Fgfbp1 promotes blood-brain barrier development by regulating collagen IV deposition and maintaining Wnt/β-catenin signaling

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DOI: 10.1242/dev.185140

Editor: James Briscoe

Review timeline

Original submission:	30 September 2019
Editorial decision:	6 December 2019
First revision received:	11 June 2020
Editorial decision:	10 July 2020
Second revision received:	17 July 2020
Accepted:	20 July 2020

Original submission

First decision letter

MS ID#: DEVELOP/2019/185140

MS TITLE: Fgfbp1 promotes blood-brain barrier development by regulating collagen IV deposition and maintaining Wnt/β-catenin signaling

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I apologise for the long time it took but 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. It seems to me there a three main issues that need to be tackled:

- Strengthening the evidence that loss of endothelial Fgfbp1 increases vasculature leakage and extending the analysis of the basement membrane composition in the mutants.

- Further characterising how Fgfbp1 is linked to Wnt signaling by strengthening the evidence of Wnt binding by Fgfbp1 and Wnt receptor engagement

- Finally, the referees also highlighted that improving the clarity of the data in Figure 3 would be helpful.

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy 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.

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

This paper describes a microarray analysis of mouse brain that identified Fgfbp1 as a previously undescribed Wnt/β-catenin-regulated gene involved in some properties of the neurovascular unit. The authors provide evidence for Fgfbp1 expression in the CNS endothelium and its secretion into the perivascular basement membrane during BBB formation. They also show that collagen IV deposition in the vascular basement membrane is reduced in mutant mice, leading to defective endothelial cell-pericyte interactions.

Comments for the author

Less convincing is their evidence that genetic ablation of endothelial Fgfbp1 results in increased leakage across the vasculature. As is often the case the reliance on immuno fluorescence microscopy makes it difficult to see the underlying morphology which makes the images difficult to interpret, For example in Figure 4F' it is not clear what the two arrows indicate or what structures the Alexa555-Cadaverine marker is associated with. If the marker has "leaked" out across the vessels it is not clear why the dye has this punctate appearance. Also Figure 4G suggests a 2-fold increase in Alexa555-Cadaverine deposits (or 3-fold for the highest single measurement in the Figure), which is much less than is apparent from a comparison of Figure 4D and 4F which are presumably examples of the images counted for Figure 4G.
In other frames in this Figure it is also unclear what the arrows point to in most cases. In Figure 4M one arrow (left) seems to indicate an intact vessel; the other (right) does not appear to be pointing at anything. The legend states that the arrows indicate foci of leakage that were counted, but what was counted is not clear.
The transmission electron microscope images in Figure 3 (C-H) are not of very good quality. The tight junction membrane leaflets are rather indistinct, but I agree that on the basis of these images the tight junctions appear similar in the WT and iEC-KO material. It is therefore puzzling that the authors' cartoon in Figure 8 shows Alexa555-Cadaverine marker passing through the tight junction of an iEC-KO mouse. To show this would require TEM of either photo conversion of the Alexa555-Cadaverine marker or use of a biotin labelled marker such as dextran of an appropriate molecular size, which can be visualized at the EM level. Such an EM study should be able to distinguish between transfer of the marker via tight junctions or across the endothelial cells, a rout that often seems not to be considered.

It is unhelpful that the Figures were not numbered.
It is inappropriate to calculate a p-value when n=2 (Figure 1C) although no doubt the computer programme used was probably able to do this.

Reviewer 2

Advance summary and potential significance to field

The pathways that promote the maturation of the BBB, and specifically roles for ECM proteins, remains largely uncharacterized. These studies will reveal fundamental mechanisms for BBB formation and physiology and may indicate one way the BBB is disrupted in various neurological diseases.

Comments for the author

In this manuscript, Cottarelli et al. have used in vitro and in vivo strategies to show that the ECM factor Fgfbp1 is regulated by Wnt/β-catenin signaling in the CNS endothelium. The authors convincingly show that Fgfbp1 is upregulated in mouse brain endothelial cells (mBECs) exposed to Wnt3a. They also validated these findings in vivo, using inducible endothelial-specific β-catenin gain-of-function (β-catenin iEC-GOF) mouse model to isolate brain and lung microvascular fragments. Fgfbp1 mRNA is significantly enriched in the brain, but not in lung microvessels isolated from β-catenin iEC-GOF as compared to wild type mice at P7 and the expression abates by P21 parallel with reduced Wnt/β-catenin signaling. Moreover they show that endothelial-specific ablation of Fgfbp1 caused subtle hypervascularization phenotypes during post-natal development of the brain and retina. The authors indicate that Fgfbp1 regulates BBB maturation by controlling deposition of Collagen IV to the vascular basement membrane, maintaining the association of ECs with pericytes, and clustering Wnt ligands on the endothelial cell surface. The experimental data are well-controlled and presented in a rational format. However, there are several major and minor issues that should be addressed.

Major issues:
1. The mechanism of action of Fgfbp1 as it relates to canonical Wnt signaling is not convincing and requires additional experimental validation and testing. For example, the authors show an Fgfbp1-dependent impact on collagen deposition in the ECM raising the possibility that the vascular basement membrane organization/composition is defective. This could lead to secondary defects in Fzd/b-cat signaling, rather than the hypothesized Wnt-receptor engagement functions by Fgfbp1. The authors should do a more thorough analysis of basement membrane composition, looking at additional ECM components such as fibronectins, vitronectin, latent-TGFbs etc. Careful ultrastructural analysis of the ECM may also be informative, as was shown with the endothelial tight junctions (Fig. 3).
2. The in vitro experiments summarized in Fig. 7 are complicated, with initial stimulation with Wnt3a followed by addition of Wnt1-HA in 293T conditioned media. Concerns about Wnt receptor occupancy and/or internalization and recycling in endothelial cells after repeated stimulation should be addressed. It is unclear about the rationale for use of Wnt3a versus Wnt1 and whether non-canonical pathways may be differentially regulated.
3. In Fig. 7 there may be Fgfbp1-dependent Wnt aggregation, but Wnt binding by Fgfbp1 and Wnt receptor engagement should be analyzed in more detail biochemically using co-immunoprecipitation and/or proximity ligation assays. In addition, immuno-EM showing Fgfbp1-dependent Wnt clustering in vivo would bolster the in vitro experiments. Also, impacts of Fgfb1 on FGFR signaling in endothelial cells should be investigated.
4. Roles for perivascular astrocytes (Fig. S2) should be more carefully analyzed using additional astrocytic markers (GLAST, GFAP, Aldh1l1, etc). Aquaporin 4 is also expressed in some vascular endothelial cells in the brain.
5. If Fgfbp1 is impacting the spatial activation of b-cat (as indicated in Fig. 7A-C) are those remaining b-gal+ signaling in the perivascular astrocyte nuclei. Also, on page 7 the authors mention that a subset of capillaries showed abnormal swelling of astrocyte end feet in Fgfbp1iEC-KO mice at P14. However, the authors did not mention if this abnormality still exist beyond P14?

