THE
EMBO
JOURNAL

Manuscript EMBO-2015-93454

# Evi1 regulates Notch activation to induce zebrafish hematopoietic stem cell emergence

Martina Konantz, Elise Alghisi, Joëlle Müller, Anna Lenard, Virginie Esain, Kelli J Carroll, Lothar Kanz, Trista E North, Claudia Lengerke

*Corresponding author: Claudia Lengerke, University Hospital Basel*

**Review timeline:**

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|---|---|
| Submission date: | 09 November 2015 |
| Editorial Decision: | 16 December 2015 |
| Additional Correspondence: | 16 December 2015 |
| Additional Correspondence: | 23 December 2015 |
| Resubmission: | 15 June 2016 |
| Editorial Decision: | 18 July 2016 |
| Revision received: | 11 August 2016 |
| Editorial Decision: | 23 August 2016 |
| Accepted: | 23 August 2016 |

Editor: Daniel Klimmeck

### 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 16 December 2015

Thank you for submitting your manuscript for consideration by the EMBO Journal. I apologize for the delay in getting back to you at this time of the year. Your manuscript has now been seen by three referees whose comments are shown below. In light of these comments, I am afraid we decided that we cannot offer publication in The EMBO Journal.

As you can see, the referees appreciate that the analysis extends previous work. However they also raise concerns with the analysis that I am afraid preclude publication here. While Referee #3 is in general positive on the study, Referee #1 outlines significant overlap with previous work, which in his/her view reduces novelty and states lack of mechanistic advance as major concern. Referee #2 shares his/her opinion that the degree of conclusiveness and conceptual advance is not compelling. In addition all referees list an extensive number of unresolved aspects, both on the mechanistic content and on the technical side that in their view undermine the strength of the results and conclusions.

Given these opinions from good experts in the field and as we require strong support from referees to move forward with a manuscript, I am afraid we cannot offer to publish it here. While the technical concerns raised might be addressed to some extent during the course of revision, this would in our view not resolve major concerns related to novelty and conceptual advance, thus would not be sufficient to warrant further steps here.

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Thank you in any case for the opportunity to consider this manuscript. I regret that we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments to be constructive and helpful.

REFEREE REPORTS

Referee #1:

In this paper, Konantz et al present the important role of evi1/pAKT/Notch axis in endothelial-to-hematopoietic transition, contributing to definitive early embryonic hematopoiesis. The authors visually describe the alterations of HSC emergence by evi1 morphant and the overall topic is of interest meets the scope of the journal. However, except for the visualization of altered endothelial-to-hematopoietic transition, the manuscript is constructed by the previously revealed Evi-1 functions in mammals.

Referee #2:

In this work, Konantz et al identify evi1 as a new player in HSC development in zebrafish. They show that evi1 is expressed in the dorsal aorta at the time of HSC generation. The presence of hematopoietic cells (myb/runx1+, globin+, CD41+, lys+, ikaros+, rag-1+)) is reduced by the injection of 2 different morpholinos while the main vasculature is not disrupted. The authors also show that in the knocked down embryos, flk+myb+ cells appear in the DA but they cannot leave and finalise the EHT process. These embryos contain less Notch activity and restoration of it leads to recovery of runx1+ cells. They further test the possibility that PI3K, P-Akt is affected. Decreased levels of P-AKT are detected in Evi MO while reactivation of this pathway can also rescue the hematopoietic development.

This is a novel preliminary observation, however the results are confusing and unclear to support the conclusions.

Major concerns:
The authors claim that Evi1 is important for flk1+/myb+ cells to accomplish the migration from the DA, but not in the specification of these cells. In figure 3B, they quantify the number of leaving HSCs, but they should show as well the number of flk+myb+ cells in the endothelium which would prove that they are specified but have a maturation/migration problem. In fact, flow cytometry determination indicates that all endothelial/hematopoietic subpopulations flk+, myb+ and double flk+myb+ are affected. On the other hand, the flow cytometry dot plots are hard to interpret because the populations are not clearly separated. Are the differences in percentages significant? Is this a representative analysis? Can the authors show the values of at least triplicates?
If as authors say in page 8, Evi1 "specifically regulates the "budding" and release of newly formed hematopoietic cells into the vasculature", they should quantify the effect on the dobule flk+myb+ cells in the different experiments (testing for Notch , AkT, etc).
There are different waves of Notch activity that have been linked to HSC development (Clements et al, 2011, Burns et al, 2005 and Zhang et al, 2015)
The authors claim that Notch is the effector downstream of Evi1, however they map this effect as early as 14hpf or at least they use this time point for Notch experiments. Thus, they could be interfering with the early non-cell autonomous effect of Notch, or the wave of Notch required for specification of EHT rather than associated to maturation. Moreover about 26 hpf Notch needs to be decreased for HSCs to mature (Zhang et al, Cell Research 2015). The authors should test which wave of Notch activity depends on Evi1.

The authors show Evi1 expression at 32 hpf in Fig1. But if it is responsible for the activation of Notch pathway in the early commitment, it should be there much earlier, 16-20hpf. The authors need to clarify this issue as well to understand what is the role of Evi1.

In Akt experiments, the authors need to show that inhibition or activation of the pathway is indeed affecting Notch activity.

Another important issue is that images are not clear in many of the figures, background in the comparable embryos in WISH staining is very different. They should show comparable background. Moreover, many cases images are not convincing for the differences that they claim (ex. Fig 1D, most of Fig 4, Fig 5E,F and supplementary figures).

Statistical analysis has not been performed through out the figures, which makes impossible to take any conclusion.

Figure 7C,D, Akt and P-Akt should be shown from the same blot/sample and the amount of P-Akt should be quantified relative to the total amount of Akt. On the other hand, it is surprising that lower levels of PTEN (90%) in the absence of Evi1, results in lower amount of P-Akt.

Referee #3:

Konantz et al. present a novel role for Evi1 in regulating HSC emergence. Overall, the story is convincing, but the paper suffers in many places from imprecision. If these issues can be addressed, the manuscript should be suitable for publication.

