Mitochondrial Metabolism in Hematopoietic Stem Cells Requires Functional FOXO3

Pauline Rimmele, Raymond Liang, Carolina L. Bigarella, Kocabas Fatih, Jingjing Xie, Madhavika N. Serasinghe, Jerry Chipuk, Hesham Sadek, Cheng Cheng Zhang and Saghi Ghaffari

Corresponding author: Saghi Ghaffari, Mount Sinai School of Medicine

Review timeline:

<table>
<thead>
<tr>
<th>Review Stage</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>09 October 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>10 November 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>11 May 2015</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>07 June 2015</td>
</tr>
<tr>
<td>Revision received</td>
<td>11 June 2015</td>
</tr>
<tr>
<td>Accepted</td>
<td>15 June 2015</td>
</tr>
</tbody>
</table>

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Barbara Pauly

1st Editorial Decision 10 November 2014

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have now received the full set of reviews on your manuscript and I am pasting them below for your information.

You will see that all reviewers appreciate the interest of your findings and are, in principle, supportive of publication of your study in our journal. However, they are also in agreement that several aspects of the data need to be improved by additional, independent lines of evidence, the inclusion of important controls and additional clarifications before the paper can be published. Especially referee 2 feels that the current data set needs to be strengthened, given that is seems to be contradicting previous observations. This reviewer also asks for further experiments addressing the mechanism by which loss of Foxo3 affects LT-HSC repopulation activity and while we consider this to be beyond the scope of the current study, some alternative possibilities to mitochondrial dysfunction, such as changes in cell cycle progression and/or cell death should at least be excluded.

Overall, and given the reviewers' constructive comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.
I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

In this manuscript, the transcription factor FOXO3 is claimed as a new regulator of metabolic adaptation of HSC. Foxo3 KO HSC exhibit defective mitochondrial oxidative phosphorylation independent of ROS levels or mTOR signaling. Instead, this defect is associated with altered expression of mitochondrial metabolic genes and compromises HSC function. This manuscript presents an interesting and important finding: long-term competitive repopulation ability of Foxo3−/− HSC is not mediated by the enhanced levels of ROS or mTOR activation are unexpected findings and should create more discussion and thoughts about the role of ROS and mTOR signaling in HSC function in the field of HSC biology.

Despite these interesting results some of the conclusions drawn or statement made may require clarification in order to aid clear understanding of general readers. Here are some examples.

1. In case of the summary within the abstract "FOXO3 controls metabolic checkpoints in HSC" it is unclear what authors mean by 'metabolic checkpoints'.

2. Page 9. Conclusion "These combined findings support the notion that the loss of HSC long-term competitive repopulation ability in Foxo3-deficient mice is likely the result of dysfunctional mitochondria in Foxo3−/− HSC." seems to be a logical leap. Results may exclude ROS as a causal factor but does not necessarily bring in mitochondria defect which could be a consequence of many other defects that FoxO3 mutant HSC have.

3. It will be helpful to clearly define different populations of HSC authors examined upfront. For example, Page 5. "we provide evidence that activation of mTOR signaling pathway mediates the abnormal mitochondrial function in the less primitive subset of HSPC". This statement is limited to HSPCs. Regardless, the statement "Foxo3 KO HSC exhibit defective mitochondrial oxidative phosphorylation independent of ROS levels or mTOR signaling" in the abstract is more generally used and may confuse the readers as the distinction among HSC, HSPC, primitive HSPC, ST-HSC, LT-HSC, LSK, and cKit+ is not clearly given.

4. Page 5, ".elevation of ROS indicates an underlying unhealthy mitochondrial state in Foxo3 KO HSC" reference is necessary for this statement. In fact, cellular ROS elevation could be due to many other reasons, to list a few, pentose phosphate pathway, glutamine metabolism, among others.

5. Page9 subtitle "Inhibition of mTOR signaling in vivo alleviates oxidative stress but does not ameliorate Foxo3−/− LT-HSC function" this may be revised to clearly deliver the conclusion.

6. Throughout the manuscript authors define increased mitochondrial membrane potential being "abnormal". This is presumably based on decreased ATP generation. Did authors look at other features of mitochondria including but not limited to resistance to apoptosis and complex IV activity, respiration property using more sophisticated and staged pharmacological challenges (e.g. seahorse platform?)

7. What is the evidence that elevated mitochondrial potential is the cause of defective HSC function? It goes hand in hand with HSC function and could be a mere correlation or secondary to mitochondrial defects of Foxo3 ko ST-HSC but not LT-HSC.

For experimental evidences presented:

8. For Figure 1B oxygen biosensor was used. This should be confirmed by more stringent method since it is central to the conclusion of 'defective oxidative phosphorylation'.

9. In Fig. 2E - experiments were performed with HSCs treated with NAC prior to the
transplantation. Did authors measure ROS or oxidative stress indices at the end of experiment or even during 16 week time period? Otherwise it would be difficult to interpret that decreasing ROS levels in vivo may not be sufficient to overcome the defects of Foxo3 KO HSC. In addition, to reinforce the conclusion it would be necessary to have additional evidence of NAC effect. Such as GSH/GSSG or oxyblot of BM cells, among others. Along the same line, lack of Rapamycin effect in long term repopulation assay in Fig 6E is presented. Could this be due to the inability of Rapamycin to keep mTORC1 inhibited over time? Were 12 - 16 wk Rapamycin-treated HSCs checked for pS6 level?

