Dhx38 is required for the maintenance and differentiation of erythro-myeloid progenitors and hematopoietic stem cells by alternative splicing

Jiayi Tu, Shanshan Yu, Jingzhen Li, Mengmeng Ren, Yangjun Zhang, Jiong Luo, Kui Sun, Yuexia Lv, Yunqiao Han, Yuwen Huang, Xiang Ren, Tao Jiang, Zhaohui Tang, Mark Thomas Shaw Williams, Qunwei Lu and Mugen Liu
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Original submission

First decision letter

MS ID#: DEVELOP/2021/200450

MS TITLE: Dhx38 regulates the maintenance and differentiation of erythro-myeloid progenitors and hematopoietic stem cells by alternative splicing

AUTHORS: Jiayi Tu, Shanshan Yu, Jingzhen Li, Yangjun Zhang, Mengmeng Ren, Jiong Luo, Kui Sun, Yuexia Lv, Yunqiao Han, Yuwen Huang, Xiang Ren, Tao Jiang, Zhaohui Tang, Mark TS Williams, Qunwei Lu, and Mugen Liu

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 the manuscript by Tu et al. entitled “Dhx38 regulates the maintenance and differentiation of erythro-myeloid progenitors and hematopoietic stem and progenitor cells by alternative splicing”, a role for the splicing factor Dhx38 in embryonic hematopoiesis was explored. The authors claim that loss of dhx38 in zebrafish impairs HSPCs and EMPs. The defects are attributed to abnormal cell cycle and cell death triggered by changes in pre-mRNA splicing. Splicing regulation is important for human health and understanding how defects in splicing factors impact tissue homeostasis is important.

Comments for the author

These mechanisms put forth by the reviewers are supported by correlative data, but an involvement of any of the alternative splicing events is not directly tested.
It is also plausible that disruption of RNA processing could trigger DNA damage independent of specific splice isoform changes. These major concerns plus other concerns spelled out below could significantly improve the presented data.
1. Embryonic hematopoiesis in 30-48 hpf zebrafish embryos is comprised of cells from both the primitive and EMP waves. The authors claim that the changes in granulocyte and erythrocyte numbers in dhx38 animals at these time points is due to EMP defects. It is also plausible that it is due to defects in differentiation of primitive progenitors or defects in the viability of the differentiated cells
(supported by data in Figure 8).
2. More rigorous quantification of in situ hybridization and fluorescence results throughout the manuscript need to be provided. This can be done using ImageJ by cell counting or region of interest quantifications. Accompanying statistical analysis of the quantitative data also need to be provided.
3. The experiment shown in figure 7D must be explained in more detail. What are the two different DHX38 siRNAs and how are they different? This can be applied to many of the experiments described in the manuscript, so please make the language clearer.
4. The authors show the expression pattern of the dhx38 promoter is enriched in PBI. Prior in situ hybridization data suggests the gene is fairly ubiquitous in developing zebrafish (Thisse et al. 2004), a finding that is consistent with the ubiquitous expression of Dhx38 in other organisms. This discrepancy should be explained.

Minor points
1. Please read through the manuscript for grammatical and spelling errors. There are many throughout the manuscript.
2. On page 3, please change the references from Mupo, Fei, and Frame and North which are either reviews or references to mouse papers instead of fish papers, to the following three primary references to properly cite those three mutants:
1) Sf3b1: De La Garza A, Cameron RC, Nik S, Payne SG, Bowman TV. Spliceosomal component Sf3b1 is essential for hematopoietic differentiation in zebrafish. Exp Hematol. 2016 Sep;44(9):826-837.e4. doi: 10.1016/j.exphem.2016.05.012. Epub 2016 Jun 1. PMID: 27260753; PMCID: PMC4992596. 2) Ddx41: Weinreb JT, Ghazale N Pradhan K, Gupta V, Potts KS, Tricomi B, Daniels NJ, Padgett RA, De Oliveira S Verma A, Bowman TV. Excessive R-loops trigger an inflammatory cascade leading to increased HSPC production. Dev Cell. 2021 Mar 8;56(5):627-640.e5. doi: 10.1016/j.devcel.2021.02.006. Epub 2021 Mar 1. PMID: 33651979; PMCID: PMC8258699.
3) U2af1: Danilova N, Kumagai A, Lin J. p53 upregulation is a frequent response to deficiency of cell-essential genes. PLoS One. 2010 Dec 31;5(12):e15938. doi: 10.1371/journal.pone.0015938. PMID: 21209837; PMCID: PMC3013139.

3. Please remove this line from the discussion, unless you can show that this is the only splicing factor involved in EMP development: “In particular, Dhx38 is identified to be the ‘missing link’ of splicing factors in EMP development.”
4. In figure 5C, in the dhx38-/- panel, how are the first 3 versus last 3 panels different from one another? Please label the figure more clearly and explain in the text as well.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Tu et al. describes the role of dhx38 in regulating erythro-myeloid progenitors (EMPs) and hematopoietic stem cells (HSCs) in zebrafish embryos. By combining genetic mutants, cell cycle analysis, and RNA-seq, authors showed abnormal splicing of cell-cycle related genes, cell-cycle arrest of EMPs and HSCs, and decrease of differentiated hematopoietic lineages in zebrafish dhx38 mutants. The hematopoietic defects were, at least partially, contributed by the p53-dependent apoptosis of EMPs and HSCs. Overall, the paper is well organized and demonstrates a novel insight of dhx38 in regulating hematopoiesis. However, there are a few questions to be addressed before publication.

Comments for the author

Essential revisions:
1. The expression of dhx38 in EMPs and HSPCs was supported by the co-localization of dhx38-mCherry and cmyb-eGFP in the PBI at 36 hpf (Figure 1B).
The co-localization of mCherry and GFP is not clear in the figure. It will be helpful to show images with higher quality to demonstrate the co-localization.
In addition, as Tg(cmyb:eGFP) could mark both HSPCs and EMPs in the PBI at 36 hpf, the co-localization of Dhx38 and cmyb-eGFP in the VDA region would be more convincing to claim its expression in HSPCs.
2. A p53 inhibitor could rescue cmyb+ or gata1+ cells in dhx38 mutants (Figure 8D), but the quantification in dhx38 mutants with p53 inhibition was missing. In addition, EMPs only transiently exist between 24-48 hpf (Bertrand et al., 2007).
Gata1+ cells at later stages may represent differentiated cells from EMPs or even HSPCs. The authors need to pay attention when claiming the defects of EMPs.
3. A rescue experiment with dhx38 mRNA would help to verify the hematopoietic defects observed in dhx38 mutants. Alternatively, morpholinos or F0 ‘crispant’ knockouts targeting other sites of dhx38 could confirm the phenotype of dhx38 mutants.

Minor comments
1. Some of the figures, especially the mpeg1 signal in Figure 1I, Figure 2E-F, and Figure 4A/B/E/F, are not clear. Images with higher magnification and less background may better demonstrate the authors’ points.
2. Pure HSCs in zebrafish are not well defined. It would be better to use hematopoietic progenitor/stem cells (HSPCs) to replace the “HSCs” in the manuscript.
3. Page177-180, “EMPs and HSCs are overlapped from the dorsal aorta and CHT region at 36 to 48 hpf, but can be sorted based on cd41+gata1+ (HSCs) and cd41+gata1- (EMPs) markers respectively as previously reported (Bertrand et al., 2008; Bertrand et al., 2010; Forrester et al., 2012).” The interpretation of previous reports is incorrect. Actually, HSPCs were sorted by CD41+ from 42 hpf trunk, whereas EMPs were sorted by LMO2+GATA1+ from 30 hpf PBI in previous studies (Bertrand et al., 2008, 2010).

First revision

Author response to reviewers' comments

Response to Review

We have performed experiments and addressed each of the insightful concerns of the Reviewers in the revised manuscript. Major changes made in the manuscript text are marked in blue color.

Reviewer 1 Advance Summary and Potential Significance to Field:

In the manuscript by Tu et al. entitled "Dhx38 regulates the maintenance and differentiation of erythro- myeloid progenitors and hematopoietic stem and progenitor cells by alternative splicing", a role for the splicing factor Dhx38 in embryonic hematopoiesis was explored. The authors claim that loss of *dhx38* in zebrafish impairs HSPCs and EMPs. The defects are attributed to abnormal cell cycle and cell death triggered by changes in pre-mRNA splicing. Splicing regulation is important for human health and understanding how defects in splicing factors impact tissue homeostasis is important.