Major issues:

1. The fact that efnb2a is decreased suggests that there may be an arterial defect in Evi1 morphants. That shh can rescue the HSC defect also supports this notion, as this input into Notch signaling is thought to be largely restricted to arterial specification. More attention should thus be given to this issue. For example, does shh overexpression rescue the reduction in efnb2a expression?
2. The HSC emergence phenotype is akin to that observed in the runx1 mutants. Might Evi1 directly regulate the expression of the runx1 gene? In the runx1 mutants, HE forms but nascent HSCs appear to die as they would normally exit the aortic endothelium. Does this occur in evi1 morphants? Imaging could reveal this, as could analysis for PCD using AO or TUNEL staining.
3. Many of the WISH images are difficult to discern. For example, I cannot see DP cells in the Figure 1B and C panels. Perhaps higher magnification would help here?
4. Similarly, numbers should be presented in the figure panels showing WISH patterns to indicate how many animals of the total showed the noted phenotypes.

Minor issues:

1. There is no AGM region in the zebrafish; it is a term that only applies to mammalian anatomy. The use of this term should thus be changed to something like VDA.
2. In the introduction, it is stated that primitive red blood cells are the first hematopoietic wave. Most would argue that primitive macrophages are the first, so the wording here should be altered.
3. It is difficult to see the appropriate bands in the gels show in EV1C. The WT and expected size changes in morphants should be noted.
4. On p. 6, cd41+ megakaryocytes are discussed. There are no megakaryocytes in fish.
5. From the data presented, it appears that there may be a primitive RBC phenotype. Can the authors comment on this?
6. Ikzf1+ lymphoid precursors are discussed on p. 7. What are these? What is the region shown in the figure? It looks like the PBI, but there are no lymphoid precursors present here.
7. On p. 7, the text states that flt1 and flt4 were analyzed. The figure shows flk1, not flt1. It also appears that there exists an ISV phenotype here in the morphants.
8. On p. 9, notch3 and notch1b should be reordered to match the order in the figure.
9. On p. 9, the authors refer to gata2. There are two gata2 genes in teleosts. Which one is referred to here? Did the authors analyze expression of gata2a and gata2b in evi1 morphants? This could help determine at what level in development the Notch inputs are required.

Additional Correspondence 16 December 2015

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Thank you for your detailed response.

I am sorry for the concerns raised by the reviewers on the technical side. I do understand all their

points and think their comments are reasonable. We can address all these points, several additional data have already been generated while the manuscript was under revision.

From your lines though, I understand that the rejection was based not on technical but on novelty concerns.

Based on the comments available to me, novelty is indeed the major concern of Referee 1, but not of Referee 2 who concludes "This is a novel preliminary observation, however the results are confusing and unclear to support the conclusions" and Referee 3 who actually supports publication providing her/his technical concerns are addressed.

Even Referee 1 states that the topic of the project is of interest and meets the scope of the journal.

Referee 1 unfortunately does not provide any information on why she/he considers that most of the data has been already shown in mice (e.g. also no publication that reports an EVI1-AKT-NOTCH axis in murine HSC specification or a role of EVI1 in endothelial-to-hematopoietic transition in mice). We are not aware of any publication showing that HSC emergence is impaired in EVI1-/- mice, and delineating WHY this happens. We have followed the whole time-window of HSC emergence by live imaging and did not observe in any of these movies apoptosis or impairment of proliferation to be the reason for the diminished HSCs. We are now growing two double transgenic kdrl/runx and fli1/cmyb lines, and will be able to visualise the effects of Notch and AKT rescue by in vivo life imaging (as also requested by Referee 2) in early January.
Again, we are not aware of any murine studies reporting this function and molecular targets of EVI1 in HSC development.

In case the Referee 1 feels that the publication showing EVI1-/- mice as having impaired HSC function is the reason for the lack of novelty, we could perhaps convince her/him by additional analyses, for example such as those suggested by Referee 2 and 3.

I was wondering if you would perhaps reconsider and eventually encourage a resubmission using the same Referees - potentially though alternating Referee 1 if she/he does not provide more information on her/his concerns so that we can truly address them.

A potential reviewer with a longstanding expertise on EVI1 but that would not not have competing interests, is for example Prof. Ruud Delwel, at the Erasmus University Medical Center (h.delwel@erasmusmc.nl<mailto:h.delwel@erasmusmc.nl>).

This paper is indeed important to us since we feel that there is much competition in this field at the moment and we would like to lose as less time as possible for publishing it.

Also, I was very happy with the way you handled the manuscript and this encouraged me to approach you this way.

If you would wish to see additional data now or later, or discuss in more detail on the phone, I would be happy to anytime.

Thank you very much for your time.

Additional Correspondence 23 December 2015

Thank you for your letter concerning our recent decision on your manuscript. I have now reviewed your arguments, re-evaluated your manuscript as well as the referees' reports and I have also discussed the work again with the journal's chief editor Bernd Pulverer (who is CCed).

I can see from your letter, that you would possibly be able to address additional experimental issues during revision. However, as pointed out, we were, besides the technical issues raised, mostly concerned about the novelty and conceptual advance needed at a level we have to expect at The EMBO Journal. These concerns were strongly supported by additional comments of reviewers #1

and #2 (enclosed below), who both found that the manuscript was too preliminary in its current form.

In addition, we have now sought advice from a fourth expert in the field, who - in line with referees #2 and #3 - stated the potential interest of the current findings, but raised additional aspects of concern, the first being the exclusive use of morpholinos in the current study as opposed to CRISPR-Cas9 based approaches commonly used to minimize off-target effects. While we see that given the controls provided in the study, CRISPR experiments are not a must-have per se, they however represent an important orthogonal approach, which would in our view significantly strengthen the credibility and impact of the study. As a second point, this referee suggested Evi-1 gain-of function experiments e.g. in an inducible setting, which could be helpful to further dissect its role in EHT and activation of Notch signaling.

I consider the comments of the referees to be generally reasonable, thus given the negative judgement of in particular referees #1 and #2, we are not convinced that experiments suggested would entirely address the concerns raised, in particular by referee #2, and lead to a sufficiently striking advance we need here. Thus, I am afraid that we have decided to maintain our decision not to proceed with the peer-review process to avoid prolonged manuscript processing without benefit here.

