the profile of cell surface heterodimer expression can be assessed by biotinylation-immunoprecipitation approaches which are commonly used in the integrin field.

Minor comments:
1. In Figure 1 there are green speckles in the Lef1 immunofluorescence panels that distract from the quality of the figure and should be corrected. Alternatively, new panels with images that lack the speckles should be added.
2. In Figure 2 data using a control RNA probe for the in situ hybridization should be shown to control for antisense/sense especificity.
3. In Figure 4 it is confusing why the retinal vascular extravasation data in Figure 4G are shown after factoring in extravasation in the liver. An explanation for this ratioed strategy should be included, or the retina data should be shown alongside the liver data.
4. In Figure 7 immunofluorescence for b1 integrin/Itgb1 should be shown in control and Apcdd1-/- mice.
5. In Supp. Figure 2 the Lama2 immunoreactive protein bands are not convincing. The immunoblots have been excessively cropped. In addition, laminins are very large glycosylated proteins and should not show distinct bands, but rather have a smeared appearance by immunoblotting. Perhaps PNGase-treated controls should be included.
6. In Supp. Figure 6 there should be better quality images shown for the cerebellar vasculature in control and mutant mice.

Reviewer 2

Advance summary and potential significance to field

This manuscript by Biswas and colleagues is focused on Norrin/B-catenin signaling in retinal mural cells driving expression of ECM Lama2. The authors showed enhanced Lama2 in Apcdd1-KO animals that have higher Norrin/B-catenin signaling, correlating with increase astrocytic Aqp-4 and Integrin-a6. Work by others has shown Lama2 deletion leads to perturbed BBB/BRB integrity however regulation of Lama2 has not been previously investigated. This work showing that it is downstream of Norrin/B-catenin in mural cells is novel. Further, it broadens the mechanism through which Norrin/B-catenin regulate BBB beyond activity in the endothelium, which has been the primary focus of research up to this point. All of this is a new and interesting facet to how the

Wnt/Norrin/B-catenin pathway regulates CNS vascular development and integrity, making it of high interest to the scientific community.

Comments for the author

Major:
1) To make images accessible to red-green color-blind readers, please select different color palettes for all fluorescence images – figures can be checked to accessibility on this website: https://www.color-blindness.com/coblis-color-blindness-simulator/
2) The author's show Pdgfrb+ and Foxc1+ pericytes in the postnatal retina and postnatal cerebellum have active Wnt signaling, the idea is that this is downstream of Norrin. To further support this, the authors should look at Lef1 or TCF-Lef1-GFP in mural cells in other brain regions where Norrin is not expressed. It is possible that mural cell Wnt/B-catenin activity is more widespread than retina/cerebellum, which would be of interest as well.
3) Fig. 3 – analysis of Lama2 expression appears limited to artery and veins (both quantification and images) in Apcdd1-KO v control however B-cat activity is also in mural cells associated w/ capillaries (likely based on pictures in Fig. 1), is increase in Lama2 see in all retinal vasculature or is this restricted to larger vessels? This is important information as the idea is that Lama2 is participating in promoting barrier integrity/vascular maturation throughout the vasculature, showing that is the case would help support this model. If the increase in Lama2 is limited to the large blood vessels in the retina, this should be addressed.
4) Please provide quantification of the Occludin expression in Fig. 3H-K 5) The authors and others have previously shown that Norrin/B-catenin signaling is increased in retinal endothelial cells in Apcdd1-KO – it would be important to look at Lef1 expression in Ng2 or Pdgfrb+ mural cells in Apcdd1-KO to test if it is also increased. This would be good support that the effect is cell autonomous and align with their culture data showing CHIR treatment of Pdgfrb+ cells increases Lama2. Further, this is also important as Apcdd1 is only expressed in a subset of mural cells (Fig. 1, ~25%) yet its loss seems to have widespread effects on Lama2 expression and astrocyte maturation.
6) The studies here are over-activation of Norrin/B-cat, in vivo with the Abcdd1-KO and the culture studies with CHIR. An outstanding question is whether Norrin/B-cat in mural cells is required for Lama2 expression in the vasculature and astrocyte maturation. There are experimental approaches to address this, culture approaches and animal models to conditionally disrupt norrin or its receptors in mural cells (or even Norrin-KO). However I recognize that these are considerable experiments to undertake and would be satisfied for this limitation of the study to be addressed in the discussion.

Minor:
1) Description of Figure 1T-X in 'results' – indicate that Pdgfra and GFAP are labeling astrocytes.
2) For resubmission, please provide pg and ln numbers to aid in review.
3) Fig 2 – Gene labels in volcano plots are very small and hard to read, please enlarge.
Signed: Julie Siegenthaler

Reviewer 3

Advance summary and potential significance to field

Biswas et al., aim demonstrating that Norrin/b-catenin signaling is active in CNS mural cells and regulates Lama2 deposition in the vascular basement membrane. The paper is well written but has important caveats. The most important one concerns the demonstration of the expression of Apcdd1 in mural cells. I would also no speak about NVU assembly since regarding astrocyte endfeet, only some markers are perturbed by the absence of Apcdd1.

