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Epigenetic *Thpok* silencing limits the time window to choose CD4$^+$ helper-lineage fate in the thymus

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(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.)

1st Editorial Decision 06 December 2012

Thank you for submitting your manuscript to the EMBO journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees find the analysis interesting. Referee #1 brings up a few minor issues, while referees #2 and 3 raise more significant ones. Referees #2 and 3 find that further data is needed to support the conclusions drawn and that some of the findings need to be extended. Should you be able to able to address the raised concerns in full then we would consider a revised version. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Referee reports:

Referee #1 (Remarks to the Author):

This is a solid from a group that has contributed significantly to this area of the role of ThPOK in lineage commitment. The study solidly demonstrates that the silencer region in ThPOK is required only during the "window" of determination and not required after silencing has occurred, a finding that relies heavily on the conditional deletion of the element after commitment to silencing has occurred. The techniques used are all well controlled and are based on well established in vivo approaches, and are actually quite sophisticated.

There are only a couple of minor issues that I would raise. The authors recognize the caveat that the epigenetic mark (H3K27me3) does not appear to be deposited in the region around promoter 2 (Fig. 1A), which is still silenced. Secondly, there is a bit of leakiness, in that promoter 1 does turn on detectibly after removal of the silencing region (Fig. 3C,D). However, these are not major problems and the authors do discuss the issues. While the authors do confirm the presence of marks seen in ChIP/chip by using conventional ChIP, I might suggest the authors confirm the absence of the marks not seen by ChIP/chip around promoter 2 by a test using conventional ChIP as well. Just to be sure.

The work involving the tandem three silencer elements was very nice, showing a dose response to the effect of turning off the ThPOK in CD4 cells, showing that the same machinery that epigenetically silences ThPOK in CD8 T cells does exist in CD4 T cells, but must be normally counteracted by other factors. The authors propose a kinetic interpretation and a threshold interpretation, in the discussion, which by the way was well written.

IT would be interesting to know what modification occur in the 3S CD4 cells (in Fig. 5) although I am not suggesting this needs to be done for publication. The data in Fig. 5D begins a characterization, and I am sure the authors are examining this. Perhaps the process would extend into the regions of the second promoter.

In summary, this is a nice study. I recommend acceptance without additional experiments.

Referee #2 (Remarks to the Author):

The authors characterized the complex regulation of Thpok gene during thymocyte development and in peripheral T cells. Analyses of the epigenetic state of Thpok showed that silencer-deleted alleles lost H3K27me3 and gained H3K4me3 in CD4+CD8+ thymocytes at the P1 promoter (Fig. 1); however, CpG methylation at selected sites across the regulatory regions was not significantly affected (Fig. 2). Conditional deletion of the silencer in CD8-committed thymocytes did not restore Thpok expression (Fig. 3) and a locus with three copies of the silencer underwent variegated repression in CD4+ cells (Fig. 4). This variegation was unaltered after conditional deletion of the triplicated silencer (Fig. 5). Activity of the monomeric silencer required two Runx binding sites, whereas the Runx-mutated triple silencer had considerable residual activity (Fig. 6). Deletion of the proximal enhancer reduced Thpok mRNA in CD4+ T cells two-fold, and conditional GATA3-deletion had no effect on Thpok activity (Fig. 7).

This manuscript contains extensive elegant genetics, including conditional deletion of the silencer, generation of the 3S locus, generation of the Runx-mutated silencer and conditional deletion of PE and GATA3. The resultant phenotypes of these mutations are also convincing, and often quite interesting. Despite this, however, the manuscript lacks a coherent mechanistic theme and therefore appears to be a mix of partially developed, but potentially interesting, stories. For example, germline deletion of the silencer causes Thpok de-regulation in both lineages, yet conditional deletion in CD8+ T cells has apparently no effect. What is the basis for this change in epigenetic state of the Thpok locus? Similarly, the demonstration that the 3S silences in CD4+ cells as well, suggests that

components mediating silencing are present in both CD4+ and CD8+ T cells. Why does it take 3S to see this effect in CD4+ T cells whereas only one copy works in CD8+ T cells? (Is there a limiting factor? Does 3S become a non-tissue-specific silencer?) Finally, what is the connection between the proximal enhancer and GATA3 (the subjects of Fig. 4) and the silencer (Figs. 3-5)? For these reasons, the present form of this manuscript is not suitable for publication in the EMBO J.

