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Nuclear Export of Histone Deacetylase 7 During Thymic Selection is required for Immune Self-tolerance

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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.)

1st Editorial Decision 13 March 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 appreciate the findings reported, but also find that some further analysis is needed to strengthen the findings reported. In particular, better support for that the autoimmunity phenotype observed is linked to a specific defect in negative selection is needed. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript that addresses the concerns raised in full. I should add that it is EMBO Journal policy to allow only a single round of revision and it is therefore important to address the raised concerns in full.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

This manuscript builds upon the known function of HDAC7 in thymic negative selection by extending these results to the in vivo context. The authors show a major defect in negative selection (with notable caveats below). While this result is in line with previous in vitro data, the major novelty of the data is in the severity of the resulting autoimmunity. B6 mice are generally highly resistant to autoimmunity, with genetic defects in Aire, Bim, Fas or other negative-selection mediators resulting in relatively mild autoimmunity. The level of autoimmunity observed in the HDAC7-delP mice is equivalent to that observed in "double deficient" mice, indicating either the higher importance of HDAC7 to negative selection, or the role of HDAC7 in additional T cell tolerance processes (such as anergy).

While the data showing the severity of dominant T cell-dependent autoimmunity (Figures 5-7) is very convincing, there are several major issues with the data linking this autoimmunity to a specific defect in negative selection:

Firstly, the method of presenting the data on negative selection in Figure 1-2 is ambiguous. The results in figure 2 clearly show a defect in negative selection in HDAC7-delP mice, using two different transgenic systems, the HY-TCR (against male antigen) and the OT-II (anti-OVA, crossed to Act-OVA). In both cases there was a clear defect in negative selection. However this needs to be shown using the context of no negative selection, ie, side-by-side with female HY-TCR transgenics and OT-II TCR transgenics without OVA. This would allow a more quantitative assessment of the defect in negative selection to be made, ie, does HDAC7-delP reduce the efficiency of negative selection by 10%, 50% or 90%? This question becomes directly relevant in the next section of the paper, which discusses the "much more subtle" effect on HDAC7-delP on positive selection. In the manuscript the effect of HDAC7-delP is said to increase SP number and have no effect on DP-SP transition, however the representative plots in Figure 2E suggest ~50% defect in positive selection efficiency. This discrepancy comes from the increase in absolute numbers of the thymus of HDAC7-delP, making the absolute numbers of SP cells slightly higher, however this is an unusual definition of positive selection, which is typically defined as the transition between DP and SP cells. Using this more conventional definition, the results would be interpreted as a ~50% defect in positive selection (assuming the representative plots are truly representative, statistical analysis is not presented for this data). Thus it becomes highly relevant to quantify the defect in negative selection, is it on the same order as the defect in positive selection, or greater?

Secondly, the transcriptional data in Figures 3-4 is suggestive of a negative selection-specific defect, but this result is muddied by the technical system used to induce negative selection (i.p. peptide injection into TCR transgenic mice). In this system the death of thymocytes seen in this system could be secondary due to the massive peripheral activation of T cells. As such, either a peripheral defect in activation or a thymic defect in negative selection could manifest with similar changes in transcriptional profile in the thymus. This experimental design is therefore hard to place in context, without unambiguous data showing the negative selection is profoundly more impeded than positive selection and peripheral activation.

Finally, on the peripheral consequence of the negative selection defect, the authors "assume" that the defect in negative selection translates into increased autoreactive peripheral T cells; it is surprisingly that they do not show peripheral data for their TCR transgenic systems which could actually demonstrate this point. An increase in CD69 expression and activated T cells in the non-TCR transgenic mice is insufficient to demonstrate this point, as there are multiple interpretations of this data beyond increased autoreactive T cells.

Referee #2

The manuscript by Kasler et al and presents an interesting transgenic new mouse model with a surprisingly strong phenotype in negative selection of thymocytes. The defect in negative selection is well documented using two different TCR transgenic mouse strains.
Further, the authors convincingly document how multi-organ autoimmunity develops in the transgenic mice as a result of defective negative selection.