Comments for the author

Images in Fig2 are not informative. Please provide more resolutive images showing that mural cells express Apcdd1. Based on these images it is very difficult to state that Apcdd1 is expressed in mural cells. It would also be important to be able to localize vessels on these images. The Pdgfbr FISH shows a huge background. The single cell Rnaseq data from Macosko et al shows a very low

level of Apcdd1 in mural cells. The rest of the paper is based on this result so it is actually very important to assess the potential expression of Apcdd1 in mural cells in a more serious way.

In the Lama2 LacZ mice, please explain why the labelling is patchy. Is it a nuclear labelling? Lama2 is also expressed by perivascular fibroblasts and endothelial cells (although not at the same level than pericytes) (Fig sup 2A). Thus it is not sure that upregulated laminin is provided by mural cells. If the lacZ signal is nuclear, crossing the Apcdd1 KO with Lama Lac2 might allow to differentiate the laminin upregulated by endothelial/fibroblast/mural cells. Another more immediate accessible method would be to perform Lama2 FISH (with coFISH staining for each vascular cell type).
It is striking that only some molecules of the astrocyte endfeet are upregulated. For instance, GlialCAM the partner of MLC1 is not upregulated. The same for Gjb6 encoding Cx30 the other gap junction protein of astrocyte perivascular processes with Cx43. It would mean that Lama2 influence only some markers and not the general maturation process of endfeet.
A more direct experiment to the assess astrocyte endfeet molecular repertoire would be to perform western blot analysis on purified microvessels (which retains endfeet). If would complement the IF study.
Reagarding NVU assembly, only some astrocytic markers are upregulated. It does not mean that assembly of NVU is dependent on Lama2. I would rather write maturation of the NVU but not assembly.
Define Apcdd1-/- in the abstract and give its full name.
Supp data sheet 1: Order the genes at P10 by their FC
Gene names in Fig 2H-M are too small.

First revision

Author response to reviewers' comments

Response to Reviewers:

We thank the reviewers for their insightful feedback on the manuscript. We are gratified that the reviewers expressed considerable interest in the original manuscript. The revised manuscript addresses three major concerns raised by reviewers namely: a) additional evidence supporting the claim that *Apcdd1* mRNA is expressed in mural cells; b) quantification of Lama2 mRNA and / or protein expression in neurovascular cell types in wild-type (WT) and *Apcdd1*$^{-/-}$ retinas; 3) ultrastructural analysis of astrocyte endfeet around blood vessels in P14 WT and *Apcdd1*$^{-/-}$ retinas. In addition, the revised manuscript addresses several additional concerns raised by the reviewers. Below, we provide a point-by-point response to reviewers' concerns about the original manuscript.

Reviewer #1
Major comments:
1) There are several concerns that should be addressed experimentally, particularly related to: (i) the premise that Norrin/ß-catenin signaling is primarily deregulated in Apcdd1$^{-/-}$ *mice, and (ii) which cells in the neurovasculature are involved in Lama2 expression and subsequent ECM adhesion.*

i) We have already published before (Mazzoni et al., 2017) that Norrin/ß-catenin signaling is upregulated in P10 *Apcdd1*$^{-/-}$ compared to WT retinas by both Western blot analysis for phospho-LRP6/total LRP6 and active ß-catenin/total ß-catenin, and immunofluorescence analysis for Sox17 protein, a transcription factor downstream of the pathway (Corada et al., 2019), in the superficial vascular plexus (see Figure S3G-O in Mazzoni et al., 2017). These previous data demonstrate collectively that Norrin/ß-catenin pathway is activated at higher levels in endothelial cells of the superficial vascular plexus in the developing *Apcdd1*$^{-/-}$ compared to WT retina. In the revised manuscript, we now show that the number of Lef1^{+} mural cells (co-labeled with Pdgfrß) is significantly increased in P14 *Apcdd1*$^{-/-}$ compared to WT retina (Supplementary Fig. 3A-C), which supports our claim that Norrin/ß-catenin signaling is also upregulated in mural cells in the *Apcdd1*$^{-/-}$ retina.
ii) Endothelial cells, mural cells and astrocytes are the three main neurovascular unit (NVU)

cell types that contribute to the formation of the vascular basement membrane (vBM) in the superficial retinal plexus by secreting various ECM proteins. A previous single cell RNA-seq study showed that mural cells (e.g. pericytes) are the predominant cell types expressing *Lama2* mRNA in the retina, whereas *Lama2* mRNA expression is much lower in astrocytes and endothelial cells [Supplementary Fig. 3D and (Macosko et al., 2015)]. We performed a detailed analysis of *Lama2* mRNA expression at both P10 and P14 WT and *Apcdd1*$^{-/-}$ retinas by fluorescence *in situ* hybridization (FISH) for *Lama2* mRNA combined with antibody staining for markers of each NVU cell type. Our FISH data show that there is minimal colocalization of *Lama2* mRNA puncta or ß-Gal protein (Lama2 reporter mice) with endothelial cells (Caveolin-1) at either P10 or P14 retina (Fig. 3B, E, I, J). In contrast, *Lama2* mRNA puncta or ß-Gal protein are present both in mural cells (NG2^{+}; Fig. 3A, D, G, H) and astrocytes (GFAP^{+}; Fig. 3C, F, K, L). Importantly, *Lama2* mRNA expression was upregulated only in mural cells (Fig. 3G, H), but not endothelial cells or astrocytes (Fig. 3I-L) of the *Apcdd1*$^{-/-}$ retinas. These data indicate that upregulation of *Lama2 mRNA* in the *Apcdd1*$^{-/-}$ retina observed from analysis of bulk RNAseq data (Fig. 2G, H, I, J) is derived specifically from mural cells, but not endothelial cells and astrocytes.