Minor issues:
1. Reduced Fgfbp1 RNA expression is shown in sorted endothelial cells (Fig. 2), but protein expression should be addressed too. It looks like there is residual Fgfbp1 protein (Fig. 2D). Is this in the endothelium (incomplete loss of expression) or in other neurovascular cell types?
2. Latent-TGFb is a critical vascular basement membrane component that control developmental angiogenesis and formation of the BBB. These data should be discussed in the Introduction with relevant references included for TGFbR2/Alk5 signaling in endothelial cells and latent-TGFb activation via integrins in the perivascular astrocyte compartment.
3. In Fig. 7 A-B'' the DAPI signal is much stronger within iEC-KO images compared to WT images. Additionally, CD31 signal is much weaker in B' as compared to A'. This likely indicate that images were captured or manipulated via different settings. Additionally, it seems that iEC-KO images (B-B'') were captured with a higher magnification than WT images (A-A''). Furthermore, iEC-KO images have a smaller field of view compared to WT images. These issues should be corrected.

Reviewer 3

Advance summary and potential significance to field

In this manuscript Cottarelli et al., described the identification of a gene fgfbp1 which was not previously characterized within the vascular CNS. They found that fgfbp1 is up-regulated by wnt3a and its expression is conserved and enriched in brain blood vessels in vivo. The authors developed an inducible and endothelial-specific fgfbp1 KO, and found that loss of fgfbp1 increased brain vascular density and diminished pericytes coverage resulting in a regional- specific loss of collagen-based matrix deposition. The authors confirmed their findings in vitro and concluded that fgfbp1 is required to mediate the function on wnt3a-bcatenin by recruiting wnt protein to cell junctions.

Overall the manuscript is well written and shows a cohesive and interesting mechanism of regulation of wnt signaling. Besides suggesting experiments to strengthen some of the main conclusions, I consider this manuscript a good candidate for publication.

Comments for the author

In this manuscript Cottarelli et al., described the identification of a gene fgfbp1 which was not previously characterized within the vascular CNS. They found that fgfbp1 is up-regulated by wnt3a and its expression is conserved and enriched in brain blood vessels in vivo. The authors developed an inducible and endothelial-specific fgfbp1 KO, and found that loss of fgfbp1 increased brain vascular density and diminished pericytes coverage resulting in a regional-specific loss of collagen-based matrix deposition. The authors confirmed their findings in vitro and concluded that fgfbp1 is required to mediate the function on wnt3a-bcatenin by recruiting wnt protein to cell junctions.

Overall the manuscript is well written and shows a cohesive and interesting mechanism of regulation of wnt signaling. Besides suggesting experiments to strengthen some of the main conclusions, I consider this manuscript a good candidate for publication.

1) In the Gene Chip experiment as well as others , the authors used either wnt3a or wnt1. Since in the introduction the authors described that most of the effects in CNS and BBB formation are linked to Wnt 7a it is not clear to me why they used different wnts for their analysis. It would help to provide a rationale for the choice of Wnt as ligand and address the relevance of Wnt3a/wnt1 in the introduction.

2) For clarity, I would suggest providing the zoom out of the figures 1E-G, or possibly a different figure. For example, in figure 2C fgfbp1 expression is much clearer than in figure F1E.

3) I suggest moving main Figure 2F-H to supplementary since this is just a validation of the mice-creRE model already published. Instead, I would include the result of the fgfbp1 loss of function phenotype in the main figure. For example, the authors report an increase in vascular density and vascular branching points in the brain vessels cortex; is this correlated with more tip cell numbers in the vessels sprouting in the brain parenchyma? This analysis was provided in the retina vessels but I think that it more relevant in the endothelial cells of the brain.

4) In figure 3, the authors report a BBB phenotype in which no tight junction defect is observed, but rather a diminishment of astrocyte end feet. What do they mean by reporting that “a subset of capillaries showed abnormal swelling”? Is this sufficient to claim a defect in astrocyte end feet? Which subset of vessels have this phenotype? Is there a rationale of why only particular vessels have the phenotype? Could the author provide quantification of this phenotype? Furthermore, they found a mild but significant decrease in pericyte numbers in the fgfbp1 loss of function CNS vessels. It seems to me that this is important data for the authors working model. I would suggest moving this result to the main figure 3, rather than in supplementary figure 2A-G. Also, since the phenotype is not very obvious I suggest rewriting the conclusive statement “Fgfbp1 is essential to maintain the association between ECs and pericytes”.

5) I found figure 4 very convincing and I’m fascinated by the spatial selectivity in which fgfbp1

functions. What is the potential rationale for that? Is fgfbp1 expressed differentially between cortical vessels? Does wnt signaling require different regulation based on the spatial location of the CNS vessels?

6) It would be interesting to test if fgfbp1 affect ECM deposition/composition in vitro, and whether that effects the BBB properties of cells. For example, if the authors provide a particular matrix substrate to the mBEC in vitro can they rescue the BBB phenotype in absence of fgfbp1 expression? This would support that the effect of fgfbp1 on the ECM is the main cause of BBB defects in the fgfbp1 loss of function model.

7) The heat-map in figure 7E is difficult to interpret without the proper scale. Is 1 to 4 a log2FC? I suggest substituting this representation with the volcano plot as in figure 1A.

8) The working model showed a positive feedback loop between wnt/bcatenin and fgfbp1. Would it be possible to perform the experiments in figure 7J-Q using the recombinant protein of fgfbp1? The prediction is that, by increasing the expression of fgfbp1, Wnt – junctional localization and b-catenin signaling should also increase.

