Jagged 2b induces intercellular signaling within the somite to establish hematopoietic stem cell fate in zebrafish

Yukino Wada, Hikaru Tsukatani, Chihiro Kuroda, Yurika Miyazaki, Miku Otoshi and Isao Kobayashi
DOI: 10.1242/dev.200339

Editor: Hanna Mikkola

Review timeline
Original submission: 5 November 2021
Editorial decision: 29 December 2021
First revision received: 9 February 2022
Accepted: 17 March 2022

Original submission

First decision letter

MS ID#: DEVELOP/2021/200339

MS TITLE: Jagged-2b induces intercellular signaling within the somite to establish hematopoietic stem cell fate in zebrafish

AUTHORS: Yukino Wada, Hikaru Tsukatani, Chihiro Kuroda, Yurika Miyazaki, Miku Otoshi, and Isao Kobayashi

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Wada et al. identified the novel Notch-regulatory pathway related to HSC specification in somites using zebrafish genetics. They showed that Notch ligand Jagged-2b induced Notch activation and its target Ephrin A1b in adjacent cells, which induced Wnt16 in next adjacent cells. Since they previously demonstrated that Wnt16 in somites induced another somitic Notch ligands Dlc and Dld, which directly instruct HSC fate in vascular precursor cells, they concluded that Jag2b-driven Notch signaling regulates Ephrin A1 expression in the somite to modulate the Wnt16 – Dlc/Dld signaling axis, which is required for HSC specification.

Overall, this paper provides important advances in understanding the relationship between somitic Notch signaling and HSC cell fate. The study is well performed, and the manuscript is well written. This interesting work seems to be suitable for publication in Development.

Comments for the author

The only things this reviewer would like to ask authors are following points:

Major points:
1. This reviewer agrees with authors' comment that genetic compensation may be the reason for phenotypic differences between jag2b mutants and jag2b crispants/morphants. To strengthen their idea, authors should perform expression analysis of jag2a and/or other jags in jag2b mutants and rescue analysis of reduced runx1 expression in jag2b crispants using jag2a and/or other jags.
2. Since this finding is novel not only for zebrafish but others. Authors should discuss the deduced roles of Jagged-2b in mammalian hematopoiesis.
3. It is quite interesting to find Ephrin-Eph signaling in somites plays roles in HSC cell fate. Since Ephrin-Eph signaling has been shown to play critical roles in mammalian hematopoiesis, authors should mention more about this topic in Discussion.
Minor comment:
"enfa1bkz5" should be "efna1bkz5" in page 7 line18.

Reviewer 2

Advance summary and potential significance to field

This is an elegant study that adds a new element to the cascade of interactions that takes place in the somite before hemogenic cells are formed and move to the floor of the aorta. The authors show that Jag2b is responsible for activating Notch, that in turn activates Efna1b, that activates Wnt16, and downstream Dlc and Dld, that will activate Gata2b through Notch. The authors have elaborated on previous data obtained in the Traver's lab. They now complete some gaps, for example with Jag2b at the top of the cascade and activaton of efna1. This cascade is very useful to understand how these cells behave.

Comments for the author

This is a very clear and elegant work from the Kobayashi group. They have prepared very clear figures, in which we can follow the sequence of events from the expression of jag2b at 11h hours to the first HSCs at 28h. The fact that signals from the somite are inducing the hemogenic endothelium and it would be nice to see a movie tracing angioblast from the somite moving to the dorsal aorta. Although the work is a follow up of previous work from Traver's lab, they complete the cascade by involving 2 different Notch signals, one from Jag2b and another one by Dlc and Dld. In addition, they fill the gaps in between with efna1 and Wnt16.
The authors show in Figure 1 that jag2b expression starts at 11hpfh but the authors should test the kinetics of other Notch ligands in order to exclude that they are also involved. Similarly, in the experiments of gRNA to knockout specific factors.

It would also be good that they speculate about the different Notch signals that are required and seem to come from different ligands. The authors could speculate on the different action of these ligands in terms of Notch activation.

