Discrete Notch signaling requirements in the specification of hematopoietic stem cells

Albert D. Kim, Chase H. Melick, Wilson K. Clements, David L. Stachura, Martin Distel, Daniela Pánáková, Calum MacRae, Lindsey A. Mork, J. Gage Crump, and David Traver

Corresponding author: David Traver, University of California, San Diego

Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>21 April 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>08 May 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>26 July 2014</td>
</tr>
<tr>
<td>Accepted</td>
<td>11 August 2014</td>
</tr>
</tbody>
</table>

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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 08 May 2014

Thank you very much for submitting your study on distinct Notch-signaling contributions in HSC-specification for consideration to The EMBO Journal editorial office.

You will find referee comments enclosed below for your kind information.

From these it becomes rather obvious that refs#1 and #2 are rather supportive conditioned on a few clarifications/rescue experiments. While raising a valid point, I believe the functionality issue brought up by ref#1 should for this paper be dealt with in a focused discussion.

In contrast, I do see the requested genetic (and thus definitive) corroboration of at least the most central findings on tNotch1a/b versus Notch3 function much more critical. I would thus encourage to focus revisions/amendments to this manuscript on this point, as it would indeed significantly boost general value and impact of your study.

Given that The EMBO Journal offers 'scooping protection' (publication of related studies from other labs would NOT impinge on a novelty of your findings during revisions), I hope to offer strong incentives to pursue such consolidating work.

Conditioned on such rationale and constructive amendments, I am delighted to invite a revised version of your dataset for final assessment/eventual publication. I realize that these are in part
challenging demands that would however translate into a much improved paper.

Please do not hesitate to get in touch with regard to feasibility and anticipated timeline for the necessary revisions (due to time constrains preferably via e-mail).

I do have to formally remind you that The EMBO Journal only considers one major round of revisions and look forward to hear from you/receive a suitably revised version of your study!

REFEREE REPORTS:

Referee #1:

The authors highlight the importance of discrete notch signalling (specifically notch 1a/b and notch 3) in embryonic HSC (claimed ... blood cell actually) generation. Notch 3 was shown to act in a non-cell autonomous manner and was instrumental for sclerotome formation (as seen by foxclb and twistl1b expression) but not dorsal aorta formation (as seen by efnb2a). However, Notch 1a/b (homologue to murine Notch 1) was shown to act cell-autonomously and was instrumental for dorsal aorta formation but not sclerotome formation. Subsequently both these receptors were important and indispensable for HSC formation (as seen by runx1 expression) at 26 hours post fertilization (hpf).

Moreover, when Notch intracellular domain (NICD) was overexpressed in tissue-specific kdrl+ endothelium cells at 20hpf, runx1+ HSC formation was rescued in notch1a/b morphants thereby elucidating the role of notch1a/b in hemogenic endothelium cells. This was in contrast to NICD induction in somite specific tissues (phlclb+) at 6-15 hpf which was the only condition to rescue runx1+ HSC formation in notch3 morphants. Later induction time points (post 20 hpf) and kdrl+ endothelium NICD induction were not able to rescue runx1+ HSC thereby elucidating a temporal non-cell autonomous role for Notch 3.

Subsequently the authors showed that notch 3 was downstream of somatic wnt16/dlc/dld by showing that notch3 morphants were not rescued by combined dlc/dld mRNA injections. The authors stated that combination of dlc/notch3 (or dld/notch3) knockdown was more severe than single dlc/dld knockdowns and that notch3 morphants were not able to rescue HSC+ in Wnt16 morphants (even with dlc/dld injections - which was previously shown to rescue HSCs in wnt16 morphants).