Reviewer 1 Comments for the Author:
These mechanisms put forth by the reviewers are supported by correlative data, but an involvement of any of the alternative splicing events is not directly tested. It is also plausible that disruption of RNA processing could trigger DNA damage independent of specific splice isoform changes. These major concerns plus other concerns spelled out below could significantly improve the presented data.
Response:
We are indebted to the reviewer for the insightful and constructive comments and suggestions. Yes, DNA damage might be caused by the alternative splicing of specific genes but also, as you mentioned, by the dysregulation of fundamental processes like transcription, DNA replication, and mitosis (Wickramasinghe and Venkitaraman, 2016; Jelluma and Kops, 2014). Therefore, it is likely that multiple regulatory mechanisms of DHX38 co-exist in cells and work together to regulate DNA damage. Mechanistically, mRNA processing factors involved in maintaining genome integrity occur at different levels, either indirectly by regulating DNA damage response or mitotic gene transcription and splicing, or more directly by preventing R-loop co-transcription (Hofmann et al., 2010). It has also been shown that mitotic errors and DNA damage are mechanistically distinct results of mRNA splicing disorders (Jimenez et al., 2019; Weinreb et al., 2021). Actually, we have another paper under review that focuses on the essential role of Dhx38 in suppressing the accumulation of R-loops.
We add discussion with Dhx38 and disruption of RNA processing on **Page 16: Line 420 - 431**.

Ref: Wickramasinghe, V. O. and Venkitaraman, A. R. (2016) RNA Processing and Genome Stability: Cause and Consequence, Mol Cell 61(4): 496-505.
Jelluma, N. and Kops, G. J. (2014) Collateral genome instability by DNA damage in mitosis, *Cancer Discov* 4(11): 1256-8.
Hofmann, J. C., Husedzinovic, A. and Gruss, O. J. (2010) The function of spliceosome components in open mitosis, Nucleus 1(6): 447-59.
Jimenez, M., Urtasun, R., Elizalde, M., Azkona, M., Latasa, M. U., Uriarte, I., Arechederra, M., Alignani, D., Barcena-Varela, M., Alvarez-Sola, G. et al. (2019) Splicing events in the control of genome integrity: role of SLU7 and truncated SRSF3 proteins, Nucleic Acids Res 47(7): 3450-3466.
Weinreb, J. T., Ghazale, N., Pradhan, K., Gupta, V., Potts, K. S., Tricomi, B., Daniels, N. J., Padgett, R. A., De Oliveira, S., Verma, A. et al. (2021) Excessive R-loops trigger an inflammatory cascade leading to increased HSPC production, *Dev Cell* 56(5): 627-640 e5.

1. Embryonic hematopoiesis in 30-48 hpf zebrafish embryos is comprised of cells from both the primitive and EMP waves. The authors claim that the changes in granulocyte and erythrocyte numbers in *dhx38* animals at these time points is due to EMP defects. It is also plausible that it is due to defects in differentiation of primitive progenitors or defects in the viability of the differentiated cells (supported by data in Figure 8).

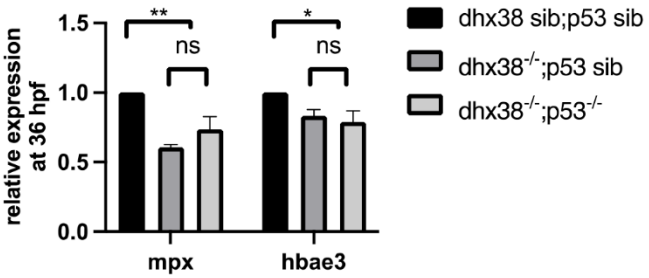
Thank you for raising this, we understand the reviewer's viewpoint here. It seems that EMPs overlap with the latter half of primitive hematopoiesis and the emergence of HSPCs from the dorsal aorta at 36 to 48 hpf, making it hard to distinguish the overlap changes. To determine to which group the defect belongs, we used a strict timing check the hematopoietic development.

The primary hematopoiesis peaks at 12 hpf and declines before 24 hpf. The granulocyte and erythrocyte numbers from primary hematopoiesis are first to test in around 24 hpf (Davidson and Zon, 2004). However, we detected no difference between wild-type siblings and *dhx38* mutants in the expression of the myelocyte markers *mpx* and *lyz* in the rostral blood island, as well as the erythrocyte marker *β-eglobin* in the intermediate cell mass, at 24 hpf, and even at 30 hpf (Fig. S1C, Fig. S2C). Based on this, we consider the differentiation of primary hematopoiesis is not significantly changed.

The EMPs' peaks at 30 hpf and begins to decline after 40 hpf (Bertrand et al., 2007). We also didn't find any defects in EMP at 30 hpf (Figure S2C). However, the expression of alpha-*globin* labeled by *hbae1* and myelocytes labeled by *mpx*, *mfap4*, and *mpeg1* is reduced in the *dhx38* mutant at 33 hpf and 36 hpf (Fig. 1H, I; Fig.S4). Meanwhile, we found that the expression of *lmo2* and *gata1*, which have previously been reported to represent EMPs, were increased dramatically at 33 hpf in the PBI of *dhx38* mutants (Fig. 1F). As a result, we suspect that changes in differentiated cell number in *dhx38* mutants at 33 hpf are mainly caused by EMP defects.

We also wondered if the changes in granulocyte and erythrocyte numbers in *dhx38* mutants at 30-48 hpf are due to defects in the viability of the differentiated cells. To answer this question, we performed TUNEL assays to determine apoptosis of EMPs and HSPCs in *dhx38*$^{-/-}$ embryos. However, there is no significant difference between *dhx38* mutants and wild-type siblings in the proportion of apoptotic EMPs and HSPCs at 36 hpf in the PBI (Fig. S4C).

Although *p53* expression is increased in the mutants' CHT at 36 hpf, inhibiting *p53* activity in *dhx38* mutants failed to rescue the phenotype of granulocyte (*mpx*) and erythrocyte (*hbae3*) reduction at 36 hpf. Therefore, we eliminate the possibility that the changes in granulocyte and erythrocyte numbers in *dhx38* mutants at 36 hpf are due to defects in the viability of differentiated cells.



Ref: Bertrand, J. Y., Kim, A. D., Violette, E. P., Stachura, D. L., Cisson, J. L. and Traver, D. (2007) Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo, Development 134(23): 4147-4156.
Davidson, A. J. and Zon, L. I. (2004) The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis, *Oncogene* 23(43): 7233-46.

2. More rigorous quantification of in situ hybridization and fluorescence results throughout the manuscript need to be provided. This can be done using ImageJ by cell counting or region of interest quantifications. Accompanying statistical analysis of the quantitative data also need to be provided.

Thank you for underlining this deficiency. At the reviewer's suggestion, we have performed a quantitative analysis of in situ hybridization and fluorescence using Image J. New results have been added to the revised manuscript in Fig. 1, Fig. 3, Fig. 8, and the corresponding figure legends.

Revised Fig. 1:

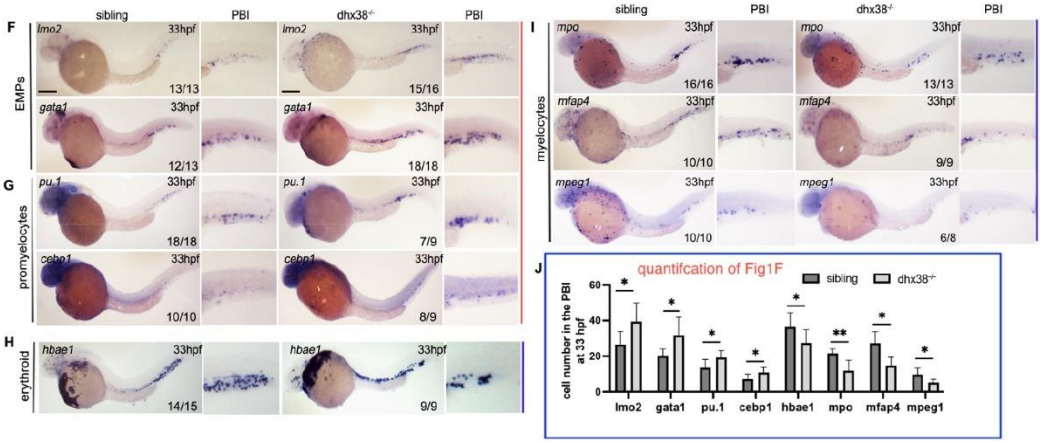
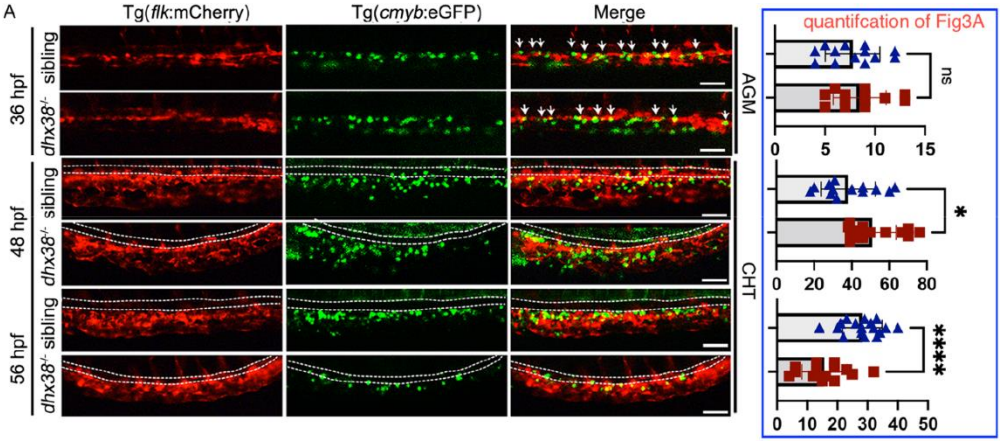