Referee #2:

Rimmele and colleagues provide data that they suggest shows a role for Foxo3 in activating HSC mitochondria to increase respiration by knocking Foxo3 out of HSCs and determining that genes regulating mitochondria and metabolism are altered for their expression, impairing mitochondrial function (lower respiration and ATP, increase lactate levels) independent of ROS production or mTOR activation.

1. The authors cite several studies that claim that HSCs are dormant in a hypoxic BM niche in the unactivated state. Yet, isolated Foxo3-/- HSCs in Figure 1A-C show lower levels of ATP and oxygen consumption and a shift to glycolysis with increased lactate production, which leads the authors to suggest that Foxo3 loss compromises mitochondrial respiration and ATP generating functions. How is this possible if quiescent BM cells, which include LSK and LT-HSC and c-Kit+ subpopulations, are already highly glycolytic, as described in multiple references cited by the authors? Are dormant HSCs more oxidative than the authors' text and citations suggest? This starts the paper out in a very confusing way and needs to be experimentally and textually rectified and clarified.

2. Text page 6 and Figure 1D legend- LT-HSC mitochondrial mass is not statistically different in WT and Foxo3-/- mice, although it is stated that it is- which is correct?

3. An increased mitochondrial membrane potential with decreased respiration and oxidative phosphorylation in Figure 1 is unexpected and just claiming it must be due to an unexplained compensatory mechanism does not provide further understanding for the results that, like point 1 above, are contradictory to what has been reported by multiple labs previously.

4. Figure 1F- JC-1 staining for measurements of mitochondrial membrane potential is not optimal because JC-1 is a relatively inaccurate reporter. JC-1 fluorescence intensity/emission spectra are not only dependent on Nernstian accumulation but also bind and quench anomalously, as discussed in reviews by Duchen or Nicholls, and thus are not consistent or reliable measures of mitochondrial membrane potential. A more reliable and non-Nernstian method uses TMRM staining.

5. Figure S1- ROS increases in Foxo3-/- HSCs but there is no reference to these figures in the main text.

6. How do the authors explain increased ROS with decreased oxidative phosphorylation, which again is a contradictory result with what has been reported by others?

7. Figure 2C, 2D- Foxo3-/- BM cells have also been reported to have reduced cell survival by increased apoptosis, which would decrease cell numbers that may not be fixable by blocking ROS with NAC. How do the authors know this is not the responsible mechanism for the failed rescue in cell numbers with NAC exposure?

8. Text page 8 and Figure 2D- I think the authors are saying they treated WT and Foxo3-/- mice with NAC, then harvested LSKCD48-CD150+ BM cells and transferred with carrier BM in competitive assays into lethally irradiated CD45.1 congenic mice and did not see an effect of NAC on long term reconstitution. If this is correct, then why didn't the recipient irradiated mice also receive NAC preconditioning and throughout the reconstitution assay?
9. Excluding ROS or mTOR does not exclude any other potential mechanism for lack of reconstitution assay beyond mitochondrial function and this is not explored further in the current work. What is even more perplexing is that the results for mitochondrial dysfunction disagree with many published studies, as discussed in the points above and by the authors in the text in several places.

10. There is no text referring to Figure 2E.

11. Figures that use NAC - where are controls to show that NAC actually did reduce ROS? How do the authors know the compound was working?

12. How does the data explain increased glycolysis with HSC loss of quiescence in Foxo3-/- HSCs when the anticipated result is to increase oxidative phosphorylation and not glycolysis?

13. Lower IF1 (Figure 4A) should lower the ATP level of respiring cells since it would increase ATP hydrolase activity in complex V ATP synthase - this is seen in Figure 1A - could this be the source for at least some of the paradoxical effects reported by the authors?

14. The authors exclude ROS accumulation or mTOR activation for the loss of LT-HSC reconstituting ability in HSCs that lack Foxo3. However, the only insight provided for this lost activity is that mitochondrial membrane potential is altered, as are ATP levels and the expression of a few genes that regulate mitochondrial metabolism. There is no insight for how Foxo3 loss affects LT-HSC repopulating activity, which is a major weakness of the study. As it stands, the effect of Foxo3 loss could be independent of mTOR, ROS and mitochondrial effects and due to something totally unrelated, such as changes in cell cycle potential or heightened apoptosis sensitivity. What data excludes possibilities beyond a mitochondrial dysfunction for the observed phenotype?

15. Overall, the text suffers from lack of clarity for explanations throughout and mainly reports descriptive observations. Although referencing seems very thorough, connections and insight beyond reporting is lacking. Re-writing of the text with clearer explanations and connections would benefit all readers.

Referee #3:

The manuscript by Rimmele et al describes analysis of the role of Foxo3 in murine hematopoietic stem cells (HSCs). The authors evaluate mitochondrial physiology as well as biological function of stem and progenitor cells in WT vs. Foxo3-/- mice. They conclude that the loss of stem cell potential that arises from Foxo3 knock-out is not due to increased oxidative state, but rather to mitochondrial dysfunction (decreased oxidative phosphorylation and altered gene expression).

This is an interesting and timely analysis of HSC metabolism as it relates to Foxo3. The authors describe several novel findings associated with Foxo3 inactivation, including reduced oxidative phosphorylation, increased glycolysis, altered mitochondrial membrane potential, and altered gene expression. While the work leaves many questions unanswered, the data provide valuable additional insights on HSC biology and should stimulate a variety of lines of investigation. However, some of the experiments appear to have significant experimental limitations. The study would be strengthened by further attention to the follow points:

Figure 2: the failure of NAC to rescue LSK and LT-HSC numbers is interesting and important. However, the choice of only two weeks of NAC treatment for this study is not explained. Previous studies have employed longer treatments (on the order of 5-6 weeks), which begs the question - would NAC have an effect if simply dosed for a longer period? Additional studies, with longer treatment would strengthen these findings. In addition, the lack of rescue upon transplantation into secondary mice (Figure 2E) was determined in absence of any further NAC treatment. Presumably, in order to manifest rescue of the phenotype, continuous NAC would be required. Consequently, interpretation of this key functional readout is difficult. Repeating this experiment with continuous NAC treatment of the secondary mice would make the findings much stronger.
Figure 3: While the failure of NAC to rescue the membrane potential phenotype observed in Foxo3-/- mice is quite interesting, the data would be stronger if the authors performed all of the same studies performed in Figure 1 for the initial characterization (i.e. oxygen consumption, lactate production, ATP levels).