Comments about paper:
- In introduction paragraph, authors state that there are 'four ordered, temporal waves' during vertebrate development. They only mention three - it would be logical to mention the fourth state which in my mind is the transition of HSCs to the fetal liver and thereafter to the bone marrow.
- Introduction (3rd paragraph) - 'Notch signalling is especially important for the formation and function of the adult hematopoietic system' - this should be clarified to elucidate HSCs made in the embryo. As it stands, makes it sound Notch is important in Bone marrow microenvironment.
- In the results when the authors state 'normal expression, mild expression (Figure S3b and 3D), significant expression (Fig S11A and 11B), etc' - is this purely based on qualitative data? Is there a way to quantify the intensity of the stain so the authors can state a % thereby allowing stats to be performed? In my opinion, this will allow the authors to convincingly state 'significant expression' over another figure.
- Zebrafish is a powerful system to study blood cell emergence. A limitation of the system is that typically no "functional assays" are used to understand the types of cells that are directly emerging. In many instances the authors state that HSC are emerging, but this reviewer is not convinced that the subsequent developmental analysis adequately determines if these cells are HSC, primitive or definitive blood (if such things exist in zebrafish), or are perhaps more restricted cells. Certainly others have considered this issue (for example Len Zon published his PGE2 paper where he first showed zebrafish data and then backed it up with mouse data (CFCs/xenotransplantation studies). It would be interesting for the authors to consider addressing or at least discuss this issue (and perhaps consequently modify claims associated with HSC).

Figure 1
- A cartoon diagram highlighting where the trunk, endothelium, mesoderm, somites would be
appreciated since it'll highlight where expression is situated in the zebrafish embryo for the preceding figures.

Figure 2
- Why is staining in 2A for N3MO kdrl much more intense compared to Figure 1 D N3MO. It would seem kdr staining is low from Figure 1D but Figure 2A shows intense staining of kdrl in N3MO.
- Could the authors show higher magnification insets for Figure 2D,2E,2F. This would allow the readers to see the tissue specific difference.

Figure 3
- Figure 3c) Can the authors explain why N3MO 20HS+ has lower percentages of embryos giving rise to runx1 cells compared to N3MOHS- (the last two columns). It would make sense that they are both similar however the difference looks significant - why would overexpression of notch at later time points be detrimental for HSC numbers?

Figure S8
- Missing caption for Figure G).

Referee #2:
The manuscript by Albert D.Kim et al, entitled: "Discrete notch signaling requirements in the specification of hematopoietic stem cells" studied the function of notch receptors in the hematopoietic stem cell (HSC) specification. The authors discovered that Notch1a and Notch1b, two orthologues of murine Notch1 receptors, are intrinsically required for the transition of hemogenic endothelium to HSCs. Intriguingly, the authors also showed that, unlike Notch1a and Notch1b, Notch3 receptors function earlier in the developing somite to regulate HSC development in a non-cell autonomous manner. Moreover, they went on to demonstrate that the Noth3 receptors act downstream of Wnt16-DeltaC/DeltaD axis in HSC development. This work provides a valuable new insight into our understanding of HSC specification, especially the role of Notch3 in HSC emergence, and it is suitable for publication in EMBO.

Major point
To strengthen the claim that Noth3 acts downstream of Wnt16-DeltaC/DeltaD axis in the regulation of HSC development, it would be nice to show that enforced expression of NICD can rescue HSC development in the Notch3 morphants.

Minor points
1. It would be nice to perform the rescue experiment to prove the notch1b morphant phenotype is specific.
2. In Figure 3 and Figure 5, it is better to include statistic quantification to calculate the significance of the data.

Referee #3:
In their manuscript "Discrete Notch signaling requirements in the specification of hematopoietic stem cells" Kim et al examine the roles that individual Notch receptors play in the formation of hematopoietic stem cells during zebrafish embryogenesis. Using morpholino-mediated gene knock-down combined with sophisticated rescue experiments they expand on previous work that had suggested a dual requirement for Notch signaling during hematopoietic stem cell formation: (a) an early non-cell autonomous requirement for Notch signaling and (b) a later cell-autonomous requirement. The authors now present data to suggest that Notch 1a and Notch 1b are essential mediators of the cell autonomous Notch requirement. By contrast, Notch 3, though expressed by the dorsal aorta endothelial cells that give rise to the hematopoietic stem cells, plays a non-cell autonomous role in their formation by ensuring the correct specification of sclerotomal cells.
While the story appears consistent there are several major important points of concern:

1. The paper is entirely based on morpholino data. Given (a) the obvious reservations against morpholinos and (b) the availability of Notch1a and Notch3 mutants (see for example Quillien et al, 2014; Development 141, 1-9) the manuscript would benefit enormously from having key findings confirmed in the mutants. Firstly, loss of runx1-positive hematopoietic stem and progenitor cells and loss of sclerotomal cells should be verified in Notch3 mutants. Secondly, loss of hematopoietic stem and progenitor cells and retention of sclerotomal cells should be confirmed in Notch1a mutants. Thirdly, differences between this manuscript and the recent Quillien et al., 2014 paper should be address. While Quillien et al saw Notch1b and Notch3 as the main Notch receptors involved in arterial specification. In this manuscript, the authors have identified Notch1a and to a lesser extent Notch1b in this process. Fourthly, since the Notch1b and Notch2 morpholinos have been used for the first time, specificity controls need to be done. Another morpholino that targets a different sequence should be designed against Notch1b and Notch2 to confirm selected key findings.

2. Notch3 is portrayed as the Notch receptor required for the specification of sclerotomal cells within the somites. The idea that Notch signaling is needed for sclerotomal specification was first put forward in the authors' previous paper (Clements et al., 2011). The suggestion was based on a few in situos for sclerotomal markers done on 24 hour embryos that were published as part of the supplementary material. This suggestion has since not been further substantiated. In the current manuscript, only two markers, foxc1b and twist1b, are used to analyze sclerotomal cells. Whether their expression at 24h is truly sclerotomal has not been critically evaluated. Foxc1b expression has previously been suggested to be endothelial at this stage (De Val et al., 2008) and twist expression as shown by Stickney et al., 2000 (Dev Dynamics 219, page 294 figure 4F) may well include both sclerotomal and endothelial cells. The authors should examine whether the expression changes described in this manuscript (Figs. 2, 4 and 6) are truly sclerotomal.

3. The authors suggest that Notch signaling is needed for sclerotomal specification at early somite stages. Thus, Notch3 expression and sclerotomal gene expression would be expected to co-localize early on. The images in Figure S8A show some coexpression of foxc1b and Notch3 in the somites. It is not clear how thick the optical slice is and whether the picture shows the ventral, medial or dorsal section of the somite. A more detailed analysis is needed to validate coexpression in the ventrally located sclerotomal progenitors. Furthermore, early loss of sclerotomal gene expression should be examined in Notch3 morphants/mutants. In this context, please note that Baxendale et al (2009; Gene Expression Patterns 9, 73-82) showed that twist1a expression in sclerotomal progenitors can nicely be distinguished from that in the rest of the somite when embryonic sections are analyzed.

Minor important issues:

1. The labeling of the diagrams 3C, 5C, S6C-D and S9C-D is misleading. It gives the impression that HSC numbers have been counted when in fact numbers of embryos with normal or reduced runx1 expression have been assessed. This needs to be corrected. Since the absolute numbers of embryos are given in the figure panels one may wonder whether these diagrams could not be dropped. The annotation of the Y axis in Fig S5B is also ambiguous.

2. In figure S4, the thickness of the optical slice needs to be given.

3. In Fig S5A, the staining seems to be superficial. Can the authors show us that NICD-Myc expression has been activated in the dorsal aorta? Is figure S5B based on actual embryo counts? The parallel lines would suggest that the diagram is not based on real data.

4. In figure S8F, Notch3 expression values are given without standard deviation and statistical analysis. Given the widespread expression of alpha-actin-egfp in the somites one may wonder how informative the outcome of this experiment is.

