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PIAS1 SUMO ligase regulates the self-renewal and differentiation of hematopoietic stem cells

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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: Thomas Schwarz-Romond

1st Editorial Decision 22 October 2012

Thank you very much for submitting your paper on the role of PIAS1 in regulating HSC self-renewal for consideration to The EMBO Journal editorial office.

Three scientists assessed your work. All three appreciate the described hematopoiesis defects that you report upon PIAS knockout. They also point to significant shortcomings when it comes to experimental substantiation and definitive insights into the underlying molecular mechanisms. Thus, ref #1 currently opposes publication while ref#2 and #3 condition their essential support on rather substantial further experimentation.

Please allow me to highlight their major concerns:
It would be essential
-to determine the frequency of functional stem cells by competitive limiting dilution assays on LT-HSCs (rather than 'sole' serial transplantations)
-parallel your mechanistic work in T-cells, to assess PIAS1 binding to DNMT-family members in bone marrow AND functional implications studied

-GATA1's functional relevance formerly be established
-PIAS1 loss/GATA1 up regulation in light of distinct lineage effects to be reconciled.

Such significant further demands illustrate the relatively preliminary state of analyses. Assuming that you might be in a strong position to address these in a timely manner, we would be prepared to offer appropriate revisions. Please do not hesitate to contact me with in case of further questions, possibly outlining the expected timeframe or indeed discuss the need for more time than the usual 3 month for a standard revision (preferably via E-mail).

I am sorry that I cannot be more encouraging at this stage but I hope that clear communication of what is needed to meet the expectations from our expert referees facilitates efficient proceedings for your study.

REFEREE REPORTS:

Referee #1:

The authors examined the effect of PIAS1 deficiency on hematopoiesis. No significant alterations in various murine myeloid precursor compartments were observed, except in the CLP population. The LSK fraction was increased and differences were mainly seen in the short-term multipotent progenitor cell fraction. No differences between WT and KO dormant HSC were found and ratios between the various HSC populations were not significantly altered. The authors found that the number of cycling Ki67 positive dormant HSC cells was increased, suggesting abrogation of quiescence in the otherwise non-proliferating d-HSC population. Competitive reconstitution assays showed strongly compromised repopulation activity of PIAS KO bone marrow cells, with defective B- and T-lymphoid and increased myeloid differentiation potential. Defects were not due to failure of engraftment, suggesting intrinsic HSC defects. Analysis of lineage priming or lineage specific transcripts in the lineage negative precursor population showed deregulation of some genes with the tendency of myelo-erythroid upregulation and B-lymphoid downregulation. In the CLP-fraction myelo-eythroid genes were found upregulated, suggesting incomplete repression of CMP/MEP/GMP specific genes. In accordance with these observations the authors show that the GATA1 promoter that binds PIAS1 in lineage negative progenitor cells is incompletely methylated, similarly to what has been found previously with the Foxp3 gene. The authors conclude that PIAS1 represses GATA1 in HSC by maintaining DNA methylation of the GATA1 promoter.

The authors have a clear point in showing that the PIAS KO affects murine hematopoiesis. Defects are found in the lymphoid and in the LSK populations. The data may indicate failure of gene suppression as the major defect in PIAS deficient LSK and, together with a previous analysis (Lin et. al., 2007, Science 330:521), may further suggest that methylation of a subset of lineage specifying genes (here GATA1) depends on PIAS1. The authors imply that PIAS1 mediated DNA methylation is important and related to what has been observed with a DNMT1 mutant. Although the authors provide convincing data on defects in the hematopoietic system, they fall short in demonstrating unequivocally that the mechanism that they imply (defective DNA methylation), is indeed responsible for the biology, as observed (in particular how failure of PIAS1 dependent DNA methylation abrogates quiescence and stem cell exhaustion). The manuscript is mostly descriptive and at this point does not provide a comprehensive insight into how PIAS1 may regulate HSC maintenance.

Referee #2:

The manuscript by Liu et al., characterizes the hematopoietic compartment of E3 ligase PIAS1 knock out mice. The authors have previously shown that PIAS1 can recruit DNMTs to specific promoters to modulate DNA methylation in regulatory T cells. Here they asked whether PIAS1 has any function in the regulation of DNA methylation in hematopoietic stem cells. The authors find a 50% decrease in the frequency of common lymphoid progenitors (CLP) associated with a minor increase in hematopoietic stem cell population in PIAS1-/- bone marrow. Bone marrow B cell

numbers are reduced while the granulocyte./monocyte population is slightly increased. The B cell defects are specific to the bone marrow since there is no change in peripheral blood or spleen B cell content while the granulocyte.macrophage population is increased significantly in all three compartments. The repopulation ability of hematopoietic stem cell (using total bone marrow and Lin-Sca-1+cKit+) population is dramatically compromised. While PIAS1-/- HSC can contribute to both lymphoid and myeloid lineages, there is a bias towards myeloid at the expense of lymphoid population in lethally irradiated transplanted animals. These results are associated with a significant reduction in CLP (common lymphoid progenitors) frequency while the bone marrow MEP, CMP and GMP are not significantly different from wild type control. These anomalies are associated with alteration of gene expression in LSK and CLP specifically, increased expression of GATA-1 in LSK and in CLP is noted. The authors use ChIP to show whether PIAS1 binds GATA-1 promoter. They further examine using bisulfite sequencing the methylation status of GATA-1 promoter and find it to be significantly reduced in PIAS1-/- bone marrow subpopulations of HSC. Based on these results the authors conclude that PIAS1 regulates hematopoietic stem cell self-renewal and differentiation via regulation of methylation dynamics in the HSC differentiation program.

While these observations are individually interesting, the connections between various cellular and molecular observations are weak and the conclusions are not fully supported by the data presented:

1) The assessment of HSC self-renewal is solely based on the phenotypic analysis of HSC which is quite insufficient. Surprisingly experiments examining self-renewal of HSC by serial transplantations were not performed.