Reviewer 3

Advance summary and potential significance to field

In this manuscript, the authors revisit the early stages of Hematopoietic Stem Cell (HSC) specification and the role of Jagged-2b (Jag2b) in this process. Using both jag2b morphant and mutant embryos, the authors demonstrate that early Jag2b expression within the somite is necessary for the downstream signaling axis of wnt16 and dlc/dld; previously shown to be required for HSC specification. Next, the authors demonstrate that jag2b-Notch signaling operates non-autonomously within the somites to regulate wnt16 by inducing the expression of efna1b. In addition, the authors performed an array of overexpression and rescue experiments that nicely complement their main findings.
The manuscript is comprehensive and well written. The experiments follow a logical trajectory and are well designed and controlled. The manuscript covers significant points and open questions in early hematopoiesis. This manuscript adds essential findings to the field. Following are several suggestions for improvement.

Comments for the author

Major concerns:
1. Much of the analysis in the paper is based on the quantification of expression levels. Please clarify how the expression levels were quantified and translated into the statistical analysis. Enumeration of cells would provide more convincing statistics. The one experiment in which this was done – quantification of gata2b+ cells in jag2bsgRNA animals – shows differences which are not very convincing.
2. An important part of the story is the cell-cell communication between cells in the developing somite.
While the sections provide some support for this model, this point is not very clear. FISH would provide a more compelling means to demonstrate non-cell-autonomy. This is particularly important for efna1b and wnt16 expression patterns.
3. The authors observed no phenotype in the complete mutants and hypothesis that it is due to compensation. How do the authors explain that in the sgRNA mutants, there is no compensation? The RNA levels are reduced by almost 96%, but that could be due to RNA decay that can still lead to compensation.
4. It was previously shown by Clements et al. that loss of Wnt16 leads to a near complete loss of the sclerotome. It is therefore puzzling that loss of efna1b does not lead to this same phenotype. How do the authors explain this discrepancy?
5. The phldb1 line shows some expression within LPM cells, leading to a concern that the NICD rescue experiment may not be acting within the somite. It is advised to employ another somitic GAL4 driver to replicate this result.
Minor concerns:
1. Figure 1 panel H: What are the positive cells along the DA? Do DA cells express jag2b? Please clarify.
2. The authors show a reduction in wnt16, dlc, and dld expression in jag2b mutants and morphants.
Later they show a reduction of wnt16 in efna1b mutants and morphants. Can the authors complement the results and show dlc/dld reduction in efna1b morphants?
3. In figure 6, panels C and D: please label the Runx1 probe.
4. Is there evidence for direct interaction between efna1b and rspo1?
5. Throughout the manuscript, the authors differentiate between different somite compartments.
Perhaps the authors can add a sketch of the compartments to their current model.

First revision

Author response to reviewers' comments

We thank the reviewers for their many helpful suggestions. We believe the manuscript is now greatly improved by the inclusion of new data generated in response to reviewer comments.

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript, Wada et al. identified the novel Notch-regulatory pathway related to HSC specification in somites using zebrafish genetics. They showed that Notch ligand Jagged-2b induced Notch activation and its target Ephrin A1b in adjacent cells, which induced Wnt16 in next adjacent cells. Since they previously demonstrated that Wnt16 in somites induced another somitic Notch ligands Dlc and Dld, which directly instruct HSC fate in vascular precursor cells, they concluded that Jag2b- driven Notch signaling regulates Ephrin A1 expression in the somite to modulate the Wnt16 - Dlc/Dld signaling axis, which is required for HSC specification.

Overall, this paper provides important advances in understanding the relationship between somitic Notch signaling and HSC cell fate. The study is well performed, and the manuscript is well written. This interesting work seems to be suitable for publication in Development.

We gratefully appreciate the constructive comments on our manuscript.

Reviewer 1 Comments for the Author:
The only things this reviewer would like to ask authors are following points:
Major points:
1. This reviewer agrees with authors' comment that genetic compensation may be the reason for phenotypic differences between jag2b mutants and jag2b crispants/morphants. To strengthen their idea, authors should perform expression analysis of jag2a and/or other jags in jag2b mutants and rescue analysis of reduced runx1 expression in jag2b crispants using jag2a and/or other jags.

