blf and the *drl* cluster synergistically regulate cell fate commitment during zebrafish primitive hematopoiesis

Xue Zhang, Yuxi Yang, Yuxuan Wei, Qingshun Zhao and Xin Lou
DOI: 10.1242/dev.200919

Editor: Hanna Mikkola

Review timeline
Original submission: 6 May 2022
Editorial decision: 9 June 2022
First revision received: 6 September 2022
Editorial decision: 27 September 2022
Second revision received: 20 October 2022
Accepted: 14 November 2022

Original submission

First decision letter

MS ID#: DEVELOP/2022/200919

MS TITLE: Blf and drl cluster synergistically regulate cell fate commitment during zebrafish primitive hematopoiesis

AUTHORS: Xue Zhang, Yuxi Yang, Qinshun Zhao, and Xin Lou

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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

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, the authors show the “synergistic” role of blf and drl in primitive hematopoiesis. Elimination of both complexes leads to erythroid hypoplasia while the myeloid population is enhanced especially the macrophages (neutrophils were diminished). The mutants also have impaired definitive hematopoiesis. Genome wide expression analysis showed that blf and drl downregulate the expression of vascular genes in ICM and monocytic genes in rostral blood island. A human zing finger protein can rescue partially the phenotypes.

Comments for the author

This is an interesting paper. Some suggestions:

-What happens to stem cells and to other differentiated cells in adulthood?
-What happens to arterial development?
-Is it necessary to delete all four drl genes? Do the authors have additional cripsr clones and what happens to them. This is especially important since individual genes can rescue the phenotype
-Since blf cannot fully rescue, the functions of drl and blf are not fully redundant What is the binding pattern of blf? What are the similarities to drl binding?
-Can the authors rescue the myeloid phenotype with irf8 knockdown? Or by manipulating any other target of drl?
-Can the human zing finger protein rescue HSPC formation?

Minor comments

-How many chip replicates did the authors do? Please fill in such details in material and methods.
-Put numbers in in situ when missing and number of independent experiments “we tested the efficacy of these 5 genes on rescuing defective hematopoiesis in blf-/-; drl 4KO embryos” mention the five genes
-Since erythrocytes are born but die of apoptosis then maybe this is an overstatement: “All of these results indicate that blf and drl cluster genes are essential for hemangioblasts to initiate primitive erythropoiesis.”

Reviewer 2

Advance summary and potential significance to field

Zhang et al. have used the zebrafish model to understand fate decisions during primitive hematopoiesis. They found that blf and drl clusters promote erythropoiesis. Blf and drl are two highly similar zinc finger proteins that have expression in primitive hematopoieic domains of the embryo. Draculin expression was discovered by Herbomel et al in 1999. Sumanas et al. in 2005 described a phenotype for blf in neurulation but not in hematopoiesis suggesting some redundancy between blf and drl genes. Pimtong et al. in 2014 found multiple similar genes: blf, drl, drl.1, drl.2, and drl.3, but only drl.3 had some function in hematopoiesis—a transient decrease in primitive myeloid cells. To deal with the redundancy in the drl cluster, in this current study Zhang et al. used CRISPR/Cas9 to delete the entire drl cluster (drl, drl.1 drl.2, and drl.3). They found synergism between the drl cluster KO and blf in regulation of primitive erythropoiesis and myelopoiesis. The blf mutant was a gene trap mutation the authors had previously identified. The conservation of drl-like genes across species has been unclear because of lost synteny, so the authors also performed a rescue screen that identified ZFN932 as a potential mammalian homolog. This high-quality study provides insight into the function of draculin cluster genes that have not been well understood beyond their expression patterns. The genetic work also highlights an early fate decision in the anterior myeloid precursor population of the zebrafish embryo. Revisions could be made to clarify several experiments in the manuscript.

Comments for the author

Major points:
1. The authors should address the differences in phenotype between this study that has mutants without drl.3 (drl-4KO) but no myeloid or erythroid changes and the study by Pimtong et al. that has drl.3 loss-of-function and a myeloid cell defect.

2. In Figure 3D the box that is used to select TUNEL+ cells is over the EMP population (end of yolk extension) not the ICM as described (above the yolk extension and along the trunk). If the ICM region is selected for TUNEL analysis is the result the same? The cellular composition of the two regions is different and should be analyzed separately.