Figure 4: The authors provide a relatively superficial interpretation of the gene expression data. Of particular note, levels of Atpif1 are markedly reduced, which should correlate with increased ATP. However, as shown in figure 1A, ATP levels are reduced in Foxo3-/- mice. Further explanation would be helpful.

Figure 6: The secondary transplantation assay shown in Figure 6E has the same limitation as noted for similar experiments in Figure 2, i.e. the drug treatment is not maintained in the secondary mice. Again, it would be preferable to perform this study in the presence of continued drug treatment.

Response to Reviewers

We are submitting our revised version of the manuscript entitled “Mitochondrial metabolism in hematopoietic stem cells requires functional FOXO3” to be considered for publication in EMBO Reports.

We were very pleased with the reviews and thank the reviewers for their constructive comments that helped us in improving our manuscript. We have addressed almost all if not all their queries; we have revised the paper accordingly.

The summary of our revisions is:

(1) A major concern of reviewers was the length of time N-Acetyl-Cysteine (NAC) was administered to mice and the continuity of NAC in post-transplanted mice. We have discussed the problem associated with post-transplant NAC; regardless, we have performed a longer NAC treatment (4 weeks pre-transplant as compared to 3 week pre-transplant treatment of ATM/-/- mice [1]) with or without post-transplant NAC and have found that the original findings stand (Figure 2F).

(2) The reviewers requested additional evidence for mitochondrial dysfunction, particularly data pertaining to apoptosis and proliferation. We have shown some data and discussed the effect of FOXO3 and ROS on HSC proliferation [2]. Loss of FOXO3 does not result in an increase in HSC apoptosis [3]. In fact, loss of FOXO3 results in a relative resistance to oxidative stress-mediated apoptosis in Foxo3-/- HSPC (Fig 1R attached), in agreement with lack of increased apoptosis despite elevated ROS, further supporting data presented in the current manuscript. We have included additional data on mitochondrial morphology in Foxo3 mutant HSC (Fig. S2). It is unclear at this time whether these Foxo3 mutant HSPC mitochondrial abnormalities are related to alterations in metabolism.

(3) We have added additional controls for mitochondria potential using TMRE as requested (Figures 1G; 3C; S1E; S4C).

(4) We also provided additional controls for NAC effect on ROS.
(Figures S3 and S4A, S4B, S4D).

(5) We attempted to perform Seahorse assay for measurement of O2 consumption. We would like to point out that our approach using O2 Biosensor is the pioneering approach used by our collaborators for the first time for the measurements of O2 consumption in HSC [4]. All other data (with graphs) reported to use Seahorse assay from HSC are generated from HSC cultured for days or from Lin- cells and not HSC; this is because Seahorse assay is a relatively insensitive assay and currently 20-30 mice per point (500,000 HSPC) are required to perform Seahorse for HSC. Nonetheless, we generated data from Lin-Kit+ cells with Seahorse that we provide for the reviewers (Fig 2R), that is in support of our findings but is derived from insufficient cell numbers (120,000 cells) and therefore not optimum and not from HSC. We have discussed this in detail in our point-by-point responses to the reviewers.

Figures 1G, 2F, 3C, S1E, S2A, S2B, S2C, S3 and S4A, S4B, S4C and S4D are all new.

Below are our point-by-point responses to the reviewers:

Referee #1:

In this manuscript, the transcription factor FOXO3 is claimed as a new regulator of metabolic adaptation of HSC. Foxo3 KO HSC exhibit defective mitochondrial oxidative phosphorylation independent of ROS levels or mTOR signaling. Instead, this defect is associated with altered expression of mitochondrial metabolic genes and compromises HSC function. This manuscript presents an interesting and important finding: long-term competitive repopulation ability of Foxo3-/HSC is not mediated by the enhanced levels of ROS or mTOR activation are unexpected findings and should create more discussion and thoughts about the role of ROS and mTOR signaling in HSC function in the field of HSC biology. Despite these interesting results some of the conclusions drawn or statement made may require clarification in order to aid clear understanding of general readers. Here are some examples.

1. In case of the summary within the abstract "FOXO3 controls metabolic checkpoints in HSC" it is unclear what authors mean by 'metabolic checkpoints'.

Response: we have modified the abstract overall; this sentence is now removed.

2. Page 9. Conclusion "These combined findings support the notion that the loss of HSC long-term competitive repopulation ability in Foxo3-deficient mice is likely the result of dysfunctional mitochondria in Foxo3-/ HSC." seems to be a logical leap. Results may exclude ROS as a causal factor but does not necessarily bring in mitochondria defect which could be a consequence of many other defects that Foxo3 mutant HSC have.

