A dual involvement of Protocadherin 18a in stromal cell development guides the formation of a functional hematopoietic niche

Anne-Lou Touret, Catherine Vivier, Anne Schmidt, Philippe Herbomel and Emi Murayama
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MS TITLE: A dual involvement of Protocadherin-18a in stromal cell development guides the formation of a functional hematopoietic niche

AUTHORS: Anne-Lou Touret, Catherine Vivier, Anne Schmidt, Philippe Herbomel, and Emi Murayama

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. 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.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing

how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The caudal hematopoietic tissue (CHT) is a tissue critical for the proliferation and differentiation of hematopoietic stem and progenitor cells (HSPCs) in zebrafish. Previous studies have shown that somite-derived stromal cells (SCPs) and the caudal venous plexus (CVP) are the two key components of the CHT niche for HSPCs. In this study, the authors identified a cell adhesion protein protocadherin-18a (Pcdh18a) is highly expressed in the somite-derived stromal cells. The authors further showed that removal of the intracellular domain of Pcdh18a impaired the migration, the number of SCPs, and their interaction with the endothelial cells in the CHT, leading to the impairment of the hematopoietic niche and resulting in the reduction of HSPC number in the CHT. This work reveals the important role of the protocadherin-18a in the regulation of the development of SCPs and the formation of CVP, which is well suited for the publication in Development upon addressing following concerns.

Comments for the author

Major concerns:
1. Although HSPC number apparently declined at the CHT in pcdh18a morphants, the evidence presented in current study could not rule out the intrinsic effect of Pcdh18a in HSPCs. It would be nice to provide additional evidence (either expression data or rescue assay) to support the HSPC reduction is indeed caused by the impairment of the niche.
2. In addition, it would be nice to reveal whether the reduction of HSPCs in pcdh18a morphants is due to the impairment of CHT colonization, survival, or proliferation of HSPCs.

Reviewer 2

Advance summary and potential significance to field

In the manuscript by Touret and colleagues entitled “A dual involvement of Protocadherin-18a in stromal cell development guides the formation of a functional hematopoietic niche”, the authors describe the requirement for protocadherin 18a in the proper development of the CHT hematopoietic niche. Specifically, they show defects in the migration of stromal cell progenitors (SCPs) that they suggest are critical for endothelial cell remodeling and HSPC seeding in the CHT. The findings are interesting and could reveal important insight into the embryonic establishment of a hematopoietic niche, if some key caveats to the experimental design and interpretation are remedied.

Comments for the author

Major Points:
1. Many of the experiments center on morpholino knockdown of pcdh18a, but appropriate controls that are standards in the field were not performed. For instance, p53 pathway activation is a common off-target effect of morpholinos. The authors must demonstrate that the diminishment of SCPs and HSPCs is not due to p53-mediated apoptosis. Additionally, reversal of the observed phenotypes by overexpression of a morpholino-resistant pcdh18a is needed to demonstrate the effects are due to the on-target loss of pcdh18a. Alternatively, a novel CRISPR/Cas9 based ‘crispant’ approach could be used to generate a mosaic mutant and phenotype can be assessed in the injected embryos much like in a morphant.
Alternative morpholinos, a mutant line, and full length overexpression constructs were described in Bosze et al. Histochemistry and Cell Biology 2020. These reagents could be used to confirm the findings in the study of SCP development. In addition, a full description of the morphant along with a picture showing the gross morphology of the morphants needs to be included.

2. The authors propose that the primary defect in the SCP causes the subsequent changes to vascular remodeling and HSPC CHT seeding. It is also possible that these are independent defects in the pcdh18a morphants. Tissue-specific rescue experiments with pcdh18a full length or ICD would strengthen these claims. The new cspg4:Gal4 line is a nice tool employed in this manuscript that would allow expression in the SCPs. Related to this transgenic, images showing the specificity of the expression pattern should be provided for the reviewers even though not included in the paper because it is part of another manuscript.

Minor Points:
1. In Figure 2A it is unclear what the 40X insert is magnifying and how it relates to 10X image.
2. Figure 4D: As the authors demonstrate altered vascular area within the CHT (Figure 5), the diminished number of perivascular stromal cells could be due to decreased area. To account for this, the perivascular cell counts should be normalized to the kdrl+ vasculature area.
3. In Figure 5: in the text the authors state that the endothelial kdrl+ cells did not “often” interact with the SCPs in morphants, but this is not quantified – how many fish out of those viewed had this? There is nothing in the figure legend about this, please include your sample size.

Reviewer 3

Advance summary and potential significance to field

This study by Touret et al. explores the role of protocadherin-18a (pcdh18a) in development of stromal cells and formation of the hematopoietic niche in the zebrafish embryo. The authors previously published an article in Nature Communications (2015) that showed stromal cells in the embryonic hematopoietic niche are derived from the ventral aspect of the somites. These stromal cell progenitors (SCPs) delaminate and migrate into the caudal hematopoietic tissue (CHT). In this current study the authors find that protocadherin-18a (pcdh18a) is expressed in the SCPs and that it has a functional role in these cells. There are fewer SCPs in the absence of pcdh18a and they have abnormal migration.
Ultimately, this results in a poorly formed CHT niche that does not functional properly to support hematopoietic cells. Interestingly, pcdh18a mediates heterotypic adhesion between endothelial and stromal cells. This study is significant because it highlights novel aspects of a poorly understood process: how the niche stromal and endothelial cells are properly assembled to support hematopoietic cells.

Comments for the author

The presented data is of very high quality and the imaging is superb. There are some additional experiments detailed below that would make the study more complete and the data more robust. It will be important to provide additional genetic data to support the conclusions drawn from morpholino data.

Major points
1. pcdh18a was the focus of this study but pcdh18b is also present in the zebrafish genome. At a minimum, whole mount in situ hybridization should be performed to check for expression of pcdh18b in stromal cells. Ideally knockdown of pcdh18a and pcdh18b together and individually should be done to check for possible synergy and/or compensation.
2. As outlined in the community resource, “Guidelines for morpholino use in zebrafish”, it is preferable to have some genetic data to support the splice-
blocking morpholino (MO) pcdh18a data. This could be significant, given the authors point out the translation blocking MO referenced from another study has a more severe phenotype than the splice blocking MO. These are several outstanding questions: 1) Do the authors have a stable genetic mutant for pcdh18a (ENU or CRISPR/Cas9)? 2) Have they tested CRISPR/Cas9 reagents for transient ‘crispant’ mutagenesis? These could be designed to create the same truncation mutation described in the study. 3) Have the authors performed a rescue experiment that would support specificity of the MO?
3. There is not much data describing the hematopoietic niche defects in the pcdh18a MO embryos. Live imaging of cd41:gfp+ cell colonization of the niche would be informative, as it would

indicate if cells: 1) home to the niche but do not lodge; 2) lodge in the niche but do not proliferate; or 3) do not home to the niche.
4. There appears to be developmental delay in the pcdh18a MO embryos shown in Fig. 6. Is this a significant issue and does it affect interpretation of the results? For example, the number of cells lodged in the CHT will be less if the embryos are delayed.