2) The quiescence of HSC is only based on the Ki-67 staining of dHSC. Given that these cells are extremely rare, it will be informative to include FACS plots. It will be important to include a full examination of cell cycle of dormant HSC combined with experiments to determine the percentage of quiescent cells.

3) The data on PIAS1 regulation of GATA-1 gene expression is interesting. However the ChIP data is suboptimal. The authors should make use of PIAS1-/- bone marrow cells as control. Also it will be important to include additional antibody and a positive control for PIAS1 activity. It is important to show whether PIAS-1 regulates GATA-1 transcriptional activity. Does loss of PIAS1 impact the expression of GATA-1-dependent genes in CLP?

4) Does the methylation of GATA-1 promoter impact GATA-1 transcriptional activity and the expression of GATA-1-dependent genes in LSK or CLP?

5) The authors show an interaction between endogenous DNMT1 and PIAS1 in thymocytes. Do these proteins interact in the bone marrow? Does DNMT1 antibody immunprecipitate PIAS1? The authors should discuss the potential outcome of PIAS1 interaction with DNMT1 (is PIAS1 required for DNMT1 function or whether PIAS1 increases the activity of DNMT1?). What is the potential function of PIAS1 in the regulation of DNMT1 methylation of GATA-1 promoter? Does overexpression of DNMT1 in CLP or total bone marrow rescue the methylation of GATA-1 promoter? It will be important to include this data in the body of the manuscript and not in the supplements.

6) What is the explanation for increased frequency of lineage negative cells?

7) Finally GATA-1 is a megakaryocyte-erythroid transcription factor. Ectopic expression of GATA-1 in CLP leads to Meg-E lineage differentiation. Why, while loss of PIAS1 results in upregulation of GATA-1 expression it does not affect erythroid/megaK lineage?

Minor points:

1) ChIP protocol should be discussed in more details as performing ChIP in LSK is not trivial.

2) The data discussed in page 5 should be presented in a more logical manner. For instance bone marrow cellularity should be presented first. The increase in Lin- cells should be included in the figures of the manuscript and not in the supplements.

3) Results should be shown as the mean of multiple experiments and not only a representative experiment.

4) What is the phenotype of PIAS1-/- mice?

Referee #3:

Liu and colleagues present new findings on the functional relevance of an epigenetic regulator, the SUMO E3 ligase, PIAS1, which they show is required for the sustained self-renewal capacity of HSCs. Absence of PIAS1 caused enhanced cell cycle entry of dormant HSCs and elevated myeloerythroid, but reduced B-lymphoid differentiation. The authors suggest a molecular mechanism through which sets of lineage-specific genes are aberrantly expressed in HSCs and CLPs in the absence of PIAS1. For one PIAS1 target gene, GATA1, Liu et al. show premature transcriptional activation through DNA demethylation at its promoter.

Several previous studies have shown that epigenetic regulation of transcriptional networks is essential for normal HSC function and hematopoietic differentiation. Furthermore, aberrations in the epigenetic regulation of hematopoietic stem and progenitor cells have been shown to play a role in the generation and maintenance of leukemia. It is of high relevance to identify specific effectors and their targets relevant for differentiation and stage-specific epigenetic regulation of hematopoiesis.

The same group reported a central role for PIAS1 in the restriction of natural regulatory T-cell differentiation a few years ago. The findings presented in their current study extend this initial study to the hematopoietic stem cell compartment and are novel and very interesting in terms of transcriptional cell fate regulation at the epigenetic level in HSCs. However, there are some gaps and important additional questions, in particular with regards to the mechanistic characterization of PIAS1 in HSCs, which need to be addressed to complete this work:

Specific major concerns which should be addressed to support the main conclusions:

1) The authors show that the number of total HSCs is increased while the number of total d-HSCs is unchanged in absence of PIAS1. This would mean a relative decrease of d-HSC in the PIAS1 KO mice. In order to quantify the number of long-term repopulating cells in absence of PIAS1 the authors should perform competitive limiting dilution assays of LT-HSCs (rather than using competitive transplantation assays -- with an equal mix of WT and KO total BM or LSK cells), to definitively determine the frequency of functional stem cells. This is of particular importance given that the entire Lin- compartment of PIAS KO mice is expanded.
2) The authors should use Pyronin Y/Hoechst in addition to Ki67 staining, as Ki67 alone is not a reliable marker for quiescence in HSCs.
3) BrdU or EdU incorporation assays on purified LT-HSCs or d-HSCs (pulse-chase) should ideally be performed in order to determine their alterations in proliferation (Fig. 1).
4) Homing and retention assays should be performed with LT-HSCs as it is not clear what type of stem and/or progenitor cell remains in the BM. This could be different for WT and KO cells and has to be clarified. Longer observation periods than 6 hrs would also be helpful (e.g. 12, 24, or 48 hrs).
5) The increase in myeloid cells in primary PIAS1 KO mice and in the BM transplantation assays could also resemble an increased production of cells at the progenitor level. It would be interesting to investigate whether L-S-K+ cells from PIAS1 KO mice give rise to more progeny than their WT counterparts in short-term reconstitution assays.
6) Fig. 6: The authors need to show gene expression profiles of LT-HSC as the difference in gene expression measured in the LSK compartment could result from differences in the multipotent progenitor compartment.
7) What confers the demethylation? The authors need to test whether PIAS1, as they have shown in T-cells, also interacts with DNMT1, 3a, 3b and/or HP1gamma. Primary HSCs need to be examined for this, and not T-cells (Fig. S3). This is a very important point as PIAS1-mediated regulation may be lineage specific.
8) The authors need to formally demonstrate functional relevance of GATA1 overexpression in

PIAS1 KO HSCs to complete this work. Does restoration/reduction of GATA1 expression in PIAS1 KO cells reduce/normalize the observed increased myeloid commitment?