Minor comments:
I would suggest removing acronyms on the abstract to facilitate the reading for non-experts in the field: e.g. Plvap, mBEC , fgfpb1

First revision

Author response to reviewers' comments

MS ID#: DEVELOP/2019/185140
MS TITLE: Fgfbp1 promotes blood-brain barrier development by regulating collagen IV deposition and maintaining Wnt/β-catenin signaling

RESPONSE TO REVIEWERS:

We thank the reviewers for their insightful comments and feedback on the original manuscript. We are gratified that the reviewers were enthusiastic about the manuscript and found the results of our study very important. The reviewers' suggestions have been extremely helpful in revising our manuscript. We have therefore performed several additional experiments to address their concerns, and have modified several sections of the manuscript and figures (main and supplemental) accordingly. Below, we provide a point-by-point response to reviewers' concerns regarding the original manuscript:

REVIEWER #1

This paper describes a microarray analysis of mouse brain that identified Fgfbp1 as a previously undescribed Wnt/β-catenin-regulated gene involved in some properties of the neurovascular unit. The authors provide evidence for Fgfbp1 expression in the CNS endothelium and its secretion into the perivascular basement membrane during BBB formation. They also show that Collagen IV deposition in the vascular basement membrane is reduced in mutant mice, leading to defective endothelial cell-pericyte interactions.

1) Less convincing is their evidence that genetic ablation of endothelial Fgfbp1 results in increased leakage across the vasculature. As is often the case, the reliance on immunofluorescence microscopy makes it difficult to see the underlying morphology which makes the images difficult to interpret. For example, in Figure 4F' it is not clear what the two arrows indicate or what structures the Alexa555-Cadaverine marker is associated with. If the marker has "leaked" out across the vessels it is not clear why the dye has this punctate appearance.

The punctate appearance of the dye corresponds to the uptake by neurons. Cadaverin-Alexa555 has been shown to be taken up by neurons upon extravasation from the blood-brain barrier (BBB) [(Armulik et al., 2010) Supplementary Figure 7)].

2) Figure 4G suggests a 2-fold increase in Cadaverine- Alexa555 deposits (or 3-fold for the highest single measurement in the figure), which is much less than is apparent from a comparison of Figure 4D and 4F which are presumably examples of the images counted for Figure 4G.

Each dot shown in Figure 4G represents the average value of the fluorescence intensity of the tracer (Cadaverine-Alexa555) from 6-9 regions of interest (ROIs) acquired throughout the cortex in each mouse. These include both ROIs where the BBB leakage was minimal (i.e. low permeability, as in panel 4E) and those where BBB leakage was very high (i.e. high permeability, as in panel 4F, F'). This makes the average fluorescence intensity of the tracer smaller than the apparent leakage shown in the panel 4F, F' for cortical regions with high BBB permeability.

To better clarify this point for the reviewer, we provide here an additional graph in which each bar represents a single mouse and each dot represents Cadaverine-Alexa555 fluorescence intensity for each ROI in that specific mouse. The dotted line represents the average fluorescence intensity for all wild-type (WT) mice. Please note that, in each Fgfbp1iEC-KO (iEC-KO) mouse, some ROIs are plotting around the "average of WT" line (i.e. low permeability regions), while other ROIs have a much higher fluorescence intensity of the tracer (i.e. high permeability regions). To make the interpretation of Figure 4G easier, we have modified the graph, figure legend and main text to clarify that the quantification of Cadaverine-Alexa555 leakage is expressed as fold change in average fluorescence intensity of the tracer compared to WT.

3) In other frames in this Figure it is also unclear what the arrows point to in most cases. In Figure 4M one arrow (left) seems to indicate an intact vessel; the other (right) does not appear to be pointing at anything. The legend states that the arrows indicate foci of leakage that were counted, but what was counted is not clear.

We apologize for the confusion. Following the reviewer's suggestion, we took out the arrows and drew dotted lines around the foci of BBB leakage. Also, the resolution of the images has been improved to display the leakage more clearly (See Figure 4J' J"' and 4M' M' ").

4) The transmission electron microscope images in Figure 3 (C-H) are not of very good quality.

We have improved the quality of TEM images shown in the revised Figure 3E-J.

5) The tight junction membrane leaflets are rather indistinct, but I agree that on the basis of these images the tight junctions appear similar in the WT and iEC-KO material. It is therefore puzzling that the authors' cartoon in Figure 8 shows Alexa555-Cadaverine marker passing through the tight junction of an iEC-KO mouse.

Previous studies have shown that Claudin-5 deletion increases paracellular permeability of CNS endothelial cells without affecting the morphology of tight junctions (TJs) by TEM (Nitta et al., 2003). Therefore, even though we could not detect any difference in tight junction appearance between WT and Fgfbp1iEC-KO animals by immunofluorescence or TEM (Fig. 3A-J), we depicted the dye as passing through cell-cell junctions to represent the increase in paracellular BBB permeability that we observe in mutant mice since we could not find any evidence for an increase in the number of caveolae indicative of increased transcellular BBB permeability.

6) To show this would require TEM of either photo conversion of the Cadaverine-Alexa555 marker or use of a biotin labelled marker such as dextran of an appropriate molecular size, which can be visualized at the EM level. Such an EM study should be able to distinguish between transfer of the marker via tight junctions or across the endothelial cells, a route that often seems not to be considered.

We have considered whether the tracer could be transported via caveolae and have found no evidence of increased transcellular transport in Fgfbp1iEC-KO mice by TEM since there were very

few caveolae in CNS endothelial cells of both genotypes. We have modified the text to clarify this point.

7) It is unhelpful that the Figures were not numbered.

We have numbered the figures in this resubmission to facilitate the review process.

8) It is inappropriate to calculate a p-value when n=2 (Figure 1C) although no doubt the computer program used was probably able to do this.

We agree with the reviewer. We were able to purify micro-vessels from 4 additional wild-type brains to compare the relative expression of Fgfbp1 mRNA between the wild-type and Fgfbp1iEC-KO mice in Figure 1C. We have found that this is significant (Figure 1C * $p<0.05$). However, due to the shutdown of our laboratories from the severe COVID-19 pandemic, we were unable to purify additional micro-vessels from lungs of wild-type mice.