Minor points
5. Pg. 6 it is mentioned that a manuscript is in preparation for characterization of the TgBAC(cspg4:GAL4) line. As it is presented in this paper, why not include that data as part of the supplement?
6. Pg. 6 Fig. 2a likewise, it is mentioned there is additional expression data not shown for the cadherin and protocadherin family members. Could this be included as a supplemental resource?
7. Typo: Pg. 6 "termination codon 13 codons downstream, hence in a protein retaining only 106 out of" should be "…hence resulting in…"
8. Movie 3 is too short and ends quickly. It is hard to make the observation described in the text. It needs to be slowed down.
9. Movie 5 needs arrows to show the point of interest 10. Fig. S4a expression negative data of other tested ECM molecules should be included as a resource (syndecan 2, cspg4, etc)
11. There is an issue with Fig. legend S4. A description for panel C e-f is missing 12.Could the authors speculate about the functional significance of fibronectin increase?
13. Is Fig. 6C referenced in the main article text?
14. The meaning of "sheer" in this sentence is not clear: "overexpression in stromal cells of the WT Pcdh18a ECD form led to a sheer inversion of this ratio"
15. It needs to be clear in the Fig. 7 legend that the results in 7C should be compared to 4D, or uninjected controls should be shown side-by-side in 7C.
16. Should it be "ECM" and not "ICM" in this sentence: "notably α5β1 that can bind to the RGD motif present on other cells or proteins of the ICM."
Additional extensions of the current study
17. As the pcdh18a phenotype appears to affect endothelial cells as well, as described in Fig. 5, there is no further data to explain how this contributes to the CHT niche phenotype. It would be helpful to understand the distinct role of pcdh18a in endothelial versus stromal cells.

First revision

Author response to reviewers' comments

Responses to the reviewers (in blue)

Reviewer 1 Advance Summary and Potential Significance to Field:
The caudal hematopoietic tissue (CHT) is a tissue critical for the proliferation and differentiation of hematopoietic stem and progenitor cells (HSPCs) in zebrafish. Previous studies have shown that somite-derived stromal cells (SCPs) and the caudal venous plexus (CVP) are the two key components of the CHT niche for HSPCs. In this study, the authors identified a cell adhesion protein protocadherin-18a (Pcdh18a) is highly expressed in the somite-derived stromal cells. The authors further showed that removal of the intracellular domain of Pcdh18a impaired the migration, the number of SCPs, and their interaction with the endothelial cells in the CHT, leading to the impairment of the hematopoietic niche and resulting in the reduction of HSPC number in the CHT. This work reveals the important role of the protocadherin-18a in the regulation of the development of SCPs and the formation of CVP, which is well suited for the publication in Development upon addressing following concerns.

Reviewer 1 Comments for the Author:
Major concerns:
1) Although HSPC number apparently declined at the CHT in pcdh18a morphants, the evidence presented in current study could not rule out the intrinsic effect of Pcdh18a in HSPCs. It would be nice to provide additional evidence (either expression data or rescue assay) to support the HSPC reduction is indeed caused by the impairment of the niche.

- No expression of the pcdh18a gene in HSPCs is detected by WISH, and very little to none by transcriptome analyses following FACS - either at the level of sorted populations (Xue et al., 2019) or of single cells (Bresciani et al. 2021, Blood Adv. 5:4949; Xia et al. 2021, PNAS 118). We have included these references in the revised manuscript.

2) In addition, it would be nice to reveal whether the reduction of HSPCs in pcdh18a morphants is due to the impairment of CHT colonization, survival, or proliferation of HSPCs.
- The fact that from the very beginning of CHT colonization by aorta-derived HSPCs (36-48 hpf), pcdh18a morphants display a large deficit in HSPC number in the CHT pointed to an impairment of CHT colonization. We have now confirmed this by a more detailed analysis, in which we separately counted CD41:GFPlow (=HSPCs) and CD41:GFPhigh (=HSPC-derived pro-thrombocytes) in the CHT at 48h, then systematically confocal time-lapse imaged the embryos and counted again GFPlow and GFPhigh cells 10 and 20 hrs later **(new Fig. 6C and Movie 7).** Among the few HSPCs found at any time in the morphant CHT, mitoses do happen, at a rate that seems similar to that of WT embryos (see the **new Movie 7**), although this is difficult to quantify due to the low number of these cells in the morphants.
Our new time-lapse imaging sequences of the CHT of *Tg(CD41:GFP; Kdrl:mCherry-F)* morphants revealed some additional distinctive features over time: i) in WT and morphants, the HSPCs first home in the most dorsal part of the CHT (just beneath the caudal artery), as first noticed by Li et al. (Nature 2018). Then in the WT CHT, they progressively spread also into the more ventral part of the CHT; in the morphants, they don't seem to do so (new Movie 7). Regarding HSPC survival, we also confirmed that apoptosis was not observed in HSPCs settled in the CHT by performing TUNEL staining on *Tg(CD41:GFP)* embryos (**new Fig. S5**).

Reviewer 2 Advance Summary and Potential Significance to Field:
In the manuscript by Touret and colleagues entitled “A dual involvement of Protocadherin-18a in stromal cell development guides the formation of a functional hematopoietic niche”, the authors describe the requirement for protocadherin 18a in the proper development of the CHT hematopoietic niche. Specifically, they show defects in the migration of stromal cell progenitors (SCPs) that they suggest are critical for endothelial cell remodeling and HSPC seeding in the CHT. The findings are interesting and could reveal important insight into the embryonic establishment of a hematopoietic niche, if some key caveats to the experimental design and interpretation are remedied.

Reviewer 2 Comments for the Author:
Major Points:
1. Many of the experiments center on morpholino knockdown of pcdh18a, but appropriate controls that are standards in the field were not performed. For instance, p53 pathway activation is a common off-target effect of morpholinos. The authors must demonstrate that the diminishment of SCPs and HSPCs is not due to p53-mediated apoptosis.
- We performed co-injection of pcdh18a MO and p53 MO in Tg(CD41:gfp; kdrl:ras-mCherry) embryos. The phenotype remained the same as upon injection of pcdh18a Mo alone (in terms of number of CD41:GFP+ cells and vascular morphology) (**see Supp. Fig. 1 for Reviewers**).
- We also assessed for apoptosis by TUNEL (Apoptag) staining and found none in the CHT of the morphants, either among pax3:GFP+ SCPs (**new Fig. S3C**), or among CD41:GFP+ HSPCs. (**new Fig. S5**)

Additionally, reversal of the observed phenotypes by overexpression of a morpholino- resistant pcdh18a is needed to demonstrate the effects are due to the on-target loss of pcdh18a.
- We first tried rescue experiments via injection of pcdh18a mRNA in the morphants, but the number of stromal cells was not recovered, nor the structure of the venous plexus.
This suggests to us that the indiscriminate overexpression of Pcdh18a was not a good solution to reverse the phenotypes. The non-rescue of stromal cell number progenitors may be due to the indiscriminate expression of Pcdh18a in the whole somites instead of specifically the ventral clusters. Simiarly, such Pcdh18a overexpression in stromal cells and also vascular endothelial cells may have adversely affected the vascular remodeling, due to homophilic interactions between the Pcdh18a ectopically present on vascular endothelial cells, and/or with the Pcdh18a present at the surface of the stromal cells.
We also tried to overexpress Pcdh18a-HA or Pcdh18a-mKate2 from a heat-shock promoter, inducing expression by heat-shock at 20 hpf. With Pcdh18a-HA, too little expression was obtained in the

ventral clusters, whereas with Pcdh18a-mKate2, the protein was found only in one small dot per expressing cell, which upon closer examination turned out to be within an intracellular, likely endosomal vesicle and was not distributed at the plasma membrane (**see Supp. Fig. 2A,B for the Reviewers**). A similar phenomenon was reported by Bosze et al, (2020). Therefore in this revised manuscript we finally moved on to the analysis of crispants, which was successful (see below).

