

RPS19 and RPL5 Haploinsufficient Models Reveal Divergent Ribosomal Subunit Controls of Fetal Hematopoiesis

Corresponding Author: Dr Lionel Blanc

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript, Tang et al tackle the question of what underlies the clinical heterogeneity in the hematopoietic defects observed in Diamond-Blackfan Anemia, with their hypothesis being that the heterogeneity is driven by mechanisms specific to the ribosomal protein that is mutated. Currently available DBA mouse models have multiple limitations, and there is a need for better models that will recapitulate important aspects of human disease biology. The authors generated two mouse models with conditional hematopoietic heterozygous deletion of either RPS19 or RPL5, both of which are commonly mutated in DBA. Clinical features of the RPS19-mutant mouse model included live birth but lethality prior to weaning, with hematopoietic defects comparable to what is observed in DBAS patients, while the RPL5-mutant mice experienced more severe defects and late-gestational lethality. The two models showed divergent effects on hematopoietic progenitors, with RPS19-mutant mice showing a depletion of HSPCs and early erythroid progenitors, and RPL5-mutant mice showing loss of later erythroid progenitors and an accumulation of HSPCs. In both models, global protein synthesis was increased in HSPCs and decreased in erythroid cells, and the authors suggest that the elevated translation in HSPCs may contribute to their exhaustion. Both mouse models showed activation of p53, and deletion of TP53 improved survival. The authors show that in RPS19-mutant mice, p53 activation leads to activation of RUNX1, which ultimately increases p21 levels to contribute to HSPC depletion. RUNX1 levels were also found to be upregulated in DBA patients. Overall, this is an important study that serves two goals: (1) It presents valuable new mouse models that will be important to the DBA, erythropoiesis, and ribosome biogenesis communities, and (2) The extensive characterization presented here demonstrates that even though the same central process is impacted in both knockouts (ribosome biogenesis), different mutated ribosomal proteins have the ability to produce qualitatively distinct phenotypic defects, mediated through different downstream pathways. My comments below are minor.

Minor comments:

- In Fig 3b, the quantification provided is of free 60S and 40S subunits. However, that isn't really reflective of the total number of subunits in the cell, because a majority of subunits are contained in the 80S and polysome fractions. It would be useful to get a quantification of the degree of discrepancy of the actual total subunits, which could be achieved by performing polysome profiling after EDTA treatment of the lysate (which would dissociate all 80S and polysomes into 40S and 60S subunits). If technically feasible, could the authors quantify this? However, this is not an essential requirement for this paper – there is already sufficient experimental data included here.
- The authors perform a lot of very interesting and rigorous experiments, but by the end of the paper, it is a bit hard to keep track of what the overall final mechanism is for each mouse model. Can the authors include graphical models in the final figure?
- The result section title “Translational alterations in Ter119+ cells are primarily a consequence of transcriptional changes” is a bit confusing. I believe the authors mean to convey that secondary transcriptional effects exceed specific direct translational impairments? Perhaps the authors could pick a better section header?
- In the end of the introduction, the authors say that they demonstrate functions of the RPs “beyond their canonical role in translation” (line 88-89), but I'm not sure that this is directly tested - consider rephrasing.

Stylistic comments:

- In Figure 1f, it appears as though there is a minor typo and both mice are labeled as Cre-
- The percentages in the table shown in Figure 3k are a bit confusing to interpret - they are supposed to be a percentage of total, but they do not add up to 100.

- Figure 5d is referenced in the text in line 287, but doesn't seem to have anything to do with S phase speed. Do the authors perhaps mean to refer to it in the next section, where they're talking about TP53 levels?
- Line 298 seems to reference the wrong figure (should be Figure 5f?)
- In Figure 7f, what is the difference between the top two tracks that are shown? The labeling of the tracks is hard to read.
- Add mouse genotype labels to the plots in Fig S9b
- In line 505, they say that there is "enhanced translation fidelity" in RPL5 mice, but I don't think that translation fidelity was directly measured. Consider rephrasing.

Reviewer #2

(Remarks to the Author)

In this interesting manuscript, the authors reveal divergent functions for the large and small subunit proteins RPS19 and RPL5 in fetal hematopoiesis in mice. The authors argue that RPL5 haploinsufficiency causes HSPC accumulation and prenatal lethality via p53-mediated ferroptosis of mature erythroid progenitors while RPS19 haploinsufficiency results in HSPC depletion and reduced erythroid expansion that is mediated by p53-dependent apoptosis. A strength of the work is the development of robust in vivo mouse models that have allowed the authors to dissect how RP haploinsufficiency dysregulates hematopoiesis to cause Diamond-Blackfan anemia syndrome.