REVIEWER #2
Major issues:

1) The mechanism of action of Fgfbp1 as it relates to canonical Wnt signaling is not convincing and requires additional experimental validation and testing. For example, the authors show an Fgfbp1-dependent impact on collagen deposition in the ECM raising the possibility that the vascular basement membrane organization/composition is defective. This could lead to secondary defects in Fzd/b-cat signaling, rather than the hypothesized Wnt-receptor engagement functions by Fgfbp1. The authors should do a more thorough analysis of basement membrane composition, looking at additional ECM components such as fibronectins, vitronectin, latent-TGFbs etc. Careful ultrastructural analysis of the ECM may also be informative, as was shown with the endothelial tight junctions (Fig. 3).

We thank the reviewer for these comments. Following reviewer's suggestions, we have now performed a more extensive analysis of the composition of the vascular basement membrane. We have analyzed expression of Fibronectin and Perlecan in the basement membrane of cortical blood vessels from wild-type and Fgfbp1iEC-KO mice at P7, P14 and P21 in addition to the previous analysis of Collagen IV and Laminins (a2, a4 and a5). We have found that the fraction of blood vessels that have either Fibronectin or Perlecan deposition in the basement membrane is increased in Fgfbp1iEC-KO compared to wild-type mice at P7 to potentially compensate for the loss of Collagen IV. However, this phenotype is transient since by P14 and P21 there is no difference in Perlecan vessel coverage by immunofluorescence analysis between two genotypes, whereas Fibronectin vessel coverage is reduced in Fgfbp1iEC-KO compared to wild-type mice by P21. These data are now shown in a new supplementary figure (Figure S4). We have not found any evidence for changes in the thickness of the basement membrane by TEM in Fgfbp1iEC-KO mice and therefore we briefly mention this point in the Results section of the manuscript. Finally, although the analysis of latent TGFbs could be important, we think that this is beyond the scope of our manuscript.

2) The in vitro experiments summarized in Fig. 7 are complicated, with initial stimulation with Wnt3a followed by addition of Wnt1-HA in 293T conditioned media. Concerns about Wnt receptor occupancy and/or internalization and recycling in endothelial cells after repeated stimulation should be addressed. It is unclear about the rationale for use of Wnt3a versus Wnt1 and whether non-canonical pathways may be differentially regulated.

We have now clarified in the Results section and figure legend that the reason for the initial stimulation of primary mouse brain endothelial cells with Wnt3a is to induce expression of Fgfbp1 mRNA, that would otherwise be very low (See Figure 1B). We have used a distinct Wnt ligand tagged with HA (Wnt1-HA) to separate the effects of untagged versus tagged Wnts on binding to the cell membrane. Both Wnt1 and Wnt3a are known to activate strongly the Wnt/b-catenin pathway in multiple cell types. Moreover, Wnt1 and Wnt3a are expressed by neuroglial progenitor cells in the developing CNS, albeit primarily in the dorsal hindbrain and spinal cord. To address the concern of the reviewer for adequate activation of the pathway, we have analyzed the levels of phosphorylated versus total LRP6 in primary mouse brain endothelial cells under stimulation

with Wnt3a, Wnt1-HA or both Wnt3a and Wnt1-HA in control and FGFBP1-knockdown cells. We found that Wnt3a, Wnt1-HA and the combination (Wnt3a and Wnt1-HA) strongly phosphorylate LRP-6 in wild-type primary mouse brain endothelial cells, indicative of pathway activation. This effect is completely abolished in FGFBP1-knockdown primary mouse brain endothelial cells strengthening our argument that FGFBP1 is required for proper activation of the pathway by phosphorylation of LRP6 receptors. These data are now shown in the revised Figure S5D.

3) In Fig. 7, there may be Fgfbp1-dependent Wnt aggregation. Wnt binding by Fgfbp1 and Wnt receptor engagement should be analyzed in more detail biochemically using co-immunoprecipitation and/or proximity ligation assays. In addition, immuno-EM showing Fgfbp1-dependent Wnt clustering in vivo would bolster the in vitro experiments. Also, impacts of Fgfb1 on FGFR signaling in endothelial cells should be investigated.

We were unable to perform co-immunoprecipitation or proximity ligation assays for Wnt receptors and FGFBP1 because of the lack of good and reliable antibodies required for these assays. Although the FGFBP1 antibody works for immunofluorescence and Western blotting studies, it has high background. Moreover, the antibodies for several Wnt ligands and Fzd receptors are notoriously non-specific, poor and unreliable. The same concerns apply for the immune-EM studies that require very good antibodies in order to be successful.

We have now performed Western blotting for total and phosphorylated FGFR1 and total and phosphorylated ERK (MAPK) in primary control of FGFBP1-knockdown primary mouse brain endothelial cells in the absence or presence of FGF-2 (10 ng/mL). We have found that phosphorylation of both FGFR1 and ERK is increased after exposure to FGF-2 in both control and FGFBP1 knockdown-primary mouse brain endothelial cells. Therefore, FGFBP1 does not affect the FGF-2/FGFR1 signaling in primary mouse brain endothelial cells. These new data are now shown in Figure S5C.

4) Roles for perivascular astrocytes (Fig. S2) should be more carefully analyzed using additional astrocytic markers (GLAST, GFAP, Aldh1l1, etc). Aquaporin 4 is also expressed in some vascular endothelial cells in the brain.

We have analyzed expression of GFAP in wild-type and Fgfbp1iEC-KO mice at P14 and have found no difference between the two genotypes similar to Aqp4. These data are now shown in Figure S2 H-M.
We politely disagree with the reviewer's comment that Aquaporin-4 is also expressed in some vascular endothelial cells. Several bulk and single RNA seq studies (Cahoy et al., 2008; Vanlandewijck et al., 2018) have shown that Aqp4 mRNA is exclusively expressed by astrocytes in the mouse brain. The graph above obtained from the single cell brain RNA seq database from the Betsholtz group (http://betsholtzlab.org/VascularSingleCells/database.html) clearly illustrates this point.

5) If Fgfbp1 is impacting the spatial activation of b-cat (as indicated in Fig. 7A-C) are those remaining b-gal+ signaling in the perivascular astrocyte nuclei. Also, on page 7 the authors mention that a subset of capillaries showed abnormal swelling of astrocyte end feet in Fgfbp1iEC-KO mice at P14. However, the authors did not mention if this abnormality still exist beyond P14?