We agree on the importance to investigate which *jagged* genes are upregulated in *jag2b* mutant embryos. According to the reviewer's suggestion, we performed expression analysis of *jag1a*, *jag1b*, and *jag2a* in $jag2b^{kz6/kz6}$ embryos. Surprisingly, we found that both *jag1a* and *jag1b*, but not *jag2a*, were upregulated in $jag2b^{kz6/kz6}$ embryos. Whole-mount *in situ* hybridization of *jag1a* and *jag1b* revealed that although *jag1a* and *jag1b* were predominantly expressed in the notochord in wild type embryos, upregulation of these two genes was observed in the whole body in $jag2b^{kz6/kz6}$ embryos (Fig. S2F, G). These data suggest that the phenotypic differences between $jag2b^{kz6/kz6}$ embryos and *jag2b* crispants/morphants may be due, at least in part, to the upregulation of *jag1a* and/or *jag1b*. In zebrafish, Jag1a has been shown to be involved in HSC development by activating Notch signaling in endothelial cells within the aortic floor (Espin-Palazon et al., *Cell* 2014; Monteiro et al., *Dev Cell*. 2016). Therefore, even if forced expression of *jag1a* (or *jag1b*) rescues *runx1* expression in $jag2b^{sgRNA}$ embryos, this could be mechanically distinct from the phenotype observed in $jag2b^{sgRNA}$ embryos. Furthermore, it is also likely that multiple Notch-related genes, including *jag1a* and *jag1b*, simultaneously compensate HSC development in *jag2b* mutant embryos. Unfortunately, we are therefore unable to determine the detailed compensation mechanisms in *jag2b* mutant embryos. Importantly, however, *jag1a* and *jag1b* expression was unchanged in $jag2b^{sgRNA}$ embryos and *jag2b* morphants (Fig. S2F, G), suggesting that the results in $jag2b^{sgRNA}$ embryos and *jag2b* morphants more precisely reflect the loss of *jag2b* than those in *jag2b* mutant embryos.

We added the expression data of *jag1a*, *jag1b*, and *jag2a* to Fig. S2F and G in the revised version of the manuscript.

2. Since this finding is novel not only for zebrafish but others. Authors should discuss the deduced roles of Jagged-2b in mammalian hematopoiesis.

Thank you very much for this suggestion. The role of Jag2 in mouse hematopoiesis was described in the introduction in the revised version of the manuscript as follow:

“Jag2 signaling has been shown to promote the survival and proliferation of hematopoietic progenitors in the murine bone marrow, whereas it is also involved in T cell differentiation in the thymus (Tsai et al., 2000; Van de Walle et al., 2011). However, the role of Jag2 in HSC development is undetermined in both mammals and zebrafish.”

3. It is quite interesting to find Ephrin-Eph signaling in somites plays roles in HSC cell fate. Since Ephrin-Eph signaling has been shown to play critical roles in mammalian hematopoiesis, authors should mention more about this topic in Discussion.

We described the role of Ephrin B2 – Eph B4 interaction in erythroid differentiation in the discussion of the revised manuscript as follow:

“In mammals, Eph B4-expressing HSCs interact with Ephrin B2-expressing mesenchymal stromal cells, and this interaction triggers detachment of HSCs from stromal cells, leading to erythroid differentiation of HSCs (Foo et al., 2006; Suenobu et al., 2002).”

Minor comment:
"enfa1bkz5" should be "efna1bkz5" in page 7 line18.

Thank you for pointing out the error. We improved this error in the revised version of the manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field:
This is an elegant study that adds a new element to the cascade of interactions that takes place in the somite before hemogenic cells are formed and move to the floor of the aorta. The authors show that Jag2b is responsible for activating Notch, that in turn activates Efna1b, that activates Wnt16, and downstream Dlc and Dld, that will activate Gata2b through Notch. The authors have elaborated on previous data obtained in the Traver's lab. They now complete some gaps, for example with Jag2b at the top of the cascade and activaton of efna1. This cascade is very useful to understand how these cells behave.

Reviewer 2 Comments for the Author:
This is a very clear and elegant work from the Kobayashi group. They have prepared very clear figures, in which we can follow the sequence of events from the expression of jag2b at 11h hours to the first HSCs at 28h. The fact that signals from the somite are inducing the hemogenic endothelium and it would be nice to see a movie tracing angioblast from the somite moving to the dorsal aorta. Although the work is a follow up of previous work from Traver's lab, they complete the cascade by involving 2 different Notch signals, one from Jag2b and another one by Dlc and Dld. In addition, they fill the gaps in between with efna1 and Wnt16.