3. The discussion of zebrafish primitive macrophages and neutrophils should be expanded and clarified. Specifically, the differences between primitive, EMP and definitive waves of myeloid cell development. This should include a thorough discussion of bipotential myeloid progenitors (spi1b+) that give rise to macrophages and neutrophils in the anterior mesoderm as shown by Le Guyader et al., 2008. Before 24 hpf the bipotential myeloid progenitors are considered pre-macrophages. In Figure 4 the phenotype is already visible at 24 hpf. To make specific claims about the role of blf/drl genes in fate decisions, lineage analysis should be done either by uncaging of caged fluorescein or photoactivatable protein. For example, lineage would need to be traced from 20 hpf and then observed later at 72 hpf.

4. Overall, there is insufficient detail regarding the myeloid phenotype. Sudan black for neutrophils is presented at 36 hpf (Figure 4B) but there is no macrophage marker. Both lineages should be considered at different developmental stages, and in the context of primitive, EMP, and definitive populations.

Additional points:

4. The authors should reanalyze existing flow cytometry data in Fig. S3B. FSC should be plotted against SSC to show 4 typical clusters in zebrafish kidney marrow (erythrocyte, lymphocyte, precursor, myelomonocyte) because the mutant may show lineage skewing not visible in the total count of cells

5. Error in Figure 4 legend. Spi1b at 8 somite stage is not pictured and is in Figure S5. Coro1a is not mentioned.

6.English language and grammar need editing throughout

First revision

Author response to reviewers' comments

Editor and Individual Reviewer Responses:

Reviewer comments are in bold font and our responses are in italics.

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript, the authors show the “synergistic” role of blf and drl in primitive hematopoiesis. Elimination of both complexes leads to erythroid hypoplasia while the myeloid population is enhanced especially the macrophages (neutrophils were diminished). The mutants also have impaired definitive hematopoiesis. Genome wide expression analysis showed that blf and drl downregulate the expression of vascular genes in ICM and monocytic genes in rostral blood island. A human zinc finger protein can rescue partially the phenotypes.

Reviewer 1 Comments for the Author:

This is an interesting paper. Some suggestions:

- **What happens to stem cells and to other differentiated cells in adulthood?**

To examine the hematopoiesis in adult blf$^{-/-}$; drl 4KO animals, we applied microscopic observation and hematological analysis. The kidney from adult blf$^{-/-}$; drl 4KO fish are moderately hypoplastic compared with control, while H&E staining revealed the escapers have abnormal accumulation of hematopoietic cells between the renal tubules. Differential cell counting for major blood cell lineages also showed precursor and lymphocyte fractions are markedly expanded in the blf$^{-/-}$; drl 4KO zebrafish. These results indicated blf and drl cluster genes played important roles for normal development of hematopoietic stem cells and definitive hematopoiesis. This part of data was included into Fig. S3 and the results part of the manuscript.

- **What happens to arterial development?**

Beside evaluating vascular development with alkaline phosphatase staining and Tg(kdrl:EGFP)^{s843} line, we examined arterial development in blf$^{-/-}$; drl 4KO embryo through probing the expression of arterial marker genes ephrin B2 and deltaC. Compare to control embryos, no overt difference was observed, this indicated arterial development in blf$^{-/-}$; drl 4KO embryos was largely not affected. This part of data was included into Fig. S4 and the results part of the manuscript.

- **Is it necessary to delete all four drl genes? Do the authors have additional cripsr clones and what happens to them. This is especially important since individual genes can rescue the phenotype.**

During the process of generating drl 4KO allele, we obtained several CRISPR alleles bearing mutants on drl or drll.3, no overt hematopoietic defects were observed in the homozygous embryos of these alleles. Considering that drl 4KO homozygous embryos display mild hematopoietic phenotype and most of them could be raised to adulthood, we believe there is strong genetic redundance exists between blf and drl cluster and this redundance is prohibitive to study the function of individual drl gene. We agree with the reviewer it is interesting to investigate how the drl cluster was evolved and whether individual drl gene have different biological functions while it is beyond the scope of current research.

- **Since *blf* cannot fully rescue, the functions of drl and blf are not fully redundant What is the binding pattern of Blf? What are the similarities to drl binding?**

To characterize the binding pattern of Blf, we applied CUT&Tag for Blf on haematopoietic progenitors. The results revealed the binding regions of Blf have similar genome-wide distribution pattern compare to Drl. Further analysis showed there was extensive overlap (63.9%) between Blf and Drl chromatin occupancy, as well as substantial overlap (65.7%) between associated genes. This part of data was included into Fig. 6 and the results part of the manuscript.