Fig. 1J Quantification of cells from (F-I), respectively; two-tailed Student's t-test; *p < 0.05, **p < 0.01; error bars, mean ± SD.

Revised Fig. 3:



Revised Fig. 8:

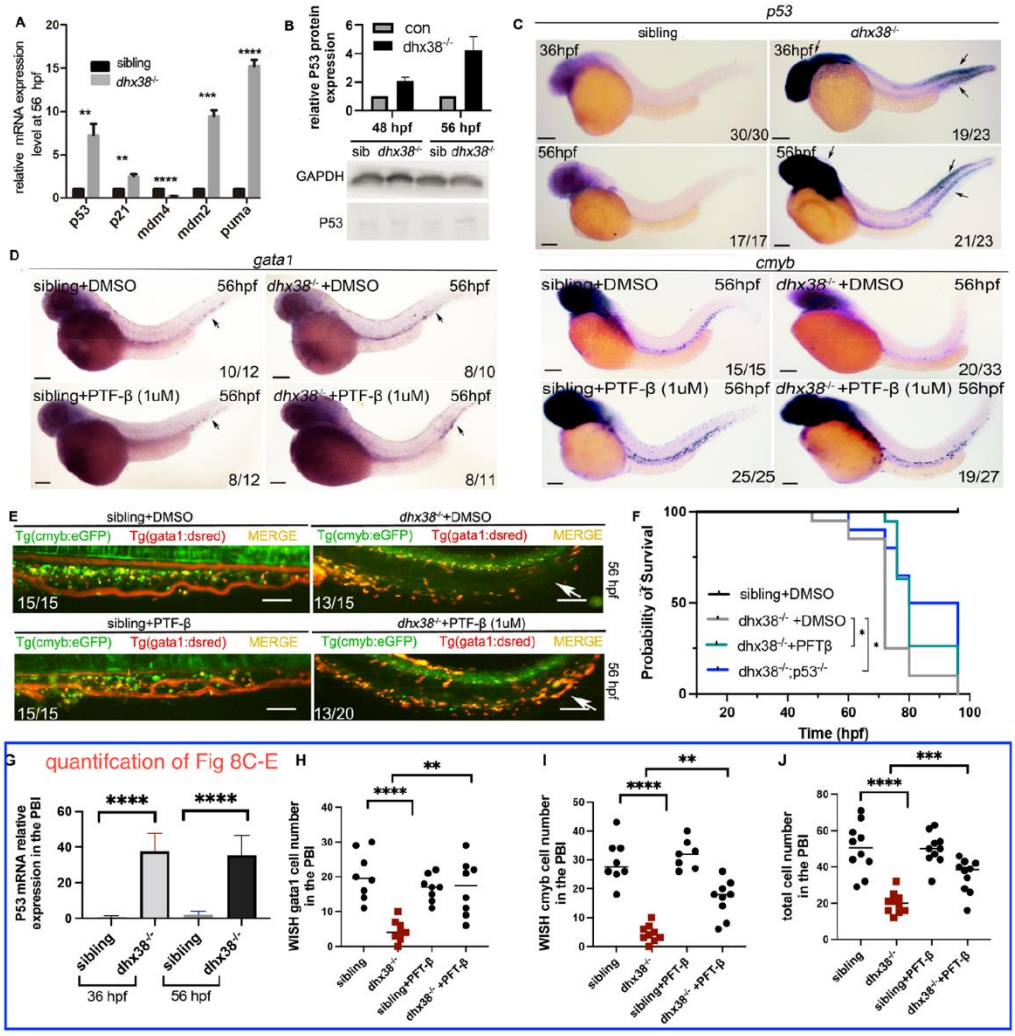


Fig. 8G-J Quantification of cells from (C-E), respectively; two-tailed Student's t-test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; error bars, mean ± SD.

3. The experiment shown in figure 7D must be explained in more detail. What are the two different DHX38 siRNAs and how are they different? This can be applied to many of the experiments described in the manuscript, so please make the language clearer.

We deeply appreciate the reviewer's suggestion. According to the reviewer's comment, we have now added this explanation to the methods and figure legend section on **Page 14: Line 359-365 and Page 26: Line 755-758**.

Small interfering RNAs (siRNAs) targeting different encoding regions of human DHX38 were designed by the RiboBio company (Guangzhou, China). Two independent siRNAs were used for the Dhx38 gene to avoid off-target effects. The siDHX38-641 RNA is at the c.641 position of the human DHX38 gene, and siDHX38-328 is at c.328. A nonspecific siRNA duplex was used as an siRNA negative control (si-NC). The silencing effects of these two siRNAs were confirmed by western blot. The siDHX38-641 has valid silencing effects, while the siDHX38-328 siRNA does not (Fig 7D).

Sequences of used these two siRNAs are listed in Table S7.

Negative control (Nc)	5'-3' UUCUCCGAACGUGUCACGUTT
	3'-5' ACGUGACACGUUCGGAGAATT
DHX38-Homo-641	5'-3' GCGGGAACAUGGUGUCUAUTT
	3'-5' AUAGACACCAUGUUCCCGCTT
DHX38-Homo-325	5'-3' GCGAGCAGCAUGUCUUCAATT
	3'-5' UUGAAGACAUGCUGCUCGCTT

Revised Fig. 7D The siDHX38-641 siRNA is at the c.641 position of the human DHX38 gene, siDHX38-328 is at c.328. The silencing effects of these two siRNAs were confirmed by western blot. Western of DHX38 shows efficiency of DHX38 knockdown in the si-DHX38-641 group, but not the si-DHX38-325 group.
As suggested by reviewers, we additionally clarify Fig.6A, Fig.6C, Fig.7A, and Fig.7D.

Page 25: Line 726-727
Fig. 6A Categories of differential spliced genes based on the changed PSI value in the *dhx38* mutants. The PSI UP indicates that exon/intron is easier to skip. PSI DOWN indicates that exon/intron is easier to retain. PSI, percent spliced in; A3SS, alternative 3' splice site; A5SS, alternative 5' splice site; SE, skipped exon; RI, retained intron; MXE, mutually exclusive exons.

Page 26: Line 732-737
Fig. 6C Sequence lengths and GC content around normal splicing sites and significant abnormal splicing sites in SE and RI events of *dhx38* mutants. In SE events, the length of PSI DOWN group of 3' and 5' introns is longer than the PSI control group, but the length of PSI UP group of introns in RI events is shorter. It means that longer introns in SE events and shorter introns in RI events are more likely to respond to the dhx38 loss function. The PSI DOWN group of GC content around the exon in differential SE events is lower in the *dhx38* mutants. It means lower GC content of exon is more likely skipping when *dhx38* knockout.

Page 26: Line 742-746
Fig7A Semi-qPCR confirms the abnormal splicing of the genes in Fig. 6D. The left band represents gene splicing events in siblings, while the right band represents splicing events in the *dhx38* mutants. PSI, percent spliced in; A3SS, alternative 3' splice site; A5SS, alternative 5' splice site; SE, skipped exon; RI, retained intron; E, exon; i, intron; PTC, premature termination codon.

