EMBO reports

Cerebrovascular endothelial cells form transient Notch-dependent cystic structures in zebrafish

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Review timeline:

Submission date:	12 September 2018
Editorial Decision:	22 November 2018
Revision received:	7 March 2019
Editorial Decision:	3 May 2019
Revision received:	7 May 2019
Accepted:	10 May 2019

Editor: Martina Rembold

Transaction Report:

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

1st Editorial Decision 22 November 2018

Thank you once more for the submission of your research manuscript to our journal. As communicated earlier, we have meanwhile received the full set of referee reports that is copied below.

All three referees consider the observation that "kugeln" form in the cerebral vasculature interesting. However, they come to different conclusions regarding the absence of functional data. Referee 1 considers more insight into their formation and function required for publication in a more general interest journal like EMBO reports while referee 3 supports publication of the work without further functional data - given that the observation is confirmed in an independent reporter line. I have discussed the reports and your paper further with the referees and both re-emphasized their viewpoints. In his/her further feedback also referee 2 pointed out that the observation is interesting but also this referee considers some more data on either the function of kugeln or on their Notch-dependence important for publication in EMBO reports.

I have forwarded these reports to you and you have submitted a detailed point-by-point response. I notice that you can address most of the referee concerns in a revision apart from their functional relevance. Overall, I think that the proposed revision might ultimately not reveal the function of kugeln but it will provide a detailed characterization of their formation and more evidence for the signals and mechanical forces that are involved. Given that all referees considered this observation as such interesting and given the support from at least two referees for potential publication in EMBO reports, we have decided to invite you to revise your study for EMBO reports.

I think that the validation of the appearance of kugeln in a second reporter line like Tg(fl1aep:eGFP-CAAX) has highest priority. If possible, data on wildtype fish without a membrane marker should be added. I notice that kugeln are rather transient and I am not sure how easy it is to catch them in immunostainings or TEM images but such data would certainly also strengthen your findings.

I think that all the points you address in your response to the referees are pertinent and should be provided (monitor the formation of kugeln in one embryo over time and beyond 9 dpf, assess the effect of actin depolymerisation or the inhibition of heart contraction, provide further insight into the role/activity of Notch and Wnt signaling) with the exception of point 4 from referee 1 (laser ablation). I agree that the laser ablation will be difficult to establish and might have severe side effects. I guess the current study is a starting point to decipher the function of kugeln in the future.

Based on this evaluation, we would thus like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Please also upload a version of the related manuscript you mentioned in your cover letter upon resubmission.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

You have currently 4 figures and I suggest to resubmit your manuscript as Scientific Report in which case the Results and Discussion section have to be combined. If the revision leads to a manuscript with more than 5 main figures these sections can stay as they are now.

Please note that the references should be numbered. You can download the respective EndNote file from our guide to authors if you wish:
https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information.
- Currently you have three movies. These should be called Movie EVx. Please provide the legend in a separate plain text README file and zip the legend together with the movie. The ZIP file is then uploaded.
- Other supplementary data can also be submitted as Expanded View figures (Figure EV1 etc). The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Please note that we can only accommodate up to 5 EV figures.
- Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
(In order to avoid delays later in the publication process please check our figure guidelines before preparing the figures for your manuscript:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS
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Referee #1:

In this paper Kugler et al use light sheet fluorescence microscopy to image the cerebral vasculature in developing zebrafish embryos. They describe a previously unreported vascular structure that they named kugeln. These are vesicular protrusions on the vessels that can only be observed in membrane-tagged vascular reporters. These vesicular structures seem to be disconnected from the vascular lumen and F-actin enrichment in the neck points to a potential role for active cytoskeletal rearrangements for formation or maintenance. Kugel number appears to be dependent on VEGF-Notch signaling, and to contain NO.

While the observation of the kugel structures is interesting, a thorough analysis of the cellular and molecular mechanism and of the biological function is lacking. The authors perform a very limited study of molecular regulators and only show an effect of VEGF and Notch manipulation, and this only by global chemical inhibition. Considering the importance of VEGF and Notch signaling in vascular development, the effect on a minor structure in the vasculature is not surprising. Additionally, minimal experiments (DAF-FM staining and LysoTracker) were used to analyze content and functional properties of kugeln, but these seem to only partially explain and suggest a potential function that is not further investigated or discussed. How does this fit with a specific function of kugeln in the cerebral vasculature?
Without a more thorough analysis of mechanism and function, the observation of these rare structures seems insufficient for publication in Embo Reports. The following comments and suggestions could help to further increase the understanding of kugel structures and their relevance.