A weaker point in the manuscript is the analysis of the molecular mechanism how dominant HDAC7-deltaP prevents apoptosis of to be negatively selected thymocytes.
Specifically, it would be useful to investigate the effect of antigenic peptide injection at earlier time points than the presented 2.5h (Fig.3) and 3h (Fig.4). Otherwise it is hard to tell whether e.g. reduced Erk phosphorylation or less Nur77 expression is the cause or the symptom. An additional option to address this would be transgenic overexpression of Bcl-2 in these investigated mice.

In addition, I have a few rather minor points in random order:

It would be interesting to see and easily tested whether development of classical CD4 single positive NKT cell is affected in the HDAC7-deltaP Tg.

Some awkward sentences should be double-checked P3 how are Tregs eliminated from the repertoire; P4 Errors in the coupling...; P5 Conversely, thymus-specific negative selection of HDAC7 ...; P9 these cells would be expected to be more likely ...

Tregs are now called "Treg cells"

Figure 5C should either show colon or no colon for both genotypes.

The survival data in Fig. 5B, 6D,E, and 7C should shown as normal Kaplan Meier curves.

In the abstract the link to AIRE deficiency and NOD mice is a far shot.

Referee #3

HDAC7 can shuttle between the nucleus and cytoplasm and TCR signaling induces nuclear export of HDAC7 in DP thymocytes. In the submitted manuscript, Verdin and colleagues investigated the importance of HDAC7 nuclear export on thymocyte development by generating a transgenic mouse in which exogenously expressed HDAC7 is constitutively localized in the nucleus (HDAC7-deltaP). The authors observed that transgenic thymocytes expressing HDAC7-deltaP had a block in negative selection, while positive selection was relatively normal. Impaired negative selection resulted in the escape of auto-reactive T cell in the periphery, leading to a lethal autoimmune syndrome. Mechanistically, gene expression arrays revealed that the induction of gene expression programs associated with negative selection are suppressed in transgenic DP thymocytes and that there is impaired activation of the MAP kinase pathway upon TCR triggering in DP thymocytes.

Overall, this is a nicely performed story that provides novel insight (i) into how negative selection during thymocyte development is regulated and (ii) about the role of HDAC7 in this process.

Specific comments:

(1) In Figure 1, the authors show the phenotype of HDAC7-deltaP tg mice. However, a more comprehensive analysis of T cell development should be provided. Are there any changes in the expression levels of TCR/CD3, CD24 (HSA), CD69 or CD5 in the various subsets? What is the distribution between CD3hi and CD3lo cells between the CD8SP gate? It would also be informative to show a CD4/CD8 dot plot on TCRb-high and TCRb-low-gated thymocytes to get a better overview about the relative presence of various thymocyte subsets.

(2) In Figure 2, the authors show negative and positive selection using various TCR transgenic mice.

The data using the H-Y or the OT-II x Act-Ova TCR systems show that there is impaired negative selection. However, there is no information provided about peripheral T cells in these mice. Are there peripheral CD8+ T cells in HY mice and CD4+ T cells in OT-II x Act-Ova mice that have been selected on the transgenic HY TCRalpha chain (detectable with the T3.70 Ab) or the tg OT-II Va2 chain? Quite similar, there is also no information provided about peripheral T cells in OT-I and OT-II tg mice. The authors state that there is not really a difference in positive selection. In order to make this statement about positive selection, the authors should show the expression of the transgenic Va2 chain in peripheral CD4+ and CD8+ T cells.

(3) The authors show that genes required for negative selection are repressed in HDAC7-deltaP tg mice upon TCR stimulation. It would be interesting to know whether constitutive nuclear HDAC7 also blocks dexamethasone-induced deletion of DP thymocytes? Thus, the authors should inject dexamethasone into wt and HDAC7-deltaP tg mice and test whether there is a difference in the deletion of DP thymocytes.