Response: Increasing literature suggests that defective mitochondrial metabolism is broadly associated with defects in the maintenance and/or lineage commitment of HSC [5-11]. In agreement with this, we wrote: "our findings support the notion (but does not prove) that the loss of competitive repopulation ability in Foxo3-deficient mice is likely the result of dysfunctional mitochondria in Foxo3-/ HSC". To make our point more clear, we have revised the sentence to: 
"Thus, loss of Foxo3-/ HSC long-term competitive repopulation is associated with impaired mitochondrial metabolism but not mediated by ROS. These combined findings raise the possibility that compromised Foxo3-/ HSC mitochondria may
be implicated in defects of Foxo3-/- HSC activity.”

3. It will be helpful to clearly define different populations of HSC authors examined upfront. For example, Page 5. “we provide evidence that activation of mTOR signaling pathway mediates the abnormal mitochondrial function in the less primitive subset of HSPC”. This statement is limited to HSPCs. Regardless, the statement “Foxo3 KO HSC exhibit defective mitochondrial oxidative phosphorylation independent of ROS levels or mTOR signaling” in the abstract is more generally used and may confuse the readers as the distinction among HSC, HSPC, primitive HSPC, ST-HSC, LT-HSC, LSK, and cKit+ is not clearly given.

Response: we have now explained the terminology upfront and made this clear.

4. Page 5, “.elevation of ROS indicates an underlying unhealthy mitochondrial state in Foxo3 KO HSC” reference is necessary for this statement. In fact, cellular ROS elevation could be due to many other reasons, to list a few, pentose phosphate pathway, glutamine metabolism, among others.

Response: we have added a reference (ref#52) and have modified the sentence.

5. Page9 subtitle “Inhibition of mTOR signaling in vivo alleviates oxidative stress but does not ameliorate Foxo3-/- LT-HSC function” this may be revised to clearly deliver the conclusion.

Response: the sentence has been modified to: “Inhibition of mTOR signaling improves ROS levels but does not ameliorate Foxo3-/- LT-HSC function in vivo”.

6. Throughout the manuscript authors define increased mitochondrial membrane potential being “abnormal”. This is presumably based on decreased ATP generation. Did authors look at other features of mitochondria including but not limited to resistance to apoptosis and complex IV activity, respiration property using more sophisticated and staged pharmacological challenges (e.g. seahorse platform?)

Response: we had analyzed membrane potential by JC-1 and Dilc-1. We have now extended and confirmed these results by analyzing the mitochondrial membrane potential with TMRE (tetramethylrhodamine, ethyl ester) to label active mitochondria. We have shown that oxygen consumption is significantly reduced in Foxo3-/- HSPC (Fig. 1B). In addition, using an antibody to Tom20, a mitochondria specific protein, we show that the mitochondrial morphology is compromised and mitochondria is hyper-fragmented in Foxo3-/- HSPC (Fig. S2). We agree it will ideal to have Seahorse data. To our knowledge, Seahorse assay has not been used for freshly isolated HSC; in few publications where Seahorse assay has been used for HSC analysis and graphs were shown, isolated hematopoietic stem and progenitor cells (LSK cells) were either cultured for many days or not used at all, instead lin- cells were used [6, 12, 13]. Despite our efforts, we were unable to perform Seahorse analysis that is much less sensitive that our approach and requires a large number of mice (over 30 mice) per point for stem cell analysis. We have attached data from Lin-Kit+ cells (120,000 cells) (Fig 2R) that although support our results, is clearly derived from insufficient cell numbers. We further provide evidence that Foxo3 mutant HSC are relatively resistant to apoptosis (attached, Fig 1R), this data is part of a separate manuscript on a distinct subject that is prepared for submission.

Response: we have now extended and confirmed these results by analyzing the mitochondrial membrane potential

7. What is the evidence that elevated mitochondrial potential is the cause of defective HSC function? It goes hand in hand with HSC function and could be a mere correlation or secondary to mitochondrial defects of Foxo3 ko ST-HSC but
not LT-HSC.

**Response:** We did not suggest that defective HSC activity is due to elevated mitochondrial potential. Elevated mitochondrial potential associated with ATP depletion and reduced mitochondrial respiration (and associated with increased glycolysis) in Foxo3−/− HSC that have lost their quiescence, was surprising. Given the amount of data pointing to defective mitochondria as being potentially implicated in defective LT-HSC competitive repopulation [5-11], our data raise the possibility (but does not prove) that lack of repopulation might be due to defective mitochondrial function. We made that clear in the manuscript now.

**For experimental evidences presented:**

8. For Figure 1B oxygen biosensor was used. This should be confirmed by more stringent method since it is central to the conclusion of 'defective oxidative phosphorylation'.

**Response:** We have used oxygen biosensor that is the first to ever measure metabolism of HSCs [4], and also the only method ever used in the literature to measure oxygen consumption in LT-HSC and that its the only feasible method, albeit indirect, but highly sensitive for mitochondrial measurements of HSPC. In few blood stem cell publications where Seahorse assay has been used and graphs shown, isolated hematopoietic stem and progenitor cells (LSK cells) were either cultured for many days or not used at all, instead lin- cells were used [6, 12, 13]. Approaches such as Seahorse and Clark type oxygen chambers require 30 mice per point, an approach that is currently extremely challenging. We have attached data from Seahorse assay of Lin-Kit+ cells (120,000 cells) ([Fig 2R]) that although support our results, is not from the cells of interest and clearly derived from insufficient cell numbers.

9. In Fig. 2E - experiments were performed with HSCs treated with NAC prior to the transplantation. Did authors measure ROS or oxidative stress indices at the end of experiment or even during 16 week time period? Otherwise it would be difficult to interpret that decreasing ROS levels in vivo may not be sufficient to overcome the defects of Foxo3 KO HSC.
   