5. The authors claim that HSCs are lost in the embryos that were treated late with the inhibitor DBZ. This is not convincing. Runx1 expression is clearly visible in the embryo shown in Fig 4A (right panel), but it is present at a lower level. The description needs to reflect that.
Responses to reviewer concerns:

We thank our reviewers for constructive criticism and a variety of suggested experiments to strengthen our manuscript. We have addressed each concern below and believe the work is now much improved. Changes to the manuscript text have been highlighted in blue.

Referee #1:

The authors highlight the importance of discrete notch signalling (specifically notch 1a/b and notch 3) in embryonic HSC (claimed ... blood cell actually) generation. Notch 3 was shown to act in a non-cell autonomous manner and was instrumental for sclerotome formation (as seen by foxc1b and twist1b expression) but not dorsal aorta formation (as seen by efnb2a). However, Notch 1a/b (homologue to murine Notch 1) was shown to act cell-autonomously and was instrumental for dorsal aorta formation but not sclerotome formation. Subsequently both these receptors were important and indispensable for HSC formation (as seen by runx1 expression) at 26 hours post fertilization (hpf).

Moreover, when Notch intracellular domain (NICD) was overexpressed in tissue-specific kdrl+ endothelium cells at 20hpf, runx1+ HSC formation was rescued in notch1a/b morphants thereby elucidating the role of notch1a/b in hemogenic endothelium cells. This was in contrast to NICD induction in somite specific tissues (phldb+) at 6-15 hpf which was the only condition to rescue runx1+ HSC formation in notch3 morphants. Later induction time points (post 20 hpf) and kdrl+ endothelium NICD induction were not able to rescue runx1+ HSC thereby elucidating a temporal non-cell autonomous role for Notch 3.

Subsequently the authors showed that notch 3 was downstream of somatic wnt16/dlc/dld by showing that notch3 morphants were not rescued by combined dlc/dld mrna injections. The authors stated that combination of dlc/notch3 (or dld/notch3) knockdown was more severe than single dlc/dld knockdowns and that notch3 morphants were not able to rescue HSC+ in Wnt16 morphants (even with dlc/dld injections - which was previously shown to rescue HSCs in wnt16 morphants).

Comments about paper:
- In introduction paragraph, authors state that there are 'four ordered, temporal waves' during vertebrate development. They only mention three - it would be logical to mention the fourth state which in my mind is the transition of HSCs to the fetal liver and thereafter to the bone marrow.

We have changed the text to better describe development of primitive erythroid cells, primitive myeloid cells, erythromyeloid progenitors, and HSCs.

- Introduction (3rd paragraph) - 'Notch signalling is especially important for the formation and function of the adult hematopoietic system' - this should be clarified to elucidate HSCs made in the embryo. As it stands, makes it sound Notch is important in Bone marrow microenvironment.

The text has been changed to 'Notch signaling is especially important for the initial specification of the adult hematopoietic system during embryogenesis.'

- In the results when the authors state 'normal expression, mild expression (Figure S3b and 3D), significant expression (Fig S11A and 11B) etc' - is this purely based on qualitative data? Is there a way to quantify the intensity of the stain so the authors can state a % thereby allowing stats to be performed? In my opinion, this will allow the authors to convincingly state 'significant expression' over another figure.
These statements are indeed garnered from qualitative in situ expression patterns. We have since quantified precisely the number of cmyb:EGFP+; kdrl:RFP+ cells in Notch3 morphants, which demonstrate strong and significant loss of HSPCs at 48 hpf. These new data are presented in Figure 1F.