- Minor concerns that should be addressed:
1) Title: typo/spelling "hematopoietic stem cells".
2) The L-S-K+ population is significantly increased in the PIAS1-/- mice (1.1% of total BM cells). Why do the subpopulations of the L-S-K+ progenitor population do not add up to 1.1% total BM cells (Fig. 1B, sum is 1.6% of total BM cells in KO mice)? Please clarify.
3) Absolute numbers of hematopoietic stem and progenitor cells should be shown for BM and SP (Fig. 1); move the relative distributions to the supplement.
5) What causes the decrease of CLPs in the absence of PIAS1? Are Gata3 and/or IL7r promoter DNA methylation affected by absence of PIAS1 ? And does normalization of expression of either gene rescue the number of CLPs/pre/pro B-cells?

Additional Author Correspondence 29 October 2012

Thank you for sending us the positive reviews and the opportunity to revise our manuscript.

We have taken time to carefully go through the reviewers' comments and your requests, and we truly appreciate your effort in facilitating efficient proceedings for our studies. We have a few questions on the major issues outlined in your email that we would like to discuss with you and ask for your advice.

1. "- to determine the frequency of functional stem cells by competitive limiting dilution assays on LT-HSCs (rather than 'sole' serial transplantations)".

This question was raised by reviewer #3 because he/she thought that there was a "relative decrease of d-HSC in the PIAS1 KO mice" in our transplantation assays since "the entire Lincompartment of PIAS1 KO mice is expanded". We regret to cause this misunderstanding and we would like to clarify it. We performed two types of transplantations assays: one is to use the same amounts of total bone marrow cells, and the other is to use the same amounts of total LSK cells. Both assays gave the similar results. As we have shown, the percentage of d-HSC in PIAS1 KO mice bone marrow is the same as that in the wild type mice. Thus, under the conditions when the same amount of bone marrow cells were used for transplantation assays, the numbers of d-HSC used for both PIAS1 KO and wild type mice were the same, thus the issue of relative difference in d-HSC did not exist in these transplantation assays. One important point that we would like to emphasize is that the difference of repopulating capacity between PIAS1 KO and wild type mice revealed by our studies (1:1 ratio of wild type and PIAS1 KO bone marrow used) was very dramatic, at least more than 10 fold. As a result, even under the conditions when equal amounts of LSK cells were used in transplantation assays, the small difference (about 50%) in LSK populations between PIAS1 KO and wild type mice did not have a significant impact on the final results due to the strong and overwhelming difference in the self-renewal potential.

This reviewer is correct that competitive limiting dilution assay is a classic way to determine QUANTITATIVE difference in the frequency of functional stem cells, which is essential when there is a difference in d-HSC cell number or when the difference in repopulating capacity is not clearly evident in qualitative assays. But as described above, this is not the case for our studies. In fact, when the difference in repopulating capacity is very clear, the data from each dilution point in the limiting dilution assays would reveal the similar difference. For example, in the studies of Gfi-1 in HSC reported by the Orkin's group (Hock et al., 2004, Nature, 431:1002), the results from each dilution point in the limiting diluting assays revealed similar qualitative difference in repopulating capacity (Fig. 2 of Hock's paper). Thus, performing limiting dilution assays for our model system would not affect our conclusion that PIAS1 KO HSC is significantly defective in self-renewal. Also, from the animal welfare point of view, three independent repeats of the limiting diluting assays require the use of over 100 recipient mice, and the entire experiment will take about 6 months to complete. Thus, if such experiments would not change the conclusion of the studies, we wonder if they are necessary. Probably for these same reasons, quantitative limiting dilution assays were not performed in many HSC studies reported in the literature.

I hope you understand our intension to clarify this issue, and we would appreciate that you evaluate our points and give us your advice. If you still believe that we should perform the limiting dilution assays, we will certainly follow your instruction to have the experiments completed. In such a case, the timeline to finish these experiments would be about 6 months.

2. "- parallel your mechanistic work in T-cells, to assess PIAS1 binding to DNMT-family members in bone marrow AND functional implications studied"

Yes, we will perform these experiments requested by the reviewers. We should be able to complete these studies within the standard 3 month revision period.

3. "-GATA1's functional relevance formerly be established"

4. "-PIAS1 loss/GATA1 up regulation in light of distinct lineage effects to be reconciled."

We would like to address these two points together since they are related. From these points raised by the reviewers, we believe that we had given the reviewers the unintended impression that the biological effect of PIAS1 knockout is mediated through GATA1 up-regulation. This is certainly not what we wished to conclude in this manuscript. The data described in our paper suggest that the PIAS1 epigenetic mechanism regulates HSC self-renewal by silencing a group of genes. GATA1 is one of many genes that are regulated by PIAS1. For example, we showed in the CLP population, other genes that play important roles in lineage determination such as Gata2, Csf1r, Mpo, and Cebpa are all up-regulated in PIAS1 KO CLP cells. It has been shown that there exists crosstalk among these lineage-associated genes. For example, the opposing effect between Gata1 and Gata2 has been reported. Also, both Gata1 and Cebpa are known to be able to suppress Ebf1, a crucial transcription factor for early B-cell development. Thus, it is the overall epigenetic program, rather than a single GATA1 gene, is responsible for the observed biological phenotype of PIAS1 KO mice. In fact, what makes this manuscript most interesting is not the discovery of the importance of a single lineage-associated transcription factor in HSC regulation, since many examples like this have already been reported in the literature. The novelty of this paper is that it reports the first known DNA methylation regulatory pathway in the epigenetic regulation of HSC. DNA methyltransferases have been shown to play important roles in HSC biology, and PIAS1 is the first known regulator of DNMTs involved in the DNA methylation of the HSC system. A related note that I would like to point out is the reported similar observation of the up-regulation of GATA1 and Cebpa in DNMT1 knockout mice. There the authors also did not assign the biological effect of DNMT1 deficiency specifically to the single effect of GATA1 upregulation (Broske et al., 2009, Nature Genetics, 41:1207; Trowbridge et al., 2009, Cell Stem Cell, 5:442).