   In addition, to reinforce the conclusion it would be necessary to have additional evidence of NAC effect. Such as GSH/GSSG or oxyblot of BM cells, among others.

**Response:** we have added data and now provide evidence that ROS are reduced during and at the end of the experiments: we show that ROS are reduced in the peripheral blood CD45+ cells from Foxo3 mutant mice treated and not the ones that were not treated with NAC ([Figure S4B]). We further show that ROS are significantly reduced in LT-HSC and LSK cells after 4 weeks of NAC ([Figure S4]). Finally we show oxyblot of NAC-treated versus untreated BM cells ([Figure S3]).

Along the same line, lack of Rapamycin effect in long term repopulation assay in Fig 6E is presented. Could this be due to the inability of Rapamycin to keep mTORC1 inhibited over time? Were 12 - 16 wk Rapamycin-treated HSCs checked for pS6 level?

**Response:** Having continuous rapamycin treatment in the recipient may have many additional effects on immune cells that could impact the HSC and interfere with rapamycin effects on intrinsic mTOR signaling in HSC. Although we did not see any effect of rapamycin (by our regimen) on LT-HSC numbers, similar or identical regimen to the one we used have been reported to normalize the number of LT-HSC in which mTOR signaling is hyperactivated, after short (10 days-2 weeks) treatment with rapamycin [5, 7-9]. The lack of effects on LT-HSC was not due to a lack of rapamycin efficiency since we showed that the levels of
pS6 in transplanted LT-HSC were normalized. In addition, LSK numbers were normalized with this regimen. Although, we can not rule out the possibility of reduced effect of rapamycin on mTOR signaling with time in HSC, our experiment is to assay the long term repopulation potential of the cell we transplanted in which mTOR signaling was normalized. As we discussed in the revised paper, the lack of mTOR involvement in Foxo3-/- HSC phenotype is not surprising since the phenotype of Foxo3-/- HSC are quite distinct from that observed in Tsc1-/- (activated mTOR in HSC) mice [5].

Referee #2:

Rimmele and colleagues provide data that they suggest shows a role for Foxo3 in activating HSC mitochondria to increase respiration by knocking Foxo3 out of HSCs and determining that genes regulating mitochondria and metabolism are altered for their expression, impairing mitochondrial function (lower respiration and ATP, increase lactate levels) independent of ROS production or mTOR activation.

1. The authors cite several studies that claim that HSCs are dormant in a hypoxic BM niche in the unactivated state. Yet, isolated Foxo3-/- HSCs in Figure 1A-C show lower levels of ATP and oxygen consumption and a shift to glycolysis with increased lactate production, which leads the authors to suggest that Foxo3 loss compromises mitochondrial respiration and ATP generating functions. How is this possible if quiescent BM cells, which include LSK and LT-HSC and c-Kit+ subpopulations, are already highly glycolytic, as described in multiple references cited by the authors? Are dormant HSCs more oxidative than the authors’ text and citations suggest? This starts the paper out in a very confusing way and needs to be experimentally and textually rectified and clarified.

Response: Foxo3-/- HSC are not quiescent. Our data show a defective oxygen consumption associated with ATP depletion and increased glycolysis in Foxo3-/- HSC. We agree that the paradoxal loss of quiescence of Foxo3 mutant HSC in the face of increased glycolysis is surprising. Our data may indicate a defect in mitochondria (perhaps in ATP synthesis) that might be compensated by increased glycolysis. Alternatively increased glycolysis might result in defective mitochondria. We are not aware of data showing that increased glycolysis results in increased quiescence of HSC. As we stated mutations that cause HSC loss of quiescence associated with increased ROS as observed in Foxo3-/- HSC are often associated with decreased glycolysis and increased oxidative phosphorylation that is the major alternative source of energy to glycolysis in HSC [5, 14, 15].

1. Text page 6 and Figure 1D legend- LT-HSC mitochondrial mass is not statistically different in WT and Foxo3-/- mice, although it is stated that it is- which is correct?

Response: we could not find any statement supporting this.

3. An increased mitochondrial membrane potential with decreased respiration and oxidative phosphorylation in Figure 1 is unexpected and just claiming it must be due to an unexplained compensatory mechanism does not provide further understanding for the results that, like point 1 above, are contradictory to what has been reported by multiple labs previously.

Response: we understand these results are surprising and do not know yet the underlying mechanism. We have revised the manuscript to reflect this clearly.
2. Figure 1F: JC-1 staining for measurements of mitochondrial membrane potential is not optimal because JC-1 is a relatively inaccurate reporter. JC-1 fluorescence intensity/emission spectra are not only dependent on Nernstian accumulation but also bind and quench anomalously, as discussed in reviews by Duchen or Nicholls, and thus are not consistent or reliable measures of mitochondrial membrane potential. A more reliable and non-Nernstian method uses TMRM staining.

**Response:** We had used both JC-1 and DilC-1 for the analysis of mitochondrial membrane potential. We have now confirmed these results by adding TMRE analysis.

5. Figure S1: ROS increases in Foxo3-/- HSCs but there is no reference to these figures in the main text.

**Response:** We have now added these.

6. How do the authors explain increased ROS with decreased oxidative phosphorylation, which again is a contradictory result with what has been reported by others?

**Response:** We agree these results are not in agreement with most cases where elevated ROS is not associated with defect in mitochondria. Our findings suggest that ROS are elevated despite a reduction in oxygen consumption and ATP levels in Foxo3 mutant HSPC; these associated with abnormal mitochondrial parameters (Figures 1-4, S2) suggest an altered mitochondrial function.