(4) The authors conclude that nuclear HDAC7 represses genes required for negative selection and they provide experimental evidence to support the conclusion. However, wouldn't it be possible that HDAC7 also has a function in the cytoplasm that is required for negative selection (e.g. that HDAC7 acetylates certain proteins in the cytoplasm)? Why is MAP kinase pathway activation impaired? The authors suggest that HDAC7 regulates a "functional cassette of genes...that are required to allow MAP kinase activation" (page 12). However, it is possible that cytoplasmic HDAC7 acetylates signaling molecules and that this modification is required for MAP kinase activation. The authors should mention in the discussion section the possibility that HDAC7 might have a cytoplasmic function to indicate the possibility of yet unknown additional activities of HDAC7 beyond repressing genes in the nucleus.

(5) The authors should show how they gated for ISP cells (Fig. S1)

Minor issue:

On page 5, last paragraph, 7th line from the bottom: the phrase "negative selection of HDAC7" in the sentence "Conversely, thymus-specific negative selection of HDAC7 results in ..." does not appear to be correct.

1st Revision - authors' response 14 September 2012

Referee #1:

While the data showing the severity of dominant T cell-dependent autoimmunity (Figures 5-7) is very convincing, there are several major issues with the data linking this autoimmunity to a specific defect in negative selection:

Firstly, the method of presenting the data on negative selection in Figure 1-2 is ambiguous. The results in figure 2 clearly show a defect in negative selection in HDAC7-delP mice, using two different transgenic systems, the HY-TCR (against male antigen) and the OT-II (anti-OVA, crossed to Act-OVA). In both cases there was a clear defect in negative selection. However this needs to be shown using the context of no negative selection, ie, side-by-side with female HY-TCR transgenics and OT-II TCR transgenics without OVA. This would allow a more quantitative assessment of the defect in negative selection to be made, ie, does HDAC7-delP reduce the efficiency of negative selection by 10%, 50% or 90%? This question becomes directly relevant in the next section of the paper, which discusses the "much more subtle" effect on HDAC7-delP on positive selection. In the manuscript the effect of HDAC7-delP is said to increase SP number and have no effect on DP-SP transition, however the
representative plots in Figure 2E suggest ~50% defect in positive selection efficiency. This discrepancy comes from the increase in absolute numbers of the thymus of HDAC7-delP, making the absolute numbers of SP cells slightly higher, however this is an unusual definition of positive selection, which is typically defined as the transition between DP and SP cells. Using this more conventional definition, the results would be interpreted as a ~50% defect in positive selection (assuming the representative plots are truly representative, statistical analysis is not presented for this data). Thus it becomes highly relevant to quantify the defect in negative selection, is it on the same order as the defect in positive selection, or greater?

Although we did include statistics for the effect of HDAC7-ΔP on positive selection in the original version of our manuscript (Fig. 2F), we acknowledge that our discussion of the effect of HDAC7-ΔP on positive vs. negative selection was not sufficiently clear. The use of OT-1 as the model for CD8 positive selection in this discussion rather than H-Y might also have caused confusion. We have therefore switched to H-Y females to show the effect on CD8 positive selection, and integrated the discussion of the effect of HDAC7-ΔP on positive and negative selection into the same figure panels. Representative flow plots (Fig. 2A) and quantification of cell numbers (Fig. 2B-C) are now provided side-by side for both the positively and negatively selecting cases of both TCR transgenes. Using this analysis and based on the reviewer's criteria for the efficiency of positive and negative selection, we determine that HDAC7-ΔP causes a 2-fold reduction in the efficiency of positive selection and a 10 to 100-fold reduction in the efficiency of negative selection. This finding is stated in the revised manuscript.

Secondly, the transcriptional data in Figures 3-4 is suggestive of a negative selection-specific defect, but this result is muddied by the technical system used to induce negative selection (i.p. peptide injection into TCR transgenic mice). In this system the death of thymocytes seen could be secondary due to the massive peripheral activation of T cells. As such, either a peripheral defect in activation or a thymic defect in negative selection could manifest with

similar changes in transcriptional profile in the thymus. This experimental design is therefore hard to place in context, without unambiguous data showing the negative selection is profoundly more impeded than positive selection and peripheral activation.