Thank you very much for your constructive comments and interesting suggestions on our manuscript. As the reviewer mentioned, the main finding of our study is the signal transduction triggered by Jag2b within the somite, providing new mechanistic explanations in the spatiotemporal regulation of HSC specification by the somite. It has been shown that the dynamics of angioblasts are controlled predominantly by cell adhesion molecules expressed between angioblasts and somites, such as Jam proteins and integrins (Kobayashi et al., *Nature* 2014; Rho et al., *Dev Cell*. 2019). Therefore, it is unlikely that the migration of angioblasts is directly regulated by Jag2b. However, the dynamics of angioblasts may also be dependent on the individual cell fate that is regulated by Notch signaling. Future studies may elucidate the relationship between cell fate, distribution, and Notch activity in angioblasts.

The authors show in Figure 1 that jag2b expression starts at 11hpfh but the authors should test the kinetics of other Notch ligands in order to exclude that they are also involved. Similarly, in the experiments of gRNA to knockout specific factors.
Following reviewer suggestions, we examined *jag1a* and *jag1b* expression in wild type embryos at 12 hpf, and neither of them was expressed in the somite (new Fig. S2G). However, Jag1a has been shown to regulate HSC development through Notch activation in endothelial cells within the

aortic floor Espin-Palazon et al., *Cell* 2014; Monteiro et al., *Dev Cell*. 2016). These observations suggest that multiple waves of Notch signaling are involved in HSC development. Indeed, at least five Notch ligand genes (*jag1a*, *jag2b*, *dlc*, *dld*, and *dll4*) have been shown to be involved in establishing the HSC program. In addition to these five, there may be additional Notch ligands that regulate HSC development, and the role of these Notch ligands should be carefully determined by future studies.

We described divergent roles of Notch ligands in HSC development in the discussion as follow:

"There are at least five Notch ligands that are involved in establishing HSC programs in the zebrafish embryo. Dlc and Dld are involved in early HSC specification during angioblast migration (Clements et al., 2011; Kobayashi et al., 2014), and we have shown here that the expression of these Notch ligand genes is regulated in part by Jag2b-driven Notch signaling in the somite. It has been shown that Delta- like 4 (Dll4) is required for specification of the arterial endothelium; however, knockdown of *dll4* also resulted in loss of HSCs, suggesting that arterial programs are required before formation of HECs (Bonkhofer et al., 2019). Within the aortic floor, Jag1a expressed by arterial endothelial cells activates Notch in neighboring endothelial cells to establish HSC programs (Espín-Palazón et al., 2014; Monteiro et al., 2016). Thus, multiple waves of Notch signaling provided by various cell types and ligands are involved in HSC development. Further studies of Notch-related genes will elucidate the spatiotemporal regulatory mechanisms of HSC development."

It would also be good that they speculate about the different Notch signals that are required and seem to come from different ligands. The authors could speculate on the different action of these ligands in terms of Notch activation.

As I mentioned above, there are multiple waves of Notch signaling in HSC development, and it may be interesting to examine which Notch ligands activate which types of Notch receptors in each case. It has previously been reported in zebrafish that three of the four Notch receptors, Notch 1a, 1b, and 3, were involved in HSC development; however, all three Notch genes were expressed in somite, lateral plate mesoderm, and dorsal aorta (Kim et al., *EMBO J*. 2014), highlighting the difficulty to determine divergent roles of Notch receptors in HSC development. On the other hand, the expression patterns of Notch ligand genes are distinct, and we agree on the importance of discussing divergent roles of Notch ligands in HSC development as described above.

Reviewer 3 Advance Summary and Potential Significance to Field:
In this manuscript, the authors revisit the early stages of Hematopoietic Stem Cell (HSC) specification and the role of Jagged-2b (Jag2b) in this process. Using both jag2b morphant and mutant embryos, the authors demonstrate that early Jag2b expression within the somite is necessary for the downstream signaling axis of wnt16 and dlc/dld; previously shown to be required for HSC specification. Next, the authors demonstrate that jag2b-Notch signaling operates non-autonomously within the somites to regulate wnt16 by inducing the expression of efna1b. In addition, the authors performed an array of overexpression and rescue experiments that nicely complement their main findings. The manuscript is comprehensive and well written. The experiments follow a logical trajectory and are well designed and controlled. The manuscript covers significant points and open questions in early hematopoiesis. This manuscript adds essential findings to the field. Following are several suggestions for improvement.