7. Figure 2C, 2D: Foxo3-/- BM cells have also been reported to have reduced cell survival by increased apoptosis, which would decrease cell numbers that may not be fixable by blocking ROS with NAC. How do the authors know this is not the responsible mechanism for the failed rescue in cell numbers with NAC exposure?

**Response:** We are confused by this comment: we are not aware of apoptosis data of Foxo3 mutant BM cells. In fact Foxo3 mutant BM cells are reported not to be apoptotic [3]. We have also included data for the reviewer (Fig. 1R) that supports that Foxo3 mutant HSPC are relatively resistant to oxidative-stress mediated apoptosis. Our data suggest that the reduction in total numbers and frequency of LT-HSC and LSK cells in Foxo3 mutant mice is not mediated by ROS.

8. Text page 8 and Figure 2D: I think the authors are saying they treated WT and Foxo3-/- mice with NAC, then harvested LSKCD48-CD150+ BM cells and transferred with carrier BM in competitive assays into lethally irradiated CD45.1 congenic mice and did not see an effect of NAC on long term reconstitution. If this is correct, then why didn’t the recipient irradiated mice also receive NAC preconditioning and throughout the reconstitution assay?

**Response:** We were asking whether reduction of intrinsic ROS levels in LTHSC that are being transplanted will have an effect on their long term reconstitution after 16 weeks as has been reported for instance for ATM-/- LTHSC [1]. We did not observe such an effect. Treatment of recipient throughout reconstitution addresses a different question and positive results might involve the impact of environmental ROS. Regardless, we have now performed these experiments to clarify these questions and our results stand: NAC treatment of mice before, or before and after transplantation does not improve Foxo3 mutant stem cell competitive repopulation activity after 16 weeks. We have included these data in the revised manuscript (Figure 2F).
9. Excluding ROS or mTOR does not exclude any other potential mechanism for lack of reconstitution assay beyond mitochondrial function and this is not explored further in the current work. What is even more perplexing is that the results for mitochondrial dysfunction disagree with many published studies, as discussed in the points above and by the authors in the text in several places.

**Response:** we are confused by this comment.

10. There is no text referring to Figure 2E.

**Response:** we have fixed this error in numbers in the text.

11. Figures that use NAC - where are controls to show that NAC actually did reduce ROS? How do the authors know the compound was working?

**Response:** The data was provided in Fig. 2B, additional data is now provided in Figs S3, S4.

12. How does the data explain increased glycolysis with HSC loss of quiescence in Foxo3-/- HSCs when the anticipated result is to increase oxidative phosphorylation and not glycolysis?

**Response:** This finding is surprising and may indicate a mitochondrial defect.

13. Lower IF1 (Figure 4A) should lower the ATP level of respiring cells since it would increase ATP hydrolyase activity in complex V ATP synthase - this is seen in Figure 1A - could this be the source for at least some of the paradoxical effects reported by the authors?

**Response:** we agree with the reviewer's interpretation that lower IF1 might mediate some of the phenotype and needs to be tested.

14. The authors exclude ROS accumulation or mTOR activation for the loss of LT-HSC reconstituting ability in HSCs that lack Foxo3. However, the only insight provided for this lost activity is that mitochondrial membrane potential is altered, as are ATP levels and the expression of a few genes that regulate mitochondrial metabolism. There is no insight for how Foxo3 loss affects LT-HSC repopulating activity, which is a major weakness of the study. As it stands, the effect of Foxo3 loss could be independent of mTOR, ROS and mitochondrial effects and due to something totally unrelated, such as changes in cell cycle potential or heightened apoptosis sensitivity. What data excludes possibilities beyond a mitochondrial dysfunction for the observed phenotype?

**Response:** our published data suggest that NAC treatment corrects Foxo3 mutant LSK cell cycle gene expression [2], however we show here that scavenging ROS does not improve repopulation ability of Foxo3 mutant LT-HSC. In addition apoptosis is not increased in Foxo3 mutant HSC [3]. We also included evidence that Foxo3 mutant HSPC are relatively resistant to oxidative stress mediated apoptosis (Fig. 1R, attached).

15. Overall, the text suffers from lack of clarity for explanations throughout and mainly reports descriptive observations. Although referencing seems very thorough, connections and insight beyond reporting is lacking. Re-writing of the text with clearer explanations and connections would benefit all readers.

**Response:** we have kept this in mind in our revised version and rewritten many sections.
Referee #3:

The manuscript by Rimmelle et al describes analysis of the role of Foxo3 in murine hematopoietic stem cells (HSCs). The authors evaluate mitochondrial physiology as well as biological function of stem and progenitor cells in WT vs. Foxo3-/- mice. They conclude that the loss of stem cell potential that arises from Foxo3 knock-out is not due to increased oxidative state, but rather to mitochondrial dysfunction (decreased oxidative phosphorylation and altered gene expression).

This is an interesting and timely analysis of HSC metabolism as it relates to Foxo3. The authors describe several novel findings associated with Foxo3 inactivation, including reduced oxidative phosphorylation, increased glycolysis, altered mitochondrial membrane potential, and altered gene expression. While the work leaves many questions unanswered, the data provide valuable additional insights on HSC biology and should stimulate a variety of lines of investigation. However, some of the experiments appear to have significant experimental limitations. The study would be strengthened by further attention to the following points:

Figure 2: the failure of NAC to rescue LSK and LT-HSC numbers is interesting and important. However, the choice of only two weeks of NAC treatment for this study is not explained. Previous studies have employed longer treatments (on the order of 5-6 weeks), which begs the question - would NAC have an effect if simply dosed for a longer period? Additional studies, with longer treatment would strengthen these findings. In addition, the lack of rescue upon transplantation into secondary mice (Figure 2E) was determined in absence of any further NAC treatment. Presumably, in order to manifest rescue of the phenotype, continuous NAC would be required. Consequently, interpretation of this key functional readout is difficult. Repeating this experiment with continuous NAC treatment of the secondary mice would make the findings much stronger.