Alternatively, a novel CRISPR/Cas9 based 'crispant' approach could be used to generate a mosaic mutant and phenotype can be assessed in the injected embryos much like in a morphant.

- So we did, despite the difficulty of assessing such mosaic phenotypes.

The morpholino we had used targeted the intron 1-exon 2 junction, i.e., the splice acceptor site, and the resulting product was a truncated protein lacking much of the cytoplasmic region of Pcdh18a due to the insertion of a premature stop caused by the retention of intron 1. Therefore, we designed gRNAs to mimic this modification and succeeded in intron1 retention with one of the three gRNAs that we tested (**new Fig. S6**). The phenotypic traits obtained were clearly similar to those obtained with the pcdh18a MO: 1) altered plexus structure, 2) reduced number of stromal cells, and 3) reduced number of HSPCs (**new Fig. S6**).

Alternative morpholinos, a mutant line, and full-length overexpression constructs were described in Bosze et al. Histochemistry and Cell Biology 2020. These reagents could be used to confirm the findings in the study of SCP development.

- As mentioned above, the protein modification derived from our MO is truncation, not deletion, so we prefered to show the phenotype obtained with crispants, as described above.
- The mutant line described by Bosze et al. is no longer extant (we had asked them).
- As mentionned above, we made our own full-length overexpression constructs, but found that they led to very mosaic expression, or endocytosis of the tagged protein (as was the case with the construct used by Bosze et al.; in their case it did not really matter, as it was only used qualitatively, to show where in the cell the protein localized).

In addition, a full description of the morphant along with a picture showing the gross morphology of the morphants needs to be included.

- Agreed. We have now added images of the whole morphant embryos at 2 and 3 dpf, which show that the gross morphology is normal by this stage (**new Fig. S2D**). Notably, even though in situ hybridization shows high expression of pcdh18a in the nervous system, we observed no neural tube atrophy in the pcdh18a morphants. In the caudal region, some differences arise past 2 dpf. In WT, as the tail progressively grows in length, the CVP progressively becomes longer and thinner (Murayama et al., Immunity 2006). This thinning is more pronounced in the pcdh18a morphants (**new Fig. S2D**).

In addition, when larvae > 2 dpf are fixed and processed for WISH (which involves rather harsh treatments, e.g. dehydratation via passage in methanol, protease treatment, heating overnight at 65°C…), the morphant tails happen to shrink much more in the process than the WT. We suspect this reflects a difference in the composition of the extracellular matrix (possibly including the excess fibronectin that we describe in the manuscript).

2. The authors propose that the primary defect in the SCP causes the subsequent changes to vascular remodeling and HSPC CHT seeding. It is also possible that these are independent defects in the pcdh18a morphants. Tissue-specific rescue experiments with pcdh18a full length or ICD would strengthen these claims.

- The *pcdh18a* gene is expressed neither in endothelial cells, nor in HSPCs (Xue et al., 2019; Bresciani et al. 2021, Blood Adv. 5:4949; Xia et al. 2021, PNAS 118). So the phenotypes observed for these cells in the pcdh18a morphants cannot be cell-autonomous.

Nevertheless, we did attempt to express full-length Pcdh18a-GFP specifically in the SCPs of pcdh18a morphants as a UAS-driven construct injected in our Tg(cspg4:Gal4) line . We found it expressed in some cells in the notochord, where cspg4 gene expression is relatively high from early development, but more rarely in the SCPs, and those that did often underwent apoptosis later on (**see Suppl. Fig. 2C for the Reviewers, and accompanying time-lapse Movie 8, also just for the reviewers**). The low level of expression overall could be due to the length of the protein (1123 aa), since when we used a similar strategy to express a Pcdh18a -GFP lacking the cytoplasmic region (Fig. 7), we obtained a high number of GFP+ stromal cells.

The new cspg4:Gal4 line is a nice tool employed in this manuscript that would allow expression in the SCPs. Related to this transgenic, images showing the specificity of the expression pattern should be provided for the reviewers even though not included in the paper because it is part of another manuscript.
- We now provide additional images of Tg(cspg4:Gal4; UAS:RFP; ET37:eGFP) embryos, which show that the cspg4:gal4 driven RFP is expressed in the somite ventral clusters earlier than ET37:eGFP, but somewhat mosaically (a typical feature of the Gal4/UAS system in zebrafish), so that later on when the stromal cells have differentiated, the RFP+ cells are a subset of the ET37:eGFP+ stromal cells (**see Supp. Fig. 3 for the Reviewers**).

Minor Points:
1. In Figure 2A it is unclear what the 40X insert is magnifying and how it relates to 10X image.
- The arrows in the 40x insert were positionned at the same place as the arrows in the 10x image. We have now added a dashed frame in the 10x image that corresponds to the 40x image.

2. Figure 4D: As the authors demonstrate altered vascular area within the CHT (Figure 5), the diminished number of perivascular stromal cells could be due to decreased area. To account for this, the perivascular cell counts should be normalized to the kdrl+ vasculature area.
- Figure 4D actually shows measurements of the non-vascular part of the CHT, namely the intervascular loops. It shows that the morphants displays lesser but larger loops. And these measurements were done on 2D confocal images. Calculating the 3D surface of the caudal pleuxs would be an enormously difficult task, as it has a sort of fractal structure, with unclear topology at many places, micro-ramifications in the dorsal-most part that are only discerned at very high magnification (Li et al. Nature 2018), etc… And it is unnecessary here, for the diminished number of perivascular cells merely follows from the diminished number of SCPs (Fig. 4A), which equally reverberates on their three derivatives, the SRCs, SPvCs, and FMCs (Fig. 4D).

3. In Figure 5: in the text the authors state that the endothelial kdrl+ cells did not “often” interact with the SCPs in morphants, but this is not quantified – how many fish out of those viewed had this? There is nothing in the figure legend about this, please include your sample size.

- We have now increased the number of control and morphant Tg(Pax3a:GFP; Kdrl- mCherry-F) larvae subjected to confocal time-lapse imaging (n=11 for each), and analysed the images more closely and systematically also in orthogonal (transverse) view with Imaris. This led us to discern that in all pcdh18a morphant embryos, already early in their migration the SCPs did not adhere to the outer wall of the primordial vein (new Fig. 5Aa-b). We also added arrows in Movie 5, pointing at the normal stromal-endothelial cell interaction in the control embryo, and the isolated/independent migration of stromal cells in the pcdh18a morphant.

Reviewer 3 Advance Summary and Potential Significance to Field:
This study by Touret et al. explores the role of protocadherin-18a (pcdh18a) in development of stromal cells and formation of the hematopoietic niche in the zebrafish embryo. The authors previously published an article in Nature Communications (2015) that showed stromal cells in the embryonic hematopoietic niche are derived from the ventral aspect of the somites. These stromal cell progenitors (SCPs) delaminate and migrate into the caudal hematopoietic tissue (CHT). In this current study the authors find that protocadherin-18a (pcdh18a)
is expressed in the SCPs and that it has a functional role in these cells. There are fewer SCPs in the absence of pcdh18a and they have abnormal migration. Ultimately, this results in a poorly formed CHT niche that does not functional properly to support hematopoietic cells. Interestingly, pcdh18a mediates heterotypic adhesion between endothelial and stromal cells. This study is significant because it highlights novel aspects of a poorly understood process: how the niche stromal and endothelial cells are properly assembled to support hematopoietic cells.