The work provides several new insights into the differential effects of large versus small subunit haploinsufficiency. In particular, the authors show in vivo that loss of early erythroid progenitors by E17.5 is unique to *Rps19* but not *Rpl5*-deficient mice. They further show that RPS19 haploinsufficiency leads to HSPC depletion, while RPL5 depletion leads to its expansion, although the erythroid progenitors are still markedly defective in generating CFU in vitro. The data indicate that RPS19 and RPL5 haploinsufficient mice exhibit divergent and differentiation stage-specific responses downstream of p53 activation, with RPS19-haploinsufficient HSPC undergoing apoptosis, while RPL5-haploinsufficient EPs undergo ferroptosis due to oxidative stress. Interestingly, they also show that Trp53 deletion rescues survival through distinct mechanisms in RPS19 and RPL5 haploinsufficient mice. The conclusions that the authors wish to draw are generally well supported by the data and I believe that this work represents an interesting and important contribution to the field of bone marrow failure, ribosomopathy and the wider ribosome biology community.

However, I have points that should be addressed below and the apparent errors in formatting, figure calling and the text within the figure legend needs to be carefully corrected (see examples listed below). I think the work would benefit from a graphical model to better communicate the findings to the wider readership of the journal.

Specific points

1. There are several issues with formatting, figure calling in the text and figure legends that made review challenging and should be carefully checked and corrected by the authors.
2. Figure S7- The observations on ferroptosis are interesting, but have the authors validated any of their ferroptosis gene expression data at the protein level? Have the authors tested the effects of ferroptosis inhibitors on hematopoiesis in their models?
3. While the manuscript has many strengths, dissection of the underlying mechanisms at the molecular level is weak in places, raising several unanswered questions. For example, I struggle to interpret the western blotting experiments in Figure 3 in terms of explaining the reported phenotypes. What might be the basis for the transient increase in the p-eIF2a/eIF2 ratio? Have the authors further validated their stated hypothesis that the ISR is indeed activated? While the observation of differential eIF5a hypusination is interesting, the data are somewhat preliminary. Perhaps some of these experiments might be better placed in the supplementary data?
4. The observation that RUNX1 may act between p53 and p21 to drive hematopoietic failure in the *Rps19lox/+* mice is potentially interesting, but as the authors acknowledge, additional work is required to validate these findings and explore their significance in the DBAS disease context.
5. I would recommend that the authors include a graphic summarizing their findings to help communicate the work to a more general readership.
6. Line 179: "*Rps19lox/+* and *Rpl5lox/+* progenitors revealed a decrease in the 40S and 60S fractions, respectively". Although the quantification in Fig 3b supports this conclusion, the polysome profile example shown on the left for *Rps19* does not look so convincing. The resolution of the pdf figures is low and there seems to be a poorly defined third curve in the two left hand panels in Fig 3a?
7. Line 185: "Surprisingly, OPP incorporation was significantly higher in *Rps19lox/+* and *Rpl5lox/+* LSK populations compared to control littermates. Interestingly, the magnitude of the increase was substantially (significantly?) greater in *Rps19lox/+*".
The figure panel is not called here in the text.
Substantially (significantly?). Please correct.
How confident can the authors be in concluding that protein synthesis is indeed higher in the *Rps19* vs *Rpl5*-deficient mice given the difference in the number of samples analyzed for the two mutants?
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10. Line 237: " \log_2 (translational changes) and \log_2 (transcriptional changes) (\log_2 ti(translation)/ti(transcription))". Is this a formatting issue?
11. Line 303 and 306: should be annotated as Fig 5g, not 5h, right?
12. Figure S7-Have the authors validated any of their ferroptosis gene expression data at the protein level?
13. Fig 7H-the legend does not correspond to what is shown in the figure. No data are shown for RPS19. What is the new information in Fig 7H vs Fig 7g?
14. Fig 7k-what are the numbers on the x-axis?

15. Line 945-formatting issue

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have satisfactorily addressed my review comments and concerns.

Reviewer #2

(Remarks to the Author)

The authors have made a good job of addressing all the concerns and corrected the formatting errors.

Minor point

Please clarify in the legend to Fig 7k that the numbers on the x-axis represent quantification of RUNX1 intensity versus actin control as this is not explicitly stated.

Reviewer #3

(Remarks to the Author)

The single-cell analyses are nicely done overall, but a few additions could make the conclusions more convincing. It would be useful to show how well the scRNA-seq biological replicates agree with each other, for example by correlating pseudobulk expression profiles or cluster abundances, to make the reproducibility more explicit.

The claim that RPS19 haploinsufficiency causes alterations in HSPC, MEP, megakaryocyte, and erythroid progenitor frequencies (Supplementary Fig 5b-c) rely on changes in cell-type or state proportions across conditions. Adding some statistical support for these compositional shifts (e.g. using a simple bootstrap or permutation approach or tools like scCODA) would help clarify which differences are robust.

The chromatin accessibility analysis relies primarily on Gigggle scores for transcription factor motif enrichment in differential peaks, an orthogonal analysis such as chromVAR could strengthen the story. In particular, chromVAR scores for TP53 and RUNX1 motifs could help assess whether their activities are correlated at the single-cell level in the relevant populations, which would support the proposed coordination between these pathways, even if it does not establish direct regulation.