Page 26: Line 755-758
Fig. 7D The siDHX38-641 siRNA is at the c.641 position of the human DHX38 gene, siDHX38-328 is at c.328. The silencing effects of these two siRNAs were confirmed by western blot. Western blot of DHX38 shows efficient DHX38 knockdown in the si-DHX38-641 group but not si-DHX38-325 group. Western blot of CCNB2 in the si-DHX38-641 group displays decreased protein expression, compared to the si-NC or the si-DHX38-325 group. n=3, *p=0.041.

4. The authors show the expression pattern of the *dhx38* promoter is enriched in PBI. Prior in situ hybridization data suggests the gene is fairly ubiquitous in developing zebrafish (Thisse et al. 2004), a finding that is consistent with the ubiquitous expression of Dhx38 in other organisms. This discrepancy should be explained.
It is true as Reviewer mentioned that Thisse et al. shows *dhx38* expression is in whole-organism (Thisse et al. 2004). To investigate the role of *dhx38* in the development of hematopoiesis, we performed WISH to examine its spatiotemporal expression in WT embryos at 36 hpf and 48 hpf (Fig. S1A). Dhx38 displayed a ubiquitous but dynamic expression pattern throughout embryo development, which is consistent with previous findings. Additionally, we found that *dhx38* was expressed in PBI at 36 hpf and further increased in expression in CHT at 48 hpf. Besides HSPCs, *dhx38* is highly expressed in the head as well. It appears that different cis-regulatory elements are likely to promote gene expression in such disparate tissues (Gottgens et al., 2004; Li et al., 2013) .

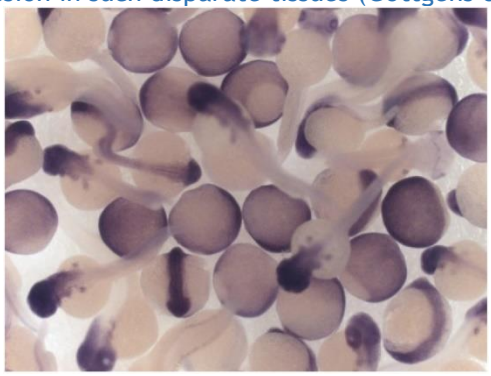


Fig Ref: Thisse et al. 2004

[NOTE: We have removed a figure which was provided for the referees in confidence.]

A WISH with a *dhx38* probe reflects *dhx38* expression in the PBI and CHT region at 36 hpf and 48 hpf, respectively. Black arrowheads indicate the expression pattern of *dhx38* markers. Scale bars, 200 μm.

Ref: Thisse, B., et al. (2004). Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. Methods Cell Biol 77: 505-519.
Gottgens, B., Broccardo, C., Sanchez, M. J., Deveaux, S., Murphy, G., Gothert, J. R., Kotsopoulou, E., Kinston, S., Delaney, L., Piltz, S. et al. (2004) The scl +18/19 stem cell enhancer is not required for hematopoiesis: identification of a 5' bifunctional hematopoietic-endothelial enhancer bound by Fli-1 and Elf-1, Mol Cell Biol 24(5): 1870-83.
Li, L., Freudenberg, J., Cui, K., Dale, R., Song, S. H., Dean, A., Zhao, K., Jothi, R. and Love, P. E. (2013) Ldb1-nucleated transcription complexes function as primary mediators of global erythroid gene activation, Blood 121(22): 4575-85.

Minor points

1. Please read through the manuscript for grammatical and spelling errors. There are many throughout the manuscript.

Response:

Thank you for the comment. We have fixed grammatical and spelling errors throughout the manuscript. The revised manuscript was critically read and modified by Dr. Mark TS Williams (Glasgow Caledonian University) in the new version of the manuscript.

2. On page 3, please change the references from Mupo, Fei, and Frame and North, which are either reviews or references to mouse papers instead of fish papers, to the following three primary references to properly cite those three mutants:

1) Sf3b1: De La Garza A, Cameron RC, Nik S, Payne SG, Bowman TV. Spliceosomal component Sf3b1 is essential for hematopoietic differentiation in zebrafish. Exp Hematol. 2016 Sep;44(9):826-837.e4. doi: 10.1016/j.exphem.2016.05.012. Epub 2016 Jun 1. PMID: 27260753; PMCID: PMC4992596.

2) Ddx41: Weinreb JT, Ghazale N, Pradhan K, Gupta V, Potts KS, Tricomi B, Daniels NJ, Padgett RA, De Oliveira S, Verma A, Bowman TV. Excessive R-loops trigger an inflammatory cascade leading to increased HSPC production. Dev Cell. 2021 Mar 8;56(5):627-640.e5. doi: 10.1016/j.devcel.2021.02.006. Epub 2021 Mar 1. PMID: 33651979; PMCID: PMC8258699.

3) U2af1: Danilova N, Kumagai A, Lin J. p53 upregulation is a frequent response to deficiency of cell- essential genes. PLoS One. 2010 Dec 31;5(12):e15938. doi: 10.1371/journal.pone.0015938. PMID: 21209837; PMCID: PMC3013139.

Response:

Thank you for your introduction to this wonderful and relatively research work. According to your suggestion, we properly cite these articles on **Page 3: Line 69-70** as follows:

Reference number 14: Danilova, N., Kumagai, A. and Lin, J. (2010) p53 upregulation is a frequent response to deficiency of cell-essential genes, *PLoS One* 5(12): e15938.

Reference number 15: De La Garza, A., Cameron, R. C., Nik, S., Payne, S. G. and Bowman, T. V. (2016) Spliceosomal component Sf3b1 is essential for hematopoietic differentiation in zebrafish, *Exp Hematol* 44(9): 826-837 e4.

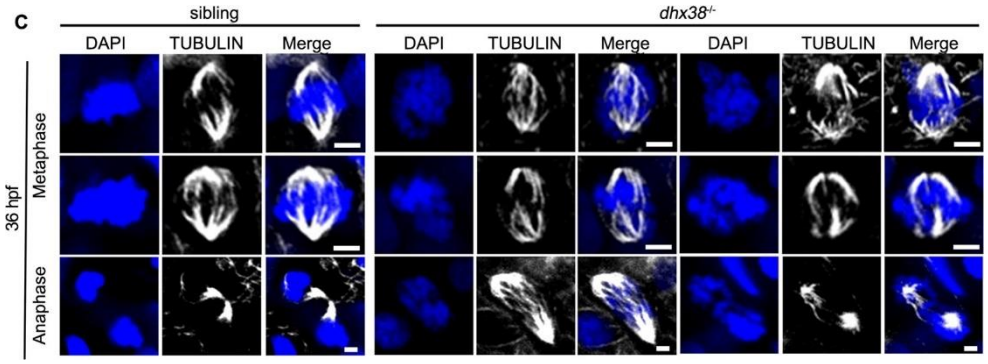
Reference number 16: Weinreb, J. T., Ghazale, N., Pradhan, K., Gupta, V., Potts, K. S., Tricomi, B., Daniels, N. J., Padgett, R. A., De Oliveira, S., Verma, A. et al. (2021) Excessive R-loops trigger an inflammatory cascade leading to increased HSPC production, *Dev Cell* 56(5): 627-640 e5.

3. Please remove this line from the discussion, unless you can show that this is the only splicing factor involved in EMP development: “In particular, Dhx38 is identified to be the ‘missing link’ of splicing factors in EMP development.”

We thank the reviewer for the good suggestion. In the revised manuscript, we have removed this sentence.