- **Zebra-fish is a powerful system to study blood cell emergence. A limitation of the system is that typically no "functional assays" are used to understand the types of cells that are directly emerging. In many instances the authors state that HSC are emerging, but this reviewer is not convinced that the subsequent developmental analysis adequately determines if these cells are HSC, primitive or definitive blood (if such things exist in zebrafish), or are perhaps more restricted cells. Certainly others have considered this issue(for example Len Zon published his PGE2 paper where he first showed zebrafish data and then backed it up with mouse data (CFCs/xenotransplantation studies). It would be interesting for the authors to consider addressing or at least discuss this issue (and perhaps consequently modify claims associated with HSC).**

Our laboratory and others have demonstrated that developmental hematopoiesis in the zebrafish proceeds through the same primitive and definitive waves observed in mammals (Davidson and Zon, 2004), Bertrand and Kim et al., 2007). In all vertebrate animals analyzed, the ventral aortic floor generates runx1+ cells that appear to contain the HSCs that go on to colonize the adult animal to sustain lifelong hematopoiesis. This statement is based upon a wealth of data from many model systems (reviewed in Cumano and Godin, 2007, Clements and Traver, 2013). In any system, including the mouse, functional data on whether or not these runx1+ cells are bona fide HSCs is scant. Transplant experiments in mouse have demonstrated that the AGM region contains HSCs, but that these HSCs are likely only a subset of runx1+ cells along the aortic floor. These transplantation assays, however, have been performed into irradiated adult recipients and thus likely markedly underestimate the true frequency of HSCs emerging from hemogenic endothelium. Thus, robust assays in even the most advanced models do not exist for nascent HSCs. Nonetheless, we feel that terming these cells “HSCs” in zebrafish is consistent with the nomenclature used in the field.

In most of our papers, we have worked hard to provide functional readouts for each of the hematopoietic precursors that emerge during embryogenesis. We developed in vitro assays to determine that lmo2+ gata1+ cells were erythromyeloid progenitors based upon their multipotent myeloerythroid potential (Bertrand and Kim et al., 2007), similar to the assays utilized in the mouse embryo by Palis, Keller and colleagues to first demonstrate the existence of EMPs. We showed by transplantation that emerging CD41:eGFP+ cells contain HSCs as evidenced by their contribution to all major hematopoietic lineages, including T lymphocytes in the thymus (Bertrand and Kim et al., 2008). More recently, we have imaged cmyb:eGFP+ cells emerging directly from kdrl:mCherry+ ventral aortic endothelium, and shown by lineage-tracing approaches that all hematopoietic cells in the adult animal derive from kdrl+ hemogenic endothelium (Bertrand and Chi et al., 2010). So, in our work the statement that “no ‘functional assays’ are used to understand the types of cells that are directly emerging” is not accurate. While we are still working to develop more precise means to assay the frequency of bona fide HSCs within our marked populations, study of HSCs in zebrafish has in fact been backed by functional readouts. From the wealth of evidence collected from all systems studied, it is reasonable to conclude that HSCs emerge from ventral aortic endothelium in an ordered progression from runx1+ → CD41/cmyb positive cells in the vertebrate embryo. Since studies in any system have failed to quantify the actual frequency of HSCs within these fractions, a more conservative term to describe these populations is hematopoietic stem and progenitor cells (HSPCs). We have modified our descriptions to include this abbreviation where appropriate.

- **A cartoon diagram highlighting where the trunk, endothelium, mesoderm, somites would be appreciated since it'll highlight where expression is situated in the zebrafish embryo for the preceding figures.**

A cartoon and legend have been added to Figure 2 that indicates the localization of relevant tissues.
Figure 2
- Why is staining in 2A for N3MO kdrl much more intense compared to Figure 1 D N3MO. It would seem kdr staining is low from Figure 1D but Figure 2A shows intense staining of kdrl in N3MO.

Kdrl transcript expression by WISH is more intense in the vessels of notch3 morphants compared to uninjected control embryos, however the effect on RFP expression in kdrl:RFP transgenic embryos is not consistently observed. It is plausible that fluorescence of RFP from this kdrl promoter is less affected than actual levels of kdrl transcript, perhaps due to missing enhancer regions in the transgenic reporter fragment utilized compared to those found in the endogenous kdrl regulatory regions.

- Could the authors show higher magnification insets for Figure 2D,2E,2F. This would allow the readers to see the tissue specific difference.