Reviewer 3 Comments for the Author:
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Major points

1. pcdh18a was the focus of this study but pcdh18b is also present in the zebrafish genome. At a minimum, whole mount in situ hybridization should be performed to check for expression of pcdh18b in stromal cells. Ideally, knockdown of pcdh18a and pcdh18b together and individually should be done to check for possible synergy and/or compensation.

- The expression pattern of Pcdh18b has been studied in detail by WISH (Kubota et al. *Int. Dev. Biol*. 2008). It is expressed in the neural tube and the pharyngeal arches, and some endodermal cells – not in the somitic areas. We have further checked that in the tail, we detected no expression in the ventral somites, and only a faint expression by 48 hpf more ventrally (see **Supp. Fig. 4A for the Reviewers**). So we had no reason to try and knock- down a non existent expression in the SCP progenitors.
Compensation effects were not expected, as in the litterature they have been observed for mutants, as opposed to morphants. Nevertheless, we have now checked by qPCR analysis using cDNA prepared from the tails of Pcdh18a Mo injected embryos, and we found no change in pcdh18b expression in pcdh18a morphants relative to control embryos (see Supp. Fig. 4B for the Reviewers).

2. As outlined in the community resource, "Guidelines for morpholino use in zebrafish", it is preferable to have some genetic data to support the splice- blocking morpholino (MO) pcdh18a data. This could be significant, given the authors point out the translation blocking MO referenced from another study has a more severe phenotype than the splice blocking MO. These are several outstanding questions: 1) 1) Do the authors have a stable genetic mutant for pcdh18a (ENU or CRISPR/Cas9)?
- One was published by Bosze et al. (Histochem. Cell Biol. 2020), but is no longer extant, as the authors did not maintain it (we asked them).

2) Have they tested CRISPR/Cas9 reagents for transient 'crispant' mutagenesis? These could be designed to create the same truncation mutation described in the study.
- We have now done crispants which mimicked the effect of the Pcdh18a Mo (retention of intron 1, leading to a shorter protein), and found that they produced the same phenotypes as the morpholino (**new Figs S6**; and see our response to Reviewer 2, 3rd item in Major Point 1).

3) Have the authors performed a rescue experiment that would support specificity of the MO?
- We had attempted to do so for the initial submission. For a discussion on this point, see our response to Reviewer 2, 2nd item in Major point 1, and the corresponding **Suppl. Fig. 2 for the Reviewers.**

3. There is not much data describing the hematopoietic niche defects in the pcdh18a MO embryos. Live imaging of cd41:gfp+ cell colonization of the niche would be informative, as it would indicate if cells: 1) **home to the niche but do not lodge**; 2) lodge in the niche but do not proliferate; or 3) do not home to the niche.
- The fact that the number of HSPCs is reduced since the very start of CHT colonization (36 hpf) pointed to a defect in the homing. We have now checked and confirmed that in more detail, via analysis of extensive time-lapse imaging sequences (**see new Fig. 6B-C, and new Movie 7**). The few HSPCs that homed there correctly lodged to the dorsal side of the venous plexus. They did undergo mitoses, but then tended not to expand to the more ventral side of the CHT. And finally, we detected no sign of apoptosis (see **new Fig. S5**). Please see also our more detailed response to Reviewer 1's Point 2 (same point).

4. There appears to be developmental delay in the pcdh18a MO embryos shown in Fig. 6. Is this a significant issue and does it affect interpretation of the results? For example, the number of cells lodged in the CHT will be less if the embryos are delayed.
- What is seen in the WISH-treated larvae in Fig.6A-d,f is not a developmental delay, but a late effect affecting the tail of morphant larvae after 2 dpf, especially following WISH treatment (see below). As explained in our response to the last item of Reviewer 2's Major point 1, we have now added images of the whole morphant embryos at 2 and 3 dpf, which show that the overall morphology is normal by and for thess stages **(Fig. S2D).** Regarding hematopoiesis, the emergence of definitive HSPCs from the aorta is not delayed in any way (Fig. 6A-a,b).
In the caudal region, some difference arises later. In WT, as the tail progressively grows in length past 2 dpf, the CVP progressively becomes longer and thinner over a few days (Murayama et al.,

Immunity 2006). This progressive thinning is more pronounced in the pcdh18a morphants (new **Fig. S2D, and new Fig. 6B**). In other words, it reflects the temporal evolution of CVP dysmorphogenesis in the morphant larvae, after the one we already documented up to 54 hpf.
The more global apparent malformation of the caudal region in the morphant larvae of Fig. 6A-d,f results from an additional phenomenon that appears to happen when larvae > 2 dpf are fixed and processed for WISH (which involves rather harsh treatments, e.g. dehydratation via passage in methanol, collagenase treatment, heating overnight at 65°C…): the morphant whole tails appear to shrink (and curve) much more in this process than the WT. We suspect this reflects a difference in the composition of the extracellular matrix (possibly related to the excess fibronectin that we have described in the manuscript?).

Minor points
5. Pg. 6 it is mentioned that a manuscript is in preparation for characterization of the TgBAC(cspg4:GAL4) line. As it is presented in this paper, why not include that data as part of the supplement?
- Precisely because the characterization of this new Tg line is part of another submitted manuscript more focused on this cspg4 gene. Nevertheless we now provide additional images of Tg(cspg4:Gal4; UAS:RFP; ET37:eGFP) embryos (**Suppl. Fig. 3 for the Reviewers**).

6. Pg. 6 Fig. 2a likewise, it is mentioned there is additional expression data not shown for the cadherin and protocadherin family members. Could this be included as a supplemental resource?
- The WISH images for these genes at the relevant developmental stages that can be found on the zebrafish community resource (ZFIN.org) are as good as ours, so it would be redundant. We now mention their utility in the manuscript.

7. Typo: Pg. 6 “termination codon 13 codons downstream, hence in a protein retaining only 106 out of” should be “…hence resulting in…”
- The “resulting in” is already present earlier in that sentence: “resulting in a frameshift past codon 734 and a premature termination codon 13 codons downstream, hence in a protein retaining only 106…”. Should we repeat it ?

8. Movie 3 is too short and ends quickly. It is hard to make the observation described in the text. It needs to be slowed down.
- We have slowed it down.

9. Movie 5 needs arrows to show the point of interest
- We have now inserted arrows showing the normal stromal-endothelial cell interaction in the control embryo and the isolated (or independent) migration of stromal cells in the pcdh18a morphant.

10. Fig. S4a expression negative data of other tested ECM molecules should be included as a resource (syndecan 2, cspg4, etc)
- We have now included them in **Fig. S4A**.

11. There is an issue with Fig. legend S4. A description for panel C e-f is missing
- The legend gave a global description of the panels in C (“WISH for *fn1b* at 23, 26 and 36 hpf in control and pcdh18a-ΔCP106 morphant embryos.“); the individual meaning of each panel (control or pcdh18a Mo, developmental stage) is indicated in the panels themselves.