Finally, the link between RUNX1 and Cdkn1a would be stronger with comparative RUNX1 ChIP data across conditions. Without this, it may be worth framing the functional relationship a bit more cautiously.

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We would like to thank the Editor and the Reviewers for their thoughtful comments and suggestions. We have taken all criticisms to heart and have made all changes suggested. Summarized below are the major changes to the manuscript:

- We have added a model as **Figure 8**, to summarize our findings and better communicate them to the broad readership of *Nature communications*. (**Reviewers 1 & 2**).
- We have validated by western blot the expression pattern of several of the pro- and anti-ferroptosis genes that were identified by scRNAseq, as suggested by **Reviewer 2**. The results are presented in **Supplemental Figure 7b-c**.
- We have tested the effects of ferroptosis inhibitors on hematopoiesis *in vitro* as suggested by **Reviewer 2**. We used β -mercapto-ethanol (BME) and ferrostatin-1 (Fer-1). BME partially rescued proliferation and erythroid differentiation in the Rpl5 haploinsufficient cells while Fer-1 did not. These results remain preliminary and are the focus of our next studies. They are provided to the Reviewers only.
- As suggested by Reviewer 2, data related to the different translation initiation and elongation factors have been moved to the supplement (**Supplemental Figure 3**).
- All typo and conversion issues have been corrected (**Reviewers 1 & 2**).

Reviewer #1 (Remarks to the Author)

In this manuscript, Tang et al tackle the question of what underlies the clinical heterogeneity in the hematopoietic defects observed in Diamond-Blackfan Anemia, with their hypothesis being that the heterogeneity is driven by mechanisms specific to the ribosomal protein that is mutated. Currently available DBA mouse models have multiple limitations, and there is a need for better models that will recapitulate important aspects of human disease biology. The authors generated two mouse models with conditional hematopoietic heterozygous deletion of either RPS19 or RPL5, both of which are commonly mutated in DBA. Clinical features of the RPS19-mutant mouse model included live birth but lethality prior to weaning, with hematopoietic defects comparable to what is observed in DBAS patients, while the RPL5-mutant mice experienced more severe defects and late-gestational lethality. The two models showed divergent effects on hematopoietic progenitors, with RPS19-mutant mice showing a depletion of HSPCs and early erythroid progenitors, and RPL5-mutant mice showing loss of later erythroid progenitors and an accumulation of HSPCs. In both models, global protein synthesis was increased in HSPCs and decreased in erythroid cells, and the authors suggest that the elevated translation in HSPCs may contribute to their exhaustion. Both mouse models showed activation of p53, and deletion of TP53 improved survival. The authors show that in RPS19-mutant mice, p53 activation leads to activation of RUNX1, which ultimately increases p21 levels to contribute to HSPC depletion. RUNX1 levels were also found to be upregulated in DBA patients. Overall, this is an important study that serves two goals: (1) It presents valuable new mouse models that will be important to the DBA, erythropoiesis, and ribosome biogenesis communities, and (2) The extensive characterization presented here demonstrates that even though the same central process is impacted in both knockouts (ribosome biogenesis), different mutated

ribosomal proteins have the ability to produce qualitatively distinct phenotypic defects, mediated through different downstream pathways. My comments below are minor.

We thank **Reviewer 1** for his/her positive comments on our manuscript.

Minor comments:

- In Fig 3b, the quantification provided is of free 60S and 40S subunits. However, that isn't really reflective of the total number of subunits in the cell, because a majority of subunits are contained in the 80S and polysome fractions. It would be useful to get a quantification of the degree of discrepancy of the actual total subunits, which could be achieved by performing polysome profiling after EDTA treatment of the lysate (which would dissociate all 80S and polysomes into 40S and 60S subunits). If technically feasible, could the authors quantify this? However, this is not an essential requirement for this paper – there is already sufficient experimental data included here.

We agree with the Reviewer that the quantification of free 40S and 60S can be impeded by not considering the subunits engaged in translation (monosomes and polysomes). Performing similar quantifications upon EDTA-mediated ribosome dissociation is an excellent suggestion. However, we want to emphasize that the main point of **Figure 3** is to highlight defects in global translation efficiency (**Figure 3b, c, Supplemental Figure 4**) as well as in mRNA-specific translation (**Figure 3d-f**). Performing such an experiment would require sacrificing a high number of embryos to confirm observations that are not essential for the manuscript as pointed out by the **Reviewer** him/herself. For ethical reasons, we therefore elected to leave this experiment aside and acknowledge the limitation of our approach in the manuscript.

- The authors perform a lot of very interesting and rigorous experiments, but by the end of the paper, it is a bit hard to keep track of what the overall final mechanism is for each mouse model. Can the authors include graphical models in the final figure?

We agree. This suggestion was also made by **Reviewer 2**. We added a model, summarizing our findings, in a new **Figure 8**.