2) Figure 1: It would be informative if Lef1 expression were quantified in different retinal endothelial cell sub-populations. In particular, is there differential Lef1 expression in endothelial tip cells versus stalk cells in the primary retinal vascular plexus at P6 and the secondary vascular plexus at P10 and P14? Do differences in Lef1 expression/activation correlate with differences in Lama2 expression?

Expression of some downstream targets for Wnt/ß-catenin signaling [e.g. Sox7, Sox17 and Sox18 (Zhou et al., 2015)] and Apcdd1 (Mazzoni et al., 2017) has been extensively analyzed at various stages of vascular development in the retina. Furthermore, we have previously reported that Wnt/ß-catenin signaling is upregulated in *Apcdd1*$^{-/-}$ compared to WT retinal endothelial cells (Mazzoni et al., 2017) (please see the response to #1). In this study, we focus on the effects of Wnt/ß-catenin signaling on retinal mural cells, not endothelial cells, which has not been studied before. Moreover, *Lama2* mRNA expression is upregulated only in *Apcdd1*$^{-/-}$ retinal mural cells (Fig. 3G, H), and not endothelial cells (Fig. 3I, J). Thus, we believe quantifying Lef1 expression in different sub-populations of retinal endothelial cells is not relevant for our study.

3) Figure 3: From the data shown in Figure 3A-D using LacZ as a surrogate marker it seems that multiple neurovascular cell types are expressing Lama2, including pericytes/vSMCs and astrocytes. The relative expression levels should be quantified in different cell types in the Lama2$^{+/-}$ *mice. Similarly, levels of Lama2 should be quantified in different cell populations in the Apcdd1*$^{-/-}$ *retinas.*

Lama2$^{Lacz/+}$ reporter mouse strain is a great tool to identify which NVU cell types express *Lama2* mRNA. However, it cannot be used to quantify the levels of Lama2 mRNA or protein expression in an NVU cell type at a particular time point, because ß-Gal protein has a different half-life compared to Lama2 protein. Thus, we used FISH to localize and measure *Lama2* mRNA expression in mural cells, endothelial cells and astrocytes of WT and *Apcdd1*$^{-/-}$ retinas. Our data show that there is minimal colocalization of *Lama2* mRNA puncta with endothelial cells in P10 and P14 WT retina (Fig. 3B, E, I, J), suggesting that endothelial cells do not express *Lama2* mRNA. In contrast, there is colocalization of *Lama2* mRNA puncta with NG2^{+} mural cells (Fig. 3A, D, G, H) and GFAP^{+} astrocytes (Fig. 3C, F, K, L), suggesting these two cell types express *Lama2* mRNA. These data are consistent with a previous single cell RNA-seq study showing that pericytes are the major source of *Lama2* mRNA in the retina whereas astrocytes and endothelial cells have much lower expression [Supplementary Fig. 3D; (Macosko et al., 2015)].

4) Figure 4: The Biocytin-TMR/BRB experiments in Apcdd1$^{-/-}$ *mice should be shown alongside the Lama2*$^{-/-}$ *results for comparative purposes. The data showing extravasation of Biocytin-TMR in Lama2*$^{-/-}$ *mice alone are incremental, since abnormal BBB integrity has already been reported in Lama2*$^{-/-}$ *mice. The authors also cite their own prior work for BRB integrity differences in Apcdd1*$^{-/-}$ *mice.*

We have already published the biocytin-TMR extravasation data in the *Apcdd1*$^{-/-}$ retina compared to WT retina (Mazzoni et al., 2017). Therefore, we do not see a point in duplicating our data in this study. We have added a schematic diagram (Fig. 4A) to show that biocytin-TMR

extravasation is lower in *Apcdd1*$^{-/-}$ compared to WT retina, suggesting a precocious paracellular BRB maturation.

Although BBB leakage has been documented in the *Lama2*$^{-/-}$ brain, the assessment of BBB leakage was performed by analysis of Evan's Blue extravasation [Fig. 2a, b in (Menezes et al., 2014)]. Evan's Blue binds to serum albumin (~60 kDa) and therefore can be a readout of both impaired paracellular (regulated by tight junctions) and transcellular (regulated by caveolae) BBB permeability; however, it is primarily a readout of increased transcellular barrier permeability regulated by caveolae. The biocytin-TMR is a smaller (~890 Da) tracer that crosses the BBB primarily through the paracellular route regulated by tight junctions. Therefore, the biocytin-TMR extravasation data in the *Lama2*$^{-/-}$ retina presented in our manuscript (Fig. 4B-G) represent a more detailed analysis of the functional impairment of paracellular BRB leakage, compared to prior studies (Menezes et al., 2014).