While we did not assess the effect of HDAC7-ΔP on thymocyte death per se in a system where massive peripheral activation occurs (i.e. we used H-Y and OT-2 X Act-Ova, in which these cells are deleted), we acknowledge that peripheral activation could be a confounding factor in the *in-vivo* stimulation we used to assess the effect of HDAC7-ΔP on the transcriptional response to strong activation and on MAP kinase activity. We have taken several steps to mitigate this concern. First, we have clarified and expanded our discussion of positive and negative selection, as detailed above, and provided data in response to another reviewer concern (Fig. 2E) that shows that CD4 T cell numbers in the periphery are near normal in OT-2 vs. OT-2 X HDAC7ΔP mice. Secondly, we have now demonstrated the defect in MAP kinase activation and Nur77 expression in HDAC7-ΔP transgenic thymocytes in an *ex-vivo* system, where influences from peripheral T cells are not an issue (Fig. 4C-D). Lastly, we have shown that both upregulation of activation markers and proliferation are normal in splenic HDAC7-ΔP X OT-2 T cells after *ex-vivo* stimulation with Ova peptide (Fig. S2G-H). We believe that taken together, these results strongly support the conclusion that the defect in negative selection caused by HDAC7-ΔP is cell-intrinsic.

Finally, on the peripheral consequence of the negative selection defect, the authors "assume" that the defect in negative selection translates into increased autoreactive peripheral T cells; it is surprisingly that they do not show peripheral data for their TCR transgenic systems which could actually demonstrate this point. An increase in CD69 expression and activated T cells in the non-TCR transgenic mice is insufficient to demonstrate this point, as there are multiple interpretations of this data beyond increased autoreactive T cells.

While we do believe that the bias towards activated and memory cells in the peripheral T cell populations observed in WT: HDAC7-ΔP chimeras is an important observation, we acknowledge that this by itself does not demonstrate the escape of autoreactive thymocytes into the periphery, and that we should have done more to show this in our initial submission. We have not been able to demonstrate this directly in the H-Y model, in which we observe fairly large numbers of clonotype-positive cells in the periphery regardless of HDAC7-ΔP. However, in the OT-2 X act-Ova model, we do see more CD4/Vα2-positive cells in both spleen and lymph node of OT-2 X act-Ova X HDAC7-ΔP mice vs. OT-2 X act-Ova controls, and we have now shown this in Fig. 2D-E.

Referee #2:

A weaker point in the manuscript is the analysis of the molecular mechanism how dominant HDAC7-deltaP prevents apoptosis of to be negatively selected thymocytes. Specifically, it would be useful to investigate the effect of antigenic peptide injection at earlier time points than the presented 2.5h (Fig.3) and 3h (Fig.4). Otherwise it is hard to tell whether e.g. reduced Erk phosphorylation or less Nur77 expression is the cause or the symptom. An additional option to address this would be transgenic overexpression of Bcl-2 in these investigated mice.

The main reason we only evaluated one time point in depth in the original submission was the considerable technical difficulty involved in obtaining enough flow-sorted thymocytes from all of the mice required to do the analysis with sufficient replicates. Therefore, in order to evaluate more time points more efficiently, we have switched to a magnetic bead sorting strategy and done the stimulation *ex-vivo*, allowing us to evaluate multiple time points with the thymocytes from one mouse.
In this format, which also addresses a concern of Reviewer #1 described above, we have now shown that the defect in P38 activation is detectable as early as 25 minutes post-stimulation, before Nur77, one of the most rapidly and strongly upregulated targets of TCR signaling, is detectably induced (Fig. 4C-D). The picture with Erk activation is less clear-cut at early time points, with significant reduction of Erk activity not being observed until 100 minutes (Fig. 4E), although we suspect that more repetitions of the experiment would show significance at 50 minutes as well ($P = 0.07$, 2-tailed paired T-test for 4 replicates). Importantly, we did not mean to imply in our narrative that all of the gene expression changes we observed were a consequence rather than a cause of the defect in MAP kinase activation, nor do we feel this finding would be in any way critical to the model we are advancing. Rather, we assert that as a consequence of the primary effect of HDAC7-ΔP on gene expression, the response of the Erk and P38 pathways is dampened in a way that significantly broadens the defect in negative selection beyond any direct targets of HDAC7.