12. Could the authors speculate about the functional significance of fibronectin increase?

- We wrote in the Discussion: “It remains unclear why and how a deficiency in the Pcdh18a- ICD causes an increase in expression of the fibronectin genes in caudal somites and endothelial cells. We speculate that unusual cell-cell interactions caused by Pcdh18a truncation in SCPs may induce a microenvironmental stress and promote the secretion of extracellular matrix, notably fibronectin, by nearby cells.” We have no additional speculation to offer.

13. Is Fig. 6C referenced in the main article text?

- It now is.

14. The meaning of “sheer” in this sentence is not clear: “overexpression in stromal cells of the WT Pcdh18a ECD form led to a sheer inversion of this ratio”

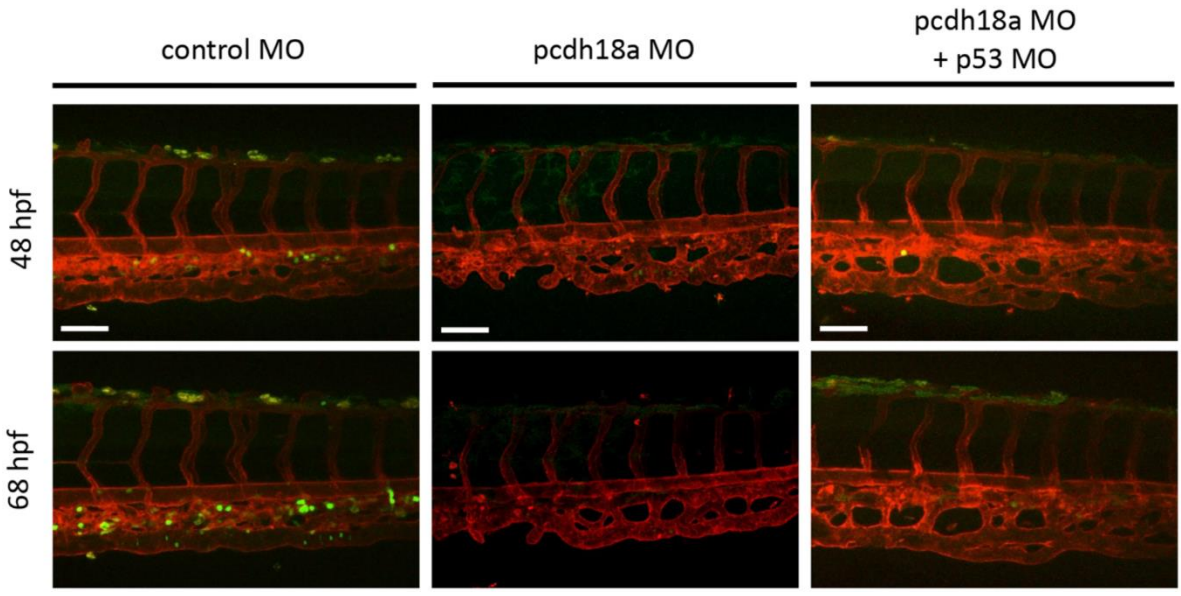
- We replaced “sheer” by “striking”

15. It needs to be clear in the Fig. 7 legend that the results in 7C should be compared to 4D, or uninjected controls should be shown side-by-side in 7C.
- We clearly stated in the main text that the results in 7C should be compared to 4D (we wrote: “…(Fig. 7C). While in normal injected embryos, the ratio of stromal reticular to perivascular cells is 80:20 (Fig. 4D)…”)

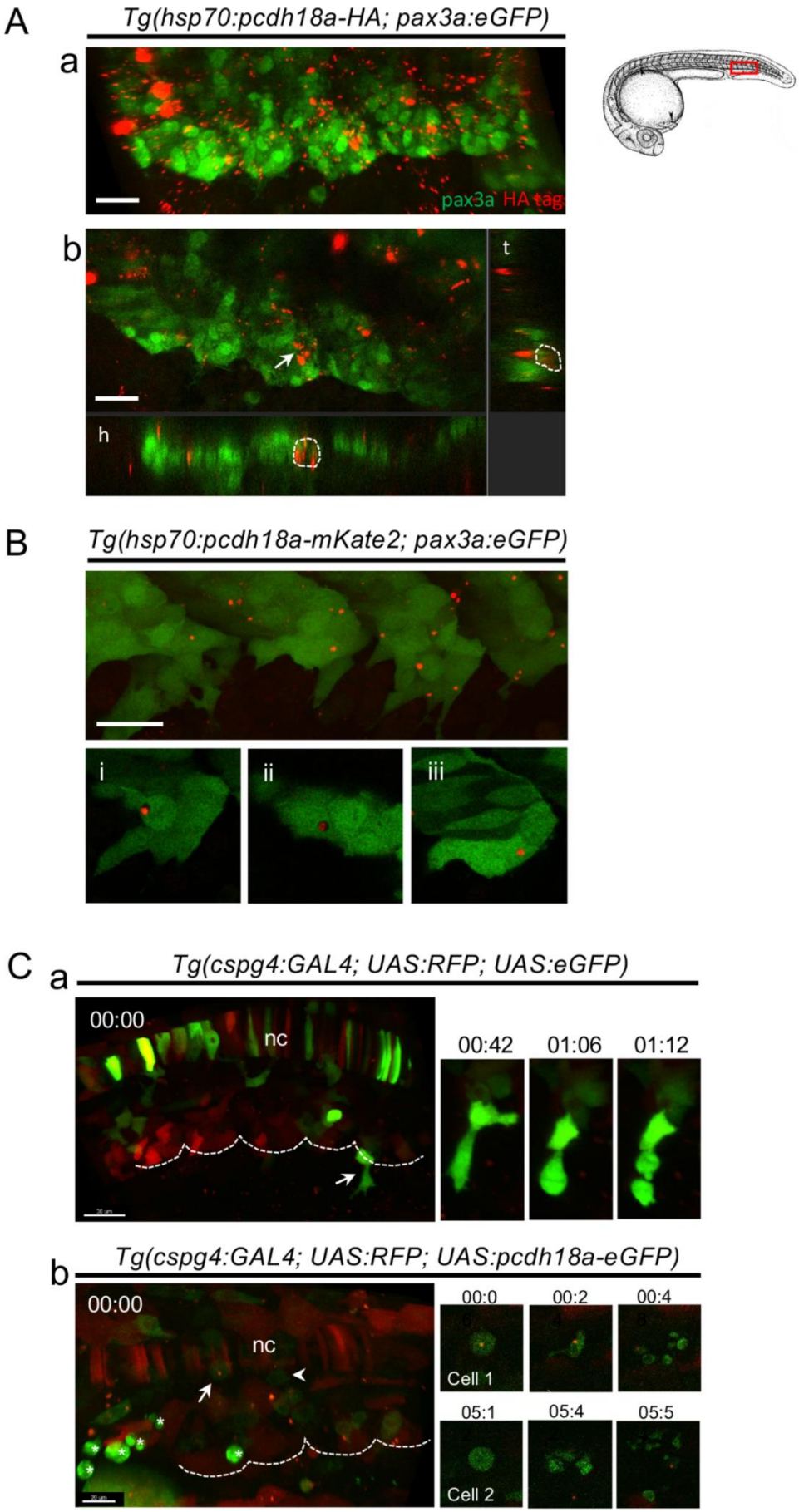
16. Should it be “ECM” and not “ICM” in this sentence: “notably α5β1 that can bind to the RGD motif present on other cells or proteins of the ICM.”
- Yes indeed. Corrected.