5a) Figure 5: The use of the GSK3β inhibitor CHIR for in vitro cell treatments is not a very specific strategy to modulate Wnt/β-catenin signaling activation, since it is a broad agonist of the canonical Wnt pathway and others. The authors should treat cultured cells with recombinant Wnts (Wnt7a/b), or Norrin, followed by quantitation of Lama2 levels.

We agree with the reviewer's comment that CHIR 99021 is a broad agonist of Wnt/β-catenin signaling. However, recombinant Wnt7a/7b or Norrin did not work in our hands to activate the pathway for *in vitro* studies. Therefore, we used recombinant Wnt3a, another potent activator of canonical Wnt/β- catenin signaling, that we have used successfully in previous studies to activate the pathway (Cottarelli et al., 2020; Mazzoni et al., 2017). Similar to the results obtained with CHIR 99021 (Fig. 5C, D, G, H, K, L), Wnt3a treatment also upregulated Lama2 expression in brain pericytes (Fig. 5M, N), but not in either cerebellar astrocytes (Fig. 5O, P), or brain endothelial cells (Fig. 5Q, R).

5b) Throughout the manuscript the authors make the general claim that Norrin/β-catenin pathway is mainly involved in Apcdd1$^{-/-}$ *mice, presumably because they are studying the retinal vasculature. However, additional Wnt family members (other than Norrin) may be involved in control of Lama2 expression. This should be substantiated by the in vitro experiments suggested above as well as in some in vivo studies to show specific involvement of Norrin over other Wnts.*

We have found that Wnt/β-catenin signaling is also activated in mural cells of the cortex, in addition to the retina and cerebellum, where Wnt7a/7b are the main Wnt ligands [Norrin is the main ligand activating the pathway in the retina and cerebellum]. These data are now included in Supplementary Fig. 2I-J' and suggest that Wnt/β-catenin signaling is activated in mural cells in various CNS regions.

Based on these additional experiments, we have modified the title and the text to reflect the broad Wnt/β-catenin-dependent induction of Lama2 expression (instead of Norrin/β-catenin-dependent activation of Lama2 expression) by CNS mural cells.

6) Figure 6: The authors claim that in Apcdd1$^{-/-}$ *mice "retinal astrocytes mature precociously" as evidence by increased levels of GFAP and AQP4 (but not PDGFRα) expression along the retinal vasculature as a result of hyperactive Wnt/β-catenin signaling. An alternative explanation is that these astrocytes have become secondarily reactive and are responding to vascular and/or neural pathologies in Apcdd1*$^{-/-}$ *mice. Indeed, GFAP is a marker for reactive astrogliosis. The authors should at least discuss this possibility to clearly explain why secondary reactive gliosis is less likely than primary changes in astrocyte differentiation.*

We agree with the reviewer that in principle GFAP upregulation in *Apcdd1*$^{-/-}$ retinal astrocytes could be due to reactive astrogliosis. However, we did not see upregulation of other established reactive astrogliosis marker genes (e.g., *Nes*, *Synm*, *Vim*, *C3*, *Aldoc*, *Blbp*, *Maob*, *Lcn2*, *Serpina3*, *S100b*, *Sox9*, *Stat3*, *Trkb*, *Eaat1*, *Eaat2*, *Kir4.1* etc), with the exception of *Tspo (Escartin et al., 2021)*. *Tspo* is also expressed by Müller cells, horizontal cells, endothelial cells, pericytes, microglia, amacrine cell and fibroblasts (Macosko et al., 2015) (https://singlecell.broadinstitute.org/single_cell/study/SCP301/c57b6-wild-type-p14-retina-by-drop-seq#study-summary). Based on this analysis, it is unlikely that astrocytes become reactive in

the *Apcdd1*$^{-/-}$ retina. This analysis supports our interpretation that upregulation of GFAP and Aqp-4 (along with other astrocyte maturation markers such as *Plcd4*, *Gja1*, *Apoe*, *Mlc1*; Fig. 6A, B, M-V) in *Apcdd1*$^{-/-}$ retinal astrocytes is indicative of precocious astrocyte maturation. We have clarified this point in the revised result (page 13, paragraph 2) and discussion (page 20, paragraph 1).

7) Figure 7: There may be a modest upregulation of Itgα6 in retinal astrocytes (as shown by GFAP co- localization) in Apcdd1$^{-/-}$ *mice, but from the data it looks like the vascular endothelial cells and/or pericytes are showing a more robust change in α6 integrin expression. These changes in Itgα6 in endothelial cells and/or pericytes should be experimentally quantified and discussed in more detail.*

We agree with the reviewer that Itgα6 expression is increased in *Apcdd1*$^{-/-}$ retinal endothelial cells and astrocytes. Perhaps, this is a potential mechanism by which *Apcdd1*$^{-/-}$ retinal endothelial cells increase Occludin expression and localization to tight junctions (Mazzoni et al., 2017). However, a detailed analysis of the role of Itgα6 in endothelial cells is beyond the scope of this study. We acknowledge this aspect in the revised discussion (page 20, paragraph 1).