Since PIAS1 regulates its target genes through epigenetic modifications, the expression of the PIAS1-target genes in a given cell lineage is also dependent on the availability of transcription factors that activate these target genes. The removal of the PIAS1-mediated epigenetic suppression is necessary, but not sufficient, to promote the transcriptional induction of these target genes. This property explains the cell type specific expression of PIAS1 target genes. For example, GATA1 was up-regulated in PIAS1 KO CLP, but not in the megakaryocyte erythroid progenitor (MEP) lineage (we will include this data in our revision). This unique feature of the PIAS1 epigenetic mechanism, together with the above mentioned involvement of multiple lingease-associated transcription factors contribute to the observed lineage specific effects of PIAS1 KO mice. We plan to address these issues by modifying our manuscript and making these points clear in order to avoid misunderstanding.

In addition, we will include a detailed point-by-point response to the reviewers' other points in the revision.

Finally, I would like to point out that the PIAS1 epigenetic pathway is likely involved in the regulation of other types of stem cells. We hope that this manuscript will be the first in reporting the importance of this newly identified epigenetic pathway in stem cell biology. Because of the highly competitive nature of this field, we wish to outline an efficient plan to address the key points in the revision in a timely manner. Please advise us of your thoughts about our proposal and let us know how we should proceed.

Thank you again for your time and effort on our behalf.

Additional Editorial Correspondence 06 November 2012

I appreciate your efforts to establish upfront what would be needed to ensure efficient proceedings of a revised version at The EMBO Journal.

It crystallizes however, that you seem reluctant to perform a characterization of the PIAS1-dependent HSC-phenotype involving serial transplantation assays. Though rather perceptive to your earlier arguments, I am not in a position to wave this completely as in a subsequent communication ref#2 remarked the following:

'A major concern is that the title claims "PIAS1 regulates self renewal of hematopoietic stem cells (HSC)". However they have not performed serial transplantation assay which is the gold standard for determining HSC self-renewal.'

Together with the still relatively vague proposal how to overcome the crucial mechanistic demands from ref#1, I do foresee major hesitations based on the amendments you currently suggest.

Please do understand that the referee comments are not as encouraging as the might seem at first glance as one explicitly recommended rejection and two others demand significant work. The offer to thoroughly revise is based on the notion that you would have all the tools and expertise to address existing major criticisms if expending the necessary time and efforts.

Though I am willing to present a suitably revised paper once more to some of the original referees, I am currently not convinced that what is on the table might suffice to win their crucial support.

I am sorry that I cannot be more encouraging at this stage, but I hope that expressing this clearly at this stage might govern further demanded experimentation, respective alternative proceedings of your study.

Please let me know how you plan to proceed.

Additional Author Correspondence 08 November 2012

I appreciate your prompt response and your effort in helping us setting a clear plan to revise this manuscript.

The key issue appears to be on HSC self-renewal assays. There are two types of assays mentioned by the reviewers: "serial transplantation assay" by reviewer #2 and "limiting dilution assay" by reviewer #3. Both assays are regarded as "Gold standard" in the HSC field. The "serial transplantation assay" involves multiple rounds of transplantation and would take a very long time to complete (especially for d-HSC analysis); thus, for practical reasons many studies use "limiting dilution assay" as an alternative approach in characterizing HSC self-renewal capacity (Gan et al., 2010, Nature, 468:703; Liu et al., 2009, Cell Stem Cell, 4:37; Zeng et al., 2004, EMBO, 23:4116; Hock et al., 2004, Nature, 431:1002). We performed competitive assays at 1:1 ratio (one dilution condition in a limiting dilution assay) and showed a clear self-renewal defect of PIAS1 knockout HSC. We believe that additional "serial transplantation assay" or "limiting dilution assay" would provide more quantitative assessment of the defect of Pias1-/- HSC in self-renewal, although such experiments would not change the key conclusion of our manuscript. However, if you and the reviewer believe that we need to perform any of such additional assays, we will certainly follow your advice. For "serial transplantation assay", we expect to carry out 3-4 rounds of transplantation in order to assay the d-HSC self-renewal potential, thus this assay would take about 1.5 to 2 years (each round takes about 16 to 20 weeks for long term HSCs). For "limiting dilution assays", it would

take about 6 to 8 months.

We hope you understand our intention is to revise this manuscript in a timely manner, and we believe that the PIAS1 epigenetic pathway plays a key role in regulating the self-renewal of multiple types of stem cells based on our preliminary studies in other biological systems. This paper is the first report to describe the unexpected involvement of PIAS1 epigenetic pathway in stem cell biology. Since these two types of assays involve extensive commitment of time and animals; thus, we would like to get a clear instruction from you regarding how we should proceed with these two types of assays. We are committed to make our best effort to satisfy your requirements.

Thank you again for your effort.

Additional Editorial Correspondence 22 November 2012

I do apologize for the slightly delayed response caused by work-related travel to the US.

I did in the meantime receive an explicit response from one of the original referees on the of serial transplantation issue.

As you will see below, this scientist rates these time-consuming tasks as necessary to substantiate the proposed function. As s/he will be involved in any future assessment of your paper at The EMBO Journal, I see no other option than to insist on these before your study can be reassessed for potential publication here.

In case you decide to embark on this, please let me know to your earliest convenience, as I will have to mark this down in our database to avoid unnecessary automatic chasers for a revised version.

I would also appreciate a short note, in case you decide to take the paper at this stage for more rapid publication to a possibly less demanding title.