- The result section title “Translational alterations in Ter119+ cells are primarily a consequence of transcriptional changes” is a bit confusing. I believe the authors mean to convey that secondary transcriptional effects exceed specific direct translational impairments? Perhaps the authors could pick a better section header?

The **Reviewer** is correct. We have now changed the section header, limiting it to 60 characters to fit *Nature communications* requirements.

- In the end of the introduction, the authors say that they demonstrate functions of the RPs “beyond their canonical role in translation” (line 88-89), but I’m not sure that this is directly tested - consider rephrasing.

We have removed this part from the sentence. The text now reads: “These results demonstrate new physiological and divergent functions for ribosomal proteins and provide insights into how disrupted RP stoichiometry alters developmental hematopoiesis and contributes to disease pathogenesis”.

Stylistic comments:

- In Figure 1f, it appears as though there is a minor typo and both mice are labeled as Cre-

Thank you. This has been corrected.

- The percentages in the table shown in Figure 3k are a bit confusing to interpret - they are supposed to be a percentage of total, but they do not add up to 100.

We apologize for the confusion. We have now edited the table.

- Figure 5d is referenced in the text in line 287, but doesn't seem to have anything to do with S phase speed. Do the authors perhaps mean to refer to it in the next section, where they're talking about TP53 levels?

The Reviewer is correct. We apologize. This has now been modified.

- Line 298 seems to reference the wrong figure (should be Figure 5f?)

Thank you. This has been rectified.

- In Figure 7f, what is the difference between the top two tracks that are shown? The labeling of the tracks is hard to read.

The first two tracks in **Figure 7f** represent the scATAC peaks in HSPC for two different genotypes- *Rps19^{lox/-}*; *p53^{lox/-}* and *Rps19^{lox/-}*. We have increased the font and labeling.

- Add mouse genotype labels to the plots in Fig S9b

Thank you. This has been done.

- In line 505, they say that there is “enhanced translation fidelity” in RPL5 mice, but I don't think that translation fidelity was directly measured. Consider rephrasing.

The Reviewer is correct. We have removed the part dealing with translation fidelity and have rephrased that sentence.

Reviewer #2 (Remarks to the Author)

In this interesting manuscript, the authors reveal divergent functions for the large and

small subunit proteins RPS19 and RPL5 in fetal hematopoiesis in mice. The authors argue that RPL5 haploinsufficiency causes HSPC accumulation and prenatal lethality via p53-mediated ferroptosis of mature erythroid progenitors while RPS19 haploinsufficiency results in HSPC depletion and reduced erythroid expansion that is mediated by p53-dependent apoptosis. A strength of the work is the development of robust in vivo mouse models that have allowed the authors to dissect how RP haploinsufficiency dysregulates hematopoiesis to cause Diamond-Blackfan anemia syndrome.

The work provides several new insights into the differential effects of large versus small subunit haploinsufficiency. In particular, the authors show in vivo that loss of early erythroid progenitors by E17.5 is unique to Rps19 but not Rpl5-deficient mice. They further show that RPS19 haploinsufficiency leads to HSPC depletion, while RPL5 depletion leads to its expansion, although the erythroid progenitors are still markedly defective in generating CFU in vitro. The data indicate that RPS19 and RPL5 haploinsufficient mice exhibit divergent and differentiation stage-specific responses downstream of p53 activation, with RPS19-haploinsufficient HSPC undergoing apoptosis, while RPL5-haploinsufficient EPs undergo ferroptosis due to oxidative stress. Interestingly, they also show that Trp53 deletion rescues survival through distinct mechanisms in RPS19 and RPL5 haploinsufficient mice. The conclusions that the authors wish to draw are generally well supported by the data and I believe that this work represents an interesting and important contribution to the field of bone marrow failure, ribosomopathy and the wider ribosome biology community.

We thank the Reviewer for his/her positive comments on our study.

However, I have points that should be addressed below and the apparent errors in formatting, figure calling and the text within the figure legend needs to be carefully corrected (see examples listed below). I think the work would benefit from a graphical model to better communicate the findings to the wider readership of the journal.

We apologize for these errors and have made every effort to correct them. As for a model, we agree with comments raised by both reviewers and have now added one as **Figure 8**.

Specific points

1. There are several issues with formatting, figure calling in the text and figure legends that made review challenging and should be carefully checked and corrected by the authors.

We apologize for the oversight. This has now been corrected.

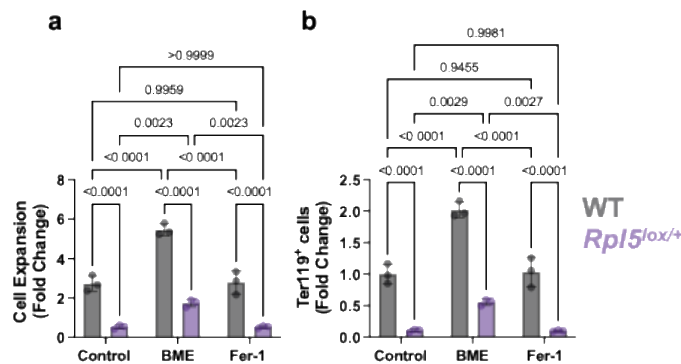
2. Figure S7- The observations on ferroptosis are interesting, but have the authors validated any of their ferroptosis gene expression data at the protein level? Have the authors tested the effects of ferroptosis inhibitors on hematopoiesis in their models?