4. In figure 5C, in the *dhx38*$^{-/-}$ panel, how are the first 3 versus last 3 panels different from one another? Please label the figure more clearly and explain in the text as well.
Thanks for pointing out this problem. The first three panels are similar to the last three panels showing different abnormal cells with multiple "graped" karyotypes and progressively disordered spindles phenotypes. We labeled the figure and explained it in the figure legends on **Page 25: Lines 712-716** as below:

Original figure 5C:



Revised figure 5C:

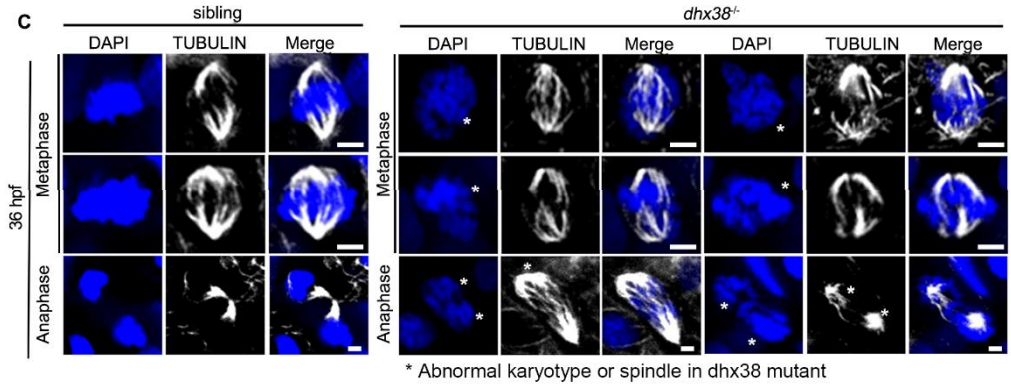
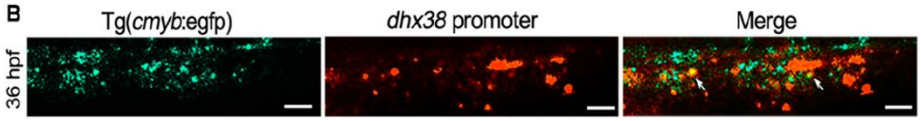


Fig 5C: Confocal image of co-immunostaining immunostaining for α-TUBULIN and DAPI. The fist 3 panel in sibling shows normal karyotype and spindles during metaphase and anaphase. The last 6 panel in *dhx38*$^{-/-}$ show a clearly 'grape' karyotype in metaphase. The 'grape' karyotype appears to be chromosome unable to align to the equatorial plate and progressing to disordered anaphase in *dhx38*$^{-/-}$ embryo. Scale bars, 5 μm.

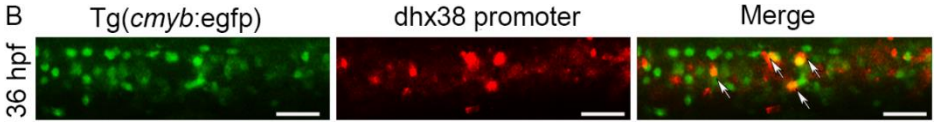
Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript by Tu et al. describes the role of *dhx38* in regulating erythro-myeloid progenitors (EMPs) and hematopoietic stem cells (HSCs) in zebrafish embryos. By combining genetic mutants, cell cycle analysis, and RNA-seq, authors showed abnormal splicing of cell-cycle related genes, cell-cycle arrest of EMPs and HSCs, and decrease of differentiated hematopoietic lineages in zebrafish *dhx38* mutants. The hematopoietic defects were, at least partially, contributed by the p53-dependent apoptosis of EMPs and HSCs. Overall, the paper is well organized and demonstrates a novel insight of *dhx38* in regulating hematopoiesis. However, there are a few questions to be addressed before publication.

Reviewer 2 Comments for the Author:
Essential revisions:
1. The expression of *dhx38* in EMPs and HSPCs was supported by the co-localization of *dhx38*-mCherry and cmyb-eGFP in the PBI at 36 hpf (Figure 1B).
The co-localization of mCherry and GFP is not clear in the figure. It will be helpful to show images with higher quality to demonstrate the co-localization.

Thank you for pointing out this unclear figure. We have replaced this figure with a clearer one.
Original figure 1B:



Revised figure 1B:



In addition, as Tg(cmyb:eGFP) could mark both HSPCs and EMPs in the PBI at 36 hpf, the co-localization of Dhx38 and cmyb-eGFP in the VDA region would be more convincing to claim its expression in HSPCs.

According to the reviewer's point, Tg (*cmyb*: eGFP) could mark both HSPCs and EMPs in the PBI at 36 hpf, which is consistent with our point of view. The expression of *dhx38* in EMPs and HSPCs was supported by the co-localization of *dhx38*-mCherry and *cmyb*-eGFP in the PBI at 36 hpf. Additionally, we detected the co-localization of *dhx38* and *cmyb* in the VDA region at 36 hpf by using immunofluorescence. Dhx38 also has a co-location with *cmyb* in the VDA region at 36 hpf (Figure S1B). But it is worth mentioning that the DHX38 expression in the PBI is greater than in the VDA. We consider *dhx38* is more highly expressed in the region that needs proliferation events.

The figure was added in **Fig S1B**.

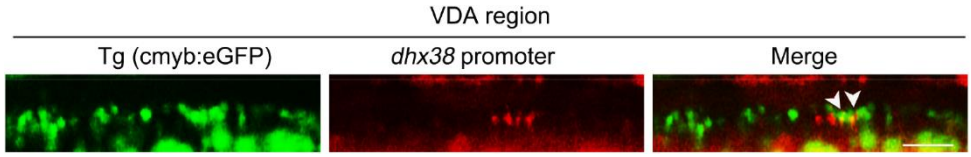
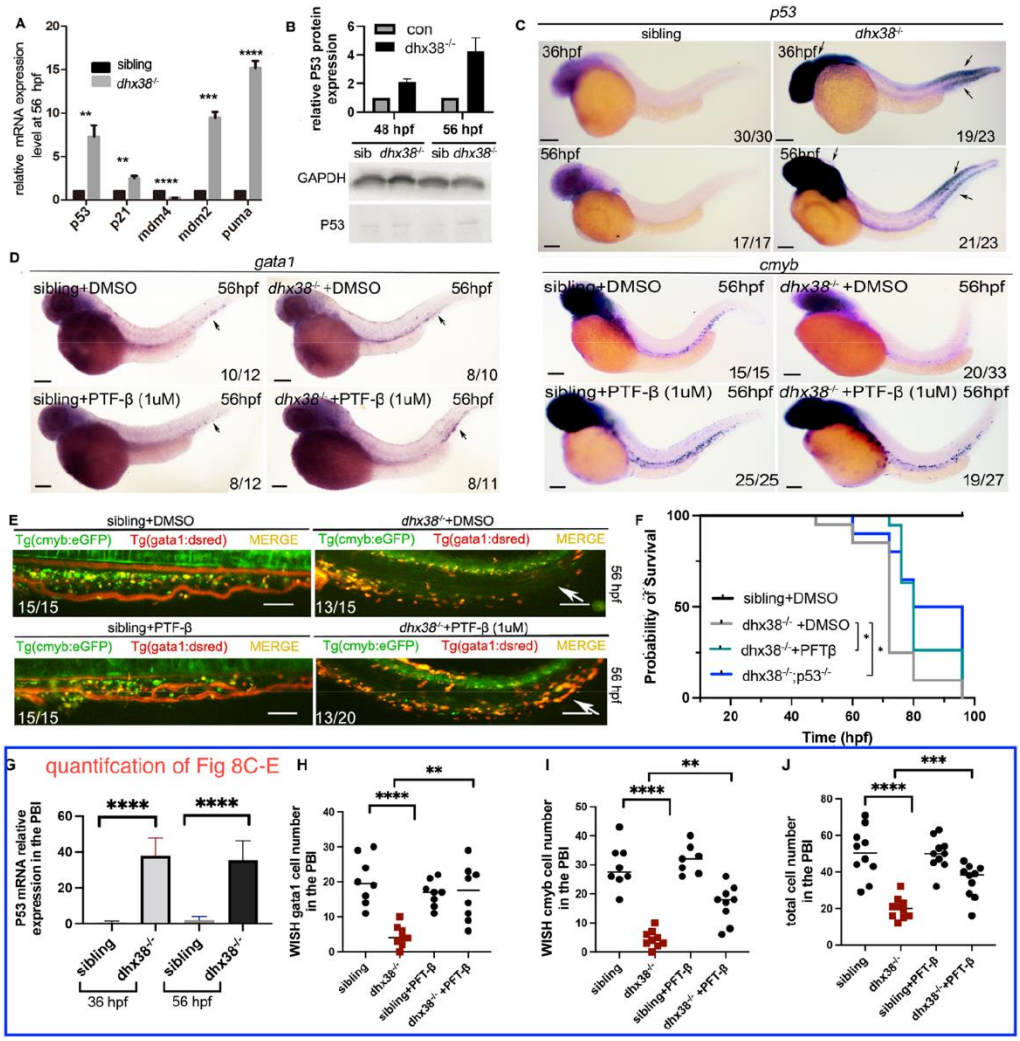


Fig S1B: Phenotype distribution plot of *dhx38* expression in Tg(*cmyb*:eGFP) in the VDA region at 36 hpf. White arrowheads indicate the co-expression of *dhx38* and *cmyb*. Scale bars, 50 μm.