I am sorry that I am unable to deliver more encouraging news, though I am sure you will agree that at the end of the day this will elevate the relevance and importance of the proposed PIAS1 biological function.

Please do not hesitate to contact me in case I can be of further assistance.

Referee:

The authors claim that they have a hematopoietic stem cell (HSC) self-renewal phenotype. The only accepted (and gold standard) measurement for self renewal of HSC is by serial transplantation (as in http://www.ncbi.nlm.nih.gov/pubmed/7622039) and multilineage repopulation. Although the experiment takes about 8-10 months (two times 16 weeks at least), it is necessary to be performed in order to establish the self-renewal phenotype. Limiting dilution combined with Poisson distribution only measures the frequency of HSC (I am happy to refer to further to more reviews by leaders in this field, if necessary). Thus, until a self-renewal phenotype is established by serial transplantation this claim should, at least in my view, neither be kept in the title nor anywhere in the paper.

Additional Author Correspondence 27 November 2012

Thank you for your advice. We will perform the suggested serial transplantation experiments, and will include the data in our revision. We will try our best to send you the revised manuscript as soon as we can.

1st Revision - authors' response 29 September 2013

I would like to thank you for the opportunity to revise our manuscript. Please allow me to summarize the key improvement to address the reviewers' major concerns, followed by our point-by-point responses to the reviewers' comments.

Major issues:

1. "To determine the frequency of functional stem cells by competitive limiting dilution assays on LT-HSCs"

We have performed competitive limiting dilution assays as suggested by the reviewers, and we found that *Pias1*$^{-/-}$ BM cells showed a 5-fold decrease in the frequency of functional HSCs (Table 1), which is consistent with our previous data from competitive reconstitution assays (Figure 3).

2. "To assess PIAS1 binding to DNMT-family members in bone marrow AND functional implications studied"

We have performed co-immunoprecipitation (Co-IP) and chromatin immunoprecipitation (ChIP) assays using bone marrow cells. We found that PIAS1 can interact with DNMT3A *in vivo*, and PIAS1 is required for the recruitment of DNMT3A to the *Gata1* promoter in BM (Figure 7).

3. "GATA1's functional relevance"

We have performed quantitative real time PCR (Q-PCR) assays and showed that the GATA1-downstream genes were also up-regulated in *Pias1*$^{-/-}$ CLP cells (Figure 6D), consistent with the increased *Gata1* transcription observed in *Pias1*$^{-/-}$ cells.

The data described in our paper suggest that the PIAS1 epigenetic mechanism regulates HSC self-renewal by silencing a group of genes, and GATA1 is one of such genes that are regulated by PIAS1. For example, we showed in the CLP population, other genes that play important roles in lineage determination such as *Gata2*, *Csf1r*, *Mpo*, and *Cebpa* are all up-regulated in *Pias1*-null CLP cells. It is known that there exists crosstalk among these lineage-associated genes. For example, both GATA1 and CEBPA are known to be able to suppress *Ebf1*, a crucial transcription factor for early B-cell development. Thus, we believe that it is the overall PIAS1-mediated gene expression program, rather than the single GATA1 gene, is responsible for the observed biological phenotype of PIAS1 KO mice.

4. "PIAS1 loss/GATA1 up regulation in light of distinct lineage effects to be reconciled"

As pointed out by one of the reviewers, "GATA-1 is a megakaryocyte-erythroid transcription factor. Ectopic expression of GATA-1 in CLP leads to Meg-E lineage differentiation." We have shown that *Gata1* transcription is upregulated in *Pias1*$^{-/-}$ BM subsets (Figure 6). After simultaneous analyses of various subpopulations of WT and *Pias1*$^{-/-}$ BM using the same sets of samples acquired on the same flow cytometer (LSR) bearing up to 7 colours, we now showed that the megakaryocyte erythrocyte progenitor (MEP) population was increased in *Pias1*$^{-/-}$ BM (Figure 1C), consistent with the up-regulation of *Gata1* transcription in *Pias1*$^{-/-}$ cells.

Point-by-point responses:

Referee #1:

As outlined above, we have provided additional experimental data to address the major concern of

this reviewer on the mechanistic aspect of the PIAS1-mediated epigenetic regulation of HSCs.

Referee #2:

Major points:

1) The assessment of HSC self-renewal is solely based on the phenotypic analysis of HSC which is quite insufficient. Surprisingly experiments examining self-renewal of HSC by serial transplantations were not performed.

Done as suggested. We have performed competitive limiting dilution assays as suggested by the reviewers, and we found that *Pias1*$^{-/-}$ BM cells showed a 5-fold decrease in the frequency of functional HSCs (Table 1), which is consistent with our previous data from competitive reconstitution assays (Figure 3).

2) The quiescence of HSC is only based on the Ki-67 staining of dHSC. Given that these cells are extremely rare, it will be informative to include FACS plots. It will be important to include a full examination of cell cycle of dormant HSC combined with experiments to determine the percentage of quiescent cells.

Done as suggested. We have performed intracellular Ki67 and Hoechst DNA staining experiments to thoroughly examine the cell cycle profiles of various subpopulations in WT and *Pias1*$^{-/-}$ BM. Our data showed that the G0 population was decreased with a concurrent increase in cells in G1 phase in HSC-enriched *Pias1*$^{-/-}$ subsets, including dormant HSCs, LT-HSC and LSK; but not differentiated progenitors (Figure 2 and S3). These data support the role of PIAS1 in the maintenance of HSC dormancy. We also included representative FACS plots of d-HSCs as requested.

3) The data on PIAS1 regulation of GATA-1 gene expression is interesting. However the ChIP data is suboptimal. The authors should make use of PIAS1-/- bone marrow cells as control. Also it will be important to include additional antibody and a positive control for PIAS1 activity. It is important to show whether PIAS-1 regulates GATA-1 transcriptional activity. Does loss of PIAS1 impact the expression of GATA-1-dependent genes in CLP?