We thank the Reviewer for his/her suggestions.

With regards to the validation, this is indeed an important point which we have addressed using western blot approaches. We isolated c-Kit⁺ and Ter119⁺ cell populations from WT and RPS19/RPL5 haploinsufficient mice at E15.5 and measured the expression levels of GPX4, SLC7A11, FTH, HO-1 (pro-ferroptotic) and PCBP1 (anti-ferroptotic). We observed that the expression of most of the pro-ferroptotic genes was increased in the Ter119⁺ cell population from RPL5 haploinsufficient mice. Conversely, the expression of PCBP1 was dramatically reduced in the same population. In the RPS19 model, the opposite was observed – except for an increase in FTH. These data are presented in **Supplemental Figure 7b-c**.

With regards to the effects of ferroptosis inhibitors, we performed some pilot experiments *in vitro*. We isolated Lin⁻ cells from *Rpl5*^{lox/+} and WT littermate control and cultured them *in vitro* in the presence or absence of β -mercapto-ethanol (BME) or ferrostatin-1 (Fer-1) towards the erythroid lineage¹. BME partially rescued proliferation (Panel a) and erythroid differentiation (Panel b) in the RPL5 haploinsufficient cells while Fer-1 did not. The results of these experiments are presented below.

These results are provided for the Reviewers only. Indeed, dissecting the signaling pathway(s) leading to Ferroptosis in the *Rpl5*^{lox/+} model is the focus of our current studies.



β -mercapto-ethanol (BME) partially rescues proliferation and erythropoiesis *in vitro*. Lin⁻ HSPCs were cultured *in vitro* in the presence of 25 μ M BME or 10 μ M Fer-1. After 48 hours of culture, proliferation (a) and erythropoiesis (b) were assessed as fold change.

3. While the manuscript has many strengths, dissection of the underlying mechanisms at the molecular level is weak in places, raising several unanswered questions. For example, I struggle to interpret the western blotting experiments in Figure 3 in terms of explaining the reported phenotypes. What might be the basis for the transient increase in the p-eIF2a/eIF2 ratio? Have the authors further validated their stated hypothesis that the ISR is indeed activated? While the observation of differential eIF5a hypusination is interesting, the data are somewhat preliminary. Perhaps some of these experiments might be better placed in the supplementary data?

We understand and agree with the Reviewer. The main point of the western blots presented in **Figure 3** was indeed rather to provide evidence of “disturbance” in translation regulatory pathways, with regards to reported findings by our own group (i.e. hypusination) as well as others (e.g. eEF2, 4E-BP1) in ribosomopathies, than necessarily deciphering underlying mechanisms. However, the Reviewer is correct, and

these western blots raise additional questions. Therefore, we moved them to **Supplemental Figure 3**.

4. The observation that RUNX1 may act between p53 and p21 to drive hematopoietic failure in the Rps19lox/+ mice is potentially interesting, but as the authors acknowledge, additional work is required to validate these findings and explore their significance in the DBAS disease context.

We agree with the Reviewer, and we are now aiming at understanding the significance of these findings in patients through our collaboration with the DBA Registry of North America.

5. I would recommend that the authors include a graphic summarizing their findings to help communicate the work to a more general readership.

Thank you for the suggestion, a point also raised by **Reviewer 1**. A model is now presented in **Figure 8**.

6. Line 179: "Rps19lox/+ and Rpl5lox/+ progenitors revealed a decrease in the 40S and 60S fractions, respectively". Although the quantification in Fig 3b supports this conclusion, the polysome profile example shown on the left for Rps19 does not look so convincing. The resolution of the pdf figures is low and there seems to be a poorly defined third curve in the two left hand panels in Fig 3a?

We apologize for the poor quality of the pdf figure and poor resolution of the polysome profile leading to the appearance of a third curve in panel a. This issue has now been resolved.

7. Line 185: "Surprisingly, OPP incorporation was significantly higher in Rps19lox/+ and Rpl5lox/+ LSK populations compared to control littermates. Interestingly, the magnitude of the increase was substantially (significantly?) greater in Rps19lox/+". The figure panel is not called here in the text. Substantially (significantly?). Please correct.

We apologize. The figure panel is now called in the text and the text now reads "the increase was significantly greater in *Rps19^{lox/+}*".

How confident can the authors be in concluding that protein synthesis is indeed higher in the Rps19 vs Rpl5-deficient mice given the difference in the number of samples analyzed for the two mutants?

We apologize for this. We meant to compare *WT* vs *Rps19^{lox/+}* and *WT* vs *Rpl5^{lox/+}*. This is now clarified in the main text.