Additional major points:

8) What the study needs is some ultrastructural analyses (transmission EM) of the retinal neurovasculature with a particular focus on the vascular basement membranes as well as perivascular astrocyte endfeet in control and Apcdd1$^{-/-}$ *mice. Immuno-EM for Lama5 could also be included using control and Apcdd1*$^{-/-}$ *retinal or cerebellar samples.*

We have performed electron microscopy studies of blood vessels from P14 WT and *Apcdd1*$^{-/-}$ retinal superficial vascular plexuses to examine both the vBM thickness and how astrocyte endfeet cover blood vessels. We found that there is more extensive astrocyte endfeet coverage around *Apcdd1*$^{-/-}$ retinal blood vessels compared to the WT shown in a new figure (Fig. 7). In contrast, there was no obvious difference in vBM thickness between *Apcdd1*$^{-/-}$ and WT retinas, most likely due to a modest change in Lama2 deposition, which is one of many ECM proteins found in the vBM. We discuss these findings in the revised result section (page 14, paragraph 1). Lama5 is not changed in *Apcdd1*$^{-/-}$ compared to WT retinas.

9) Astrocytes from control and Apcdd1$^{-/-}$ *mice should be analyzed in ECM assays to determine if they have adhesion defects. Based on the Itgα6 data (Figure 7), Apcdd1*$^{-/-}$ *astrocytes should show enhanced ECM adhesion. Also, using control and Apcdd1*$^{-/-}$ *cultured astrocytes, the profile of cell surface heterodimer expression can be assessed by biotinylation-immunoprecipitation approaches which are commonly used.*

It is well established that astrocytic expression levels of Integrins depend upon the amount of ECM substrates (e.g. Laminins) that they are exposed to during development (Gnanaguru et al., 2013). Therefore, astrocytic integrin expression levels are not determined in a cell-autonomous manner. *Apcdd1*$^{-/-}$ retinal astrocytes express higher level of Itgα6 *in vivo* because they are exposed to increased Lama2 levels in the vBM. Thus, it is unlikely that *Apcdd1*$^{-/-}$ retinal astrocytes will have increased adhesion *in vitro* compared to WT astrocytes when both these cells are plated on the same amount of Lama2 protein. In that experiment the *Apcdd1*$^{-/-}$ retinal astrocytes will likely change their Itgα6 expression levels based on the amount of Lama2 protein that they are plated on a dish, which should be similar to the WT retinal astrocytes plated on the same amount of Lama2 protein.

A more biologically relevant experiment would be to examine whether Lama2-Itgα6 interactions are responsible for astrocyte maturation and polarization. We have inhibited Lama2-Itgα6 interaction in astrocytes cultured on Laminin-211 with an Itgα6 blocking antibody effectively and we now demonstrate that it reduces GFAP and Aqp-4 expression in cultured astrocytes (new Fig. 8R-V). These data strongly indicate that Lama2-Itgα6 interaction is responsible for astrocyte maturation and polarization. We discuss these findings in the revised result section (page 16, paragraph 1).

Minor comments:

9) In Figure 1 there are green speckles in the Lef1 immunofluorescence panels that distract from

the quality of the figure and should be corrected. Alternatively, new panels with images that lack the speckles should be added.

The levels of nuclear Lef1 expression is much lower in mural cells compared to endothelial cells. Therefore, we need to increase the gain during image acquisition to detect Lef1 expression in mural cells. Unfortunately, as it is the case with most immunofluorescence staining, there are some non-specific background fluorescence speckles in these images. However, the nuclear Lef1 staining is clearly recognizable, which is quantified in our study.

10) In Figure 2 data using a control RNA probe for the in-situ hybridization should be shown to control for antisense/sense specificity.

The specificity of the antisense mRNA probe against *Apcdd1* has been established previously (Mazzoni et al., 2017).

11) In Figure 4 it is confusing why the retinal vascular extravasation data in Figure 4G are shown after factoring in extravasation in the liver. An explanation for this strategy should be included, or the retina data should be shown alongside the liver data.

Liver does not have a functional blood-tissue barrier. Thus, tracer extravasation in the liver is a measure of the efficiency of the tracer injection in the blood stream, and serves as an internal control for the tracer extravasation in the retina with a functional BRB. Thus, the ratio of tracer extravasation in the retina over liver is a better quantification of functional BRB integrity than measuring the tracer extravasation just in the retina. We have used this method to assess changes in BBB permeability in the context of various neurological diseases (e.g. ischemic stroke, experimental autoimmune encephalomyelitis) in previous studies (Lengfeld et al., 2017; Lutz et al., 2017; Mazzoni et al., 2017; Platt et al., 2020). We have made this point clear in the method section of our revised manuscript (page 25, *in vivo* tracer injection).

12) In Figure 7 immunofluorescence for β1 integrin should be shown in control and Apcdd1$^{-/-}$ mice.

Based on our bulk RNA-seq data, the expression of β1-Integrin does not change in the *Apcdd1*$^{-/}$ retina. Therefore, the rationale for measuring β1-Integrin immunofluorescence intensity is unclear. The expression of α6-Integrin does change in the *Apcdd1*$^{-/-}$ retina, which provides a rationale to analyze the α6-Integrin levels by RNA-seq and immunofluorescence and perform functional studies *in vitro* with an Itgα6-blocking antibody.

13) In Supp. Figure 2 the Lama2 immunoreactive protein bands are not convincing. The immunoblots have been excessively cropped. In addition, laminins are very large glycosylated proteins and should not show distinct bands, but rather have a smeared appearance by immunoblotting.