Response: We were asking whether reduction of ROS levels in LT-HSC at the time of transplantation will have an effect on their long term reconstitution after 16 weeks as has been observed for instance for ATM-/- LT-HSC even in the absence of any further treatment [1]. We did not observe such an effect. A positive response to the treatment of recipient throughout reconstitution may not be due to LT-HSC intrinsic levels of ROS. Nonetheless, we have now performed these experiments to clarify the answer to this question and the results stand: NAC treatment of mice only before (for 4 weeks, longer than for instance the ATM-/- mice NAC treatment) or before and after transplantation does not improve Foxo3 mutant stem cell competitive repopulation after 16 weeks. We have included these data in the revised manuscript (Figure 2F).

Figure 3: While the failure of NAC to rescue the membrane potential phenotype observed in Foxo3-/- mice is quite interesting, the data would be stronger if the authors performed all of the same studies performed in Figure 1 for the initial characterization (i.e. oxygen consumption, lactate production, ATP levels).

Response: Although we agree it would be great to have such results, practically it is a highly challenging experiment to carry. Metabolic assays on transplanted HSC at the end of experiment, with very few left Foxo3-/- HSC (less than 15% chimera of CD45+ peripheral blood) in transplanted mice make this experiment almost impossible.

Figure 4: The authors provide a relatively superficial interpretation of the gene expression data. Of particular note, levels of Atpip1 are markedly reduced, which
should correlate with increased ATP. However, as shown in figure 1A, ATP levels are reduced in Foxo3-/- mice. Further explanation would be helpful.

Response: ATPIF1 is an inhibitor of mitochondrial F1F0-ATPase that limits the ATP depletion, therefore its reduced expression is associated with decreased rather than increased ATP. We have added a reference that confirms this statement. We have also revised the paragraph related to the gene expression.

Figure 6: The secondary transplantation assay shown in Figure 6E has the same limitation as noted for similar experiments in Figure 2, i.e. the drug treatment is not maintained in the secondary mice. Again, it would be preferable to perform this study in the presence of continued drug treatment.

Response: Having continuous rapamycin treatment in the recipient may have many additional effects on immune cells that could impact the HSC and interfere with rapamycin effects on intrinsic mTOR signaling in HSC. Although we did not see any effect of rapamycin (by our regimen) on LT-HSC numbers, similar or identical regimen to the one we used have been reported to normalize the number of LT-HSC in which mTOR signaling is hyperactivated, after short (10 days-2 weeks) treatment with rapamycin [5, 7-9]. Our lack of effects on LT-HSC was not due to lack of rapamycin effect since we showed that the levels of pS6 in transplanted LT-HSC were normalized. In addition, LSK numbers were normalized with this regimen. Although, we can not rule out the possibility of reduced effect of rapamycin on mTOR signaling with time, our experiment is to assay the long term repopulation potential of the cell we transplanted in which mTOR signaling was normalized. As we discussed in the revised paper, the lack of mTOR involvement in Foxo3-/- HSC phenotype is not surprising since the phenotype of Foxo3-/- HSC are very distinct of that observed in Tsc1-/- (activated mTOR in HSC) mice [5].

References
stem/progenitor cell defects, mitochondrial dysfunction, ROS overproduction, and a rapid aging-like phenotype. *Blood* **120**: 2589-2599

![Fig. 1R](image)

Flow cytometry analysis of apoptosis using annexin V-binding of freshly isolated LSK cells treated for 1h with or without 100uM H$_2$O$_2$. 
Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. While referee 3 is satisfied with the way in which you have addressed his/her concerns, referee 2 still raises issues, but since s/he does not specifically request additional experiments, I would like you to address his/her concerns in writing only before submitting the final version of the study.

Please also specify your response to this reviewers' concerns in a separate letter to me.

Seahorse plots of oxygen consumption rates (OCR) in FACS sorted LIN- cKIT+ cells from the bone marrow of mice treated with NAC or vehicle for 6 weeks. 1.2 x 10^5 purified LIN+, cKIT+ cells were seeded in 96 well plates with Seahorse assay media. Lines denote time points of administering (A) Oligomycin, (B) FCCP, (C) Rotenone, and Antimycin A.
Finally, we are in the process of updating the way in which we display additional/supplementary information. In essence, all supplementary figures are now called Expanded View Figures and should be labeled and referenced as Figure EV1, Figure EV2 etc. in the main text of the manuscript. The legends for the EV figures should be incorporated in the main body of the text after the legends for the main figures. Please modify your additional figures accordingly.

I look forward to seeing the final version of your manuscript as soon as possible.

REFeree REPORT:

Referee #2:

For this reviewer, the paper retains confusing and sometimes contradictory or unclear text in multiple places. Also, the inability to identify how Foxo3 knockout in HSCs affects mitochondria beyond descriptive, often self-inconsistent observations without a unifying theme or underlying connection remains problematic since many of the reported observations contradict and therefore could overturn prior published results, raising the bar. ROS is reasonably excluded from defects in Foxo3-/- HSC repopulation assays (which is a different result than for ROS dependence of Atm-/- HSCs for repopulation assays, which is interesting) and if this is the main point of the paper, then it seems reasonable and important to report.