8. Line 813: formatting issue

The formatting issue has now been corrected.

9. Fig 3b-why on the y-axis do we have 40S/60S ratio for Rps19 but 60S/40S ratio for Rpl5?

Apologies. Due to the 40S defect in Rps19 and the 60S defect in Rpl5, we had initially decided to present the data as 40S/60S ratio for Rps19 but 60S/40S ratio for Rpl5. But the Reviewer is right, and we have now modified panel 3b to both reflect the 40S/60S ratio.

10. Line 237: “ti(translational changes) and ti(transcriptional changes) (Log2 ti(translation)/ti(transcription))”. Is this a formatting issue?

Apologies. It is indeed a formatting issue. It should read Δ . This has been modified.

11. Line 303 and 306: should be annotated as Fig 5g, not 5h, right?

Yes, the Reviewer is right. We apologize for the mistake, and this has now been corrected.

12. Figure S7-Have the authors validated any of their ferroptosis gene expression data at the protein level?

We thank the Reviewer for his/her suggestion. We have validated by western blot the expression pattern of several of the pro- and anti-ferroptosis genes that were identified by scRNAseq. The results are presented in **Supplemental Figure 7b-c**.

13. Fig 7H-the legend does not correspond to what is shown in the figure. No data are shown for RPS19. What is the new information in Fig 7H vs Fig 7g?

We apologize for the confusion raised by Figure 7h. Figure 7h is the quantification of the western blot presented in Figure 7g: Quantification of the western blots for p53 and p21 in *Rps19*^{lox/+}, *Rps19*^{lox/+}; *Runx1*^{lox/+} and *Rps19*^{lox/+}; *Runx1*^{lox/lox} ckit+ cells, normalized to control.

14. Fig 7k-what are the numbers on the x-axis?

We thank the Reviewer for the question. The numbers represent the quantification of the RUNX1 band intensity normalized to Actin. Healthy control signal intensity was arbitrarily set up as 1.

15. Line 945-formatting issue

The formatting issue has now been corrected.

Reference:

1. Ji P, Jayapal SR, Lodish HF. Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. *Nat Cell Biol* **10**, 314–321 (2008).

We would like to thank the Editor and the Reviewers for being pleased with our revisions. Summarized below are the changes relevant to the remaining concerns, brought by the new Reviewer:

- We have added the correlation between biological replicates as new **Supplementary Figure 5d** show a strong correlation between biological replicates (Spearman $r=0.92-0.98$), clearly conveying reproducibility between the cell-type/cluster fractions.
- We have performed scCODA as suggested by the Reviewer, and provided the results as **Supplementary Table 1**,
- We provided the chromVAR scores for Runx1 and tp53 motifs below and as part of **Supplementary Figure 9f-g**.

Reviewer #1 (Remarks to the Author)

The authors have satisfactorily addressed my review comments and concerns.

We thank **Reviewer 1** for his/her positive comments on our revised manuscript.

Reviewer #2 (Remarks to the Author)

The authors have made a good job of addressing all the concerns and corrected the formatting errors.

We thank **Reviewer 2** for his/her positive comments on our revised manuscript.

Minor point

Please clarify in the legend to Fig 7k that the numbers on the x-axis represent quantification of RUNX1 intensity versus actin control as this is not explicitly stated.

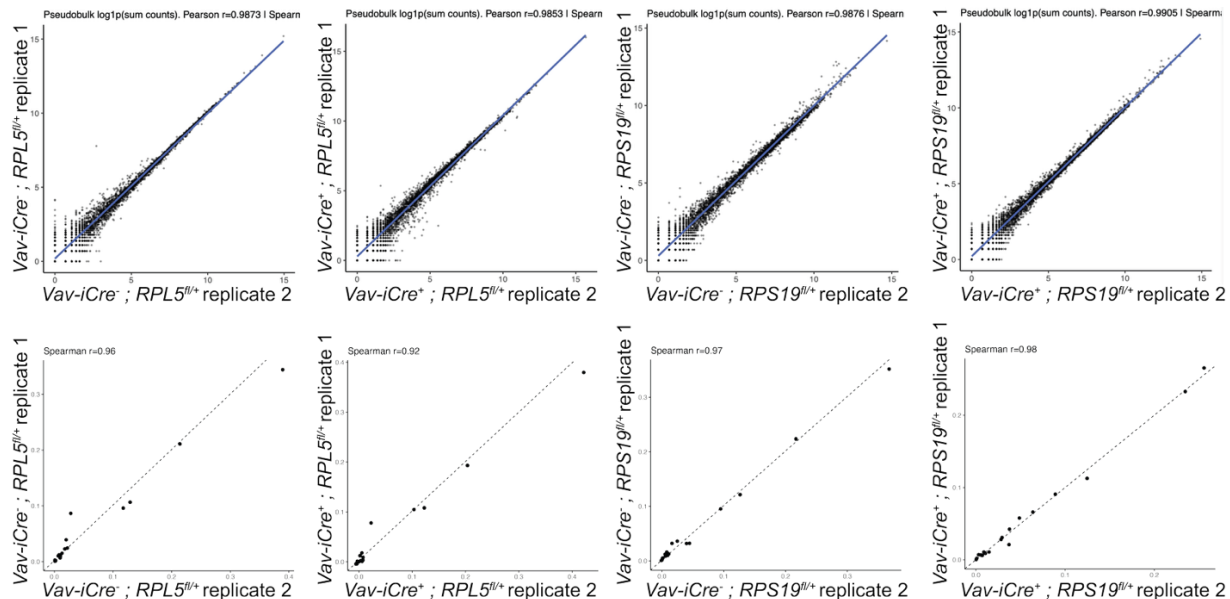
We apologize. The Reviewer is correct. This is now clearly stated.