Referee #3 (Remarks to the Author):

The manuscript by Tanaka et al details an in-depth molecular analysis of ThPOK silencing and expression by both genetic and epigenetic mechanisms during positive selection and lineage commitment in the thymus. The manuscript is an impressive technical tour-de-force that is highly informative, providing significant new information about ThPOK silencing and expression during thymocyte development. Based on the findings they report in this manuscript, the authors speculate that persistent TCR signaling during positive selection is needed to prevent spontaneous ('default') ThPOK silencing, and that it is the prevention of 'default' ThPOK silencing that up-regulates ThPOK expression and results in commitment to the CD4 helper lineage. While I think their speculation is quite intriguing, the authors have chosen to highlight their speculation in the manuscript's title and abstract. However, I think the manuscript documents the molecular effects of ThPOK silencer and enhancer cis-elements on ThPOK expression during lineage commitment, but I do not think the manuscript compellingly links those observations to changes in thymocyte lineage commitment. Consequently, I think the authors might seriously consider altering the writing of the manuscript to limit its focus to the cis-elements mediating ThPOK silencing and expression, rather than over-interpreting their data to convince the reader that those molecular observations are the basis for helper versus cytotoxic lineage choice during thymocyte development. Moreover, the writing of the current manuscript would probably be improved by a narrower focus as it is now very densely written and unnecessarily difficult to read.
I don't see how the data in this manuscript compellingly demonstrate either that: (a) 'default' silencing occurs in TCR signaled thymocytes, or that (b) 'default' silencing of ThPOK must be prevented for helper lineage commitment. The authors use their data with the 3S silencer construct to propose 'default' silencing, but their data also indicate that silencing by the 3S construct is dependent on Runx binding sites and, hence, is presumably dependent on Runx protein expression. If 'default' silencing is dependent on Runx protein expression, why is it called 'default' silencing? Are Runx proteins expressed by thymocytes in sufficient amounts during MHC class II-specific positive selection to silence ThPOK?
Overall, I think the data in this manuscript make a significant contribution to our understanding of the molecular events that occur during lineage commitment and positive selection in the thymus. However, I think these data do not provide compelling support for the conclusions that the authors draw in the manuscript as it is currently written. I think the authors should alter the writing of the manuscript to either: (a) better justify the conclusions they currently draw, or (b) to modify and limit the conclusions drawn.

1st Revision - authors' response 15 January 2013

Point-by-point responses:

We thank reviewers for their helpful comments, suggestions, and criticisms that have substantially improved the manuscript

Referee #1:

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We thank the referee for appreciating the quality of our work and for your thoughtful comments.

There are only a couple of minor issues that I would raise. The authors recognize the caveat that the epigenetic mark (H3K27me3) does not appear to be deposited in the region around promoter 2 (Fig. 1A), which is still silenced. Secondly, there is a bit of leakiness, in that promoter 1 does turn on detectibly after removal of the silencing region (Fig. 3C,D). However, these are not major problems and the authors do discuss the issues. While the authors do confirm the presence of marks seen in ChIP/chip by using conventional ChIP, I might suggest the authors confirm the absence of the marks not seen by ChIP/chip around promoter 2 by a test using conventional ChIP as well. Just to be sure.

This is an excellent suggestion. We have added analytical ChIP/qPCR results in Figure 1, which well-reproduced the ChIP/chip results.

The work involving the tandem three silencer elements was very nice, showing a dose response to the effect of turning off the ThPOK in CD4 cells, showing that the same machinery that epigenetically silences ThPOK in CD8 T cells does exist in CD4 T cells, but must be normally counteracted by other factors. The authors propose a kinetic interpretation and a threshold interpretation, in the discussion, which by the way was well written.

We are grateful for this positive comment on our discussion.

IT would be interesting to know what modification occur in the 3S CD4 cells (in Fig. 5) although I am not suggesting this needs to be done for publication. The data in Fig. 5D begins a characterization, and I am sure the authors are examining this. Perhaps the process would extend into the regions of the second promoter.