I want to emphasize though that given the potential interest stated by the referees, we would in principal be open to a re-submission of a sufficiently complemented manuscript at a later time point, which would then however be treated as a fresh submission and which I would most likely send out to referees #2 and #3 plus the additional referee we sought advice from.

I hope that this letter has clarified the rationale for our decision and re-emphasized the strong demands that we have to apply to satisfy the aim and scope of the journal. I appreciate your thorough discussion of the context and findings and that you approached us further regarding this decision.

ADDITIONAL REFEREE COMMENTS

Additional comment referee #1:

As referee #2 pointed out, it is not a novel finding at least in mammals that Notch pathway is acting in different waves in early hematopoiesis. In addition, It was also reported that Evi-1 promotes para-aortic splanchnopleural hematopoiesis through up-regulation of GATA-2 in mice. Also, several groups reported the interaction between Evi-1 and PI3K/AKT pathway. I also agree with the other referees' opinions that WISH images is difficult to interpret due to different background and the lack of statistical analysis, although a large proportion of the manuscript is based on these data. Taken together, the paper does not reach the enough quality in its present form.

Additional comment referee #2:

This is an interesting observation since Evi1 has not been connected to HSC development. The authors make an effort to decipher the mechanism but they fail in putting it into the context of what is know in zebrafish HSC development. There is a general agreement that Notch is acting in different waves and this work does not contemplate this issue. Altogether it makes the whole mechanism quite hard to integrate into what is known. Last, but not least, the images are not publication quality and also raise lots of concerns. In my opinion this work is too preliminary for publication. I agree that the novelty is an important issue as pointed out by other referees. The fact that Evi1 has already been investigated in the mammalian embryo requires at least some more conceptual advance.

Resubmission 15 June 2016

**Rebuttal letter**

We very much appreciate the detailed comments of the reviewers, which we think have helped us improve the quality of our study significantly. Here we provide a point-by-point response addressing their concerns.

**Referee 4**

Referee 4 raised additional aspects of concern, the first being the exclusive use of morpholinos in the current study as opposed to CRISPR-Cas9 based approaches commonly used to minimize off-target effects.

> We appreciate the reviewer's suggestion. Unfortunately, in spite of testing multiple gRNAs for various regions of *evi1*, we were not able to detect any positive mutations, even by collaborating with the lab of Christian Moosiman, who has nicely shown how to maximize mutagenesis with solubilized CRISPR-Cas9 ribonucleoprotein complexes (Burger et al., Development 2016). However, since we observe dead fish after injection, we assume that potential mutations might be lethal even at mosaic levels (as also observed in *Evi1* knockout mice, which die intrauterinally early in development (Hoyt et al., 1997, Yuasa et al., 2005)). This may also explain, why there is, as of yet to our knowledge, no zebrafish *evi1* mutant available from other sources (e.g. from the Sanger Zebrafish Mutation Project).
>
> We hope that the controls provided in our study (second morpholino, MO, showing the same phenotype and rescue experiments with *evi1* mRNA or UAS:*mEvi1* plasmid DNA) as well as the newly added gain-of-function experiments (see below), also performed in response to the referee's suggestion, provide further evidence that we are not observing off-target effects, but specific functions of *evi1* in our MO experiments. Especially the gain-of-function experiments, which nicely show increased *runx1/c-myb* expression in the VDA after *Evi1* induction, independently reflect opposite results to the loss-of-function MO studies.

As a second point, this referee suggested Evi-1 gain-of function experiments e.g. in an inducible setting, which could be helpful to further dissect its role in EHT and activation of Notch signaling.

> As mentioned above and following the reviewer's suggestion, we performed two conditional *Evi1* overexpression studies. One set targeted endothelial cells only by injecting a murine *Evi1* construct (*UAS:mEvi1*, a codon-optimized version of the murine *Evi1* gene, which is highly conserved to zebrafish *evi1* (Konantz et al., Leukemia 2013)) into Tg(*fli.1:Gal4FF;UAS:RFP)* embryos. The second utilized the same con-

struct in Tg(*-1.5hsp70l:Gal4*) embryos, with global heat-shock induction at 14 hpf (Figure 1I-K). Both experiments showed increased *runx1/c-myb* expression in the VDA following *Evi1* induction. The data have been included in the manuscript and are now shown as Figure 1I-K. Additionally, as hypothesized, *evi1* induction in endothelial cells enhanced *notch1b* levels at this site (see Appendix Figure S7), providing a mechanistic explanation for the enhanced *runx1/c-myb* expression in the VDA and re-inforcing the role of *evi1* in the regulation of Notch at this developmental stage. To our knowledge, although Notch has been strongly implicated in HSC emergence (re-viewed e.g. in Butko et al., Dev Biol. 2016; Kanz, Konantz et al., Ann N Y Acad Sci. 2016), its inductive effects on EHT have not been previously documented using live imaging as shown here.

Furthermore, we have used the overexpression system to explore whether *evi1* regu-lates Notch via or in parallel to its known upstream regulator Vegf. Wild-type and *evi1* inducible fish (generated as described above) were treated with a Vegf receptor inhib-itor (SU5461) at different concentrations (Appendix Figure S8). While in wild-type fish, Vegf inhibition suppressed *notch1b* and *runx1/c-myb* expression, treatment of fish overexpressing *Evi1* in the endothelial compartment resulted in a rescue of both *notch1b* levels and HSPCs in the VDA (see Fig 6B&C). In sum, these gain-of-function data reinforce the results of our MO studies, showing that *evi1* regulates EHT via modulating Notch levels independently of Vegf signaling (see Fig. 7).

**Referee 2**

Major concerns:

The authors claim that Evi1 is important for flk1+/myb+ cells to accomplish the migration from the DA, but not in the specification of these cells. In figure 3B, they quantify the number of leaving HSCs, but they should show as well the number of flk+myb+ cells in the endothelium which would prove that they are specified but have a maturation/migration problem.