Additional extensions of the current study
17. As the pcdh18a phenotype appears to affect endothelial cells as well, as described in Fig. 5, there is no further data to explain how this contributes to the CHT niche phenotype. It would be helpful to understand the distinct role of pcdh18a in endothelial versus stromal cells.
- Pchd18a is not expressed in endothelial cells. Perturbing its expression in the SCPs and their SC derivatives affects SCP/SC number and behaviour, which in turn affects their interaction with the endothelial cells and consequently the structure of the venous plexus. So there is no “distinct role of pcdh18a in endothelial cells” per se. We therefore can’t separate the impact on niche function of pcdh18a perturbation in the stromal cells from the impact of the consequent alteration in venous endothelial cell behaviour.

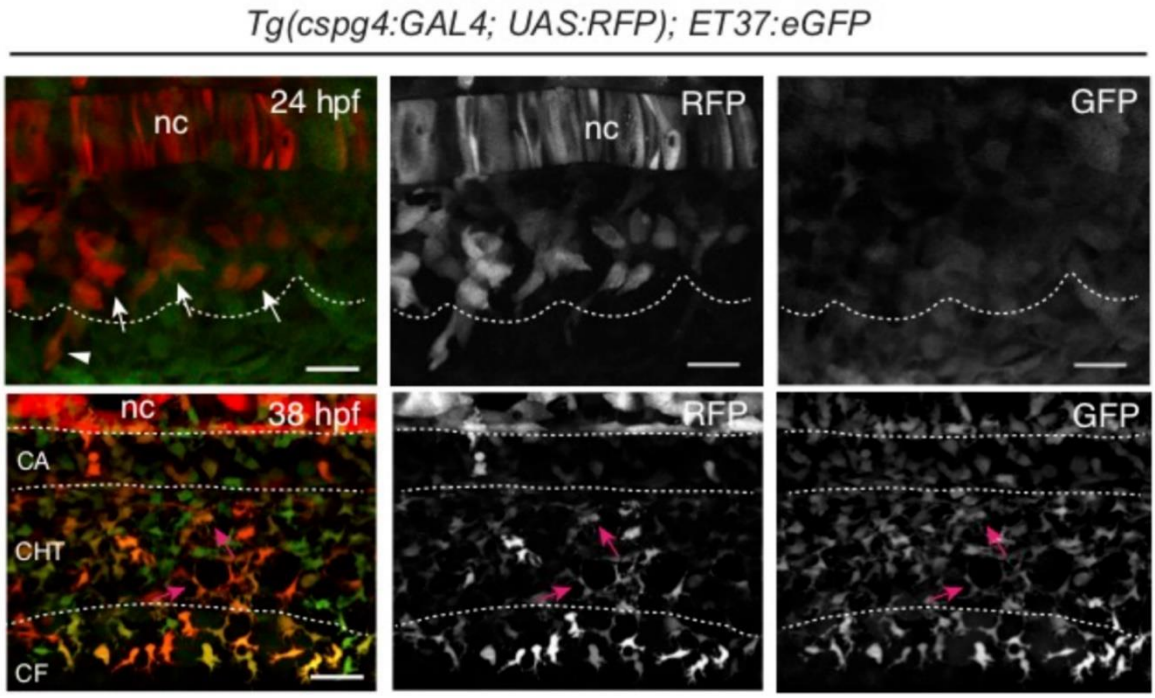
Supplemental Figures for the Reviewers



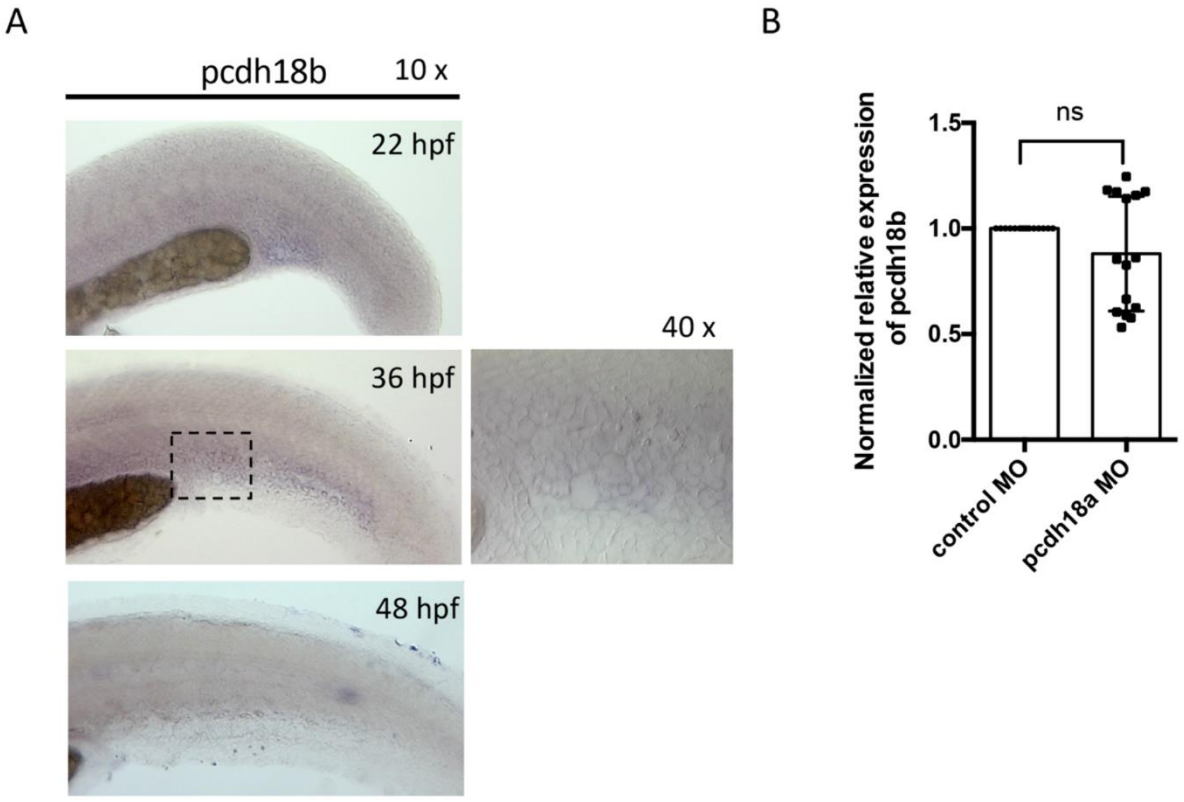
Suppl. Fig. 1 for Reviewers. p53 MO does not rescue the pcdh18 morphant phenotype. Confocal maximum projections extracted at the beginning (48 hpf) and end (68 hpf) of time- lapse imaging sequences of control, pcdh18a MO, and pcdh18a MO + p53 MO (4 ng each) injected *Tg(CD41:GFP; kdrl:ras-mCherry)* embryos. Scale bars, 100 µm.