2. A p53 inhibitor could rescue cmyb+ or gata1+ cells in *dhx38* mutants (Figure 8D), but the quantification in *dhx38* mutants with p53 inhibition was missing.
Sorry for this missing part. We have added all the quantifications in Figures 1-8 in the revision. Please see our response to the comment (2) of Reviewer #1.



In addition, EMPs only transiently exist between 24-48 hpf (Bertrand et al., 2007). Gata1+ cells at later stages may represent differentiated cells from EMPs or even HSPCs. The authors need to pay attention when claiming the defects of EMPs.

Thank you for your kind reminder. To clearly describe the EMP, we mainly noted the EMPs with *cmyb*$^{+}$*gata1*$^{+}$ after 48 hpf.

3. A rescue experiment with *dhx38* mRNA would help to verify the hematopoietic defects observed in *dhx38* mutants. Alternatively, morpholinos or F0 'crispant' knockouts targeting other sites of *dhx38* could confirm the phenotype of *dhx38* mutants.

Thanks for this valuable comment. Another F0 'crispant' knockout, *dhx38* del7, was tested in hematopoietic development. It exhibits a phenotype that is identical to the *dhx38* del10 we used in this study. This figure has been added as **Fig. S9**, and it is described as follows on **Page 7: line 185-187**.

Using an F0 strain, *dhx38* del7 (c.14_21delCTCCCTG), we also confirm the role of *dhx38* in hematopoiesis, as this mutant phenocopies that of the *dhx38* del10 mutant confirming (Fig. S9).

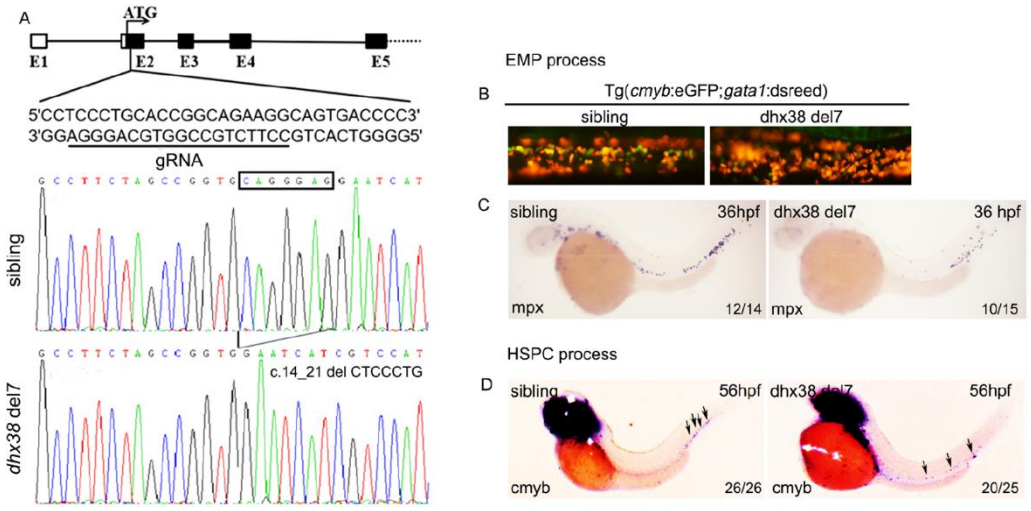
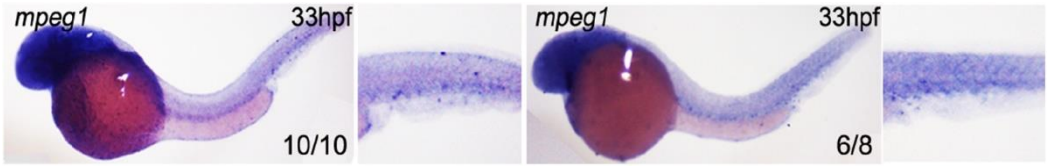


Fig S9: Enhanced EMP progenitor and impaired HSPC maintenance in *dhx38* del 7 mutants.
A A schematic diagram of the *dhx38* gRNA locus. DNA sequencing identified a 7 bp deletion of cDNA (c.14_21delCTCCCTG), which predicts a truncated protein (p.Ser5_ThrfsTer8). **B** In vivo imaging of EMPs and HSPCs in the PBI region of Tg(*cmyb*:eGFP;*gata1*:dsred) fish at 36 hpf. The number of EMPs and HSPCs in the *dhx38*$^{-/-}$ zebrafish is higher than that in wild-type siblings. **C** WISH results reflecting the expression of mature myelocytes (*mpx*) is decreased in the *dhx38* del7 mutant at 36 hpf. **D** The expression of the hematopoietic progenitor cells marker *cmyb* at 56 hpf in siblings and *dhx38* del7 mutants. The black arrows indicate the position of the *cmyb* gene expression.

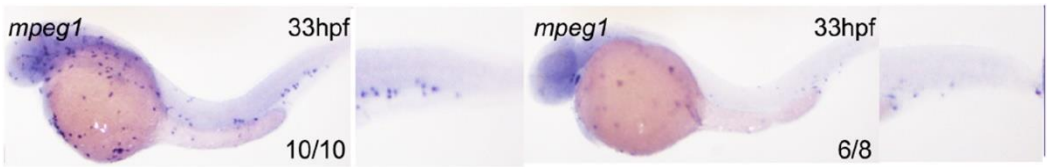
Minor comments

1. Some of the figures, especially the mpeg1 signal in Figure 1I, Figure 2E-F, and Figure 4A/B/E/F, are not clear. Images with higher magnification and less background may better demonstrate the authors' points.
Thank you for pointing out these unclear figures. We have already replaced the old figures with clearer ones.

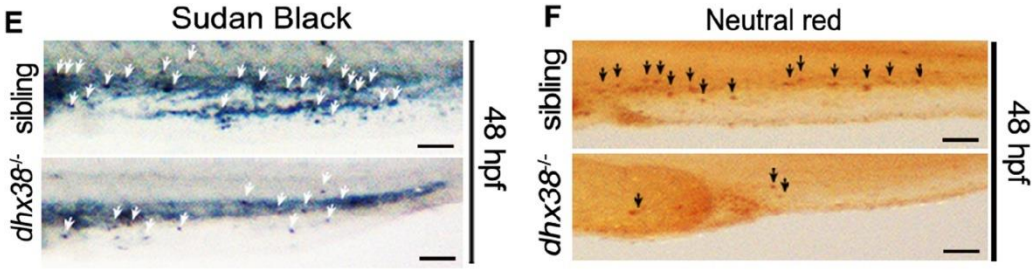
Original Fig. 1l:



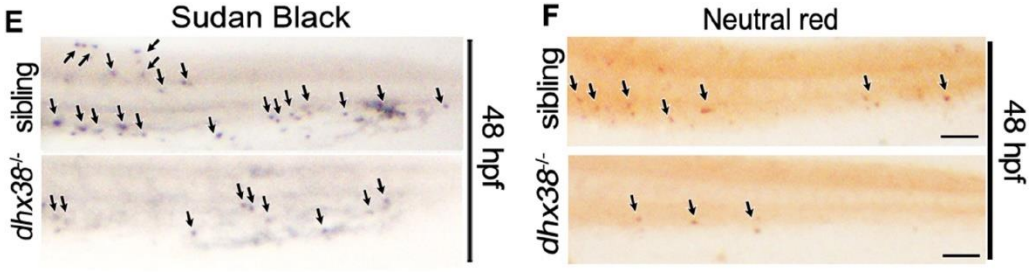
Revised Fig. 1l:



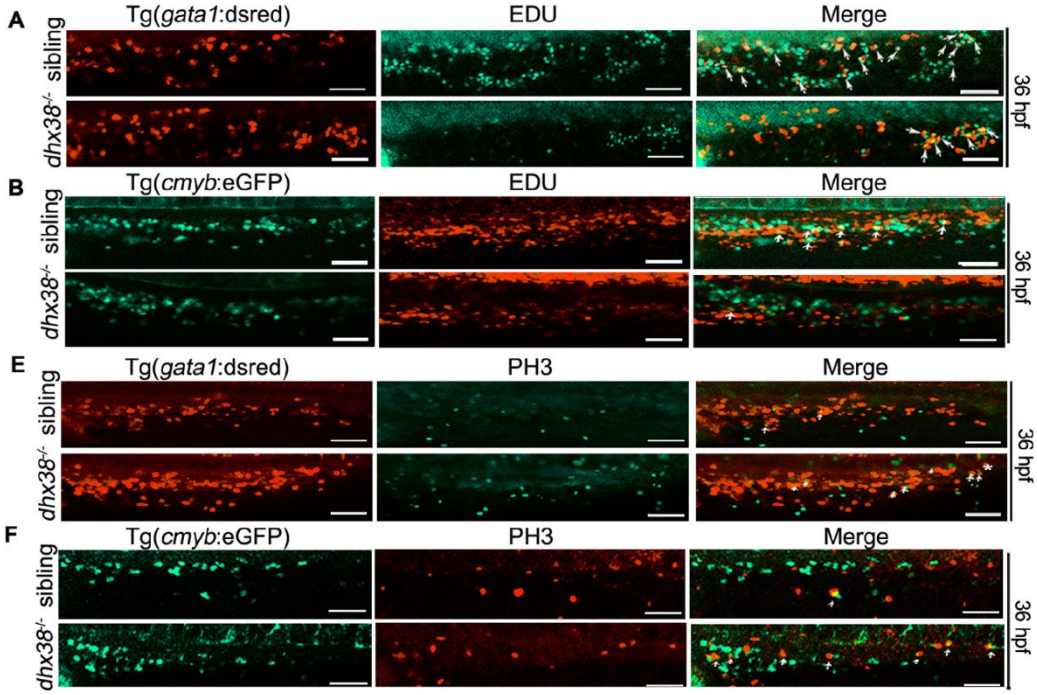
Original Fig. 2E-F:



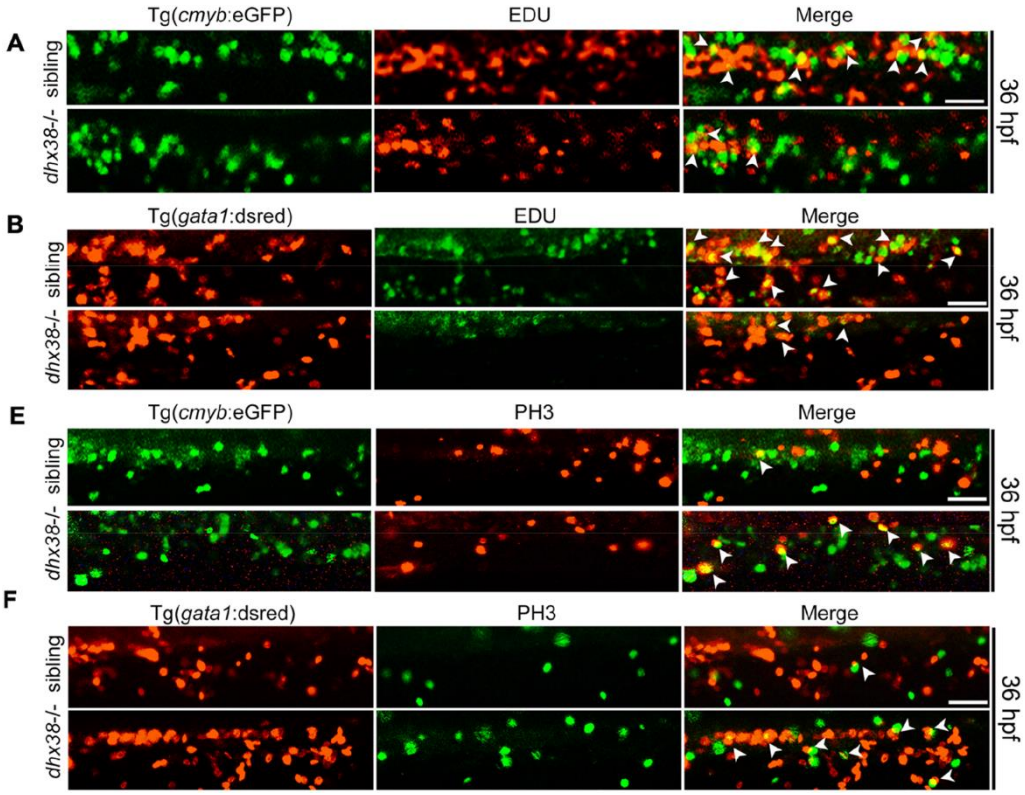
Revised Fig. 2E-F



Original Fig. 4A/B/E/F:



Revised Fig. 4A-F



2. Pure HSCs in zebrafish are not well defined. It would be better to use hematopoietic progenitor/stem cells (HSPCs) to replace the "HSCs" in the manuscript.

We have exchanged "HSCs" with "HSPCs" throughout the text.

3. Page177-180, "EMPs and HSCs are overlapped from the dorsal aorta and CHT region at 36 to 48 hpf, but can be sorted based on cd41+gata1- (HSCs) and cd41+gata1- (EMPs) markers respectively as previously reported (Bertrand et al., 2008; Bertrand et al., 2010; Forrester et al., 2012)." The interpretation of previous reports is incorrect. Actually, HSPCs were sorted by CD41+ from 42 hpf trunk, whereas EMPs were sorted by LMO2+GATA1+ from 30 hpf PBI in previous studies (Bertrand et al., 2008, 2010).

Thank you for pointing this out. Based on the research of Bertrand et al. and Xia et al., HSPCs were labeled by *CD41*$^{+}$*gata1*$^{-}$ (Bertrand et al., 2008; Xia et al., 2021). And Bertrand et al. claimed *gata1*$^{+}$ *lmo2*$^{+}$ or *cd41*$^{+}$ cells in the PBI are committed erythromyeloid progenitors (Bertrand et al., 2007). we correct this sentence on **Page 7: Line 178-179** as follows.

It seems EMPs overlap with the HSPCs in the PBI region from at 30 to 40 hpf, but can be labeled based on *cd41+gata1+* (HSPCs) and *cd41+gata1-* (EMPs) markers respectively as previously reported (Bertrand et al., 2008; Bertrand et al., 2010; Forrester et al., 2012; Xia et al., 2021).

Ref: Bertrand, J. Y., Kim, A. D., Teng, S. and Traver, D. (2008) CD41+ cmyb+ precursors colonize the zebrafish pronephros by a novel migration route to initiate adult hematopoiesis, Development 135(10): 1853-62.

Bertrand, J. Y., Kim, A. D., Violette, E. P., Stachura, D. L., Cisson, J. L. and Traver, D. (2007) Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo, Development 134(23): 4147-4156.

Xia, J., Kang, Z., Xue, Y., Ding, Y., Gao, S., Zhang, Y., Lv, P., Wang, X., Ma, D., Wang, L. et al. (2021) A single-cell resolution developmental atlas of hematopoietic stem and progenitor cell expansion in zebrafish, Proc Natl Acad Sci U S A 118(14).

Second decision letter

MS ID#: DEVELOP/2021/200450

MS TITLE: Dhx38 regulates the maintenance and differentiation of erythro-myeloid progenitors and hematopoietic stem cells by alternative splicing

AUTHORS: Jiayi Tu, Shanshan Yu, Jingzhen Li, Mengmeng Ren, Yangjun Zhang, Jiong Luo, Kui Sun, Yuexia Lv, Yunqiao Han, Yuwen Huang, Xiang Ren, Tao Jiang, Zhaohui Tang, Mark TS Williams, Qunwei Lu, and Mugen Liu

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 describe a role for the splicing factor Dhx38 in embryonic hematopoiesis showing that it impairs HSPCs and EMPs. The defects are attributed to abnormal cell cycle and cell death triggered by changes in pre-mRNA splicing.

Comments for the author

The authors were very responsive to the critiques. There are only a few minor additions to the manuscript requested.
1. Pleas add the data on dhx38 mutant;tp53 mutant included for the reviewers into Figure S3.
2. There is some data on siDHX38 presented in Figure 4I, but the western blot validation of the effectiveness of the RNAi is not presented until Figure 7. This data should be moved up.
3. The language describing the PSI is still confusing. Do you mean "PSI UP indicate exon/intron that are found to be skipped more in the mutants and PSI DOWN are exon/introns that are more frequently retained in the mutants?

Reviewer 2

Advance summary and potential significance to field

The revised manuscript has improved a lot. There are several points to be clarified by the authors as listed below.