We have performed chromatin immunoprecipitation (ChIP) assays using WT and *Pias1*$^{-/-}$ bone marrow cells, as well as 2 independent PIAS1 antibodies and showed that PIAS1 binds to the *Gata1* promoter (Figure 7A). We have also performed quantitative real time PCR (Q-PCR) assays and showed that GATA1-downstream genes were upregulated in *Pias1*$^{-/-}$ CLP cells (Figure 6D), consistent with the increased *Gata1* transcription observed in *Pias1*$^{-/-}$ cells.

4) Does the methylation of GATA-1 promoter impact GATA-1 transcriptional activity and the expression of GATA-1-dependent genes in LSK or CLP?

We believe that the promoter demethylation of *Gata1* allows *Gata1* gene expression, which has also been observed in *Dnmt1* knockout mice (Broske et al., 2009). Consistently, we observed the up-regulation of GATA1-dependent genes in CLP cells (Figure 6D).

5) The authors show an interaction between endogenous DNMT1 and PIAS1 in thymocytes. Do these proteins interact in the bone marrow? Does DNMT1 antibody immunprecipitate PIAS1? The authors should discuss the potential outcome of PIAS1 interaction with DNMT1 (is PIAS1 required for DNMT1 function or whether PIAS1 increases the activity of DNMT1?). What is the potential

function of PIAS1 in the regulation of DNMT1 methylation of GATA-1 promoter? Does overexpression of DNMT1 in CLP or total bone marrow rescue the methylation of GATA-1 promoter? It will be important to include this data in the body of the manuscript and not in the supplements.

As suggested by this reviewer, we have performed co-immunoprecipitation (Co-IP) assays using bone marrow cells. We found that PIAS1 interacted with DNMT3A in BM cells. We also performed chromatin immunoprecipitation (ChIP) assays and demonstrated that PIAS1 is required for DNMT3A binding to the *Gata1* promoter in BM. As requested by this reviewer, these results are included in the body of the manuscript (Figure 7).

6) What is the explanation for increased frequency of lineage negative cells?

We observed minor increase in the frequency of Lineage negative (Lin^-) cells in *Pias1*$^{-/-}$ BM (Figure S2B), while the cell number (Figure 1B) and the cell cycle profiles (Figure S3C) of *Pias1*$^{-/-}$ Lin^- cells were not altered. It is possible that in *Pias1*-null mice, more d-HSCs exit dormancy and enter into the differentiation pool, which may cause the minor increase in the frequency of Lin^- cells.

7) Finally GATA-1 is a megakaryocyte-erythroid transcription factor. Ectopic expression of GATA-1 in CLP leads to Meg-E lineage differentiation. Why, while loss of PIAS1 results in upregulation of GATA-1 expression it does not affect erythroid/megaK lineage?

We have shown that *Gata1* transcription is upregulated in *Pias1*$^{-/-}$ BM subsets (Figure 6). After simultaneous analyses of various subpopulations of WT and *Pias1*$^{-/-}$ BM using the same sets of samples acquired on the same flow cytometer (LSR) bearing up to 7 colours, we now showed that the megakaryocyte erythrocyte progenitor (MEP) population is increased in *Pias1*$^{-/-}$ BM (Figure 1C), consistent with the upregulation of *Gata1* transcription in *Pias1*$^{-/-}$ cells.

Minor points:

1) ChIP protocol should be discussed in more details as performing ChIP in LSK is not trivial.

Done as suggested. We have re-written the ChIP protocol in more details.

2) The data discussed in page 5 should be presented in a more logical manner. For instance bone marrow cellularity should be presented first. The increase in Lin- cells should be included in the figures of the manuscript and not in the supplements.

Done as suggested. We have re-written this part of the Results section.

3) Results should be shown as the mean of multiple experiments and not only a representative experiment.

Done as suggested. We have revised the data presentation and pooled multiple experiments to show the results as the mean of multiple experiments.

4) What is the phenotype of PIAS1-/- mice?

Pias1 knockout mice display various defects in the gene expression program in the immune system.

We have published our work on the phenotypes of *Pias1*$^{-/-}$ mice in several papers (Liu et al., Nature Immunology 2004; Liu et al., MCB 2005; Liu et al., Cell 2007; Liu et al., Science 2010).

Referee #3:

Major points:

1) The authors show that the number of total HSCs is increased while the number of total d-HSCs is unchanged in absence of PIAS1. This would mean a relative decrease of d-HSC in the PIAS1 KO mice. In order to quantify the number of long-term repopulating cells in absence of PIAS1 the authors should perform competitive limiting dilution assays of LT-HSCs (rather than using competitive transplantation assays -- with an equal mix of WT and KO total BM or LSK cells), to definitively determine the frequency of functional stem cells. This is of particular importance given that the entire Lin- compartment of PIAS KO mice is expanded.

Done as suggested. We have performed competitive limiting dilution assays as suggested by the reviewers, and we found that *Pias1*$^{-/-}$ BM cells showed a 5-fold decrease in the frequency of functional HSCs (Table 1), which is consistent with our previous data from competitive reconstitution assays (Figure 3).

2) The authors should use Pyronin Y/Hoechst in addition to Ki67 staining, as Ki67 alone is not a reliable marker for quiescence in HSCs.

Done as suggested. We have performed intracellular Ki67 and Hoechst DNA staining experiments to thoroughly examine the cell cycle profiles of various subpopulations in WT and *Pias1*$^{-/-}$ BM. Our data showed that the G0 population was decreased with a concurrent increase in cells in G1 phase in HSC-enriched *Pias1*$^{-/-}$ subsets, including dormant HSCs, LT-HSC and LSK; but not differentiated progenitors (Figure 2 and S3). These data support the role of PIAS1 in the maintenance of HSC dormancy. We also included representative FACS plots of d-HSCs as requested.