Specific comments

1. In embryos where no kugeln were observed, do they arise later or not at all? If not, this indicates that kugeln don't have an important function in cerebral vasculature. Have the same embryo's without kugeln been followed over time to check later development? At 3, 4 and 5 days embryos were screened for the presence of kugeln, and at every stage several fish were found without kugeln. It is not clear from the explanation whether these were the same embryos.
2. fig 3D: gata1:dsRed label is mentioned on the figure panel, but not mentioned in the text, the legend nor the methods section. It is also not clear what imaging of blood cells would add.
3. The mechanism of formation should be investigated in more detail. The role of F-actin should be confirmed. Is myosin II involved? Loss of function studies could be performed to determine the importance of cytoskeletal rearrangements for kugel formation.
4. Laser ablation of kugeln could be performed to test the importance of these structures and the effect of their absence on the vasculature. Or does ablation of the kugel cause too much damage the mother cell?
5. The role of VEGF and Notch should be investigated further, as well as other molecular regulators. Considering the importance of VEGF and Notch signaling in vascular development, the effect on a minor structure in the vasculature is not surprising. Therefore, the specific effect of these pathways on kugel formation should be tested. e.g. the Notch reporter Tg(TP1:kaede) could be used to specifically test the Notch activity status of kugeln at different moments after conversion of the kaede protein. Also, what is the effect of (mosaic) Notch activation or mosaic Notch inhibition?
6. p8: the concluding part of a sentence is missing: "We performed similar experiments with LysoTracker to visualize acidic cell compartments and found that 23% of kugeln... (missing)".
7. The experiments should be better motivated in the manuscript. VEGF and Notch involvement were analyzed merely because they are central orchestrators of vascular development. DAF-FM staining and LysoTracker were used to analyze the content of kugeln and were found to be positive. Have other components been tested and found negative but not described, or were the authors just 'lucky'? Why did the authors specifically choose to check for NO and lysosomal compartments?
8. Fig 3D: the enrichment of F-actin in the neck region is not very clear from this example. Also in movie 3, the lifeact signal is not very strong. Separated channels could improve the clarity.
9. Fig 4A-F: mean kugel number in control embryos should be around 10 according to the graphs (panel B,E), but in the images (panel A,D) only 1-3 kugeln are indicated
10. Fig 4H: LysoTracker positive kugel: the structure labeled positive by LysoTracker doesn't look like a kugel structure in the kdrl:HRAS-mCherry panel (no vesicular structure can be observed)
11. Fig 4G,H: positive and negative examples are explicitly shown, but in the text it is not discussed why both cases occur together or what could be the meaning.

Minor comments
12. p6: ..., which is far larger than microvesicles or other previously described membrane derived structures,... : references are missing to previous publications
13. Supplemental video legends should contain more information explaining what is shown. They also don't contain information about developmental stage and time interval. Also scale bars are missing.
14. fig 1B: rotated 3D views from movie 1 are not separately explained, and have minimal added value. These panels should also be colour inverted.
15. Fig 1D: Colour coding could make these panels more clear. Similar colours can be used in panels D and F. Better distinguishable colours should be used
16. Fig 2A: difference between black and grey arrows is not clear
17. Fig 2C: kugel indicated by triangle seems to disappear instead of expanding

Referee #2:

This is a fascinating story, again showing that our understanding of the ontogenesis and the physiology of the cerebral vasculature is far from complete. I have a few questions:

The authors describe a "large vesicle like" structure (termed kugeln) connected to cerebral vessels. These structures are derived from cerebral vascular endothelium but lack a direct connection to the vessel lumen. Using fli1a transgenics the authors show that kugeln lack endothelial cytoplasm; in actin reporter fish they observe actin expression at the "neck" of the kugeln.

What is the earliest time point at which kugeln can be observed in the cerebral circulation. Do they

remain beyond 5 dpf?