We agree with the reviewer that Laminins are large glycosylated proteins and should show smeared appearance by Western blotting due to various levels of glycosylation. We now show all the glycosylated products for Lama2 and Lama1 proteins (Supplementary Fig. 3L). The approximate molecular weights of the highest glycosylation bands are shown in the figure.

14) In Supp. Figure 6, there should be better quality images shown for the cerebellar vasculature in control and mutant mice.

We now show all channels separately along with merged images in the cerebella of WT and *Apcdd1*$^{-/-}$ mice for Aqp-4 and Itgα6 staining to improve their visualization (Supplementary Fig. 7).

Reviewer #2
Major comments:
1) The author's show Pdgfrb^{+} and Foxc1^{+} pericytes in the postnatal retina and cerebellum have active Wnt signaling, the idea is that this is downstream of Norrin. To further support this, the authors should look at Lef1 or TCF-Lef1::GFP in mural cells in other brain regions where Norrin is not expressed. It is possible that mural cell Wnt/β-catenin activity is more widespread than

retina/cerebellum, which would be of interest as well.

We thank the reviewer for this important suggestion. We now show that Wnt/ß-catenin signaling is also active in mural cells in the cerebral cortex, where Wnt7a/b are the main Wnt ligands (Supplementary Fig. 2I-J'). These data suggest a broader role of mural cell Wnt/ß-catenin signaling in regulation of Lama2 expression throughout the CNS.

2) Fig. 3 – Analysis of Lama2 expression appears limited to artery and veins (both quantification and images) in Apcdd1-KO vs. control. However, ß-cat activity is also in mural cells associated w/ capillaries (likely based on pictures in Fig. 1). Is the increase in Lama2 seen in all retinal vasculature, or is this restricted to larger vessels? This is important information as the idea is that Lama2 is participating in promoting barrier integrity/vascular maturation throughout the vasculature. Showing that is the case would help support this model. If the increase in Lama2 is limited to the large blood vessels in the retina, this should be addressed.

We thank the reviewer for the suggestion. We now show that vBM deposition of Lama2 is not significantly different between WT and *Apcdd1*$^{-/-}$ retinal capillaries (Supplementary Fig. 3G-K). These data are consistent with our findings that the number of Lef1^{+} mural cells is significantly lower in retinal capillaries, compared to arteries and veins (Supplementary Fig. 1B-E). Therefore, Wnt/ß-catenin activity is lower in capillary pericytes compared to arterial or venous mural cells. Thus, the effect of Apcdd1 deletion (upregulation of Wnt/ß-catenin signaling) is more robust in arteries and veins, compared to capillaries.

3) Please provide quantification of the Occludin expression in Fig. 3H-K.

We now provide the quantification of Occludin expression in the WT and *Lama2*$^{-/-}$ retinal vessels (Figure. 4H-L) and show that Occludin localization is significantly decreased in *Lama2*$^{-/-}$ retinal endothelial cells.

4) The authors and others have previously shown that Norrin/ß-catenin signaling is increased in retinal endothelial cells in Apcdd1-KO. It would be important to look at Lef1 expression in Ng2 or Pdgfrb+ mural cells in Apcdd1-KO to test if it is also increased.

We thank the reviewer for the suggestion. We now show that the number of Lef1^{+} mural cells is significantly increased in the *Apcdd1*$^{-/-}$ retina (Supplementary Fig. 3A-C), supporting the upregulation of Wnt/ß-catenin activity in *Apcdd1*$^{-/-}$ retinal mural cells.

5) The studies here are over-activation of Norrin/ß-cat, in vivo with the Apcdd1-KO and the culture studies with CHIR. An outstanding question is whether Norrin/ß-cat in mural cells is required for Lama2 expression in the vasculature and astrocyte maturation. There are experimental approaches to address this, culture approaches and animal models to conditionally disrupt Norrin or its receptors in mural cells (or even Norrin-KO). However, I recognize that these are considerable experiments to undertake and would be satisfied for this limitation of the study to be addressed in the discussion.

We greatly appreciate the reviewer's consideration that some of these experiments may take a long time to complete within the revision timeframe. Since, these experiments are relevant for the story, we acknowledge the limitations of the current study and suggest future directions in the revised discussion of the manuscript (page 21, discussion section).

Minor comments:

1) Description of Figure 1T-X in 'results' – indicate that Pdgfrα and GFAP are labeling astrocytes.
We clarify this point in the revised manuscript.

2) For resubmission, please provide pg and ln numbers to aid in review.
We added the page and lane numbers in the manuscript and provide the page and paragraph numbers in the response to reviewers to facilitate the revisions.

3) Fig 2 – Gene labels in volcano plots are very small and hard to read, please enlarge.
We have now enlarged the gene labels in the volcano plots.

Reviewer #3

Major Comments:

1) Images in Fig2 are not informative. Please provide more resolutive images showing that mural cells express Apcdd1. Based on these images it is very difficult to state that Apcdd1 is expressed in mural cells. It would also be important to be able to localize vessels on these images. The Pdgfrb FISH shows a huge background.