- **Can the authors rescue the myeloid phenotype with irf8 knockdown? Or by manipulating any other target of drl?**

When expression of irf8 was knock-downed with morpholino injection, neutrophil development in blf$^{-/-}$; drl 4KO embryos was restored. This indicated mis-regulated irf8 expression contribute to the skewed myeloid lineage commitment in blf$^{-/-}$; drl 4KO embryos. This part of data was included into Fig. 6 and the results part of the manuscript.

- **Can the human zinc finger protein rescue HSPC formation?**

Overexpression of Zfp932 mRNA in blf$^{-/-}$; drl 4KO embryos could partially rescue the expression of runx1 and cmyb, this suggest Zfp932 are capable to restore HSPC formation. This part of data was included into Fig. 7 and the results part of the manuscript.

Minor comments

- **How many chip replicates did the authors do? Please fill in such details in material and methods.**

We performed CUT&Tag against Blf and Drl on two pools of sorted RFP+ cells as biological replicates. Detailed information for sample handling and data processing has been added into material and methods.

- **Put numbers in in situ when missing and number of independent experiments "we tested the efficacy of these 5 genes on rescuing defective hematopoiesis in blf-/-; drl 4KO embryos" mention the five genes**

The sample numbers of in situ hybridization and number of experiments were added into figure legends. The texted was revised as reviewer's suggestion.

- **Since erythrocytes are born but die of apoptosis then maybe this is an overstatement: "All of these results indicate that blf and drl cluster genes are essential for hemangioblasts to initiate primitive erythropoiesis."**

This statement was revised as "All of these results indicate that blf and drl cluster genes are essential for differentiation and survival of primitive erythrocytes."

Reviewer 2 Advance Summary and Potential Significance to Field:

Zhang et al. have used the zebrafish model to understand fate decisions during primitive hematopoiesis. They found that blf and drl clusters promote erythropoiesis. Blf and drl are two highly similar zinc finger proteins that have expression in primitive hematopoieic domains of the embryo. Draculin expression was discovered by Herbomel et al in 1999. Sumanas et al. in 2005 described a phenotype for blf in neurulation but not in hematopoiesis, suggesting some redundancy between blf and drl genes. Pimtong et al. in 2014 found multiple similar genes: blf, drl, drl.1, drl.2, and drl.3, but only drl.3 had some function in hematopoiesis—a transient decrease in primitive myeloid cells. To deal with the redundancy in the drl cluster, in this current study Zhang et al. used CRISPR/Cas9 to delete the entire drl cluster (drl, drl.1, drl.2, and drl.3). They found synergism between the drl cluster KO and blf in regulation of primitive erythropoiesis and myelopoiesis. The blf mutant was a gene trap mutation the authors had previously identified. The conservation of drl-like genes across species has been unclear because of lost synteny, so the authors also performed a rescue screen that identified ZFN932 as a potential mammalian homolog. This high- quality study provides insight into the function of draculin cluster genes that have not been well understood beyond their expression patterns. The genetic work also highlights an early fate decision in the anterior myeloid precursor population of the zebrafish embryo. Revisions could be made to clarify several experiments in the manuscript.

Reviewer 2 Comments for the Author:

Major points:

1. The authors should address the differences in phenotype between this study that has mutants without drl.3 (drl-4KO) but no myeloid or erythroid changes, and the study by Pimtong et al. that has drl.3 loss-of-function and a myeloid cell defect.

As described in the manuscript, drl 4KO embryos displayed mild erythropoiesis defect (Fig. 1 A and B), so it is possible drl cluster genes act in a dosage-dependent fashion and knocking down drll.3

could modestly impair hematopoiesis. Considering that individual drl cluster gene and blf can rescue the myeloid and erythroid phenotype in $blf^{-/-}$; drl 4KO embryos, we believe there is strong genetic redundance exists among these genes and knock down one gene is unlikely to cause severe defects. During the process of generating drl 4KO allele, we also obtained two CRISPR alleles bearing mutants on drll.3, no overt hematopoietic defects were observed in the homozygous embryos of these alleles. The discussion part has been expanded to include this information.

2. In Figure 3D the box that is used to select TUNEL+ cells is over the EMP population (end of yolk extension) not the ICM as described (above the yolk extension and along the trunk). If the ICM region is selected for TUNEL analysis is the result the same? The cellular composition of the two regions is different and should be analyzed separately.

The viability of hematopoietic progenitors in ICM and EMP was separately analyzed, quantification of TUNEL+ cells showed both two populations undergo enhanced programmed cell death. Fig. 3 and related text have been revised accordingly.