At this specific time-point in development, select endothelial cells of the VDA undergo EHT (endothelial-to-hematopoietic transition) and then emerge as HSCs. This pro-cess requires inhibition of the endothelial and concomitant activation of the hemato-poietic molecular program. Our live imaging experiments visualize the transition be-tween these two cell fates; cells that are in between states retain red fluorescence protein expression (indicating that until shortly they had expressed the endothelial gene *kdrl*, Figure 3 or *fli.1*, Figure 5), but have also started to up-regulate *c-myb* driv-ing green fluorescent protein expression, as a sign for their transition to hematopoietic fate. The gene expression data on double positive *kdrl:mKate+/c-myb+* cells indicate

that indeed these cells express both endothelial and hematopoietic programs, but show lower endothelial gene expression than endothelial cells only, and lower hematopoietic gene expression than *c-myb* cells only (Bertrand et al., Nature 2010). As HSCs emerge, they retain some red signal indicative of their endothelial origin (because of the half life of the protein, detectable with even more sensitivity by flow cytometry) (Appendix Figure S3) than by microscopy (Figure 3).

In the revised manuscript version, we have included the data suggested by the reviewer and now show numbers of *kdrl+/c-myb+* double positive cells in the VDA, dividing double positive cells next to quantification of emerging HSCs. Note that double positive *kdrl+/c-myb+* cells are detected at almost equal numbers, and divide similarly to controls, but do not emerge from the VDA when we knockdown *evi1*, as previously indicated (Figure 3).

In fact, flow cytometry determination indicates that all endothelial/hematopoietic subpopulations flk+, myb+ and double flk+myb+ are affected. On the other hand, the flow cytometry dot plots are hard to interpret because the populations are not clearly separated. Are the differences in percentages significant? Is this a representative analysis? Can the authors show the values of at least triplicates?

We agree that the data are not easy to interpret and populations not clearly separated. Gates were, however, set according to dissociated non-transgenic and single transgenic (*kdrl+/c-myb-* and *kdrl-/c-myb+*) fish. The original version of the manuscript showed one representative analysis. We now provide a graph of summarized data from flow cytometry analyses of three independent biological experiments, summarizing the percentages double-positive cells (Appendix Figure S3). Indeed, as noted by the reviewer, double-positive cells are reduced in *evi1* morphants compared to control injected fish (Fig. 3B). However, the cells recognized as double positive by flow cytometry include not only double positive *krdl+/c-myb+* cells detectable in the VDA (Fig. 3B), but also a large proportion (if not all) emerging HSCs that have left this anatomical site (Fig. 3B), since these retain some fluorescent red signal due to the half life of the protein (see also above). This residual red signal is also often detected in the live imaging experiments, but even more readily by the more sensitive flow cytometry method. Therefore, the reduction in double positive cells as detected by flow cytometry is consistent with our live imaging data. Interestingly, in addition to quantification, flow cytometry allowed further investigation of the mechanisms by which *evi1* knockdown reduces numbers of emerging HSCs. Indeed, in *evi1* morphants, double positive *kdrl+/c-myb+* cells have increased endothelial and reduced hematopoietic gene expression as compared to double positive *kdrl+/c-myb+* cells from control in-

jected animals, suggesting that the transition between the two fates is compromised (Figure 3C and Appendix Figure 3). Nevertheless, we believe that the live imaging experiments provide more precise information on the processes involved, especially because EHT can be followed over time (unlike the snapshot provided by FACS analysis), and therefore show these results in the main figure (Figure 3A-B) and the flow cytometry data as part of the supplementary material (Appendix Figure S3).

If as authors say in page 8, Evi1 "specifically regulates the "budding" and release of newly formed hematopoietic cells into the vasculature", they should quantify the effect on the dobule flk+myb+ cells in the different experiments (testing for Notch , AkT, etc).

In order to answer this question, we crossed *Tg(fli.1:Gal4FF;UAS:RFP*) to *Tg(c-myb:EGFP* to generate a double transgenic line that labels both the HSCs as well as endothelial cells and additionally carries a UAS-construct (*Tg(fli.1:Gal4FF;UAS:RFP;c-myb:EGFP)).* This line we then crossed to *Tg(5xUAS-E1b:6xMYC-notch1a)* fish to specifically induce Notch in endothelial cells and to perform *in vivo* live cell imaging experiments after injection of the *evi1* MO. The results are now summarized in Figure 5. Interestingly, endothelial specific induction of Notch not only restored *runx1/c-myb* expression, consistent with our prior observations (see Figure 4B), but also rescued the reduced numbers of emerging HSCs in the VDA observed after *evi1* knockdown.

There are different waves of Notch activity that have been linked to HSC development (Clements et al, 2011, Burns et al, 2005 and Zhang et al, 2015).

The authors claim that Notch is the effector downstream of Evi1, however they map this effect as early as 14hpf or at least they use this time point for Notch experiments. Thus, they could be interfering with the early non-cell autonomous effect of Notch, or the wave of Notch required for specification of EHT rather than associated to maturation. Moreover about 26 hpf Notch needs to be decreased for HSCs to mature (Zhang et al, Cell Research 2015). The authors should test which wave of Notch activity depends on Evi1.

We agree with the reviewer and thank for this helpful comment. In the early rescue shown in the first manuscript version (now in Appendix Figure S5), NICD induction persists for more than 24 hours as shown by Scheer et al. (Development, 2001). Therefore, induction at this early time-point will provide a rescue for both the early and the late Notch waves and cannot distinguish between the different Notch signaling requirements for HSC development (Kim et al., EMBO J, 2014). To address this, we have now performed the rescue experiments at later time-points (20 hpf) and observe a similarly robust rescue of *runx1/c-myb* expression in the VDA following both

NICD (Figure 4A) and VEGF (Figure 4C) induction. This is further supported by the notion that we do not see any changes in the expression of *wnt16* (EVFig. 4C), which regulates somatic expression of Notch ligands, or the sclerotome markers *foxc1b* and *twist1b* (EVFig. 4D-E) (Clements et al., Nature 2011; Kim et al., EMBO J, 2014). From this data, we conclude that *evi1* plays a role during the late window of Notch requirement in the endothelium. We did not specifically perform induction experiments at even later time-points, beyond EHT, where Notch activation has been shown to decrease HSCs.