Higher magnification inserts have been added for these panels now moved to Figure 2F-H.

Figure 3
-Figure 3c) Can the authors explain why N3MO 20HS+ has lower percentages of embryos giving rise to runx1 cells compared to N3MOHS- (the last two columns). It would make sense that they are both similar however the difference looks significant - why would overexpression of notch at later time points be detrimental for HSC numbers?

Recent studies have shown that relief of Notch signaling may be necessary at relatively late timepoints to amplify HSC numbers in the DA (Richard et al., 2014), despite the fact that multiple studies have shown that Notch signaling is needed earlier to specify HSC fate. Our findings show that Notch3 is required earlier than Notch1a/Notch1b. It is thus possible that induction of Notch signaling at an inappropriate timepoint may inhibit amplification of rare ‘escapee’ HSCs in notch3 morphants, consistent with the observations of Richard et al., 2014. This is speculation, however, and will require further inquiry as to what may be happening at these later timepoints.

Figure S8
- Missing caption for Figure G).

Figure S8 has been modified.

Referee #2:

The manuscript by Albert D. Kim et al, entitled: "Discrete notch signaling requirements in the specification of hematopoietic stem cells" studied the function of notch receptors in the hematopoietic stem cell (HSC) specification. The authors discovered that Notch1a and Notch1b, two orthologues of murine Notch1 receptors, are intrinsically required for the transition of hemogenic endothelium to HSCs. Intriguingly, the authors also showed that, unlike Notch1a and Notch1b, Notch3 receptors function earlier in the developing somite to regulate HSC development in a non-cell autonomous manner. Moreover, they went on to demonstrate that the Notch3 receptors act downstream of Wnt16-DeltaC/DeltaD axis in HSC development. This work provides a valuable new insight into our understanding of HSC specification, especially the role of Notch3 in HSC emergence, and it is suitable for publication in EMBO.

Major point
To strengthen the claim that Notch3 acts downstream of Wnt16-DeltaC/DeltaD axis in the regulation of HSC development, it would be nice to show that enforced expression of NICD can rescue HSC development in the Notch3 morphants.

Figure 3 and Figure 5 demonstrate that ectopic NICD induction under specific temporal and spatial control is sufficient to rescue HSPCs in notch3 morphants.
**Minor points**

1. It would be nice to perform the rescue experiment to prove the notch1b morphant phenotype is specific.

In our experience mRNA rescue of Notch receptor genes have proven difficult for technical reasons, likely due to the large ORFs of each. However, induction of NICD rescues HSPC defects in Notch1b morphants demonstrating that the loss of HSPCs is due to loss of Notch signaling. We have since utilized another published notch1b morpholino from Quillien et al., 2014. This study demonstrated that Notch3 and Notch1b receptors are required together to specify dorsal aorta, while loss of either receptor alone (notch3fh332 mutant or notch1b by morpholino) does not impair arterial fate. We have since utilized the notch3fh332 mutant and notch1b morpholino utilized in Quillien et al., 2014 and show that these new reagents closely phenocopy our results. These new data are presented in our revised Figure S5E-F. Taken together, these results strongly support the conclusion that our Notch1b MO is specific.

2. In Figure 3 and Figure 5, it is better to include statistic quantification to calculate the significance of the data.

By runx1 WISH it is difficult to quantify precisely the number of HSPCs. We therefore performed counts of total numbers of embryos that fall into unaffected or lower than wildtype-level expression of runx1. To address this concern, we have now quantified HSPC number by enumerating cmyb:EGFP+;kdrl:RFP+ cells and performed statistical analysis on these data. These new results are presented in Figure 1F.