3) BrdU or EdU incorporation assays on purified LT-HSCs or d-HSCs (pulse-chase) should ideally be performed in order to determine their alterations in proliferation (Fig. 1).

We have deleted the BrdU incorporation assays of LSK (original Figure 2C) in this revised manuscript, which mainly measure DNA synthesis (S phase) during the cell cycle. By using Ki67 and Hoechst DNA staining, we showed that the effect of PIAS1 appears to restrict dormant HSCs from exiting G0 and entering G1 phase of the cell cycle, while the S+G2/M phase is largely unchanged.

4) Homing and retention assays should be performed with LT-HSCs as it is not clear what type of stem and/or progenitor cell remains in the BM. This could be different for WT and KO cells and has to be clarified. Longer observation periods than 6 hrs would also be helpful (e.g. 12, 24, or 48 hrs).

Done as suggested. We have performed homing assays with FACS-sorted LT-HSCs (Figure 4C). We also performed the experiments at longer time points (12, 24 h) as suggested (Figure 4A-C). These results showed that there is no defect in *Pias1*$^{-/-}$ LT-HSCs to home to BM.

5) The increase in myeloid cells in primary PIAS1 KO mice and in the BM transplantation assays could also resemble an increased production of cells at the progenitor level. It would be interesting to investigate whether L-S-K+ cells from PIAS1 KO mice give rise to more progeny than their WT

counterparts in short-term reconstitution assays.

Done as suggested. We have performed short-term competitive reconstitution assays with FACS-sorted $L^-S^-K^+$ cells from WT or *Pias1*$^{-/-}$ littermates to address whether PIAS1 affects the differentiation of $L^-S^-K^+$ cells into their progeny *in vivo* (Figure S4). Surprisingly, *Pias1*$^{-/-}$ $L^-S^-K^+$ cells showed defects in differentiating into $Mac1^+$ cells as compared to WT controls, indicating that PIAS1 is important for the proper differentiation of $L^-S^-K^+$ cells into their progeny. These data also suggest that the increase in myeloid cells in *Pias1*$^{-/-}$ BM-reconstituted mice in the transplantation assays is not likely due to the enhanced differentiation from $L^-S^-K^+$ progenitors; rather the increase in myeloid cells may result from the increased pool of $L^-S^-K^+$ cells generated from upstream progenitors/stem cells, as well as the disrupted balance between myeloid and lymphocyte differentiation.

6) Fig. 6: The authors need to show gene expression profiles of LT-HSC as the difference in gene expression measured in the LSK compartment could result from differences in the multipotent progenitor compartment.

Done as suggested. We have performed Q-PCR assays with LT-HSC and replaced LSK data (Figure 6C).

7) What confers the demethylation? The authors need to test whether PIAS1, as they have shown in T-cells, also interacts with DNMT1, 3a, 3b and/or HP1gamma. Primary HSCs need to be examined for this, and not T-cells (Fig. S3). This is a very important point as PIAS1-mediated regulation may be lineage specific.

We have performed co-immunoprecipitation (Co-IP) and chromatin immunoprecipitation (ChIP) assays using bone marrow cells, and found that PIAS1 can interact with DNMT3A and PIAS1 is required for DNMT3A binding to the Gata1 promoter in BM (Figure 7).

8) The authors need to formally demonstrate functional relevance of GATA1 overexpression in PIAS1 KO HSCs to complete this work. Does restoration/reduction of GATA1 expression in PIAS1 KO cells reduce/normalize the observed increased myeloid commitment?

We have performed quantitative real time PCR (Q-PCR) assays and showed that the GATA1-downstream genes were also up-regulated in *Pias1*$^{-/-}$ CLP cells (Figure 6D), consistent with the increased Gata1 transcription observed in *Pias1*$^{-/-}$ cells.

The data described in our paper suggest that the PIAS1 epigenetic mechanism regulates HSC self-renewal by silencing a group of genes, and GATA1 is one of such genes that are regulated by PIAS1. For example, we showed in the CLP population, other genes that play important roles in lineage determination such as *Gata2*, *Csf1r*, *Mpo*, and *Cebpa* are all up-regulated in PIAS1-null CLP cells. It is known that there exists crosstalk among these lineage-associated genes. For example, both GATA1 and CEBPA are known to be able to suppress *Ebf1*, a crucial transcription factor for early B-cell development. Thus, we believe that it is the overall PIAS1-mediated gene expression program, rather than the single GATA1 gene, is responsible for the observed biological phenotype of PIAS1 KO mice.

Minor points:

1) Title: typo/spelling "hematopoietic stem cells".

Corrected as suggested.

2) The L-S-K+ population is significantly increased in the PIAS1-/- mice (1.1% of total BM cells). Why do the subpopulations of the L-S-K+ progenitor population do not add up to 1.1% total BM cells (Fig. 1B, sum is 1.6% of total BM cells in KO mice)? Please clarify.

The data in the original manuscript were from different sets of flow cytometry analyses. Now we have re-done all the relevant flow cytometry experiments with simultaneous analyses of various subpopulations of WT and *Pias1*$^{-/-}$ BM using the same sets of samples acquired on the same flow cytometer (LSR) bearing up to 7 colours, and we plotted the data in the same figure (Figure 1C and S2C).

3) Absolute numbers of hematopoietic stem and progenitor cells should be shown for BM and SP (Fig. 1); move the relative distributions to the supplement.

We have revised the data as suggested (Figure 1, S1 and S2).

5) What causes the decrease of CLPs in the absence of PIAS1? Are Gata3 and/or IL7r promoter DNA methylation affected by absence of PIAS1? And does normalization of expression of either gene rescue the number of CLPs/pre/pro B-cells?

