nature portfolio

Peer Review File

Mutations in linker-2 of KLF1 impair expression of membrane transporters and cytoskeletal proteins causing hemolysis

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Huang et al., provides insights into how the mutations in linkers of zinc finger transcription factors result in different phenotypes compared to loss of function, through their studies on KLF1. The authors show, using a mouse model, that a linker2 mutation (H350R) does not alter sequence specificity, but reduces affinity of DNA binding, and only a small subset of the KLF1-dependent transcriptome is sensitive. Using in vitro analysis, they show that human KLF1 linker2 disease mutations (T348A, G349R) also have a similar reduced DNA binding affinity, similar to what was observed in the mouse context. This well written manuscript describes rigorously designed experiments with convincing data. There are no major concerns, and some minor ones are listed below:

1. In figure 2A, the authors show that out of 228 differentially expressed genes between Klf1+/- and Klf1H350R/- fetal liver RNAs, 168 overlap with KLF1 dependent gene list and 60 do not. Since approximately one fourth of the differentially expressed genes do not overlap, it will be useful to discuss these genes and pathways that are unique to the Klf1H350R/- genotype and whether this adds to or affects the conclusions of the study.

2. The authors mention that for RNA-Seq studies on splenic erythroblasts, they used CD71+ Ter119+ (S3) erythroblast population for all five genotypes, to enable appropriate comparison, since the WT mice have only a few cells at this S3 stage. On the other hand, although E14.5 fetal livers are used for both RNA-Seq and ChIP-Seq studies, data on how the CD71 and Ter119 profile in fetal liver erythroblasts compares between the genotypes is lacking. In addition, it will be useful to show the morphology of these fetal liver cells by MGG staining for all the genotypes, since dysregulation of membrane genes in H350R/- genotype could lead to altered membrane morphology in the fetal liver erythroblasts.

3. The authors describe partial rescue of NSHA after supplementation with L-leucine, which was lost by 8 weeks. As evidence for this rescue, they show Hb levels in figure 7. The authors could include RBC count, and also add MGG staining of peripheral blood similar to figure 1A, for comparison.

4. In the discussion on the partial rescue of NSHA after supplementation with L-leucine, the authors speculate that this could be due to improved translation of globins and other erythroid genes ie., a secondary translational problem in H350R/- genotype. There are no references provided here- therefore, it is not clear if this inference is based on the data from the current study or from previous studies. If this is based on their data from the current study, the authors could show/discuss this for clarity.

5. There is a typo in the last line prior to the discussion section- This should be KLF+/- not KLF+/+ as per figure 7. One other typo is in the end of the third paragraph of discussion: the sentence, “In this case the L-glutamine would need to enter erythroid progenitors … has low cells of expression of the receptor”- perhaps should be “low levels of expression of the receptor”.

Reviewer #2 (Remarks to the Author):

This is a detailed analysis of the genetic mutation and phenotype of KLF1, one of the erythrocyte-specific transcription factors, using a mouse model. KLF1 is one of the C2H2-type zinc finger proteins, and 25 SP/KLF proteins are known to exist in humans and mice. They have three zinc fingers separated by a linker exhibiting conservation across species, ie, five amino acid residues represented by TGEKP, at the C-terminus.
KLF1 regulates the expression of genes encoding proteins that play important roles in cell cycle regulation, heme biosynthesis, globin production, and erythrocyte membrane/membrane cytoskeleton, and mutations (polymorphism) in the second linker is known to be common among

residents of southern China and the Mediterranean coastal areas.
A review by the authors (Blood 2016) states that compound heterozygotes between class 2 missense mutations and class 3 nonsense mutations or frameshift mutations cause severe NSHA. and compound heterozygotes for loss-of-function mutations in another allele were also shown to cause NSHA.
Although it is understandable that the linker mutation H350R affecting only a part of the KLF-dependent transcriptome does not cause thalassemia due to globin gene downregulation, it is difficult to understand what the direct cause of the mild NSHA is. It is not mentioned which of the down-regulated genes namely Glut1, Glut4, Asct2, Lat3, and Ebp49, is the main causative gene of NSHA. Do mutant mouse erythrocytes show osmotic fragility? Or does it indicate over-hydrated stomatocytosis? Are there any abnormalities in electrophoretic patterns of membrane proteins?
The authors explain splenomegaly in mutant mice as extramedullary hematopoiesis, and the underlying phenomenon is stress response. Was there evidence of early cell death in the erythroblasts in the spleen? Isn't it considered ineffective hematopoiesis?

We thank the two reviewers for the insightful and generous comments. Here are our responses (in red) to the specific questions.

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Thank you for raising this. The majority of the 60 genes in the Klf1$^{H350R/-}$ fetal liver are involved in immune activation. However, we did not identify any KLF1 bound sites near these genes, suggesting they are most likely indirectly regulated.