In addition, I have a few rather minor points in random order:

 # It would be interesting to see and easily tested whether development of classical CD4 single positive NKT cell is affected in the HDAC7-deltaP Tg.

We agree that this is an interesting question and fairly easy to answer. We have now stained wild type and HDAC7-ΔP transgenic thymocytes and splenocytes with ceramide-loaded CD1d tetramers, and found that classical CD1d-restricted NKT cells appear to be totally absent from the HDAC7-ΔP transgenic mice (figure removed in the RPF). This is obviously an interesting and important finding, and as such it merits significant further investigation before it is published. We therefore ask that we be allowed to exclude this finding from the current manuscript, so that we may give it the further investigation it requires and publish it at a later time, when we understand the apparently categorical nature of this defect better.

 # Some awkward sentences should be double-checked P3 how are Tregs eliminated from the repertoire; P4 Errors in the coupling ...; P5 Conversely, thymus-specific negative selection of HDAC7 ...; P9 these cells would be expected to be more likely ...

These sentences have now been clarified.

 # Tregs are now called "Treg cells"

We have now changed all instances to “Tregs”

 # Figure 5C should either show colon or no colon for both genotypes.

The colon is in fact quite a bit smaller in the WT animal, but we agree that it seems to have been cut somewhat closer to the caecum than in the HDAC7-ΔP animal. We have now truncated the image of the HDAC7-ΔP colon at what we estimate is the same location.

 # The survival data in Fig. 5B, 6D, E, and 7C should shown as normal Kaplan Meier curves.

This has been corrected in the revised manuscript.

 # In the abstract the link to AIRE deficiency and NOD mice is a far shot.

This language has been removed from the abstract.

Referee #3:

(1) In Figure 1, the authors show the phenotype of HDAC7-deltaP tg mice. However, a more comprehensive analysis of T cell development should be provided. Are there any changes in the

expression levels of TCR/CD3, CD24 (HSA), CD69 or CD5 in the various subsets? What is the distribution between CD3hi and CD3lo cells between the CD8SP gate? It would also be informative to show a CD4/CD8 dot plot on TCRb-high and TCRb-low-gated thymocytes to get a better overview about the relative presence of various thymocyte subsets.

We had already performed some of these analyses and chosen not to include them for reasons of space, but we have now put them in the manuscript and added analysis of TCRβ and CD24, which we had not already done (Fig 1C, S1C). The overall conclusion of this analysis is that while CD5 expression is universally suppressed, the pattern of expression of the other markers suggests a bias towards greater maturity in HDAC7-ΔP transgenic thymocytes, particularly in the CD8 SP population.

(2) In Figure 2, the authors show negative and positive selection using various TCR transgenic mice. The data using the H-Y or the OT-II x Act-Ova TCR systems show that there is impaired negative selection. However, there is no information provided about peripheral T cells in these mice. Are there peripheral CD8+ T cells in HY mice and CD4+ T cells in OT-II x Act-Ova mice that have been selected on the transgenic HY TCRalpha chain (detectable with the T3.70 Ab) or the tg OT-II Va2 chain? Quite similar, there is also no information provided about peripheral T cells in OT-I and OT-II tg mice. The authors state that there is not really a difference in positive selection. In order to make this statement about positive selection, the authors should show the expression of the transgenic Va2 chain in peripheral CD4+ and CD8+ T cells.