The authors show Evi1 expression at 32 hpf in Fig1. But if it is responsible for the activation of Notch pathway in the early commitment, it should be there much earlier, 16-20hpf. The authors need to clarify this issue as well to understand what is the role of Evi1.

Indeed, *evi1* is also expressed earlier in the endothelium of the VDA (20 hpf, as now shown in the revised Figure 1A, left), the time-point where Notch levels are required to be present to induce EHT. Figure 1A (middle) shows *evi1* expression at later time-points in the hemogenic endothelium.

In Akt experiments, the authors need to show that inhibition or activation of the pathway is indeed affecting Notch activity.

Thank you for this suggestion. These data are now shown in the revised Figure 7B.

Another important issue is that images are not clear in many of the figures, background in the comparable embryos in WISH staining is very different. They should show comparable background. Moreover, many cases images are not convincing for the differences that they claim (ex. Fig 1D, most of Fig 4, Fig 5E,F and supplementary figures).

We understand the reviewer's concerns and have repeated several *in situ* analyses to show higher quality and/or more representative pictures. Double-ISHs in Fig. 1B-C were repeated and are e.g. now shown at 40x magnification for better visualization (Revised Figure 1A). Since double positive cells in the WISH analysis for both *c-myb* and *evi1* are indeed hard to see, we provide this picture now at 40x magnification in the supplementary information (Appendix Figure 1). Double positive cells are marked with black arrowheads. Additionally, we also repeated the *gata2* mRNA co-injection experiment and provide new pictures (Figure 4D), and show now for rescue experiment WISH analysis of *runx/c-myb* in both *evi1* MO and corresponding rescued animals (Figure 4A-C).

Statistical analysis has not been performed through out the figures, which makes impossible to take any conclusion.

We have now completed statistical analysis by performing a Fisher´s exact test to calculate statistical significance for differences in the *in situ* hybridization patterns (normal vs. decreased gene expression, Figures 1, 2, 4, 6 & 7 as well as EV Figure 2 and Supplementary Figures 1, 3-7) and indicated these numbers on all the respective figures. A p-value of $< 0.05$ was considered to be statistically significant.

Figure 7C,D, Akt and P-Akt should be shown from the same blot/sample and the amount of P-Akt should be quantified relative to the total amount of Akt. On the other hand, it is surprising that lower levels of PTEN (90%) in the absence of Evi1, results in lower amount of P-Akt.

Akt and pAkt show overlapping migratory behavior in standard SDS gels due to the low molecular weight of the phospho-modification. This hampers co-detection of both signals in one blot. Since stripping with our protocols does not eliminate the Akt band completely, we therefore decided to analyze the same samples on different blots, each time using the same amount of protein and actin as a loading control.

Quantification has been performed as follows: first, band intensities have been measured for both Akt and pAkt and the respective actin controls using ImageJ software. Intensities for Akt and pAkt were then normalized to actin levels. The indicated values in the original version of the manuscript reflect indeed the total amount of Akt and pAkt relative to the corresponding controls. We now also provide the data of pAkt levels in relation to the total amount of Akt for each experiment.

We observe slightly increased (110% versus 100%) PTEN levels in evi1 morphants versus control embryo (Figure 7E). The trend in enhancement of PTEN, as a suppressor of pAKT, is consistent with the depleted pAKT levels that we observe in *evi1* morphants. However, we speculate that this modest change is not sufficient to mediate the profound suppression of pAKT and that perhaps other factors play a role. This is now discussed in the revised manuscript version.

**Referee #3:**

Konantz et al. present a novel role for Evi1 in regulating HSC emergence. Overall, the story is convincing, but the paper suffers in many places from imprecision. If these issues can be addressed, the manuscript should be suitable for publication.

Major issues:

1. The fact that efnb2a is decreased suggests that there may be an arterial defect in Evi1 morphants. That shh can rescue the HSC defect also supports this notion, as this input into Notch signaling is thought to be largely restricted to arterial specification. More attention should thus be given to this issue. For example, does shh overexpression rescue the reduction in efnb2a expression?

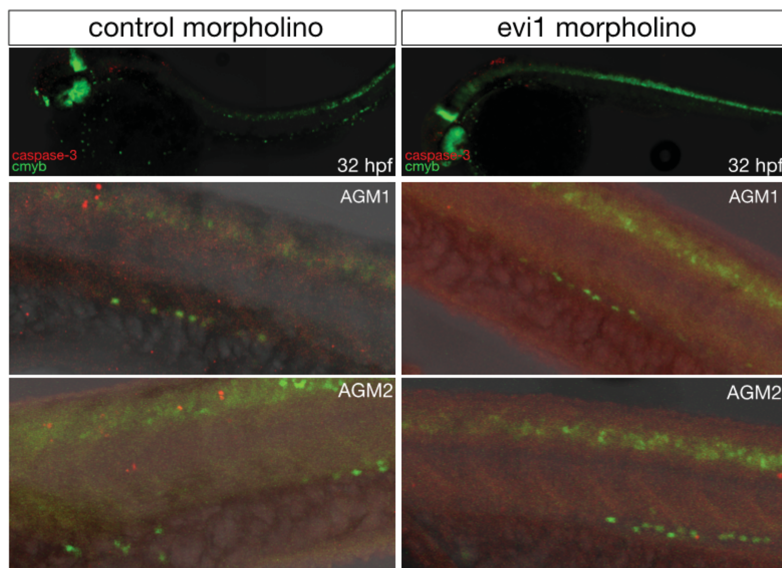
With the attempt of performing the rescue experiments proposed by the reviewer, and following the suggestions on image quality, we performed additional analyses of *efnb2a* in *evi1* morphants and control injected fish using a new *in situ* probe, which provides less background (see Figure EV4B). In a total of 11 analyzed fish from 2 independent experiments, we could not see convincing down-regulation of *efnb2a* using this probe (Figure EV4B). We propose that *evi1* regulates the emergence of HSPCs from VDA cells and is rather not involved in specification of arterial fate itself. In line with that hypothesis, according to our *in vivo* imaging, double positive *kdlr+/c-myb+* or *fli.1+/c-myb+* cells respectively are formed at similar rates, however HSPC emergence is significantly impaired in *evi1* morphants versus control-injected fish (Figures 3 and 5). Furthermore, rescue of the hematopoietic phenotype can be achieved in *evi1* morphants also following NICD induction performed at a time-point previously shown to not be required or alter *efnb2a* expression (Burns et al, 2005). Indeed, in this publication, Burns and colleagues show that induction of NICD during this time window dose-dependently expands HSCs independent of aortic cell fate.