We gratefully acknowledge for constructive comments on our manuscript.

Reviewer 3 Comments for the
Author: Major concerns:
1. Much of the analysis in the paper is based on the quantification of expression levels. Please clarify how the expression levels were quantified and translated into the statistical analysis. Enumeration of cells would provide more convincing statistics. The one experiment in which this was done - quantification of gata2b+ cells in jag2bsgRNA animals - shows differences which are not very convincing.
We apologize for our inadequate description of the quantification of expression data. In some experiments, only half of the embryos displayed the indicated phenotype. For example, as shown

in Fig. 2A, 72 out of 140 *jag2b*^{sgRNA} embryos showed lower levels of *runx1* expression, whereas some embryos showed the same level with wild type embryos. In this case, we classified embryos into three categories based on the signal intensity of *runx1* expression (low, middle, or high), showing the phenotypic distributions of each group in the graph. Statistical significance of the phenotypic distributions was tested using Pearson's chi square test. However, due to the limited figure space, we cannot display all data with the graph of phenotype distributions. As shown in Fig. 2E, the majority of *jag2b*^{sgRNA} embryos (20 out of 23) displayed indicated *efnb2a* expression in the dorsal aorta, whereas the rest (3 out of 23) showed slightly lower expression levels. Thus, two classifications were applied when most embryos (> 70%) showed equivalent expression levels.

Expression levels of *runx1* were evaluated by whole-mount *in situ* hybridization (WISH) as described above, whereas the number of HSPCs was evaluated by counting *gata2b*+ cells in the ventral floor of the dorsal aorta (Fig. 2C). As the reviewer mentioned, although statistically significant, the number of *gata2b*+ cells was reduced by only 27% in *jag2b*^{sgRNA} embryos compared to wild type embryos. In this case, we evaluated the absolute number of GFP+ cells in the dorsal aorta, but not the intensity of GFP expression, providing a distinct assessment in HSC development. In the present study, we observed that knockdown of *jag2b* decreased expression levels of *wnt16* and *runx1*, whereas forced expression of *jag2b* increased these expressions, suggesting that Jag2b-driven Notch signaling controls the expression levels of downstream target genes. Therefore, we mainly evaluated the expression levels of each tested gene by WISH to determine phenotypic effects in each embryo type.

We described the detailed quantification methods of expression data in the materials and methods in the revised version of the manuscript as follow:

“Quantification and statistical analyses
Data were analyzed for statistical significance after at least two repeated experiments. To quantify the expression levels of each tested gene, individual embryos were classified into three (high, middle, or low) or two categories (high or low) based on the signal intensity. Statistical differences of phenotypic distributions between groups were determined using Pearson's chi square test. The statistical difference in count data between groups was determined by unpaired two-tailed Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.”

2. An important part of the story is the cell-cell communication between cells in the developing somite. While the sections provide some support for this model, this point is not very clear. FISH would provide a more compelling means to demonstrate non-cell-autonomy. This is particularly important for efna1b and wnt16 expression patterns.

We appreciate an excellent suggestion for cell-cell communication in the somite. According to the reviewer’s suggestion, we performed double-fluorescent whole-mount *in situ* hybridization using *efna1b* and *wnt16* probes. As shown in the new Fig. 5K, we observed that *efna1b*-expressing cells were laterally and anteroposteriorly adjacent to *wnt16*-expressing cells at 15 hpf. This data strongly suggests that *wnt16* expression is induced by neighboring *efna1b*-expressing cells within the somite.
These new data have been added to Fig. 5K in the revised version of the manuscript.

3. The authors observed no phenotype in the complete mutants and hypothesis that it is due to compensation. How do the authors explain that in the sgRNA mutants, there is no compensation? The RNA levels are reduced by almost 96%, but that could be due to RNA decay that can still lead to compensation.

