Zebrafish Hif3α modulates erythropoiesis via regulation of *gata1 to* facilitate hypoxia tolerance

Xiaolian Cai, Ziwen Zhou, Junji Zhu, Qian Liao, Dawei Zhang, Xing Liu, Jing Wang, Gang Ouyang and Wuhan Xiao
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Original submission

First decision letter

MS ID#: DEVELOP/2019/185116

MS TITLE: Zebrafish hif-3α modulates erythropoiesis via gata-1 regulation to facilitate hypoxia tolerance

AUTHORS: Wuhan Xiao, Xiaolian Cai, Ziwen Zhou, junji zhu, Qian Liao, Dawei Zhang, Xing Liu, Jing Wang, and Gang Ouyang

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.

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

This is a very timely MS by Xiao and colleagues on the in vivo function of hypoxia-inducible factor 3. Using a zebrafish model, the authors provided genetic, biochemical, and molecular data showing that HIF-3a1 alters erythropoiesis by up-regulating gata-1 expression. This in turn impairs hypoxia tolerance. Compared with HIF1a and HIF-2a, the physiological functions of HIF-3a is less well understood. Therefore, the findings reported in this MS add new information to hypoxia-inducible factor biology. Overall, the MS is well written and experiments appeared to be competently performed.

Comments for the author

The authors may want to consider to the following points to further improve this nice MS.

#1) The nature of M1 and/or M2 mutations. M1 contains the bHLH domain and M2 contains bHLH-PAS-PAC-ODD domains. The bHLH domain is known to be important for DNA binding and dimerization with HIF-b. The PAS- A/B and PAC domains are also involved in HIF-b for dimerization. One wonders whether M1 and/or M2 mutant proteins may act in a dominant-negative manner. This may explain the observed up-regulation of EPO, Runx1, c-Myb expression. This can be tested easily by co-transfection with HIF1a, HIF2a, and HIF3a1 and a HRE-luciferease reporter.

#2) Since there are multiple HIFa genes in zebrafish, it will be nice to measure other hifa genes in the mutant fish by qRT-PCR.

#3) Is it possible to include some biological data on the heterozygous fish (survival rate, red blood cells etc.)?

#4) Key information is missing in most figures/legends (maybe except Fig. 4). Please provide the sample number (n), how many times the experiments were repeated, and what the error bars in these figures mean.

#5) In multiple occasions (abstract, Introduction Page 4 and page 5), the authors emphasized that whether HIF3a is a transcription factor is still unclear. This is an outdated view. The HIF3a gene generates a number of HIF3a isoforms. But several recently published studies clearly show that the full-length HIF3a functions as a transcription factor. In addition to the loss- and gain-of-functional studies reported in zebrafish by Zhang et al., 2014, l loss-of-functional studies have been reported in mammalian system (Zhou et al., Mol Cancer Res. 2018; Huang et al., 2013. PLoS One; Heikkila et al., 2011, Cell Mol. Life Sci.) These have been reviewed by Duan, 2016, which the authors have cited. These statements should be revised.

#6) In the method section, it was stated that gene expression was measured by real-time PCR. But it was also mentioned that semi-quantitative PCR in figure legends. Please provide more details how the mRNA expression data were actually measured? Were the data normalized?

#7) The hypoxia treatment/tolerance experiments (Fig.1) were done by manipulating air O2 levels using Invivo2 Hypoxia workstation, which is fine. Since zebrafish live in water, it will be nice to add the actual levels of dissolved O2.

#8) Supplementary Fig. S1 should be deleted as it only contains published information by others.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Cai et al. examines the functional role of Hif3a in erythropoiesis in the zebrafish embryo. The authors indicate that Hif3a is required during normal embryonic erythropoiesis at steady-state, and both embryonic and adult hematopoiesis in response to acute hypoxic stress.