Examples of continuing text problems for this reviewer include:

1. The text on page 6 states: "Mitochondrial mass was increased in primitive hematopoietic stem cell compartment of Foxo3-/- mice including in Foxo3-/- LT-HSC and LSK cells as compared to controls according to the mitotracker green probe that measures mitochondrial mass independently of membrane potential (Figure 1D)." YET, the figure legend 1 D states: "One representative FACS plot of the mitochondrial mass measured by the geometric mean fluorescence intensity of Mitotracker green of 2 (LT-HSC, LSKCD48+CD150-) or 3 (LSK and c-Kit+) independent experiments (n=3 mice per genotype) is shown: LT-HSC, p <0.324; LSK, p <0.021; and c-Kit+, P <0.092. Doesn't the p<0.324 mean a lack of statistical significance at the threshold 5% by chance for a change in mitochondrial mass for LT-HSC, yet the text says that there is a change, so which is correct?

2. A minor comment is that text on page 3 states that "blowd is ablated"- to ablate blood does not make much sense, but to ablate HSCs and impair the differentiation and replacement of lineages that derive from HSCs to repopulate the blood makes more sense.

3. Another example that adds to confusion: Text on page 6- in the same paragraph it is stated: "Alternatively, lower respiration rates may indicate that Foxo3 mutant HSC use glycolysis for energy production instead of oxidative phosphorylation", implying that HSCs use OXPHOS as main energy source. Then later in the same paragraph, it is stated: "These results were highly unexpected as HSC use glycolysis as their main source of energy [7, 9, 28, 55]". So which is it? HSCs use OXPHOS or glycolysis as their main energy source?

4. In general, strongly suggest that all instances of misleading and imprecise language be identified and corrected throughout the manuscript, which will make the story much easier to follow.

Response to Referee #2:

For this reviewer, the paper retains confusing and sometimes contradictory or unclear text
in multiple places. Also, the inability to identify how Foxo3 knockout in HSCs affects mitochondria beyond descriptive, often self-inconsistent observations without a unifying theme or underlying connection remains problematic since many of the reported observations contradict and therefore could overturn prior published results, raising the bar. ROS is reasonably excluded from defects in Foxo3-/- HSC repopulation assays (which is a different result than for ROS dependence of Atm-/- HSCs for repopulation assays, which is interesting) and if this is the main point of the paper, then it seems reasonable and important to report.

Examples of continuing text problems for this reviewer include:

1. The text on page 6 states: "Mitochondrial mass was increased in primitive hematopoietic stem cell compartment of Foxo3-/- mice including in Foxo3-/- LT-HSC and LSK cells as compared to controls according to the mitotracker green probe that measures mitochondrial mass independently of membrane potential (Figure 1D)." YET, the figure legend 1D states: "One representative FACS plot of the mitochondrial mass measured by the geometric mean fluorescence intensity of Mitotracker green of 2 (LT-HSC, LSKCD48+CD150-) or 3 (LSK and c-Kit+) independent experiments (n=3 mice per genotype) is shown: LT-HSC, p <0.324; LSK, p <0.021; and c-Kit+, P <0.092. Doesn't the p<0.324 mean a lack of statistical significance at the threshold 5% by chance for a change in mitochondrial mass for LT-HSC, yet the text says that there is a change, so which is correct?

Response: to clarify this statement we modified it to: “Mitochondrial mass was increased in primitive hematopoietic stem cell compartment of Foxo3-/- mice including in Foxo3-/- LSK cells and LT-HSC (although in these latter cells the increase did not reach significance in the limit of the number of replicates used) as compared to controls according to the mitotracker green probe that measures mitochondrial mass independently of membrane potential (Figure 1D).

2. A minor comment is that text on page 3 states that "blood is ablated"- to ablate blood does not make much sense, but to ablate HSCs and impair the differentiation and replacement of lineages that derive from HSCs to repopulate the blood makes more sense.

Response: we have modified this to the standard language of "bone marrow is ablated".

3. Another example that adds to confusion: Text on page 6- in the same paragraph it is stated: "Alternatively, lower respiration rates may indicate that Foxo3 mutant HSC use glycolysis for energy production instead of oxidative phosphorylation", implying that HSCs use OXPHOS as main energy source. Then later in the same
paragraph, it is stated: "These results were highly unexpected as HSC use glycolysis as their main source of energy [7, 9, 28, 55]". So which is it? HSCs use OXPHOS or glycolysis as their main energy source?

Response: normal HSC use glycolysis but Foxo3 mutant HSC, as they have exited quiescence, are anticipated to increase their use of oxidative phosphorylation but they do not. The prior sentences that lead to the sentence the referee 2 selected out the context of the paragraph, clearly stated: “Lower rates of mitochondrial respiration may reflect lower energy requirements. That is unlikely since Foxo3 mutant HSC in contrast to their wild type counterparts have exited the quiescence state and are likely subject to higher energy demand [15, 16].” Following sentences in the same paragraph stated: “These results were highly unexpected as HSC use glycolysis as their main source of energy [7, 9, 28, 55]…. Mutations that cause HSC loss of quiescence associated with increased ROS as observed in Foxo3−/− HSC are often associated with decreased glycolysis and increased oxidative phosphorylation that is the major alternative source of energy to glycolysis in HSC [18-20].” To further clarify the selected sentence we have modified the sentence to: “Alternatively, lower respiration rates may indicate that despite loss of quiescence Foxo3 mutant HSC increase glycolysis for energy production instead of increasing oxidative phosphorylation.”

4. In general, strongly suggest that all instances of misleading and imprecise language be identified and corrected throughout the manuscript, which will make the story much easier to follow.

Response: This has now been done.

3rd Editorial Decision 15 June 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.