Question: are these vesicle like structures/kugeln somehow communicating or interacting with the population of cerebral "scavenger or BLEC cells" that were recently described by the groups of Weinstein, Hogan and Schulte-Merker? Do these kugeln possess any "scavenging like" properties? Do kugeln perhaps contribute to local inflammatory responses, or interactions with tissue resident immune cells.
What determines the polarization of kugeln formation as they seem to form only at the abluminal side.

Nitric oxide can diffuse from endothelial cells and affect VSMC contractile behavior. Can the authors speculate why delivery of nitric oxide via kugeln to other vascular cells is more beneficial than just having nitric oxide diffuse from the endothelium.

It is very surprising that "kugeln" have not been reported previously as many groups have used Tg(kdrl:HRAS-mCherry)s916 before to study brain vascularisation. To substantiate the data the authors are encouraged to repeat the experiments in another line with a membrane tagged fluorescent marker (for example Tg(fli1:Myr-mCherry)).

The authors show that loss of Vegf or Notch signaling affects kugeln number and size. What about the Wnt signaling pathway - does LOF/GOF for the Wnt signaling pathway impact kugeln? Given the role of nitric oxide in shear stress adaptation, and despite kugeln not being connected to the vessel lumen, is kugeln formation/regression influenced by flow?

Referee #3:

The manuscript submitted by Kugler, Chico and colleagues describes a novel phenomenon is vascular biology, the formation of large spherical structures of membrane pocketing out of endothelial cells in the brain vasculature of zebrafish embryos. These novel structures are only found in the brain vasculature, show oscillatory growth/regression, are influenced by Vegf and N signalling inhibition, and contain NO. The role of these structures is of course not clear, since it is not possible to selectively remove them.

The study is sound and the result are novel and intruiging. My major worry is that these structures might actually be induced by the CAAX reporter the authors used. Is there any way to use another driver and ask whether these structures still develop? Can these structures be found in fixed embryos, or with a transmembrane-teathered fluorescent proteins? Since these structures have not been seen before in any other study, I feel that it is important to reassure the reader that this does not represent an artefact.

The Introduction is rather short, hardly has any references and does not really bring the reader to the point where he is prepared to what comes in the result section. The authors should invrst some more time to write a well-researched introduction.

In general, the text is not helping the reader to appreciate the experiments done. In the chemical treatment section, the authors should describe when and for how long they treated the embroys, since this of course matter with regard to the outcome. The reader should not have to go to Materials and Methods to look for these important informations.

Second last sentence of the Result section is incomplete ...

1st Revision - authors' response 7 March 2019

Specific responses to reviews

We here provide a point-by-point response to your and the reviewer’s comments.
Please note that I have grouped and re-ordered these to address similar points. I have

initially responded to the comments for which we we have performed additional experimentation, with other points listed towards the end.

Editor comment: "I think that the validation of the appearance of kugeln in a second reporter line like Tg(fl1aep:eGFP-CAAX) has highest priority. If possible, data on wildtype fish without a membrane marker should be added. I notice that kugeln are rather transient and I am not sure how easy it is to catch them in immunostainings or TEM images but such data would certainly also strengthen your findings."
Reviewer #2: "To substantiate the data the authors are encouraged to repeat the experiments in another line with a membrane tagged fluorescent marker (for example Tg(fli1:Myr-mCherry))."
Reviewer #3: "My major worry is that these structures might actually be induced by the CAAX reporter the authors used. Is there any way to use another driver and ask whether these structures still develop? Can these structures be found in fixed embryos, or with a transmembrane-teathered fluorescent proteins? Since these structures have not been seen before in any other study, I feel that it is important to reassure the reader that this does not represent an artefact."
Our response: We entirely accept this critical point. We used two additional approaches to visualise endothelial *kugeln*. In addition to the original description of kugeln in the *Tg(kdrl:HRAS-mCherry)s916* transgenic, we have now examined the transgenic *Tg(fli1aep:eGFP-CAAX)* which uses a different promoter and method of labelling the endothelial membrane and find these also develop *kugeln* (Figures 1E, EV1). Furthermore, we induced transient mosaic expression of *Tg(fli1a:myr-mCherry)* and find *kugeln* also form in these animals. This excludes the possibility that *kugeln* arise as an artefact of the original promoter construct, the transgenic integration site, or the specific method of labelling the endothelial membrane.
We have not yet attempted to identify *kugeln* using TEM or immunostaining in fixed embryos due to the technical difficulty of establishing this and the anticipated difficulty of distinguishing *kugeln* from the parent vessels. We have attempted to obtain preexisting datasets that we could examine within the timeframe for resubmission but have not been successful. However, we point out that if *kugeln* arise as a general artefact of tagging endothelial membrane with a fluorescent reporter this would not explain why *kugeln* are restricted to the cerebral vessels and are not found in other vascular territories. We therefore are confident that *kugeln* are a real phenomenon.