2. The authors mention that for RNA-Seq studies on splenic erythroblasts, they used CD71+ Ter119+ (S3) erythroblast population for all five genotypes, to enable appropriate comparison, since the WT mice have only a few cells at this S3 stage. On the other hand, although E14.5 fetal livers are used for both RNA-Seq and ChIP-Seq studies, data on how the CD71 and Ter119 profile in fetal liver erythroblasts compares between the genotypes is lacking. In addition, it will be useful to show the morphology of these fetal liver cells by MGG staining for all the genotypes, since dysregulation of membrane genes in H350R/- genotype could lead to altered membrane morphology in the fetal liver erythroblasts.

Thank you. Klf1$^{-/-}$ fetal liver cells have very low expression levels of Ter119 and markedly reduced CD71 as previously published (Hodge et al. 2006; Pilon et al.2006). This is due to the fact the genes encoding these surface markers are likely direct targets of Klf1. Thus, we are not able to sort cells at equivalent stages of maturation in the fetal liver. This has been discussed extensively by the field over the past two decades. It is generally well accepted that Ter119 and CD71 as differentiation makers cannot be used in Klf1$^{-/-}$ cells. A comment about this has been added to the methods. We have prepared MGG-stained cytospins of blood and fetal liver from the three genotypes as requested but we don't think they are particularly informative. They are included in Supplementary Figure….

3. The authors describe partial rescue of NSHA after supplementation with L-leucine, which was lost by 8 weeks. As evidence for this rescue, they show Hb levels in figure 7. The authors could include RBC count, and also add MGG staining of peripheral blood similar to figure 1A, for comparison.

The RCCs have been added as requested but we did not collect blood smears.

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It is based on the publication from the team of Barry Paw (Chung et al. 2015), that we cited as evidence. We have not done any globin translation work.

5. There is a typo in the last line prior to the discussion section- This should be KLF+/- not KLF+/+ as per figure 7. One other typo is in the end of the third paragraph of discussion: the sentence, “In this case the L-glutamine would need to enter erythroid progenitors … has low cells of expression of the receptor”- perhaps should be “low levels of expression of the receptor”.

Thank you. We have fixed these two typos.

Reviewer #2 (Remarks to the Author):

This is a detailed analysis of the genetic mutation and phenotype of KLF1, one of the erythrocyte-specific transcription factors, using a mouse model. KLF1 is one of the C2H2-type zinc finger proteins, and 25 SP/KLF proteins are known to exist in humans and mice. They have three zinc fingers separated by a linker exhibiting conservation across species, ie, five amino acid residues represented by TGEKP, at the C-terminus.
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We believe it is a combination of down regulation of a suite of cell transmembrane and cytoskeletal proteins that is responsible for the haemolytic phenotype. The cell morphology is interesting. There is marked aniso-poikilocytosis with micro-spherocytes and fragments (Figure 1A). There are no obvious stomatocytes. We did not do an osmotic fragility test. The phenotype is not dissimilar from the deletion of the headpiece domain of Ebp49 (Lu et al. Blood 2016 Jul 7;128(1):93-103), so down regulation of this gene might be partly responsible for the haemolysis. This has been referenced. There are likely far too many target genes to attempt a rescue.

The authors explain splenomegaly in mutant mice as extramedullary hematopoiesis, and the underlying phenomenon is stress response. Was there evidence of early cell death in the erythroblasts in the spleen? Isn't it considered ineffective hematopoiesis?

Yes, we base the claim on the expanded red pulp and FACS profiles with increased CD71+/TER119+ cells some DEGs suggestive of a response to hypoxia or EPO stimulation (Figure 3E – new addition to the Figure). We did not look at apoptosis directly but viability in the fetal livers (by FV700 viability staining) was comparable across all genotypes (not shown). We would expect rapid clearance of dying or dead cells by the reticuloendothelial system. We suggest the phenotype is mostly haemolysis and erythroid expansion in response to anaemia, rather than ineffective erythropoiesis. There is certainly no significant imbalance in globin gene expression, as one would find in thalassaemia. A couple of sentences have been added to the relevant section of the paper.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed the concerns adequately.