Comments for the author

1. The co-localization of cmyb-eGFP and dhx38 in the PBI at 36 hpf is nice.
However, their co-localization in the VDA region is not convincing. Does this mean
HSPCs only express dhx38 after leaving the VDA region?
2. Cmyb+ HSPCs exist in dhx38 del7 at 56 hpf. This phenotype is different from the dhx38 del10 mutant in which cmyb+ HSPCs completely lost at 56 hpf. How to explain the discrepancy?
3. Page 7: 181-182, cd41+gata1+ should be EMP marker, and cd41+gata1- should be HSPC marker.

Second revision

Author response to reviewers' comments

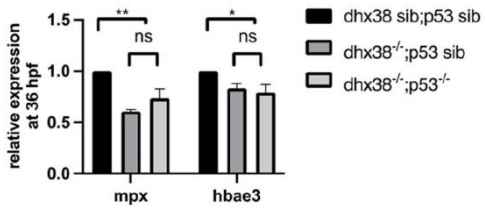
Response to Review

We have addressed each of the insightful concerns of the Reviewers in the revised manuscript. Major changes made in the manuscript text are marked in red color.

Reviewer 1 Advance Summary and Potential Significance to Field:
The authors describe a role for the splicing factor Dhx38 in embryonic hematopoiesis showing that it impairs HSPCs and EMPs. The defects are attributed to abnormal cell cycle and cell death triggered by changes in pre-mRNA splicing.

Reviewer 1 Comments for the Author:
The authors were very responsive to the critiques. There are only a few minor additions to the manuscript requested.

1. Pleas add the data on dhx38 mutant; tp53 mutant included for the reviewers into Figure S3.
Thanks for the kind reminder, we have added the P53 data to Figure S4. And added a description on Page 8 Line 209-212 as below.
"Although there is no significant difference between *dhx38* mutants and wild-type siblings, with regards to the proportion of apoptotic EMPs at 36 hpf in the PBI (Fig. S4C), apoptosis increased significantly in the PBI of *dhx38*$^{-/-}$ embryos at 56 hpf (Fig. 4K-N). Also, inhibiting p53 activity in *dhx38* mutants failed to rescue the phenotype of granulocyte (*mpx*) and erythrocyte (*hbae3*) reduction at 36 hpf (Figure 8C; Figure S3C). Therefore, we eliminate the possibility that the changes in granulocyte and erythrocyte numbers in *dhx38* mutants at 36 hpf are due to defects in the viability of differentiated cells. But it strongly suggests that the observed reduced number of EMPs and HSPCs at 56 hpf are the result of increased apoptosis."



2. There is some data on siDHX38 presented in Figure 4I, but the western blot validation of the effectiveness of the RNAi is not presented until Figure 7. This data should be moved up.
We have added the western blot of RNAi efficacy to Figure 4I and the relative Figure legend at Page 25 Line 711-715.

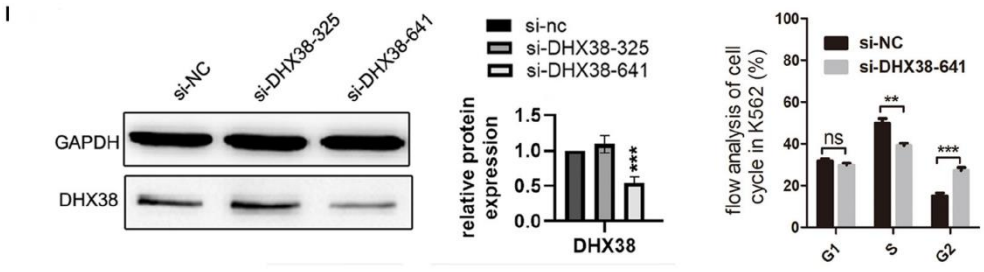


Figure 4I: The siDHX38-641 siRNA is at the c.641 position of the human DHX38 gene, siDHX38-328 is at c.328. The silencing effects of these two siRNAs were confirmed by western blot. Western of DHX38 shows efficiency of DHX38 knockdown in the si-DHX38-641 group, but not the si-DHX38-325 group. Flow analysis cell cycle after treatment with si-DHX38-641, showing a decrease of cells in the S phase and an increase of cells in the M phase. performed with six replicates; two-tailed Student's t-test, ns p=0.18, **p=0.001, ***p=0.0001.

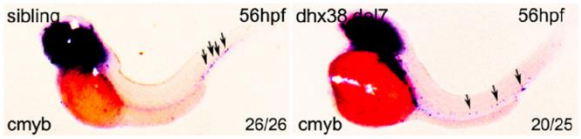
3. The language describing the PSI is still confusing. Do you mean "PSI UP indicate exon/intron that are found to be skipped more in the mutants and PSI DOWN are exon/introns that are more frequently retained in the mutants?
Sorry for the confusing we caused. Thank the reviewer for understanding and making this language clearer. We have changed this description on Page 26 Line 747-751.
"The percent spliced in index (PSI) indicates the efficiency of splicing a specific exon into the transcript population of a gene. Exons/intron that are constitutively retained in all transcripts and never skipped have a PSI of 100. So, the PSI UP indicate exon/intron that are more likely to be retained in the mutants and PSI DOWN are the exon/introns that more frequently skipped in the mutants."

Reviewer 2 Advance Summary and Potential Significance to Field:
The revised manuscript has improved a lot. There are several points to be clarified by the authors as listed below.

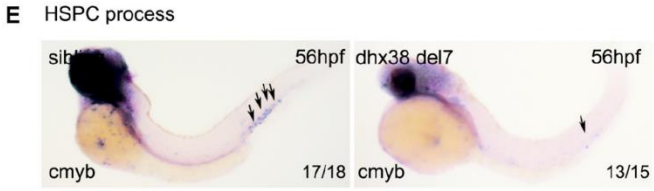
Reviewer 2 Comments for the Author:
1. The co-localization of cmyb-eGFP and dhx38 in the PBI at 36 hpf is nice.
However, their co-localization in the VDA region is not convincing. Does this mean HSPCs only express dhx38 after leaving the VDA region?
We agree with the reviewer. We also found that DHX38 expression in the PBI is greater than in the VDA. Also, in the WISH of *dhx38*, *dhx38* expression seems more enriched in the CHT region than in the VDA region (Figure S1A and Figure 1A). HSPC are generated in the VDA through endothelium-hematopoiesis transition and homing to the CHT region and proliferate there. We consider *dhx38* to be more highly expressed in the region that needs proliferation events. We remove this figure from Figure S1B.

2. Cmyb+ HSPCs exist in dhx38 del7 at 56 hpf. This phenotype is different from the dhx38 del10 mutant in which cmyb+ HSPCs completely lost at 56 hpf. How to explain the discrepancy?
Thanks for your clear check and kind reminder. We double-checked the phenotype of the *dhx38* del7 and del10 models. They both lost almost all *cmyb* expressions at 56 hpf compared with their siblings. But we consider that the del7 and del10 cannot be compared because it depends on the different staining times of WISH at the different benches. The del7 also has a darker background. We used another figure showing del7 mutants, which have the same backgrounds yet the same phenotype as del 10, that loss of dhx38 will impair the maintenance of HSPC.

Original Figure:



Revised Figure:



3. Page 7: 181-182, cd41+gata1+ should be EMP marker, and cd41+gata1- should be HSPC marker.
Thanks for the reviewers' reminder. We have changed this description on Page 7: 183 as below and checked all the similar descriptions.
Revised sentence
It seems EMPs overlap with the HSPCs in the PBI region from at 30 to 40 hpf, but can be labeled based on *cd41*$^{+}$*gata1*$^{+}$ (EMPs) and *cd41*$^{+}$*gata1*$^{-}$ (HSPCs) markers respectively as previously reported.

Third decision letter

MS ID#: DEVELOP/2021/200450

MS TITLE: Dhx38 regulates the maintenance and differentiation of erythro-myeloid progenitors and hematopoietic stem cells by alternative splicing

AUTHORS: Jiayi Tu, Shanshan Yu, Jingzhen Li, Mengmeng Ren, Yangjun Zhang, Jiong Luo, Kui Sun, Yuexia Lv, Yunqiao Han, Yuwen Huang, Xiang Ren, Tao Jiang, Zhaohui Tang, Mark TS Williams, Qunwei Lu, and Mugen Liu
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 study explored a role for the splicing factor Dhx38 in embryonic hematopoiesis.

Comments for the author

All minor points were sufficiently addressed.

Reviewer 2

Advance summary and potential significance to field

All my previous comments have been successfully addressed.

Comments for the author

All my previous comments have been successfully addressed.