Reviewer #2: "It is very surprising that "kugeln" have not been reported previously as many groups have used Tg(kdrl:HRAS-mCherry)s916 before to study brain vascularisation."
Our response: Kugeln can be mistaken for vascular cross sections but once their existence is known it is easier to recognise them retrospectively. For example a previously published micrograph (see Fig 1D' in https://doi.org/10.7554/eLife.25932.001 by Max van Lessen et al.) clearly shows a *kugel*. Retrospective analysis of datasets generated by van Lessen confirms *kugeln* are frequently present (examples shown in Figure EV3).

Reviewer #1: In embryos where no kugeln were observed, do they arise later or not at all? If not, this indicates that kugeln don't have an important function in cerebral vasculature. Have the same embryo's without kugeln been followed over time to check later development? At 3, 4 and 5 days embryos were screened for the presence of kugeln, and at every stage several fish were found without kugeln. It is not clear from the explanation whether these were the same embryos.
Our response: We understand the reviewer considers *kugeln* "rare" and agree that if *kugeln* are never present in some animals this would make a functional role less likely. We therefore performed additional timelapse imaging and find that animals with no *kugeln* at the start of imaging develop them later on (Figure 2D). Given a mean number of five to ten *kugeln* per animal at a single timepoint, and a mean lifespan of 23 minutes, it might be predicted that a proportion of animals would have no *kugeln* at a single timepoint. We cannot yet prove that all animals develop *kugeln* at some point, but the fact that we have retrospectively identified *kugeln* in previous datasets from other colleagues in Münster and Dresden (once it is known what to look for) suggests *kugeln*

are not particularly “rare”. We expect that if our paper is published, this will lead others to examine their own datasets and predict they will find *kugeln* easily and commonly.

Reviewer #1: The mechanism of formation should be investigated in more detail. The role of F-actin should be confirmed. Is myosin II involved? Loss of function studies could be performed to determine the importance of cytoskeletal rearrangements for kugel formation.
Our response: We have tested the reviewer’s suggestions pharmacologically. The suggested investigations were addressed by inhibition of F-actin polymerization via application of Latrunculin. We indeed found that inhibition of actin polymerization by latrunculin treatment increased *kugel* number (**Figure 3E)**, while *kugel* diameter was significantly decreased (**Figure 3F**). We similarly tested the role of Myosin II by inhibition via Blebbistatin treatment and found a statistically significant decrease in *kugel* number (**Figure 3G)** while *kugel* diameter was unaffected (**Figure 3H)**. Together these data supports the reviewer’s suspicion that cytoskeletal rearrangement is required for *kugel* formation.

Reviewer #1: The role of VEGF and Notch should be investigated further, as well as other molecular regulators. Considering the importance of VEGF and Notch signaling in vascular development, the effect on a minor structure in the vasculature is not surprising. Therefore, the specific effect of these pathways on kugel formation should be tested. e.g. the Notch reporter Tg(TP1:kaede) could be used to specifically test the Notch activity status of kugeln at different moments after conversion of the kaede protein. Also, what is the effect of (mosaic) Notch activation or mosaic Notch inhibition?
Our response: We agree the relationship between *kugeln* and the VEGF and Notch pathways needs further examination. We examined expression of the transgenic notch pathway reporters *Tg(dll4in3:eGFP)* and *Tg(TP1glob:venusPest)s940* (**Figure 6C** and **6D**, respectively) but this did not indicate any differential local or parent-vessel specific expression, and are associated with a significant amount of non-vascular expression. We performed antisense morpholino oligonucleotide-mediated knockdown of *dll4*, *notch1b, jagged-1a* and *jagged-1b* and found that knockdown of *notch1b* significantly reduced *kugeln* number without affecting diameter, while jagged-1a knockdown significantly increased *kugel* diameter without affecting *kugeln* number. However, time did not allow us to perform these experiments in stable mutants or to induce mosaic notch activation or inhibition.