Mutants exhibit severely compromised erythroid differentiation in the embryo, as shown by phenotypic and marker gene analysis, and demonstrate anemia in adults. The authors correlate expression of gata1 with that of hif3a during embryogenesis, and demonstrate a functional transcriptional interaction between the two TFs whereby wild-type Hif3a was shown to promote activation of a gata1 promoter construct in vitro. While the observations regarding erythropoietic maturation and number are intriguing, the study seems somewhat superficial in regard to how Hif3a may interact or intersect with the hypoxia responsive erythroid regulatory roles of the related Hif1a protein. Similarly, due to gaps in the methodological details provided it is difficult to independently interpret much of the data shown, making the strong conclusions feel somewhat premature.

Major critiques:
1. The description of experimental methods is very scant in the text of the results, legends or methods themselves, and lacks key experimental details needed to interpret the findings and determine experimental rigor. For instance, with exception of some in situ panels, it is difficult to determine how many embryos were used to quantify a particular observation (like gene expression) versus the number of experimental replicates performed in almost all figures.

2. Given the well-known role for Hif1a in regulating erythropoiesis, the authors need to clarify whether the response of Hif1a to hypoxia is functional, or potentially up or downregulated in the absence of Hif3a. Would the authors expect Hif1a overexpression to rescue loss of Hif3a? It is important to examine Hif1a expression/protein levels in the various assays, and potentially conduct analysis of their epistatic relationships.

3. Related, the authors mention the role of PHD enzymes and VHL protein in regulating HIF activity in the introduction. Is HIF3a controlled by these same regulators in this developmental context? A more detailed explanation/graphical depiction of the differences in zebrafish Hif3a and Hif1a, as well as potential interplay, may be helpful.

4. The authors demonstrate that the embryos are severely anemic with fewer red cells, and suggest a reduction in gata1 as the likely mechanism. However, expression of epo, a classic target of Hif1a, was not evaluated in the embryos as it was in the adult kidney. Thus, it remains unclear if a reduction in Epo production also contributes to the reduction in gata1 and anemic phenotype? If gata1 is indeed controlled by Hif3a independently of Epo (and perhaps Hif1a), then Epo overexpression would not rescue the anemic phenotype in mutant embryos.

5. Zhang et al., 2014 indicated that Hif3a is degraded during normoxia in zebrafish embryos. However, in mutants generated for this study, defects in what is presumably steady-state erythropoiesis are observed. Are the authors able to visualize Hif3a stabilization with their antibody in embryos in vivo or via immunocytochemistry to strengthen the argument that Hif3a is required for steady-state erythropoiesis in the embryo and/or adult? Cell autonomy of the Hif-3a/Gata1 mechanism would significantly improve the impact of this study.

Minor critiques:
1. Abbreviations used in the figures are often not defined or explained in the figure legends.

2. Details/citations on the EPC cell line are not given in the methods.

3. The interpretation of the IP assays depend heavily on the fidelity of the HIF3a antibody. Could the authors cite and/or provide more information about the antibody generated? Does it cross-react with other HIF proteins? The authors also do not say what cell type was used for the IP experiments. The Western blot in figure S2E indicates some residual protein expression in Hif3a mutants, but do not indicate if this was done in adults, and/or under hypoxic conditions?

4. Do erythroblasts from adult Hif3a mutant fish also exhibit basophilic cytoplasm as in the mutant embryos?

5. The authors need to cite the software used and/or appropriately explain the methodology used by said software to quantify RNA expression and cell counts in the embryos for in situ hybridization analysis where counts of individual cells are impossible (for example globin stain).

Related, due to the small size of the individual figure panels, it is difficult to see the Â“boxedÂ” regions that was scored.

6. Somewhat related, it is unclear why control in situ panels in the same figure do not have similar expression levels. For example, in Figure 3A the gata1 and alas2 patterns are quite strong in controls, while in the rescue experiments in Figure 3D the gata1 and alas2 expression patterns are low. While some variability between samples is expected in zebrafish, controls should exhibit a roughly similar pattern with increased or decreased expression being scored from that WT baseline.

7. Please label the different bands in the HMA Figure S2C.

8. The references in the introduction should be consolidated to facilitate the flow of the text.

9. Could the authors clarify what is meant by semi-quantitative RT-PCR? Are PCR products quantitated on agarose gels or with fluorescent detection methods in a real-time PCR machine? There is no mention of the equipment used to collect data.