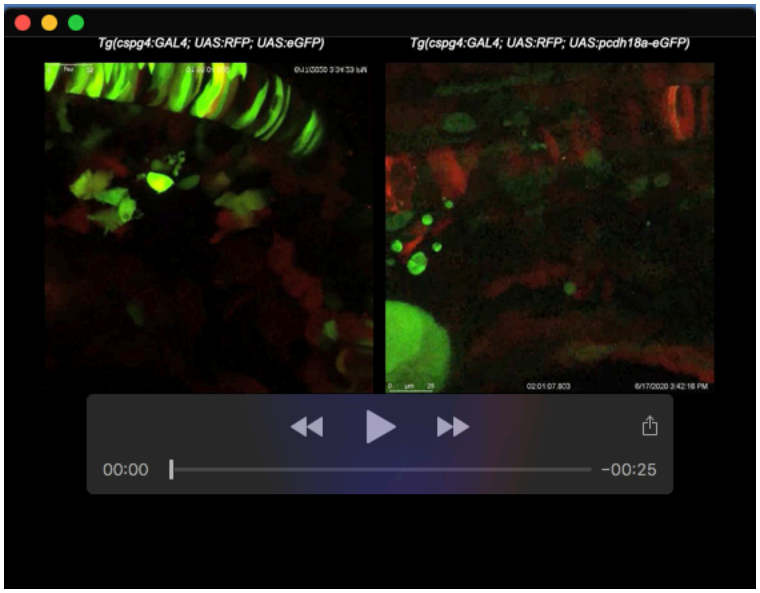
Suppl. Fig. 2 for the Reviewers. Overexpression and visualization of Pcdh18a protein in vivo in the caudal region using various genetic tools. (A) Tg(pax3a:eGFP) embryos were injected with a hsp70:pcdh18a-HA plasmid construct, then heat-shocked at 22 hpf, and immunostained for HA-tag (red) and pax3a:eGFP (green) at 26 hpf. **a,** Confocal maximum projection; **b,** single plane, with horizontal (h) and transverse (t) views focusing on a cell containing several spots of HA-tag signal (arrow). Scale bars, 20 µm. **(B)** Confocal projections of live *Tg(hsp70:pcdh18a-mKate2; pax3a:eGFP)* embryo at 26 hpf. The global view shows sparsely scattered dot-like mKate2 signals; at higher magnification, almost all of these mKate2 dot-like signals are detected within intracellular vesicles (i-iii). **(C)** Confocal projections of *Tg(cspg4:GAL4; UAS:RFP)* embryos injected with UAS:eGFP **(a)** or UAS:pcdh18a-eGFP **(b)** constructs. In the embryos injected with UAS:eGFP construct, the overall GFP intensity in expressing cells was high, whereas in embryos injected with the UAS:pcdh18a-eGFP construct, GFP signals were faint. Asterisks indicate GFP+ apoptotic bodies. The arrow (cell 1) and arrowhead (cell 2) point at the original location of the apoptotic cells shown in the right panel. The timestamps were extracted from time-lapse confocal images acquired every 6 minutes, with the left panel as 00 (hour):00 (minute). Scale bars, 20 **(a)** and 30 **(b)** µm.



Suppl. Fig. 3 for the Reviewers. Tg(cspg4:GAL4;UAS:RFP) embryos highlight stromal cell progenitors even before their migration. Confocal projections of live *Tg(cspg4:GAL4; UAS:RFP; ET37:eGFP)* embryos at 24 and 38 hpf. White arrows and arrowhead in the panel of 24 hpf embryo point at ventral cluster cells and an emerging stromal cell progenitor, respectively; the dashed line outlines the ventral border of caudal somites. In the 38 hpf panels, magenta arrows point at RFP/GFP double-positive cells in the CHT, and dashed lines indicate the boundaries of caudal artery (ca), caudal hematopoietic tissue (CHT) and caudal fin (CF). Scale bars, 20 µm.



Suppl. Fig. 4 for the Reviewers. Expression pattern of pcdh18b in the caudal region, and its expression level in pcdh18a morphant. A, WISH for pcdh18b at 22, 36 and 48 hpf. Faint signals were detected at 36 hpf more ventral than the caudal somites. Dashed square in the 36 hpf panel (x10) indicates the area magnified in the right panel (x40). **B,** qPCR analysis of pcdh18b expression in control and pcdh18a-ΔCP106 morphant embryos at 23 hpf (n=9 for each, from 3 independent experiments). Mean±SD, Student's t-test.



Movie 8, for the reviewers. Time-lapse confocal imaging of *Tg(cspg4:GAL4; UAS:RFP)* embryos injected at the 1-cell stage with UAS:eGFP plasmid (left) or UAS:pcdh18a-eGFP plasmid (right). Arrows in the UAS:pcdh18a-eGFP embryo indicate GFP^+ cells that undergo apoptosis.

Second decision letter

MS ID#: DEVELOP/2021/200278

MS TITLE: A dual involvement of Protocadherin-18a in stromal cell development guides the formation of a functional hematopoietic niche

AUTHORS: Anne-Lou Touret, Catherine Vivier, Anne Schmidt, Philippe Herbomel, and Emi Murayama

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

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the remaining referees' comments (reviewers 2 and 3) can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

The authors have addressed my concerns in the revised manuscript.

Comments for the author

I have no further question.

Reviewer 2

Advance summary and potential significance to field

The authors present novel data on the requirement for protocadherin 18a in the proper development of the CHT hematopoietic niche

Comments for the author

The revised manuscript is significantly improved. We only request that the p53 MO data provided for the reviewers be included in the manuscript as it is a standard experiment expected in the field when a morpholino is used.

Reviewer 3

Advance summary and potential significance to field

The authors have sufficiently addressed the reviewers’ major comments, with only some minor comments that should be addressed to further clarify the manuscript.

Comments for the author

Additional minor points:

Has the data in Fig. S3C been quantified? From the single representative image it looks like TUNEL staining could be higher in the mutant. Also, the WT and MO panels need to be labeled. The need for quantification also applies to Fig. S5.
Could the authors explain the meaning of “conditions” in this sentence? “Pcdh18a cytoplasmic domain conditions stromal cells guidance of venous plexus morphogenesis”
For the crispant data in Fig. S6, the methods state individual embryos were genetically checked. Do they all have the 3 bp deletion at the exon-intron boundary? A variety of different mutations would be predicted among crispant F0 embryos. Were pooled embryos sequenced at any point followed by TIDE or CRISPResso analysis to estimate the gene editing efficiency but also range of mutations?
In the following statement, what does 50% refer to? “Single guide RNAs (sgRNAs) were designed to target around the intron 1 of the pcdh18a gene, and we found one that induced at high frequency (~50%) a deletion of 3 bp at the 3' end of exon1”. Is it the % of embryos that have a phenotype, or is it quantification of the editing efficiency of the guide based on sequencing?
Fig. 7. If the WT REDV ECD of pcdh18a is preferential for perivascular adhesion of SCPs, could the authors speculate in the discussion section about how reticular SCPs are specified in vivo? Is there another protein expressed in reticular SCPs with a domain similar to AEAV?
What does the dotted line in Fig. S6Ab represent?
Should be “It is still not…”, “It still not established whether they can engage heterophilic trans interactions.”
Should be “plane”, Figure 2 label “plan”
Should be “resulting”, “Following injection of this MO at the 1-cell stage, the resutling embryos and swimming larvae displayed a globally normal morphology
(Fig. S2D).”