The quantification in Figure 7G refers only to b-gal (b-galactosidase) signals in brain ECs. The remaining b-gal positive nuclei in 7B are probably neurons or astrocytes.

We have observed some abnormal swelling of astrocyte endfeet in Fgfbp1iEC-KO mice compared to wild-type mice by P14 with TEM. These are illustrated in Figure 3H, I, J (red asterick). However, we did not find any abnormalities in either GFAP or AqP4 expression in Fgfbp1iEC-KO mice (Figure S2).

Minor issues:
1) Reduced Fgfbp1 RNA expression is shown in sorted endothelial cells (Fig. 2), but protein expression should be addressed too. It looks like there is residual Fgfbp1 protein (Fig. 2D). Is this in the endothelium (incomplete loss of expression) or in other neurovascular cell types?

The residual Fgfbp1 protein present in Fgfbp1iEC-KO brains (Figure 2D) results from expression and secretion from other cells types such as neurons or astrocytes. We were unable to perform a Western blotting for FGFBP1 in CD31+ vs CD31- cells from wild-type and Fgfbp1iEC-KO mice at P7 due to the lack of additional animals and closure of research laboratories from the COVID-19 pandemic over the past three months.

2) Latent-TGFb is a critical vascular basement membrane component that controls developmental angiogenesis and formation of the BBB. These data should be discussed in the Introduction with relevant references included for TGFbR2/Alk5 signaling in endothelial cells and latent-TGFb activation via integrins in the perivascular astrocyte compartment.

We have included a brief description about the role of latent-TGFb signaling as a critical vascular basement membrane component that regulates both CNS angiogenesis and BBB formation in the revised Introduction. However, we feel that analysis of latent TGFb signaling is beyond the scope of this study.

3) In Fig. 7 A-B'' the DAPI signal is much stronger within iEC-KO images compared to WT images. Additionally, CD31 signal is much weaker in B' as compared to A'. This likely indicate that images were captured or manipulated via different settings. Additionally, it seems that iEC-KO images (B-B'') were captured with a higher magnification than WT images (A-A''). Furthermore, iEC-KO images have a smaller field of view compared to WT images. These issues should be corrected.

We have corrected the images in the revised Figure 7A-B" as suggested by the reviewer. Both images are captured with the same field of view and magnification.

REVIEWER #3

1) In the Gene Chip experiment as well as other experiments, the authors used either Wnt3a or Wnt1. Since in the Introduction the authors described that most of the effects in CNS and BBB formation are linked to Wnt7a it is not clear to me why they used different Wnts for their analysis. It would help to provide a rationale for the choice of Wnt as ligand and address the relevance of Wnt3a/Wnt1 in the Introduction.

Both Wnt1 and Wnt3a are known to activate strongly the Wnt/β-catenin pathway in multiple cell types. We have previously shown in multiple studies that Wnt3a activates the canonical Wnt signaling in mouse or rat brain endothelial cells (Liebner et al., 2008; Mazzoni et al., 2017; Paolinelli et al., 2013). Moreover, Wnt1 and Wnt3a are expressed by neuroglial progenitor cells in the developing CNS, albeit primarily in the dorsal forebrain, hindbrain and spinal cord. Therefore, these Wnt ligands are relevant for the current study since Fgfbp1 is expressed throughout the CNS blood vessels where Wnt/b-catenin signaling is active. In addition, the secretion of Wnt7a-HA tag protein from the HEK-293 cells was very poor to perform the in vitro experiments described in Figure 7.

2) For clarity, I would suggest providing the zoom out of the figures 1E-G, or possibly a different figure. For example, in figure 2C fgfbp1 expression is much clearer than in figure F1E.

We now provide new lower and higher magnification images showing expression of Fgfbp1 protein in brain endothelial cells (these vessels are located in the cortex) and very low expression in liver endothelial cells at P7 in the revised Figure 1E-G.

3) I suggest moving main Figure 2F-H to supplementary since this is just a validation of the mice. VE-cadherin-CreERT2 model has already been published.

Based on the suggestion from the reviewer, we have removed these data from the paper since they have been published before in multiple studies.

4) Instead, I would include the result of the Fgfbp1 loss of function phenotype in the main figure. For example, the authors report an increase in vascular density and vascular branching points in the brain vessels cortex. Is this correlated with more tip cell numbers in the vessels sprouting in the brain parenchyma? This analysis was provided in the retina vessels but I think that it more

relevant in the endothelial cells of the brain.

To address the concern of the reviewer, we performed immunofluorescence staining for the tip cell markers (e.g. Dll4) as well as Glut-1 [Glut-1 has been reported to label tip cells in the brain (Tang et al., 2017)] in P7, P14 and P21 wild-type and Fgfbp1iEC-KO brains to determine if we could visualize tip cells. However, in our experience, tip cells were notoriously difficult to visualize/quantify in the brain with either Dll4 or Glut-1 immunofluorescence. Therefore, we could not address this issue. The tip cells are easier to analyze in the retina, which is the reason that we performed the analysis in the retina as shown in Figure S1. However, we have decided to keep the retina angiogenesis data as a supplemental figure since the main body of the work is focused on the brain (CNS angiogenesis and blood-brain barrier).

5) In Figure 3, the authors report a BBB phenotype in which no tight junction defect is observed, but rather a diminishment of astrocyte endfeet. What do they mean by reporting that “a subset of capillaries showed abnormal swelling”? Is this sufficient to claim a defect in astrocyte endfeet? Which subset of vessels have this phenotype? Is there a rationale of why only particular vessels have the phenotype? Could the author provide quantification of this phenotype?

We have observed some abnormal swelling of astrocyte endfeet in Fgfbp1iEC-KO mice compared to wild-type mice at P14 with TEM. These are illustrated in Figure 3H, I. However, they are not present in all vessels. Since the BBB phenotype is not uniform throughout the whole cortex in Fgfbp1iEC-KO mice (there are regions of low and high BBB permeability), the abnormal swelling of astrocyte endfeet is very difficult to quantify from the TEM studies and correlate it with BBB leakage. In addition, we did not find any abnormalities in either GFAP or Aqp4 expression as well as Aqp4 vascular coverage in Fgfbp1iEC-KO compared to wild-type mice at P14. We believe that this swelling may correspond to regions of the vasculature where Collagen IV is not deposited in the basement membrane (See Figure 4H”-J’) and Wnt/b-catenin activation is lower. However, this is difficult to ascertain given that the phenotype (BBB leakage and abnormalities in Collagen IV deposition) is not uniform throughout the cortex.