Comments for the author

see above

First revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field
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#1) The nature of M1 and/or M2 mutations. M1 contains the bHLH domain and M2 contains bHLH-PAS-PAC-ODD domains. The bHLH domain is known to be important for DNA binding and dimerization with HIF-b. The PAS- A/B and PAC domains are also involved in HIF-b for dimerization. One wonders whether M1 and/or M2 mutant proteins may act in a dominant-negative manner. This may explain the observed up-regulation of EPO, Runx1, c-Myb expression. This can be tested easily by co-transfection with HIF1a, HIF2a, and HIF3a1 and a HRE-luciferease reporter.
Response: We performed these experiments exactly following the reviewer’s suggestion and made Fig. S2A-S2C. Based on the results, we found that M1 and/or M2 mutant proteins had no obvious effects on the activity of hif1a, hif2a, and hif3a.

#2) Since there are multiple HIFa genes in zebrafish, it will be nice to measure other hifa genes in the mutant fish by qRT-PCR.
Response: In these revision, we examined expression of the hif-1a down-stream targets glut1, pdk1 and the hif-2a down-stream targets pou5f1, pai1 (Fig. S10C-S10F in this revision).

#3) Is it possible to include some biological data on the heterozygous fish (survival rate, red blood cells etc.)?
Response: Yes, in this revision, we added some biological data on the heterozygous fish (Fig. S2F and S2G in this revision)

#4) Key information is missing in most figures/legends (maybe except Fig. 4). Please provide the sample number (n), how many times the experiments were repeated, and what the error bars in these figures mean.
Response: Yes, we agree with the reviewer. In this revision, we have tried our best to provide all of the missing information.

#5) In multiple occasions (abstract, Introduction Page 4 and page 5), the authors emphasized that whether HIF3a is a transcription factor is still unclear. This is an outdated view. The HIF3a gene generates a number of HIF3a isoforms. But several recently published studies clearly show that the full-length HIF3a functions as a transcription factor. In addition to the loss- and gain-of-functional studies reported in zebrafish by Zhang et al., 2014, l loss-of-functional studies have been reported in mammalian system (Zhou et al., Mol Cancer Res. 2018; Huang et al., 2013. PLoS One; Heikkila et al., 2011, Cell Mol. Life Sci.) These have been reviewed by Duan, 2016, which the authors have cited. These statements should be revised.
Response: Yes, we agree with the reviewer. In this revision, we revised these statements and added the references.

#6) In the method section, it was stated that gene expression was measured by real-time PCR. But it was also mentioned that semi-quantitative PCR in figure legends. Please provide more details how the mRNA expression data were actually measured? Were the data normalized?
Response: In this revision, we provided the information. Actually, we used quantitative RT-PCR assays, but not semi-quantitative PCR.

#7) The hypoxia treatment/tolerance experiments (Fig.1) were done by manipulating air O2 levels using Invivo2 Hypoxia workstation, which is fine. Since zebrafish live in water, it will be nice to add the actual levels of dissolved O2.
Response: Yes, we agree with the reviewer. In this revision, we provided the actual levels of dissolved O2 (Fig. S2D and S2E in this revision).

#8) Supplementary Fig. S1 should be deleted as it only contains published information by others.
Response: Yes, we revised accordingly.

Reviewer 2 Advance summary and potential significance to field
The manuscript by Cai et al. examines the functional role of Hif3a in erythropoiesis in the zebrafish embryo. The authors indicate that Hif3a is required during normal embryonic erythropoiesis at steady-state, and both embryonic and adult hematopoiesis in response to acute hypoxic stress. Mutants exhibit severely compromised erythroid differentiation in the embryo, as shown by phenotypic and marker gene analysis, and demonstrate anemia in adults. The authors correlate expression of gata1 with that of hif3a during embryogenesis, and demonstrate a functional transcriptional interaction between the two TFs whereby wild-type Hif3a was shown to promote activation of a gata1 promoter construct in vitro. While the observations regarding erythropoietic maturation and number are intriguing, the study seems somewhat superficial in regard to how Hif3a may interact or intersect with the hypoxia responsive erythroid regulatory roles of the related Hif1a protein. Similarly, due to gaps in the methodological details provided, it is difficult to independently interpret much of the data shown, making the strong conclusions feel somewhat premature.