3. Q.12 The discussion of zebrafish primitive macrophages and neutrophils should be expanded and clarified. Specifically, the differences between primitive, EMP, and definitive waves of myeloid cell development. This should include a thorough discussion of bipotential myeloid progenitors (spi1b+) that give rise to macrophages and neutrophils in the anterior mesoderm as shown by Le Guyader et al., 2008. Before 24 hpf the bipotential myeloid progenitors are considered pre-macrophages. In Figure 4 the phenotype is already visible at 24 hpf. To make specific claims about the role of blf/drl genes in fate decisions, lineage analysis should be done either by uncaging of caged fluorescein or photoactivatable protein. For example, lineage would need to be traced from 20 hpf and then observed later at 72 hpf.

The introduction of primitive myelopoiesis in zebrafish has been expanded and related references were included into the revised manuscript.
To clarify the role of blf and drl cluster genes in fate decision of primitivemyeloid progenitors, lineage analysis was carried out by photoactivable fluorescein labeling as the reviewer suggested (trace the cell from 20 hpf and then observe at 72 hpf). The data showed, in $blf^{-/-}$; drl 4KO embryos, vast majority of myeloid progenitors from rostral blood islands give rise to macrophages and only very small fraction of them take the neutrophil fate. These results are consistent with our previous observations and have been incorporated into Fig. 4.

4. Q.13 Overall, there is insufficient detail regarding the myeloid phenotype. Sudan black for neutrophils is presented at 36 hpf (Figure 4B) but there is no macrophage marker. Both lineages should be considered at different developmental stages, and in the context of primitive, EMP, and definitive populations.

Based on the review's suggestion, we expanded the analysis on the myeloid phenotype. In situ hybridization for neutrophil and macrophage marker genes at 36 hpf and 96 hpf revealed both the EMP and definitive wave of myelopoiesis are abolished in $blf^{-/-}$; drl 4KO embryos (Fig. S6 and Fig. S7). This observation is consistent with the data that formation of EMP (Fig S5 and Fig 3) and HSC (Fig 1) are severely disrupted in $blf^{-/-}$; drl 4KO embryos.

Additional points:

5. Q.14 The authors should reanalyze existing flow cytometry data in Fig. S3B. FSC should be plotted against SSC to show 4 typical clusters in zebrafish kidney marrow (erythrocyte, lymphocyte, precursor, myelomonocyte) because the mutant may show lineage skewing not visible in the total count of cells

To answer this question, we repeated hematological analysis and applied histological staining on adult fish kidney. Differential cell counting for major blood cell lineages showed precursor and lymphocyte fractions are markedly expanded in the $blf^{-/-}$; drl 4KO zebrafish. This result is consistent with the H&E staining for head kidney which revealed the escapers have accumulation of abnormal hematopoietic cells between the renal tubules. This part of data was included into

Fig. S3 and the results part of the manuscript.

6. Q.15 Error in Figure 4 legend. Spi1b at 8 somite stage is not pictured and is in Figure S5. Coro1a is not mentioned.
This error has been corrected.

7. Q.16 English language and grammar need editing throughout.

The manuscript was edited by native English speaker.

Second decision letter

MS ID#: DEVELOP/2022/200919

MS TITLE: Blf and drl cluster synergistically regulate cell fate commitment during zebrafish primitive hematopoiesis

AUTHORS: Xue Zhang, Yuxi Yang, Yuxuan Wei, Qinshun Zhao, and Xin Lou

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

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

Reviewer 1

Advance summary and potential significance to field

The authors have answered all my concerns.

Comments for the author

The authors have answered all my concerns.

Reviewer 2

Advance summary and potential significance to field

The authors have mostly addressed all of the reviewers' comments. There are a few minor revisions suggested below.

Comments for the author

1. The authors need to provide some quantification of either the flow cytometry or histology data to make this statement:

“The kidney from blf-/-; drl 4KO fish are moderately hypoplastic compared with control, while H&E staining revealed the escapers have abnormal accumulation of hematopoietic cell between the renal tubules (supplemental Figure 3A).”

2. The authors should provide quantification of both the numbers and percentages of different populations in the kidney marrow (KM) and peripheral blood (PB).
This would help resolve questions such as why erythrocytes numbers are down in PB but proportions are not, and why proportions are down in KM. It is not clear from the images in S3A what populations are contributing to the accumulation and if they are significant across multiple mutant animals.