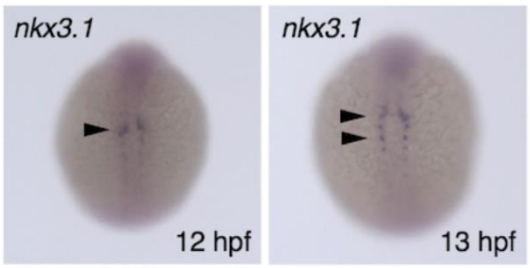
This is an important question to convince readers that Jag2b regulates HSC development. In this revision, we examined the expression of *jag1a*, *jag1b*, and *jag2a* in wild type embryos, *jag2b* homozygous mutant embryos (*jag2b*$^{kz6/kz6}$), *jag2b*^{sgRNA} embryos, and *jag2b* morphants (*jag2b* MO) by qRT-PCR. The expression levels of *jag1a* and *jag1b*, but not *jag2a*, were increased in *jag2b*$^{kz6/kz6}$ embryos compared to wild type embryos. This upregulation was also confirmed by whole-mount *in situ* hybridization (WISH) of the *jag1a* and *jag1b* probes at 12 hpf, raising the possibility that the phenotypes of *jag2b* mutant embryos were compensated, at least in part, by the upregulation of these two *jag* genes. More importantly, despite the large reduction in

jag2b mRNA, no or only slight upregulation of these genes was observed in *jag2b*^{sgRNA} embryos. These data suggest that the effect of genetic compensation is very small in *jag2b*^{sgRNA} embryos, and that the results in *jag2b*^{sgRNA} embryos and *jag2b* morphants more precisely reflect the loss of *jag2b* than those in *jag2b* mutant embryos.

We have added these expression data to Fig. S2F and G in the revised version of the manuscript.

4. It was previously shown by Clements et al. that loss of Wnt16 leads to a near complete loss of the sclerotome. It is therefore puzzling that loss of efna1b does not lead to this same phenotype. How do the authors explain this discrepancy?

Thank you very much for pointing this issue out. As mentioned by the reviewer, morpholino knockdown of *wnt16* resulted in a reduction in the expression of sclerotome marker genes (Clements et al., *Nature* 2011). In contrast, we observed intact expression of the sclerotome marker *nkx3.1* in *jag2b*^{sgRNA} and *efna1b*^{sgRNA} embryos at 15 hpf despite a reduction in *wnt16* expression (Fig. 4B and S6G). We believe that early sclerotome formation at the ventromedial surface of the somite is independent of *wnt16* expression. As shown in Fig. 4F-J, *wnt16* expression begins from 14 hpf, whereas *nkx3.1* expression can be detected from 12 hpf (Response Fig). Clements et al. showed reduced expression of sclerotome markers in *wnt16* morphants and *dlc/dld*-deficient embryos at 22 hpf, a time point after sclerotome migration adjacent to the neural tube and notochord, while no data were provided in the ventromedial sclerotome. Because angioblasts received Dlc/Dld signaling from the ventromedial sclerotome during migration from the lateral plate mesoderm to the midline (15 – 18 hpf), it is important for us to show that sclerotomes are intact at 15 hpf in *jag2b*^{sgRNA} and *efna1b*^{sgRNA} embryos.



We described these explanations in the discussion of the revised manuscript as follow:

"Clements et al. reported that loss of *wnt16* led to reduced expression of sclerotome maker genes at 22 hpf, a time point after sclerotome migration adjacent to the neural tube and notochord (Clements et al., 2011). In contrast, we observed intact expression of the sclerotome marker *nkx3.1* in *jag2b*^{sgRNA} and *efna1b*^{sgRNA} embryos at 15 hpf despite a reduction in *wnt16* expression. Because *wnt16* expression begins from 14 hpf, it is likely that early sclerotome formation at the ventromedial surface of the somite is independent of *wnt16* expression."

5. The phldb1 line shows some expression within LPM cells, leading to a concern that the NICD rescue experiment may not be acting within the somite. It is advised to employ another somitic GAL4 driver to replicate this result.

The somite-specific Gal4 line is very limited, and unfortunately, we are currently unable to employ other Gal4 lines for NICD rescue experiments. However, the leakage of *phldb1:Gal4-mCherry* expression to angioblasts is very limited (Kobayashi et al., *Nature* 2014). In addition, Kim et al. reported that loss of *runx1* expression in *notch3* morphants can be rescued by using *phldb1:Gal4- mCherry* in combination with *UAS:NICD*, whereas this was not rescued by *kdrl:Gal4* (Kim et al., *EMBO J.* 2014), which induces Gal4 expression in angioblasts/vascular endothelial cells. These observations suggest that *phldb1:Gal4-mCherry* does induce Gal4 expression in somites.