Referee #2: What is the earliest time point at which kugeln can be observed in the cerebral circulation. Do they remain beyond 5 dpf?
Our response: We have never observed *kugeln* at 32hpf, and so believe they develop between 32hpf and 3dpf and discuss this in the revised manuscript. In the revised manuscript we include data from 28dpf fish showing that *kugeln* exist even at this late stage. However, we are unable to image older animals for technical reasons.

Referee #2: Question: are these vesicle like structures/kugeln somehow communicating or interacting with the population of cerebral "scavenger or BLEC cells" that were recently described by the groups of Weinstein, Hogan and Schulte-Merker? Do these kugeln possess any "scavenging like" properties?
Our response: We have investigated whether *kugeln* interact with BLECs or scavenge in collaboration with Professor Schulte-Merker. We examined co-localisation of kugeln and BLECs using either double *Tg(kdrl:HRAS-mCherry)s916*, *Tg(fli1a:Lifeact-mClover)sh467* transgenics, or double *Tg(kdrl:HRAS-mCherry)s916*, *Tg(flt4BAC:mCitrine)hu7135* transgenics and have never observed co-localisation; kugeln tend to form on vessels which are remote from the sites of BLECs. Furthermore, we knocked down ccbe1 which prevents BLEC formation and found this had no effect on kugeln number (Figure EV3). We also examined whether kugeln can scavenge IgG-conjugated Alexa 647 injected into the tectum and found no evidence for this (Figure EV3E)

Referee #2: Do kugeln perhaps contribute to local inflammatory responses, or interactions with tissue resident immune cells.

Our response: We studied macrophages and *kugeln* simultaneously in the transgenic reporter line *Tg(fms:GAL4.VP16)i186, Tg(UAS-E1b:nfsB.mCherry)il149, Tg(kdrl:HRASmCherry)s916*. No interaction of macrophages with *kugeln* was observed (**Figure 6**).

Referee #2: Nitric oxide can diffuse from endothelial cells and affect VSMC contractile behavior. Can the authors speculate why delivery of nitric oxide via kugeln to other vascular cells is more beneficial than just having nitric oxide diffuse from the endothelium.
Our response: Presently we can only speculate that storage of NO in *kugeln* would allow release of a larger quantity of NO than basal production would allow, though cannot explain under what circumstances this would be required.

Referee #2: The authors show that loss of Vegf or Notch signaling affects kugeln number and size. What about the Wnt signaling pathway - does LOF/GOF for the Wnt signaling pathway impact kugeln?
Our response: As suggested we examined the effect of pharmacological inhibition and activation of the Wnt pathway on kugeln and find that both significantly increased the number of *kugeln* without affecting *kugel* diameter (**Figure 8**).

Referee #2: Given the role of nitric oxide in shear stress adaptation, and despite kugeln not being connected to the vessel lumen, is kugeln formation/regression influenced by flow?
Our response: We performed further experiments to examine the role of blood flow on *kugel* formation. We induced temporary cessation of cardiac contraction by Tricaine and found that *kugeln* could form in the absence of blood flow (**Figure 4**), so we conclude blood flow is not necessary for *kugel* formation. We also examined *tnnt2a* morphants that never develop blood flow and found that kugeln did not form in these animals (**Figure 4F**) but this may be due to abnormal formation of cerebral vessels. Overall, we feel that blood flow is not required at the time of initiation of *kugel* formation, and that this is probably independent of blood flow.

Referee #3: The Introduction is rather short, hardly has any references and does not really bring the reader to the point where he is prepared to what comes in the result section. The authors should invest some more time to write a well-researched introduction.
Our response: We have expanded both introduction and discussion.

Referee #3: In general, the text is not helping the reader to appreciate the experiments done. In the chemical treatment section, the authors should describe when and for how long they treated the embryos, since this of course matter with regard to the outcome. The reader should not have to go to Materials and Methods to look for these important informations.
Our response: We have amended the manuscript as suggested.

Referee #3: Second last sentence of the Result section is incomplete ...
Our response: We apologise, this has been corrected.