3. For the uncaging experiment in 4B, do the authors have an image of the embryo at the time of uncaging to confirm the region that was illuminated? At a minimum, could the authors provide a schematic of the experimental design and region targeted?

4. The following sentence is confusing because it names myelopoiesis but gata1 is an erythrocyte lineage marker and lmo2 is more broadly hematopoietic. Instead the authors could focus on erythro-myeloid progenitors (EMPs).

“To evaluate the roles of blf and drl cluster genes in intermediate myelopoiesis, we examined the expression of EMP marker genes gata1 and lmo2 at 30 hpf.”

5. The following sentence is confusing:

“We further analyzed the definitive wave of myelopoiesis in blf-/-; drl 4KO mutants, WISH results showed at 96 hpf no significant increase on mpx positive neutrophils compare to earlier stages and the number of mpeg1 positive macrophages is lower than which in controls (supplemental Figure 7).”

The text should focus on the significant decrease in mpx positive cells, as was observed in earlier stages.

6. The following may be overstated given many of these genes may not be direct targets and could be reworded:

“this suggested blf and drl cluster genes primarily act as transcription repressor”

7. In Figure S9 and the methods could the authors provide more information about the number of sorted cells collected for each experiment (RNA-seq and CUT&TAG)?

8. Could the authors provide quantification of the results to support the following statement:

“expression of irf8, a master driver of monocytopoiesis, was profoundly up-regulated in rostral blood island (Figure 6 G and H).”

9. typos: estrp should be etsrp

10. In figure 7A the text described 25 candidates tested but the figure shows 14--are the other results shown elsewhere?

11. This sentence is confusing because it suggests etsrp was over-expressed, but it seems the authors are referring to the increase in etsrp expression due to loss of blf;drl1-4 in the KO.

“Ectopic expression of estrp in intermediate cell mass did not induce excess endothelialization in blf-/-; drl 4KO homozygous embryos (supplemental Figure 4).

12. The following conclusion is difficult to make without more evidence. Could the authors make it more speculative in its wording?

"We believe that the differentiation of hemangioblasts to hematopoietic cells is suspended, and immature hemangioblasts that possess endothelial characteristics went into apoptosis."

13. There are still grammatical and spelling errors that need correction

---

Second revision

Author response to reviewers' comments

Editor and Individual Reviewer Responses:

Reviewer comments are in **bold** font and our responses are in *italics*.

Reviewer 1 Advance Summary and Potential Significance to Field: The authors have answered all my concerns.

Reviewer 1 Comments for the Author:

The authors have answered all my concerns.

Reviewer 2 Advance Summary and Potential Significance to Field:

The authors have mostly addressed all of the reviewers' comments. There are a few minor revisions suggested below.

Reviewer 2 Comments for the Author:

1. The authors need to provide some quantification of either the flow cytometry or histology data to make this statement: "The kidney from blf-/-; drl 4KO fish are moderately hypoplastic compared with control, while H&E staining revealed the escapers have abnormal accumulation of hematopoietic cell between the renal tubules (supplemental Figure 3A)."

The quantification data for kidney hypoplastic and accumulation of hematopoietic cell has been added into Figure S3 A and B.

2. The authors should provide quantification of both the numbers and percentages of different populations in the kidney marrow (KM) and peripheral blood (PB). This would help resolve questions such as why erythrocytes numbers are down in PB but proportions are not, and why proportions are down in KM. It is not clear from the images in S3A what populations are contributing to the accumulation and if they are significant across multiple mutant animals.

The percentages of different populations in the kidney marrow and peripheral blood is shown in Fig S3C.

3. For the uncaging experiment in 4B, do the authors have an image of the embryo at the time of uncaging to confirm the region that was illuminated? At a minimum, could the authors provide a schematic of the experimental design and region targeted?

Images were added as Figure S6 to demonstrate uncaging of photoactivatable fluorescein on the rostral blood island of embryo at 20 hpf.

4. The following sentence is confusing because it names myelopoiesis but gata1 is an erythrocyte lineage marker and lmo2 is more broadly hematopoietic. Instead the authors could focus on erythro-myeloid progenitors (EMPs). "To evaluate the roles of blf and drl cluster genes in intermediate myelopoiesis, we examined the expression of EMP marker genes gata1 and lmo2 at 30 hpf."

The sentence was revised as "To evaluate the roles of blf and drl cluster genes in intermediate myelopoiesis, we examined the expression of gata1 and lmo2 at 30 hpf."