Could the authors use a different word here? Perhaps “preventing” instead of “refraining”?
“possibly by refraining them to fuse too rapidly”
From previous review: 7.Typo: Pg. 6 “termination codon 13 codons downstream hence in a protein retaining only 106 out of” should be “…hence resulting in…”
- The “resulting in” is already present earlier in that sentence: “resulting in a frameshift past codon 734 and a premature termination codon 13 codons downstream, hence in a protein retaining only 106…”. Should we repeat it ?
Could the authors use “generating” in this sentence? “…hence generating a protein retaining only 106 out of the 399 amino acids of its cytoplasmic domain”

Second revision

Author response to reviewers' comments

Reviewer 2 Comments for the Author:
The revised manuscript is significantly improved. We only request that the p53 MO data provided for the reviewers be included in the manuscript as it is a standard experiment expected in the field when a morpholino is used.

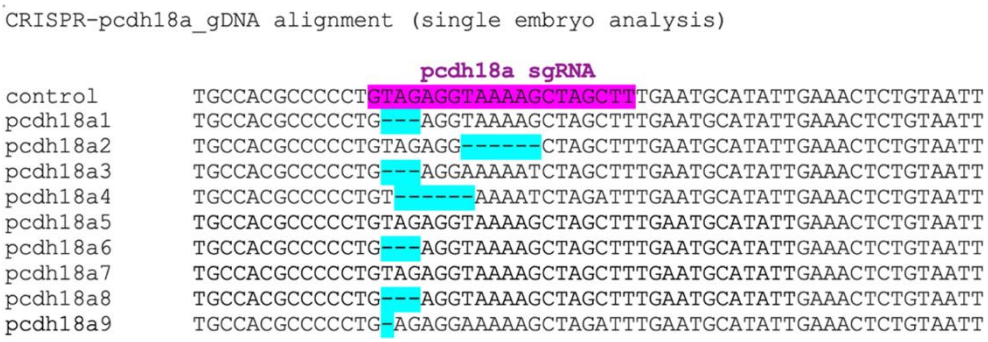
- OK. We have now added it to Fig. S5.

Reviewer 3 Advance Summary and Potential Significance to Field:
The authors have sufficiently addressed the reviewers’ major comments, with only some minor comments that should be addressed to further clarify the manuscript. Additional minor points:
Has the data in Fig. S3C been quantified? From the single representative image, it looks like TUNEL staining could be higher in the mutant. Also, the WT and MO panels need to be labeled. The need for quantification also applies to Fig. S5.

- These experiments demonstrate that apoptosis was not observed in HSPCs nor in stromal cells within the CHT of morphant or control larvae. No statistical treatment was necessary because we detected no TUNEL+/GFP+ double-positive cell. Therefore, we have now only added the number of observed larvae in the legend of Figs. S3C and S5A.

Could the authors explain the meaning of “conditions” in this sentence? “Pcdh18a cytoplasmic domain conditions stromal cells guidance of venous plexus morphogenesis”
- By this we simply mean that without the cytoplasmic domain of Pcdh18a, stromal cells are unable to guide the formation of the venous plexus properly.

For the crispant data in Fig. S6, the methods state individual embryos were genetically checked. Do they all have the 3 bp deletion at the exon-intron boundary? A variety of different mutations would be predicted among crispant F0 embryos. Were pooled embryos sequenced at any point followed by TIDE or CRISPResso analysis to estimate the gene editing efficiency but also range of mutations?
- We have not performed either TIDE or CRISPResso analysis. Genomic DNA was extracted and sequenced from each injected F0 embryo. Nine embryos were successfully analyzed, and individuals with 1, 3 and 6 base deletions were identified in 1, 4 (same deletion site) and 2 (different deletion sites) embryos, respectively. The remaining two embryos showed wild-type sequences (see attached image below).



In the following statement, what does 50% refer to? “Single guide RNAs (sgRNAs) were designed to target around the intron 1 of the pcdh18a gene, and we found one that induced at high frequency (~50%) a deletion of 3 bp at the 3' end of exon1”. Is it the % of embryos that have a phenotype, or is it quantification of the editing efficiency of the guide based on sequencing?

- It is the editing efficiency based on sequencing; the frequency of occurrence of the 3 bp deletions based on sequence analysis was 4/9 (approximately 50%).

Fig. 7. If the WT REDV ECD of pcdh18a is preferential for perivascular adhesion of SCPs, could the authors speculate in the discussion section about how reticular SCPs are specified in vivo? Is there another protein expressed in reticular SCPs with a domain similar to AEAV?

- During development, SCPs gradually develop a reticular type shape as they mature. According to our observations, the peri-vascular type emerges a little later. So the reticular phenotype may be the default differentiation state of these stromal cells. So far we do not know whether SRC-specific CAMs exist. We don’t wish to speculate more on this topic in the Discussion section as our manuscript is already slightly above the size limit specified by the journal.
- AEAV merely is the way we chose to mutate the REDV motif so as to change two of these four amino acids while retaining the overall 3D structure of the protein domain. So there is no reason to look for an AEAV motif in other proteins.

What does the dotted line in Fig. S6Ab represent?
- There are two dotted lines there, one red and one black; their meaning was given in the legend.

Should be “It is still not…”, “It still not established whether they can engage heterophilic trans interactions.”
- Indeed! Corrected.

Should be “plane”, Figure 2 label “plan”
- Indeed! Corrected.

Should be “resulting”, “Following injection of this MO at the 1-cell stage, the resutling embryos and swimming larvae displayed a globally normal morphology (Fig. S2D).”
- OK, corrected.

Could the authors use a different word here? Perhaps “preventing” instead of “refraining”? “possibly by refraining them to fuse too rapidly”
- “Preventing” is fine to us.

From previous review: 7.Typo: Pg. 6 “termination codon 13 codons downstream, hence in a protein retaining only 106 out of” should be “…hence resulting in…”
- The “resulting in” is already present earlier in that sentence: “resulting in a frameshift past codon 734 and a premature termination codon 13 codons downstream, hence in a protein retaining only 106…”. Should we repeat it ?
Could the authors use “generating” in this sentence? “…hence generating a protein retaining only 106 out of the 399 amino acids of its cytoplasmic domain”
- OK, done.

Third decision letter

MS ID#: DEVELOP/2021/200278

MS TITLE: A dual involvement of Protocadherin-18a in stromal cell development guides the formation of a functional hematopoietic niche.

AUTHORS: Anne-Lou Touret, Catherine Vivier, Anne Schmidt, Philippe Herbomel, and Emi Murayama
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.

Reviewer 3

Advance summary and potential significance to field

This study by Touret et al. explores the role of protocadherin-18a (pcdh18a) in development of stromal cells and formation of the hematopoietic niche in the zebrafish embryo. The authors previously published an article in Nature Communications (2015) that showed stromal cells in the embryonic hematopoietic niche are derived from the ventral aspect of the somites. These stromal cell progenitors (SCPs) delaminate and migrate into the caudal hematopoietic tissue (CHT). In this current study the authors find that protocadherin-18a (pcdh18a) is expressed in the SCPs and that it has a functional role in these cells. There are fewer SCPs in the absence of pcdh18a and they have abnormal migration.
Ultimately, this results in a poorly formed CHT niche that does not functional properly to support hematopoietic cells. Interestingly, pcdh18a mediates heterotypic adhesion between endothelial and stromal cells. This study is significant because it highlights novel aspects of a poorly understood process: how the niche stromal and endothelial cells are properly assembled to support hematopoietic cells.

Comments for the author

The authors have sufficiently addressed the reviewers’ comments.