Reviewer #1: fig 3D: gata1:dsRed label is mentioned on the figure panel, but not mentioned in the text, the legend nor the methods section. It is also not clear what imaging of blood cells would add.
Our response: We apologise for not making this clear, this has been corrected.

Reviewer #1: Laser ablation of kugeln could be performed to test the importance of these structures and the effect of their absence on the vasculature. Or does ablation of the kugel cause too much damage the mother cell?
Our response: We discussed this experiment with the Editor who agreed it would be too difficult to interpret at this stage and so was not attempted.

Reviewer #1: p8: the concluding part of a sentence is missing: "We performed similar experiments with LysoTracker to visualize acidic cell compartments and found that

23% of kugeln... (missing)".
Our response: We apologise, and this has been corrected.

Referee #2: What determines the polarization of kugeln formation as they seem to form only at the abluminal side.
Our response: We have only observed *kugeln* at the abluminal side but cannot exclude the possibility that *kugeln* could form on the luminal side of endothelial cells since they would be within the lumen and hard to distinguish. We think this unlikely, although do not currently know the reason for their abluminal position.

Reviewer #1: The experiments should be better motivated in the manuscript. VEGF and Notch involvement were analyzed merely because they are central orchestrators of vascular development. DAF-FM staining and LysoTracker were used to analyze the content of kugeln and were found to be positive. Have other components been tested and found negative but not described, or were the authors just 'lucky'? Why did the authors specifically choose to check for NO and lysosomal compartments?
Our response: We agree that VEGF and Notch are obvious pathways to test although we were surprised to find VEGF inhibition increases *kugeln* number as we hypothesised the opposite. We have not tested dyes other than DAF-FM and Lysotracker, so we were indeed just lucky! We would like to reassure the reviewer that this is the first time we have experienced good luck and do not expect it to continue. However, we have made clearer that we examined Lysotracker to determine whether acidic contents might have caused the DAF-FM reactivity. Since the proportion of *kugeln* positive for Lysotracker is low, this confirms that *kugeln* do truly contain NO.

Reviewer #1: Fig 4A-F: mean kugel number in control embryos should be around 10 according to the graphs (panel B,E), but in the images (panel A,D) only 1-3 kugeln are indicated
Our response: We have removed these micrographs to accommodate other data.

Reviewer #1: Fig 3D: the enrichment of F-actin in the neck region is not very clear from this example. Also in movie 3, the lifeact signal is not very strong. Separated channels could improve the clarity.
Our response: We have improved the micrographs and movie as suggested.

Reviewer #1: Fig 4H: LysoTracker positive kugel: the structure labeled positive by LysoTracker doesn't look like a kugel structure in the kdrl:HRAS-mCherry panel (no vesicular structure can be observed)
Our response: The previous Figure 4H was replaced by a more representative image of a kugel positive for LysoTracker (**Figure 7E**)

Reviewer #1: Fig 4G,H: positive and negative examples are explicitly shown, but in the text it is not discussed why both cases occur together or what could be the meaning.
Our response: this has been corrected.

Reviewer #1: Minor comments
p6: ..., which is far larger than microvesicles or other previously described membrane derived structures,... : references are missing to previous publications
Supplemental video legends should contain more information explaining what is shown. They also don't contain information about developmental stage and time interval. Also scale bars are missing.
fig 1B: rotated 3D views from movie 1 are not separately explained, and have minimal added value. These panels should also be colour inverted.
Fig 1D: Colour coding could make these panels more clear. Similar colours can be used in panels D and F. Better distinguishable colours should be used
Fig 2A: difference between black and grey arrows is not clear
Fig 2C: kugel indicated by triangle seems to disappear instead of expanding
Our response: We accept all these points and have addressed them as suggested.

2nd Editorial Decision 3 May 2019

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for the unusual delay in handling your manuscript. I was hoping to receive feedback from former referee 1 as well but unfortunately, this referee was not available anymore. Since however both, referee 2 and 3, support publication of your revised study in EMBO reports, I have decided to move forward with your manuscript. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- Please provide the conflict of interest statement in the article, i.e., in a separate section after the Author Contributions paragraph.

- Abstract: please use present tense to describe your findings.