Regarding the rescue, we believe that the reviewer is referring to the vegf induction experiments. *shh* expression was not altered in *evi1* morphants as compared to control fish, suggesting that *evi1* is not modulating Notch levels via *shh* (Figure EV3). Given our data with NICD and the fact that Vegf is an upstream regulator, we believe that in this experimental setting, the Vegf-mediated rescue of the hematopoietic phenotype (Figure 4C) is due to restoration of Notch levels in the VDA of *evi1* MO injected fish (Figure 6A). Importantly, new data added during revision show that *evi1* overexpression restores Notch and HSPCs in embryos treated with the Vegf-inhibitor SU5461 (Figure 6C). Overall, since both *evi1* and Vegf regulate Notch levels, but in-

duction of either can compensate inhibition of the other, we conclude that they represent parallel mechanisms of Notch induction used in conjunction during HE transition to HSC fate.

2. The HSC emergence phenotype is akin to that observed in the runx1 mutants. Might Evi1 directly regulate the expression of the runx1 gene? In the runx1 mutants, HE forms but nascent HSCs appear to die as they would normally exit the aortic endothelium. Does this occur in evi1 morphants? Imaging could reveal this, as could analysis for PCD using AO or TUNEL staining.

To our knowledge, *runx1* has not been identified as a direct target of *Evi1* in healthy HSPCs. In leukemic cells, it has been suggested that RUNX1 activity is repressed by Evi1, but no direct molecular interaction was shown (Senyuk et al, Cancer Res, 2007). During HSPC specification, *runx1* expression is controlled via the Notch pathway. Our data, particularly from the NICD rescue experiments, suggest that *evi1* induces *runx1* via up-regulation of Notch signaling (Figure 4A-B). In regard to comparisons with the cell death seen in *runx1* mutants, analysis of the live imaging data (see Appendix movies 1-3) and anti-activated Caspase-3 staining in *Tg(c-myb:eGFP)* embryos did not reveal dying cells (see RFig1) below), suggesting important differences to the *runx1* mutants. These data suggest that these cells do not attempt to complete EHT, the point at which cells "explode" in the *runx1* line with loss of function mutation, which is consistent with our data showing normal numbers of specified double positive cells.




**RFig1:** Anti-activated Caspase-3 staining in control and *evi1* MO injected Tg(*c-myb:eGFP)* embryos.

3. Many of the WISH images are difficult to discern. For example, I cannot see DP cells in the Figure 1B and C panels. Perhaps higher magnification would help here?

We have now included double ISH pictures with higher magnification (revised Figure 1A and Appendix Figure S1) and repeated several WISH images. Please see also comment to Referee 2.

4. Similarly, numbers should be presented in the figure panels showing WISH patterns to indicate how many animals of the total showed the noted phenotypes.

Embryo numbers have now been added to all the figures. Arrows indicate up- or down-regulation of specific genes in each context. Additionally, graphs have been added to depict differences in the expression patterns (see e.g. Fig 1, 2 and 4) across a particular cohort.

Minor issues:

1. There is no AGM region in the zebrafish; it is a term that only applies to mammalian anatomy. The use of this term should thus be changed to something like VDA.

We have changed this accordingly.

2. In the introduction, it is stated that primitive red blood cells are the first hematopoietic wave. Most would argue that primitive macrophages are the first, so the wording here should be altered.

We have changed this accordingly.

3. It is difficult to see the appropriate bands in the gels show in EV1C. The WT and expected size changes in morphants should be noted.

We now show inverted pictures of the gels for better visualization. The expected size changes are mentioned in the corresponding figure legend (Fig. EV1).

4. On p. 6, cd41+ megakaryocytes are discussed. There are no megakaryocytes in fish.

We have changed this now to CD41+ cells.

5. From the data presented, it appears that there may be a primitive RBC phenotype. Can the authors comment on this?

Following the reviewer's suggestion, we have investigated the effects on primitive erythropoiesis. As shown below, *gata1* expression is unaltered at 12 somite stage (ss) when primitive erythropoietic progenitors are formed. Moreover, in contrast to 6 dpf, where globin expression is strongly reduced in *evi1* MO fish (Figure 1F), at earlier

time-points (36-38 hours, when most erythrocytes are of primitive origin), *globin* expression and respectively numbers of globin positive cells from dissociated whole embryos (measured by flow cytometry) both appear unaltered (Figure RFig 2 - Figure for Referees not shown). Together, these data indicate that *evi1* inhibition is not associated with a primitive RBC phenotype.

6. Ikzf1+ lymphoid precursors are discussed on p. 7. What are these? What is the region shown in the figure? It looks like the PBI, but there are no lymphoid precursors present here.

We had originally included this staining based on the publication by Willet et al., 2001, who showed a band of *ikaros* staining in this region at slightly earlier time-points and assumed this staining to be *ikzf1+* lymphoid precursor cells. As there seems controversy about the identity of these cells, we have removed these data and the accompanying text from the revised version of the manuscript.

7. On p. 7, the text states that flt1 and flt4 were analyzed. The figure shows flk1, not flt1. It also appears that there exists an ISV phenotype here in the morphants.

We thank the reviewer for the correction. Indeed we used probes for *flt1* here and changed this information in the figure legend accordingly (now Figure EV3B). We have carefully analyzed all *in situ* and live imaging data and do not to see an obvious ISV phenotype. Furthermore, blood flow seems to be unaffected (see Appendix movies) and O-dianisidine staining did not reveal pooling as an effect of the MO injection (see Extended Figure EV1D).