6) Furthermore, they found a mild but significant decrease in pericyte numbers in the Fgfbp1 loss of function CNS vessels. It seems to me that this is important data for the authors working model. I would suggest moving this result to the main figure 3, rather than in supplementary figure 2A-G. Also, since the phenotype is not very obvious I suggest rewriting the conclusive statement “Fgfbp1 is essential to maintain the association between ECs and pericytes”.

Based on the suggestion from the reviewer, we have moved the pericyte data in the main Figure 3K-Q.

7) I found Figure 4 very convincing and I’m fascinated by the spatial selectivity in which Fgfbp1 functions. What is the potential rationale for that? Is Fgfbp1 expressed differentially between cortical vessels? Does Wnt signaling require different regulation based on the spatial location of the CNS vessels?

We thank the reviewer for this comment. Although we do not have a clear answer for the phenotype observed in Fgfbp1iEC-KO mice, we have found that Fgfbp1 protein is expressed at higher levels in some cortical blood vessels that others at P7, consistent with variable activation of Wnt/b-catenin signaling in these cells (the pathway is not activated at the same time in ALL brain endothelial cells). Moreover, during the early phase of postnatal development, there is increased endothelial cell proliferation and sprouting from P5 - P10, followed by a gradual decline and quiescence by P25 when endothelial cell proliferation ceases in the cortex (Harb et al., 2013). This second postnatal wave of angiogenesis (P5-P10) in the developing cortex may be driven by activity-dependent mechanisms (neuronal activity), which have been shown to play a role in refining the CNS vasculature to match the metabolic demands of neurons (Lacoste et al., 2014). Therefore, we postulate that the cortical regions with higher BBB permeability may in fact correspond to angiogenic vessels that express higher levels of Fgfbp1, which fail to form a mature BBB during the second wave of postnatal angiogenesis. We discuss this issue in more detail in the revised Discussion.

8) It would be interesting to test if Fgfbp1 affect ECM deposition/composition in vitro, and

whether that affects the BBB properties of cells. For example, if the authors provide a particular matrix substrate to the mBEC in vitro can they rescue the BBB phenotype in the absence of Fgfbp1 expression? This would support that the effect of Fgfbp1 on Collagen IV is the main cause of BBB defects in the Fgfbp1 loss of function model.

We have performed two experiments to address this point. First, we have analyzed expression of Fibronectin and Perlecan in wild-type and Fgfbp1iEC-KO mice at P7, P14 and P21 in addition to Collagen IV and Laminins (2, 4 and 5). We have found that the fraction of blood vessels that have either Fibronectin or Perlecan deposition in the basement membrane is increased in Fgfbp1iEC-KO compared to wild-type mice at P7 to potentially compensate for the loss of Collagen IV. However, this phenotype is transient since by P14 and P21 there is no difference in Perlecan vessel coverage by immunofluorescence analysis between two genotypes, whereas Fibronectin vessel coverage is reduced in Fgfbp1iEC-KO compared to wild-type mice by P21.

Second, we analyzed if addition of Wnt3a to primary mouse brain endothelial cells, cultured in Fibronectin rather than Collagen IV substrate, was sufficient to increase transendothelial electrical resistance (TEER), a readout of tight junction function, and reduce transcytosis (uptake of albumin-Alexa594) in control or FGFBP1-knockdown cells. We found that Wnt3a was not able to increase TEER (Response Fig. 4A, B) and reduce the uptake of albumin-Alexa594 (Response Fig. 4C) in either wild-type or FGFBP1-knockdown cells. These findings contrast with the data shown in Figure 6 where primary mouse brain endothelial cells (control or FGFBP1-knockdown cells) cultured in plates coated with Collagen IV can respond to Wnt3a and the response is reduced by elimination of FGFBP1 protein. These data demonstrate that the basement membrane composition plays a critical role in the response of primary mouse brain endothelial cells to Wnt3a ligand and the effects that FGFBP1 has on Wnt/b-catenin signaling activation in these cells. Since the data with the Fibronectin substrate were negative (there was no Wnt effect), we have decided to leave them outside the revised manuscript. We could not test other substrates (e.g. Perlecan) since our laboratories were shut down due to the severe COVID-19 pandemic.

9) The heat-map in Figure 7E is difficult to interpret without the proper scale. Is 1 to 4 a log2FC? I suggest substituting this representation with the volcano plot as in Figure 1A.

We have modified the panel (Figure 7E) to include the scale. The heatmap is intended to show that brain ECs have different responses to Wnt/b-catenin activation depending on the presence or absence of FGFBP1. This will be very difficult to show with a volcano plot.

10) The working model showed a positive feedback loop between Wnt/b-catenin and Fgfbp1. Would it be possible to perform the experiments in Figure 7J-Q using the recombinant protein of Fgfbp1? The prediction is that, by increasing the expression of Fgfbp1, Wnt – junctional localization and b-catenin signaling should also increase.

We were not able to generate a recombinant Fgfbp1 protein in order to perform the experiments requested by the reviewer. However, to address the concerns of the reviewer, we have analyzed the levels of phosphorylated versus total LRP6 in primary mouse brain endothelial cells under stimulation with Wnt3a, Wnt1-HA or both Wnt3a and Wnt1-HA in control and FGFBP1-knockdown primary mouse brain endothelial cells. We found that either Wnt3a, Wnt1-HA or a combination (Wnt3a and Wnt1-HA) strongly phosphorylates LRP-6 in wild type primary mouse brain endothelial cells, indicative of pathway activation. This effect is completely abolished in FGFBP1-knockdown primary mouse brain endothelial cells strengthening our argument that FGFBP1 is required for proper activation of the pathway by phosphorylation of LRP6 receptors. These data are now shown in the revised Figure S5D.

Minor comments:
1) I would suggest removing acronyms on the abstract to facilitate the reading for non-experts in the field: e.g. Plvap, mBEC , Fgfpb1.

This is difficult to accomplish since there is a word limit for the abstract.