**Referee #3:**

In their manuscript "Discrete Notch signaling requirements in the specification of hematopoietic stem cells" Kim et al examine the roles that individual Notch receptors play in the formation of hematopoietic stem cells during zebrafish embryogenesis. Using morpholino-mediated gene knock-down combined with sophisticated rescue experiments they expand on previous work that had suggested a dual requirement for Notch signaling during hematopoietic stem cell formation: (a) an early non-cell autonomous requirement for Notch signaling and (b) a later cell-autonomous requirement. The authors now present data to suggest that Notch 1a and Notch 1b are essential mediators of the cell autonomous Notch requirement. By contrast, Notch 3, though expressed by the dorsal aorta endothelial cells that give rise to the hematopoietic stem cells, plays a non-cell autonomous role in their formation by ensuring the correct specification of sclerotomal cells.

While the story appears consistent there are several major important points of concern:

1. The paper is entirely based on morpholino data. Given (a) the obvious reservations against morpholinos and (b) the availability of Notch1a and Notch3 mutants (see for example Quillien et al, 2014; Development 141, 1-9) the manuscript would benefit enormously from having key findings confirmed in the mutants. Firstly, loss of runx1-positive hematopoietic stem and progenitor cells and loss of sclerotomal cells should be verified in Notch3 mutants. Secondly, loss of hematopoietic stem and progenitor cells and retention of sclerotomal cells should be confirmed in Notch1a mutants. Thirdly, differences between this manuscript and the recent Quillien et al., 2014 paper should be address. While Quillien et al saw Notch1b and Notch3 as the main Notch receptors involved in arterial specification. In this manuscript, the authors have identified Notch1a and to a lesser extent Notch1b in this process. Fourthly, since the Notch1b and Notch2 morpholinos have been used for the first time, specificity controls need to be done. Another morpholino that targets a different sequence should be designed against Notch1b and Notch2 to confirm selected key findings.
We have now conducted extensive analysis of the notch3fh332 mutant, notch1a b420 mutant, notch2el517 mutant, and notch1b morpholino utilized in Quillien et al., 2014. We find that the phenotypes observed in these knockdown animals to be consistent with our morphant phenotypes and are presented in revised figures.

Genotyped notch3fh332 mutant embryos showed a comparable reduction of runx1 and foxc1b to notch3 morphants (affected/total numbers indicated in bold), consistent with our observations that approximately 25% of the progeny from notch3fh332 heterozygotes have comparable defects to notch3 morphants (affected/total numbers indicated in parenthesis) (Figures S1).

Progeny of mutant notch1a b420 heterozygotes can be screened by visible somite boundary defects (Figure S5A and B). Mutant embryos (in bold) show reduction in runx1 and efnb2a compared to WT progeny. Mutant numbers were Mendelian (in parenthesis) and showed phenotypes similar to notch1a morphants.

We recently obtained an unpublished notch2el517 mutant (A gift from the laboratory of Dr. Gage Crump) from which genotyped mutant embryos showed little change in runx1 expression but showed loss of intersomitic vessel expression of dlc (in bold). 25% of embryos from heterozygote crosses (in parenthesis) had affected dlc expression similar to our Notch2 morphants (Figure S5C and D).

As stated above in our response to reviewer 1, we utilized the notch1b morpholino from Quillien et al., 2014 (that we refer to as N1bMO2 in figure S5) and found that our notch1b morphant phenotype is comparable with respect to loss of runx1 and synergy with loss of Notch3 in (notch3fh332 mutants and Notch3 morpholino) for efnb2a, demonstrating that our findings are consistent (Figure S5E and F).

2. Notch3 is portrayed as the Notch receptor required for the specification of sclerotomal cells within the somites. The idea that Notch signaling is needed for sclerotomal specification was first put forward in the authors' previous paper (Clements et al., 2011). The suggestion was based on a few in situ for sclerotomal markers done on 24 hour embryos that were published as part of the supplementary material. This suggestion has since not been further substantiated. In the current manuscript, only two markers, foxc1b and twist1b, are used to analyze sclerotomal cells. Whether their expression at 24h is truly sclerotomal has not been critically evaluated. Foxc1b expression has previously been suggested to be endothelial at this stage (De Val et al., 2008) and twist expression as shown by Stickney et al., 2000 (Dev Dynamics 219, page 294 figure 4F) may well include both sclerotomal and endothelial cells. The authors should examine whether the expression changes described in this manuscript (Figs. 2, 4 and 6) are truly sclerotomal.