9. On p. 9, the authors refer to gata2. There are two gata2 genes in teleosts. Which one is referred to here? Did the authors analyze expression of gata2a and gata2b in evi1 morphants? This could help determine at what level in development the Notch inputs are required.

In this publication, we used full-length *gata2a* mRNA (at that time known as *gata2*) for the rescue experiments. During course of the revisions we obtained the recently published *gata2b* probe (Butko et al., Development, 2015). Indeed, evi1 morphants ver-

sus control injected embryo show downregulation of gata2b in the hemogenic endothelium. These data have been added to the revised Figure 1 (as Fig 1I). *gata2a* and *gata2b* show homologous zinc finger domains (see also Butko et al. 2015), and *gata2a* overexpression might thus compensate the effects of *evi1*-mediated *gata2b* suppression.

2nd Editorial Decision 18 July 2016

Thank you for sending us your complemented manuscript. It has now been seen by three referees - two of the original referees (#2 and #3), as well as the additional expert advisor - and we have received reports from all of them, which I enclose below.

As you will see all referees find that their concerns have been sufficiently addressed and are broadly in favour of publication, pending satisfactory minor revision, and a few editorial issues concerning text and figures that I need you to address.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript using the link enclosed below, addressing the comments of all reviewers.

Please contact me if you have any questions related to the referee comments or if you anticipate any problems.

Please see below for more information on how to revise your manuscript as well as the link for upload.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #2:

The manuscript is greatly improved. All genetic experiments look clear to me and they are publication quality.
However, my only remaining concern is about the biochemistry as a proof for p-AKt induction to be responsible for Notch activation. I acknowledge the effort to show levels from the embryos, but the conclusions are overstated because they rely in complex measures of signals, which is always tricky (for example comparing lanes that are wider than other and actin levels are different), but more important they are not visually obvious. I suggest that they make an effort to load identical levels of actin in all blots, show lower exposure of Akt and show p-Akt and PTEN in panels above. It will be easier to compare and extract conclusions. However, actin should be load equally.

In addition, in Figure 7F, can the authors show the expression of notch1b in the Evi1MO+myr-Akt condition? That is important to show the Evi1-Akt-notch axis and understand the mechanism.

Minor:
Specify what is dlc.

Referee #3:

The authors have now performed a very thorough set of experiments to respond to all of the concerns we previously raised. The paper is greatly improved from inclusion of these new data. We feel the conclusions are now very well supported by the results obtained and that the work is suitable for publication in the EMBO Journal.

Referee #4:

The authors answered satisfactorily to my comments. The text and figures are very clear and easy to understand.
I would ask a modification related to my request of using Crispr/Cas9 to knockout Evi1. They did not manage to generate an Evi1 mutant fish line suggesting "that potential mutations might be lethal

even at mosaic levels". I think this information is interesting enough to be included in the manuscript especially in the light that the Evi1 morpholino did not trigger such as a strong effect.

1st Revision - authors' response 11 August 2016

Responses to the Reviewers' concerns:

Referee #2:

The manuscript is greatly improved. All genetic experiments look clear to me and they are publication quality.
However, my only remaining concern is about the biochemistry as a proof for p-AKt induction to be responsible for Notch activation. I acknowledge the effort to show levels from the embryos, but the conclusions are overstated because they rely in complex measures of signals, which is always tricky (for example comparing lanes that are wider than other and actin levels are different), but more important they are not visually obvious. I suggest that they make an effort to load identical levels of actin in all blots, show lower exposure of Akt and show p-Akt and PTEN in panels above. It will be easier to compare and extract conclusions. However, actin should be load equally.

We have repeated the western blots as requested. We agree that the signals measured by the western blot might be difficult to interpret since they were performed on whole embryo lysates; to investigate expression changes specifically in the VDA, we tried immunohisto- as well as -cytochemical stainings, which unfortunately for both AKT and pAKT did not work in our hands. As suggested by the Reviewer, we analyzed the effect of endothelial specific myr-AKT induction on notch1b expression in evi1 MO transgenic Tg(fli.1:Gal4FF$^{ubs3}$; UAS:RFP)$^{rk}$ embryos. Indeed, induction of myr-AKT in endothelial cells could restore notch1b in the VDA of evi1 morphants, further supporting the notion of an Evi1-Akt-notch molecular axis. We thank the reviewer for this important remark. These data are now shown in the revised Figure 7D and the corresponding Results part on page 12 "(…) Finally, forced endothelial pAKT expression, using a UAS:myr-AKT construct injected into *Tg(fli.1:Gal4FF$^{ubs3}$; UAS:RFP)$^{rk}$* embryos, rescued both *runx1/c-myb* and *notch1b* expression (..)".
Please note that we also repeated immunoblots for Wortmannin and control treated embryos and that western data are now shown as Appendix Figure 9.

Minor:
Specify what is dlc.

We are sorry for the misunderstanding and have now included this information in the results part (see page 9: "….. expression of the Notch ligand *delta C* (*dlc*) and the Notch target gene *efnb2a*…").

Referee #3:

The authors have now performed a very thorough set of experiments to respond to all of the concerns we previously raised. The paper is greatly improved from inclusion of these new data. We feel the conclusions are now very well supported by the results obtained and that the work is suitable for publication in the EMBO Journal.

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The authors answered satisfactorily to my comments. The text and figures are very clear and easy to understand.
I would ask a modification related to my request of using Crispr/Cas9 to knockout Evi1. They did not manage to generate an Evi1 mutant fish line suggesting "that potential mutations might be lethal
even at mosaic levels". I think this information is interesting enough to be included in the manuscript especially in the light that the Evi1 morpholino did not trigger such as a strong effect.

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This information was now added on page 12 of the discussion section. "(…) Notably, embryos injected with multiple gRNAs died suggesting that introduction of mutations using the CRISP-Cas9 system might be lethal even at mosaic levels. (…)"

Responses to the Formatting changes required for a revised manuscript:

>> Please provide Appendix Figure S9B in improved quality

We have repeated the immunoblot for AKT and pAKT after myr-AKT overexpression and provide the new data now as Appendix Figure S9D in better quality.