Reviewer #2 (Remarks to the Author):

We would like to thank the authors for their thoughtful responses to our reviewers' comments.
The morphology of the mutant mouse RBCs shown in Figure 1A shows significant anisopoikilocytosis with fragmented red cells, suggesting that the RBC membrane cytoskeleton is significantly unstable. As the authors state, it can be understood that gene mutations in KLF1, an erythrocyte-specific transcription factor, reduce the expression of multiple membrane protein genes. In Figure 1B, H350R/- mutant mice exhibit anemia accompanied by a marked reticulocytosis, and a decrease in MCV is also evident. I understood that the decrease in MCV was caused not by thalassemia but by the previously mentioned disrupted RBCs.
Figure 1C shows FCM findings of CD71/Ter119 double staining in the bone marrow, but have you also added Annexin V staining to examine the percentage of apoptotic cells? If you have data, please provide it.
In Figure 8, the authors state that decreased expression of GLUT1 and ASCT2 in H350R/- mice causes impaired nucleotide synthesis in RBCs and is involved in the establishment of anemia. I am commenting on the clinical similarities with G6PD deficiency, but in G6PD deficiency, although Heinz body formation is observed due to increased oxidative stress within RBCs, abnormal RBC morphology is usually not observed. A decrease in NAPDH production through the pentose phosphate pathway indicates an acute hemolytic attack due to a decrease in the concentration of reduced glutathione, and in severe cases, chronic hemolysis. Although I think this is an interesting working hypothesis for the authors to use this mutant mouse to develop new drug treatments in the future, I think it would be better not to show Figure 8 in this paper.

Thursday, 8 February 2024

We thank the two reviewers for their comments on our revised manuscript, NCOMMS-23-10908A

We are pleased reviewer 1 is satisfied with our revision

For reviewer 2, we have prepared a point by point response to the new comments and questions.

1. The morphology of the mutant mouse RBCs shown in Figure 1A shows significant anisopoikilocytosis with fragmented red cells, suggesting that the RBC membrane cytoskeleton is significantly unstable. As the authors state, it can be understood that gene mutations in KLF1, an erythrocyte-specific transcription factor, reduce the expression of multiple membrane protein genes.

   Yes, we show the H350R/- compound heterozygous mutations in Klf1 reduce expression of many transmembrane and cytoskeletal genes (Figure 2).

2. In Figure 1B, H350R/- mutant mice exhibit anemia accompanied by a marked reticulocytosis, and a decrease in MCV is also evident. I understood that the decrease in MCV was caused not by thalassemia but by the previously mentioned disrupted RBCs.

   Yes, there are micro-spherocytes that resemble those found in severe membrane disorders that we believe explain the low MCV. Alpha and beta globin genes are normally expressed.

3. Figure 1C shows FCM findings of CD71/Ter119 double staining in the bone marrow, but have you also added Annexin V staining to examine the percentage of apoptotic cells? If you have data, please provide it.

   I am sorry, but we did not perform Annexin V staining

4. In Figure 8, the authors state that decreased expression of GLUT1 and ASCT2 in H350R/- mice causes impaired nucleotide synthesis in RBCs and is involved in the establishment of anemia. I am commenting on the clinical similarities with G6PD deficiency, but in G6PD deficiency, although Heinz body formation is observed due to increased oxidative stress within RBCs, abnormal RBC morphology is usually not observed. A decrease in NAPDH production through the pentose phosphate pathway indicates an acute hemolytic attack due to a decrease in the concentration of reduced glutathione, and in severe cases, chronic hemolysis. Although I think this is an interesting working hypothesis for the authors to use this mutant mouse to develop new drug treatments in the future, I think it would be better not to show Figure 8 in this paper.

   We agree with the reviewer that G6PD deficiency can mimic different types of non-immune haemolysis. We are haematologists and manage patients with G6PD and KLF1 deficiencies. Although some forms of G6PD deficiency can have moderate red cell morphological changes and chronic mild haemolysis rather than sudden oxidant stress haemolysis (e.g. after fava bean ingestion or drugs such as dapsone), we don't think the severe blood film abnormalities in the adult *Klf1* H350R/- mice

resemble G6PD deficiency. The G6PD expression levels are normal in the erythrocytes of these mice. We have not measured G6PD enzyme activity in the red cells, so we cannot be absolutely sure they are normal and we cannot be sure of the levels of NADPH if glucose transport into the RBCs is impaired. That is why we included these sentences in our discussion.

"Glucose (via G6P) is also used to generate reducing power in the form of NADPH via G6PD, so *Klf1*$^{H350R/-}$ erythrocytes are also likely to be susceptible to oxidative hemolysis due to lack of glucose (Figure 8). Indeed, the red blood cell abnormalities found in the blood smears of *Klf1*$^{H350R/-}$ mice are reminiscent of those found in oxidative hemolysis, or G6PD deficiency."

These are however, quite speculative and we think we should remove them to satisfy reviewer 2 and not confuse the readers with something we think is unlikely to be the major issue. We have removed these two sentences from the second revision of the manuscript and submitted an updated version.

We would like to keep Figure 8 as it is a nice summary of the major findings and novelty of the paper.

Yours sincerely

Andrew Perkins

Corresponding author