We now added new images of FISH for *Apcdd1* mRNA long with immunostainings for Caveolin- 1 (endothelial cells), PdgfrB (mural cells) or GFAP (astrocytes) (Fig. 2A-C). The background for PdgfrB in these new images is significantly lower and the colocalization of *Apcdd1* mRNA with a subset of PdgfrB^{+} mural cells (solid arrowheads) is very clear.

2) In the Lama2-LacZ$^{+/-}$ mice, please explain why the labelling is patchy. Is it a nuclear labelling?

We agree with the reviewer that there is extravascular B-Gal expression in *Lama2$^{Lacz/+}$* reporter retinas. This extravascular B-Gal expression is most likely due to expression by neuronal cell bodies in the retina. This interpretation is supported by published P14 retinal single cell RNA-seq data showing that *Lama2* mRNA is expressed by horizontal cells, bipolar cells, amacrine cells as well as Müller glia (Macosko et al., 2015). However, Lama2 protein produced by these neuronal subtypes does not contribute to the vBM composition of the retinal superficial plexus. We now clarify this point in the result section of our revised manuscript (page 9, paragraph 1).

3) Lama2 is also expressed by perivascular fibroblasts and endothelial cells. Thus, it is not sure that upregulated laminin is provided by mural cells. Another more immediate accessible method would be to perform Lama2 FISH (with coFISH staining for each vascular cell type).

Please see the response to Reviewer #1, major comment #1.

3) It is striking that only some molecules of the astrocyte endfeet are upregulated. For instance, GlialCAM the partner of MLC1 is not upregulated. The same for Gjb6 encoding Cx30 the other gap junction protein of astrocyte perivascular processes with Cx43. It would mean that Lama2 influence only some markers and not the general maturation process of endfeet. Regarding NVU assembly, only some astrocytic markers are upregulated. I would rather write maturation of the NVU but not assembly.

We agree with the reviewer's comment. We have made the changes from "NVU assembly" to "NVU maturation" in the title of the manuscript and throughout the text.

4) Define Apcdd1$^{-/-}$ in the abstract and give its full name.

We now define Apcdd1 both in the abstract (page 2) and introduction (page 4, paragraph 2).

5) Gene names in Fig 2H-M are too small.

Please see the response to Reviewer #1, minor comment #3.

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Second decision letter

MS ID#: DEVELOP/2022/200610

MS TITLE: Mural Wnt/β-catenin signaling regulates Lama2 expression to promote neurovascular unit assembly.

AUTHORS: Saptarshi Biswas, Sanjid Shahriar, Nicholas P Giangreco, Panos Arvanitis, Markus Winkler, Nicholas P Tatonetti, William J Brunken, Tyler Cutforth, and Dritan Agalliu

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. The referees have a small number of suggestions to help improve the accessibility of your study. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

The study probes functional links between perivascular mural cells and endothelial cells during neurovascular development. The authors identify how the canonical Wnt signaling pathway promotes laminin (Lama2) deposition in the vascular basement membrane in control of BBB maturation and integrity. This adds new and important knowledge to the field of neurovascular biology.

Comments for the author

In this revised manuscript, Biswas et al. have shown that canonical Wnt signaling in mural cells (pericytes and astrocytes) within neurovascular units of the mouse retina is linked to Itga6-lama2 laminin expression to control endothelial barrier integrity. The authors were quite responsive to the extensive Reviewer critiques. New experimental data was added and portions of the manuscript text were clarified and elaborated. As a result, the revised manuscript is a significant improvement over the original submission.
The study will make an important contribution to the field of neurovascular biology. There are a couple of minor issues that should be addressed in a revision (without additional experimentation) that will make the paper more understandable for the general reader and strengthen the scientific conclusions, as detailed below:

Figure 4. The model in panel A is showing paracellular differences in BBB permeability between wild type control and Apcdd1-/- mice that have a “tighter” BBB. However, all the other data in the figure are focused on the BBB in Lama2-/- mice. This makes interpretation of data (connected to the model) somewhat confusing for the reader. The authors should add a panel in Fig. 4A to show that the Lama2-/- BBB is more permeable (paracellularly) in comparison to the control and Abcd1-/- BBB.

Figure 7. The TEM data are very nice and show increased astrocyte coverage of blood vessels in Apcdd1-/- mice, which is supportive of the IF data. The authors mention that the Apcdd1-/- vascular basement membrane has no obvious ultrastructural differences; however, what about the endothelial cell tight junctions. Are the tight junctions more abundant and/or ultrastructurally different in the Apcdd1-/- retinas?
This could be addressed in the text, but preferably arrows could be added to indicate tight junctions in the images. More preferably, higher magnification images could be added to highlight the tight junctions and paracellular flaps with any differences between control and knockouts.

Figure 8W. This model could be simplified. There are many molecules indicated with arrows that are shown but not clearly explained. There is no description of the model or its components in Figure legend 8. The Figure 8 legend ends at panel V and does not detail panel W.