Major critiques:
1. The description of experimental methods is very scant in the text of the results, legends or methods themselves, and lacks key experimental details needed to interpret the findings and determine experimental rigor. For instance, with exception of some in situ panels, it is difficult to determine how many embryos were used to quantify a particular observation (like gene expression) versus the number of experimental replicates performed in almost all figures.
Response: Yes, we agree with the reviewer. In this revision, we have tried our best to provide all of the missing information, including the number of embryos and the number of experimental replicates.

2. Given the well-known role for Hif1a in regulating erythropoiesis, the authors need to clarify whether the response of Hif1a to hypoxia is functional, or potentially up or downregulated in the absence of Hif3a. Would the authors expect Hif1a overexpression to rescue loss of Hif3a? It is important to examine Hif1a expression/protein levels in the various assays, and potentially conduct analysis of their epistatic relationships.
Response: According to the suggestion, we performed these experiments and made new figures (Fig. S10 in this revision). In fact, Hif1a is upregulated and functional under hypoxia in hif3a mutant. It seems that Hif1a overexpression can partially rescue the defects of erythropoiesis in hif3a mutant.

3. Related, the authors mention the role of PHD enzymes and VHL protein in regulating HIF activity in the introduction. Is HIF3a controlled by these same regulators in this developmental context? A more detailed explanation/graphical depiction of the differences in zebrafish Hif3a and Hif1a, as well as potential interplay, may be helpful.
Response: According to the reviewer's suggestion, we performed these experiments and made new figures (Fig. S11 in this revision). Yes, similar to hif1a and hif2a. zebrafish hif3a is also regulated by PHD enzymes and VHL protein.

4. The authors demonstrate that the embryos are severely anemic with fewer red cells, and suggest a reduction in gata1 as the likely mechanism. However, expression of epo, a classic target of Hif1a, was not evaluated in the embryos as it was in the adult kidney. Thus, it remains unclear if a reduction in Epo production also contributes to the reduction in gata1 and anemic phenotype? If gata1 is indeed controlled by Hif3a independently of Epo (and perhaps Hif1a), then Epo overexpression would not rescue the anemic phenotype in mutant embryos.
Response: Yes, we agree with the reviewer. In this revision, according to the reviewer's suggestion, we performed these experiments and made new figure (Fig. S5 in this revision). Epo overexpression could not rescue the defects of erythropoiesis in hif3a mutant. In addition, epo is increased in hif3a mutant (Fig S4C). So, it appears that gata1 is indeed controlled by Hif3a independently of Epo.

5. Zhang et al., 2014 indicated that Hif3a is degraded during normoxia in zebrafish embryos. However, in mutants generated for this study, defects in what is presumably steady-state erythropoiesis are observed. Are the authors able to visualize Hif3a stabilization with their antibody in embryos in vivo or via immunocytochemistry to strengthen the argument that Hif3a is required for steady-state erythropoiesis in the embryo and/or adult? Cell autonomy of the Hif-3a/Gata1 mechanism would significantly improve the impact of this study.
Response: In this revision, we examined Hif3a protein level from larvae to adult tissues by Western blot analysis using an anti-Hif3a antibody (Zhang et al., 2012; provided by Dr. Cunming Duan at University of Michigan) and found that Hif3a is stable from embryo to adult (Fig. S1 and S9).

Minor critiques:
1. Abbreviations used in the figures are often not defined or explained in the figure legends.
Response: In this revision, we have tried to provide the information.