Reviewer #3 (Remarks to the Author):

1. The single-cell analyses are nicely done overall, but a few additions could make the conclusions more convincing. It would be useful to show how well the scRNA-seq biological replicates agree with each other, for example by correlating pseudobulk expression profiles or cluster abundances, to make the reproducibility more explicit.

We thank the Reviewer for their positive comments on our -omics analyses and for their helpful suggestions. We have now performed correlation analyses as suggested and show that pseudobulk gene-expression profiles (Pearson $r > 0.98$) and cluster

abundances (Spearman $r = 0.92$ – 0.98) are highly concordant across biological replicates, indicating robust reproducibility of scRNA-seq annotations and cell-type frequency estimates. Tight 1:1 alignment indicates no major replicate-specific bias, arguing against any single replicate driving the WT–het differences. These results are presented below and as new **Supplementary Figure 5d**. In addition, we would like to emphasize that we had made every effort to validate our single-cell analyses using confirmatory and/or functional experiments.



2. The claim that RPS19 haploinsufficiency causes alterations in HSPC, MEP, megakaryocyte, and erythroid progenitor frequencies (Supplementary Fig 5b-c) rely on changes in cell-type or state proportions across conditions. Adding some statistical support for these compositional shifts (e.g. using a simple bootstrap or permutation approach or tools like scCODA) would help clarify which differences are robust.

We thank the Reviewer for this suggestion. We have now applied scCODA compositional modeling to both comparisons (4 samples in each comparison, 18 cell types). In the case of Rps19 (WT vs. het), scCODA identified robust and credible compositional shifts, including increased proportions of EP, MEP, Mk, and Primitive_Erythrocyte populations, along with decreased proportions of late erythroid states (OrthoE, PolyE) and a reduction in the Unknown1 cluster.

In contrast, in the case of Rpl5 (WT vs. het) scCODA returned zero non-zero effects across all cell types, indicating that—given the current level of biological replication—there were no compositional changes.

We thus concluded that the compositional changes are specific and strong in the case of Rps19 haploinsufficiency, while there is no detectable compositional shift under the same statistical framework in the Rpl5 haploinsufficient model.

The results of scCODA are presented below, and as **Supplementary Table 1**.

Rps19 Effects:

Covariate	Cell Type	Final Parameter
Condition[T.LioRps19het]	BasoE	0
Condition[T.LioRps19het]	Basophil	0
Condition[T.LioRps19het]	EP	0.568684
Condition[T.LioRps19het]	GMP	0
Condition[T.LioRps19het]	Gran	0
Condition[T.LioRps19het]	HSPC	0
Condition[T.LioRps19het]	Hepatocyte	0
Condition[T.LioRps19het]	Kupffer	0
Condition[T.LioRps19het]	LEC	0
Condition[T.LioRps19het]	MEP	0.662706
Condition[T.LioRps19het]	Mk	0.571598
Condition[T.LioRps19het]	OrthoE	-0.578451
Condition[T.LioRps19het]	PolyE	-0.705538
Condition[T.LioRps19het]	Primitive_Erythrocyte	0.72023
Condition[T.LioRps19het]	ProE	0
Condition[T.LioRps19het]	Stellate	0
Condition[T.LioRps19het]	Unknown1	-0.435799
Condition[T.LioRps19het]	Unknown2	0

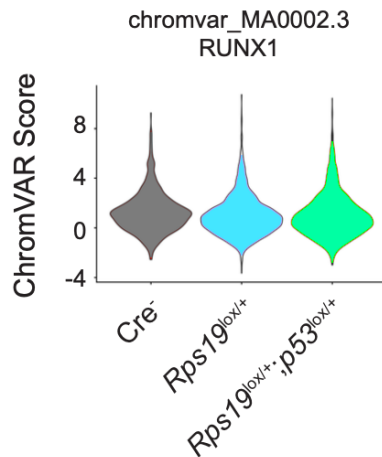
Rpl5 Effects:

Covariate	Cell Type	Final Parameter
Condition[T.LioRpl5het]	BasoE	0
Condition[T.LioRpl5het]	Basophil	0
Condition[T.LioRpl5het]	EP	0
Condition[T.LioRpl5het]	GMP	0
Condition[T.LioRpl5het]	Gran	0
Condition[T.LioRpl5het]	HSPC	0
Condition[T.LioRpl5het]	Hepatocyte	0
Condition[T.LioRpl5het]	Kupffer	0
Condition[T.LioRpl5het]	LEC	0
Condition[T.LioRpl5het]	MEP	0
Condition[T.LioRpl5het]	Mk	0
Condition[T.LioRpl5het]	OrthoE	0
Condition[T.LioRpl5het]	PolyE	0
Condition[T.LioRpl5het]	Primitive_Erythrocyte	0
Condition[T.LioRpl5het]	ProE	0
Condition[T.LioRpl5het]	Stellate	0
Condition[T.LioRpl5het]	Unknown1	0
Condition[T.LioRpl5het]	Unknown2	0

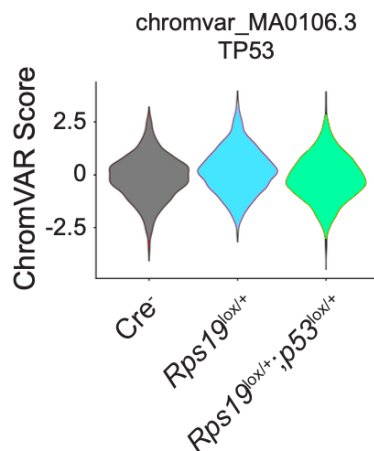
3. The chromatin accessibility analysis relies primarily on Giggle scores for transcription

factor motif enrichment in differential peaks, an orthogonal analysis such as chromVAR could strengthen the story. In particular, chromVAR scores for TP53 and RUNX1 motifs could help assess whether their activities are correlated at the single-cell level in the relevant populations, which would support the proposed coordination between these pathways, even if it does not establish direct regulation.

We thank the Reviewer for their comment and suggestion. We agree and have now included the chromVAR scores for Runx1 and tp53 motifs below and as part of **Supplementary Figure 9f-g**.



The RUNX1 (MA0002.3) motif exhibits a slight skewing towards negative in both *Rps19^{lox/+}* HSPCs and *Rps19^{lox/+}; tp53^{lox/+}* HSPCs. However, the presence of longer tails suggests an increase in variability; a small subset showing enhanced accessibility.



The TP53 (MA0106.3) motif exhibits modest positive skewing in *Rps19^{lox/+}* HSPCs relative to control, indicating enhanced accessibility at TP53-bound regulatory elements. This pattern suggests preferential engagement of TP53-associated sites in the *Rps19^{lox/+}* background, consistent with activated p53 stress signaling under ribosomal haploinsufficiency. In contrast, *Rps19^{lox/+}; tp53^{lox/+}* HSPCs display a broader distribution with a prominent negative tail, revealing subpopulations exhibiting suppressed TP53 motif accessibility.

4. Finally, the link between RUNX1 and Cdkn1a would be stronger with comparative RUNX1 ChIP data across conditions. Without this, it may be worth framing the functional relationship a bit more cautiously.

We agree with the Reviewer. Unfortunately, the low number of cells precluded us from performing these experiments, at least in the haploinsufficient conditions. While we have performed *in vivo* experiments using the *Runx1^{fl/fl}* mice to mechanistically attempt to frame the functional relationship (**Figure 7**), we acknowledge that ChIP data would be ideal. Thus, to fit with the previous point mentioned by the Reviewer, we have modified the results section which now read: “Published ChIP-seq data from mouse bone marrow and HSPCs^{45,46} confirmed the binding of RUNX1 on the *Cdkn1a* gene (**Supplementary Fig. 9e**). Further analyses of the scATACseq results showed that the RUNX1 (MA0002.3) motif exhibits a slight skewing towards negative in both *Rps19^{lox/+}* HSPCs and *Rps19^{lox/+}; tp53^{lox/+}* HSPCs. However, the presence of longer tails suggests an increase in variability; a small subset showing enhanced accessibility (**Fig. 7f, Supplementary Fig.9f, g**). Taken together, these results demonstrate that RUNX1 could play a role in the mechanism of HSPC failure in RPS19 haploinsufficiency through direct binding to the *Cdkn1a* promoter. Since we could not perform ChIP-seq analyses due to the low number of cells in the haploinsufficient conditions, we also carried out western blot assays on the c-Kit⁺ fraction at E15.5. We observed that while the p21 expression levels were dramatically reduced in the absence of *Runx1*, the levels of p53 remained unchanged (**Fig. 7g, h**). Conversely, in the *Rps19^{lox/+}; Trp53^{lox/lox}* c-Kit⁺ cells, the levels of RUNX1 were back to baseline levels, suggesting that Trp53 is upstream of Runx1 (**Fig. 7i**). Together, these results indicate that RUNX1 acts between p53 and p21 in the mechanism leading to hematopoietic failure in *Rps19^{lox/+}*”.

Point by point Response to Reviewers' Comments

N/A. Only Editorial changes requested.