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Second decision letter

MS ID#: DEVELOP/2019/185140

MS TITLE: Fgfbp1 promotes blood-brain barrier development by regulating collagen IV deposition and maintaining Wnt/Î²-catenin signaling

AUTHORS: Azzurra Cottarelli, Monica Corrada, Galina V Beznoussenko, Alexander A. Mironov, Maria A. Globisch, Saptarshi Biswas, Hua Huang, Anna Dimberg, Peetra U Magnusson, Dritan Agalliu, Maria Grazia Lampugnani, and Elisabetta Dejana

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. The referees agree that the additional data and revisions you have made strengthen your manuscript. Referee 1 continues to have some concerns about the possibility of transendothelial or other routes of entry to explain the phenotype. It would seem appropriate to acknowledge and address these caveats in the Discussion.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.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 1

Advance summary and potential significance to field

I have already commented on this in my original review and have nothing to add.
Significance is in the eye of the beholder and I do not thinks is a relevant factor in a decision to publish a manuscript.

Comments for the author

I thank the authors for their detailed responses to comments in my review, in particular for clarifying the nature of the fluorescent staining that is interpreted as evidence that the blood-brain barrier "leaked" Alexa555-Cadaverine marker in the brains of animals in which endothelial Fgfbp1 had been deleted. The identification of the marker in neurons does not establish the route by which the marker reached that site. Have the authors considered the possibility that the marker was taken up by the choroid plexuses, transferred into CSF and then taken up by neurons.
The quantification of marker localized in neurons establishes that more marker reached neurons in the cortex. It does not define the route.

The quality of the electron micrographs is improved and confirms the point made by the authors about the integrity of the tight junctions.

The paper of Nitta et al (2003) is widely cited as evidence that small molecular weight markers can pass through the tight junction paracellular pathway. However, in what is otherwise an excellent paper the authors missed one essential examination which was to do ultrastructural studies of material containing the low molecular markers. Without such evidence the possibility of transendothelial transfer of marker cannot be excluded. Indeed, I think this is a plausible explanation of the findings of Nitta et al. Their finding of "leakage" of Evans blue perhaps supports this suggestion. Evans blue is widely (but incorrectly) believed to bind exclusively to plasma albumin so the dye is held to be a marker for albumin. If albumin has crossed the vessel walls in Nitta et al.'s experiments where the tight junctions appear intact then a dye labelled protein has clearly not entered the brain via the tight junctions.

The clarification that there were very few caveolae in CNS endothelial cells of both Genotypes is evidence that in these experiments transendothelial transfer is unlikely, but a definitive conclusion would require the observation to be made in the presence of the Alexa555-Cadaverine marker (and preferably its visualisation at the EM level) since the marker could be inducing transcellular transfer.

I am relieved to hear that the authors agree that n=2 is an inappropriate basis for calculating a p value.

In summary, I am not convinced that the authors have demonstrated blood-brain barrier leakage via the paracellular pathway in the brains of animals in which endothelial Fgfbp1 had been deleted. They have not excluded the possibility of transendothelial transfer of the marker, for reasons outlined above. They also have not considered other routes of entry.
For these reasons I think it is not appropriate to include in their Figure 8 cartoon a depiction of the cadaverine crossing via the tight junctions. They have no evidence for this.

Particularly given the difficulties caused by the coronavirus epidemic for every aspect of life including continuing lab work I do not think t would be reasonable to ask for more experiments to be done to clarify the above concerns. However, I suggest that the authors should be more circumspect in their claim that the only explanation for their findings with Alexa555-Cadaverine is that there was blood-brain barrier “leakage”. As outlined above there are at least two other explanations for their findings.

Reviewer 2

Advance summary and potential significance to field

The data in this paper identifies a new signaling component (Fgfbp1) in regulation of neurovascular unit/blood-brain barrier integrity by linking proteins in the extracellular matrix to canonical Wnt signaling.

Comments for the author

The authors have submitted a thorough, measured and very clear rebuttal to the extensive critiques from the three Reviewers. New experimental data have been added and clarifications to the text have been made. I recommend publication without any additional revisions.

Reviewer 3

Advance summary and potential significance to field

This manuscript provides an insightful understanding of how the matrix protein Fgfbp1 controls Wnt/β-catenin-regulated gene in mouse brain endothelial cells.
Discovering the mechanism of Fgfbp1 will help elucidate how maturation of blood brain barrier properties in vivo is mediated by cell-cell interactions and Wnt/β-catenin activity.

Comments for the author

I have no further comments and I'm satisfied with the author additional experiments.

Second revision

Author response to reviewers' comments

MS ID#: DEVELOP/2019/185140

MS TITLE: Fgfbp1 promotes blood-brain barrier development by regulating collagen IV deposition and maintaining Wnt/β-catenin signaling

RESPONSE TO REVIEWERS:

We thank the reviewers for their feedback on the revised manuscript. We are gratified that Reviewers # 2 and # 3 were satisfied with our revised manuscript and have endorsed the publication of the study in Development. We are pleased that Reviewer # 1 was also satisfied with the majority of the experiments that we performed for the revisions of the manuscript. However, Reviewer # 1 is still not convinced that “we have demonstrated blood-brain barrier leakage via the paracellular pathway in the brains of animals in which endothelial Fgfbp1 had been deleted” since in his/her opinion “we have not excluded the possibility of trans-endothelial transfer of the marker”. Below, we provide a point-by-point response to this reviewer’s concerns regarding the revised manuscript.

REVIEWER # 1

1. I thank the authors for their detailed responses to comments in my review, in particular for clarifying the nature of the fluorescent staining that is interpreted as evidence that the blood-brain barrier "leaked" Alexa555-Cadaverine marker in the brains of animals in which endothelial Fgfbp1 had been deleted. The identification of the marker in neurons does not establish the route by which the marker reached that site.
Have the authors considered the possibility that the marker was taken up by the choroid plexuses, transferred into CSF and then taken up by neurons? The quantification of marker localized in neurons establishes that more marker reached neurons in the cortex. It does not define the route.