2. Details/citations on the EPC cell line are not given in the methods.
Response: We revised accordingly.

3. The interpretation of the IP assays depend heavily on the fidelity of the HIF3a antibody. Could the authors cite and/or provide more information about the antibody generated? Does it cross-react with other HIF proteins? The authors also do not say what cell type was used for the IP experiments. The Western blot in figure S2E indicates some residual protein expression in Hif3a mutants, but do not indicate if this was done in adults, and/or under hypoxic conditions?
Response: Yes, we totally agree with the reviewer that the reliability of Chromatin IP (ChIP) assays is depended heavily on the fidelity of the HIF3a antibody. Actually, this antibody was kindly provided by Dr. Cunming Duan (University of Michigan) (Zhang et al., 2012). It was generated against zebrafish HIF3a antibody specifically and its fidelity has been confirmed previously (Zhang et al., 2012). In this revision, we provided the information about this antibody and the conditions used for the sample collection and Western bolt analysis (figure S1E in this revision).
Yes, sometimes, we noticed some residual protein expression in Hif3a mutants (Fig. S1E; Fig.S9C), but its signal is much weaker than that in wildtype zebrafish. We think that it might be non-specific background.

4. Do erythroblasts from adult Hif3a mutant fish also exhibit basophilic cytoplasm as in the mutant embryos?
Response: No, it was only observed in zebrafish larvae.

5. The authors need to cite the software used and/or appropriately explain the methodology used by said software to quantify RNA expression and cell counts in the embryos for in situ hybridization analysis where counts of individual cells are impossible (for example globin stain). Related, due to the small size of the individual figure panels, it is difficult to see the “boxed” regions that was scored.
Response: In this revision, we provided the information accordingly (Materials and Methods). In addition, we highlighted the “box” in Figure 3.

6. Somewhat related, it is unclear why control in situ panels in the same figure do not have similar expression levels. For example, in Figure 3A the gata1 and alas2 patterns are quite strong in controls, while in the rescue experiments in Figure 3D the gata1 and alas2 expression patterns are low. While some variability between samples is expected in zebrafish, controls should exhibit a roughly similar pattern with increased or decreased expression being scored from that WT baseline.
Response: Sorry, we did not explain these figures in figure legends clearly, resulted in bringing the confusion for the reviewer. Actually, the controls in Figure 3A were shown the staining results of wildtype embryos (hif-3a+/+), but the controls in Figure 3D were shown the staining results of mutant embryos (hif-3a-/-), they should be different. Thus, it is understandable that the gata1 and alas2 expression patterns in Figure 3D (hif-3a-/-) is lower than those in Figure 3A (hif-3a+/+), in agreement with the main conclusion of this manuscript.

7. Please label the different bands in the HMA Figure S2C.
Response: We revised accordingly.

8. The references in the introduction should be consolidated to facilitate the flow of the text.
Response: We have tried to improve it in this revision.

9. Could the authors clarify what is meant by semi-quantitative RT-PCR? Are PCR products quantitated on agarose gels or with fluorescent detection methods in a real-time PCR machine? There is no mention of the equipment used to collect data.
Response: In this revision, we provided the information in Materials and Methods. Actually, we performed quantitative RT-PCR assays, not semi-quantitative RT-PCR. We corrected it in manuscript.

Second decision letter

MS ID#: DEVELOP/2019/185116

MS TITLE: Zebrafish hif-3α modulates erythropoiesis via gata-1 regulation to facilitate hypoxia tolerance

AUTHORS: Wuhan Xiao, Xiaolian Cai, Ziwen Zhou, junji zhu, Qian Liao, Dawei Zhang, Xing Liu, Jing Wang, and Gang Ouyang

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, referee #2 has remaining questions and concerns regarding the integration of your new data into the revised manuscript that will need to be addressed before we can consider it for 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.

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

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

Reviewer 1

Advance summary and potential significance to field

The authors have added additional data and addressed all my comments.

Comments for the author

The MS is improved and I have no more concern.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Cai et al examines the impact of loss of Hif3a on hypoxia tolerance in zebrafish. The authors made a strong effort to address the concerns of the prior review. However, rather than use those points to further clarify the mechanism and impact of Hif3a function, that material is unfortunately simply added to the text without explanation or integration. This is problematic as some of the data spoke to the biology of the system - for example Hif1a OE partially rescuing the mutants and should have lead to follow-up studies from the authors (is it the number or the maturation that is fixed? did it rely on Gata1 upregulation?) that would have added significant impact to the story. It is clear from the new studies that Hif3a can directly regulate Gata1, but the why and when remains unresolved. Does it get induced prior to Hif1a under hypoxic conditions? Is it transcriptionally regulated or post-transcriptionally?
Does it have an impact on Hif1a expression? Is it simply a back-up for acute stress? It is expressed in the RBCs themselves or in the niche? Likewise, while the authors added additional numbers as requested by both reviewers, they did not clarify what they meant in each assay. Does a notation of "n=3" mean that only 3 embryos were evaluated to draw a conclusion (a very low number in zebrafish studies) or did that mean 3 replicates? Similarly, the n=2000 notation in the antibody studies presumably indicates how many embryos were pooled to achieve a signal, however it doesn't indicate if the assay itself was repeated.
The data presented are quite intriguing and likely would be of interest to the developmental biology and hematology community, but unfortunately the current manuscript still feels preliminary in scope for publication in Development.