- Our data editors from Wiley have already inspected the Figure legends for completeness and accuracy. Please see their suggested changes in the attached Word file.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Once you have made these minor revisions, please use the following link to submit your corrected manuscript:

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If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS

Referee #2:

I now reviewed the revised manuscript entitled "Cerebrovascular endothelial cells form transient Notch-dependent cystic structures in zebrafish" by Elisabeth Kugler for publication in EMBO Reports.

The authors revised their manuscript along the lines of my suggestions. In particular they repeated the experiments in different vascular reporter lines, and were able to identify Kugel in these lines as well. Moreover, Kugel could be identified on previously published micrographs.

The authors furthermore substantiated the role of endothelial cytoskeleton rearrangements in regulating Kugel number and Kugel size.

In response to my questions, the authors performed loss and gain of function experiments for the Wnt signaling pathway. Interestingly, Wnt signaling promoted Kugel formation however had no impact on Kugel size. Moreover, Kugel did not interact with BLEC or macrophages, and did not show any "scavenging properties". Kugel structures thus clearly differ (in morphology/function/regulation) from any of the other recently discovered "novel" vessel structures

in the cerebral vasculature.

Overall, and taking into account the additional experiments performed in response to the other reviewers, I believe that this manuscript is suitable for publication in EMBO reports. Kugel are intriguing structures.

Referee #3:

The authors have responded to all my querries and have done additional experiments, thereby strengthening their conclusions. I feel that the paper can now be published.

2nd Revision - authors' response 7 May 2019

The authors performed all minor editorial changes.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Tim Chico and Elisabeth Kugler
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-47047V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Based on the mean/SD of the data in the control groups in the assays used, previous post hoc power calculations have shown that these assays have at least 80% power to detect an effect size of 30% difference between groups when group sizes are 12/group (alpha = 0.05).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See above; we also increased group sizes to attempt to detect smaller effect sizes where appropriate
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Samples were excluded from analysis if image quality did not allow for reliable quantification of kugeln.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animals were allocated to treatment groups randomly without selection. Imaging and data analysis was performed unblinded to treatment allocation, often because the effect of treatment was easily deduced from the appearance of the micrograph.
For animal studies, include a statement about randomization even if no randomization was used.	Chemical treatments were randomly applied to individual embryos housed in 96 well plates to avoid batch effects.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Animals were allocated to treatment groups randomly without selection. Imaging and data analysis was performed unblinded to treatment allocation, often because the effect of treatment was easily deduced from the appearance of the micrograph.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Data analysis was performed without blinding.
5. For every figure, are statistical tests justified as appropriate?	Statistical analysis of normally distributed data was performed using a One-way ANOVA to compare multiple groups or Student's t-test to compare two groups. Non-normally distributed data were analysed with a Kruskal-Wallis test to compare multiple groups, or Mann-Whitney test to compare two groups. Diameter of kugeln is shown as average of all kugeln per embryo, unless otherwise indicated. Analysis was performed in GraphPad Prism Version 7 (GraphPad Software, La Jolla California USA). P values are indicated as follows: p<0.05 *, p<0.01 **, p<0.001 ***, p<0.0001 ****.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normality of data was tested using D'Agostino-Pearson omnibus test.
Is there an estimate of variation within each group of data?	Data represents mean and standard deviation (s.d.), if not otherwise stated.

USEFUL LINKS FOR COMPLETING THIS FORM

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http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	yes.

C- Reagents

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

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Zebrafish strains used are described on p5 in Material and Methods, as well as in the appropriate sections/figures (including age). Husbandry - p5 - Maintenance of adult zebrafish in all three fish facilities was conducted according to previously described husbandry standard protocols at 28°C with a 14:10 hours (h) light:dark cycle 21. Embryos, obtained from controlled pair- or group-mating, were incubated in E3 buffer (5mM NaCl, 0.17mM KCl, 0.33mM CaCl2, 0.33mM MgSO4) with or without methylene blue.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Experiments performed at the University of Sheffield conformed to UK Home Office regulations and were performed under Home Office Project Licence 70/8588 held by TJAC. Experiments performed at the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden and the WWU Münster Institute for Cardiovascular Organogenesis and Regeneration conformed to guidelines of the relevant German animal ethics committees.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliance confirmed.

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Data are available on request.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Data are available on request, as light sheet data exceed normal data volumes.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n.a.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	n.a.

G- Dual use research of concern

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