5. The following sentence is confusing: "We further analyzed the definitive wave of myelopoiesis in blf-/-; drl 4KO mutants, WISH results showed at 96 hpf no significant increase on mpx positive neutrophils compare to earlier stages and the number of mpeg1 positive macrophages is lower than which in controls (supplemental Figure 7)." The text should focus on the significant decrease in mpx positive cells, as was observed in earlier stages.

The sentence was revised as "We further analyzed the definitive wave of myelopoiesis, WISH results showed at 96 hpf, both the number of mpx positive neutrophils and mpeg1 positive macrophages in blf-/-; drl 4KO mutants are lower than which in controls (supplemental Figure 8)."

6. The following may be overstated given many of these genes may not be direct targets and could be reworded: "this suggested blf and drl cluster genes primarily act as transcription repressor"

The statement has been revised as "this suggested blf and drl cluster genes may primarily act as transcription repressor".

7. In Figure S9 and the methods could the authors provide more information about the number of sorted cells collected for each experiment (RNA-seq and CUT&TAG)?

The number of sorted cells collected for RNA-seq and CUT&TAG have been added into Figure S10.

8. Could the authors provide quantification of the results to support the following statement: "expression of irf8, a master driver of monocytopoiesis, was profoundly up-regulated in rostral blood island (Figure 6 G and H)."

The expression of irf8 in blf$^{-/-}$; drl 4KO embryo (measured by RNA-seq and qPCR) was added into Figure 5D.

9. typos: estrp should be etsrp

These typos have been corrected.

10. In figure 7A the text described 25 candidates tested but the figure shows 14--are the other results shown elsewhere?

The results from other candidates were included into supplementary data as Fig S15.

11. This sentence is confusing because it suggests etsrp was over-expressed, but it seems the authors are referring to the increase in etsrp expression due to loss of blf;drl1-4 in the KO. "Ectopic expression of estrp in intermediate cell mass did not induce excess endothelialization in blf-/-; drl 4KO homozygous embryos (supplemental Figure 4).

The text should be refer to Figure 6, the error has been corrected.

12. The following conclusion is difficult to make without more evidence. Could the authors make it more speculative in its wording?
"We believe that the differentiation of hemangioblasts to hematopoietic cells is suspended, and immature hemangioblasts that possess endothelial characteristics went into apoptosis."

The sentence was revised as "One possible explanation for this observation is the differentiation of hemangioblasts to hematopoietic cells is suspended, and immature hemangioblasts that possess endothelial characteristics went into apoptosis."

13. There are still grammatical and spelling errors that need correction.

The manuscript has been processed by scientific editing services provided by Springer Nature.

Third decision letter

MS ID#: DEVELOP/2022/200919

MS TITLE: Blf and drl cluster synergistically regulate cell fate commitment during zebrafish primitive hematopoiesis

AUTHORS: Xue Zhang, Yuxi Yang, Yuxuan Wei, Qingshun Zhao, and Xin Lou
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

The authors have answered all the questions of the reviewers

Comments for the author

The authors have answered all the questions of the reviewers

Reviewer 2

Advance summary and potential significance to field

Zhang et al. have used the zebrafish model to understand fate decisions during primitive hematopoiesis. They found that blf and drl clusters promote erythropoiesis. Blf and drl are two highly similar zinc finger proteins that have expression in primitive hematopoieic domains of the embryo. Draculin expression was discovered by Herbomel et al in 1999. Sumanas et al. in 2005 described a phenotype for blf in neurulation but not in hematopoiesis suggesting some redundancy between blf and drl genes. Pimtong et al. in 2014 found multiple similar genes: blf, drl, drl.1, drl.2, and drl.3, but only drl.3 had some function in hematopoiesis—a transient decrease in primitive myeloid cells. To deal with the redundancy in the drl cluster, in this current study Zhang et al. used CRISPR/Cas9 to delete the entire drl cluster (drl, drl.1 drl.2, and drl.3). They found synergism between the drl cluster KO and blf in regulation of primitive erythropoiesis and myelopoiesis. The blf mutant was a gene trap mutation the authors had previously identified. The conservation of drl-like genes across species has been unclear because of lost synteny, so the authors also performed a rescue screen that identified ZFN932 as a potential mammalian homolog. This high-quality study provides insight into the function of draculin cluster genes that have not been well understood beyond their expression patterns. The genetic work also highlights an early fate decision in the anterior myeloid precursor population of the zebrafish embryo.

Comments for the author

The authors have addressed all of the reviewers' comments.