Minor concerns:
1. Figure 1 panel H: What are the positive cells along the DA? Do DA cells express jag2b? Please clarify.

We examined *jag2b* expression in a section of the trunk region of the embryo at 24 hpf. As shown in the new Fig. 1K, *jag2b* was expressed in the ventral domain of the neural tube, lateral line, pronephros, but not in the dorsal aorta. The *jag2b*-positive domain along the dorsal aorta in Fig. 1H is thus part of the neural tube. This new data was added and described in the results in the revised version of the manuscript.

2. The authors show a reduction in wnt16, dlc, and dld expression in jag2b mutants and morphants. Later they show a reduction of wnt16 in efna1b mutants and morphants. Can the authors complement the results and show dlc/dld reduction in efna1b morphants?

In the original version of the manuscript, we showed reduced expression of *dlc* and *dld* in *efna1b*^{sgRNA} embryos, but not in *efna1b* mutant embryos (*efna1b*^{kz5}). As suggested by the reviewer, we complemented the *dlc* and *dld* expression data of *efna1b*^{kz5} embryos in the revised version of the manuscript. Consistent with *efna1b*^{sgRNA} embryos, *efna1b*^{kz5} embryos also showed reduced expression of *dlc* and *dld* in the somite. These new data have been added to Fig. S7E and F.

3. In figure 6, panels C and D: please label the Runx1 probe.

Thank you very much for pointing out these errors. We have labeled "*runx1*" in these figures in the revised version of the manuscript.

4. Is there evidence for direct interaction between efna1b and rspo1?

We have no evidence that Ephrin A1b directly interacts with Rspo1 to regulate *wnt16* expression in the somite. Therefore, we have removed the word of "cooperatively" from the sentence in the discussion as follow:

Original sentence
"These observations suggest that spatiotemporal expression of *wnt16* is predominantly regulated by Jag2b-driven Notch signaling, and Rspo1 may cooperatively boost *wnt16* expression in the somite."

Modified sentence
"These observations suggest that spatiotemporal expression of *wnt16* is predominantly regulated by Jag2b-driven Notch signaling, and Rspo1 may boost *wnt16* expression in the somite."

5. Throughout the manuscript, the authors differentiate between different somite compartments. Perhaps the authors can add a sketch of the compartments to their current model.

According to the reviewer's suggestion, a cross-hatched region was added to the adaxial region of somites in Fig. 7, where *jag2b*-expressing slow muscle precursors distribute.

Second decision letter

MS ID#: DEVELOP/2021/200339

MS TITLE: Jagged-2b induces intercellular signaling within the somite to establish hematopoietic stem cell fate in zebrafish

AUTHORS: Yukino Wada, Hikaru Tsukatani, Chihiro Kuroda, Yurika Miyazaki, Miku Otoshi, and Isao Kobayashi
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Wada et al. identified the novel Notch-regulatory pathway related to HSC specification in somites using zebrafish genetics. They showed that Notch ligand Jagged-2b induced Notch activation and its target Ephrin A1b in adjacent cells, which induced Wnt16 in next adjacent cells. Since they previously demonstrated that Wnt16 in somites induced another somitic Notch ligands Dlc and Dld, which directly instruct HSC fate in vascular precursor cells, they concluded that Jag2b-driven Notch signaling regulates Ephrin A1 expression in the somite to modulate the Wnt16 – Dlc/Dld signaling axis, which is required for HSC specification. Overall, this paper provides important advances in understanding the relationship between somitic Notch signaling and HSC cell fate.

Comments for the author

The revision of the manuscript by authors has addressed the comments raised by this referee. This interesting work seems now suitable for publication in the Development.

Reviewer 2

Advance summary and potential significance to field

The authors identify Jag2b as responsible for activating Notch, that in turn activates Efna1b,that activates Wnt16 in the first signals in the somite that will
impact on hemogenic cells. The authors have complete some gaps from the previous work, for example with Jag2b at the top of the cascade and activaton of efna1.

Comments for the author

The authors have answered all raised questions.

Reviewer 3

Advance summary and potential significance to field

The authors have made outstanding contributions to fleshing out the signaling pathways in the somite required for HSC specification. These findings should be of high impact to the field.

Comments for the author

The authors have done a thorough and convincing job revising their paper. Our concerns are now sufficiently addressed.