We believe that it is highly unlikely that "the marker was taken up by the choroid plexuses, transferred into CSF and then taken up by neurons" in Fgfbp1ECKO mice for three reasons. 1) The choroid plexi have a leaky vasculature and they are filled with tracer both in wild-type and Fgfbp1ECKO mice; however, we do not see a lot of tracer accumulation in the brains of wild-type mice, in particular by P14 and P21 (There is some tracer accumulation in P7 wild-type brains as the blood-brain barrier has not fully matured by this developmental stage; please see Figure 1 below). Therefore, we predict that the tracer is present in the CSF of both genotypes but we see more tracer uptake by cortical neurons in Fgfbp1ECKO mice. 2) We provide in Figure 1 below the fluorescence images of the tracer distribution in the cortex of wild-type and Fgfbp1ECKO P7 mice. The tracer leakage is higher in Fgfbp1ECKO cortexes; however, the blood-brain barrier leakage appears patchy in the upper layers of the cerebral cortex away from the ventricular zone (see white arrows in the figure). If the marker was taken up by neurons via the CSF, we would have anticipated to see that all neurons close to the ventricles and throughout the cortex to be filled with the marker. However, this is not the case since we see neurons in the upper layers of the cortex filled with tracer whereas neurons in the lower layers of the cortex are devoid of the tracer. In addition, the diffusion of the tracer from the ventricles to the brain parenchyma cannot explain the patchy distribution of the tracer in the cortex in particular in layers 2-4 (white arrows). 3) The CNS parenchyma provides a physical barrier to free diffusion of molecules. Thus, it would be impossible that neurons located in the upper layers of the cortex receive the tracer from the ventricular zone, whereas the neurons in the deeper layers do not receive the tracer. Please keep in mind that the tracer was administered only 2 hours prior to perfusion of mice. This time is not sufficient for re-distribution of the tracer. For these reasons, we do NOT think that this route of tracer diffusion in the brain can explain the increased presence of Cadaverin-Alexa555 in Fgfbp1ECKO mutant mice.

2. The quality of the electron micrographs is improved and confirms the point made by the authors about the integrity of tight junctions.

We are pleased that the reviewer is satisfied with the quality of transmission electron microscopy (TEM) images in the revised Figure 3.

3. The paper of Nitta et. al. (2003) is widely cited as evidence that small molecular weight markers can pass through the tight junction via the paracellular pathway. However, in what is otherwise an excellent paper the authors missed one essential examination which has to do with ultrastructural studies of material containing the low molecular weight markers. Without such evidence, the possibility of trans-endothelial transfer of marker cannot be excluded. Indeed, I think this is a plausible explanation of the findings of Nitta et al. (2003). Their finding of "leakage" of Evans blue perhaps supports this suggestion. Evans blue is widely (but incorrectly) believed to bind exclusively to plasma albumin so the dye is held to be a marker for albumin. If albumin has crossed the vessel walls in Nitta et., al., (2003) experiments where the tight junctions appear intact then a dye labelled protein has clearly not entered the brain via the tight junctions.

We agree with the reviewer's comment that the study by Nitta et. al., (2003) did not examine in detail whether Claudin-5 deficient mice have increased number of caveolae in brain endothelial cells that could provide a mechanism for increased blood-brain barrier permeability via the transcellular route. However, our point was that Claudin-5 mutant mice have "normal-appearing" tight junctions by TEM, albeit these junctions are functionally aberrant.

4. The clarification that there were very few caveolae in CNS endothelial cells of both genotypes is evidence that in these experiments trans-endothelial transfer is unlikely, but a definitive conclusion would require the observation to be made in the presence of the Alexa555-Cadaverine marker (and preferably its visualization at the TEM level) since the marker could be inducing transcellular transfer.

In Figure 2, we provide TEM images of a CNS capillary from the cortexes of wild-type and Fgfbp1ECKO mice. These TEM images that shows very clearly the presence of very few caveolae in CNS endothelial cells in both genotypes (2 caveolae in wild-type and 3 in Fgfbp1ECKO ECs; black arrows). Multiple studies have shown that the increase in bulk transcytosis associated with a leaky transcellular blood-brain barrier requires a large number of caveolae as seen in the context of animal models for ischemic stroke, Experimental Autoimmune encephalomyelitis (EAE) or aberrant blood-brain barrier development (e.g., Mfsd2a-/- mice). Therefore, if Fgfbp1ECKO mice do not show any changes in the number of caveolae, it is unclear to us how the transcellular pathway could explain the leakage of the tracer across the blood-brain barrier (i.e., CNS endothelial cells) in mutant mice.

5. In summary, I am not convinced that the authors have demonstrated blood-brain barrier leakage via the paracellular pathway in the brains of animals in which endothelial Fgfbp1 had been deleted. They have not excluded the possibility of trans-endothelial transfer of the marker, for reasons outlined above. They also have not considered other routes of entry. For these reasons, I think it is not appropriate to include in their Figure 8 cartoon a depiction of the cadaverine crossing via the tight junctions. They have no evidence for this.

We have revised Figure 8 to show that the tracer (Cadaverine-Alexa555) leaks from the blood into the brain without implying a potential route of passage of the tracer across the blood-brain barrier since we have not formally proved this in vivo. Our in vitro studies demonstrate that Fgfbp1 is required in primary brain endothelial cells to mediate the effects of Wnts in reducing the transcellular transport of albumin, suggesting a critical role in the transcellular route. However, we have no evidence that the number of caveolae is increased in CNS endothelial cells of Fgfbp1ECKO mice. Therefore, we do not illustrate more caveolae filled with the tracer in mutant endothelial cells in the model figure.

6. Particularly given the difficulties caused by the coronavirus epidemic for every aspect of life including continuing lab work I do not think it would be reasonable to ask for more experiments to be done to clarify the above concerns. However, I suggest that the authors should be more circumspect in their claim that the only explanation for their findings with Alexa555-Cadaverine is that there was blood-brain barrier “leakage”. As outlined above there are at least two other explanations for their findings.

We thank the reviewer for being reasonable considering the circumstances of the restrictions in research due to the COVID-19 pandemic in our respective institutions. We now discuss the possibility that the transcellular route may be affected in the mutant mice in the Discussion (See Pages 11-12, Lines 343-348 of the revised manuscript).

Third decision letter

MS ID#: DEVELOP/2019/185140

MS TITLE: Fgfbp1 promotes blood-brain barrier development by regulating collagen IV deposition and maintaining Wnt/Î²-catenin signaling

AUTHORS: Azzurra Cottarelli, Monica Corrada, Galina V Beznoussenko, Alexander A. Mironov, Maria A. Globisch, Saptarshi Biswas, Hua Huang, Anna Dimberg, Peetra U Magnusson, Dritan Agalliu, Maria Grazia Lampugnani, and Elisabetta Dejana

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.