We understand that further analysis of epigenetic changes on the three copies silencer allele is very important. However, in our experimental system, only the regions near the silencer can be separately analyzed between the 3S allele and the control allele, because other regions are identical in their sequences. Although using homozygous mice harboring the 3S allele may solve this problem, CD4 T cell development is impaired in these mice due to the low levels of ThPOK (Fig. 4), making interpretation of such data very complicated. We are sorry for such a limitation in our experimental system, but we believe that our results shown in Fig. 5 provide significant insight into epigenetic modifications induced by increased silencer activity.

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We thank the referee for positive comments on the quality and significance of our data.

Despite this, however, the manuscript lacks a coherent mechanistic theme and therefore appears to be a mix of partially developed, but potentially interesting, stories. For example, germline deletion of the silencer causes Thpok de-regulation in both lineages, yet conditional deletion in CD8+ T cells has apparently no effect. What is the basis for this change in epigenetic state of the Thpok locus? Similarly, the demonstration that the 3S silences in CD4+ cells as well, suggests that components mediating silencing are present in both CD4+ and CD8+ T cells. Why does it take 3S to see this effect in CD4+ T cells whereas only one copy works in CD8+ T cells? (Is there a limiting factor? Does 3S become a non-tissue-specific silencer?) Finally, what is the connection between the proximal enhancer and GATA3 (the subjects of Fig. 4) and the silencer (Figs. 3-5)? For these reasons, the present form of this manuscript is not suitable for publication in the EMBO J.

We thank the reviewer for pointing out the need to keep a coherent theme. We agree that the enhancer and GATA3 section was not well-connected with the main body of this work, and have removed these results.
Instead we have addressed how mechanisms of the silencer-mediated Thpok repression differ between immature DP thymocyte precursors and cytotoxic lineage committed $CD8^+$ T cells. We have added new results in revised Fig. 7, including data showing that the VWRPY motif in the Runx proteins, which recruits TLE corepressors, has a distinct role in Thpok repression at these two stages. We have revised the text on page14 accordingly and have added a discussion of these results.
We understand that it is important to unravel how the epigenetic state is different between those cells at distinct stages for better understanding of the basis of silencer-independent inheritance of Thpok repression. However, we feel this would be beyond a scope of this manuscript, which has provided significant insight into epigenetic regulation of Thpok gene during lineage commitment based on solid genetic evidence.
In response to the question of tissue specificity of the 3S silencer, we have compared Thpok-gfp expression in non-T cells, and have shown that the effect of increasing silencer copy is specific to the T-lineage (Fig 3). We have also discussed the mechanism of how the three copies silencer might influence the silencer activity (page15). We agree that it is very important to unravel mechanism that limit the Thpok silencing to $CD8^+$ T cells on the one copy silencer locus. However, given our current knowledge, it is not even clear yet whether limiting factors are present or not in this regulation. Therefore it is almost impossible to address whether limiting factors are involved in the observed differences between the three and one copy silencers. However, our results shown in Fig. 6E showed that half dosage of Bcl11b, a silencer binding protein, affects silencer activity, providing supportive information that the dosage of silencer binding proteins can be a factor that modulates silencer activity.

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the authors draw in the manuscript as it is currently written. I think the authors should alter the writing of the manuscript to either: (a) better justify the conclusions they currently draw, or (b) to modify and limit the conclusions drawn.

We thank the referee for appreciating our in-depth molecular analysis of ThPOK silencing and for your thoughtful comments. According to the suggestion of re-writing the manuscript either by better justification of the conclusions or by modification and limiting the conclusions, we have revised the discussion section and have changed the title and abstract. Since we made changes in many places, we do not list them individually here. Instead changes are highlighted in yellow in the revised manuscript. Essentially, we paid attention to the reviewer's comments to avoid over-interpretation of our data and to limit the conclusions to those that are well justified with presented results. We believe that our re-writing improved the manuscript and hopefully will satisfy referee#3.

Acceptance letter 06 February 2013

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been re-reviewed by referee #1 and 3. As you can see below the referees appreciate the introduced changes and support publication here. I am therefore very pleased to proceed with the acceptance of the study for publication here.

One last point: We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential.

Thank you for contributing to the EMBO Journal