We thank the reviewer for this helpful suggestion, and have performed WISH analysis of sclerotome markers at earlier timepoints to better assess sclerotome specification. We find that at 13 and 17 hpf effects on sclerotome markers are downregulated in Notch3 deficient embryos (Figure S2).

3. The authors suggest that Notch signaling is needed for sclerotomal specification at early somite stages. Thus, Notch3 expression and sclerotomal gene expression would be expected to co-localize early on. The images in Figure S8A show some coexpression of foxc1b and Notch3 in the somites. It is not clear how thick the optical slice is and whether the picture shows the ventral, medial or dorsal section of the somite. A more detailed analysis is needed to validate coexpression in the ventrally located sclerotomal progenitors. Furthermore, early loss of sclerotomal gene expression should be examined in Notch3 morphants/mutants. In this context, please note that Baxendale et al (2009; Gene Expression Patterns 9, 73-82) showed that twist1a expression in sclerotomal progenitors can nicely be distinguished from that in the rest of the somite when embryonic sections are analyzed.

In Notch3 morphants at 13 hpf there is significant reduction of foxc1b, twist1a, and twist1b expression lateral to the midline in the somites:
At 17 hpf, reduction of foxc1b, twist1a, and twist1b expression is likewise observed in notch3<sup>W332</sup> mutant embryos (genotyped in bold, from clutches of heterozygote parents in parenthesis) and notch3 morphants (Figure S2).

Minor important issues:
1. The labeling of the diagrams 3C, 5C, S6C-D and S9C-D is misleading. It gives the impression that HSC numbers have been counted when in fact numbers of embryos with normal or reduced runx1 expression have been assessed. This needs to be corrected. Since the absolute numbers of embryos are given in the figure panels one may wonder whether these diagrams could not be dropped. The annotation of the Y axis in Fig S5B is also ambiguous.

The figure labels have been changed to clearly state percentage of embryos with wild-type or affected phenotypes.

2. In figure S4, the thickness of the optical slice needs to be given.

This image was obtained with instrument settings that predict a calculated optical section thickness of 1.47 µm. The legend (now Figure S6) has been updated with this information.

3. In Fig S5A, the staining seems to be superficial. Can the authors show us that NICD-Myc expression has been activated in the dorsal aorta? Is figure S5B based on actual embryo counts? The parallel lines would suggest that the diagram is not based on real data.

In this revised manuscript, we have focused mainly upon validating our morpholino results with mutants, and honing our lineage-specific rescue experiments, which we believe are more convincing than refining the hsp70-based rescues. We have thus removed what was Figure S5, as we did not feel it added much to the paper. To respond to these queries: Following heatshock induction, NICD-cmyc is detected throughout the entire embryo including the vasculature. Convincing demonstration of this, however, would require co-staining with a vascular marker. Figure S5B was not based on embryo counts, but on qualitative observation that peak staining is achieved by 3 hours and detectable by 1 hour as previously observed in Clements et al., 2011. We have removed this figure and updated the text with this information.

4. In figure S8F, Notch3 expression values are given without standard deviation and statistical analysis. Given the widespread expression of alpha-actin-egfp in the somites one may wonder how informative the outcome of this experiment is.

We agree, and believe that inclusion of this data is not central to the message of the paper and have removed this figure.
5. The authors claim that HSCs are lost in the embryos that were treated late with the inhibitor DBZ. This is not convincing. Runx1 expression is clearly visible in the embryo shown in Fig 4A (right panel), but it is present at a lower level. The description needs to reflect that.

The description has been changed to more accurately state that runx1 levels are reduced in DBZ-treated embryos.