Comments for the author

See above.

Second revision

Author response to reviewers' comments

The manuscript by Cai et al examines the impact of loss of Hif3a on hypoxia tolerance in zebrafish. The authors made a strong effort to address the concerns of the prior review. However, rather than use those points to further clarify the mechanism and impact of Hif3a function, that material is unfortunately simply added to the text without explanation or integration.

Response: In this revision, we have tried to add more explanation in the text and clarify why and what we have done for the revision (marked by red fonts).

This is problematic as some of the data spoke to the biology of the system - for example Hif1a OE partially rescuing the mutants and should have led to follow-up studies from the authors (is it the number or the maturation that is fixed? did it rely on Gata1 upregulation?) that would have added significant impact to the story.

Response: According to the suggestion, we performed May–Grunwald–Giemsa staining for blood cells in larvae (2dpf) with hif3a-/- and hif3a-/- injected with hif1a (Fig.S10M in this revision) and examine the expression of gata1 in these larvae (Fig.S10N and S10O in this revision). In addition, we also counted the red blood cell numbers in larvae (2dpf) with hif3a-/- and hif3a-/- injected with hif1a even though it was really tough (Fig.S10J, S10K and S10L in this revision).

It is clear from the new studies that Hif3a can directly regulate Gata1, but the why and when remains unresolved. Does it get induced prior to Hif1a under hypoxic conditions? Is it transcriptionally regulated or post-transcriptionally? Does it have an impact on Hif1a expression? Is it simply a back-up for acute stress? It is expressed in the RBCs themselves or in the niche?

Response: In this revision, we tried to present clearly about the relationship between Hif1a and Hif3a, Hif1a and gata1, Hif1a and erythropoiesis and also discussed more about the role of hif3a in erythropoiesis and the underlying mechanism.
Yes, disruption of hif3a could cause redundant upregulation of hif1a and ectopic expression of hif1a could also induce gata1 expression and rescue defects of erythropoiesis exhibited in hif3a-null larvae. However, the fact was that disruption of hif3a eventually caused defects of erythropoiesis even though hif1a was upregulated. Therefore, the regulation of gata1 by hif3a may represent a main mechanism for hif3a in modulating erythropoiesis.

Likewise, while the authors added additional numbers as requested by both reviewers, they did not clarify what they meant in each assay. Does a notation of "n=3" mean that only 3 embryos were evaluated to draw a conclusion (a very low number in zebrafish studies) or did that mean 3 replicates? Similarly, the n=2000 notation in the antibody studies presumably indicates how many embryos were pooled to achieve a signal, however it doesn't indicate if the assay itself was repeated.

Response: In this revision, we re-described these numbers. Actually, “n=3” means 3 replicates, not the number of embryos. The “n=2000” means 2000 embryos. The ChIP assay needs a lot of embryos for extracting enough DNA. We used 2000 embryos in total for ChIP assay (about 700 embryos for each, three replicates).

Third decision letter

MS ID#: DEVELOP/2019/185116

MS TITLE: Zebrafish hif-3α modulates erythropoiesis via gata-1 regulation to facilitate hypoxia tolerance

AUTHORS: Wuhan Xiao, Xiaolian Cai, Ziwen Zhou, junji zhu, Qian Liao, Dawei Zhang, Xing Liu, Jing Wang, and Gang Ouyang
ARTICLE TYPE: Research Report

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

Please see my previous comments.

Comments for the author

I am satisfied with the revisions.