Reviewer 2

Advance summary and potential significance to field

This manuscript by Biswas and colleagues identifies a previously unknown role of Norrin/Wnt/B-catenin signaling in CNS mural cells driving expression of mural cell ECM Lama2 and subsequent functional astrocyte/BRB maturation. The authors showed enhanced mural cell Lama2 in Apcdd1-KO animals that have higher Norrin/B-catenin signaling, correlating with increase astrocytic Aqp-4 and Integrin-a6. Using in vivo genetic models and culture experiments, the authors provide rigorous experimental support for their model. Work by others has shown Lama2 deletion leads to perturbed BBB/BRB integrity however regulation of Lama2 has not been previously investigated. This work showing that it is downstream of Wnt/Norrin/B-catenin in mural cells is novel. Further, it broadens the mechanism through which Wnt/Norrin/B-catenin regulate BBB beyond activity in the endothelium, which has been the primary focus of research up to this point. All of this is a new and interesting facet to how the Wnt/Norrin/B-catenin pathway regulates CNS vascular development and integrity, making it of high interest to the scientific community.

Comments for the author

I appreciate the authors addressing all my comments (major and minor) with new data and analysis. The one thing that I requested last time that was not addressed was to make images accessible to red-green color-blind readers, this can be done by selecting different color palettes for all fluorescence images – figures can be checked to accessibility on this website: https://www.color-blindness.com/coblis-color-blindness-simulator/. I recognize that this is not an trivial undertaking and that given the large number of experiments that were requested by reviewers in the first review, this was not a focus of revision efforts. I recommend that the authors discuss this with the Development editorial staff to determine if their figures adhere to publication

guidelines, which may include specific color combinations that are visible to all readers of this journal. Beyond this, I have no further critiques of this work.

Reviewer 3

Advance summary and potential significance to field

Authors answered to my comments adequatly.

Comments for the author

I have no other comments.

Second revision

Author response to reviewers' comments

Response to Reviewers:

We thank the reviewers for their feedback on the revised manuscript. We are gratified that the reviewers were satisfied with the revised manuscript and support the publication of the study in Development. Below, we provide a point-by-point response to reviewers' concerns about the revised manuscript.

Reviewer 1:
We thank the reviewer for finding our response to the initial comments "quite responsive to the extensive Reviewers critiques" and for acknowledging that 'the study will make an important contribution to the field of neurovascular biology". Below, we addressed the minor changes asked by the reviewer.

1. Figure 4. The model in panel A is showing paracellular differences in BBB permeability between wild type control and Apcdd1$^{-/-}$ mice that have a "tighter" BBB. However, all the other data in the figure are focused on the BBB in Lama2$^{-/-}$ mice. This makes the interpretation of data somewhat confusing for the reader.

We have now reformatted the figure and added the model at the end of the figure after the data (Fig. 4L), where we show side-by-side comparison of tight junction integrity and biocytin-TMR leakage between WT, *Apcdd1*$^{-/-}$ and *Lama2*$^{-/-}$ retinas. We have changed the text (lines 223-234) and figure legend (lines 917-925) accordingly to reflect the reorganization of the figure.

2. Figure 7. Are the tight junctions more abundant and/or ultrastructurally different in the Apcdd1$^{-/-}$ retinas? This could be addressed in the text, but preferably arrows could be added to indicate tight junctions in the images. More preferably, higher magnification images could be added to highlight the tight junctions.

We have revised Figure 7 and added higher magnification images of endothelial tight junctions in WT and *Apcdd1*$^{-/-}$ retinas (Fig. 7G, H). In these panels, we show that there is no obvious difference between WT and *Apcdd1*$^{-/-}$ tight junctions. We have changed the text (lines 311-312) and figure legend (Lines 957- 959) accordingly.

3. Figure 8W. This model could be simplified. There is no description of the model or its components in Figure legend 8.

We now mention in the figure legend (Fig. 8W; line 973-974) that the model by which Wnt/β-catenin signaling in mural cells regulates Lama2 deposition in the vBM and NVU maturation is described in detail in the Discussion (Lines 449-461).

5. The Figure 8 legend ends at panel V and does not detail panel W.

We have now corrected the mistake in the figure legend (Fig. 8W; Line 974-976).

Reviewer 2:
We thank the reviewer for appreciating our response to all the initial comments "with new data and analysis" and acknowledging that our study is "of high interest to the scientific community". Below, we addressed the final changes asked by Reviewer 2.

1. The one thing that I requested last time that was not addressed was to make images accessible to red-green color-blind readers. This can be done by selecting different color palettes for all fluorescence. I recognize that this is not a trivial undertaking and that given the large number of experiments that were requested by reviewers in the first review, this was not a focus of revision efforts. Beyond this, I have no further critiques of this work.

We discussed this issue with the editor of the journal and we were encouraged to change the color format in the main figures to make it accessible to red-green colorblind readers in response to Reviewer's 2 concern and the policy of the journal. We have now changed all the *in vivo* multichannel images in the main figures (Figs. 1, 2, 3, 4, 6 and 8) from red-green-blue to magenta-green-blue format. We hope these changes will be sufficient to comply with the accessibility to red-green colorblind readers.

Reviewer 3:
We thank the reviewer for appreciating that "authors answered to my comments adequately".

Third decision letter

MS ID#: DEVELOP/2022/200610

MS TITLE: Mural Wnt/β-catenin signaling regulates Lama2 expression to promote neurovascular unit assembly.

AUTHORS: Saptarshi Biswas, Sanjid Shahriar, Nicholas P Giangreco, Panos Arvanitis, Markus Winkler, Nicholas P Tatonetti, William J Brunken, Tyler Cutforth, and Dritan Agalliu
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.