>> Expanded view figures need to be submitted as individual figure files.

ok

>> Renaming videos: each video should become an 'Expanded View' file => "Movie EV1".... and has to be provided zipped along with its legend as Readme-file > please then upload as Expanded View. The callouts in the article need to be adjusted accordingly to "Movie EV1"... and their mention + legends removed from the Appendix file

ok

Accordingly, a new revised Appendix File needs to be provided.

ok

>> Call-outs to Appendix Figure S4 in the article need to be added

This has been added accordingly (see page 9).

3rd Editorial Decision 23 August 2016

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

FYI, please find enclosed below the final comment of referee #2 who felt that all concerns have been sufficiently addressed and accordingly recommends publication.

Thank you for your contribution to The EMBO Journal.

REFEREE REPORT

Referee #2:

All my comments have been addressed. I recommend publication.

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: Claudia Lengerke


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Manuscript Number: EMBOJ-2016-xx

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## Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

**Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

**In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).**

### 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? | Sample size was chosen according to general assumptions made for zebrafish experimentations: usually 5-10 embryos for detection of differences after treatment, see also "The Zebrafish: disease models and chemical screens" Methods in Cell Biology, Volume 105 |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | See above |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | No fish were excluded from the analysis unless mentioned otherwise |
| 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. | For all experiments, embryos were collected and mixed from all dishes and afterwards randomly assigned to the different analyses. |
| For animal studies, include a statement about randomization even if no randomization was used. | See above |
| 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. | Group allocation: Animals developing in different dishes were mixed and afterwards without any further assessment randomly distributed to the groups. A selection bias during this step can be therefore excluded. Result assessment: individual steps of the experiments were performed by different co-authors, partially (for ca. 30% of the results) in a blinded manner. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | See above. |
| 5. For every figure, are statistical tests justified as appropriate? | Yes, to our knowledge. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | Normal distribution was not observed at this stage during zebrafish development. Therefore, different statistical analyses were used |
| Is there an estimate of variation within each group of data? | NA |
| 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). | All antibodies were purchased from cell signaling technology: rabbit anti-human phospho-Akt (Ser473) (#4060S) = reactive to zebrafish according to the cell signaling data sheet; rabbit anti-human pan-Akt (C67E7) (#4691S) = see "Cep55 regulates embryonic growth and development by promoting Akt stability in zebrafish", rabbit anti-human PTEN (138G6)(#9559S) = see "Altering PI3K—Akt signalling in zebrafish embryos affects PTEN phosphorylation and gastrulation", b-actin antibody (#4967) = see "Inhibition of endothelial ERK signalling by Smad1/5 is essential for haematopoietic stem cell emergence" |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | NA |

* 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. | All experiments were performed on zebrafish embryos. Embryos were obtained from crossing as specified in the manuscript. Transgenic lines were originally obtained from other zebrafish facilities as embryos and then raised to adulthood in our own facility. Zebrafish were bred and maintained as described in Zebrafish – A practical approach (Oxford University Press, 2002) at 28 °C. Following zebrafish strains were used in this study: Tg(lyz:dsred)1, Tg(cd41:eGFP)2, Tg(UAS:myc-Notch1a-intra)kca3,3, Tg(hsp70l:gal4)1.5kca4,4, Tg(hsp:70l:vegfaa,myl7:EGFP)5, Tg(cdh5BAC:gal4ff)mu101, 6, Tg(BAC:kdrl:mKate2- CAAX)UBS16,7, Tg(Tp1bglob:eGFP)um14,8 and Tg(fli.1:Gal4FFubs3; UAS:RFP)rk,9. References for transgenic lines are: 1) Hall, C., Flores, M. V., Storm, T., Crosier, K. & Crosier, P. The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish. BMC Dev Biol 7, 42, doi:10.1186/1471-213x-7-42 (2007). 2) Lin, H. F. et al. Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. Blood 106, 3803-3810, doi:10.1182/blood-2005-01-0179 (2005). 3) Scheer, N., Groth, A., Hans, S. & Campos-Ortega, J. A. An instructive function for Notch in promoting gliogenesis in the zebrafish retina. Development 128, 1099-1107 (2001). 4) Scheer, N. & Campos-Ortega, J. A. Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. Mech Dev 80, 153-158 (1999). 5) Wiley, D. M. et al. Distinct signalling pathways regulate sprouting angiogenesis from the dorsal aorta and the axial vein. Nature cell biology 13, 686-692, doi:10.1038/ncb2232 (2011). 6) Bussmann, J., Wolfe, S. A. & Siekmann, A. F. Arterial-venous network formation during brain vascularization involves hemodynamic regulation of chemokine signaling. Development 138, 1717-1726, doi:10.1242/dev.059881 (2011). 7) Lenard, A. et al. In vivo analysis reveals a highly stereotypic morphogenetic pathway of vascular anastomosis. Dev Cell 25, 492-506, doi:10.1016/j.devcel.2013.05.010 (2013). 8) Parsons, M. J. et al. Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. Mech Dev 126, 898-912, doi:10.1016/j.mod.2009.07.002 (2009). 9) Herwig, L. et al. Distinct cellular mechanisms of blood vessel fusion in the zebrafish embryo. Curr Biol 21, 1942-1948, doi:10.1016/j.cub.2011.10.016 (2011). |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | An approval for zebrafish maintainance has been issued. Experiments were performed only on zebrafish embryos until 7 days post fertilization. Until this stage, they are not considered yet as living animals according to Swiss federal law and therefore procedures undertaken at this stage do not require additional approval via specific animal protocols. |

| | |
|---|---|
| 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. | Yes |

**E- Human Subjects**

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | NA |
| 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. | NA |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | NA |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | NA |
| 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. | NA |
| 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. | NA |

**F- Data Accessibility**

| | |
|---|---|
| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | NA |
| 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). | NA |
| 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). | NA |
| 21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.<br><br>Examples:<br>**Primary Data**<br>Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462<br>**Referenced Data**<br>Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208 | NA |
| 22. 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. | NA |

**G- Dual use research of concern**

| | |
|---|---|
| 23. 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 |