Our data suggest that the increased cell death may account for the decrease of CLPs in the absence of PIAS1 (Figure 2A). It is likely that PIAS1 may regulate the promoter methylation of genes other than *Gata1*. Further studies are needed to understand how PIAS1 affects the survival of CLPs.

In summary, we believe that we have addressed all the concerns of the reviewers. Although it took almost one year to finish the experiments suggested by the reviewers (largely due to the competitive limiting dilution assays that are time-consuming in nature), we are pleased to say that the inclusion of these new experimental data has significantly improved the overall quality of this manuscript. We thank the reviewers for their very helpful comments and suggestions.

We hope to hear from you positively about this revised manuscript soon.

2nd Editorial Decision 15 October 2013

Thank you very much for your extensive revisions that have been very favorably re-reviewed by some of the original referees.

Please find enclosed a few constructive remarks that I kindly ask you to consider. I would encourage you to elaborate/specify on how the ChIP-data in LSK cells were acquired.

To not delay publication of the study any further, I would only ask for possible integration of potential rescue results (ref#2), if you were to have relevant experimental results already at hand!

Please use the enclosed link to provide us with any amended files to your earliest convenience.

I take the liberty to congratulate already at this point to such an insightful study. Be assured that the editorial office will soon be in touch soon AFTER we heard back from you on possible further amendments to formally accept an ultimate version for publication in The EMBO Journal!

REFEREE REPORTS:

Referee #1:

The manuscript by Liu et al., is highly improved and very interesting. I have few minor comments.

In some instances the authors do not fully display their finding. For instance the sentence "In addition, reduced Pre-B populations were observed in Pias1 / BM" on page 5, although true was not followed by a figure number.

Page 6: it seems that cell cycle defects are specific to LSK and LT-HSC but not seen in MPP. If that is the case they should specify.

Page 10: "Interestingly, enhanced engraftment of WT C57SJL cells into Pias1 / mice was observed at 4 weeks, while no difference was observed at later time points (Figure 5A)." It seems they have an increased engraftment after 18 weeks as well. If that is the case they should correct.

Page 11: "The erythroid factor GATA1 can directly repress the transcription of Ebf1" GATA-1 is not only erythroid, although key to erythropoiesis.

Would be good to clarify Slc4a1 is (Band 3) an erythroid specific protein.

It is remarkable that the authors were able to perform ChIP with LSK cells (Figure 7B). It is critical to precisely indicate how many cells from how many animals they used for this assay. If they have optimized their assay for LSK cells, it is important to share their protocol.

Referee #2:

Overall, this revised manuscript has significantly improved and establishes PIAS1 as a novel regulator of HSC dormancy and repressor of an EMK expression program. Liu et al. have extensively revised their manuscript and include several new data now showing convincingly that PIAS-deficient stem cells have an impaired reconstitution capability. It would have been very interesting to further determine whether the suppression of the EMK program in HSC is functionally linked with their retention in dormancy.

Mechanistically the authors added data depicting that PIAS-/- stem cells are less quiescent, although they chose not to conduct BrdU/EdU pulse-chase experiments, which would not only have measured DNA synthesis, but most importantly allowed for the quantification of cell cycling over time which would have provided ultimate proof of reduced dormancy of PIAS-/- stem cells. However, the observed phenotype and the results from the Ki67/Hst. snap shot cell cycle analyses are highly consistent with reduced dormancy in these HSC. The authors further clarified the molecular mechanism by which PIAS1 regulates gene expression relevant for HSC function regulation. They now show data demonstrating that PIAS1 interacts with DNMT3A and that this interaction is required to recruit the DNA methyltransferase to the Gata1 locus. This is a very valuable addition to the manuscript, which could have been even further extended towards formal rescue experiments. While it is possible/likely that PIAS1 also regulates the expression of other key regulators, one would expect at least a partial rescue of PIAS1-/- mediated HSC defect given the central role of GATA1 in early hematopoiesis. The descriptive data added to Figs. 1C and 6 are in line with the known function of GATA1 in E/Mk lineage commitment and highly suggest a critical role for GATA1 overexpression in PIAS1-/- HSC.

2nd Revision - authors' response 15 October 2013

Referee #1:

1. - In some instances the authors do not fully display their finding. For instance the sentence "In addition, reduced Pre-B populations were observed in Pias1−/−BM" on page 5, although true was not followed by a figure number.

Corrected as suggested.

2. - Page 6: it seems that cell cycle defects are specific to LSK and LT-HSC but not seen in MPP. If that is the case they should specify.

Specified as suggested.

3. - Page 10: "Interestingly, enhanced engraftment of WT C57SJL cells into Pias1−/− mice was observed at 4 weeks, while no difference was observed at later time points (Figure 5A)." It seems they have an increased engraftment after 18 weeks as well. If that is the case they should correct.

Corrected as suggested.

4. - Page 11: "The erythroid factor GATA1 can directly repress the transcription of Ebf1" GATA-1 is not only erythroid, although key to erythropoiesis.

Corrected as suggested.

5. - Would be good to clarify Slc4a1 is (Band 3) an erythroid specific protein.

Clarified as suggested.

6. - It is remarkable that the authors were able to perform ChIP with LSK cells (Figure 7B). It is critical to precisely indicate how many cells from how many animals they used for this assay. If they have optimized their assay for LSK cells, it is important to share their protocol.

Done as suggested. We have provided details about ChIP with LSK cells under "Material and Methods" section (page 20).

Referee #2:

We thank this reviewer for his/her encouragement and helpful suggestions. We agree with this reviewer that the reduction of GATA1 in Pias1 null mice may partially rescue some defects, which is an interesting hypothesis that can be tested in the future.

We wish to thank the Editor and the reviewers for their encouragement and insightful suggestions during the revision process of this manuscript.