As we stated in our response to the similar concern raised by reviewer #1, we have now provided information about escape of Vα2-positive, CD4-positive T cells into the periphery of OT-2 X act-Ova X HDAC7-ΔP vs. OT-2 X act-Ova animals (Fig. 2D-E). These figure panels also show a minimal (not statistically significant) reduction in the numbers of CD4/Vα2-positive T cells in the spleens of OT-2 vs. OT-2 X HDAC7-ΔP Mice (Fig. 4D). This is consistent with the small (i.e. 2-fold) effect on positive selection we show in Fig. 2B-C. As to the number of CD8 T cells in the OT-1 X HDAC7-ΔP mice, we have not included this analysis in the manuscript for reasons of space, but we have provided data for 3 animals in Fig. R2 above, for the benefit of the reviewers (figure removed in RPF). Clearly the number and proportion of CD4 and CD8 T cells in these spleens is very similar to those of otherwise wild-type OT-1 animals.

(3) The authors show that genes required for negative selection are repressed in HDAC7-deltaP tg mice upon TCR stimulation. It would be interesting to know whether constitutive nuclear HDAC7 also blocks dexamethasone-induced deletion of DP thymocytes? Thus, the authors should inject dexamethasone into wt and HDAC7-deltaP tg mice and test whether there is a difference in the deletion of DP thymocytes.

Our preliminary results with dexamethasone treatment showed no difference, so we did not pursue the question further. We have now done this analysis in more depth, using *ex-vivo* treatment of thymocytes with dexamethasone for 5 WT-HDAC7-ΔP tg pairs, and confirmed that there is no difference in dexamethasone-induced death (Fig. S2C). We have also included data showing that there is no difference in the rate of spontaneous cell death *ex-vivo* either (Fig. S1B).

(4) The authors conclude that nuclear HDAC7 represses genes required for negative selection and they provide experimental evidence to support the conclusion. However, wouldn't it be possible that HDAC7 also has a function in the cytoplasm that is required for negative selection (e.g. that HDAC7 acetylates certain proteins in the cytoplasm)? Why is MAP kinase pathway activation impaired? The authors suggest that HDAC7 regulates a "functional cassette of genes...that are required to allow MAP kinase activation" (page 12). However, it is possible that cytoplasmic HDAC7 acetylates signaling molecules and that this modification is required for MAP kinase activation. The authors should mention in the discussion section the possibility that HDAC7 might have a cytoplasmic function to indicate the possibility of yet unknown additional activities of HDAC7 beyond repressing genes in the nucleus.

Although we acknowledge that our inability to pin down the observed defects in MAP kinase activation to a specific molecular mechanism constitutes a weak point in the study as it stands,

and furthermore that we do not specifically address any putative cytoplasmic role of HDAC7, we do not feel that the data we have obtained point to a cytoplasmic mechanism for HDAC7 in regulating MAP kinase function. This is because expression of the HDAC7-ΔP transgene is in trans to the endogenous, wild-type HDAC7 gene, and therefore does not affect its normal response to TCR signaling. The enforced presence of nuclear HDAC7 in our transgenic thymocytes does not therefore imply a loss of HDAC7 from the cytoplasm. We have however added a statement to our discussion to acknowledge that this study does not answer the question of what cytoplasmic function HDAC7 may have in thymocytes.

(5) The authors should show how they gated for ISP cells (Fig. S1)

Gating for ISP cells was done using expression of CD3ε as the marker for mature vs. ISP cells (shown in Fig. S1C).

On page 5, last paragraph, 7th line from the bottom: the phrase "negative selection of HDAC7" in the sentence "Conversely, thymus-specific negative selection of HDAC7 results in ..." does not appear to be correct.

Reviewer #2 also spotted this error, which has been fixed. We meant to say “deleted”.

Acceptance 02 October 2012

Thank you for submitting your manuscript to the EMBO Journal. Your revision has now been seen by the three referees. As you can see below, the referees appreciate the introduced changes. I am therefore pleased to accept the paper for publication here.

Thank you for submitting your manuscript to us. I am pleased to see the study published in the EMBO Journal!

Your sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

In the revised version of the manuscript, all technical issues raised in the first review have been resolved through the addition of new data.

Referee #2

It is a pity that EMBO does not impose highlighting or tracking of the actual changes made in the revised manuscript. However, the authors have satisfactorily addressed all the concerns raised by three reviewers. I am looking forward to read more on the role of HDAC7 in agonist selection in future follow-up studies.

Referee #3

The authors responded properly to my comments

